P. Narayanasamy

Microbial Plant Pathogens-Detection and Disease Diagnosis:

Viral and Viroid Pathogens Vol. 3

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Viral and Viroid Pathogens, Volume 3

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Cover illustration:

Peanut bud necrosis (Volume 3)
Infected plants exhibit chlorotic ringspots on the young leaves and they show necrosis of the terminal buds later. The plants may be killed, if they are infected in the early stages of growth.
(Courtesy of P. Narayanasamy, Tamil Nadu Agricultural University, Coimbatore, India)

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Dedicated to the Memory of my Parents
for their Love and Affection

Preface

Viruses, although minute in size and primitive in structure, are capable of infecting all life forms from prokaryotic bacteria to highly evolved humans. Occurrence of diseases such as small pox and influenza, now known to be of viral origin, was reported to affect human beings even before the recorded history. However, the viral origin of diseases could be established only during the last decade of the 19th century by the researchers like Iwanowsky and Beijerinck working on tobacco mosaic disease. The controversial status of virus, whether living or nonliving, attracted the attention of not only the plant pathologists, but also biochemists, biophysicists and biotechnologists. Viruses and viroids form distinct groups of obligate parasites and they have to be considered differently, while diagnosing the diseases induced by them, because all steps of the Koch's postulates cannot be applied to prove them to be the causative agents of newly observed disease(s), as in the case of fungal and bacterial plant pathogens.

As the viruses and viroids have only elementary constitutional features and no physiological functions, application of methods based on the biological, morphological and biochemical characteristics may not yield precise results. Immunoassays have been effectively employed for the detection, identification and differentiation of viruses infecting various crops. However, the antigenic properties of the viruses are governed by only a small portion of the viral genome. Hence, the differences in the other segments of the viral genome cannot be detected by the immunoassays. This situation makes the application of nucleic acid-based techniques to be more appropriate for the detection, differentiation and quantification of plant virus populations/strains in different host plant species and in different locations and periods. Likewise, viroids, lacking the protein component, do not provide any better option other than the nucleic acid-based techniques for their detection and diagnosis of the diseases caused by them. Although bioindexing employing diagnostic plant species has been adopted for long time, this approach requires large greenhouse space and long time, in addition to the results being inconclusive frequently. Nucleic acid-based techniques are preferred for the detection, identification, differentiation and quantification of viruses and viroids because of their high levels of sensitivity, specificity, reproducibility and rapidity. Furthermore, the feasibility of automation to handle large number of samples and the possibility of simultaneous

detection of several plant viruses by employing nucleic acid-based methods have enhanced their utility for detection of plant viruses and viroids and diagnosis of the diseases caused by these pathogens.

This volume presents exhaustive information based on extensive literature search on various methods of detection of viral and viroid pathogens and diagnosis of diseases caused by them. Comparative effectiveness of different techniques is discussed critically to enable the researchers, teachers, extension specialists and graduate students to choose the suitable procedures for their investigations. Several protocols are presented as appendix in appropriate chapters to meet this requirement. This volume is expected to provide the necessary platform for planning more critical studies that may improve the efficiency and reliability of the existing methods that have been employed for simultaneous detection of two or more kinds of pathogens, without compromising the sensitivity and specificity of detection of viral and viroid pathogens and diagnosis of the diseases caused by them.

Coimbatore, India P. Narayanasamy

Acknowledgement

With a deep sense of humility, gratitude and reverence, I bow to my Alma Mater that continues to remain a source of inspiration for me for over five decades. I wish to place on record my appreciation to my colleagues and graduate students of the Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore, India for their suggestions and critical comments. I am thankful to Dr. T. Ganapathy, Professor of Plant Pathology, for providing technical assistance requiring enormous patience and skill. Permission granted by different copyright holders to reproduce the figures published in various journals is gratefully acknowledged.

It is with great pleasure, I thank profusely my wife Mrs. N. Rajakumari who showers her love and kindness, enabling me to devote my attention exclusively for the preparation of this book. Expression of abundant affection and endless encouragement of my family members Mr. N. Kumar Perumal, Mrs. Nirmala Suresh, Mr. T. R. Suresh and Mr. S. Varun Karthik has been the source of support for all my academic efforts during my career.

Contents

Table of Contents for Volumes 1 and 2

Volume 1

Volume 2

Chapter 1
Introduction

Abstract Viral and viroid pathogens have great potential for inducing several economically important crop diseases as the fungal and bacterial plant pathogens, in spite of their extremely small size and elementary structure. They can infect whole plants, seeds and vegetatively propagated plant materials causing immense quantitative and qualitative losses. Hence, application of effective methods of detection, differentiation, quantification and identification has become necessary to prevent the incidence and spread of the diseases through infected plants and plant materials and through their natural vectors. To achieve this goal several methods based on the biological, physico-chemical, immunological and nucleic acid characteristics of these pathogens have been employed with different levels of sensitivity, specificity and reliability. Disease diagnostic centers, plant quarantines and certification programs are primarily involved in the prevention of introduction of viruses and viroids into new areas, production of disease-free seeds and planting materials and providing necessary advice to the growers for taking up measures to effectively manage the diseases caused by them. Establishment of adequately equipped laboratories to facilitate efficient functioning of the personnel of these centers and programs is emphasized.

1.1 Microbial Plant Pathogens as a Major Limiting Factor of Crop Production

Microbial plant pathogens including virus and viroid pathogens have the ability to infect a few or a wide range of plant species causing varying magnitude of quantitative and qualitative losses in crops cultivated in different ecosystems. Global losses caused by crop diseases have been estimated to range from 9% to 14.2% of potential yield (Orke et al. 1994). Assessment of losses made in a later investigation indicated that about 14.1% of produce may be lost due to crop diseases with a monetary value of $220 billions per annum, the developing countries suffering more losses compared with developed countries (Agrios 2005). The loss assessments have been made for different types of diseases with different levels of accuracy. However, irrespective of the levels of accuracy, the estimates of losses underscored the imperative need for

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measures to be taken urgently to minimize the losses to the extent possible. To achieve this aim, three principles of crop disease management viz., exclusion, eradication and immunization are applied for the development of short- and long-term disease management strategies (Narayanasamy 2002). The effectiveness of crop disease management systems depends heavily on the rapid, reliable and sensitive detection of microbial plant pathogens and accurate diagnosis of the diseases caused by the putative pathogen(s) detected in infected plants.

1.2 Discovery of Viruses and Viroids as Plant Pathogens

Plant diseases were considered as God-sent curses for the sins committed by people as punishment, due to lack of scientific and analytical examinations and interpretation. However, scientific thoughts and analytical experiments of early researchers such as Micheli (1729), Tillet (1755) and Prévost (1807) laid strong foundation for modern investigations on fungal plant pathogens. de Bary (1861) was able to establish for the first time that the fungus *Phytophthora infestans* was the causative agent of the potato late blight disease which ruined the potato crops. His remarkable contributions earned him the honor of being referred as the father of plant pathology (Horsfall and Cowling 1977). Plant viruses are extremely small in size and possess simple structural features consisting of protein and genomic nucleic acid which may be either an RNA or DNA molecule. However, they have the potential for independent replication and induction of diseases in susceptible plant species as the well-developed fungal and bacterial pathogens. As the viruses are entirely obligate, intracellular parasites, no structure characteristic of virus infection is produced outside the host plant cells. In addition, their extremely small size make the application of some techniques that are useful for the detection of fungal and bacterial pathogens unsuitable. These factors have been responsible for the failure of recognizing the viruses as a distinct group of pathogenic entities different from fungal and bacterial pathogens for over two decades, after the demonstration of the fungus, *Phytophthora infestans*, being the cause of the destructive potato late blight disease of historical importance. Establishment of viruses as distinct group of pathogens was possible by the innovative experiments performed by Iwanowski (1892) and Beijerinck (1898). They showed that the virus causing the tobacco mosaic disease could pass through the porcelain filter candles that retained bacteria. The filtrate was found to be infectious to tobacco, inducing typical mosaic symptoms on the inoculated tobacco plants. Hence, the pathogenic entity was designated '*contagium vivum fluidum*' (contagious living fluid) by Beijerinck. These two researchers provided strong evidences to clear the mystery surrounding the nature of the cause of tobacco mosaic disease. Thus the new branch, plant virology sprouted from the parent tree of plant pathology. Stanley (1935), honored with a nobel prize and Bawden et al. (1936) demonstrated that *Tobacco mosaic virus* (TMV) inducing tobacco mosaic disease could be crystallized like other chemical compounds and the virus particles are composed of nucleoproteins. These findings stimulated

vigorous discussions whether the viruses should be considered as living or nonliving entities. However, it has been accepted that the viruses may be classified into families and species as other living organisms (Mayo and Brunt 2007).

1.3 Detection of Viral and Viroid Plant Pathogens and Disease Diagnosis

Occurrence of crop diseases caused by different kinds of microbial plant pathogens has been observed at different stages of crop growth. Different diagnostic methods have been developed to detect and identify the causative agent(s) of newly observed disease or disease complex. As the viruses and viroids have primitive structural features compared to other pathogens, the range of characteristics available for detection, identification and classification is considerably limited. Virus particle morphology and structural variations have limited application for differentiation and identification of viruses and diagnosing the diseases caused by them. Furthermore, the viruses with obligate mode of parasitism, have not been cultured on cell-free media. Hence, it will not be possible to fulfill all the steps of Koch's postulates. However, constant and consistent association of a putative viral pathogen with the newly observed disease will provide the reasonably realistic evidence indicating the nature of the cause of the disease.

Biological tests to determine the pathogenicity and host range of the virus isolated and the mode of transmission – mechanical/graft inoculation, seeds and vectors – are performed to have a clue about the identity of the virus causing the disease. These tests are time-consuming, labor-intensive and require large greenhouse space. Furthermore, the results may not be useful to reliably differentiate and identify the strains of a virus which show distinct differences in the virulence (pathogenic potential). As the need for the development of more precise, sensitive and specific diagnostic techniques for viruses was realized, various approaches were made to solve the virus disease problems. Diagnostic methods based on the physico-chemical, immunological and nucleic acid characteristics have been demonstrated to be efficient for the detection, differentiation and quantification of viruses in the infected whole plants, seeds and planting materials, facilitating the formulation of effective disease management systems suitable for various crops grown in different ecosystems (Chapter 2).

As the plant viruses and viroids are obligate parasites, their existence in a free state in the environment is not possible except for a few highly stable viruses. They have to be within the cells of the different crop/weed plant host species or the vectors that allow their replication as in the case of propagative viruses. The assay/diagnostic host plant species have been useful to detect and identify certain viruses especially those viruses that need long incubation period in the tree plant species. Application of immunological and nucleic acid-based methods has been demonstrated to be more sensitive and specific for the detection of viruses in additional

host plant species which may be able to serve as the potential sources of inoculum for infection of crops (Chapter 3).

Differentiation of strains of plant viruses by employing biological tests may be useful to select the mild strains that can be used for providing protection against severe strains of the same virus. However, application of immunological and/or nucleic acid- based techniques are preferred, because of their potential to provide sensitive, specific and precise results rapidly (Chapter 4). Diagnosis of virus and viroid diseases has been the primary responsibility of disease diagnostic centers (DDCs), plant quarantines and certification programs. Several investigations have indicated the possibility of diagnosing infections of plants and plant materials by two or more viruses/viroids simultaneously by employing hybridization-based or polymerase chain reaction (PCR)-based techniques which offer the advantage of saving time and reduced cost of testing. The aim of attaining higher production levels relies primarily on the use of disease-free seeds or planting materials, prevention of introduction of new virus/viroid disease(s) and provision of timely advice to the growers based on the accurate diagnosis of the disease problems. It is essential that the laboratories manned by the personnel of DDCs, plant quarantines and certification programs are adequately equipped to meet the requirements (Chapter 5).

The information reflecting extensive literature search is presented in an easily understandable style. It is expected that the various aspects of detection, differentiation, quantification and identification of viral and viroid pathogens, as well diagnosis of the diseases caused by these pathogens discussed in this volume will be highly useful to the researchers, teachers and graduate students in the Departments of Plant Pathology, Microbiology, Plant Protection, Molecular Biology and Plant Breeding. In addition, the extension plant pathologists in disease diagnostic centers and personnel of plant quarantine and certification programs will find the information to have practical utility. Presentation of several protocols appended as appendices in appropriate chapters will assist in selecting the right procedures for reaching their research targets.

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Chapter 2
Detection of Virus and Viroid Pathogens in Plants

Abstract Plant viruses differ significantly from other microbial pathogens in their simple structure and minute size, method of replication and obligate parasitism. Despite their simple structure, they cause numerous economically important plant diseases that still remain as threat to crop production in several countries. Depending on the biological, morphological, immunological and genomic properties, several approaches have been made for the detection, identification, quantification and differentiation of the viruses. Histochemical methods employing light and electron microscopy have been shown to be useful for detecting and studying the nature of intracellular inclusions characteristic of virus infection. Certain groups of viruses can be putatively identified by the type and nature of inclusion bodies in infected plant cells. Electron microscopy using appropriate stains has been useful to study the ultrastructure of viruses. Immunological techniques have been shown to provide more rapid, sensitive and precise detection of viruses compared to biological methods that depend on inoculation to diagnostic/assay hosts that exhibit characteristic symptoms. Nucleic acid-based techniques represent significant advancement in pathogen diagnostics. They offer more reliable, reproducible, sensitive and specific results for the detection, identification and quantification of the viruses in different host plant species that are symptomatic or asymptomatic. Attempts to detect two or more viruses simultaneously present in infected plants have been successful, resulting in saving time, labor and costs of testing. In spite of all the advantages, many nucleic acid-based techniques have not yet been demonstrated to be applicable in different locations, cost-effective and suitable for large scale use under field conditions.

Viroids represent a group of most primitive pathogenic entities constituting exclusively of nucleic acids that are capable of independent replication and inducing diseases when introduced into susceptible plant cells. Biological methods have been useful in detecting the viroids based on their reaction on diagnostic host plants. Nucleic acid-based techniques are the ones that can provide reliable and rapid results for their detection and identification. As it is very difficult to cure the plants already infected by viruses or viroids, the practical option available for the management of diseases caused by them is the removal of infected plants. Such an eventuality will result in devasting adverse effect on crop production. To avoid such a catastrophic possibility, adequate attention has to be bestowed for the early and reliable detection of viruses and viroids

P. Narayanasamy, *Microbial Plant Pathogens-Detection and Disease Diagnosis: Viral and Viroid Pathogens, Vol. 3*, DOI 10.1007/978-90-481-9754-5_2,

in infected seeds and vegetatively propagated materials and eliminate all infected materials to build virus/viroid-free nucleus stocks for distribution to growers.

Viruses are known to cause diseases in all living organisms from highly evolved human beings to primitive prokaryotes. However, they were established as the causative agents of plant diseases only in the late nineteenth century. Because of the extremely small size, viruses remained as elusive and enigmatic causes of several disease problems defying human efforts to contain the incidence and spread of the diseases caused by them. Although significant advancement has been made in the knowledge on different aspects, management of virus diseases is still restricted largely to elimination of infected plant materials and reducing the population of vectors spreading the viruses from infected plants to healthy plants. This fact brings into focus the imperative need for the development of techniques for rapid, reliable, specific and sensitive detection of viruses in infected plants and planting materials that can serve as sources of infection for the crops to be planted in the subsequent seasons.

Plant viruses are intracellular obligate parasites with limited variations in the particle morphology that can be used for reliable identification of individual viruses. They are simple in their constitution, primarily possessing a protein coat which forms a protective covering for viral genomic nucleic acid. The viral nucleic acid may be of either ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) molecule(s). Viruses replicate in a manner that is distinctly different from other living organisms. The viral components – coat protein and nucleic acid – are synthesized separately in different sites in susceptible host cells. When these components reach the required concentrations, they are assembled together to form progeny virus particles. Except a few, all plant viruses depend on different kinds of vectors such as insects, mites, nematodes and fungi for transmission from infected plants to healthy plants. Under natural conditions, transmission through parasitic dodder may occur to some extent. Mechanical inoculation and grafting are useful to transmit the viruses to healthy plants under experimental conditions.

Since the diagnostic techniques are based on the different characteristics of plant viruses, information on the biological, physical, chemical, immunological and genomic properties has to be gathered. The usefulness of various diagnostic techniques developed for detection of plant viruses are discussed below.

2.1 Detection of Plant Viruses in Plant Organs

2.1.1 Plant Virus Taxonomy

Plant viruses were named earlier based on the host plant on which the virus was first described and the major symptoms induced by the virus concerned in that host plant species (e.g., *Tobacco mosaic virus*). Since the proposal of Beijerinck (1898) on the nature of the disease causing agent in tobacco as *contagium vivum fluidum*, a little

over a century ago, enormous and fascinating information has accumulated about these ultramicroscopic, diminutive pathogenic agents, capable of infecting all organisms living on the earth. But this system of naming the viruses based on the symptoms became inadequate, as the number of viruses reported on plants increased rapidly indicating that a comprehensive system of nomenclature and classification for viruses was essential. The International Committee on Nomenclature of Viruses (ICNV) was established in 1966 to develop a universal taxonomy for viruses. The name of the Committee was later changed in 1973 as International Committee on Taxonomy of Viruses (ICTV) to develop systems for naming and classification of viruses. With increasing knowledge on the properties of plant viruses, they have been classified into 20 families and 71 genera, with another 17 genera yet to be assigned to appropriate families (Mayo and Brunt 2007). Viruses can be divided into two groups based on the nature of viral genome as RNA viruses and DNA viruses. The RNA viruses are divided into single-stranded (ss) + sense RNA viruses, double-stranded (ds) + sense RNA viruses and single-stranded (ss) – sense RNA viruses. The DNA viruses which are less numerous compared to RNA viruses, are divided into ss-DNA viruses and reverse transcribing ds-DNA viruses. Particle morphology and presence of membranous envelope, immunological properties and nucleotide and amino acid sequences of the capsid protein and presence of virus-associated protein are the other important characteristics used for identification, classification and differentiation of plant viruses and their strains (Table 2.1; Mayo and Brunt 2007).

2.1.2 Biological Methods

2.1.2.1 Symptomatology

The ability to infect one or more plant species and induce characteristic symptoms indicates the pathogenic potential of the virus under investigation. Obvious manifestation of symptoms of infection may not occur in certain plant species which carries the virus without any recognizable symptom in the case of latent infection. The viruses may induce macroscopic, external symptoms and also microscopic internal symptoms characteristic of infection. The severity of symptoms may vary on different cultivars of a crop plant depending on the levels of susceptibility or resistance to the virus concerned. The virus may be localized in the inoculated leaves which may exhibit chlorotic or necrotic local lesions (primary symptoms). In such plant species, the virus is restricted to the lesions and it cannot be detected in other plant parts. The number of local lesions present on the inoculated leaves reflect the viral concentration of the inoculum used for inoculation. As the local lesions are considered to be initiated by a single virus particle, the host plant species reacting with local lesions are used as diagnostic hosts for detecting and quantifying the virus concentrations. Plant species that have been employed commonly as indicator/diagnostic hosts are *Nicotiana glutinosa*, *Gomphrena globosa*, *Phaeolus vulgaris*, *Vigna unguiculata*, *Datura metel*, *D. ferox*, *Lablab niger*, and *Chenopodium amaranticolor* (Narayanasamy and Doraiswamy 2003).

Table 2.1 List of families, genera and type species of plant viruses (Mayo and Brunt 2007)

Virus genome	Family	Genus	Type species
(+) sense ss-RNA viruses	Potyviridae	*Potyvirus*	*Potato virus Y*
		Rymovirus	*Ryegrass mosaic virus*
		Macluravirus	*Maclura mosaic virus*
		Tritimovirus	*Wheat streak mosaic virus*
		Ipomovirus	*Sweet potato mild mottle virus*
		Bymovirus	*Barley yellow mosaic virus*
	Sesquiviridae	*Sesquivirus*	*Parsnip yellow fleck virus*
		Waikavirus	*Rice tungro spherical virus*
	Comoviridae	*Comovirus*	*Cowpea mosaic virus*
		Fabavirus	*Broad bean wilt virus*
		Nepovirus	*Tobacco ringspot virus*
	Luteoviridae	*Luteovirus*	*Barley yellow dwarf virus-PAV*
		Polerovirus	*Potato leafroll virus*
		Enomovirus	*Pea enation mosaic virus*
	Tymoviridae	*Tymovirus*	*Turnip yellow mosaic virus*
		Marafivirus	*Maize rayado-fino virus*
		Maculavirus	*Grapevine fleck virus*
	Tombusviridae	*Tombusvirus*	*Tomato bushy stunt virus*
		Carmovirus	*Carnation mottle virus*
		Necrovirus	*Tobacco necrosis virus A*
		Machlomovirus	*Maize chlorotic mottle virus*
		Dianthovirus	*Carnation ringspot virus*
		Aureusvirus	*Pothas latent virus*
		Panicovirus	*Panicum mosaic virus*
	Bromoviridae	*Bromovirus*	*Brome mosaic virus*
		Alfamovirus	*Alfalfa mosaic virus*
		Cucumovirus	*Cucumber mosaic virus*
		Ilarvirus	*Tobacco streak virus*
		Oleavirus	*Olive latent virus 2*
	Closteroviridae	*Closterovirus*	*Beet yellows virus*
		Crinivirus	*Lettuce infectious yellows virus*
		Ampelovirus	*Grapevine leafroll-associated virus 3*
	Flexivirdae	*Carlavirus*	*Beet yellows virus*
		Potexvirus	*Potato virus X*
		Capillovirus	*Apple stem grooving virus*
		Trichovirus	*Apple chlorotic leaf spot virus*
		Foveavirus	*Apple stem pitting virus*
		Allexivirus	*Shallot virus X*
		Vitivirus	*Grapevine virus A*
		Mandarivirus	*Indian citrus ringspot virus*
	Unassigned genera	*Tobamovirus*	*Tobacco mosaic virus*
		Tobravirus	*Tobacco rattle virus*

(continued)

Table 2.1 (continued)

Virus genome	Family	Genus	Type species
		Hordeivirus	*Barley stripe mosaic virus*
		Furovirus	*Soilborne wheat mosaic virus*
		Pomovirus	*Potato mop-top virus*
		Pecluvirus	*Peanut clump virus*
		Benyvirus	*Beet necrotic yellow vein virus*
		Sobemovirus	*Southern bean mosaic virus*
		Idaeovirus	*Raspberry bushy dwarf virus*
		Ourmiavirus	*Ourmia melon virus*
		Umbravirus	*Carrot mottle virus*
		Sadwavirus	*Satsuma dwarf virus*
		Cheravirus	*Cherry rasp leaf virus*
(−) sense ss-RNA viruses	Rhabdoviridae	*Cytorhabdovirus*	*Lettuce necrotic yellows virus*
		Nucleorhabdovirus	*Potato yellow dwarf virus*
	Bunyaviridae	*Tospovirus*	*Tomato spotted wilt virus*
	Unassigned genera	*Ophiovirus*	*Citrus psorosis virus*
		Tenuivirus	*Rice stripe virus*
		Varicosavirus	*Lettuce big-vein associated virus*
ds-RNA viruses	Reoviridae	*Phytoreovirus*	*Wound tumor virus*
		Fijivirus	*Fiji disease virus*
		Oryzavirus	*Rice ragged stunt virus*
	Partitiviridae	*Alphacryptovirus*	*White clover cryptic virus 1*
		Betacryptovirus	*White clover cryptic virus 2*
	Unassigned virus	*Endornavirus*	*Vicia fabe endornavirus*
ss-DNA viruses	Geminiviridae	*Mastrevirus*	*Maize streak virus*
		Curtovirus	*Beeet curly top virus*
		Topocuvirus	*Tomato pseudo-curly top virus*
		Begomovirus	*Bean golden mosaic virus*
	Nanoviridae	*Nanovirus*	*Subterranean clover stunt virus*
		Babuvirus	*Banana bunchy top virus*
Reverse transcribing viruses	Caulimoviridae	*Caulimovirus*	*Cauliflower mosaic virus*
		Soymovirus	*Soybean chlorotic mottle virus*
		Cavemovirus	*Cassava vein mosaic virus*
		Petuvirus	*Petunia vein clearing virus*
		Badnavirus	*Commelina yellow mottle virus*
		Tungrovirus	*Rice tungro bacilliform virus*
	Pseudoviridae	*Pseudovirus*	*Saccharomyces cerevisiae Ty1 virus*
		Sirevirus	*Glycine max SIRE 1 virus*
	Metaviridae	*Metavirus*	*Saccharomyces cerevisiae Ty3 virus*

Pepper mild mottle virus (PMMoV), formerly referred to as *Tobacco mosaic virus*-pepper strain, is transmitted through seeds of pepper (chilli). The infected seeds are homogenized and the extracts are inoculated onto the leaves of *Nicotiana glutinosa*, the indicator plant species for PMMoV. Countable necrotic local lesions develop on the leaves within 2 days after inoculation. *N. glutinosa* is used for detection and also for quantification of PMMoV after chemical treatment or dry sterilization of seeds to assess the effect of treatment on the infectivity of PMMoV in the seeds (Toyoda et al. 2004). An indicator method using sugar beet roots was adopted for the detection of *Tobacco rattle virus* (TRV) infecting sugar beets. The infected roots are stored in sterilized sand for 5 months in winter and then parts of these roots are allowed to grow 2-year flowering plants. Symptoms develop in the leaves as yellow mosaic. The racemes formed in these plants are tested by immunoassays for the presence TRV (Dikova 2005).

When the virus becomes systemic, leaves and other organs away from the site of infection (inoculation) develop secondary symptoms which may be grouped as color changes, teratological symptoms, necrosis and abnormal growth forms. Mosaic patterns, chlorosis, leaf roll, leaf curl, enations, galls and tumors are some of the secondary symptoms induced by viruses, in addition to general stunting and deformation of leaves and other organs of the infected plants (Figs. 2.1–2.3). The external symptoms may provide a clue as to the nature of the causative agent. The absence of fungal spores or bacterial ooze may be the basis for excluding fungal or bacterial pathogen(s) as the possible cause(s) of the disease under investigation. Symptoms due to nutritional deficiency may show some similarities to mosaic or mottling symptoms due to virus infection. Restoration of normal foliage color, when the infected plants are planted in soils with adequate nutrients will indicate the nature of the cause of the disease.

Internal symptoms induced by viruses may be of anatomical and cytological changes in infected tissues. Of these changes, production of virus-specific intracellular inclusion bodies is characteristic of virus infection and the inclusion bodies have diagnostic value. Members of 20 virus groups are known to induce the formation of inclusion bodies which constitute one of the principal characteristics of the respective virus groups. These inclusions are useful for diagnostic and taxonomic purposes and for identification and characterization of virus-specific non-capsid proteins and possible sites of virus synthesis (Hiebert et al. 1984). The presence of intracellular inclusions in virus-infected plant tissues can be detected by using stains such as trypan blue, phloxine and a combination of pyronin and methyl green or Azure A and orange-green. Details of these techniques have been described in the earlier publication (Narayanasamy 2001). Polyclonal antisera against inclusion bodies induced by viruses belonging to the genera *Potyvirus* and *Caulimovirus* have been produced. The antisera produced against nonstructural virus-associated proteins were found to be useful for the detection of homologous viruses in different host plants (Falk et al. 1987; Hammond 1998). The epidermal strips from leaves and stems of squash and watermelon plants infected by *Squash vein yellowing virus* (SqVYV) were stained with Azure A (AA) and Luxol Brilliant Green (OG). By using the light microscope, cylindrical inclusions were seen in the SqVYV-infected watermelon and squash along the plasmalemma of the cell wall of epidermal tissue stained

Fig. 2.1 Symptoms of peanut bud necrosis disease (Courtesy of Dr. P. Narayanasamy)

Fig. 2.2 Symptoms of rice tungro disease (Courtesy of Dr. P. Muthulakshmi, Tamil Nadu Agricultural University, Coimbatore, India)

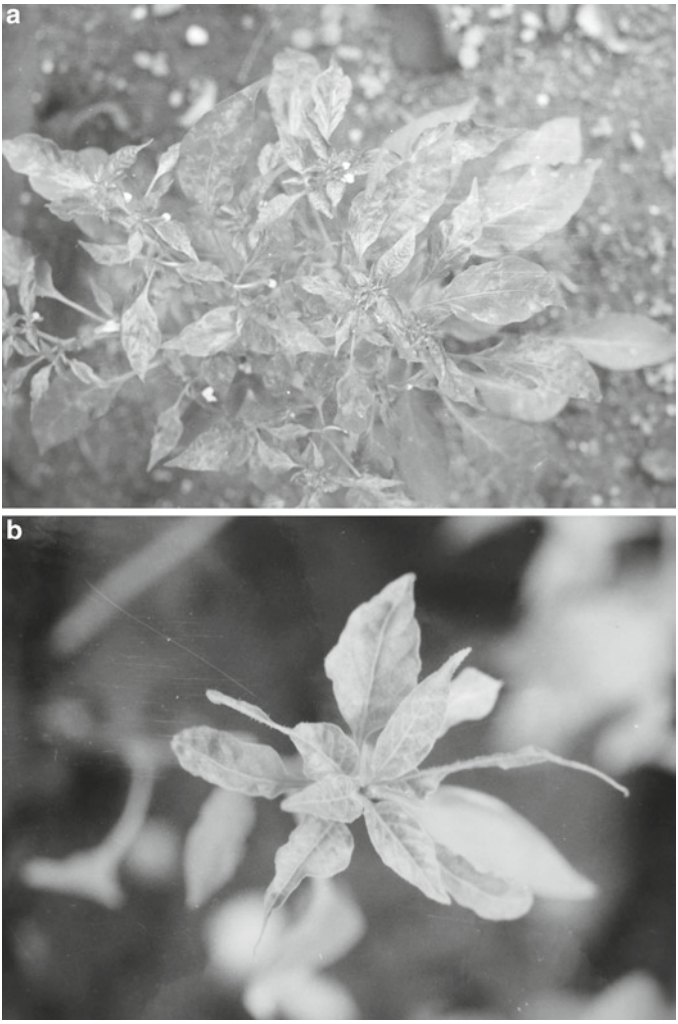

Fig. 2.3 (**a**, **b**) Symptoms of pepper mosaic disease (Courtesy of Dr. P. Narayanasamy)

with OG. These inclusions were not observed in epidermal tissue stained with AA. However, additional inclusions that stained with OG and light pink with AA were observed in vascular tissue, indicating the presence of protein and RNA respectively (Fig. 2.4; Adkins et al. 2007).

2.1.2.2 Mode of Transmission

Plant viruses differ from fungal and bacterial pathogens in the methods of dissemination/transmission from infected plants to healthy plants for infection

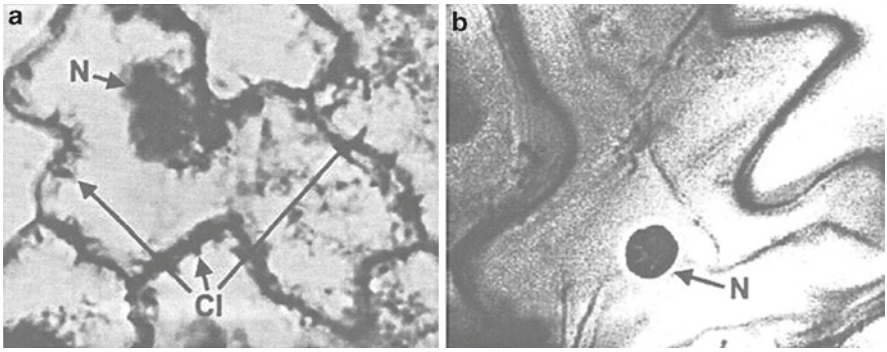

Fig. 2.4 Detection of infection by *Squash vein yellowing virus* (SqVYV) by light microscopy using stains. (**a**) Virus-induced inclusions in squash leaf strip from SqVYV-infected plant stained with Orange-Green (OG); (**b**) watermelon leaf strip (uninfected); N, nucleus. Note the presence of viral inclusions (CI) in infected leaf strip (**a**) but not in uninfected leaf strip (**b**) (Courtesy of Adkins et al. 2007; The American Phytopathological Society, MN, USA)

to be initiated. Infection by viruses cannot occur through unwounded plant surfaces. In contrast, fungi and bacteria can infect the plants when they reach a suitable host plant by producing enzymes or toxins that aid in the penetration of or ingestion through plant surfaces. Plant viruses have to be introduced and deposited inside the susceptible cells of host plants by grafting, budding or mechanical inoculation (under experimental conditions or normal nursery practices adopted for propagation) or through parasitic dodders. In a few cases, natural root grafts may be possible, when the roots of infected and healthy plants come together, providing the avenue for the entry of the virus into healthy plants. Abrasion of leaf surface following leaf contact and contamination of agricultural implements or tools with stable viruses may be responsible for the transmission of some viruses under field conditions. The most common method of transmission of plant viruses occurs through the agency of different kinds of vectors such as insects, mites, fungi and nematodes that acquire the virus(es) while feeding on infected plants and transmit, when they feed on healthy plants.

Grafting and Budding

Common horticultural methods of propagation may be sufficient for transmission of a virus by inserting a scion tissue or bud from infected plants into healthy test plant. Most of the viruses infecting dicot plants have been successfully transmitted to healthy plants experimentally. However, special techniques may be necessary for the transmission of some viruses. A leaf-grafting technique was adopted for the transmission of grapevine viruses (Kuniyuki et al. 1998). Indexing mother plants and planting materials for the presence of viruses is a general practice adopted for certification and quarantine purposes. Indexing by graft inoculation of indicator

plants was found to be an effective method for detection and identification of viruses and their strains, as in the case of *Plum pox virus* (Fuchs et al. 1995) and *Apple stem pitting*, *stem grooving* and *vein yellowing viruses* (Ramel et al. 1998). A clone of *Malus micromalus* (GMAL 273a) exhibited diagnostic foliage symptoms when graft-inoculated with apple viruses. This indicator plant showed positive reaction within 2–4 weeks, while a period of 6–8 months was required by the cultivar Virginia Crab (*M. domestica*) which was earlier used as a bioindicator (Howell et al. 1996). *Prunus tomentosa* (hybrid selection IR 473 × IR 474) produced diagnostic symptoms following graft-inoculation with *Plum pox virus* (PPV) and several *Prunus* viruses. *P. tomentosa* was shown to be useful for maintenance of stock cultures of *Prunus* viruses in the glasshouses. The strains M and D of PPV could be differentiated based on the type of symptoms induced on *P. tomentosa* (Damsteegt et al. 1997, 1998). In a later investigation, the peach seedling GF 305 was shown to be a more sensitive host plant for detection of PPV, because of its very high susceptibility to the virus than *P. tomentosa* (Desvignes et al. 1999).

Grapevine viruses such as *Grapevine fan leaf virus* (GFLV), *Grapevine fleck virus* (GFV), *Grapevine asteroid mosaic-associated virus* (GAMaV) and *Rupestris stem pitting-associated virus* (RSPaV) can be detected by graft-inoculating *Vitis rupestris* St. George. *V. vinifera* cv. Cabernet Franc is reported to be suitable for detecting *Grapevine leafroll-associated viruses* (Rowhani et al. 2005). In certification schemes, biological testing seems to be the most reliable and practical method that is being used to test propagating materials. However, biological testing (indexing) program needs dedicated facilities in terms of large greenhouse space provided with the required climatic conditions, cold chamber, seeds and supplies (Gentit 2006).

Mechanical Transmission

The stability of the virus in the sap extracted from virus-infected plant tissues determines the ability to be transmitted by mechanical (sap) inoculation on the susceptible leaves of healthy plants. Viruses such as *Tobacco mosaic virus* (TMV), *Potato virus X* (PVX) and *Potato virus Y* (PVY) are easily transmitted by mechanical inoculation, whereas the unstable virus *Tomato spotted wilt virus* (TSWV) requires special condition for successful transmission. Ice-tray method was shown to be effective in transmitting the yellow mosaic virus infecting leguminous plant *Lablab niger* (Subramanian and Narayanasamy 1978). Sap transmissible viruses like *Grapevine fan leaf virus* infecting grapevine, have been indexed on assay hosts such as *Chenopodium quinoa*, *C. amaranticolor*, *Nicotiana clevelandii* and *Cucumis sativus.* Likewise, members of the genera *Nepovirus*, *Ilarvirus* and *Trichovirus* infecting *Prunus* spp. have been detected by sap inoculation to *C. quinoa*, *Nicotiana occidentalis*, *N. benthamiana* and *C. sativus* (Rowhani et al. 2005). Several factors such as virus concentration, physiological conditions of test plants, use of abrasives and the environmental conditions in which donor and receptor plants are grown may influence the transmission of viruses by mechanical inoculation. In the case of TMV and PVX, mechanical transmission may be possible through direct leaf contact

and contaminated implements/tools during agricultural operations. *Blueberry leaf mottle virus* (BBLMV) was reported to be transmitted mechanically through pollen present on the legs of bees that visit the flowers for the nector. The presence of BBLMV could be detected by enzyme-linked immunosorbent assay (ELISA) test (Childress and Ramsdell 1987).

Biological indexing is essential for viruses like *Citrus psorosis virus* (CPsV) that spread primarily by propagation of infected buds. CPsV has no known vector that can spread the disease under natural conditions. The first certification program in California was based on biological indexing of budwood sources (Hildbrand 1957). Indexing is performed by grafting tissues to sensitive young seedlings of sweet orange (*Citrus sinensis*) which develop characteristic symptoms. Budsticks taken from glasshouse-grown trees were used to graft-inoculate two Pineapple sweet orange and two *C. excelsa* seedlings. CPsV was detected in 41 of 47 trees tested. The indicator plants showed characteristic symptoms (Martin et al. 2002b). Isolates of *Zucchini yellow mosaic virus* (ZYMV) infecting cucurbits could be detected and differentiated based on type of symptoms induced by them on nine host plant species which produced local lesions or systemic symptoms (Table 2.2; Bananej et al. 2002). *Cucumber mosaic virus* (CMV) isolates associated with Banana mosaic disease (BMD) were grouped into four pathotypes based on the symptoms induced in banana, *Nicotiana glutinosa* and cowpea. CMV strains for the banana symptom types also induced recognizable symptoms in *N. glutinosa*, suggesting that CMV strains within each BMD symptom group share similar biological properties in inducing symptoms in both banana and *N. glutinosa* (Table 2.3; Chou et al. 2009).

The physical properties of the viruses in the expressed sap, such as dilution end point (DEP), thermal inactivation point (TIP) and stability in vitro have been determined under laboratory conditions for sap-transmissible viruses. A large number of

Table 2.2 Variations in symptoms induced in different plant species by *Zucchini yellow mosaic virus* isolates (Bananej et al. 2002)

	Isolate number					
Plant species	A[a]	2	3	4	5, 8	6
Cucumis melo	M[b]	M, Y	M, Y	M, Y	M	M
Cucurbita sp.	M	M	M, SY	M	M	M
Cucumis sativus	M	M	M, Y	M	M	M
Cucurbita pepo	M	M	M, Y	M	M	M, Y
Chenopodium amaranticolor	NL	NL	NL	NL	NL	NL
Gomphrena globosa	O	NL	NL	O	NL	O
Phaseolus vulgaris	O	O	O	O	O	O
Vicia faba	O	O	O	O	O	O
Vigna unguiculata	O	O	O	O	O	O

[a] A represents isolates 1, 7, 9, 10, 11, 12 which induce identical symptoms on the host plant species tested

[b] M mosaic, Y yellowing, NL necrotic local lesions, SY severe yellowing
O, no visible symptoms

Table 2.3 Identification of pathotypes of *Cucumber mosaic virus* (CMV) based on symptoms on different host plants (Chou et al. 2009)

Pathotype	CMV isolates	Banana symptom Type[a]	Ng symptom type[b]	Cowpea symptom type[c]
I	16	I-s	A	L
	20	I-s	A	L
	21	I-d	A	L
	22	I-s	A	L
	24	I-s	A-f	L
	25	I-s	A	L
	29	I-d	A	L
II	28	I-s	A	S
III	7	II	B	L
IV	31	III	C	S

[a] I-d, severe green mosaic with deformed leaves; I-s, with slender leaves; II, yellow mosaic with necrosis; III, severe green mosaic only

[b] Ng, *Nicotiana glutinosa*; Am, severe green mosaic with puckering or leaf distortion; B, with leaf necrosis; C, mild mottling

[c] L, local lesions; S, systemic symptoms

plant viruses for which these physical properties have been determined, is listed in the earlier publication (Narayanasamy 2001). Though the usefulness of the physical properties is limited, they may be useful to develop purification procedures suitable for different viruses. Mechanical inoculation has been adopted to locate the presence of target virus in different fractions during the purification of the virus concerned.

Assay hosts/indicator plants that react with formation of countable local lesions are used for detection and quantification of the target virus in different inocula prepared from leaves at different positions, stems, roots, floral parts and parts of seeds. The number of local lesions indicates the difference in virus titers (concentrations) in different plants. The effects of environments, chemicals and antiviral principles (AVPs) on the virus infectivity may also be determined by using assay hosts (Narayanasamy and Ganapathy 1986).

Transmission by Vectors

Plant viruses are dependent on vectors for transmission from infected plants to healthy plants under natural conditions, although they can be experimentally transmitted by grafting/budding or mechanical inoculation. Viruses are transmitted by various kinds of vectors such as insects, mites, nematodes and fungi. Based on the nature of relationship between the virus and its vector, they may be grouped into three categories: nonpersistent (mechanical), semipersistent (circulative) and persistent (propagative) viruses. The nonpersistent viruses can be acquired and transmitted by vector insect after a short acquisition access period (lasting for a few minutes) on

infected source plants followed by similar short inoculation access period on a healthy plant. Inoculativity of the vector of nonpersistent virus is retained for short period (less than 24 h) and after this period the vector cannot transmit the virus, unless it is reacquired again from an infected plant. Most of the plant viruses transmitted by aphids have nonpersistent relationship with their vectors. Semipersistent viruses require long periods (several hours) of feeding on the infected source and healthy plants for acquisition and transmission respectively by their vectors. After the acquisition of the virus, a definite period of time (incubation/latent period) has to lapse before the vector is able to transmit the virus to a healthy plant. These viruses are transmitted by leafhoppers or aphids. Persistent viruses are transmitted by their vectors after long acquisition feeding period and completion of incubation period. These vectors may retain their infectivity till their lifetime. In some cases, the viruses may be transmitted to subsequent generations through the eggs (transovarial transmission). The type of relationship between the virus and vector and vector specificity may help in the putative identification of plant viruses. However, these procedures require long time and are cumbersome, although they provide the basic information on the biological characteristics of plant viruses (Narayanasamy 2001; Narayanasamy and Doraiswamy 2003).

2.1.2.3 Cross-Protection

The phenomenon of cross-protection of plants infected by one virus against infection by its strains or related virus has been shown to be useful for establishing the relationship between the strains or related viruses. Cross-protection has been demonstrated to be an effective approach for the management of some virus diseases (Narayanasamy 2001, 2005). The development of cross-protection has been studied at molecular level by employing enzyme-linked immunosorbent assay (ELISA) and Western blotting techniques. The soilborne *Beet mosaic virus* protects beet plants against *Beet necrotic yellow vein virus* (BNYVV). The accumulation of coat protein (CP) of the challenging virus BNYVV was affected, as the level of cross-protection increased. The CP of BNYVV could not be detected by ELISA in the protected beet plants, although the RNA of BNYVV could be detected by reverse transcription (RT)-polymerase (PCR) technique. This study suggests that replication of the challenging virus/strain may be affected, if the CP synthesis is reduced or prevented by the protecting (challenged) virus (Mahmood and Rush 1999).

The efficacy of three mild isolates of *Citrus tristeza virus* (CTV) in protecting grapefruit cv. Ruby Red on sour orange root stock was assessed by visual observations and ELISA tests at 16 years after inoculation with mild isolates. The mild strain DD102 provided better protection against the severe strain to grapefruit trees (Powell et al. 1999). The incidence of decline due to CTV in Kagzi lime trees could be reduced by cross-protection by inoculating the plants with mild strain (Chakraborty and Ahlawat 2001). Specific diagnosis for *Citrus psorosis virus* (CPsV) required a cross-protection test to be performed. Psorosis B was used as challenge inoculum to demonstrate cross-protection against psorosis A in the

protected citrus plants. Healthy sweet orange seedlings inoculated with psorosis B exhibited the characteristic symptoms within 6 months, whereas plants already infected with psorosis A were protected and did not show the symptoms due to psorosis B. Cross-protection against psorosis B was observed in 41 of 47 citrus plants as revealed by symptom observations (Martin et al. 2002a).

2.1.3 Morphological Characteristics

Virus particle morphology can be studied only by employing the electron microscopes, because the size of the plant viruses is below the resolution power of light microscopes. It has been possible to study the ultrastructure of biological materials such as individual cells and molecules such as proteins, nucleic acids, nucleoproteins whose structure could be determined earlier only through biochemical analyses. Morphological characteristics of plant viruses and that of intracellular inclusions containing virus particles have been studied by viewing the purified virus preparations or ultrathin sections of infected plant tissues. Presence of virus particles in the tissues of natural vectors also has been detected by electron microscopy. *Clover yellow mosaic virus* was detected in the extracts of individual local lesions induced by the transcripts of a full-length complementary DNA (cDNA) copy of the virus (Holy and Abou Haidar 1993). By using the electron microscope, virus particles have been observed in the extracts from plants to be indexed. *Peach mosaic virus* (PMV) was detected in the extracts from symptomatic leaves of *Chenopodium amaranticolor* mechanically inoculated with PMV. Confirmatory results were obtained by Western blot analysis using polyclonal antibodies (Gispert et al. 1998). Furthermore, electron microscopy has been used extensively to track the viruses during the process of purification and to study the structural features of purified virus particles. Various techniques for preparing the purified virus preparations and ultrathin sections of plant tissues infected by viruses are described in detail in an earlier publication (Narayanasamy 2001).

2.1.3.1 Negative Staining

The virus preparations are stained with 2% aqueous solutions of sodium or potassium phosphotungstate (PTA), adjusted to pH 7.0 with NaOH or KOH. Floating the grids with adsorbed virus particles on a drop of 0.1% glutaraldehyde for 5 min before staining with PTA may reduce the damage to the virus particles due to PTA (Milne 1984). The use of negative stains for high resolution and good particle preservation was recommended by Milne and Lesemann (1984). For viruses such as *Alfalfa mosaic virus* and *Tomato spotted wilt virus* which are labile in PTA, 2% ammonium molybdate at pH 6–7 can be used for staining the virus particles (Roberts 1981). *Soybean dwarf virus* (SbDV) strains – dwarfing (SbDV-D) and yellowing (SbDV-Y) – were examined under the electron microscope. The purified preparations were stained with 2% uranyl acetate. Isometric particles with similar morphology and mean diameters

of 25–26 nm were observed. High concentrations of virions were present in a few phloem sieve elements and companion cells of vascular bundles, but not in xylem parenchyma or leaf mesophyll cells of diseased plants (Damsteegt et al. 1999).

Blackcurrant reversion-associated virus (BRAV) belonging to the genus *Nepovirus* and family *Comoviridae* causes two forms of blackcurrant reversion disease, the common (European, E-form) and severe (Russian, R-form). Electron microscopic observations revealed the presence of *Rhabdovirus*-like particles in ultrathin sections of flower stalks, measuring 200–347 × 64–90 nm. These particles were present mostly within the nucleus of parenchyma cells of vascular bundles as single particles, rafts of particles and aggregates (Fig. 2.5; Přibylová et al. 2002). *Apricot latent virus* (ApLV), a definitive species in the genus *Foveavirus* and family *Flexiviridae* was detected by using electron microscopy. The presence of filamentous virus particles scattered in the cytoplasm of cells of infected *Nicotiana occidentalis* plants (Ghanem-Sabanadzovic et al. 2005). A new carmovirus infecting Angelonia plants (*Angelonia angustifolia*) was examined under the electron microscope. Isometric particles were observed in leaf dips and also in purified virus preparations and in thin sections. The virus was identified as *Angelonia flower breaking virus* (AnFBV) belonging to the genus *Carmovirus* (Adkins et al. 2006). A new virus infecting chickpea and faba bean causing chickpea chlorotic stunt disease was purified. Electron microscopic observations of the purified preparations revealed the presence of isometric particles measuring 28 nm with smooth surface and slightly hexagonal outline (Fig. 2.6). The virus was designated *Chickpea chlorotic stunt virus-FB* (CpCSV-FB) (Abraham et al. 2006).

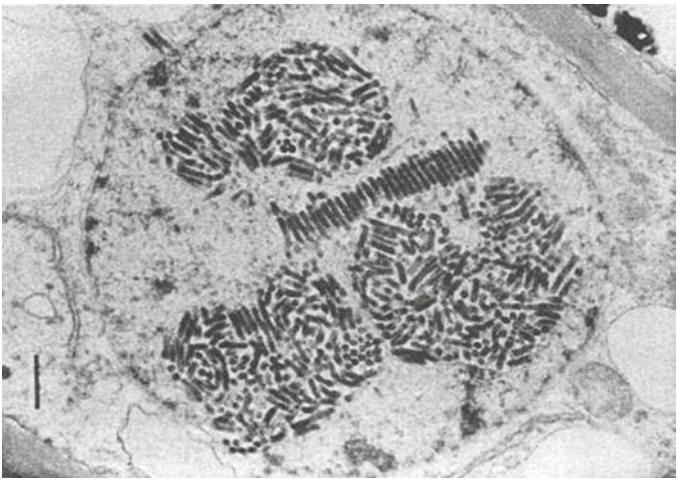

Fig. 2.5 Detection of rhabdovirus-like particles in blackcurrant plants showing reversion disease symptoms by electron microscopy. Note the presence of membrane-associated rhabdovirus-like particles forming rafts or dispersed singly in the nucleus and in perinuclear space of parenchyma cells of vascular bundle; bar = 500 nm. (Courtesy of Přibylová et al. 2002; Acta Virologica, Bratisalva, Slovak Republic)

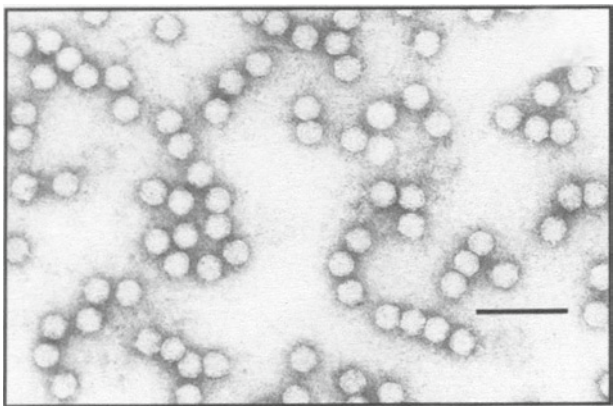

Fig. 2.6 Detection of isometric particles of *Chickpea chlorotic stunt virus* (CpCSV)-FB in purified preparations (bar = 100 nm) (Courtesy of Abraham et al. 2006; The American Phytopathological Society, MN, USA)

Samples of crown, stem and leaves of *Squash vein yellow virus* (SqVYV)-infected and mock-inoculated squash and watermelon plants were examined under the electron microscope. Pinwheel-like inclusion bodies, typical of the family *Potyviridae* were observed in the thin sections. Cylindrical inclusions were seen at the cell periphery attached to the plasmalemma along the cell wall of SqVYV infected squash tissues. All inclusions were present in the phloem parenchyma and companion cells (Adkins et al. 2007).

Two new viruses infecting *Phalaenopsis* orchids were investigated to establish their identities. Ultrathin sections of infected leaves of *Phalaenopsis* from fields and inoculated leaves of *Nicotiana benthamiana* and *Datura stramonium* with isolate 91-orchid-1 fixed in 2% glutaraldehyde and stained with 2% uranyl acetate were examined under the electron microscope. Roughly spherical enveloped virions measuring 70–100 nm in diameter were observed. The morphology of observed particles was similar to the tospovirus. The tospovirus-like particles were present also in *N. benthamiana* and *D. stramonium* inoculated with this virus isolate (Fig. 2.7). Based on immunological properties and sequence analysis, the virus was identified as an isolate of *Capsicaum chlorosis virus* (CaCV) known to infect tomato and capsicum plants in Australia and Thailand and designated *Capsicum chlorosis virus-Ph* (CaCV-Ph) (Zheng et al. 2008b). Another isolate causing chlorotic spots in *Phalaenopsis* orchids was also studied using electron microscope. The ultrathin sections of infected leaves of Phalaenopsis and inoculated leaves of *Chenopodium quinoa* and *N. benthamiana*, processed as mentioned above, were examined. Long flexuous filamentous particles measuring about 12–15 × 750–800 nm were observed in all infected tissues. In addition, the presence of cylindrical inclusions appearing as pinwheels characteristic of potyviruses was also

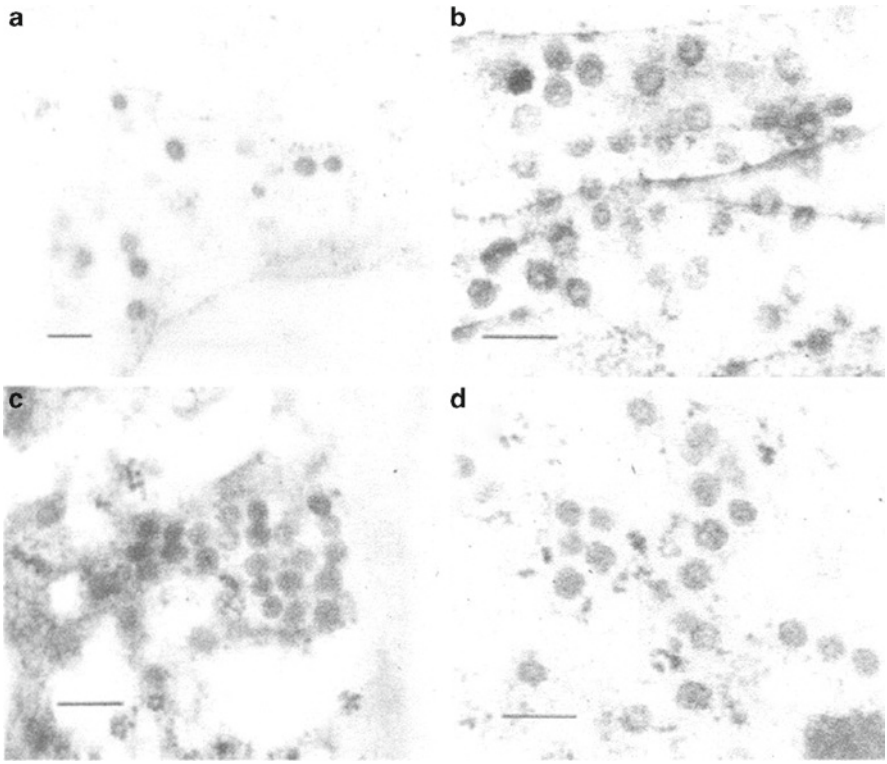

Fig. 2.7 Detection of tospvirus particles in *Capsicum chlorosis virus* (CaCV)-infected plant species by electron microscopy. (**a**) chlorotic spots with concentric rings in the leaves of *Phalaenopsis* orchid; (**b**) *Nicotiana benthamiana*; (**c**) *Datura stramonium* and (**d**) *Phalaenopsis* orchid plant (Courtesy of Zheng et al. 2008b and with kind permission of Springer Science and Business Media, Heidelberg, Germany)

seen in the infected cells (Fig. 2.8). Results of immunoassays and molecular cloning and sequence analysis confirmed that the isolate 7–2 was a potyvirus. The virus was named as *Phalaenopsis chlorotic spot virus* (PhCSV) (Zheng et al. 2008a).

2.1.3.2 Dip Method

The dip method is simple and useful for spot-checking the presence of the target virus rapidly. A freshly cut leaf surface or an epidermal strip is passed through the surface of a drop of water placed on a glass slide, facilitating the flow of cellular contents into the drop of water. Particulate materials such as the virus particles form a thin film over the surface of the water drop. A filmed grid is then touched to the surface water drop and negatively stained. Alternatively, the cut leaf may be passed directly through a drop of negative stain placed on the grid. The grid is then drained

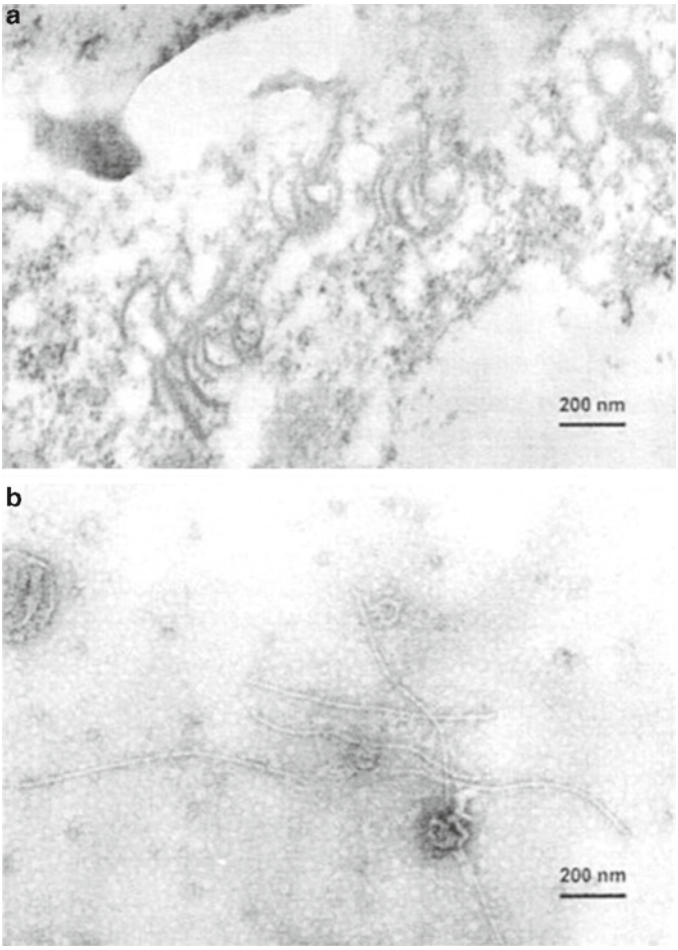

Fig. 2.8 Detection of *Phalaenopsis chlorotic spot virus* (PhCSV) by electron microscopy. (**a**) Pin wheel inclusion bodies characteristic of poytviruses in ultra-thin sections of PhCSV-infected *Nicotiana benthamiana* plants; (**b**) long flexuous filamentous particles in the crude sap of PhCSV-infected *Chenopodium quinoa* plants (Courtesy of Zheng et al. 2008a and with kind permission of Springer Science and Business Media, Heidelberg, Germany)

of the excess fluid and dried. Dip preparation is useful for rapid identification of viruses as in the case of watermelon infected by a strain of *Tomato spotted wilt virus* (Honda et al. 1989). A new *Potato virus Y* strain infecting chickpea was detected by examining leaf-dip preparations under the electron microscope. Long slightly flexuous particles ca. 760 nm in length, characteristic of *Potyvirus*, were observed, providing a clue to the identity of the causal virus which was confirmed by enzyme-linked immunosorbent assay (ELISA) (Larsen et al. 2003).

2.1.4 Physical and Biochemical Techniques

Proteins and nucleic acids are major components constituting virus particles. During the process of virus purification from plant extracts or suspensions, several cycles of centrifugation/ultracentrifuation are performed to separate contaminant particulate materials progressively to have higher concentrations of the virus particles. Plant virus suspensions in appropriate buffers are layered into buffered density gradients of sucrose formed in a tube. Separation of components of suspension takes place over a period of several hours in a zonal density gradient. Methods based on the principles of electrophoresis, use of pH gradients or paper curtains have also been employed for purification of *Southern bean mosaic virus* and for separation of *Tobacco mosaic virus* strains. In the case of unstable viruses like *Citrus infectious variegation virus*, electrophoretic technique is especially valuable (Narayanasamy and Doraiswamy 2003).

Virus infection induces several changes in physiological activity of the host plants, although the viruses themselves do not have any physiological functions. Hence, by studying variations in the physiological functions of healthy and virus-infected plants, infection by viruses can be detected. Virus infection could be detected by differences in the isozyme patterns of healthy and infected leaves determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) technique. Eight dominant chitinase izozymes were detected in tobacco extracts. One of the isozymes was present only in the *Tobacco mosaic virus* (TMV)-infected leaves, while another isozyme was present in significantly higher concentration in TMV-infected leaves than in mock-inoculated leaves (Pan et al. 1991). Induction of synthesis of several proteins both structural and nonstructural proteins of the virus is observed in several virus-infected plants. Presence of a unique protein band (32–34 kDa) in the leaves infected by *Wheat yellow head virus* (WYHV) was detected by employing SDS-PAGE technique. This protein band was absent in the extracts of comparable healthy plants. The amino acid sequences of this protein was most closely related to the nucleoprotein of *Rice hoja blanca virus*, suggesting that WYHV might belong to tenuivirus group (Seifers et al. 2005).

The SDS-PAGE technique has been shown to be useful in determining the physical properties of the purified viruses that may indicate the identity of the virus and its strains. The molecular mass of the coat protein (CP) in purified preparations of *Peanut chlorotic streak virus* were determined to be 51 and 58 kDa by applying SDS-PAGE technique (Reddy et al. 1993). The relative molecular mass of the CP of *Apricot latent virus* was determined by using SDS-PAGE procedure. The dissociated CP migrated as a single band with an estimated size of about 50 kDa (Ghanem-Sabanadzovic et al. 2005). Likewise, the molecular masses of the dissociated nucleoproteins (NPs) of *Calla lily chlorotic spot virus* (CCSV), *Tomato spotted wilt virus* (TSWV), *Impatiens necrotic spot virus* (INSV) and *Watermelon silver mottle virus* (WSMoV) were determined by SDS-PAGE procedure. The differences in this property could be used to detect and differentiate these viruses (Lin et al. 2005). *Chickpea chlorotic stunt virus-FB* (CpCSV-FB) was purified using sucrose density

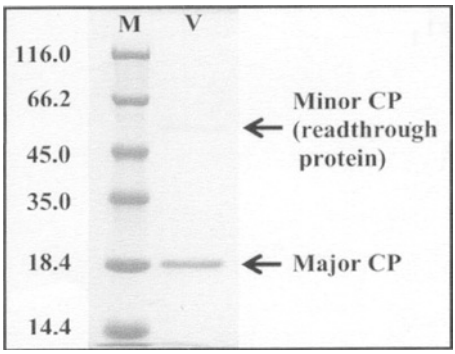

Fig. 2.9 SDS-PAGE analysis of purified preparations of *Chickpea chlorotic stunt virus* (CpCSV). Note the presence of a major (20 kDa) and a minor (50 kDa) coat protein (CP) fractions. M, marker proteins with different molecular masses (Courtesy of Abraham et al. 2006; The American Phytopathological Society, MN, USA)

gradient in which virus sedimented as a single component. SDS-PAGE analysis of purified virions produced a strong and a faint protein bands with molecular mass of ≈20 and 50 kDa corresponding to the major CP and the presumably proteolytically degraded readthrough protein respectively (Fig. 2.9; Abraham et al. 2006).

The CP gene of a virus may be expressed in the bacterium *Escherichia coli*. SDS-PAGE technique is employed to purify the recombinant protein expressed in *E. coli* and the purified protein is used for generating antibodies specific to the target virus. The antisera specific to *Bean golden mosaic virus* Brazil isolate (BGMV), *Cabbage leaf curl virus* (CabLCV), *Tomato yellow leaf curl virus* (TYLCV) and *Tomato mottle virus* (ToMoV) were produced using the CP protein purified by SDS-PAGE technique (Abouzid et al. 2002). Tricine-SDS-PAGE system was employed for the purification of the CP of *Grapevine leaf roll-associated virus-8* (GLRaV-8) with a MW of 37 kDa which was found to be serologically distinct from the CP of GLRaV-1 by Western blot analysis (Monis 2000). The bacterially expressed (*E. coli*) CP of *Faba bean necrotic yellows virus* (FBNYV) moved as a distinct band (approximately 23 kDa). This protein when injected in the animal system generated specific polyclonal antibodies (PAbs) against FBNYV (Kumari et al. 2001). The CP gene sequence of *Citrus psorosis virus* (CPsV) was expressed in *E. coli* BL21 as a fusion protein with maltose binding protein. The recombinant protein was purified using affinity chromatography. The fractions were checked for the presence of recombinant protein by SDS-PAGE in 10% gel and stained with Coomassie brilliant blue (Loconsole et al. 2006). The purified recombinant fusion N protein of *Tomato spotted wilt virus* (TSWV) was analyzed by SDS-PAGE technique followed by Western blot analysis. This procedure confirmed that the recombinant protein was successfully expressed in *E. coli* BL21 strain and it could be used for the production of PAbs (Wu et al. 2009).

2.1.5 Immunoassays

Immunoassays have been demonstrated to be useful for detection, identification, quantification and differentiation of microbial pathogens in general and viruses in particular. Because of the structural simplicity, antisera specific to plant viruses can be prepared more easily compared to fungal and bacterial pathogens that have complex antigens whose nature may vary depending the stage of development in their life cycle. The possibility of developing immunoassays for the detection of plant viruses was indicated much earlier than for bacterial and fungal pathogens. The exquisite specificity and sensitivity of mammalian and avian immune systems have been effectively and extensively used for the development of antisera containing antibodies specific to the target viruses. When the virus preparation is injected into an animal, the immune system responds by producing antibodies that specifically recognize and bind to specific sites on viral coat protein known as epitopes. The molecular viral protein used to induce the production of antibodies is called as immunogen and the molecules recognized by the antibodies are designated antigens. The lymphocytes circulating in the blood secrete antibodies in response to the presence of an immunogen and the cell-free component of the blood containing antibodies is the antiserum.

A number of antibodies may be induced, when a purified virus preparation is injected into an animal system and these antibodies may recognize different binding sites on the same virus protein molecule. But some of the binding sites or epitopes may be similar to those that are present on similar or unrelated molecules. Such antibodies are said to show cross-reaction. Hence, they are considered to be nonspecific to the antigen concerned. On the other hand, if all epitopes recognized by the antibodies are present on only the immunogen employed for the production of the antibodies, then the antibodies are termed monospecific. The antiserum containing monospecific antibodies is the most useful for the detection of the target virus and diagnosis of the disease caused. Plant viruses generally induce a highly specific response in immunized animal system and the resulting antisera can be expected to provide specific and sensitive detection of the target virus.

The advent of hybridoma technology introduced by Kohler and Milstein (1975) has been hailed as a remarkable milestone in the process of antibody production that overcomes many problems associated with polyclonal antibodies (PAbs). This technology offers the principal advantage of obtaining an unlimited supply of monoclonal antibodies (MAbs). Hybridomas are somatic cell hybrids formed by the fusion of ß-lymphocytes (antibody producing cells) with myeloma cells (capable of indefinite multiplication). The hybrids possess the ability to produce a specific antibody from the lymphocyte and to be cultured indefinitely in vitro from the myeloma cells. Each hybridoma clone produces identical antibodies that are specific for a single epitope of the immunogen. The details of PAb and MAb production are available in the earlier publications (Narayanasamy 2001, 2005). The structure and characteristics of antibodies have been discussed (Volume 1, Chapter 2).

A novel method of producing antisera has been developed and it will be useful when purified viruses or virus proteins are not available. The coat protein (CP) gene of the target virus is cloned and expressed in *Escherichia coli* cells and the polypeptide(s) produced in the bacterial cells are used as immunogen for production of polyclonal antibodies (PAbs) in rabbits. PAbs specific to *Tomato spotted wilt virus* (TSWV) (Vaira et al. 1996), *Beet necrotic yellow vein virus* (BNYVV) (Li et al. 1996), *Garlic virus A* (Gar V-A) (Helguera et al. 1997) and *Potato virus Y* (Arabatova et al. 1998) have been generated and employed for the detection of the respective viruses. Another efficient method of producing antibodies was developed for the detection of luteoviruses which reach only low concentrations and are difficult to purify. Antibodies specific to the coat protein (22 kDa) of *Barley yellow dwarf virus* (BYDV-PAV) were generated by cloning a cDNA into a mammalian expression vector (pcDNA 22K) entrapped in liposomes followed by intramuscular injection into BALB/c mice. The antibody titers of the mouse serum were substantially increased by giving a booster injection of DNA-immunized mice with purified BYDV-PAV strain (Pal et al. 2000).

A significant improvement in the production of antibodies specific for plant viruses was provided by the in vitro production of recombinant antibodies using the phage display libraries of antibody genes expressing scFv fragments. A panel of recombinant single-chain antibodies (scFvs) against structural proteins of plant virus (*Tomato spotted wilt virus*) can be retrieved from a human combinatorial scFv antibody library using the novel phage display technique. The DNA from antibody-producing ß-lymphocytes or hybridomas is genetically linked to the gene 3 DNA of the M13 phage. When the M13 phage carrying the gene fusion infects *Escherichia coli*, the proteins encoded by the antibody DNA and the gene 3 DNA are coexpressed in the bacterial cells. Any antibody DNA linked to the phage DNA and any antibody protein fused to the phage proteins will be assembled and secreted in the same manner as the phage. Recombinant antibodies have been produced using phage display technology for the detection of *Potato leaf roll virus* (Harper et al. 1997; Toth et al. 1999), *Potato virus Y* (Boonham and Barker 1998), *African cassava mosaic virus* (Zeigler et al. 1998), *Potato virus A* (Merits et al. 1998), *Cucumber mosaic virus* Fny strain (He et al. 1998), *Tobacco mosaic virus* (Fischer et al. 1999), *Tomato spotted wilt virus* (Griep et al. 2000) and *Citrus tristeza virus* (Terrada et al. 2000). Recombinant polyclonal antibodies (PAbs) to *Potato virus X* CP were demonstrated to be very efficient in detecting the virus in plant tissues. The reverse transcription-polymerase chain reaction (RT-PCR) assay was used to amplify the PVX-CP gene. The amplicon was cloned and expressed in *Escherichia coli*. The CP fractions obtained from the lysates of *E. coli* were purified and used to prepare the polyclonal antiserum by immunizing the rabbits. The effectiveness of different enzyme-linked immunosorbent assay (ELISA) format was assessed by using the polyclonal antibodies (PAbs). The PVX-CP antibodies reacted effectively in indirect plate-trapped antigen (PTA)-ELISA format and immunoblot assay and these tests were more effective in detecting a broad spectrum of PVX isolates (Cerovska et al. 2010).

The principal advantage of using scFv fusion proteins for the routine detection of plant viruses is the ability to produce large quantities of reagents cheaply in bacterial fermenters and to incorporate them in standard immunoassays. Production of recombinant antibodies using phage display technology offers other advantages such as identification of strains precisely by selecting suitable scFv antibody fragment, detection of viruses present in stored produce after harvest such as sugar beet tubers, possibility of expressing scFv fragments in plants such as tobacco for use in biological and medical applications and cost-effectiveness in antibody production. The antisera produced against recombinant viral coat protein form an additional or alternative tool for manufacturing standardized kits for serological detection of viruses like *Citrus psorosis virus* (CPsV) that are difficult to purify. Further, in the indirect DAS-ELISA format, the PAb developed against the recombinant CP protein was efficiently and successfully employed in place of monoclonal antibody used earlier for the detection of CPsV (Loconsole et al. 2006).

The DNA-based immunization for producing antiserum to plant viruses can constitute an effective alternative procedure, when conventional methods of protein based immunization provide unsatisfactory results. This approach is a time-saving method that does not require virus purification from plants or protein purification and expression from bacteria. The purification of virions may damage particles, whereas purification of recombinant proteins may modify their structure, both altering the antigenic properties of epitopes. These limitations can be avoided by DNA immunization where the antigen is directly expressed in vivo. Although DNA immunization has been applied commonly for vaccine production against animal and human pathogens (Donnelly et al. 1997), there is only a single report available on the application of DNA-based immunization procedure for plant viruses involving cloning of DNA for generating antibodies to coat protein (CP) of *Tobacco mosaic virus* (TMV) and P1 protein of *Potato virus Y* (Hinrichs et al. 1997).

DNA prime and protein boost immunization protocol was developed for the detection of *Little cherry virus* (LChV), a component of Little cherry disease complex which is considered to be due to at least two different viruses. Earlier attempt to produce antisera to recombinant proteins expressing entire viral CP gene and the C-terminal part of the minor CP (CPm) gene did not provide satisfactory results (Keim-Konrad and Jelkmann 1996). In a later investigation, the DNA-CP-boost antiserum to LChV-1 raised with coated DNA intramuscular immunization of rabbits, proved to be promising for serological detection of LChV-1, because it discriminated healthy from LChV-1 infected tissues by Western blot analysis in in vitro propagated plants and dot immunobinding assay (DIBA) and ELISA in field-grown plants. On the other hand, antiserum produced using partially purified virus preparations or using bacterially-expressed recombinant CP protein did not give satisfactory results. The antibodies against recombinant CP was probably less efficient in recognizing native CP epitopes, though it was able to react with the bacterially-expressed protein. The efficacy of detection of LChV-1 by one-step PCR and ELISA techniques was compared. Of the 50 stone fruit trees that were positive by one-step RT-PCR, the ELISA could detect LChV-1 only in 20 trees (40%), indicating the superiority

Table 2.4 Comparative efficacy of one-step RT-PCR and ELISA for detection of *Little cherry virus 1* (LChV-1) in stone fruit species (Matic et al. 2009)

Stone fruit species	No. of infected samples detected by		Detection rate by ELISA compared to PCR (%)
	One-step RT-PCR	ELISA	
Plum	44	17	39
Cherry	4	3	75
Almond	1	0	–
Peach	1	0	–
Total	50	20	40

of detection by the molecular method (Table 2.4). Thus, DNA-CP-boost antiserum may be useful for the serological detection of LChV-1, as no antiserum to LChV-1 is available commercially. However, this method needs more improvement for enhancement of sensitivity and specificity of viral detection in field-infected plants (Matic et al. 2009).

Immunoassays depend on the visualization/recognition either directly or indirectly of the binding of a specific antibody to its homologous antigen. The immunoassays used earlier, such as immunoprecipitation of antibody/antigen complex are performed in narrow tubes. Other methods depend on the ability of antigen/antibody to diffuse through agar and the formation of visible precipitin lines indicates the positive interaction between the reactants. Electrophoresis of antigen mixtures followed by immunodiffusion of the antibodies has been used for the identification of virus strains. Agglutination of antigens with related antibodies adsorbed onto a protein A-coated surface of cells of the bacterium *Staphylococcus aureus* or covalently bound to protein A-coated Sepharose beads was used in the virobacterial agglutination test (VBA) for the detection of viruses such as *Potato virus Y* (Walkey et al. 1992) and *Cocoa swollen shoot virus* (Hughes and Ollennu 1993). These immunoassays require large volumes of antiserum and high concentrations of antigen in plant tissues for providing positive results. Hence, these tests are performed less frequently compared with techniques based on labeled antibodies.

Various immunological techniques have been employed for detection and identification of as many as 270 viruses infecting a wide range of plant species (Van Regenmortel 1982). Labeled antibody techniques have been developed by attaching labels or markers such as enzymes, fluorescent dyes or radioactive materials to either antigens or antibodies. Currently these methods are being extensively used, since their specificity, sensitivity, reliability and rapidity have been significantly improved with the use of MAbs. The possibility of automation for handling large number of samples has enhanced their utility. Among the labeled antibody techniques, enzyme-linked immunosorbent assay (ELISA) and its variants have been extensively employed for the detection, identification and differentiation of plant viruses (Clark et al. 1976; Clark and Bar-Joseph 1984).

The immunoassays may be employed in combination with electron microscopy and nucleic acid-based techniques for visualization of the viruses in situ in plant tissues and for enhancing the sensitivity of detection of viruses.

2.1.5.1 Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent assay (ELISA) has remarkably improved the sensitivity of detection and identification of viruses, in addition to providing the results rapidly when compared to the immunoassays available earlier. Three formats of ELISA, the double antibody sandwich (DAS)-ELISA, triple antibody sandwich (TAS)-ELISA and plate trapped antigen (PTA)-ELISA are commonly used for the detection of plant viruses (Clark and Adams 1977; Appendix 1). Alkaline phosphatase (AP) is quite stable and it has been used more frequently for labeling the antibody (IgG) using glutaraldehyde as the coupling agent to prepare the conjugate for detecting the reaction between the antigen and antibody. Use of maleimide for conjugation provided more sensitive detection of *Bamboo mosaic virus* (BMV) than when glutaraldehyde was used (Chen and Lu 2000).

In order to reduce the cost of testing, attempts were made to replace AP for labeling the antibody without losing the efficiency of the test. Horseradish peroxidase (HRP) was found to be as sensitive and specific as AP system for the detection of *Turnip yellow mosaic virus*, *Potato virus Y*, and *Potato leaf roll virus* in infected plants (Polak and Kristek 1988). ß-Glucosidase isolated from *Escherichia coli* was shown to be more effective in labeling antibodies to *Potato virus X* than HRP, as it decreased the background reaction (Neustroeva et al. 1989). Penicillinase (PNC)-based ELISA had sensitivity similar to AP and HRP-based ELISA for the detection of *Maize mosaic virus*, *Peanut mottle virus* and *Tomato spotted wilt virus.* Further, penicillinase and penicillin are readily available and are substantially less expensive compared to AP (Sudharsana and Reddy 1989; Abraham and Albrechsten 2001).

The sensitivity levels of detection of *Maize streak virus* (MSV) in the maize plants was assessed by DAS-ELISA format using different enzyme systems viz., alkaline phosphatase (AP), horseradish peroxidase (HRP), alkaline phosphatase amplification (AMP) and penicillinase (PNC) and avidin–biotin penicillinase amplification. The AP and AMP systems were able to detect MSV in plants, but they could not detect the virus in the vector leafhoppers. The HRP and PNC systems, on the other hand, were capable of detecting MSV in maize plants and also in the insect vectors. The color change from blue to yellow (positive) was pronounced in PNC system and there was no need to use the ELISA plate reader which may not be available in all laboratories in the developing countries (Afolabi and Thottappilly 2008).

Urease conjugate was found to be superior to AP conjugate, since in the DAS-ELISA, the color change from yellow to violet could be more easily recognized than the color change from colorless to yellow in the AP system. The visual detection threshold was 0.5 ng/ml of TMV with urease, while a concentration of 500 ng/ml was necessary for detection of TMV with phosphatase system, indicating

the ease with which the color change could be discerned in urease system (Gerber and Sarkar 1988). In another investigation, the inorganic pyrophosphatase (PPase) from *Escherichia coli* was conjugated with antibodies specific to target virus and tetrazolium pyrophosphate was used as the substrate in the ELISA test. High sensitivity, negligible level of background reactions in the controls, formation of bright blue-greenish color allowing easy visualization of positive reactions and high stability of PPase-conjugated antibodies are the distinct advantages of this system. The viruses detected by PPase system include *Potato viruses X*, *Y*, *M*, *S* and *leaf roll*, *Carnation mottle virus*, *Barley stripe mosaic virus*, *Tobacco necrosis virus*, *Cucumber mosaic virus* and *Soybean mosaic virus.* Further, the sensitivity of detection of *Raspberry ringspot virus*, *Strawberry latent ringspot virus*, *Tomato black ring virus* and *Arabis mosaic virus* was significantly enhanced by PPase system, compared with HRP system in DAS-ELISA format (Surguchova et al. 1998).

Among the immunoassay employed for detection of plant viruses, ELISA and its variants have been more frequently used not only for virus detection, but also for studying different aspects of plant viruses, such as serological relationship between viruses, identification of different epitopes on virus coat protein, epidemiology and host range of viruses, assessment of levels of resistance/susceptibility of crop cultivars to viruses and virus–vector relationships (Narayanasamy 2008). Detection of viruses in the mother plants, asexually propagated plant materials and seeds is the important basic strategy that is adopted for the management of virus diseases. ELISA tests have been extensively employed for the detection of large number of viruses in the plant materials.

Biological indexing required 2–3 years for symptom development on the indicator plants inoculated with *Grapevine leaf roll-associated viruses* (GLRaV) *1 to 4*. This procedure was compared with detection by ELISA test. Presence of GLRaV-3 was detected in all infected plants by ELISA test. But biological indexing detected the virus successfully only in 47 of 57 grapevine plants tested (Rowhani et al. 1997). Detection and resolution of etiology of grapevine leaf roll and rugose wood complexes have been greatly improved by application of ELISA and other immunoassays (Martin et al. 2000). Mixed infection of grapevine by two or more viruses leads to development of complex diseases and consequent difficulty in identifying the components of the disease complex. By using specific MAb in ELISA test, *Grapevine virus D* (GVD) was detected efficiently in the cortical shavings from mature grapevine canes (Bosica et al. 2001).

Five hybridoma cell lines immunoreactive with *Grapevine leaf roll associated viruses* were cloned and characterized. A novel 37-kDa protein was found associated with grapevine leaf roll disease in a mixed virus infection. The polypeptide was separated from the 38-kDa polypeptide associated with GLRaV-1 using Tricine SDS-PAGE system. Concentrated p37 preparations were used to immunize the mice for MAb production. The DAS-ELISA format using the 15F1 MAb and the LR102 PAb allowed detection of GLRaV-5 and -8 in crude extracts of infected tissues. The use of the MAbs produced in this investigation increased the specificity and sensitivity of detection of GLRaV-5 and -8 by reducing the background due to the presence of host-specific antibodies in polyclonal antiserum (Monis 2000). The genes encoding

RNA-dependent RNA polymerase (RdRp) and coat protein (CP) of a Chinese isolate of GLRaV-2 were cloned and expressed in *E. coli*. The resulting recombinant proteins were used to raise antisera in rabbits. The PAbs so generated were able to detect infection by GLRaV-2 in all (using CP antiserum) or in most (using RdRp antiserum) of plant extracts by DAS-ELISA format. The antiserum to recombinant CP protein appeared to be more efficient in recognizing native virus CP and had a greater potential for use in the routine detection of GLRaV-2 in field-infected vines (Xu et al. 2006).

Grapevine leaf roll associated virus-3 belongs to the genus *Ampelovirus* and the infected plant material is the main source of long distance dissemination of this virus. A single-chain antibody fragment (scFvLR3cp-1) specific for the coat protein (CP) of GLRaV-3 was selected from phage display library. The antibody bound specifically to the entire length of GLRaV-3 particles and exhibited a high binding affinity. Capture ELISA format demonstrated that the scFv fragment was produced and retained its antigen-binding capacity in the plant cytosol. The selected scFv recombinant antibody could recognize four members of *Closteroviridae*. The horseradish peroxidase (HRP) labeled scFvLR3cp-1 was the ideal basis for a fully recombinant ELISA Kit capable of detecting mixed infections in grapevine plants in a consistently reproducible manner (Orecchia et al. 2008).

The single-chain fragment variable (scFv) antibody (scFvLR3) directed against the CP of *Grapevine leaf roll associated virus-3* (GLRaV-3) was expressed in *E. coli*. This recombinant protein was used to develop a diagnostic ELISA kit. The antibody was fused to the light chain constant domain of human immunoglobulin to create the bivalent reagent C_L-LR3. Indirect ELISA format showed that C_L-LR3 bound efficiently to the IgGLR3 and captured the target virus particles from infected grapevine sap. The recombinant protein captured the virion as efficiently as the commercially available antibody. The sensitivity and specificity were similar to a commercial diagnostic kit, with a detection limit of 33 ng of C_L-LR3 per well, a quantity comparable to the 50 ng recommended for the commercial kit. A fully recombinant kit was developed with the inclusion of a recombinant GLRaV-3 CP expressed in *E. coli.* This protocol avoided the problems associated with virus propagation and purification representing a rapid, simple, sensitive and standardized diagnostic protocol for GLRaV-3 detection (Cogotzi et al. 2009; Appendix 2).

Barley yellow mosaic disease is caused by *Barley mild mosaic virus* (BaMMV) and *Barley yellow mosaic virus* (BaYMV). Both viruses are transmitted by the fungal vector *Polymyxa graminis.* Standard DAS-ELISA procedure was applied employing monoclonal antibodies. BaYMV was detected in leaves and stems of infected barley plants at dilutions of 1/2560 and 1/160 respectively. Variations in the virus titers of infected tissues was indicated in detection thresholds (Ma et al. 1997). Barley cultivars with *rym4* and *rym5* genes possessed resistance to BaMMV and BaYMV-1. A pathotype of BaMMV was, however, able to infect cultivars with *rym5* resistance gene. DAS-ELISA test was applied to confirm the presence of BaMMV in six plants of cv. Tokyo, showing clear mosaic symptoms. BaMMV was detected in these plants with absorbance values at 405 nm ranging from 1.75 to 2.02, while no BaYMV could be detected in these plants, absorbance values of 0.00–0.04 at 405 nm (Habekuss et al. 2008).

The sensitivity of detection of *Indian tobacco leaf curl virus* was at a higher level when the virus-specific MAbs were employed than when PAbs were used (Swanson et al. 1998). ELISA format was developed for the detection of *Cymbidium mosaic virus* infecting orchids by using specific MAb as the coating antibody and egg yolk immunoglobulin (IgY) as the detecting antibody. It was possible to detect the virus in leaf extracts diluted up to 1,024-folds (Vejaratpimol et al. 1998). In the case of *Citrus psorosis-associated viruses*, all plants that were psorosis positive by indexing on orange cv. Madame Vinous and Dweet Tangor were also ELISA-positive. In addition, ELISA procedure detected the virus in four more citrus accessions that indexed negative. A high percentage of plants were ELISA-positive compared to biological indexing, suggesting that ELISA tests might provide reliable results rapidly, helping the certification and quarantine programs effectively (D'Onghia et al. 1998).

A DAS-ELISA protocol was developed for the detection of seven mechanically transmissible *Potato viruses A*, *Y*, *V*, *M*, *S*, *X* and aphid-transmitted *Potato leaf roll virus* (PLRV) in in vitro generated potato plantlets. Six potato varieties were mechanically inoculated and the presence of viruses was monitored over 3, 4, 5 and 6 weeks after inoculation. The potato viruses were detected by employing virus-specific antisera in DAS-ELISA format in plants regenerated in vitro. The limit of detection of these viruses in host tissue extracts was between 1 and 10 ng/ml (Khan et al. 2003). ELISA formats were evaluated for their reliability and sensitivity of detection of a novel tobamovirus infecting hibiscus in Florida. Indirect ELISA was more sensitive than DAS-ELISA for the detection of *Florida hibiscus virus* in all tested samples of sap of leaves from *Chenopodium quinoa.* End-point dilutions of sap for detection by DAS-ELISA and indirect ELISA were 1:6,400 and 1:51,200 respectively indicating the eightfold greater sensitivity of indirect ELISA format. Similar greater levels of sensitivity of indirect ELISA were observed, when extracts of bark tissues were tested. End-point dilutions of partially purified virus preparation required for detection by indirect ELISA and DAS-ELISA format were 4 and 31 ng/ml respectively. The usefulness of cocktail ELISA for detection of the virus in leaf samples of *C. quinoa* was assessed. The absorbance values for cocktail ELISA were three to four times less than those for DAS-ELISA and indirect ELISA formats. The results demonstrated the superiority of indirect ELISA test for the detection of *Florida hibiscus virus* (Kamenova and Adkins 2004).

The ELISA technique has been useful for detecting and identifying new viruses. Polyclonal antibodies (PAbs) raised against nucleoproteins of members of the genus *Tospovirus* belonging to the family *Bunyaviridae* have been employed frequently for detection by ELISA tests (Takeuchi et al. 2001; Tanina et al. 2001). *Tomato yellow ring virus* occurring in Iran was detected using PAbs targeting viral nucleoprotein. As there was significant cross-reaction between antisera generated against other tospoviruses such as *Tomato spotted wilt virus*, *Tomato chlorotic spot virus*, *Groundnut ringspot virus*, *Impatiens necrotic spot virus*, *Iris yellow spot virus* and *Watermelon silver mottle virus*, this new virus was considered to be serologically distinct from the viruses compared (Hassani-Mehraban et al. 2005).

Calla lilies (*Zantedeschia* spp.) were infected by another new tospovirus. Indirect ELISA format and immunoblotting assay were employed using PAbs and MAbs specific to *Calla lily chlorotic spot virus* (CCSV) and *Watermelon silver mottle virus* (WSMoV). The results indicated that CCSV was a distinct member in the genus Tospovirus, belonging to WSMoV serogroup (Lin et al. 2005). The 29-kDa nucleocapsid protein gene of TSWV was expressed in *Escherichia coli* and the recombinant protein was used to generate polyclonal and monoclonal antibodies capable reacting with TSWV. The dilutions of PAb at 1:5,000, MAbs at 1:5,000 and plant sap at 1:20 were found to be the optimal concentrations for obtaining positive results with TAS-ELISA format. A total of 250 leaf samples were tested for the presence of TSWV. As none of the samples tested gave positive results, it was concluded that TSWV was not present in Yunnan and Zhejiang provinces of China (Wu et al. 2009).

The DAS-ELISA format was applied for the detection of *Potato virus Y* in tobacco plants. The limit of detection of the virus corresponded to 10 ng/ml of purified virus preparation (Varveri 2000). The effectiveness of the indirect ELISA procedure for the detection and differentiation of the newly observed *Wheat yellow head virus* from *Wheat soilborne mosaic virus*, *Wheat American striate mosaic virus*, *Agropyron mosaic virus* and *High plains virus* was demonstrated (Seifers et al. 2005). Field observation and laboratory tests were performed to evaluate the sanitary status of cloned Genebank collection of stone fruit trees in Harrow, Ontario, Canada. *Prunus* accessions were screened for the presence of *Prunus necrotic ringspot virus* (PNRSV) and *Prune dwarf virus* (PDV) by DAS-ELISA format. This investigation showed that 131 of the tested trees were infected by at least one virus. The infection rates of different stone fruits were 21.6% of plum and 4.5% of nectarine, 34.9% of sweet and sour cherry, 20.7% of apricot and 24.5% of other cherry species. *Plum pox virus* (PPV) was not detected in any of the tested trees (Table 2.5; Michelutti et al. 2005).

The presence of a recombinant isolate of *Plum pox virus* (PPV) in apricot trees was detected by DASI-ELISA format using four strain-specific MAbs and this appears to be the first report on the natural occurrence of a recombinant isolate of PPV in Italy (Myrta et al. 2005). The ELISA test was employed to detect the

Table 2.5 Viruses detected by ELISA test in clonal Genebank *Prunus* accessions (Michelutti et al. 2005)

Species	No. of trees infected/tested	Viruses detected			
		PPV	PNRSV	PDV	PDV + PNRSV
Peach and nectarine	9/197	0	4	5	0
Sweet and sour cherry	64/183	0	23	15	26
Plum	23/106	0	9	10	4
Apricot	22/106	0	19	2	1
Other cherry species	13/53	0	9	2	2
Total	131/645	0	64	34	33

infection of rose geraniums (*Pelargonium* spp.) by *Prunus necrotic ringspot virus* (PNRSV). The identity of PNRSV infecting rose geraniums was established by using antibodies generated against different Ilarviuses in ELISA format. The diagnosis of the causative agent was confirmed by performing Northern hybridization, reverse transcription (RT)-polymerase chain reaction (PCR) assay, restriction fragment length polymorphism (RFLP) analysis and sequencing of PCR amplicons (Kulshrestha et al. 2005). ELISA test was applied for the detection of PNRSV in a cherry orchard of *Prunus cerasus* cv. Monmorency and *P. avium* cv. Hedelfingen in New York. The assay indicated an eightfold higher infection rate in sour cherry (33%) than in sweet cherry (4%). The infection of cherry trees by PNRSV was confirmed by performing reverse transcription (RT)-polymerase chain reaction (PCR) assay (Oliver et al. 2009).

Citrus psorosis virus (CPsV), considered to be the putative agent of psorosis, a widespread economically important citrus disease. Serological detection of CPsV was accomplished by performing the TAS-ELISA test. The antiserum A322 to CPsV was used to plate coating, while the MAb13C5 was used as CPsV-specific primary antibody and the rabbit anti-mouse IgG (whole molecule) immunoglobulins conjugated with AP were used as secondary antibody. CPsV was detected in extracts of citrus plants infected with CPsV isolates PL3, PL4 or PL5. The absence of CPsV in some source plants could be confirmed by ELISA and also by RT-PCR assay and electron microscopy, indicating the suitability of these source plants for vegetative propagation (Martin et al. 2000).

As the *Cirus psorosis virus* (CPsV) reaches low concentrations in plants, it is difficult to have purified preparation of the virus. In order to overcome this problem, the coat protein (CP) gene of an Italian virus isolate was cloned and sequenced. The recombinant protein strategy was formulated to raise a polyclonal antiserum targeting a recombinant viral CP. The CP sequence of the Italian isolate showed 97% identity at amino acid level with a Florida isolate of CPsV. The CP was expressed in *E. coli* BL21 as a fusion protein with maltose-binding protein. This problem was used as an antigen for immunization for producing PAbs. The PAbs As-Ps.Rc1 successfully detected CPsV in citrus leaves in DAS-ELISA and indirect ELISA formats (Table 2.6). All 74 isolates were detected at least once by both MAb Ps-29 and PAb As-Ps.Rc1, during the field survey. To check on the reliability of CPsV detection by ELISA throughout the year, samples of old leaves, young leaves and flowers collected every month were tested by both ELISA formats using MAb P-29 and PAb As-Ps.Rc1. The results showed that detection of CPsV was virtually was possible throughout the year and there was no signal difference in the performance of the two ELISA systems. Highest detection level was noted in spring from flowers and young leaves and in autumn from young flush vegetation (Loconsole et al. 2006).

Citrus tristeza virus (CTV) isolates show great biological diversity and the available means of distinguishing them, other than biological indexing, are not satisfactory. Decline-inducing (DI) isolates and stem-pitting (SP) isolates of CTV are recognized by the type of symptoms induced. The epitopes for a panel of 30 MAbs specific for CTV were mapped on the CTV coat protein (CP) expressed in *E. coli* cells. Nine overlapping CP gene fragments were expressed in bacterial

Table 2.6 Detection of *Citrus psorosis virus* (CPsV) by two ELISA formats using PAbs and MAbs in citrus plants (Loconsole et al. 2006)

	Indirect ELISA (PAb)		DAS-ELISA (PAb)		DAS-ELISA[a] (MAb)	
Samples	A_{405}[b]	I/H[c]	A_{405}[d]	I/H	A_{405}[e]	I/H
ps 110	1.6	26.7	0.9	15.0	2.0	33.3
ps 101	0.4	6.7	0.14	2.3	0.235	3.92
Healthy	0.06	1	0.06	1	0.06	1

[a] Routine DAS-ELISA with MAb Ps-29 as reference control

[b] Absorbance values at 405 nm recorded after 30 min of substrate incubation

[c] Ratio of absorbance values of infected/healthy control (I/H); values below 2 considered as negative reactions

[d,e] Absorbance values recorded at 2 h of substrate incubation

cells. The resulting fusion proteins were tested by ELISA and immunoblotting with 30 MAbs generated against different CTV isolates. The MCA-13 MAb has been widely employed for detection of DI isolates and differentiating them from mild isolates in Florida (Nikolaeva et al. 1996). A highly specific MAb capable of reacting with extracts of tissues infected by CTV was developed. This MAb did not react with the non-infected citrus plant extracts, revealing the specificity of the MAb in ELISA test (Öztürk and Cirakolu 2003). CTV isolates (36) occurring in CTV-eradicative and non-eradicative districts in central California did not react with MCA 13 MAb that detected presumptive CTV strains as revealed by DAS-ELISA protocol. However, the standard control isolates of CTV T36, T3 and VT reacted with this MAb. The results indicated that the presence of CTV in citrus trees should be determined by a viral detection procedure, such as ELISA rather than by visual examination of symptoms induced (Yokomi and De Borde 2005).

A survey was undertaken in commercial groves in Cyprus, for selecting elite mother plants of different citrus and lemon cultivars. Freedom from infection by *Citrus tristeza virus* (CTV) and *Citrus variegation virus* (CVV) of citrus cultivars was tested by ELISA and biological indexing. All mother plants tested were found to be free of CTV and CVV. However, some mother plants were found to be infected by *Citrus psorosis virus* (CPsV). The standard procedure of shoot-tip grafting technique was employed for eliminating CPsV in the selected citrus plants. Three 'Polyphori' lemon, six 'Arakapas' mandarin and 20 'Jaffa' orange plants became free of CPsV. These plants were used as primary source material for the basic citrus plantations in Cyprus (Kaparia-Isaia et al. 2007).

The recombinant coat protein (CP) of the *p25* gene of *Citrus tristeza virus* (CTV) was employed to produce antibodies in goats and rabbits. The antibodies were evaluated for use in a complete kit for the detection of CTV in infected plants. The combination of goat T1 used as a primary (coating) and rabbit C3 as intermediate (detecting) rCP antibodies reacted efficiently, with OD at 405 nm values between 0.250 and 2.000 with samples from an international collection of diverse CTV isolates. The OD_{405} values for healthy tissue were less than 0.100. The combination of goat T1 and rabbit C3 rCP antibodies also provided consistent

results for samples from CTV-infected and healthy citrus plants. The combination of goat T1 and rabbit C3 rCP antibodies showed suitability for large scale indexing with samples collected in commercial groves as part of the Central California Tristeza Eradication Agency's (CCTEA) monitoring program. A total of 41,195 samples from 301 commercial groves from three districts were tested. Infection by CTV in 26 trees (0.063%) was detected using the T1/C3 rCP antibody combination in DAS-ELISA procedure (Iracheta-Cárdenas et al. 2009).

A serological survey was undertaken in Chile to assess the extent of incidence of virus diseases affecting raspberry, red currant and blueberry. Double antibody sandwich (DAS)-ELISA format was employed using PAbs and alkaline phosphatase conjugates. Infection of raspberry by *Apple mosaic virus* (87–98%), *Arabis mosaic virus* (29–86%), *Raspberry bushy dwarf virus* (35–68%) and *Tomato ringspot virus* (63–85%) was detected. Blueberry infection by *Tomato ringspot virus* (0–14%) and *Tobacco ringspot virus* (6–19%) was at low levels. Incidence of *Arabis mosaic virus* and *Tomato ringspot virus* in red currant was low and it was recorded in one location only. *Tomato ringspot virus* and *Raspberry bushy dwarf virus* were observed for the first time during the survey. The survey may be useful to locate the areas with high and low levels of virus disease incidence. This information may assist in the establishment of certification programs to produce virus-free stocks (Medina et al. 2006a).

A recombinant antibody fusion protein V3HCL showing specific reactivity for *Potato leaf roll virus* (PLRV) was labeled with biotin using standard coupling procedure. A fully recombinant ELISA was adapted incorporating V3HCL, V3HCL-biotin and streptavidin–ALP conjugate. This protocol provided results for PLRV detection in potato comparable to the assay based on immunoglobulins. The V3HCL-biotin preparations were stable and retained specific activity for more than 1 year, when stored at 4°C or −20°C. The results indicated that scFv reagents derived from the synthetic phage display platforms can provide effective alternatives to assays employing immune reagents (Al-Mrabeh et al. 2009).

The effectiveness of including a blocking step in improving the reliability of results of ELISA test was demonstrated, since this step removes the false positive results. Reliable detection of *Iris yellow spot virus* (IYSV) in leaf samples was hampered, because extracts of healthy onion, leek and tulips caused false positive A_{405} values in ELISA. When tested by ELISA using IYSV-specific antibodies and a blocking step using 5% skim milk powder that improved the test reliability, the virus was detected in 11 of 21 leek and 2 of 26 onion plantings with apparent 1–7% and 7% infection respectively. The detection frequency in leek crops appeared to be a significant underestimate, because of the localization of the virus within individual plants. The patchy distribution of IYSV in naturally infected leek plants, low incidence of infection in young basal leaf closest to the meristem and absence of the virus in other plant parts indicated that IYSV did not move systemically in this host plant species (Table 2.7). Reliable estimates of IYSV during large scale survey can be obtained, if the randomly collected samples tested by ELISA test

Table 2.7 Detection and quantification of *Iris yellow spot virus* (IYSV) by ELISA based on absorbance values (OD) at 405 nm in the leaves of naturally infected leek plants (Smith et al. 2006)

Leaf position	Leaf subsection		
	Top	Middle	Base
Leaf 1	0.33[a] (6)[b]	0.43 (4)	–
Leaf 2	0.49 (12)	0.48 (5)	0.14 (1)
Leaf 3	0.86 (7)	0.58 (7)	0.55 (2)
Leaf 4	0.63 (13)	0.67 (10)	0.31 (2)
Leaf 5	0.61 (11)	0.55 (11)	0.23 (1)
Leaf 6	0.71 (7)	0.46 (6)	–
Leaf 7	0.48 (4)	0.44 (4)	1.21 (1)
Leaf 8	0.22 (2)	0.48 (5)	0.24 (1)
Healthy leaf	0.01	0.01	0.01

[a] Mean absorbance values for infected leaf subsections of partially infected plants; values >10 times those of healthy control samples considered as positive reactions

[b] Values in parantheses are number of leaf sections in which IYSV was detected

consist of combined tissue subsections from the tops and middles of several leaves from each plant sampled (Smith et al. 2006; Appendix 3).

Watermelon silver mottle virus (WSMoV) belongs to the genus *Topovirus* included in the family *Bunyaviridae* enclosing a large and diverse group of viruses infecting plants and animals. Virions of WSMoV are enveloped quasi-spherical particles containing three single-stranded nucleic acid segments designated L, M, and S RNAs. The complete WSMoV genomic sequence has been determined. The SRNA encodes a nucleocapsid protein (NP) which encapsidates viral RNAs in its viral complementary strand. A nonstructural NSs protein encoded from the viral strand of WSMoV SRNA forms filamentous inclusion bodies in the cytoplasm of infected plant cells. The NSs protein of WSMoV was expressed by *Zucchini yellow mosaic virus* (ZYMV) vector in squash. The expressed NSs protein with a histidine tag and an additional NIa protease cleavage sequence was isolated. After SDS-PAGE separation, the NSs protein was used for production of PAbs and MAbs in rabbits and mice respectively. The PAbs reacted strongly with NSs crude antigen of WSMoV and weakly with *Capsicum chlorosis virus* (CaCV), but not with *Calla lily chlorotic spot virus* (CCSV) belonging to the same serogroup. On the other hand, all three MAbs reacted with NSs proteins of members of WSMoV serogroup in the indirect ELISA format. The results indicated that CaCV was closely related, but CCSV was distantly related to WSMoV and yet they shared common antigenic determinants revealed by the MAbs. Further, the ZYMV vector system was shown to be an excellent tool that could be used as a heterologous protein expressor. The MAbs against the conserved region of NSs protein were found to be useful for the serological identification of tospoviruses belonging to the WSMoV serogroup (Chen et al. 2006).

A new virus disease affecting the orchid *Phalaenopsis* exhibiting chlorotic ringspots was investigated to establish the identity of the virus. The nucleocapsid protein (NP) of the virus isolate 91-orchid-1 was purified and used to raise the PAbs in the rabbits. This virus isolate reacted slightly with the antiserum of *Watermelon silver mottle virus* (WSMoV), but not with those of *Tomato spotted wilt virus* (TSWV), *Impatiens necrotic spot virus* (INSV) and *Groundnut ringspot virus* (GRSV) belonging to the genus *Tospovirus* in the indirect ELISA test. The results of electron microscopy, Western blotting and sequence analysis showed the isolate 91-orchid-1 was a member of the genus *Tospovirus* and closely related to WSMoV and *Peanut bud necrosis virus* (PBNV) and identified as an isolate of *Capsicum chlorosis virus* (CaCV) (Zheng et al. 2008b).

The occurrence of an unknown chickpea chlorotic stunt disease was investigated by employing DAS-ELISA and TAS-ELISA formats. A rabbit antiserum raised against *Chickpea chlorotic stunt virus* (CpCSV) cross-reacted unexpectedly with *Beet western yellows virus*-like viruses in the DAS-ELISA format. Hence, murine MAbs were produced. The PAb to CpCSV-FB was used as capture antibody and the MAb were employed as detecting antibodies in the triple antigen sandwich (TAS)-ELISA format, permitting more sensitive detection of CpCSV antigen. The results indicated that the mono- and poly-clonal antibodies could permit both sensitive and specific detection by TAS-ELISA procedure and they have the potential for use in disease diagnosis in legume crops (Abraham et al. 2006).

Nine MAbs were employed for the detection of *Cucumber mosaic virus* (CMV) by TAS-ELISA in infected tobacco plants. The sensitivities of the TAS-ELISA format varied depending on the MAbs specific to strains of CMV. The MAbs may not be equally suited as reagents in the different steps of ELISA format, since some may lose their activity after absorption to plastic or after labeling. The specific reactivity of different MAbs revealed that it was possible to detect as little as 10 pg/well of purified Pepo-CMV strain, while as little as 1 ng/well for M2-CMV strain. The M2-CMV strain reached very low concentrations in tobacco, whereas Pepo-CMV strain accumulated at a fast rate reaching high concentrations requiring only about 5 days giving an yield of 1 mg/g of leaves as against 30 days to give an yield of 2 mg/100 g of leaves required by M2-CMV strains (Zein and Miyatake 2009).

Rapid detection and identification of new viruses has been accomplished by applying ELISA and its variants. Grapevine leaf roll disease is a complex involving two or more of the five molecularly distinguishable viruses. The association of sixth virus designated *Grapevine leaf roll-associated virus-6* (GLRaV-6) with leaf roll complex in a Chassela clone was revealed by ELISA test. GLRaV-6 was detected using a new MAb in the extracts of leaf blades and petioles of infected plants (Gugerli et al. 1997). Another GLRaV-associated virus (GLRaV-2) occurring in low concentrations in infected grapevines was detected by DAS-ELISA using virus-specific antibodies (Köklü 1999). A new mechanically transmissible virus infecting mashua (*Tropaeolum tuberosum*) was detected and identified and named *Tropaeolum mosaic virus* (TropMV) based on the results of ELISA tests. TropMV was shown to be distinct, but closely related to *Potato virus Y* (Soria et al. 1998).

Isolates of *Potato virus Y* (PVY^N) causing tobacco veinal necrosis disease could be detected with PAbs and MAb4C3 by ELISA tests. Use of MAbs 1F5 and 295-5 confirmed the presence of PVY^O and PVY^N serotypes among the PVY^N pathotype isolates. Several of the PVY^N isolates appeared to be recombinant $PVY^{N:O}$. The results confirmed that isolates from western USA could not be recognized as the PVY^N strain (Crosslin et al. 2005). The occurrence of a virus disease inducing chlorotic spots on leaves of Phalaenopsis orchids was observed in Taiwan. The putative virus established in *Chenopodium quinoa* and *Nicotiana benthamiana* was purified and the isolate 7–2 was used to raise PAbs by immunizing the rabbits. The isolate 7–2 reacted positively with POTY monoclonal antibody for detecting *Potato virus Y* in indirect ELISA format indicating that this new virus might be a potyvirus. The presence of this isolate in inoculated plant species that reacted with cholorotic spots was detected by employing the PAbs-specific to the isolate 7–2 in indirect ELISA procedure. The possibility of these host plant species serving as additional hosts under natural conditions was indicated by this study. The isolate 7–2 was identified as a member of *Potyvirus* and designated *Phalaenopsis chlorotic spot virus* (PhCSV), based on the results of electron microscopy, immunoblotting and sequence analysis (Zheng et al. 2008a).

A new strain of *Potato virus Y* infecting *Dioscorea alata* was identified based on the immunological properties determined by ELISA test, in addition to the biological characteristics of the new strain (Odu et al. 1999). A new bacilliform virus infecting *Dioscorea alata* was investigated and it was found to be serologically related to *Sugarcane bacilliform virus* and *Banana streak virus.* The *Dioscorea bacilliform virus* (DaBV) was found to be a distinct badnavirus (Phillips et al. 1999). A new virus infecting tobacco was identified as a member of the genus *Phytoreovirus*. The PAb raised against *Wound tumor virus* (WTV), the type virus of *Phytoreovirus*, exhibited strong cross-reaction to the virus infecting tobacco indicating the serological relatedness of the new virus (Rey et al. 1999). A mechanically transmissible unidentified virus infecting maize has icosahedral particles (approximately 28–30 nm diameter) and positive sense single-stranded RNA as the genome. The virus was identified as *Maize mild mottle virus* based on the immunological characteristics determined by ELISA and immunoblot assays in addition to the virus particle morphology revealed by electron microscopy (Thottappilly et al. 1999). The identities of *Chrysanthemum stem necrosis virus* (CSNV) and *Zucchini lethal chlorosis virus* (ZLCV) were established by performing ELISA and Western immunoblot tests (Bezerra et al. 1999).

Ensuring freedom of plants and plant materials from infection by viruses is one of the basic disease management strategies. ELISA is the commonly employed method for certification of plants and planting materials and also during field surveys to assess the extent of infection, especially in asymptomatic plants. *Prune dwarf virus* (PDV), a major virus infecting stone fruits was efficiently detected by applying DAS-ELISA test (Abou-Jwdah et al. 2004). Variants of ELISA have been shown to be more effective in detecting some of the viruses. Plate-trapped antigen (PTA)-ELISA was employed to screen the hybridoma cell lines producing MAbs specific to *Sugarcane yellow leaf virus* (ScYLV) and for routine detection of

ScYLV in infected plant tissues (Korimbocus et al. 2002). The PTA-ELISA format was found to be effective in detecting *Cucumber mosaic virus* (CMV) in a wide range of crops and in different locations in Bulgaria, employing specific MAbs (Hristova et al. 2002). *Sugarcane mosaic virus* was reliably detected in maize plants showing dwarf symptoms by employing the specific MAb 2B5 in indirect antigen-coated plate-ELISA format (Jiang et al. 2003). *Banana bunchy top virus* (BBTV) and CMV were detected in banana plants by employing DAS-ELISA protocol (El-Dougdoug et al. 2006).

New derivatives of a virus may arise by selection or mutation with different infection potential. It is necessary to rapidly recognize such strains to make necessary changes, if any, in the disease management strategies. A new mutant strain of *Potato virus M*, designated *Potato virus M-ID* (PVM-ID), differing in the coat protein sequences in the amino-terminus region was identified using ELISA by Cavileer et al. (1998). In the case of wheat, unusual virus-like symptoms were observed. A protein fraction obtained from the infected plants was used to prepare the antiserum. Positive reaction was revealed by ELISA, when extracts from symptomatic plants were tested. The new virus designated *High Plains virus* (HPV) was distinct from already existing *wheat streak virus* (Seifers et al. 1997). The systemic infectivity of *Potato virus Y* (PVY, isolate T01) is influenced by the presence of *Cucumber mosaic virus* (CMV) in tobacco. Accumulation of PVY and CMV in singly and doubly infected tobacco was determined by ELISA test. CMV 2b protein was significantly lower than in plants doubly infected with PVY and CMV or in singly infected plants. The results suggested that the CMV 2b protein was necessary to assist systemic spread of PVY reaching greater concentrations in doubly infected tobacco plants (Ryang et al. 2004).

Cowpea (*Vigna unguiculata*) is infected by several viruses of which *Cowpea aphidborne mosaic virus* (CABMV), *Bean southern mosaic virus* (SBMV) and *Cowpea mottle virus* (CMeV) are economically important. The concentrations of these viruses in artificially (mechanically)-inoculated cowpea plants were determined by employing antigen-coated plate (ACP)-ELISA format. The age of plants at the time of infection (inoculation) had marked effect on the titer of CABMV. The absorbance values at 405 nm ranged from 0.11 to 0.46. Similar trend was observed for the other two viruses also. Absorbance values for all viruses in plants inoculated at 10 days after planting (DAP) were significantly higher than those from plants inoculated at 30 DAP. Differences in the virus titers due to cultivars were noted probably because of variations in their susceptibility/resistance levels. When cowpea infected by CMeV showed increases, while the titers of CABMV remained unchanged. Multiple infections resulted in severe symptoms (Taiwo et al. 2007).

Modifications of standard ELISA test have been made with a view to simplifying the procedure, making it less expensive and reducing the volume of antisera and labor needed. The fluorogenic ultramicro-enzyme immunoassay (DAS-UM-ELISA) was developed for the detection of *Citrus tristeza virus* (CTV). This test required only 10 μl of reagents without compromising the sensitivity which was equal to DAS-ELISA format and it provides the results within 5 h (Peralta et al. 1997). In the petridish-agar dot immunomagnetic assay (PADIA), polystyrene

petridish is used in place of microtiter plates with wells. Circular areas outlined with hydrophobic cryomarker pen are marked on the inner surface of the petridish. The antiserum and alkaline phosphatase conjugate are added into the marked areas, incubated and washed as in standard ELISA protocol. The substrate solution mixed with warm agar is added to cover the inner surface of the petridish. PADIA procedure requires 5–10 times less quantities of reagents. The sensitivity level as that of standard ELISA format is maintained. But the cost of the test is reduced by four times of the ELISA test (Abraham and Albrechsten 2000). The petridish-ELISA similar to PADIA, is also performed in polystyrene petridish. The inner surface of the dish is divided into many squares or circles using a wax pen. Drops of the reagents (50 µl) are placed within the marked areas. All steps as in standard ELISA protocol are followed and the absorbance values at 405 nm are recorded. *Tomato mosaic virus*, *Cowpea mosaic virus* and *Blackeye cowpea mosaic virus* were detected in seeds by this modified protocol (Abdalla and Albrechten 2001).

2.1.5.2 Dot Immunobinding Assay

In principle, ELISA and dot immunobinding assay (DIBA) are similar. The microtiter plates are replaced by nitrocellulose or nylon-based membranes on which the antigen is immobilized. The free protein-binding sites present in the membranes have to be blocked, because of their high affinity for proteins. Nonfat dry milk (skimmed) powder is commonly used because it is less expensive compared to bovine serum albumin (BSA) and gelatin. In addition, the milk powder is readily available and equally effective as the other blocking agents. The unconjugated virus-specific antibody is allowed to react with the immobilized antigen which is probed with AP-, HRP-labeled protein A, anti-Fc, or anti-IgG. Visual detection of the colored product is achieved using appropriate substrate.

Dot immunobinding assay (DIBA) was found to be more sensitive in detecting some plant viruses. The DIBA test was shown to be eight times and five times more sensitive than ELISA for the detection of *Tomato spotted wilt virus* (TSWV) and potyviruses respectively (Berger et al. 1985). Seedborne infection of *Barley stripe mosaic virus* in barley and *Bean common mosaic virus* in French bean could be detected by DIBA test in single seed or flour (Lange and Heide 1986). Satisfactory level of sensitivity of detection of *Peanut mottle virus* by DIBA test was achieved, only if specific monoclonal antiserum was used. On the other hand, ELISA test provided more sensitive detection than DIBA test even when polyclonal antiserum was used (Sherwood et al. 1987). But in the case of *Cherry mottle leaf virus* (CMLV), DIBA test employing MAbs or PAbs proved to be more efficient than TAS-ELISA or Western blot analysis in detecting CMLV. Young leaves and flowers of cherry were the most suitable tissues for CMLV detection (James and Mukerji 1996). *Lily symptomless virus* (LSV), *Tulip breaking virus-lily* (TBV-L) and *Cucumber mosaic virus* (CMV) were effectively detected in the scale segments of *Lilium* sp. (Nümi et al. 1999).

Enhancement of detection sensitivity of DIBA test may be achieved by using a dissecting microscope to observe the color development in the spots where the

reactants were immobilized. DIBA test could be performed using a chemiluminescent compound [disodium-3-(4-dimethoxyspirol) 1,2-dixoetane-3-2′-trichloro-(3,3,1,1) decan-(4,4,1) phenyl phosphate] or a chromogenic compound (nitroblue tetrazolium or 5-bromo-1-chloro-3-indobutyl phosphate) as a substrate to detect TSWV, *Bean yellow mosaic virus* (BYMV) and LSV in infected leaf extracts or purified virus preparations (Mansky et al. 1990; Chahal and Nassuth 1992; Makkouk et al. 1993). The *Grapevine rupestris stem-pitting-associated virus* (GRSPaV) was detected by using the antiserum against bacterially expressed coat protein. GRSPaV was detected in leaf petioles and cortical scrapings from dormant canes during the whole vegetative season. DIBA was found to be useful for virus detection during large scale field survey, since ELISA test was not effective in detecting GRSPaV (Minafra et al. 2000). The possibility of using plain paper in place of membranes for performing DIBA test was demonstrated in the attempt to simplify the DIBA test. This test offers several advantages over ELISA procedure that include requirement of only small volumes of reactants, ease of processing the samples, storing and transporting the membranes without loss of reactivity, reduction of test duration, simplicity and cost-effectiveness (Lange et al. 1991).

Florida hibiscus virus belonging to *Tobamovirus* genus was detected by applying DIBA test in sap extracted from *Chenopodium quinoa* with a dilution end point of 1:12,800. But the virus could be detected in extracts of hibiscus leaves and bark only at lower dilutions of 1:800 and 1:400 respectively. When partially purified virus preparations were used, the virus was detected by DIBA at a concentration of 125 ng/ml (Fig. 2.10). Comparison of the sensitivity of detection by DIBA with ELISA formats showed that DAS-ELISA and indirect ELISA formats were more sensitive than DIBA test for the detection of *Florida hibiscus virus*. However, DIBA test provides certain advantages over ELISA, especially when large number of samples is to be tested, as indicated earlier (Kamenova and Adkins 2004). *Soybean mosaic virus* (SbMV) belonging to the family *Potyviridae* was detected by DIBA test. Blotted membranes exhibited dark purple color following treatment with virus-specific antibodies and the substrate solution NBT/BICP, indicating the presence of SbMV in the samples tested (Fig. 2.11; Ahangaran et al. 2009).

The DIBA test was found to be useful in detecting *Banana bunchy top virus* (BBTV) and *Cucumber mosaic virus* (CMV) in naturally infected bananas, facilitating eradication of infected plants as a disease management strategy (El-Dougdoug et al. 2006). A rapid diagnostic assay applicable for the detection of CMV in both laboratory and field was developed. DIBA procedure was followed using nitrocellulose or PVDF membrane. The immobilized viral proteins could be analyzed by subsequent binding of specific antibodies and appropriate detection molecules. CMV was readily detectable in purified virus preparations and in crude sap of infected tobacco leaves by DIBA on PVDF membrane. The limits of detection were 128 ng/ml with purified virus preparation of Pepo-CMV and at a dilution of 10^5 of leaf extracts. The detectability level of M2-CMV was lower (3.2 μg/ml) than that of Pepo-CMV. DIBA test required only 2 μl aliquots and it could be applied for determining CMV concentrations higher than 128 ng/ml. Although DIBA test was less sensitive compared to TAS-ELISA format, it offers the advantages of simplicity,

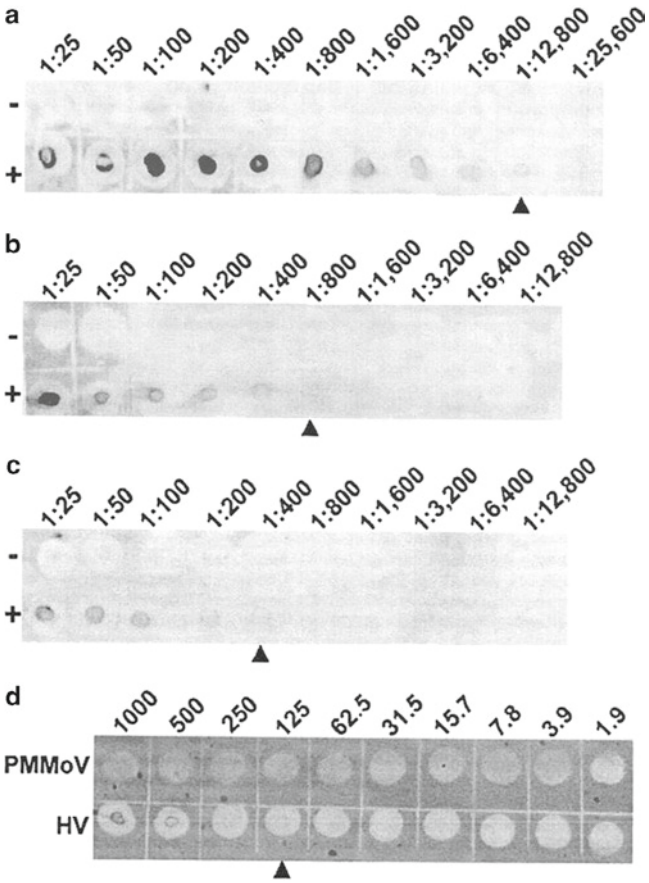

Fig. 2.10 Detection of *Florida hibiscus virus* (HV) by dot blot immunoassay (DIBA) in different plants and partially purified preparations. (**a**) End-point dilutions of extracts of uninfected (–) and infected (+) leaves of *Chenopodium quinoa*; (**b**) end-point dilutions of extracts of uninfected (–) and infected leaves of hibiscus; (**c**) end-point dilutions of extracts of uninfected (–) and infected bark tissues of hibiscus; (**d**) partially purified preparations of *Pepper mild mottle virus* (PMMoV) and *Florida hibiscus virus* (HV) in nannograms; *triangles* indicate the detection limits (Courtesy of Kamenova and Adkins 2004; The American Phytopathological Society, MN, USA)

applicability in the field conditions during surveys and storability of blots for long periods without loss of reactivity (Zein and Miyatake 2009; Appendix 4).

2.1.5.3 Tissue Blot Immunoassay

Viral antigens from freshly cut plant tissue surface are transferred to nitrocellulose membranes for performing tissue blot immunoassay (TBIA). Slight pressure is applied on the cut surface and thus a tissue imprint is made on the membrane. The unbound protein binding sites present in the membrane are blocked, using a blocking agent,

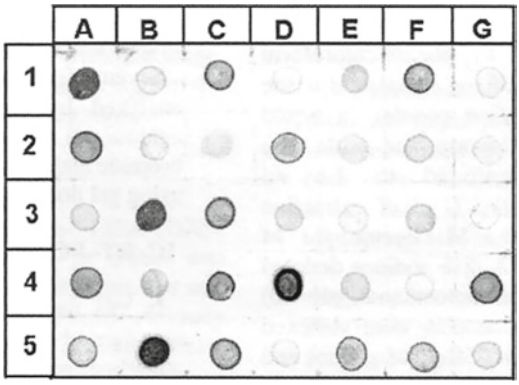

Fig. 2.11 Detection of *Soybean mosaic virus* (SbMV) by DIBA test in infected plant tissues after incubation of the blotted membranes in the substrate solution NBT/BCIP. Development of dark violet spots indicates positive reaction which can be viewed under a binocular microscope (Courtesy of Ahangaran et al. 2009; J Agricultural Science and Technology, Iran)

followed by probing the blots with specific antibodies generated against the target viruses labeled with enzymes. In the indirect method, the immobilized antigens are allowed to react first with primary antibodies specific to the virus and then with enzyme labeled secondary antibodies. This method does not require any sample preparation and reaction with host plant proteins can be avoided. Further, this method is simple, sensitive and fast, providing results in 3 h. The imprinted membranes can be stored for long periods and transported to laboratories where further processing can be done.

The direct tissue blotting immunoassay DIBA procedure was applied for the detection of *Tomato spotted wilt virus* (TSWV) in infected leaves of *Nicotiana benthamiana* and leaf and stems of *Eustoma (Lisianthus)* and *Impatiens* sp. plants showing virus-like symptoms (Hsu and Lawson 1991). TBIA test was employed to detect TSWV in *Ranunculus asiaticus* tubers and other ornamental plants using MAbs specific to NSs protein of the virus. Strong positive reactions (purple color development) were observed in the membranes with all infected plants viz., *R. asiaticus*, *Begonia* × *hiemalis*, *Chrysanthemum* spp. *Eustonia* sp. *Impatiens* sp. *Senecio cruentus*, *Tropaeolum* sp., *Datura stramonium* and *Emilia fosbergii.* The results indicated that TBIA is a reliable alternative to ELISA for detection of TSWV. There was a 96.5% agreement of results for the *Ranunculus* tuber samples. The viral NSs protein was stable on membranes and could be detected even after 84 days of storage. As TBIA and DAS-ELISA were equivalent in accuracy and reliability, the ease of sampling and processing makes TBIA a more suitable tool for growers and disease management advisors. Further, the tuber tissues were found to be the most suitable for detection by TBIA tests (Table 2.8). The assays can be performed in disposable bottles without any laboratory facilities. This possibility could enhance the efficiency of indexing and become an integral part of TSWV management programs (Whitefield et al. 2003; Appendix 5).

Table 2.8 Detection of *Tomato spotted wilt virus* (TSWV) in *Ranunculus asiaticus* by tissue blot immunoassay (TBIA) (Whitefield et al. 2003)

Percentage of infected rootlets [a,b]	Percentage of infected stems [b]	Number of tubers
100	100	31
99–75	100	26
74–50	100	14
49–25	100	13
24–1	100	13
0	0	62

[a] Wide variations (5–26) in the number of rootlets/tuber existed; so percentage of infected roots is presented
[b] TBIA was applied for all tuber materials

Several viruses *Apple chlorotic leaf spot virus*, *Prunus necrotic ringspot virus*, *Plum pox virus*, *Citrus tristeza virus*, *Tomato spotted wilt virus* and *Lettuce mosaic virus* were detected by employing direct- or indirect-TBIA format (Cambra et al. 1994). *Pineapple mealy bug wilt-associated virus* could be detected in infected plants (Sether et al. 1998). During a field survey, TBIA test was employed for the detection of *Pineapple closterovirus* (PCV). It was possible to test 2,000 samples by one person in 5 days, indicating the rapidity, reliability and amenability of TBIA for large scale testing (Hu et al. 1997). The effectiveness of TBIA procedure for testing *Garlic virus A* (Gar V-A) resulted in its recommendation for use in certification for virus-free garlic (Helguera et al. 1997). The presence of *Plum pox virus* (PPV) in different tissues of apricot was detected by TBIA procedure (Dicenta et al. 2000). A variant of TBIA, designated tissue print-ELISA was shown to be effective for the detection of *Citrus tristeza virus* (CTV) using genetically engineered scFv antibody proteins (Terrada et al. 2000).

Barley yellow mosaic virus type 2 (BaYMV-2) was detected by TBIA in the leaf samples from winter barley (Kuntze et al. 2000). In a later study, leaf and stem samples were tested by TBIA to detect *Barley mild mosaic virus* (BaMMV) and *Barley yellow mosaic virus* (BaYMV) in greenhouse-inoculated and field-collected plants. The stem tissue was found to be more suitable for direct blotting on nitrocellulose membranes, because it had no background material that could interfere in the reaction. Hence, this technique was designated direct stem blot immunoassay (DSBIA). The virus from infected leaf tissue could be detected, but color signal was not as clear as with those of stem tissue. Based on DSBIA test, out of seven fields with unclear symptoms, five fields were found to be infected by one or both viruses. The field with 90% leaf yellowing was positive for both BaMMV and BaYMV, indicating that DSBIA procedure was efficient in detecting these barley viruses in the field. For routine indexing of barley genotypes, DSBIA was suitable for providing reliable detection of the target virus(es). It was very economical to use, since it could test four to eight times more number of samples than ELISA test. However, for quantitative estimations, ELISA could give more precise results (Jonson et al. 2007).

Comparative efficacy of TBIA and ELISA techniques in detecting some plant viruses has been assessed. ELISA test was more efficient than TBIA procedure in detecting *Tuberose mild mosaic virus* (TMMV) which was widespread in Taiwan, whereas TBIA test was more sensitive of indexing tuberose bulbs stored at 5°C. TBIA detected TMMV in all infected bulbs (100%) as against detection of TMMV only in 30% of the infected bulbs by ELISA test (Chen and Chang 1999). *Florida hibiscus virus*, a *Tobamovirus* was detected readily in tissue blots from infected leaves of hibiscus and *Chenopodium quinoa*. Direct TBIA was evaluated by probing the blotted membranes with only virus-specific alkaline phosphatase-conjugated IgG. No difference was observed in the sensitivity between the direct and indirect TBIA procedures. The possibility of applying TBIA and ELISA tests for routine detection of the virus was assessed. The results indicated that TBIA was sufficiently sensitive for detection of *Florida hibiscus virus* and was effective with both leaf and bark samples. Further, TBIA provided information on the distribution of the virus in plant tissues/organs, in addition to being faster and less labor-intensive than ELISA formats. Hence, TBIA may be more suitable for large scale application in certification programs and during field surveys (Kamenova and Adkins 2004).

Direct TBIA protocol was applied for monitoring the movement and distribution of *Tomato spotted wilt virus* (TSWV) in *Capsicum* spp. (Soler et al. 1999). In another study, movement of *Potato leaf roll virus* (PLRV) from infected tubers to the leaves of potato plant was tracked by TBIA and ELISA techniques. TBIA was shown to be more effective for the detection of PLRV in the foliage of plants (secondary PLRV infection), but it was less accurate for detection of primary PLRV infection (Whiteworth et al. 2000). *Faba bean ncecrotic yellows virus* (FBNYV) was more efficiently detected than DAS-ELISA test by employing the bacterially-expressed CP protein for generating PAbs (Kumari et al. 2001). TBIA was found to provide results similar to that of RT-PCR assay for the detection of *Sugarcane yellow leaf virus* (ScYLV) (Chatenet et al. 2001).

Detection of viruses reliably and rapidly assumes greater importance for the management of viruses primarily spread by propagation of buds as in the case of *Citrus psorosis virus* (CPsV). Comparative efficacy of DTBIA, double (DAS)- and triple (TAS)-sandwich ELISA tests was assessed for the detection of CPsV using samples from various citrus varieties growing in the glasshouse and in the fields. In young shoots and leaves, CPsV was detected readily by all three methods. But DTBIA detection was less consistent when old leaves were tested. Selection of the correct tissue was critical for DTBIA detection. CPsV was consistently detected in tissue prints of young tender leaves or shoots. CPsV could be readily and specifically detected by ELISA any season and by DTBIA when young tissues were available. This would limit the use of DTBIA, when suitable tissue is not available particularly in winter season. DTBIA detection and ELISA readings in nine different citrus varieties were similar. In infected field trees CPsV was consistently detected by TAS-ELISA format, even in samples of old leaves in winter. Detection of CPsV by DTBIA, DAS- and TAS-ELISA tests in previously untested field trees correlated well with psorosis diagnosis by biological indexing. DTBIA has the advantage of being simpler, cheaper and faster than ELISA and

can provide sensitive and specific detection, if proper tissues are used for tests (Martin et al. 2002a; Appendix 6).

Transversely cut tender shoots or whole flowers or rolled old and young leaf blades of citrus trees infected by *Citrus psorosis virus* (CPsv) were gently pressed onto nitrocellulose membranes. After adopting standard steps of TBIA, the blotted membranes were incubated with alkaline phosphatase (AP) conjugated with As-Ps. Rc1 IgGs at a dilution of 1:500. Membranes were incubated in substrate solution of BCIP-NBT Sigma fast in distilled water for the development of purple color, indicating the presence of CPsV antigen. CPsV was readily detected in tissue samples from tender shoots, flowers and young leaves collected from spring, but not from hardened old leaves. The results obtained by TBIA method were similar to those of various ELISA protocols and immunosorbent electron microscopy. All these tests showed that As-Ps.Rc1 antiserum was specific in detecting CPsV in citrus samples, irrespective of the technique applied (Loconsole et al. 2006).

Application of a diagnostic assay becomes increasingly feasible, if the procedure is simplified. An in situ immunoassay (ISIA) developed for the detection of *Citrus tristeza virus* (CTV) involves essentially, fixation of stems, petioles or leaf veins of healthy and infected plants with 70% ethanol. Then the tissues are incubated with PAbs or MAbs followed by application of enzyme-conjugated with secondary antibodies and exposure to a substrate mixture consisting of nitroblue tetrazolium (NBT) and 6-bromo-4-chloro-3-indolyl phosphate (BCIP). Positive reaction was inferred by the development of a purple color that could be seen under a light microscope. Thus the presence of CTV antigen could be detected directly and precisely by ISIA in both fresh and in samples stored in plastic bags at 4°C or frozen for 4 weeks. ISIA providing reliable results comparable to DIBA is simple, rapid and specific. This protocol has the potential for large scale applications (Lin et al. 2000b). The mild and severe isolates of CTV infecting grapefruit trees were detected and differentiated by using ISIA test which was more sensitive compared to ELISA. CTV isolates were differentiated by employing isolate-specific MAbs (Lin et al. 2002).

Improvement in the sensitivity and reliability of detection of *Citrus tristeza virus* (CTV) was achieved by printing of fresh young stems of healthy and infected plants by gently and evenly pressing the fresh cut surface of the stems onto a nitrocellulose membrane. After air-drying for 5 min, the tissue blots are incubated with pre-reaction solutions of CTV-specific antibodies and labeled secondary antibodies, goat anti-mouse IgG (H + L)-alkaline phosphatase conjugate or goat anti-rabbit IgG-AP conjugate for up to 2 min, rinsed with PBST buffer for 5 min and immersed into NBT-BCIP substrate solution for 15–20 min. After rinsing the blots in water for a few seconds, the reaction results are recorded by observations under a light microscope. This protocol allowed detection of both CTV + decline-inducing isolate T-36 by employing a CTV-specific PAb PCA1212 and MAbs 17G11 and MCA 13. The reliability of this procedure was similar to that of PCR for detecting CTV in field samples, but this test provided the results within 1 h because of the use of a pre-reaction of CTV-specific antibodies and labeled secondary antibodies in solution prior to their application to the tissue blots (Lin et al. 2006).

Sugarcane yellow leaf virus (ScYLV) could be detected by employing MAbs 3A2E3 and 2F7H5 in TBIA test (Korimbocus et al. 2002). Bacterially expressed CP protein (BE-CP) of *Faba bean necrotic yellows virus* (FBNYV) was used to generate PAbs. By using the PAbs, FBNYV was detected reliably, as the PAbs gave strong specific reaction in TBIA test. The background reaction with control tissues was very weak providing significant contrasting color reaction comparable to that was obtained with MAbs specific to FBNYV. Thus the need for generating MAbs requiring long time could be avoided (Kumari et al. 2001). The TBIA procedure was applied to track the distribution of *Turnip mosaic virus* (TuMV) and its chimeric viruses in the cotyledons and leaves of cabbage and radish at 12 days post-inoculation. While chimeric viruses showed very low systemic infectivity. TuMV was found to move systemically to uninoculated leaves (Suehiro et al. 2004). Hammerblotting technique, another simple technique, was also shown to be useful for the detection of TuMV infection in two ecotypes of *Arabidopsis thaliana* (Co1.0 and Ler). The patterns of distribution of this virus differed in these ecotypes of *A. thaliana* (Kaneko et al. 2004).

A necrotic strain of *Potato virus Y* (isolate T01) causes necrotic symptoms in older leaves of systemically infected tobacco plants, but not in younger leaves. But in tobacco plants doubly inoculated with *Cucumber mosaic virus* (CMV) along with PVY, distinct symptoms developed in the young leaves also. Tissue blot immunoassay (TBIA) test was applied to visualize the distribution of viral antigen in cross-sections of stems and petioles. PVY was detected at high levels in almost all sections including apical tissues in doubly infected tobacco plants. But PVY was not detected in the shoot apex of plants infected only by PVY. In contrast, CMV was detected in all sections of both singly and doubly infected plants. This investigation indicated that the systemic spread of PVY was assisted by 2b protein of CMV which facilitated PVY to spread to the apical tissues. In the absence of CMV, PVY was restricted to the lower leaves of tobacco (Ryang et al. 2004). The International Center for Agricultural Research in the Dry Areas has developed diagnostic kit based on TBIA which was shown to be efficient in detecting 19 viruses infecting legumes. As TBIA protocol is simple, sensitive, rapid and less labor-intensive compared to ELISA, this procedure was recommended for field survey, enabling the personnel to analyze 1,000–2,000 samples per day (Webster et al. 2004). Tissue print immunoassay (TPIA) was employed for the detection of *Soybean mosaic virus* in infected soybean plants. The tissue prints on nitrocellulose membranes were treated with antibody–enzyme conjugate followed by incubation with substrate solution containing NBT/BCIP or Fast red. The processed membranes were examined under a light microscope. Development of color indicating positive results could be visualized clearly (Ahangaran et al. 2009).

2.1.5.4 Immunoblot Assay

Immunoblot analysis is based on the transfer of electrophoresed viral proteins from gel matrix onto a membrane and allowing access subsequently for specific

immunoprobes. This technique permits detection of viral capsid protein or nonstructural proteins present in the infected plants. *Passionfruit woodiness virus* (PWV) causes serious losses in production in Taiwan. This virus belonging to *Potyviridae*, produces a 51-k protein that forms the major component of amorphous, cylindrical inclusion proteins (CIPs) (67–73-k) and two nuclear inclusion (NI) proteins (54- and 49-k) in the infected cells. Purified PWV coat protein (CP), CIP and amorphous inclusion protein (AIP) were used for immunoblotting. The antiserum against CP, CIP and AIP reacted only with their homologous antigens and no cross-reactions were observed in heterologous combinations. Further, the antisera only reacted with homologous antigens in crude sap of PWV-infected golden passionfruit and *Nicotiana benthamiana* plants. The results confirmed that the AIP was a specific protein of PWV-infected plant tissue and this protein was serologically distinct from CP and CIP of this virus. In addition, the antiserum to PWV-AIP did not react with the crude sap from tissues infected with any of 14 potyviruses tested, indicating the specificity of antibodies that could be employed for specific detection of PWV (Jan and Yeh 1995).

The linear continuous epitope for an MAb that distinguished Florida quick decline-inducing (DI) isolate of *Citrus tristeza virus* (CTV) from mild isolates was expressed in *Escherichia coli* cells as a peptide fused to a maltose-binding protein (MBP). PAbs were produced using this protein and CP fusion protein containing amino acids (aa) 118–228. The proteins extracted from sweet orange bark tissue infected by *Citrus tristeza virus* (CTV) were subjected to electrophoresis in 8–20% gradient PAGE in a discontinuous Tris-glycine-SDS system. The separated proteins were transferred to nitrocellulose membranes and then they were probed with different MAbs and PAbs. Both antibodies readily reacted with severe isolate (T36) and distinguished it from mild isolates (T30 and T55) of CTV (Fig. 2.12; Nikolaeva et al. 1996).

A procedure was developed to purify rapidly and easily a sufficient quantity of native p25 coat protein (CP) of CTV to allow comparison of five isolates of CTV by serological analysis of peptide maps using both MAbs and PAbs. The CTV-CP peptides were electroblotted to polyvinylidene difluoride (PVDF) membrane. Specific MAbs were allowed to react with immobilized peptides. The migration of bands was measured and the color intensity was estimated in comparison to a 0–4 scale (0-indicating no color and 4-indicating a dark blue color). Some of the CTV isolates were indistinguishable by indirect DAS-ELISA, ds RNA pattern and biological characteristics. But the serological analysis of peptide maps allowed accurate comparison of CTV isolates and proved to be superior to other techniques for discriminating CTV isolates (Albiach-Marti et al. 2000).

Citrus psorosis virus (CPsV) is attributed to the psorosis disease causing serious losses. The coat protein (CP) gene of an Italian virus isolate was cloned and sequenced. The CP sequence was expressed in *E. coli* BL21 as a fusion protein with maltose-binding protein (MBP). Antibodies to this recombinant protein were generated and employed in Western blotting method at a dilution of 1:1,000. A protein band of expected size for CPsV coat protein (49-kDa) was detected in total protein extracts from CPsV-infected *Chenopodium quinoa* and also in virus preparation

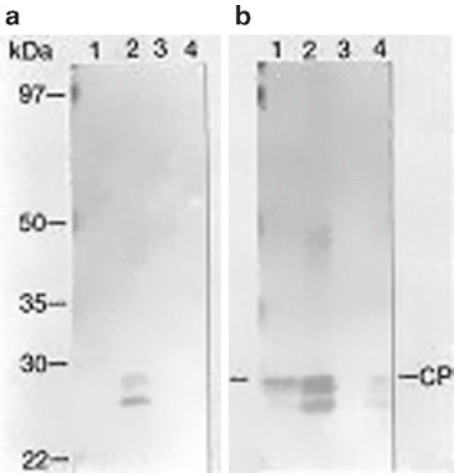

Fig. 2.12 Detection of coat protein of *Citrus tristeza virus* (CTV) in extracts of infected sweet orange bark tissue by immunoblotting technique. (**a**) Immobilized proteins treated with PAb against CP fusion protein containing amino acids 118–228; (**b**) immobilized proteins treated with PAb against maltose-binding protein-CP fusion protein. *Lane 1*, mild isolate T55; *Lane 2*, decline-inducing isolate T36; *Lane 3*, healthy plant tissue; and *Lane 4*, mild isolate T30; marker proteins are indicated to reveal relative positions of target proteins (Courtesy of Nikolaeva et al. 1996; The American Phytopathological Society, MN, USA)

purified from leaves of *C. quinoa* inoculated with CPsV ps110 isolate. There was no reaction with extracts from healthy *C. quinoa* leaves, indicating the specificity of antibodies to CPsV CP fusion protein (Loconsole et al. 2006; Appendix 7).

Using Western blot analysis with PAbs and MAbs, the P1 protein and a protein of approximately 25 kDa (P1-C25) of *Potato leaf roll virus* (PLRV) accumulating readily in sufficient levels in PLRV-infected plants could be detected. P1-C25 represents the C-terminus of P1 and it is a proteolytic cleavage product produced during P1 processing (Prüfer et al. 1999). Infections of plants by two viruses (mixed infection) may be recognized by performing immunoblot analysis. Infection by serologically related and unrelated *Grapevine leaf roll-associated viruses* (GLRaVs) and *Grapevine bark-associated virus* (GeBaV) results in complex symptoms. The components of the complex disease of grapevine were resolved by applying immunoblot technique (Monis and Bestwick 1997).

The strains of *Soybean dwarf virus*, dwarfing (SbDV-D) and yellowing (SbDV-Y) were characterized by employing Western blot analysis, using SbDV-D polyclonal antisera. The CPs of isolates MD-2 and VA-20 were very similar to SbDV-D and SbDV-Y, but not to *Bean leaf roll virus* (BLRV) or *Beet western yellows virus* (BWYV). The SbDV-D, SbDV-Y and VA-20 isolates produced distinctive 51- to 52-kDa band that may represent the readthrough protein. The less intense 51- to 52-kDa band and two additional 44- and 31-kDa bands seen with MD2 isolate could possibly be the result of proteolytic cleavage of the readthrough protein during isolation (Fig. 2.13; Damsteegt et al. 1999). An uncharacterized virus infecting cucumber plants was investigated.

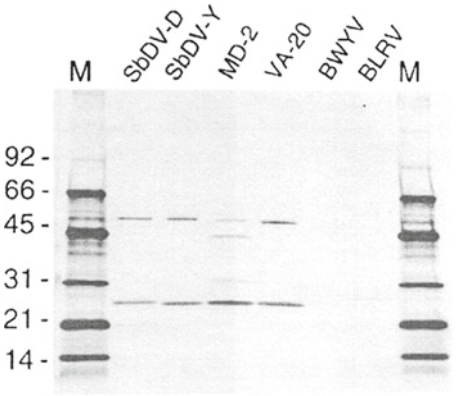

Fig. 2.13 Detection of *Soybean mosaic virus*-dwarfing (SbMV-D) by immunoblot analysis. M, molecular weight markers; SbDV-Y, *Soybean dwarf virus*-yellowing; MD-2, isolate from Maryland; and VA20, isolate from Virginia; *Lanes 1* and *2*, type strains; *Lanes 3* and *4*, endemic isolates; *Lanes 5* and *6*, *Beet western yellows virus* (BYWV); and *Bean leaf roll virus* (BLRV) respectively (Courtesy of Damsteegt et al. 1999; The American Phytopathological Society, MN, USA)

In Western blot analysis, antiserum against *Tomato mosaic virus* (ToMV) reacted strongly with its homologous CP and cross-reacted strongly with the CPs of *Cucumber fruit mottle mosaic virus* (CFMMV) and *Pepper mild mottle virus* (PMMV), but weakly with *Cucumber green mottle mosaic virus* (CGMMV-Is). Antisera against CV4 and CGMMV-Is reacted specifically with the CP band of CGMMV-Is, whereas the antisera against PMMV and CFMMV reacted only with their homologous virus respectively (Antignus et al. 2001).

Grapevine leaf roll-associated virus 2 (GLRaV-2) is a member of the genus *Closterovirus*. GLRaV-2 has a positive sense single-stranded RNA genome that consists of eight open reading frames (ORFs). ORF1b encodes RNA-dependent RNA polymerase (RdRp) and ORF6 encodes the viral coat protein (CP). Bacterially expressed proteins were used to produce specific antisera against RdRp and CP of GLRaV-2. In Western blots, antisera against recombinant RdRp diluted to 1:5,000 or recombinant CP diluted to 1:10,000 reacted strongly with homologous proteins expressed in *Escherichia coli.* The antisera were able to recognize the corresponding recombinant proteins in Western blots. CP antiserum was able to detect infection by GLRaV-2 in all samples, whereas antiserum to RdRp detected the virus in most of the samples obtained from petioles of grapevine plants (Xu et al. 2006).

Two ampeloviruses serologically distinct from all grapevine leaf roll-associated viruses were detected in two Greek grapevine varieties Prevezamko (isolate GLRaV-Pr) and Debina (isolate GLRaV-De). The isolate *Grapevine leaf roll-associated virus*-Pr (GLRaV-Pr) was investigated to determine its molecular identity. A virus-specific antibody was raised against the recombinant CP protein of GLRaV-Pr by immunizing the New Zealand rabbit. The antiserum was employed in Western blotting. A band of ca. 37-kDa recognized by the antiserum was consistently detected in all preparations from GLRaV-Pr-infected plants. Such a band was not detectable in extracts from

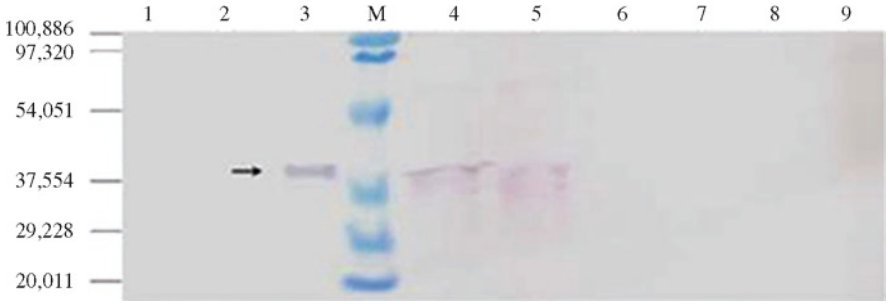

Fig. 2.14 Detection of *Grapevine leaf roll-associated virus*-Pr (GLRaV-Pr) by Western blot analysis. *Lane 1*, GLRaV-6-infected grapevine cv. Skiadopoulo; *Lane 2*, GLRaV-4-infected grapevine cv. Robolla; *Lane 3*, purified GLRaV-Pr coat protein; *Lane 4*, GLRaV-Pr-infected grapevine cv. Mantilaria (clone M3); *Lane 5*, GLRaV-Pr-infected grapevine cv. Prevezaniko; *Lane 6*, healthy grapevine cv. Kontokladi; *Lane 7*, GLRaV-5-infected cv. Fraoula; *Lane 8*, GLRaV-9 infected grapevine cv. unknown; *Lane 9*, GLRaV-De-infected grapevine cv. Kolokithas; *Lane M*, prestained low-range standard markers with different molecular masses (Courtesy of Maliogka et al. 2009 and with kind permission of Springer Science and Business Media, Heidelberg, Germany)

healthy grapevine tissues or other tissues singly infected with related GLRaV-4, -5, -6 and -9 viruses. Western blot analysis showed that the pattern expressed from the CP coding ORF (ORF5) had an electrophoretic mobility indistinguishable from the GLRaV-Pr CP expressed from infected tissues (Fig. 2.14). The genome of GLRaV-Pr has been entirely sequenced and this is the first fully sequenced virus of the subgroup I grapevine lineage. This virus was considered to be a new virus species within the genus *Ampelovirus* (Maliogka et al. 2009; Appendix 8).

Virus-associated proteins in infected plants may be detected and identified by employing Western blot analysis. Plants infected by *Potato virus Y* (PVY) contain cytoplasmic inclusions. The presence of P1 protein associated with inclusion bodies could be detected by Western blot technique (Arabatova et al. 1998). The genome of *Beet yellows virus* (BYV) encodes a 295-kDa polyprotein with domains of papain-like cystein proteinase, methyl transferase (MT) and helicase (HEL). Monoclonal antibodies (MAbs) specific to MT and HEL were produced by injecting the mice with bacterially expressed fragments of BYV-1a product encompassing the MT and HEL domains. Four MAbs specific for MT recognized a 63-kDa proteins and two MAbs specific to HEL, reacted with a 100-kDa protein on immunoblots prepared using the extracts of BYV-infected *Tetragonia expansa* plants. Both MT-like and HEL-like proteins were associated with mainly in the fractions of large organelles (PI) and membranes (P30) of the infected plants. These virus-related enzymes seem to be associated with membrane components in the host cells (Erokhina et al. 2000).

A new potyvirus infecting chickpea in Bolivia was characterized and identified by Western blot analysis as *Chickpea yellow mosaic virus* (CYMV). The total protein sample preparations of CYMV were probed with the *Potyvirus* MAb on immunoblots. After silver staining the gel, a single band of ca. 32 kDa was identified in the virus

protein preparation purified from chickpea. CYMV was considered as a distinct member of *Potyvirus*, since no positive reaction was observed with antisera raised against five members of this family (Larsen et al. 2003). Infection of *Phalaenopsis* orchid by a potyvirus was revealed by electron microscopic observations on the ultrathin sections of infected leaves. Potyvirus-like long flexuous filamentous particles were visible in the infected cells, in addition to the presence of pinwheel inclusions characteristic of potyvirus infection. Leaf extracts of mock-inoculated and virus-infected *Chenopodium quinoa* were separated by SDS-PAGE technique and then transferred to membranes. Following incubation with antiserum containing virus-specific PAbs and treatment with alkaline phosphatase (AP) conjugated IgG, color development was recorded after treatment with chromogenic substrates. The presence of a band representing a 34-kDa CP protein was observed in the immunoblots (Fig. 2.15). The virus infecting *Phalaenopsis* orchid was detected and identified as *Phalaenopsis chlorotic spot virus* (PhCSV) (Zheng et al. 2008a).

Bacterially expressed coat proteins (CPs) have been used to generate polyclonal antisera against Begomoviruses such as *Bean golden mosaic virus*-Brazil isolate (BGMV-B), *Cabbage leaf curl virus* (CabLCV), *Tomato yellow leaf curl virus* (TYLCV) and *Tomato mottle virus* (ToMV). The PAbs so raised could be employed in different immunoassays. The antisera to BGMV, TYLCV and ToMV reacted specifically with target antigens in Western blots as well as in leaf imprint blots (Abouzid et al. 2002). Likewise, the PAbs generated against the bacterially expressed CP of *Barley yellow dwarf virus* (BYDV-PAV) were employed for the detection of this virus. In Western blot analysis the PAbs specifically reacted with

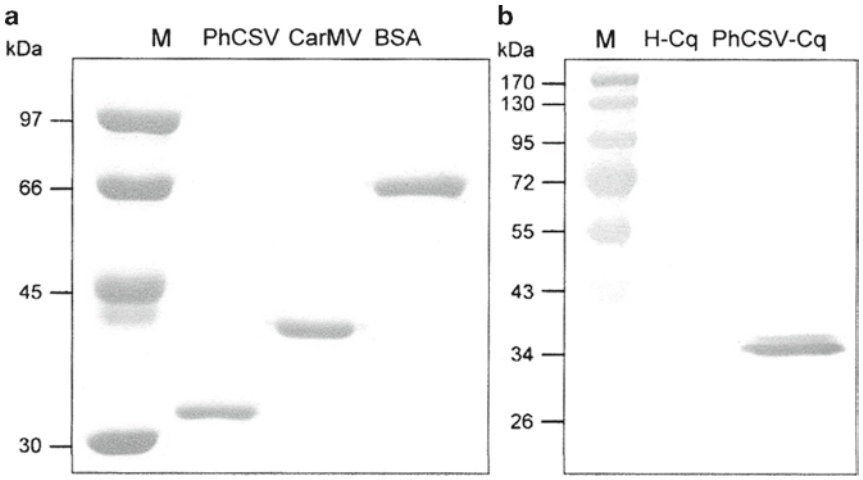

Fig. 2.15 Detection of *Phalaenopsis chlorotic spot virus* (PhCSV) by SDS-PAGE and Western blot analysis. (**a**) The structural protein present in purified preparation of PhCSV (~34 kDa), *Carnation mosaic virus* (CarMV) (~40 kDa) and bovine serum albumin (BSA) (76 kDa). (**b**) Note the presence of a protein (~34 kDa) present in the leaf extracts from PhCSV-infected *Chenopodium quinoa* (Cq) plants, but not in healthy plants (H-Cq) (Courtesy of Zheng et al. 2008a and with kind permission of Springer Science and Business Media, Heidelberg, Germany)

viral antigens present in the total protein extracts from BYDV-infected plants and recognized the 99-kDa fusion protein expressed from ORF1 and 2 of the viral genome (Formitcheva et al. 2005).

The genus *Tospovirus* is included in the family *Bunyaviridae* containing viruses infecting plants and animals including human beings. *Watermelon silver mottle virus* (WSMoV) has enveloped virions which are quasi-spherical in shape. A non-structural NSs protein encoded from the viral strand of WSMoV SRNA forms filamentous inclusion bodies in the cytoplasm of infected cells. The NSs protein of WSMoV was expressed by a *Zucchini yellow mosaic virus* (ZYMV) vector in squash. The antiserum produced against the NSs protein strongly reacted with the NSs crude antigen of WSMoV. The rabbit antiserum and mouse MAbs produced against ZYMV-expressed WSMoV NSs protein reacted differently with various tospoviral NSs antigens. In Western blots, all three MAbs reacted with the NSs proteins of members of the WSMoV serogroup including WSMoV, *Capsicum chlorosis virus* (CaCV) and *Calla lily chlorotic spot virus* (CCSV). The results indicated that the three MAbs could recognize common epitopes present on the NSs molecules of the WSMoV serogroup and that CaCV was closely related to WSMoV and distantly related to CCSV, yet they shared common antigenic determinants revealed by MAbs (Chen et al. 2006).

The purified recombinant protein obtained by expressing the NSs protein of *Tomato spotted wilt virus* (TSWV) in *Escherichia coli*, was analyzed by Western blotting technique. The viral protein was transferred to a nitrocellulose membrane, using a Trans-blot SD Semi-Dry transfer cell. Anti-TSWV MAb1 (1:1,000) or anti-His tag MAb (1:1,000) was employed. The MAbs reacted strongly with TSWV N protein with a MW of ca. 29-kDa and recombinant TSWV N protein with a MW of 48-kDa. No reactive bands could be seen in the negative controls including healthy plant leaves or cell lysates of *E. coli* (Wu et al. 2009). Occurrence of a tomato strain of *Parietaria mottle virus* (PMoV) belonging to the genus *Ilarvirus*, family *Bromoviridae* was observed in Italy, Greece, France and Spain. The complete CP gene of PMoV-AC1 isolate was cloned in pETDuet.1 protein expression plasmid to obtain the PMoV CP sequence fused in frame to an N-terminal hexahistidine tag (6 × His). The recombinant 6 × His-CP was expressed in *E. coli* BL21(DE3) cells. The purified recombinant protein was used for producing PAbs in rabbits. This polyclonal antiserum consistently detected PMoV specifically in extracts of tomato leaves by indirect (I)-ELISA and direct tissue-printing immunoassay (DTBIA). PMoV could be detected at a dilution of 1:10,000 of leaf extracts. The suitability of the antiserum for detection of PMoV in field-grown tomato plants was tested. All samples that were I-ELISA-positive, were also positive for DTBIA. However, DTBIA test provided satisfactory results with fresh plant tissues only. Healthy control samples stored at −20°C for long periods gave a background that may lead to false interpretations (Aparicio et al. 2009a).

Phalaenopsis orchid plants were infected by a virus inducing chlorotic ringspots on leaves. Evidence for a tospovirus being the cause of the disease was provided by examination of ultrathin sections under electron microscope. Immunoblot analysis of

total proteins of infected leaves was performed (Appendix 9). PAbs specific to the orchid virus and PAbs specific *Watermelon silver mottle virus* (WSMoV) reacted strongly with crude sap of infected leaves. A protein band measuring about 30-kDa was recognized in Western blotting using the antisera of WSMoV or the orchid isolate. The virus was identified as an isolate of *Capsicum chlorosis virus* (CaCV) belonging to *Bunyaviridae* family, based on the sequence analysis of viral N gene determined from a cDNA clone (Zheng et al. 2008b).

2.1.5.5 Cytofluorimetric Technique

The cytofluorimetric technique is based on the translation of biological properties into measurable fluorescence intensity. Plant virus particles in plant extracts are adsorbed onto latex particles and the fluorescence intensity is assessed by flow cytometry. *Cucumber mosaic virus* (CMV) present in plant samples is incubated successively with rabbit anti-CMV antibodies and anti-rabbit IgG labeled with fluorescein. The virus particles are detected by the laser of the cytometer. The limit of detection of the virus is 10 pg/ml compared to 2.5 ng/ml required for ELISA test. By using latex particles with different sizes and differential fluorescent dyes, it is possible to detect simultaneously two or more number of viruses present in plant extracts. *Cucumber mosaic virus* (CMV), *Potato virus Y* (PVY) and *Tomato mosaic virus* (ToMV) could be detected by sensitizing latex particles of 3, 6, 10 μm diameter separately with virus-specific antibodies (Iannelli et al. 1996, 1997).

2.1.5.6 Immunochromatographic Assay

Many sensitive and specific diagnostic techniques are available for the detection of viruses in seeds and planting stocks. Yet their utility is restricted due to the requirement of expensive equipments and reagents and qualified personnel. Hence, large amounts of cultivated and imported vegetables, berries, ornamental and horticultural crops are still beyond the scope of phytosanitary and quarantine regulations. In order to address this problem, an immunochromatographic assay (ICA) was developed based on the interaction between the target virus and immunoreagents (antibodies and their conjugates with colored colloidal particles). The test strip is dipped into the sample extract suspension. Then the sample liquid flows through membranes and triggers immunochemical interactions resulting in visible coloration in test and reference lines. The main advantages of this approach are its high sensitivity and ease of both sample preparation and analysis.

Immunochromatographic test system was applied for rapid detection of plant viruses with varying shapes and sizes of virions such as spherical *Bean mild mosaic virus* (BMMV), *Carnation mottle virus* (CarMV), rod-shaped *Tobacco mosaic virus* (TMV) and filamentous *Potato virus X* and *Potato virus Y* (PVX, PVY).

Multimembrane composites (test strips) with immobilized PAbs specific to target viruses and colloidal gold-conjugated antibodies were used for the analysis. The immunochromatographic test strips were able to detect the viruses both in purified preparations and in leaf extracts of infected plant species with a sensitivity ranging from 0.08 to 0.05 μg/ml for 10 min. ICA could be used for rapid diagnosis of virus diseases under field conditions also (Byzova et al. 2009).

2.1.5.7 Microscopy-Based Immunoassays

Both light and electron microscopes have been employed to visualize the interaction between antigen and antibody in the immunological reactions. Virus-specific antisera are used to enhance the specificity and sensitivity of the detection of the target virus.

Light Microscopy

Viruses belonging to the genus *Potyvirus* induce the formation of amorphous inclusion (AI) and cylindrical inclusion (CI) in the cytoplasm and two kinds of intranuclear inclusions (small and large) in the nuclei of cells of infected tissues. An antiserum was produced against AI proteins after purification by SDS-PAGE technique. A light microscopic immunostaining method was employed to verify, if the 51-kDa AI protein was detectable in the epidermal cells of passionfruit plants infected by *Passionfruit woodiness virus* (PWV). When the epidermal strips were allowed to react with the antiserum to the 51-kDa protein, the cytoplasmic AIs of infected cells were positively immunostained. The results indicated that the major location of the 51-kDa protein is at the cytoplasmic AIs in the PWV-infected cells (Jan and Yeh 1995; Appendix 10).

Immunohistochemistry was applied for the detection and determination of the distribution of *Potato virus Y* (PVY) in tobacco plants infected singly by PVY and doubly with *Cucumber mosaic virus* (CMV). PVY antigen was confined to external phloem cells and was not detected in internal phloem cells in singly infected tobacco plants. However, PVY was uniformly distributed in whole tissues including the external phloem, xylem parenchyma and internal phloem cells in doubly infected cells. The results indicated that CMV assisted the accumulation of PVY in stem tissues which could be associated with enhanced spread of PVY into younger tissues (Ryang et al. 2004; Appendix 11).

Leaf-Dip Serology

Leaf-dip serology is based on the interaction between virus particles released from the cut end of the plant tissue and the antibodies specific to the target virus in the antiserum kept on the filmed electron microscope grid. The antiserum is diluted with 0.001 M ammonium acetate solution to produce a 1:1,000 dilution. A drop of this antiserum is placed on a carbon-backed collodion film on a specimen grid. Pieces of

leaf tissue (1.5–2.0 × 0.5 cm) are cut from diseased leaves. The narrowest end is drawn through the antiserum drop for 1 or 2 s. The drops are then air-dried and a drop of solution containing 1 part of 1% vanadatomolybdate, pH 3.0 + 3 parts of 2% potassium phosphotungstate is used to stain the virus–antiserum mixture and then dried. *Tobacco mosaic virus* and *Barley stripe mosaic virus* were detected in infected leaves by this procedure (Ball 1971). The serological relationship between strains of elongated virus could be determined by this simple test (Langenberg 1974).

Immunoelectron Microscopy

Clarity in visualization and sensitivity and specificity of detection was increased by the development of immunosorbent electron microscopy, decoration and gold-labeling techniques which are being widely used, because of the many advantages offered by these methods.

Immunoelectron microscopy (IEM) may be employed to detect the structural and nonstructural viral proteins in infected plant tissues. Electron microscopy technique was applied to visualize the reaction between *Tobacco mosaic virus* (TMV) and the MAbs in different ELISA formats. The use of the buffer at pH 9.6 during the coating of ELISA plates resulted in dissociation of virions into subunits which bound preferentially to the solid phase. The MAbs that reacted with both virions and subunits in ELISA bound to one of the two extremities of viral rods. In contrast, MAbs reacting only with virions in ELISA were found to bind over the entire surface of the virus particles (Dore et al. 1988). Barley yellow mosaic disease is caused by *Barley mild mosaic virus* (BaMMV) and *Barley yellow mosaic virus* (BaYMV) which are transmitted by the fungal vector *Polymyxa graminis*. The plants of barley cultivar Tokyo carrying the resistance gene *rym5* showed mosaic symptoms. In order to confirm the identity of the virus inducing the symptoms, decoration technique was applied using the antiserum specific to BaMMV and BaYMV. Electron microscopic observations showed that BaMMV present in the barley plants was decorated by the antibodies specific to BaMMV and the virus particles were clearly visible. In contrast, with BaYMV antibodies, the virus particles of BaMMV remained undecorated, indicating the specificity of antibodies to these related viruses. In addition, the results showed that barley cultivars with *rym5* gene may not provide protection to BaMMV (Habekuss et al. 2008).

Immunosorbent Electron Microscopy and Gold Labeling Techniques

Immunosorbent electron microscopy (ISEM) earlier named as serologically specific electron microscopy (SSEM) by Derrick (1972, 1973), has been used for detecting the presence of target viruses in situ (Roberts and Harrison 1979). The grids are coated with Formvar (polyvinyl formaldehyde) strengthened with a layer of evaporated carbon. Crude antiserum raised against the target virus or fractionated α-globulins may be employed to sensitize the coated grids. The grids coated with specific antibodies are

treated with virus suspension at appropriate dilution and incubated at room temperature. The virus particles adsorbed to the grids are stained with 2% uranyl acetate and observed under electron microscope. Several viruses have been detected rapidly in purified virus preparations and in crude plant tissue extracts.

The sensitivity and effectiveness of ISEM may be enhanced to some extent by coating the grids with protein A obtained from *Staphylococcus aureus* which has specific affinity of binding to the Fe portion of the IgG molecule. A significant increase in the number of virus particles trapped by protein A coated grid was observed (Gough and Shukla 1980). This procedure will be effective for the detection of viruses present in low concentrations. ISEM technique offers some advantages such as direct visualization of viruses in the tissues or extracts, rapidity of providing the results, sensitivity comparable to and even better than ELISA test, possibility of using unfractionated crude antiserum, requirement of only very small volume of antiserum or antigen samples (5–10 μl), noninterference by antibodies to host proteins and the possibility of establishing relationship of viruses.

The presence of *Onion yellow dwarf virus* and *Carnation latent virus* infecting garlic was efficiently detected by ISEM in tissue-culture derived garlic plants, demonstrating the potential usefulness of this technique for production of virus-free garlic stocks (Maeso et al. 1997). Further, *Garlic virus A* was also detected by ISEM technique, employing the antiserum against virus CP (Helguera et al. 1997). Extensive infection of sugarcane germplasm entries by *Sugarcane bacilliform virus* was revealed by ISEM (Viswanathan and Premachandran 1998). For the detection of *Cymbidium mosaic virus* (CyMV) isolates, ISEM was found to be more sensitive than ELISA test. Detection of CyMV by ISEM was shown to be two times more sensitive in detecting the virus in the crude sap of infected orchid leaves than ELISA procedure (Hsu et al. 1992). In an another investigation, ISEM could detect CyMV in 74.43% of orchid plants suspected to be infected, whereas biological indexing and electron microscopy procedures showed positive results only in 51.43% and 42.86% respectively for CyMV infection in orchid plants (Vejaratpimol et al. 1999).

The involvement of an *Ampelovirus* species, *Pineapple mealybug wilt-associated virus 2* (PMWaV-2) was investigated to establish the identity of the etiology of one of the most destructive diseases of pineapple. The relationship between an undescribed *Ampelovirus* sharing highest homology with PMWaV-1 and a putative deletion mutant sharing highest homology with PMWaV-2 was established by ISEM and reverse transcription (RT)-PCR assay (Sether et al. 2005a). ISEM technique was employed to detect PMWaV-1, -2 and -3 in pineapple germplasm collections. All the three viruses were detected by ISEM and the results were similar to those obtained with TBIA and RT-PCR tests. In addition, PMWaV-5 was also detected in three accessions (Gambley et al. 2008).

A Nigerian isolate of *Banana streak virus* (BSV) was detected in greater number of asymptomatic BSV-infected plants by ISEM than by triple antigen sandwich (TAS)-ELISA format (Thottappilly et al. 1998). In a later investigation, ISEM procedure was shown to be effective in detecting BSV in various plant samples obtained from different locations in Uganda where the virus caused serious losses to banana production (Harper et al. 2002). ISEM technique was applied for the efficient detection of

Citrus psorosis virus (CPsV) in cross-protected cirus plants (Martin et al. 2004). In a later study, CPsV was extracted from infected leaves of *Chenopodium quinoa.* Electron microscope grids were sensitized by floating them on drops of crude antiserum and then they were floated on plant extracts at 4°C overnight. The adsorbed virus particles were stained with 2% uranyl acetate and examined under the electron microscope. The CPsV particles trapped from crude plant extracts were observed in infected leaves of *C. quinoa* (Loconsole et al. 2006).

The occurrence of an unknown virus belonging to the family *Luteoviridae* infecting chickpea and other food legumes in Euthopia was investigated. A rabbit antiserum raised against the virus preparation cross-reacted with *Beet western yellows virus*-like viruses. ISEM with purified *Chickpea chlorotic stunt virus* (CpCSV) or extracts of infected leaves was carried out using a mixture of antisera to CpCSV-FB and *Turnip yellows virus* (TuYV). Trapping of virions from CpCSV-FB- and TuYV-infected faba bean and oilseed rape plants was allowed. Distinct differences in the reactions between virions and homologous and heterologous antisera were noted (Abraham et al. 2006).

ISEM and decoration techniques were performed for the detection of and assessment of serological relationship between *Artichoke yellow ringspot virus* (AYRSV) and other viruses belonging to the genus *Nepovirus.* The crude sap extracts of *Nicotiana benthamiana* plants infected with the onion isolate of AYRSV did not react with the antisera produced against any of the 18 nepoviruses tested. Sequence analysis produced evidence that the nepovirus AYRSV was the causative agent of the disease affecting onion crops (Maliogka et al. 2006).

Decoration Technique

A combination of ISEM and decoration provides greater resolution of the target virus particles, facilitating the reliable identification of viruses under investigation. This procedure in principle, consists of trapping the virus particles on sensitized grids followed by staining of the viruses with a negative stain and incubation with the same or a different antibody to give a visible antibody halo (decoration). The decoration may be further improved with gold particles conjugated to a secondary antibody or protein A (PA) (Milne 1972). The decoration technique may be applied both for detection and quantification of viruses present in the samples. In addition, detection of virus mixtures and partially degraded coat protein may also be possible by employing decoration technique.

Grapevine virus A (GAV) and another virus with filamentous, flexuous particles that was mechanically transmissible to *Nicotiana benthamiana* and *N. occidentalis* were detected rapidly by decoration with antibodies raised against GAV and *Grapevine B virus* (GBV) respectively (Credi 1997). *Shallot yellow stripe virus* (SYSV) and *Welsh onion yellow stripe virus* (WoYSV) were shown to be serologically related by decoration technique using MAbs raised against SYSV as well as the antisera to *Onion yellow dwarf virus* (OYDV) and *Leek yellow stripe virus* (LYSV). SYSV was identified as a distinct member of *Potyvirus*, while WoYSV was considered

as a strain of SYSV (van der Vlugt et al. 1999). Decoration technique has been found to be useful for the detection of viruses or establishing serological relatedness between viruses such as *Pineapple mealybug wilt-associated virus* (Sether et al. 1998), *Barley yellow dwarf virus* (Du et al. 2000) and *Wheat yellow mosaic virus* and *Barley yellow mosaic virus* (Ye et al. 2000).

A new carmovirus infecting Angelonia plants (*Angelonia angustifolia*) was detected by decoration test. The antiserum generated against *Angelonia flower break virus* (AnFBV) was highly specific and reacted strongly with the polypeptide corresponding to the CP of the virus. The antiserum showed no reactivity with other carmoviruses *Pelargonium flower break virus* (PFBV), *Saguaro cactus virus* (SgCV) and *Carnation mottle virus* (CarMV) in either ELISA or Western blot tests (Adkins et al. 2006). Decoration technique was applied for the detection and identification of a new virus *Chickpea chlorotic stunt virus* (CpCSV) belonging to the family *Luteoviridae*. The antiserum to the virus strongly decorated purified CpCSV-FB isolate. The antisera to *Barley yellow dwarf virus* (BYDV), *Bean leaf roll virus* (BLRV), *Pea enation mosaic virus* (PEMV) and *Soybean dwarf virus* (SbDV) did not decorate the CpCSV-FB virions indicating the specificity of serological reaction of the antiserum to target virus (Abraham et al. 2006). The bivalent reagent C_L-LR3 was produced by fusing the bacterially expressed antibody specific to *Grapevine leaf roll-associated virus 3* (GLRaV-3) to the light chain constant domain of human immunoglobulin. C_L-LR3 bound strongly to GLRaV3 and the protein either captured or decorated GLRaV-3 conspicuously (Fig. 2.16). In contrast, this protein fusion did not decorate GLRaV-1, revealing the usefulness of decorating procedure for the detection of closely related viruses infecting the same host plant species, inducing complex disease symptoms (Cogotzi et al 2009; Appendix 12).

The decoration technique may be sufficient for detection of many plant viruses. However, labeling the decorating antibody with gold particles may be required for the detection of larger viruses. The gold label antibody decoration (GLAD) procedure for gold labeling of virus suspensions was introduced by Pares and Whitecross (1982).

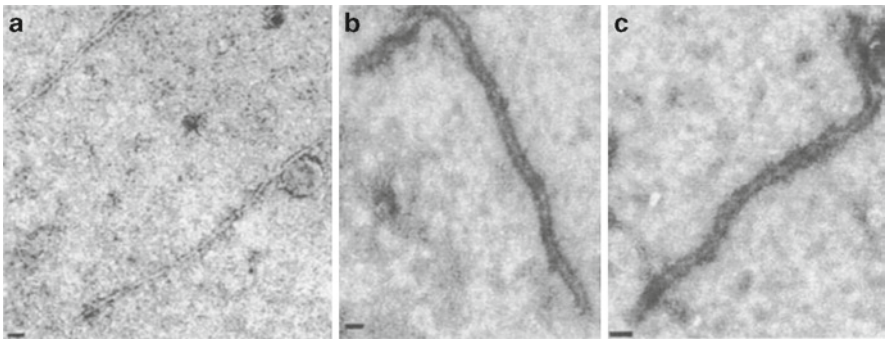

Fig. 2.16 Decoration of *Grapevine leafroll-associated viruses* (GLRaVs) in infected grapevine extracts by immunosorbent electron microscopy (ISEM) (**a**) GLRaV-1; (**b**) GLRaV-7; (**c**) GLRaV-3; decoration is performed with C_L-LR3 reagent. (Courtesy of Cogotzi et al. 2009 and with kind permission of Springer Science and Business Media, Heidelberg, Germany)

Gold labels with varying diameters ranging from 5 to 20 nm have been used. Gold particles may be conjugated directly to the primary or coating antibody. It is attached more commonly to protein A (PA) or a secondary antibody (goat anti-rabbit IgG). The virus particles are adsorbed onto filmed grids and bovine serum albumin is used to block nonspecific protein adsorption sites. The grids are incubated with protein A gold (PAG) and stained with 1% uranyl acetate to enhance the contrast.

Bacterially expressed recombinant fusion protein labeled with protein A gold (PAG) was used for the detection of *Tomato spotted wilt virus* (TSWV) in both greenhouse-grown and field-collected plant samples (Vaira et al. 1996). The presence of *Potato virus Y* (PVY) in cytoplasmic inclusions was inferred by labeling of N-terminal P1 proteinase protein of PVY expressed in *Escherichia coli* (Arabatova et al. 1998).

The distribution of the coat protein (CP) of *Tomato yellow leaf curl Sardinia virus* (TYLCSV) was monitored in tomato and *Solanum nigrum* plants using immunogold labeling technique. A heterologous antiserum generated against *African cassava mosaic virus* (ACMV) that could cross-react with TYLCSV was employed. Labeling with ACMV-CP antiserum produced very specific signal with no or very little background noise. By using gold-labeled ACMV-CP antiserum, the label was detected mostly inside the mature and immature sieve elements and the nuclei of companion and phloem parenchyma cells of TYLCSV-infected leaves. No difference in the pattern of immunogold label (IGL) distribution was seen between TYLCSV-infected tomato and *S. nigrum* and ACMV-infected *Nicotiana benthamiana* plants. In companion cells, the label consisted of electron dense structures in the nucleus. No labeling was seen in palisade and spongy parenchyma cells, indicating TYLCSV was phloem-limited (Medina et al. 2006).

2.1.5.8 Artificial Neural Network Selection Technique

A sensitive system for the detection of plant viruses was developed by employing biosensors and artificial neural networks (ANN). A biosensor may be defined as a device incorporating a biological sensing element connected to a transducer. Biosensor can play an important role in diagnostics. Cell biosensors are based on the measurement of cellular responses to various compounds, such as measuring the cellular electrophysiological properties, in particular the electric potential, which reflects changes in a network of inter-related metabolic reactions. The bioelectric recognition assay (BERA) is a technology that detects the electric response of culture cells, suspended in a gel matrix, to various ligands, which bind to the cell and/or affect its physiology. As a result of the interaction between the virus and host cells, the presence of virus may be detected. The major application of BERA technology is the detection of viruses and metabolic changes linked to disease. Specialized artificial neural networks (ANN) that are trained to recognize plant viruses according to biosensor responses, were developed. The combination of simplicity, sensitivity and reliability has made the BERA technology as the assay of choice for mass screening programs and environmental monitoring (Frossyniotis et al. 2008).

The BERA biosensors were employed to detect plant viruses *Tobacco rattle virus* (TRV) and *Cucumber green mottle mosaic virus* (CGMMV) using appropriate plant cells as the sensing elements. Each virus exhibits a unique pattern of biosensor response over a specific range of concentrations such as a 'signature'. Individual viruses leave each one a characteristic signature which can be read as a graphical curve. Three types of BERA sensors, one with antibodies specific to TRV, one with antibodies specific to CGMMV and one with no antibody, were prepared. Each experiment consisted of recording of the response of each one of the biosensors to a specific virus. For each virus 300 experiments were performed, producing 900 time-series per virus which were used as training patterns of the neural networks. In this investigation, an user-friendly software that will allow rapid and reliable recognition of the signature like response of a BERA sensor against a sample containing a virus under investigation, was developed (Frossyniotis et al. 2008).

2.1.6 Nucleic Acid-based Techniques

The nucleic acids of all organisms are the storehouses of genetic information which determines the characteristics of an organism that is to be investigated. Nucleic acid-based techniques are either based on the specificity by which nucleic acids hybridize to form double-stranded molecules or detection of similarities between nucleic acids by using restriction enzymes to cleave genomic DNA into fragments at or near defined recognition sequences. Closely related organisms share a greater nucleotide sequence similarity compared to distantly related ones. A highly specific nucleotide sequence present in a virus may be used for the detection and identification of the virus or its strain concerned. Generally nucleic acid-based assays are more sensitive and specific giving results reliably and rapidly compared to immunoassays. The antigenic determinants of viral coat proteins (CPs) represent only 2–5% of viral genomes. Hence, the differences in characteristics of the virus governed by a major portion of viral genome cannot be assessed by immunoassays. Diagnostic methods based on the detection of similarities between nucleic acids of viruses and their strains have been shown to be useful for identification of new or unusual viruses or strains. Various kinds of assays such as nucleic acid hybridization, polymerase chain reaction (PCR), reverse transcription (RT)-PCR, real-time PCR and DNA array technology have been applied with varying degrees of success for the detection, identification, quantification and differentiation of plant viruses affecting a wide range of crops and the vector carrying plant viruses.

2.1.6.1 Nucleic Acid Hybridization Techniques

Two complementary strands in a nucleic acid duplex or double helix can be denatured and separated by heat and then annealed/hybridized under conditions favoring hydrogen bonding of base pairs, and under suitable conditions of temperature and salt

concentration. Complementary sequences of single-stranded (ss) molecules may anneal to form stable double-stranded (ds) structures/hybrids. For diagnostic purposes, the target nucleic acid is fixed usually to a solid support. The probe (complementary nucleotide sequence) carrying a label that provides recognizable signal is allowed to anneal with immobilized target molecule. Hybridization is recognized by detecting signal from the probe. As majority of plant viruses have RNA as their genome, RNA probes are frequently employed. RNA:RNA hybrids are more stable than RNA:DNA or DNA:DNA hybrids. Use of highly stringent hybridization conditions is possible because of the stability of RNA:RNA hybrids, resulting in enhancement of probe specificity and reduction in background problems due to interference of plant sap. Nucleic acid hybridization formats have been demonstrated to be useful to study plant viral genome organization and virus–host interactions, in addition to detection and identification of plant viruses.

DNA probes labeled with either a radioactive (^{32}P) or nonradioactive reporter group (biotin/digoxigenin) are commonly employed in hybridization tests. The cDNA probes with appropriate common or specific nucleotide sequences may be prepared and labeled indifferent ways. The cDNA copies of viral RNA are produced by employing a retrovirus reverse transcriptase and labeled radioactively. The probes are useful for the identification of the virus and its strains. Double stranded (ds) cDNA after cloning in *Escherichia coli* is labeled by nick translation. In this procedure, some nucleotides are replaced, after excision with enzymes, followed by insertion with appropriately labeled nucleotides. In another method, the ds-cDNA is prepared by heating and randomly primed with short synthetic oligomers. ds-molecules, completely new, are produced by using DNA polymerase and then labeled. Cloned cDNA probes have been employed for the detection of several plant viruses such as *Plum pox virus* (Varveri et al. 1988), *Peanut mottle virus* and *Peanut stripe virus* (Bijaisoradat and Kuhn 1988) and *Potato leaf roll virus* (Smith et al. 1993).

Dot blot hybridization technique provides qualitative detection indicating whether a plant is infected by a virus or not. Dot blotting does not distinguish between the number and size of hybridized molecules, since hybridization signal is the sum of all sequences recognized by the probe. But this procedure is able to provide results rapidly by specific nucleic acid sequences in samples ranging from crude plant sap to highly purified preparation. This makes the dot blotting to be suitable for routine and large scale testing, as required for certification and quarantine programs, where results for large number of samples have to be made available rapidly. Southern and Northern blot hybridization methods provide more quantitative results than dot blot procedure, since this format precisely identifies the molecule recognized by the probe. Total nucleic acid preparations or viral nucleic acids extracted from purified virions can be used for analysis. This format is useful primarily for the determination of the pattern of viral nucleic acid and detection of non-encapsidated nucleic acids such as satellite RNAs. In situ hybridization (ISH) is employed to detect either specific viral sequences or proteins. It is combined with microscopy to visualize the localized viral nucleic acid with which the probe

hybridizes. This format provides information on the distribution of the target nucleic acid within the infected cells/tissues of host plants. This procedure is also useful to detect the integration of viral sequences in plant chromosomes as in *Banana streak virus* in *Musa* spp. (Harper et al. 1999).

Dot blot hybridization procedure was adopted for the detection of *Banana bunchy top virus* (BBTV) using BBTV-specific clones and radioactive or nonradioactive probes. This protocol was as sensitive as ELISA, indicating the presence of the virus in the infected plants (Xie and Hu 1995). Digoxigenin (DIG)-labeled probes and colorimetric visualization of the positive reactions in a dot blot hybridization system were effective for the detection of *Beet curly top virus*, *Beet yellow virus* and *Squash leaf curl virus*, *Lettuce infectious yellows virus* and *Zucchini yellow mosaic virus.* This protocol shows promise for routine diagnosis of the above mentioned viruses (Harper and Creamer 1995). The *Bamboo mosaic virus* (BaMV) and its associated satellite RNA were detected by employing ^{32}P or DIG-labeled probes synthesized from cDNA clones of BaMV genomic (L probe) and sat BaMV (S probe) RNA. ^{32}P-labeled L and S probes were more sensitive (25-fold) than DIG-labeled probes in detecting the virus in infected leaf extracts (Hsu et al. 2000).

DIG-labeled sense and anti-sense cRNA probes were employed for the detection of *Cymbidium mosaic virus* (CyMV) and *Odontoglossum ringspot virus* (ORSV) in crude leaf extracts or total RNA from infected leaves. The probes remained stable for more than 1 year, providing sensitive and reliable detection for routine diagnosis of these viruses (Hu and Wong 1998). A ^{32}P-labeled cDNA probe prepared from N gene of *Calla lily chorotic spot virus* (CCSV) was employed to hybridize with ds-RNAs of CCSV, *Watermelon silver mottle virus* (WSMoV) and *Tomato spotted wilt virus* (TSWV). A weak signal was observed when the probe hybridized with ds-RNA SRNA of WSMoV, whereas the probe did not hybridize with ds-RNA of TSWV, indicating the relatedness between these two viruses under test (Lin et al. 2005).

Probes labeled with nonradioactive chemicals, are preferred because of the hazards associated with radioactive labels. Dot blot assays using digoxigenin-labeled probes for the detection of *Indian peanut clump virus* had similar sensitivity levels as the assays employing ^{32}P labeled probes (Wesley et al. 1996). Several viruses *Alfalfa mosaic virus* (AMV), *Cucumber mosaic virus* (CMV), *Potato virus* Y (PVY), *Tomato mosaic virus* (ToMoV) and *Tomato yellow leaf curl virus* and TSWV were detected effectively by employing DIG-labeled probes. A total of 400–500 samples representing approximately 1.15 million tomato seedlings could be tested using this system per day, indicating its potential for use in large scale testing (Saldarelli et al. 1996). The sensitivity of detection of *Potato leaf roll virus* (PLRV) in potato leaf extracts could be enhanced by 2,000 times by employing DIG-labeled cDNA probe of approximately 2,100-bp, when compared to ELISA test. PLRV present in dormant potato tuber tissues was readily detected and no cross-reaction was seen between probes and other two important potato viruses PVY and *Potato virus X* (PVX) (Loebenstein et al. 1997).

Whitefly-transmitted *Tomato chlorosis virus* (ToCV) observed for the first time, was detected by employing DIG-11-UTP-labeled riboprobes derived from cDNA

clones representing positions of RNAs 1 and 2 in infected tomato. This virus was differentiated from the already known *Tomato infectious chlorosis virus* by the absence of cross-reaction in dot blot hybridization assays (Wisler et al. 1998). A new virus, *Sweet potato leaf curl virus* (SPLCV-US) transmitted by *Bemisia tabaci* showed sequence homology with other geminiviruses. The DNA virus *Bean golden mosaic virus* and *Tomato mottle virus* hybridized with a 2.5-kb DNA band present in the DNA extracts from plants infected by SPLCV-US isolate. Probes prepared with the B component of the geminiviruses, however, did not hybridize with the DNA extracts from sweet potato (Lotrakul et al. 1998).

The sensitivity of cRNA probes labeled with DIG was assessed using dot blot, Northern blot and microplate hybridization procedures. In dot blots, the probe could detect up to 10 fg of *Cucumber mosaic virus* (CMV)-RNA, whereas Northern blot analysis revealed the hybridization of the probe to all CMV genomic RNAs in the total RNAs extracted from inoculated leaves. These probes can be employed also to monitor the accumulation of CMV-RNA in the inoculated leaves of bottle gourd (*Lagenaria siceraria*) (Takeshita et al. 1999). The 3′ ends of RNAs of various isolates of *Apricot latent virus* have been sequenced, facilitating the development of specific riboprobes for the detection of the virus. In dot blot assays, the riboprobe pApr-47 specifically hybridized with the total nucleic acid (TNA) extracts from infected apricot and *Nicotiana occidentalis* (indicator plant species) plants, but not with extracts from healthy controls. In addition, there was no cross-reaction with TNA extracts from plants infected with *Apple stem pitting virus* (ASPV), *Cherry green ring mottle virus* (CGRMV) or *Cherry necrotic rusty mottle virus* (CNRMV) isolates (Ghanem-Sabanadzovic et al. 2005).

The usefulness of Northern hybridization technique for the detection of another virus infecting ornamental plant species was demonstrated. Northern hybridization was used to confirm the infection of rose geraniums (*Pelargonium* spp.) by *Plum necrotic ring spot virus* (PNRSV) which was earlier detected by ELISA format (Kulshrestha et al. 2005). *Angelonia angustifolia*, an ornamental plant species was infected by a new carmovirus designated *Angelonia flower break virus* (AnFBV). This virus was mechanically transmitted to *Nicotiana benthamiana*. The dsRNA was isolated from the infected *N. benthamiana* plants and fractionated on an agarose gel. Four major bands were detected on the gel. The largest band (ca. 4-kbp) co-migrated with equivalent dsRNA of *Carnation mosaic virus* (CarMV) and this band was considered to be putative dsRNA form of AnFBV genomic RNA. Three additional dsRNA species of about 1.75-, 1.60- and 1.31-kbp were also present on the gel. In a comparable Northern blot of total RNA from AnFBV-infected *N. benthamiana* and *Angelonia*, the AnFBV CP-specific DIG-labeled probe hybridized with a 3.96-kbp RNA. A similar sized RNA was located in northern blots of AnFBV viral RNA (Adkins et al. 2006).

Plant virus transcripts can be localized by a simple, rapid and inexpensive hybridization procedure. This method is based on fluorescent in situ hybridization using short DNA oligonucleotides complementary to an RNA segment representing a virus transcript in the infected plant. *Tomato yellow leaf curl virus* (TYLCV) and *Potato leaf roll virus* (PLRV) were tested using this protocol. Transcripts of both

viruses were localized specifically to the phloem sieve elements of infected plants. No signal was observed in negative controls. The presence of TYLCV and PLRV was also detected in *Bemisia tabaci* and *Myzus persicae*, the vectors respectively of these viruses (Ghanim et al. 2009).

A dot blot hybridization procedure was developed for the detection of five viruses infecting cucurbitaceous crops. DIG-labeled probes specific to *Cucumber mosaic virus* (CMV), *Papaya ring spot virus*-watermelon strain (PRSV-W), *Squash mosaic virus* (SqMV) and *Zucchini yellow mosaic virus* (ZYMV) were synthesized by PCR with specific primers. Crude extracts from infected leaves were used for testing by dot blot hybridization. The sensitivity of detection was assessed by using different dilutions of the crude extracts. The limits of detection for ZYMV, WMV, CMV, PRSV-W and SqMV were 1:160, 1:160, 1:320, 1:160 and 1:320 respectively. DIG-labeled probes prepared from PCR amplifications were specific and sensitive in rapid and precise identification of the target viruses infecting cucurbits (Meng et al. 2007).

Infection of *Pepino mosaic virus* (PepMV), a *Potexvirus* in tomato crops was observed extensively limiting tomato production in several European countries. The efficacy of dot blot hybridization and DAS-ELISA were compared with the multiplex RT-PCR assay. Serially diluted leaf extracts from PepMV-infected tomato plants were tested. Total RNA extractions of three infected field samples were fivefold diluted using total RNA extracted from healthy plant tissues. One microliter of non-diluted RNA extract and each dilution was denatured with formaldehyde and then directly applied to a nylon membrane. Dot blot hybridization was performed using a DIG-RNA probe complementary to a fragment of RNA-dependent RNA polymerase (RdRp) of PepMV. Dot blot hybridization or ELISA detected PepMV in extracts up to 5^{-2} dilution (2 mg/ml). The sensitivity levels of detection by these two techniques were less sensitive (3,125 times) than multiplex RT-PCR format developed in this investigation by Alfaro-Fernández et al. 2009).

Simultaneous detection of two or more viruses has been achieved by using nonisotopic molecular hybridization technique employing a cocktail of specific single probes against viruses infecting vegetable crops (Saito et al. 1995), ornamental plants (Sánchez-Navarro et al. 1999) and stone fruit crops (Saade et al. 2000). Later molecular hybridization approach was applied for simultaneous detection of up to six viruses. The sequences of two, four or six viruses were fused in tandem and transcribed to be employed as unique riboprobes designated “polyprobes”. Polyprobe four (poly 4) was able to detect four ilarviruses, *Apple mosaic virus* (ApMV), *Prunus necrotic ringspot virus* (PNRSV), *Prunus dwarf virus* (PDV) and *American plum line pattern virus* (APLPV). Polyprobe two (poly 2) could be employed to detect *Plum pox virus* (PPV) and *Apple chlorotic leaf spot virus* (ACLSV). Polysix (poly 6) was capable of detecting any one of the above mentioned viruses. The usefulness of the probes for detection of all the six viruses in 46 field-collected samples was demonstrated. Poly 2, poly 4 and poly 6 probe combinations were equally effective in detecting the group of viruses as the respective individual riboprobe specific for the particular virus. The simultaneous detection is helpful in saving time as well as cost of testing (Herranz et al. 2005).

A simple and sensitive nucleic acid hybridization technique, the microplate hybridization is performed in multiwell microplate in place of nitrocellulose membrane. The denatured nucleic acids are directly immobilized on polystyrene microplate and then hybridized with cRNA probe labeled with DIG. The target nucleic acid is then detected via hydrolysis of *p*-nitophenyl phosphate by alkaline phosphatase (AP)-conjugated with an anti-DIG antibody. This direct microplate hybridization procedure can detect very low concentrations (below picogram level) of viral RNA or viroid cDNA. The possibility of quantitative estimation of virus concentration and potential for routine diagnosis are the distinct advantages of the microplate hybridization technique. DIG-labeled synthetic oligonucleotide probes were used for subgroup specific and quantitative detection of genomic RNA of *Cucumber mosaic virus* (CMV). The RNAs of CMV are adsorbed to the microplate wells and then hybridized with probes specific for respective subgroups of CMV. The detection limit of the procedure was ca. 1 ng of CMV-RNA under optimal conditions without any nonspecific reaction (Uchiba et al. 1999). Tomato and potato crops are affected by several viruses either singly or in combination causing variable reduction in yield, depending on the pathogenic potential of the virus concerned. It is essential that the virus(es) present in a location has to be detected rapidly and identified precisely to develop effective disease management strategy. Detection of *Tomato yellow leaf curl virus* (TYLCV) and *Potato leaf roll virus* (PLRV) was accomplished by applying the fluorescent in situ hybridization (FISH) method. Short DNA oligonucleotides complementary to an RNA segment representing a virus transcript in the infected plant were employed in this method. The DNA probe contained a fluorescent molecule at its 5′ and 3′ ends. The viral transcripts were localized in the phloem tissues of tomato and potato plants. This protocol is simple, rapid, inexpensive and provides reliable and convincing, background-free results (Ghanim et al. 2009).

Another nucleic acid hybridization format, the tissue-print hybridization involves the transfer of the viral nucleic acid from infected plant tissues directly onto nitrocellulose or nylon membrane, followed by hybridization of the printed membrane with radioactive or nonradioactive chemiluminescent DIG-labeled probes. This procedure was applied for studying the localization pattern of the virus in specific host plant tissues. The DIG-labeled probes were effective in detecting and differentiating isolates of *Citrus tristeza virus* (CTV) from greenhouse and field (Narváez et al. 2000). *Artichoke latent virus* could be reliably detected in Globe artichoke field samples by employing either tissue imprint hybridization or one-step RT-PCR procedures (Lumia et al. 2003). For field survey to assess the incidence of *Citrus psorosis virus* (CPsV), tissue print hybridization format was found to be the most suitable, because of the advantages such as absence of sample processing and risks for handling in plant quarantines, possibility of transporting imprinted membranes to far away laboratories and storing the imprinted membranes without loss of activity. The results from samples stored for 2–3 years were similar to those obtained with fresh or desiccated citrus tissues (Martin et al. 2004).

Prunus necrotic ringspot virus (PNRSV), *Prune dwarf virus* (PDV) and *Apple mosaic virus* (ApMV) belonging to the genus *Ilarvirus*, family *Bromoviridae* are the most economically important viruses infecting stone fruit trees. In order to

monitor the presence of these viruses in different seasons in various species of stone fruits, DAS-ELISA and tissue-printing (TP) hybridization procedures were applied. The extracts (0.1 ml/sample) were directly loaded in antibody-coated microtiter plate wells in duplicate for ELISA test. Samples were considered positive, if the absorbance values were at least three times higher than that of healthy control after 1 h incubation. In the case of tissue-printing hybridization, prints of two different leaves and their petioles were made by gently pressing the transversely cut petioles or tightly rolled leaves cut in the middle onto the membranes. During winter season, dormant buds were tested by both tests for the presence of the viruses. Optimal detection was accomplished in different months for different viruses. All PNRSV-infected trees gave positive reactions in January and March–April using TP and during November or January using ELISA. In the case of PDV, all trees gave positive results in March–April with both techniques and in May with ELISA. ApMV was more difficult to detect among the three viruses. Both techniques detected the viruses in all trees only in November. The highest detection rate was achieved for the three viruses by testing the buds of dormant trees in the winter, regardless of the test applied (Table 2.9). Overall, tissue-printing technique was more successful for detecting ApMV, while ELISA was more effective for the detection of PNRSV and PDV in stone fruit trees (Matic et al. 2008).

Tomato crops are infected by *Parietaria mottle virus*-T (PMoV-T) belonging to the genus *Ilarvirus* in Catalonia, Spain. A DIG-labeled riboprobe was synthesized from the nucleotide sequence of amplified cDNA and employed in dot blot and tissue-printing hybridization methods. All samples that were ELISA-positive, also yielded a clear signal by dot blot and tissue-printing hybridization techniques. No cross-reaction was evident with healthy control samples. Dot blot and tissue-printing hybridization procedures detected PMoV-T in tomato samples (56) collected from five tomato fields. Differences in signal intensities were noted in all samples

Table 2.9 Detection of stone fruit tree viruses in different seasons by ELISA (E) and tissue-printing (TP) hybridization techniques (Matic et al. 2008)

	Sampling time			
Viruses detected	Winter (Nov.–Feb.)	Spring–early summer (March–June)	Late summer–autumn (Aug.–Sept.)	Total
PNRSV-tested samples	33	33	22	88
Positive samples (E/TP)	33/27	25/26	11/9	69/62
Detection rate E/TP (%)	96.9/81.8	75.7/78.7	50/40.9	77.2/70.5
PDV-tested samples	27	27	18	72
Positive samples (E/TP)	23/23	25/17	7/1	55/41
Detection rate E/TP (%)	85.1/85.1	86.2/62.9	38.8/5.5	76.3/56.9
ApMV-tested samples	15	15	10	40
Positive samples (E/TP)	10/11	5/7	1/1	16/19
Detection rate E/TP (%)	66.6/73.3	33.3/46.6	10/10	40/47.5

PNRSV *Prunus necrotic ring spot virus*, PDV *Prunus dwarf virus*, ApMV *Apple mosaic virus*

from tomato fields. Tissue-printing hybridization was consistent and as robust as dot blot hybridization. This method reduces sample processing to a minimum and can become the method of choice for large scale applications (Galipienso et al. 2005). *Grapevine virus A* (GVA), type member of the genus *Vitivirus*, is associated with rugose wood disease of grapevine (Martelli and Walker 1998). A dot blot hybridization assay was developed using an in vitro transcribed non-radioactive RNA probe for routine detection of GVA for large scale surveys. Phloem scrapings of dormant wood were used for isolation of total RNA. Of the 83 samples tested, seven grapevines were found to be positive for GVA employing the riboprobe. The results showed that RT-PCR and ELISA assays failed to detect GVA in two samples, while the riboprobe did not detect GVA in one sample only. This improved detection may be due to the use of probe longer than the relatively short oligonucleotide PCR primers used in RT-PCR assay (Komínek et al. 2008).

Tomato and potato crops are affected by several viruses either singly or in combination causing variable reduction in yield, depending on the pathogenic potential of the virus concerned. It is essential that the virus(es) present in a location has to be detected rapidly and identified precisely to develop effective disease management strategy. Detection of *Tomato yellow leaf curl virus* (TYLCV) and *Potato leaf roll virus* (PLRV) was accomplished by applying the fluorescent in situ hybridization (FISH) method. Short DNA oligonucleotides complementary to an RNA segment representing a virus transcript in the infected plant were employed in this method. The DNA probe contained a fluorescent molecule at its 5′ and 3′ ends. The viral transcripts were localized in the phloem tissues of tomato and potato plants. This protocol is simple, rapid, inexpensive and provides reliable and convincing, background-free results (Ghanim et al. 2009).

Six viruses infecting tomato crops viz., *Tomato spotted wilt virus* (TSWV), *Tomato mosaic virus* (ToMV), *Pepino mosaic virus* (PepMV), *Cucumber mosaic virus* (CMV), *Potato virus Y* (PVY) and *Parietaria mottle virus* (PMoV) are responsible for appreciable losses in the Mediterranean area including Spain. Hence, a polyprobe was developed for the simultaneous detection of any of these six viruses infecting tomato. Tissue-print hybridization employing individual probe for specific virus and a polyprobe capable of detecting all the six viruses was developed. No significant differences in sensitivity were observed between single and the ribopoly-6 hybridizations for CMV, PepMoV, PMoV and PVY. The end-point detection limit ranged between 5 and 0.5 pg of RNA transcripts of these viruses. The detection limit of ribopoly-6 for ToMV and TSWV was slightly lower than its corresponding individual riboprobe. The results indicated that the ribopoly-6 could detect RNA transcripts corresponding to the CP of any of the six viruses tested without loss of sensitivity at concentrations of 50 pg and above of viral RNA (Fig. 2.17). When field-collected tissue-printed 72 tomato samples were tested, all ELISA-positive samples were also positive with ribopoly-6. The polyprobe/tissue-printing technique was able to detect all six viruses, revealing its potential for large scale adoption as a reliable alternative to ELISA test (Aparicio et al. 2009b).

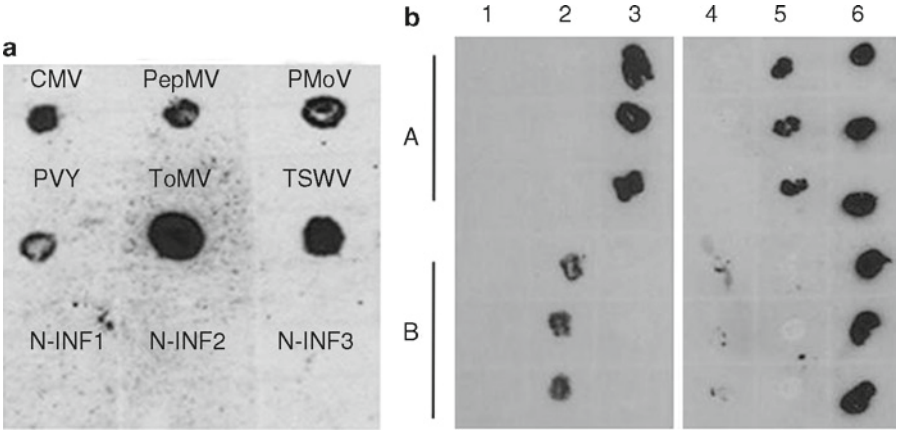

Fig. 2.17 Detection of six viruses using the polyprobe by tissue printing and non-isotopic molecular hybridization technique. (**a**) Tissue-printings from tomato plants singly infected by CMV, PepMV, PMoV, PVY, ToMV and TSWV and three healthy plants (N-INF1, N-INF2 and N-INF3). (**b**) Ribopoly-6 probe detects any one of the six viruses present in tissue-printing membrane on which freshly cut petioles are applied in triplicates. Note the positive reactions in samples A3, A5, A6, B2, B4 and B6 (Courtesy of Aparicio et al. 2009 and with kind permission of Springer Science and Business Media, Heidelberg, Germany)

2.1.6.2 Polymerase Chain Reaction-Based Methods

Development of polymerase chain reaction (PCR) is considered as one of the most significant advancement contributing to the remarkable progress achieved in all branches of biological sciences, especially in the field of pathogen diagnostics. Various aspects of biological entities from primitive viroids to highly developed human beings have been studied by adopting PCR or its variants. PCR has been extensively employed in the study of plant viruses and number of novel applications arising from PCR appears to increase progressively as the new problems are encountered demanding suitable solutions. Principal application of PCR relates to the detection, identification and differentiation of viruses in infected plants and plant materials, especially in asymptomatic plant materials that form the primary sources of infection for subsequent crops. PCR is an in vitro technique in which a desired DNA fragment with known end sequences can be amplified exponentially into billions of copies that can be detected more reliably.

A major advantage of PCR-based assay is that it can be used to increase the concentration of pathogen-related DNA sequences present in naturally infected hosts at concentrations below detection level. This situation may be often seen, because they are present in very low concentration or they may be localized in certain tissues such as phloem or they are irregularly distributed in the infected plants. Since an initially very low number of DNA molecules can be amplified to reach a concentration that becomes detectable, only a few nanograms of initial template DNA is necessary either in the form of discrete molecule or as part of a larger one. Both double-stranded (ds)-DNA and single-stranded (ss)-DNA can be

directly amplified. Majority of plant viruses have RNA genomes and they need a reverse transcription (RT) step to generate complementary DNA (cDNA) prior to PCR amplification (RT-PCR) format. PCR assays, as a diagnostic tool, provide distinct advantages of high level of sensitivity, speed, specificity and versatility. Availability of good quality total RNA from plants, especially from woody plants, is an important factor that is likely to affect the sensitivity of detection of RNA viruses infecting plants by PCR assay. An efficient protocol was developed for the extraction of high quality RNA from woody plants without the use of phenol, organic solvents or alcohol precipitation. This procedure involves the use of commercially available spin-column matrices resulting in the reduction of inhibitory effects of plant polysaccharides and polyphenolic compounds frequently observed subsequently on polymerase chain reaction amplification of targeted DNA fragments. Several viruses infecting woody plants such as *Apple stem grooving virus* (ASGV), *Apple stem pitting virus* (ASPV), *Arabis mosaic virus* (ArMV), *Prunus necrotic ringspot virus* (PNRSV), *Grapevine fanleaf virus* (GFLV), and *Grapevine leaf roll-associated virus 3* (GLRaV-3). The method also provided efficient extraction of total RNA from budwood, leaves or flower blossoms for the detection of ASGV and PNRSV in these tissues (MacKenzie et al. 1997).

Extraction of total RNA efficiently from virus-infected tissues, especially from woody plants is of great importance for the successful diagnosis by PCR-based assays. A procedure of drying plant tissues to overcome phenolic inhibitors of viral RNA was developed. Infected leaves from different host plant species were dried at 65°C for 2 days and conserved at 4°C under air-proof conditions. The treated leaves were found to be good sources of *Apricot latent virus* (ApLV), *Plum bark necrosis stem-pitting-associated virus* (PBNSPaV), *Prunus necrotic ringspot virus* (PNRSV), *Potato virus Y* (PVY) and *Apple scar skin viroid* (ASSVd). The viruses ApLV, PNRSV, PVY and the viroid could be easily detected by reverse transcription-polymerase chain reaction (RT-PCR) assay, while nested-RT-PCR format was successful for the detection of PBNSPaV from infected leaves processed by drying at 65°C. No significant difference was noted between ethidium bormide-stained band profiles of dried and fresh leaves from infected plants. This simple and inexpensive procedure can be adopted for long-term preservation of plant tissues infected with viruses (Sipahioglu and Ocak 2006).

The PCR protocol primarily involves heat denaturation of the target ds-DNA and hybridization of a pair of synthetic oligonucleotide primers to both strands of the target DNA, one to the 5′ end of the sense strand and another to the 5′ end of the anti-sense strand by an annealing step. A thermostable *Taq*DNA polymerase enzyme from *Thermus aquaticus* (*Taq*) can be used at temperatures between 60°C and 80°C. A new DNA is synthesized on templates to produce twice the number of target DNAs. Newly synthesized DNA strands are used as targets for subsequent DNA synthesis and these steps are repeated up to 50 times. The number of target DNA molecules is increased exponentially. The DNA sequences between the primers are reproduced with high fidelity with an efficiency level up to 85% per cycle (Weier and Gray 1988). The PCR products may be employed as a target for hybridization for direct sequencing of the DNA to assess variations in virus strains and as a probe.

PCR assay has been demonstrated to be more sensitive than immunoassays in the detection of many viruses. *Citrus tristeza virus* (CTV) which could be detected throughout the year and even during the seasons when CTV concentration was at levels below for detection by ELISA test (Mathews et al. 1997). However, there are several conditions/factors that may affect the outcome of PCR assays. Plants, insect vectors and soils contain several compounds that can inhibit PCR amplification of target DNA. High quality purified DNAs or target sequences are essential and contamination from extraneous sources or when handling many samples may result in false positive results. Further, sample preparation is more labor-intensive and equipments and supplies are more expensive. Although PCR assay has been shown to be very sensitive and specific, its application of routine diagnosis has been hampered by its lack of robustness and by the complexity of the post-amplification steps needed. Specificity of PCR is determined by use of appropriate primer design required for detection of one or more virus species, subgroups, strains, isolates or pathotypes. Primers in conserved regions of the coat protein (CP) and viral RNA polymerase genes have been employed for the detection of several viruses. A region of high nucleotide sequence divergence in CP gene among isolates of *Plum pox virus* (PPV) facilitated the application of a cherry subgroup-specific RT-PCR in certification and quarantine programs (Nemchinov and Hadidi 1998). PCR-based techniques have become preferred ones because of the reliability and rapidity of obtaining results of the assays. A wide range of plant viruses has been detected and identified by employing PCR directly or after reverse transcribing the viral RNA genome into corresponding cDNAs (Narayanasamy 2008).

A good quality DNA is the essential requirement for reliable detection of viruses by PCR-based assays. The efficiency of extraction of DNA, quality and stability of the viral genome could be improved by the addition of sodium sulfite as in the case of *Citrus yellow mosaic virus* (CYMV), a non-enveloped bacilliform DNA virus infecting sweet oranges in India. In addition, the viral DNA extracted with sodium sulfite showed greater stabilities at various temperatures compared with those extracted with commercial kit (Qiagen) DNeasy Kit (Baranwal et al. 2003). The nature of universal, broad specificity and also species-, genus-, and family-specific primers may affect the specificity of PCR amplification. The primers may be designed from conserved nucleotide or amino acid motifs shared by all or several members of the same taxonomic group. It is possible to enhance the sensitivity and specificity of PCR by employing a second pair of primers nested within the original PCR amplicons. Information on viral nucleotide sequences is rapidly accumulating and this is likely to facilitate the development of diagnostic kits for the reliable detection of several viruses in future.

Detection and establishment of the identity of a new virus has to be accomplished rapidly in order to prevent the incidence and further spread of the disease induced by the virus concerned. *Pineapple bacilliform virus* (PBV) was detected by employing specific primers designed from PBV sequences which yielded a 403-bp product after PCR amplification. The presence of the PCR product was detected in the extracts from infected pineapple plants, but not from extracts of plants infected with *Sugarcane bacilliform virus* or *Banana streak virus* (BSV) (Thompson et al. 1996). Likewise, the citrus mosaic disease was considered to be due to a ds-DNA

virus, as indicated by PCR amplification of degenerate oligonucleotide primers based on the conserved badna virus genomic sequences (Ahlawat et al. 1996).

PCR assay was applied for the detection of *Banana bunchy top virus* (BBTV) in different plant parts. It was also possible to infer the sites of BBTV replication using the PCR amplification of viral DNA (Hafner et al. 1995). In a later investigation, three pairs of primers were designed based on the sequences of DNA1 component of BBTV containing viral putative replicase gene. These primer pairs amplified DNA fragments of 436–446- and 447-bp from the extracts of banana leaves infected by BBTV, in vitro tissue-culture-derived banana and viruliferous aphids (Shamloul et al. 1999). Likewise, BBTV was detected in field-collected bananas, as well as in vector aphids using the primer pair that amplified 1-kb fragment of the viral DNA (Manickam 2000). Two specific primers were designed based on the sequences of BBTV-Eg-DNA 2.2 (component 2). PCR assay using these primers amplified a fragment of about 1,056-bp from the DNA extracts from BBTV-infected banana plants. No amplification occurred in the extracts from healthy banana plants. BBTV was detected successfully in all banana samples tested including the asymptomatic plants. The PCR protocol has the potential for testing the tissue culture-generated banana plants (Soweha 2005). BBTV was detected by preparing total DNA extracts from infected banana leaves and using the primers designed based on the CP sequences of BBTV. A major DNA fragment of the expected size 500-bp was amplified from extracts of leaves from infected banana plants. No amplification occurred in the extracts from uninfected plants (El-Dougdoug et al. 2006).

Reverse transcription (RT)-PCR assay has been performed for the detection of plant RNA viruses in purified virus preparations, extracts of plant tissues and vectors of these viruses. *Cucumber mosaic virus* (CMV) was detected in crude extracts of infected plants using primers complementary to the conserved sequences of CMV RNA3 for broad-spectrum detection of isolates belonging to subgroups I and II from different geographical locations (de Blas et al. 1994). Transmission of *Plum pox virus* (PPV) is known to occur through pollen from infected plants. The stone fruit anthers are transported from one country to another for breeding purposes. By applying RT-PCR protocol, using DNA primers from the 3′ noncoding region (NCR) of PPV, the anthers were considered to be a source of PPV dissemination during international movement of *Prunus* germplasm (Levy et al. 1995). PCR assay was shown to be more sensitive than ELISA formats and hybridization with chemi-luminescent or chromogenic labeled cDNA probes for the detection of *Barley yellow dwarf virus* (SaYDV-PAV-IL). The detection limit of the PCR assay was 0.1 pg of RNA extracted from purified virus and viral RNA from 0.5 g of infected leaf tissue (Figueira et al. 1997).

Polymerase chain reaction (PCR) was applied to detect and determine the genomic nucleic acid sequence divergence of *Citrus tristeza virus* (CTV) isolates which were grouped based on this characteristic. The cDNA sequences of the Florida CTV isolates T3 and T30 showed a relatively consistent symmetrical distribution of nucleotide sequence identity in both the 5′ and 3′ regions of the 19.2-kb genome. Selective amplification of these isolates occurred when primers designed from cDNA sequence for PCR were used, indicating that this characteristic can be considered as

similar to T3, T30 or T36. PCR primers designed from the T36-CP gene sequence amplified efficiently from all CTV isolates (Hilf et al. 1999).

Reverse transcription (RT)-PCR assay has been applied extensively for the detection of plant RNA viruses infecting a wide range of herbaceous plant species and also in different types of host tissues including asexually propagated planting materials. Further, it is also possible to detect viruses in crude plant extracts by employing RT-PCR assay avoiding the need for purification of viruses. However, detection of viruses infecting woody plants was found to be difficult, because of absence of efficient method of extracting the viruses from these plants. Hence, procedures such as direct binding (DB)-RT-PCR (Rowhani et al. 1998) and tube capture (TC)-RT-PCR (James et al. 1999) methods were helpful to overcome the limitation of the earlier methods. These procedures were found to be easy and useful for efficient and reliable detection of viruses infecting woody plants.

A modified RT-PCR procedure was effective for the detection of *Prunus necrotic ringspot virus* (PNRSV) in dormant peach trees. This protocol was found to be useful for screening imported budwood materials in post-entry quarantine (PEQ) programs and also for ensuring the freedom of planting materials from virus infection (Spiegel et al. 1996). Another modified RT-PCR technique was developed for the detection of PNRSV in dormant peach and almond trees. Two different pairs of primers yielding short (70-bp) and long (200-bp) products were employed. Amplification of short PCR product was effective for the detection of PNRSV in plant tissues with low virus titer as in dormant trees, while the long PCR product was amplified in tissues with high virus titer (Rosner et al. 1997).

Application of RT-PCR assay widely has become feasible, as the number of viruses for which nucleotide sequence data are available, is progressively increasing (Uga et al. 2007). RT-PCR assay was shown to be more sensitive than ELISA test in detecting PNRSV and *Prune dwarf virus* (PDV). These viruses could be readily detected at any time throughout the growing season (Mekuria et al. 2003). Location of PDV in young leaves and flower buds could be determined by in situ RT-PCR technique. The CP gene of PDV was used as a target to produce a cDNA copy that was amplified by PCR and visualized using a direct detection method using digoxigenin-labeled nucleotide probes (Silva et al. 2003). A survey was taken up to assess the incidence of PNRSV in cherry orchard of *Prunus cerasus* cv. Montomorency and *Prunus avium* cv. Hedelfingen in New York. The presence of PNRSV was confirmed by RT-PCR assay and amplification of the viral CP gene in total RNA extracted from infected leaves. Further, latent infection of a majority of trees (87%) by PNRSV was also revealed by RT-PCR assay. Asymptomatic PNRSV-infected trees clustered in spatial proximity of symptomatic trees (Oliver et al. 2009).

Plum pox virus (PPV) was detected in cultivated plum in germplasm entries and wild apricots in Kazakhstan by employing RT-PCR assay. Primers based on the sequences of PPV CP gene amplified a 243-bp fragment in the C-terminus coat protein coding region. The PPV isolates infecting plum cultivars and wild apricot were identified as D strain of PPV (Spiegel et al. 2004). The extent of prevalence and identity of strains of PPV in Cyprus was investigated by using molecular methods.

A total of 72 leaf samples from peaches, apricots and plums with or without symptoms were first tested by standard DAS-ELISA procedure. Total RNA from all these samples was extracted using a commercial kit and RT-PCR assay was performed using the broad activity PPV-specific primers P1/P2. In addition, PPV-M and PPV-D strain-specific primers P1/PM and P1/PD were employed. Of the 72 samples tested, 42 were ELISA-positive. In the RT-PCR assay, 0.24-kb fragment was amplified from 56 samples, including the 42 ELISA-positive samples. RT-PCR assay detected PPV in peach (73%), apricot (88%) and plum (75%) trees with generic P1/P2 primer pair. Strain-specific primer results showed that 49 PPV isolates gave an amplification product with primers specific for M type, whereas seven peach cultivars gave positive reaction with D-specific primer pair. Thus RT-PCR assay was found to be more sensitive and precise in detecting PPV and its strains compared to DAS-ELISA test (Papyiannis et al. 2007).

RT-PCR assay specific for the detection of *Peach mosaic virus* (PcMV) was developed by using PcMV-derived primers PM16AF and PM16AFR. This format was applied for screening a range of virus isolates representing the different genera within the family *Flexiviridae*. These primers targeted the 3′ terminus of the coding region of the replication protein (ORF1) and amplified a fragment of 419-bp. No cross-reaction was observed with *Apple chlorotic leaf spot virus* (ACLSV) isolates in the conventional RT-PCR procedure. Hence, a semi-nested RT-PCR protocol, using cDNA generated by PM16AFR for amplification with the PM-AF and PM-AFR primers were formulated. The PcMV-specific fragments were amplified making this procedure to be very specific for detection of PcMV virus and its strains accurately (James et al. 2006).

During the survey of apple growing areas in Turkey, the incidence of *Apple mosaic virus* (ApMV), *Apple stem grooving virus* (ASGV), *Apple chlorotic leaf spot virus* (ACLSV) and *Apple stem pitting virus* (ASPV) was assessed by applying RT-PCR assay. A total of 174 samples collected from apple cultivars and genotypes in different locations were tested. High incidence of latent apple viruses like ACLSV, ASGV and ASPV in commercial orchards was observed. High infection rates of these viruses in varietal collections also indicated that they might spread very rapidly and extensively through infected budwoods taken from mother plants to commercial orchards. Infection rate of ApMV was fairly low compared to the other three viruses. The RT-PCR procedure employed in this investigation, offers very effective and reliable detection of the targeted viruses infecting apple cultivars and hence, may help establish virus-free stocks which is the best strategy to effectively manage these virus diseases (Çağlayan et al. 2006).

A simplified single tube RT-PCR protocol for the detection of *Little cherry virus1* and *2* (LChV-1 and LChV-2) was employed. This protocol was effective in reducing unspecific amplification that resulted in false positive results. Both viruses LChV-1 and LChV-2 were detected reliably by employing this technique (Rybak et al. 2004). A field survey was undertaken to determine the presence and extent of infection of *Apricot latent virus* (ApLV) and *Plum bark necrosis stem-pitting associated virus* (PBNSPaV) in eastern Anatolia (Turkey). RT-PCR assay was applied for the detection of ApLV. Virus-specific primers were employed for PCR amplification.

Amplicons (200-bp) were separated by gel electrophoresis on 2% agarose gel and bands were detected after staining with ethidium bromide. Three of 224 apricot samples tested positive for ApLV. All infected samples showed the presence of the unique band of 200-bp. For the detection of PBNSPaV, nested RT-PCR format was employed. Of the 45 sweet cherry and plum samples, 35 were positive for PBNSPaV yielding a DNA fragment of 190-bp with primer pair ASPn1/ASPn2. Sweet cherry showed high incidence of PBNSPaV and its incidence was low in plum (one sample) and in prune cv. Italian Prune (seven samples). Further, it was possible to detect latent infection by PBNSPaV in cherry and prune trees (Usta et al. 2007).

PCR primers were designed based on the sequences of the fragments of cDNA of the bottom component RNA of *Citrus psorosis ringspot virus* (CtRSV-4) for using in RT-PCR assay. It was possible to detect CtRSV-4 in citrus leaves at a tissue dilution of 1:12,800 representing as little as 2 μg of tissue indicating the high sensitivity of RT-PCR procedure (Garcia et al. 1997). Detection of *Citrus tristeza virus* (CTV) was accomplished only by using phloem-rich tissues such as petioles or midribs in ELISA test. On the other hand, RT-PCR assay could detect CTV in all tissue types. Furthermore, RT-PCR protocol could be successfully applied for the detection of CTV even during the months when CTV titer dropped below levels that were not detectable by ELISA test (Mathews et al. 1997). The RT-PCR technique, along with the immunoassays ELISA and DTBIA, was evaluated for their efficacy in detecting decline-inducing and non-inducing isolates of CTV in sweet orange and grapefruit trees. The results revealed that RT-PCR assay was more sensitive than the immunoassays. RT-PCR assay was not only able to detect the CTV isolates more efficiently, but also it could differentiate the decline-inducing and non-inducing isolates of CTV. Both isolates could be detected in a single field-infected sweet orange or grapefruit tree (Huang et al. 2004).

Grapevine fanleaf virus (GFLV) is transmitted by the nematode *Xiphinema index* under natural conditions. But long distance dissemination of the disease occurs through movement of vine stock, especially tolerant cultivars without overt symptoms of infection. Virus-infected vines are generally propagated by cuttings, a process that perpetuates infections, making detection of the virus(es) difficult in these planting materials. The primers amplifying a 605-bp fragment containing a part of the coat protein (CP) of GFLV were employed for the detection of 20 isolates of this virus occurring in Tunisia. RFLP analysis indicated sequence variations that could be used as a basis for differentiation of isolates of GFLV (Fattouch et al. 2005a). In another investigation, 82 grapevine samples were tested by RT-PCR assay using the primer sets S2515/A3300 or CP433V/912C. DNA fragments of 810- and 480-bp were amplified respectively by these two primer pairs. The results of detection of GFLV by RT-PCR and ELISA techniques were compared. ELISA-positive samples (22) tested negative by RT-PCR using one or both primer sets. No ELISA-negative samples tested positive by RT-PCR assay. Forty one samples tested negative by both ELISA and RT-PCR procedures. RT-PCR assay appeared to be less robust as a diagnostic method for the detection of GFLV (Bashir and Hajizadeh 2007; Appendix 13).

Production and use of virus-free planting materials is essential for improving the performance of grapevine cultivars and this approach is the basic step for effective management of viruses transmitted through asexually propagated plant materials (Meng et al. 1999). *Grapevine leaf roll-associated virus 1* (GLRaV-1) and *Grapevine rupestris stem pitting-associated virus* (GRSPaV) were eliminated from *Vitis vinifera* cv. Agiorgitiko by combining in vitro thermotherapy and tissue culture methods. The effectiveness of virus elimination was assessed by applying nested RT-PCR procedure for detection of GLRaV-1 and GRSPaV in treated explants cultured in woody plant medium. Meristem or shoot tip cultures coupled with thermotherapy were successfully used to eliminate both GLRaV-1 and GRSPaV to different extent. Relatively high percentages of plantlets were found to be virus-free. It appeared that elimination of GLRaV-1 was easier than GRSPaV. Elimination of GLRaV-1 could be achieved up to 91.2% and 73.8% by meristem tip and shoot tip culture methods respectively. On the other hand, meristem tip and shoot tip cultures eliminated GSPaV up to 61.8% and 37% respectively (Skiada et al. 2009).

The spot-PCR assay, a variant of PCR was developed for the detection of *Grapevine A virus* (GAV), *Grapevine B virus* (GBV) and *Grapevine leaf roll-associated virus 3* (GLRaV-3). Specific amplification of genomic fragments of these viruses was accomplished by RT-PCR on total nucleic acid solubilized from small pieces of charged nylon membrane on which a drop of crude sap of infected grapevine had already been spotted. The heat treatment of the membrane at 95°C for 10 min enhanced the ease of release of viral template from the spot on nylon membrane. The spot-PCR and standard PCR assays exhibited equivalent detection limit. But the spot-PCR format provides the advantage of storability of the blots up to 1 month after spotting (La Notte et al. 1997). Molecular variants of *Grapevine virus A* (GVA) were detected reliably and specifically by employing RT-PCR assay. Isolates of GVA obtained from various grapevines and locations were inoculated on *Nicotiana benthamiana*. These isolates of GVA were detected by RT-PCR in infected *N. benthamiana* plants (Goszczynski and Jooste 2003). RT-PCR assay employing the CP-specific primers detected *Rupestris stem-pitting virus* (RSPaV) in all grapevine samples including those from asymptomatic plants. In contrast, the primer pair designed from the sequences of replicase gene could detect RSPaV only in symptomatic plants (Habili et al. 2006).

Mealybug wilt disease (MWD) is a major disease of pineapple with which two species of mealy bugs *Dysmicoccus brevipes* and *D. neobrevipes* are commonly associated. Filamentous virions typical of members of the family *Closteroviridae* were detected in infected pineapple plants (Gunasinghe and German 1989). Later, sequence analysis indicated the involvement of two viruses designated *Pineapple mealybug wilt-associated virus1* (PMWaV-1) and *Pineapple mealybug wilt-associated virus 2* PMWaV-2) belonging to the genus *Ampelovirus* whose type species is *Grapevine leaf roll-associated virus 3* (GLRaV-3) (Melzer et al. 2001; Sether et al. 2005a). Later, the presence of two more ampeloviruses *Pineapple mealybug wilt-associated virus 3* (PMWaV-3) and *Pineapple mealybug wilt-associated virus 4* (PMWaV-4) was recognized (Sether et al. 2005b). By using degenerate oligonucleotide primers designed based on conserved sequences of related viruses in RT-PCR,

the putative PMWaV-3 was detected in pineapple. Sequence analysis of the C-terminal portion of the RNA-dependent RNA polymerase (RdRp), the complete ORF for a small hydrophobic protein and the N-terminal portion of the HSP-70-like ORF suggest that putative PMWaV-3 was a distinct virus and not a strain of PMWaV-1. This specific RT-PCR protocol was able to efficiently detect the putative PMWaV-3 (Sether et al. 2005a).

In another investigation, a previously undescribed ampelovirus, *Pineapple mealybug wilt-associated virus 5 (*PMWaV-5) was detected in infected plants in Australia. A survey was undertaken to assess the incidence of these viruses in Australian commercial pineapple crops. An RT-PCR protocol was developed to detect four viruses (PMWaV-1, -2, -3 and -5) associated with PMWD. Primers were designed based on the conserved sequence of the helicase and RNA-dependent RNA polymerase (RdRp) regions of ORF1 of the viruses. All four viruses, whether present as single or mixed infections, were amplified using these primers, giving products of similar size (ca. 350-bp). An RT-PCR microplate detection (MTPD) assay was developed to differentiate individual viruses. PMWaV-1 was the most commonly found of the four viruses (78.5%), while PMWaV-5 was present only occasionally (25%) in PMWD-infected pineapple plants in Australia (Gambley et al. 2008).

The diagnostic techniques RT-PCR and ELISA were evaluated for their efficacy in detecting *Banana bract mosaic virus* (BBrMV) and other members of the family *Potyviridae.* Plate-trapped antigen (PTA)-ELISA gave weak serological signals for BBrMV and other potyviruses tested. In contrast, the RT-PCR products from BBrMV and *Abaca mosaic virus* (AbaMV) were subjected to Southern hybridization using virus-specific DIG-labeled DNA probes. The reactions were specific without any cross-reaction (Thomas et al. 1997). Polyvalent degenerate oligonucleotide (PDO)-RT-PCR assay was adopted to the detection of *Banana mild mosaic virus* (Ban MMV) and *Banana virus X* (BVX) belonging to the family Flexiviridae, infecting *Musa* spp. PDO inosine-containing primers were found to be well suited to the detection of BanMMV, but not to BVX. Hence, species-specific primers were designed for the detection of BVX. Sampling and sample processing steps were optimized in order to avoid nucleic acid purification prior to reverse transcription step. A polyclonal anti-BanMMV antiserum was raised and successfully used for the immunocapture (IC) of BanMMV virions from leaf extracts, leading to the development of a PDO-IC-RT-nested PCR assay. For the detection of BVX, direct binding (DB) format was found to be more efficient method for processing BVX-infected samples and a PDO-DB-RT-nested PCR was developed for the detection of BVX from banana leaf extracts (Teycheney et al. 2007).

Blackcurrant (*Ribes nigrum*) crops suffer appreciably due to blackcurrant reversion disease (BRD) caused by *Blackcurrant reversion-associated virus* (BRAV) belonging to the genus *Nepovirus*, family *Comoviridae*. Two forms of the disease – the common European form (E) which does not induce visible symptoms on black currant and the more severe R-form inducing malformed flowers and/or malformed leaves – have been recognized. RNeasy Plant Mini Protocol for Isolation of Total RNA from Plant Cells and Tissues (Qiagen) was

Fig. 2.18 Detection of *Blackcurrant reversion-associated virus* (BRAV) by RT-PCR based on amplification of virus-specific 215-bp fragment from extracts of infected plants. *Lane M*, 100 bp DNA ladder; *Lane 1*, negative control (healthy plant extract); *Lanes 2–6*, symptomatic plants of different cultivars; and *Lane 7*, BRAV positive control (Courtesy of Přibylová et al. 2002; Acta Virologica, Bratisalva, Slovak Republic)

applied and the extracts were subjected to RT-PCR analysis. Using BRAV-specific primers the presence of a 215-bp product was detected in the extracts from all symptomatic plants of two blackcurrant cultivars, but not from negative controls (Fig. 2.18; Přibylová et al. 2002). In a later investigation, *Black currant reversion virus* (BRV) was detected in all symptomatic plants by RT-PCR employing the primer pair P1/P2 which amplified an expected product of 215-bp. The average sequence identity between BRV isolates was about 93.8%. Using BRV-specific primers, the whole of BRV RNA 2 3′ NTR in 15 black, red and white currant plants showing symptoms of either BRD or full blossom disease (FBD) of red currant plants. The results suggested that BRV isolates associated with two forms of BRD might be associated with FBD considered to be due to a phytoplasma infecting red currant plants (Přibylová et al. 2008).

Black raspberry (*Rubus occidentalis*) plants infected by black raspberry decline disease (BRD) were investigated to establish the nature of the causative agent. Double-stranded RNA extraction from symptomatic plants revealed the presence of two high molecular weight bands. Sequence analysis indicated that a novel virus involved in the BRD shared many biological properties with *Black raspberry necrosis virus* (BRNV) described earlier. For the detection of BRNV strain infecting black raspberry plants, an RT-PCR assay was developed, using two sets of primers in tandem. Primer set 1 amplified a 417-nt fragment of RNA-dependent RNA polymerase (RdRp) region of RNA1 and primer set 2 amplified a 350-nt fragment further downstream at the very 3′ end of RdRp region. BRNV was detected in all (9/9) plant samples obtained from commercial black raspberry fields in Oregon. BRNV-specific amplicons were not generated from the extracts of healthy black raspberry controls. All sequenced BRNV amplicons were virus-specific (Fig. 2.19). Further Koch's postulates adoptable for obligate pathogens were tested to prove BRNV was the cause of black raspberry decline disease (Halgren et al. 2007).

Potato virus Y (PVY) was detected by applying the PCR-microplate hybridization procedure. A cDNA fragment from CP region of PVY-RNA genome was amplified by RT-PCR protocol, followed by detection of the amplicons employing DIG-labeled cDNA probe. The protocol was capable of detecting 10 fg of PVY genomic RNA and the sensitivity of detection was 10,000 times greater than ELISA test (Hataya et al. 1994).

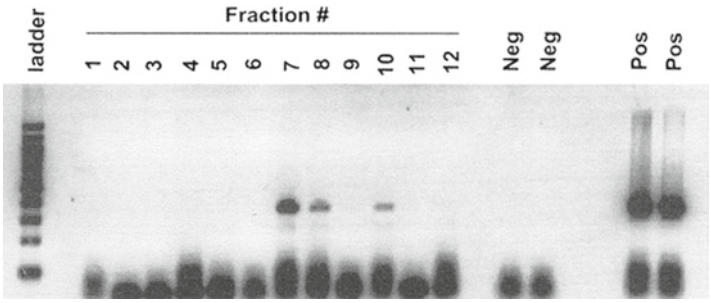

Fig. 2.19 Detection of *Black raspberry necrosis virus* (BRNV) in 12 different fractions collected from sucrose gradients during purification. *First lane*, 100 bp DNA ladder; note the presence of virus-specific fragment in fractions 7, 8 and 10 and in two positive samples at the last two lanes. (Courtesy of Halgren et al. 2007; The American Phytopathological Society, MN, USA)

Detection of immobilized amplified product in a one-phase system (DIAPOPS) was shown to be effective for the detection of four strains of PVY. The primers designed for RT-PCR procedure were employed to detect PVY strains O, N, NTN and C specifically. *Potato V* and *A* viruses were not detected indicating the specificity of DIAPOPS system. However, ELISA tests could detect PVY strains in some samples which gave negative results with DIAPOPS system. This may be due to smaller sample size (less than 10 times required for ELISA) used for DIAPOPS system (Nielson et al. 1998). PVY isolates (PVY^N), causing veinal necrosis in tobacco were detected by RT-PCR procedure. Coupled, one-tube RT-PCR (cRT-PCR) in a duplex reaction using primers Anti, S3 and S7 amplified a product of 281-bp from PVY^O strain. Under these conditions, a product of 745-bp was amplified from all the necrosis-inducing isolates tested. Two-tube RT-PCR tests with primers Anti, S3 and S7 gave results identical to the cRt-PCR tests The cRT-PCR format simplifies the detection and identification of the recombinant viruses, since the standard techniques employed earlier required ELISA and bioindexing on tobacco to identify PVY^N pathotype. The procedure developed in this investigation provided results rapidly leading to actions for reducing the spread of $PVY^{N:O}$ strains that were misidentified as PVY^O by standard ELISA tests (Crosslin et al. 2005). RT-PCR assay was applied for the detection of *Potato mop top virus* (PMTV) by employing the primers targeting the coat protein (CP) gene in RNA3 of PMTV. This protocol was highly effective for detecting the virus in potato seed tuber lots and ware potato during surveillance in the United States and Canada (Xu et al. 2004).

The genus *Tobravirus* encloses three species, *Tobacco rattle virus* (TRV), *Pepper ringspot virus* (PepRSV) and *Pea early browning virus* (PEBV) which infect a wide range of crop plants including beet root, pepper (chilli), potato, beans and tobacco. Detection of tobraviruses by PCR is complicated by the bipartite genome of these viruses, since only one of the RNAs can be detected at a time by simplex PCR format. Many PCR-based detection methods target the CP gene, since this often contains conserved segments that can be used to design generic primers. However, this approach is not ideal for detecting tobraviruses, as the CP gene is on RNA2 which is not required for viral replication. The 194K RNA polymerase gene

in RNA1 has regions with sufficient conservation across all three tobravirus species. Three forward and two reverse primers were designed to five conserved motifs. PCR was performed in triplicates on cDNA from four virus infected samples: TRV (two samples), PEBV (one sample) and PepRSV (one sample). Extracts from all samples produced a band of expected size, although some variations in the intensity of bands could be seen. All six primer combinations produced a band of the expected size. The test could be performed using fresh or freeze-dried tissue, using as little as 50 ng of RNA. The primer pair Tobra-R2 was able to detect all three tobraviruses using a single PCR protocol (Jones et al. 2007).

RT-PCR assay has been applied to assess the effectiveness of using cross-protection phenomenon as a disease management strategy. Sugar beet plants can be protected by *Beet soilborne mosaic virus* (BSBMV) against closely related, but serologically distinct *Beet necrotic yellow vein mosaic virus* (BNYVMV). The presence of RNAs of both protecting and challenging viruses could be detected by RT-PCR assay, indicating that the RNA of challenging virus was also present in the protected beet plants. But the coat protein of BNYVMV was not detectable by ELISA in the protected plants. Possibly encapsidation of BNYVMV cannot take place in the absence of CP, resulting in inactivation of the RNA of the challenging virus (Mahmood and Rush 1999). The presence of viruses causing symptoms in pepper plants similar to those induced by *Beet curly top virus* (BCTV) was detected by employing primers designed to detect a portion of CP gene and primers capable of detecting a portion of replication associated protein (rep) gene. Field isolates exhibiting homology to *Beet mild curly topvirus* (BMCTV) and *Beet severe curly top virus* (BSCTV) were identified (Creamer et al. 2005).

Sweet potato is affected by several viruses for which a simple and sensitive diagnostic procedure was developed. PCR assay based on degenerate primer pair SPG1/SPGG was used to detect nine uncharacterized isolates of geminiviruses infecting sweet potato including *Sweet potato leaf curl virus* (SPLCV-Taiwan). This assay detected the viruses in highly diluted samples ($10^{\wedge-9}$) proving that this protocol was very sensitive and specific (Li et al. 2004). RT-PCR assay was applied in combination with restriction analysis of the PCR amplification products using restriction enzymes *Hind*III and *Pvu*II. The *Sweet potato virus 2* (SPV2; synonymous with *Sweet potato virus Y* and *Ipomea vein mosaic virus*), a tentative member of the genus *Potyvirus* and *Sweet potato feathery mottle virus* (SPFMV) were detected and differentiated by using oligo T(25) primer for reverse transcription and combination of degenerate primers for PCR amplification (Tairo et al. 2006).

Mixed infections of sweet potatoes by two or more viruses are frequently observed. In Australia, incidence of diseases due to *Sweet potato caulimo-like virus* (SPCaLV), *Sweet potato feathery mottle virus* (SPFMV), *Sweet potato virus 2* (SPV2) and *Sweet potato chlorotic fleck virus* (SPCFV) was observed. SPCFV was frequently found in mixed infection along with SPFMV and SPV2. As the concentration of viruses in sweet potato was low generally, *Ipomoea setosa* (a universal indicator host for sweet potato viruses), was top-grafted onto sweet potato plants with symptoms of virus infection. The leaves of *I. setosa* scion shoots were then

tested for the presence of respective viruses. RT-PCR assays were performed using virus-specific primer pairs capable of amplifying CP gene sequences of target viruses. Presence of SPCFV, SPFMV-O and SPV2 was detected by employing virus-specific primers. Use of primers of SPFMV that differentiated RC strains from other strains revealed the mixed infection of at least two strains of the virus. This investigation revealed the incidence of SPCFV and SPFMV-O in Australia for the first time (Jones and Dwyer 2007).

Geographical distribution of *Tomato leaf curl virus* (ToLCV) inducing severe and mild symptoms in tomato was studied by employing PCR assay. DNA of ToLCV was amplified from tomato plants exhibiting mild and severe symptoms by PCR. An isolate of the bipartite *Tomato leaf curl New Delhi virus-Severe* (ToLCVNDV-Svr) induced severe symptoms in tomato. On the other hand, a monopartite virus (*Tomato leaf curl Joydepur virus-Mild* (ToLCJV-Mild) was detected in tomato plants showing mild symptoms. Further investigation is necessary to determine the relationship between the viruses causing severe and mild forms of the disease in two different locations in India (Maruthi et al. 2005). Occurrence of virus-like symptoms on tomatoes with a high frequency was observed in the Andean states of Venezuela. PCR amplification using degenerate primers detected begomoviruses in 18% of tomato leaf samples collected from commercial fields. Sequences of amplified DNA products were compared. Two of the four groups of sequences were clustered based on BLAST, GAP and phylogenetic analyses. They were found to be closely related to *Potato yellow mosaic virus*-Venezuela strain-tomato and *Tomato venezuela virus*. The other two groups of sequences of isolates appeared to belong to two new begomovirus species (Nava et al. 2006).

RT-PCR protocol in combination with restriction endonuclease analysis based on the nucleotide sequences was developed for the detection and differentiation of *Bean common mosaic virus* (BCMV) and *Bean common mosaic necrosis virus* (BCMNV) and their pathogroups (PGs). Two virus-specific primer pairs that could amplify a PCR product specific for each virus were designed. With RT-PCR format, four BCMV-PG V isolates were differentiated from isolates of BCMV pathogroups PGI, PGII, PGIV and PGVII. By employing restriction enzyme *Xba*I for digestion of BCMNV-PCR products, two BVMNV pathogroups PGII and PGIV were differentiated (Xu and Hampton 1996). A simple-direct tube (SDT) method was developed for rapid and easy extraction of viral RNA for cDNA synthesis. This protocol involves grinding of plant tissues in phosphate-buffered saline (PBS) containing Tween-20 (PBST) and placing the extract in a microfuge tube for a few minutes and allowing adsorption of virus particles to the tube wall. SDT method was successfully applied for the detection of *Turnip mosaic virus*, *Cucumber mosaic virus* and *Cucumber green mottle mosaic virus* in infected plants (Suehiro et al. 2005).

A modified RT-PCR protocol was employed for the detection and identification of *Cucurbit yellow stunting disorder virus* (CYSDV). The dsRNA templates were used for amplification. The sequence analysis of the amplified and cloned fragments

of CYSDV RNA2 revealed that the CP was in a contiguous arrangement similar to that of *Lettuce infectious yellows virus* (LIYV). These two viruses were considered as members of a new genus *Crinivirus* in *Closteroviridae* (Liveratos et al. 1999). An RT-PCR assay was developed for the detection of *Squash vein yellowing virus* (SqVYV) infecting squash plant (*Cucurbita pepo*). Primers were designed based on the sequences of coat protein (CP) gene and the product of expected size 957-bp was amplified. This unique band was detected on agarose gels. No amplification products were observed from uninfected plants. However, amplification products of a shorter length (787-bp) were obtained from *Papaya ringspot virus* type W (PRSV-W) virion or total RNA from PRSV-W infected plants (Fig. 2.20). This protocol was found to be useful for detecting mixed infection of SqVYV) and PRSV-W in cucurbits. Comparison of the SqVYV CP gene and protein sequences with those of recognized members of the family *Potyviridae* indicate that it is a novel member of the genus *Ipomovirus* (Adkins et al. 2007).

Parietaria mottle virus (PMoV-T), a member of the genus Ilarvirus, infecting tomato crops was detected by a one-step RT-PCR procedure. Specific cDNA was amplified by RT-PCR from mechanically inoculated *Chenopodium quinoa* plants and cloned into a plasmid vector. A specific set of primers PMoV-1F/PMoV-1R was designed for one-step RT-PCR assay. PMoV-T was detected in necrotic apical shoots, fruits with symptoms and symptomless shoots grown from old necrotic branches of infected tomato plants. The assay was highly specific and no cross-reactions were noted with other related ilarviruses. PMoV-T could be detected in

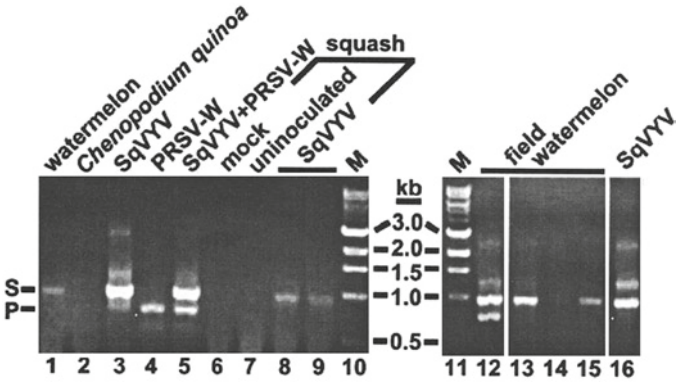

Fig. 2.20 Detection of *Squash vein yellowing virus* (SqVYV) by RT-PCR assay in mechanically- and whitefly-inoculated greenhouse and naturally infected field plants. *Lanes 1* and *2*, RT-PCR products from host range studies by mechanical inoculation with SqVYV; *Lanes 3*, *4* and *16*, positive control RT-PCR products from watermelon plants mechanically inoculated with SqVYV or *Papaya ringsopt virus* type W (PRSV-W); *Lane 5*, positive control RT-PCR products from a mixture of SqVYV and PRSV-W RNA template used to generate the products in *lanes 3 and 4*: *Lanes 6–9*, RT-PCR products from squash test plants in a whitefly transmission experiment; *Lanes 10* and *11*, markers size in kb; *Lanes 12–15*, RT-PCR products from watermelon plants in fields with vine decline symptoms (Courtesy of Adkins et al. 2007; The American Phytopathological Society, MN, USA)

all tomato plants collected from commercial fields in Catalonia, Spain. One-step RT-PCR assay was, however, found to be less robust than molecular hybridization (Galipienso et al. 2005).

Onion crops are infected by more than 20 viruses belonging to the genera *Potyvirus*, *Carlavirus*, *Allexivirus* and *Tosopovirus*. Occurrence in Greece of a virus with isometric virions with similarities to nepoviruses in host range and symptomatology was observed. Application of the nested RT-PCR format developed for the generic detection of *Comoviridae* species gave the expected amplicon (750-bp) for the onion virus and *Artichoke yellow ringspot virus* (AYRSV-Atg) and AYRSV-F isolates (Maliogka et al. 2004). An RT-PCR assay using degenerate primers specific for the family *Comoviridae* allowed amplification of an RNA-dependent RNA polymerase sequences which upon sequence analysis and comparison with AYRSV isolates from *Cynara scolymus* (AYRSV-Atg) and *Vicia faba* (AYRSV-F) were highly similar, thus providing evidence that the nepovirus AYRSV could infect onion in the field. All AYRSV isolates from different host plants were specifically detected by RT-PCR using the primers designed for amplifying a 530-bp DNA fragment (Maliogka et al. 2006).

Garlic and onion crops are severely affected by *Onion yellow dwarf virus* (OYDV) in many commercial production areas in India. An RT-PCR assay was developed for the detection of OYDV in the leaves and bulbs of garlic and leaves of onion plants. Specific primers were designed from the sequences of conserved region of RNA-dependent RNA polymerase (RdRp) gene and 3′-UTR region of viral RNA of OYDV isolates. Total RNA was extracted from leaves/bulbs of infected plants using RNeasy Plant Mini Kit (Qiagen) as per the manufacturer's recommendations. After amplification of templates, PCR products were detected on agarose gels after staining with ethidium bromide. OYDV was detected in naturally infected garlic bulbs and leaves and onion leaves based on the presence of 1.1-kb band. Sequence analysis of this PCR product showed that it had identity with OYDV isolates in amino acids of coat protein (CP) gene from 74.9% to 96.1%. OYDV isolate examined, shared maximum sequence identity with a Brazil isolate (Arya et al. 2006).

A duplex RT-PCR procedure was developed for the simultaneous detection of *Onion yellow dwarf virus* (OYDV) and *Shallot latent virus* (SLV; synonym *Garlic latent virus*). Total RNA was extracted from tissues of infected or healthy garlic leaves or cloves. Virus-specific primers successfully amplified a 1.1-kb product specific to OYDV and a 308-bp product specific to SLV. The duplex RT-PCR format was applied to detect the presence of OYDV and SLV in 10 garlic selections. Duplex RT-PCR assay detected both viruses in all selections of garlic. In the case of two selections (17 and 34) of garlic, one sample of each selection gave negative result. No amplification occurred in the extracts of tissue culture-derived healthy garlic plants. The sensitivity of duplex RT-PCR format was in equivalence with standard RT-PCR in detecting individual viruses. Duplex RT-PCR procedure could be used for simultaneous detection of OYDV and SLV in extracts of both leaves and bulbs of garlic plants reliably and rapidly (Majumder et al. 2008).

The RT-PCR assay has been found to be effective and more sensitive in detecting the plant viruses infecting ornamental crops compared to immunoassays. *Cymbidium*

mosaic virus (CyMV) and *Odontoglossum ringspot virus* (ORSV) were detected more efficiently by RT-PCR assay compared to ELISA tests. Detection limit of RT-PCR assay was 1,000 times lower than that of ELISA, in addition to the results of RT-PCR assay being more reliable and becoming rapidly available (Park et al. 1998). In another investigation, the possibility of detecting both CyMV and ORSV simultaneously by employing a pair of common primer in a single PCR was demonstrated. Sequence alignment and primer analysis were used for primer design (Seoh et al. 1998). This approach was further enlarged by the GPIME package, a computer program developed for identifying the best regions of aligned viral genes. RT-PCR assays were effective for the detection of CyMV, ORSV and *Ceratobium mosaic virus* (Gibbs et al. 1998).

Angelonia flower break virus (AnFBV), a new virus infecting Angelonia plants caused mild foliar and flower break symptoms. Electron microscopic observations revealed the presence of ca. 30 nm isometric particles. The genomic sequences of the Florida AnFBV isolate was used to design primers flanking the deduced CP gene for amplification of the corresponding genomic regions from the Israeli and Maryland isolates. The virus infecting Angelonia plants was detected by the formation of AnFBV-specific product which was also amplified with degenerate carmovirus primers. In addition, AnFBV was detected in symptomless hosts by RT-PCR and/or ELISA tests (Adkins et al. 2006).

Degenerate primers were employed for detection of *Lily symptomless virus* (LSV). These primers were designed based on the amino acid sequences of coat protein (CP) in four LSV strains and used to amplify a 483-bp fragment from LSV. Lilies generated by repeated shoot meristem excision method were tested before and during in vitro culture and thermoptherapy applied to bulblets in vitro. The amplified banding patterns by RT-PCR confirmed the absence of LSV in the genotype '409' after first meristem isolation. But the presence of LSV was detected in plants formed directly from the scale or after shoot tip culture in line '509'. The protocol developed in this investigation may find wider application in commercial tissue culture laboratories (Nesi et al. 2009).

Rice tungro disease (RTD) is caused by combined infection by two viruses, *Rice tungro spherical virus* (RTSV) (with RNA genome) and *Rice tungro bacilliform virus* (RTBV) (with DNA genome). RTBV induces major symptoms of RTD, while RTSV assists the transmission of RTBV by different species of green leafhoppers RTSV was detected by RT-PCR assay using specific oligonucleotide primers to amplify the coat protein gene fragments of RTSV. Differentiation of two strains of RTSV was possible by variations at the nucleotide positions 2,556 and 3,032 based on published sequence data. RT-PCR assay could be applied to study RTSV CP variations in natural field populations (Yambao et al. 1998). The presence of RTBV was detected by PCR assay in RTD affected rice leaves, but not in the leafhopper vector *Nephotettix* spp. PCR assay was found to be 1,000–10,000 times more sensitive than ELISA test (Takahashi et al. 1993). But in a later investigation, a small fragment of RTBV-DNA was successfully amplified by PCR from the total nucleic acid extract of a single viruliferous leafhopper and this PCR product was detected on agarose gel (Venkitesh and Koganezawa 1995). Transgenic *japonica* rice plants

expressing the full length RTSV replicase (Rep) gene in the sense orientation were highly resistant to RTSV. Accumulation of RTSV-RNA was low in the resistant plants as revealed by RT-PCR assay. The transgenic resistance was effective against geographically distinct RTSV isolates tested (Huet et al. 1999).

The RT-PCR protocol suitable for the detection of *High Plains virus* (HPV), a potential threat to cereals was developed for adoption by quarantine programs. A procedure for inspecting plants and testing seedlings employing RT-PCR was standardized (Lebas et al. 2005). A broad-spectrum RT-PCR assay was applied for detecting *Soilborne cereal mosaic virus* (SBCMV) isolates, employing primers targeting the highly conserved 3′-untranslated region of RNA-1 and RNA-2 of SBCMV. The 3′-end region is a privileged target for the detection of a wide range of isolates, because of sequence conservation of the tRNA-like structure, the major role in virus replication and signal amplification due to the presence of numerous genomic and subgenomic RNAs. European isolates of SBCMV from Belgium, France, Germany, Italy and the UK were detected using the protocol developed in this investigation (Vaïnopoulos et al. 2009).

Detection and differentiation of *Sugarcane mosaic virus* strains (A, B, D and E) and *Sorghum mosaic virus* strains (SCH, SCI and SCM) was accomplished by employing group specific primers for RT-PCR-based random fragment length polymorphism (RFLP) analysis. As the molecular technique was rapid and reliable, the use of differential hosts producing characteristic symptoms was dispensed with for the first time (Yang and Mirkov 1997). A combination of RT-PCR and restriction enzyme analysis was employed for the detection and differentiation of four potyviruses viz., *Maize dwarf mosaic virus* (MDMV), *Sugarcane mosaic virus* (ScMV) *Johnsongrass mosaic virus* (JGMV) and *Sorghum mosaic virus* (SrMV). Total RNA extracts from infected plants was subjected to RT-PCR assay and a 327-bp fragment of capsid protein gene was amplified. Following digestion with restriction enzymes *Alu*I and *Dde*I, virus-specific band patterns were obtained. Characterized strains and field-collected isolates of potyviruses could be reliably identified (Marie-Jeanne et al. 2000). The presence of *Sugarcane yellow leaf virus* (SCYLV) was detected by using RT-PCR assay in leaves, shoots and roots of all sugarcane cultivars tested. In addition, SCYLV was also detected in the aphid vector *Melanaphis sacchari* (Rassaby et al. 2004). Specific primer pairs were employed in the RT-PCR format for the detection of SCYLV genotypes BRA-(Brazil), CUB-(Cuba), PER-(Peru) and REU-(Réunion Island). The presence of these SCYLV genotypes was detected in 245 samples collected from 18 different sugarcane growing locations in the world. One of these three genotypes was detected in most of the samples tested and mixed infection by more than one genotype also could be detected in some samples (Ahmad and Royer 2006).

Infection of *Phaseolus* spp. by geminiviruses is seen frequently and the virus(es) involved in the diseases has to be detected and differentiated reliably, in order to formulate effective disease management practices. French bean infected by a geminivirus in Brazil had sequences nearly identical to that of *Bean golden mosaic virus* (BGMV-BZ), whereas lima bean was found to be infected by a new species of geminivirus which caused symptoms similar to those due to BGMV-BZ. The new

virus species was identified based on the differences in the sequences of geminivirus DNA fragments, comprising part of the *rep* gene, the common region and part of the CP gene. All sequences from bean isolates clustered with BGMV-BZ, whereas the sequences of lima bean isolate were distinctly different (Faria and Maxwell 1999). Specific primers were designed from RNA replicase cDNA sequence for the detection of *Cowpea mottle virus* (CPMoV). The RT-PCR protocol efficiently detected CPMoV in the germplasm of *Vigna* spp. and it was 10^5 times more sensitive than DAC-ELISA format. In addition, false negative reaction, as observed sometimes with ELISA test, was entirely absent with detection by RT-PCR assay (Gillaspie et al. 1999).

Variants of RT-PCR have been developed with aim of simplifying the procedure for the standard format and enhancing the level of reliability to facilitate their wide applicability. The print or spot capture PCR (PC-PCR) assay was developed for the detection of viruses in their plant hosts and also in the vector species. The print capture (PC)-RT-PCR format, analogous to tissue blot immunoassay (TBIA), was shown to be effective for the detection of *Plum pox virus* (PPV) (Olmos et al. 1996). But this format was found to be efficient for the detection of PPV in glasshouse-grown plants only and it was less effective for the detection of PPV in field-collected samples (Varveri and Boutiska 1998). On the other hand, PC-PCR was found to be suitable for the detection of PPV, *Apple chlorotic leaf spot virus* (ACLSV), *Prunus necrotic ringspot virus* (PNRSV) and *Apple mosaic virus* (ApMV) in tissue imprints of different stone fruit tree tissues. The immobilized target nucleic acids on imprinted papers can be transported to testing centers for amplification by PCR. The PC-PCR assay could be employed in conjunction with hemi-nested print-PCR and PCR-ELISA combinations for simultaneous detection and typing of PPV isolates to serotypes M or D in tissues (Cambra et al. 1998).

Detection of geminiviruses was efficiently achieved by employing the print capture PCR (PC-PCR) format. Direct amplification of viral DNA from infected plants or whitefly tissues imprinted directly on Whatman 3 MM paper was performed. This protocol eliminates tissue processing, incubation and washing steps prior to PCR amplification. Further, there is no cross-contamination between samples that is likely to occur when standard PCR procedure is followed. Detection and differentiation of *Tomato yellow leaf curl virus* (TYLCV) isolates TYLCV-Sv and TYLCV-Is was accomplished by PC-PCR format (Navas-Castillo et al. 1998). Viruses infecting olive trees were detected and identified by employing one-step RT-PCR procedure. *Arabis mosaic virus* (ArMV), *Cherry leaf roll virus* (CLRV), *Cucumber mosaic virus* (CMV), *Olive leaf yellowing-associated virus* (OLYaV), *Olive latent virus-1* (OLV-1), *Olive latent virus-2* (OLV-2) and *Strawberry latent ringspot virus* (SLRSV) were detected in olive trees in Italy. Among these viruses, OLYaV was more predominant in southern parts, whereas SLRSV was more frequently encountered in the central Italy (Faggioli et al. 2005).

Nested PCR technique is particularly useful in the case of viruses present in low concentrations in the host plant tissues and when the inhibitors of PCR are present in host tissue extract. The first round PCR amplification is performed by employing one primer pair and the amplicons obtained are again amplified by the second

primer pair resulting in high concentrations of the unique band characteristic of the target virus, leading to increase in the sensitivity of detection of the virus concerned. A combination of degenerate deoxyinosine (dI) substituted primers amplified part of the RNA-dependent RNA polymerase (RdRp) domain, followed by a semi-nested PCR amplification that is adopted. In the absence of viral genomic dsRNA or viral RNA, RNA extracts from infected plants can be used for detection of target virus. Nested PCR assay was employed for efficient detection of *Potato black ring-spot virus* (PBRSV) and *Cherry leaf roll virus* (CLRV) (Maliogka et al. 2004).

The usefulness of nested PCR format for the detection of viruses belonging to *Vitivirus* and *Foveavirus* infecting grapevines was reported by Dovas and Katis (2003). In a later study, a nested RT-PCR assay was developed that allowed generic detection of a subgroup of genetically related viruses with a distinct evolutionary history within the genus *Ampelovirus*. *Grapevine leaf roll-associated virus* (GLRaV)-4, -5, -6, -9 and two isolates (GLRaV-De and GLRaV-Pr) that represented new species belong to this lineage. This method involves a one-step RT-PCR for generic detection of *Closteroviridae* species using degenerate primers that target the heat shock protein HSP70h gene followed by a nested PCR which detects all virus-members of the lineage and differentiates them from other grapevine closteroviruses. The 490-bp nested PCR amplicons, corresponding to a phylogenetically informative region can be sequenced directly to obtain initial genetic information for their partial characterization and rapid classification. Additional primers were required for the specific detection of GLRaV-4, -5, -6, -Pr and -De for respective single or multiplex nested PCR assays. All isolates of GLRaV-4, -5, -6, -9, -De and -Pr, either in single or in multiple infections were successfully amplified when the degenerated primers LR5-ClusdoL and dHSP-nest2 were employed. No amplicon was obtained for the isolates of GLRaV-1, -2 and -3 or healthy control. The samples (52) that were found positive using the generic nested PCR for the detection of all subgroup I ampeloviruses, were further analyzed using specific primers for GLRaV-4, 5, -6, -De and -Pr. The most common means of grapevine leaf roll disease dissemination are vegetative propagation and grafting. Hence, management of this disease is primarily based on clonal selection and production of healthy propagating materials for which the reliable diagnostic method developed in this investigation will be useful (Maliogka et al. 2008).

Among the nine *Grapevine leaf roll-associated virus 1-9* (GLRaV-1-9), GLRaV-2 was found to be one of the most variable species of this group. As GLRaV-2 occurred at low concentrations with variable populations, an improved sensitive method that could detect all known variants was considered essential. New universal primers targeting the sequences in the 3′ end of the virus genome were designed. These primers were combined with a one-step SYBR Green real-time RT-PCR assay to achieve quantitative detection. All 43 GLRaV-2 isolates tested in this investigation were detected and identified rapidly and reproducibly, regardless of their geographical origin or variety of grapevine. The sensitivity of the protocol was enhanced by 125 times compared to conventional single-tube RT-PCR format. This real-time RT-PCR procedure has the potential for selection of virus-free grapevine plants for propagation and disease monitoring (Beuve et al. 2007).

Nested RT-PCR assay has been shown to be effective for the detection and identification of *Comoviridae* species. Degenerate primers containing inosine were employed in a polyvalent RT-PCR assay for the detection of filamentous virus belonging to the genera *Trichovirus*, *Capilovirus* and *Foveavirus*. An expected PCR product of 362-bp was amplified from nucleic acid extracts from *Prunus* and *Malus* leaf samples. It was possible to detect all targeted viruses by this nested RT-PCR format (Foissac et al. 2005). An RT-PCR protocol was developed for the detection of *Peach mosaic virus* (PcMV). PcMV-derived primers PM16AFF and PM16AFR were employed in this RT-PCR format for screening a range of virus isolates representing different genera within the family *Flexiviridae.* These primers targeted the 3′ terminus of the coding region of the replication protein (ORF1) and amplified a fragment of 419-bp. Cross-reactions with some isolates of *Apple chlorotic leaf spot virus* (ACLSV) were seen when conventional RT-PCR procedure was followed. Hence, a semi-nested RT-PCR was employed using cDNA generated by PM16AFR for amplification with the PM-AF1 and PM-AFR primers. The specificity of detection of PcMV was enhanced by the nested RT-PCR procedure (James et al. 2006).

Tomato yellow leaf curl disease (TYLCD) is caused by mixed infections by *Tomato yellow leaf curl virus*-Israel (TYLCV-IL), *Tomato yellow leaf curl virus*-Mild (TYLCV-Mld) and *Tomato yellow leaf curl Sardinia virus* (TYLCSV). As tomato is intercropped with cucurbits in Jordan, the role of cucumber, squash and cantaloupe (*Cucumis melo*) in TYLCD perpetuation was studied. By employing nested PCR (nPCR) procedure, the infection by TYLCV-IL, TYLC-Mld and TYLCSV was detected in artificially inoculated cucumber, squash, melon and watermelon plants. These plants did not exhibit any visible symptoms. Virus-specific products of expected size of 634-, 323- and 314-bp were detected following amplification by primers for TYLCV-IL, TYLCSV and TYLCV-Mld respectively in the nPCR format. The TYLCV-IL full-length clone was used to inoculate cucumber, squash, melon, watermelon, tomato and *Nicotiana benthamiana* plants. All inoculated plants tested positive for direct PCR assay. The results indicated for the first time the natural infection of cucurbits by TYLCD-causing viruses and the possibility of these host plant species serving as sources of infection for tomato crops (Anfoka et al. 2009).

Infection of one plant species by two or more viruses occurs more frequently in the case of perennial crops, because of their exposure to viruses for longer periods extending to several years. In addition, the asexual propagation methods also increase the chances of getting multiple infections by different viruses. In such cases, it is desirable to apply a single test that can detect multiple viruses to reduce the time and cost of testing that may be required if one test is performed for each virus involved in the disease complex. Multiplex RT-PCR technique can be employed to amplify multiple viruses present in a single sample in single reaction. In this technique, multiple primer sets in place of one primer pair are used. Amplification of PCR product by one template may be influenced in the multiplex format. An internal amplification control is incorporated into the multiplex to produce additional confidence in the results obtained.

The most important disease management strategy applicable for grapevine virus diseases is the use of virus-free propagation materials. Reliable and specific detection

techniques are essential to attain the above mentioned objective. As the virus concentration reached in grapevine was low, immunoassays were found to be unsatisfactory. A multiplex procedure was applied for the detection of *Grapevine leaf roll-associated virus 3* (GLRaV-3) and *Grapevine B virus* (GBV) (Minafra et al. 1993; Hadidi et al. 1995). RT-PCR procedure was found to be effective for simultaneous synthesis of cDNA of viral and viral RNAs in plant extracts. GLRaV-1, -2 and -4, *Grapevine viruses A* and *B*, *Grapevine fleck virus* and *Grapevine fan leaf virus* were efficiently detected up to a 10^{-3}-fold or higher dilution of the original plant extract. In addition, two viroids infecting grapevines, *Hop stunt viroid* and *Grapevine yellow speckle viroid* were also amplified to the same level as the viruses. This protocol can be applied reliably for the simultaneous detection of the viruses and viroids infecting grapevines. It is possible to provide diagnosis for a large number of samples at reduced cost for the tests (Nakaune and Nakano 2006).

A multiplex RT-PCR (mRT-PCR) assay capable of detecting nine grapevine-infecting viruses was developed. Primers were designed from conserved regions of each of *Arabis mosaic virus* (ArMV), *Grapevine fan leaf virus* (GFLV), *Grapevine fleck virus* (GFV), *Grapevine leaf roll-associated virus1*, *2*, *3* (GLRaV-1, -2, -3). The specificity of amplification by the primers was confirmed by sequencing PCR products. In addition, a plant RNA internal control was included as an indicator of the RNA extraction and RT-PCR amplification. One to nine fragments specific for viruses were simultaneously amplified from infected samples and identified by their molecular sizes in agarose gel electrophoresis. In the two-step mRt-PCR format, the detection limits were 10^{-3} to 10^{-4} extract dilutions depending on the virus concerned (Gambino and Gribaudo 2006). Multiplex nested PCR assays were evaluated for their efficacy for the simultaneous detection of GLRaRV-4, -5 and -6 and isolates GLRaV-De and -Pr . The results were consistent, but the sensitivity of detection of GLRaV-5 and -6 was poor in two samples tested. This kind of preferential amplification of one target sequence over others was attributed to the competitive nature of multiplex PCR due to the differences in the efficacy of primer pairs (Maliogka et al. 2008).

Differential and simultaneous detection of nepoviruses infecting grapevines was achieved by employing degenerate and species-specific primers. Three sets of degenerate primers, one for each of the three subgroups A, B and C of the genus *Nepovirus* were designed based on the nucleotide sequence homology of RNA-1 and RNA-2 of nepoviruses isolated from grapevines. A fragment of 255-bp was amplified from grapevine samples infected by GFLV, ArMV, *Tobacco ringspot virus* (TRSV) and *Grapevine deformation virus* (GdefV) belonging to subgroup A, but no amplification occurred from the viruses of B and C subgroups. Similarly, subgroup B nepoviruses *Grapevine chrome mosaic virus* (GCMV), *Tomato black ring virus* (TBRV), *Grapevine Anatolian ringspot virus* (GARSV) and *Artichoke Italian latent virus* (AILV) yielded an amplification product of 390-bp from grapevine samples infected by these viruses only. The subgroup C viruses *Tomato ringspot virus* (ToRSV), *Grapevine Bulgarian latent virus (*GBLV) and *Grapevine Tunisian ringspsot virus* (GTRSV) were detected by using a third set of primers which amplified a 640-bp product from grapevine plants infected by these viruses alone.

Multiplex PCR detection of subgroups A and B nepoviruses was possible by employing a specific primer (sense for subgroup A and anti-sense for subgroup B) for each of the species of the same group in combination with the degenerate subgroup specific primers. Four different viral species could be detected in single grapevine samples containing mixtures of viruses of the same subgroup, using sense and anti-sense primers. PCR products amplified for TRSV, GFLV, ArMV and GDefV (subgroup A) were respectively 190-, 259-, 301- and 371-bp, whereas GCMV, AILV, GARSV and TBRSV produced respectively amplicons of 190-, 278-, 425-, and 485-bp. The one-step RT-PCR format can be applied for rapid resolution of compound of mixed infections in grapevines (Digiaro et al. 2007).

Prunus necrotic ringspot virus (PNRSV) and *Plum pox virus* (PPV) were detected by both conventional RT-PCR and multiplex RT-PCR assays which were equivalent in the efficiency of detection. Multiplex RT-PCR may be applied for testing a limited number of samples to verify the health status of plant materials especially, if reliable results are not obtained by employing ELISA test (Kölber et al. 1998). Eight important stone fruit viruses, *Apple mosaic virus* (ApMV), PNRSV, *Prune dwarf virus* (PDV), *American plum line pattern virus* (APLPV), *Plum pox virus* (PPV), *Apple chlorotic leaf spot virus* (ACLSV) and *Plum bark necrosis stem-pitting-associated virus* (PBNSPaV) were detected by employing a one-step RT-PCR procedure. A large number of virus combinations could be detected and presence of up to three different viruses was revealed in five samples. However, the sensitivity of detection by this protocol was reduced, when the primer cocktail contained more than five different pairs of primers. But the detection sensitivity levels were more than those of ELISA and hybridization methods (Sánchez-Navarro et al. 2005).

Pear and apple cultivars are seriously affected by *Apple stem-pitting virus* (ASPV), *Apple chlorotic spot virus* (ACLSV) and *Apple stem grooving virus* (ASGV). As infection by these viruses remain latent in commercial pear and apple cultivars, it was necessary to develop reliable diagnostic techniques to ascertain the presence of these viruses even in dormant trees. Two multiplex RT-PCR procedures with coamplification of an internal control from a plant mRNA sequence were employed for the detection of ASPV, ACLSV and ASGV. Of the different plant tissues tested, bark chips of 1–2-year old pear branches were found to be most suitable for extracting RNA and allowing successful detection of the viruses all year round even in dormant trees. A specific internal RNA control which could be amplified from total nucleic acids was coamplified with target viruses. Three virus-specific primer pairs were designed to allow two multiplex RT-PCR amplifications accompanied by one pair of primers of the mitochondrial *nad5* gene. Four amplification products specific for the three viruses and internal control were sequenced to reveal the specificity of all primer pairs. The *nad5* gene in mitochondrial mRNA is present even in dormant trees, because the products of this gene belong to the basic metabolism cycle of plants. Hence, inclusion of *nad5* gene sequence as internal control facilitated the detection of the viruses in pear trees successfully in all seasons (Ma et al. 2008).

Prune dwarf virus (PDV) and *Prunus necrotic ringspot virus* (PNRSV) are often present in mixed infections. It has become essential for the phytosanitary certification

programs to ensure the absence of these two viruses in plants and planting materials. An inter-laboratory evaluation involving nine European laboratories of a duplex RT-PCR method using plant crude extracts as template for the molecular diagnosis of PDV and PNRSV. The primer pair PNRSV-10F/PNRSV-10R amplified a 348-bp fragment of PNRSV coat protein, while the primer pair PDV-17F/PDV12R amplified a 303-bp fragment of PDV CP protein. A total of 576 samples from *Prunus* trees with known sanitary status were tested. The specificity of the duplex RT-PCR protocol was 87% for PDV and 94% for PNRSV, while sensitivity was estimated to be 98% and 90% respectively for PDV and PNRSV. The sensitivity and specificity values of simultaneous detection of the two viruses using crude extracts were equal or higher than the average values published in the literature for these viruses in simplex assays using purified RNA or DNA, revealing the superiority of the duplex assay system evaluated by the European laboratories (Massart et al. 2008).

Multiplex RT-PCR (mRT-PCR) assay was applied for the detection of seven viruses (belonging to six genera), *Citrus leaf rugose virus* (CLRV), *Citrus psorosis virus* (CPsV), *Citrus tatter leaf virus* (CTLV), *Citrus tristeza virus* (CTV), *Citrus variegation virus* (CVV), *Citrus yellow mosaic virus* (CYMV) and *Indian citrus ringspot virus* ICRSV). Degenerate primers were designed based on the sequences of respective virus isolates. PCR products with 245–943-bp specific for each virus were amplified and the viruses were identified on the basis of molecular sizes of the amplicon. Reduced contamination risk and cost of test, reduction in test duration and rapidity of obtaining results are advantages over other diagnostic methods (Roy et al. 2005).

Multiple infection of *Citrus* spp. by two or more viruses and viroids may occur frequently, resulting in progressive decline and ultimate death of infected trees. *Citrus tristeza virus* (CTV) with an RNA genome and *Citrus moaic badna virus* (CMBV) with a DNA genome infect sweet oranges together in India. Two primer pairs specific for ORF8 of CTV genome and ORF3 of CMBV genome were able to generate PCR products of expected sizes of 510- and 245-bp respectively from extracts of infected citrus leaf samples. No amplification was observed in the extracts from healthy leaves. Detection limits of duplex and simplex assays were compared. The duplex PCR format had detection limits of 10^{-6} and 10^{-5} for CTV and CMBV respectively, whereas the simplex assay detected CTV and CMBV at dilutions of 10^{-6} and 10^{-4} respectively. The duplex PCR procedure provided the advantage of detecting both viruses in a single test with higher sensitivity, thus saving time and cost of testing (Ghosh et al. 2008).

Multiplex RT-PCR assays have been developed for simultaneous detection of viruses infecting strawberry. Isolates (18) of *Strawberry crinkle virus* (SCV), *Strawberry mild yellow edge virus* (SMYEV), *Strawberry mottle virus* (SMoV) and *Strawberry vein banding virus* (SVBV) present in different combinations could be detected by the multiplex format. The upper limit of detection of tissue extract was 1/200. However, problems such as poor sensitivity for one or more targets may be encountered, especially when the concentrations of templates in the reaction are different (Thompson et al. 2003). Three viruses infecting strawberry, *Raspberry ringspot virus* (RpRSV), *Strawberry latent ringspot virus* (SLRSV) and *Tomato bushy stunt virus* (ToBSV),

along with a plant internal amplification control (PIAC) were employed as targets to demonstrate that primer interaction is a crucial factor affecting the development of reliable multiplex PCR. Approaches to mitigate primer interactions and eliminate PCR inhibitors including reduction in primer concentration and extension temperature, use of bovine serum albumin (BSA), in combination with extension time and optimum number of PCR cycles were useful to develop a reliable multiplex PCR protocol for the detection of the four strawberry viruses. This protocol had the same specificity and comparable sensitivity as the simplex PCR assay (Wei et al. 2008).

Multiple infection of strawberry by *Strawberry mild yellow edge virus* (SMYEV), *Strawberry mottle virus* (SMoV) (viruses with RNA genome) and *Strawberry vein banding virus* (SVBV) (virus with DNA genome) can cause severe losses. Availability of good quality RNA is the primary requirement for detection of RNA viruses by RT-PCR assay. Detecting strawberry viruses by PCR that depends on the quality of the extracted DNA was not easy, since the secondary metabolites of strawberry were found to have direct inhibitory effects. A reliable protocol for the isolation of nucleic acid adaptable for a large-scale indexing was developed. Total nucleic acid extracted from strawberry leaves was compared with total RNA as the template for detection of two RNA viruses and one DNA virus by both single and multiplex RT-PCR assays. Primer pairs D1/D3, I2/SM2 and YT1/Y2 were selected for the amplification of expected fragments respectively from SMoV, SVBV and SMYEV by multiplex RT-PCR using total nucleic acid as the template. Total nucleic acid extract obtained with the modified CTAB method and total RNA extracts obtained with modified RNeasy and modified CTAB methods, when examined by electrophoresis, resulted in a high quality ribosomal 28S and 18S RNA. When total RNA and total nucleic acid were used as templates, detection efficiency for SMYEV and SMoV was equivalent and intensity of band amplified by single or duplex RT-PCR format was of the same order. On the other hand, with total nucleic acid as template, detection results of SMYEV and SMoV by multiplex RT-PCR showed good correlation with those of single and duplex RT-PCR assays. SVBV could be detected with equal sensitivity and reliability as the direct PCR using pure DNA as the template. Extraction of total nucleic acid using the modified CTAB method was found to be fast, easy and quite economical, costing less than $ 0.2 per sample, approximately 50 times cheaper than modified RNeasy method. The protocol developed in this investigation can be effectively used for simultaneous detection of both RNA and DNA viruses infecting strawberry plants (Chang et al. 2007; Appendix 14).

A multiplex RT-PCR was developed using primers targeting the P1 cistron of *Potato virus Y* (PVY). Based on the results, PVY isolates were placed in two main groups: isolates producing leaf necrosis in tobacco (European PVY^{N}, European PVY^{NTN}, $PVY^{N:O}$ and North American PVY^{N} and North American PVY^{NTN}) and isolates that did not produce leaf necrosis in tobacco (PVY^{O}) (Nie and Singh 2002). Later, the multiplex assay was used in conjunction with the P1 assay to detect and identify PVY isolates. The recombinants PVY^{NTN} and $PVY^{N:O}$ were identified based on amplification of three amplicons or one amplicon around the respective

recombination junctions (Nie and Singh 2003). Another multiplex based on *Hinc*II restriction digests of P1 amplicons was developed to differentiate PVY^{O} from necrotic strains. This assay could detect strain mixtures of PVY^{O} and PVY^{N}, PVY^{NTN}, NA-PVY^{N} or NA-PVY^{NTN} (Crosslin et al. 2005). These assays were limited in their ability to detect some combinations of mixed strain infections. Hence, a single multiplex RT-PCR assay that could assign PVY strain type and detect mixed infections with respect to the major strain types was developed. The multiplex identified 17 samples with strain mixtures from 118 previously characterized isolates considered to be single-strain samples. It was possible to distinguish 16 mixed infections that had gone earlier undetected. This multiplex assay is robust, inexpensive and the results have been validated in four additional laboratories. Further, the assay enables characterization of major PVY strains and detection of strain mixtures in a single assay that may be useful for certification agencies (Lorenzen et al. 2006).

A multiplex (m)-RT-PCR assay was developed for detection of three viruses *Potato virus Y* (PVY), *Potato virus X* (PVX) and *Potato leaf roll virus* (PLRV) in one reaction, using three specific primers designed to amplify the full-length of the CP gene of each virus. The fragments of expected size 548-, 700-, and 810-bp were amplified from the cDNAs of PLRV, PVX and PVY respectively. The specificity of the three primers was confirmed by the nested PCR format using three internal specific primer pairs designed to amplify 360-, 420- and 480-bp from the internal sequence of the CP gene of PVX, PLRV and PVY respectively. The mRT-PCR protocol developed in this investigation was able to detect all three viruses in a single reaction in naturally infected field-grown potatoes. The nested PCR assay was employed to confirm the presence of individual virus in infected plants without the requirement of performing sequence analysis. Multiplex procedure saves time and test cost and it may be suitable for quarantine and certification programs (Shalaby et al. 2002). Simultaneous detection of *Potato virus A* (PVA), *Potato virus S* (PVS), PVX and PLRV was achieved by applying a multiplex RT-PCR assay. Virus-specific primers and an internal control designed from the sequences of 18S RNA were employed in this protocol. These viruses individually and in different combinations could be detected by this assay which was 100 times more sensitive for detection of PVX than the commercial DAS-ELISA format. Positive results were obtained in the same samples that were ELISA-negative, indicating the greater reliability and sensitivity of the mRT-PCR format developed in this investigation (Du et al. 2006).

A multiplex PCR procedure was developed using published primers for the simultaneous detection of three viruses, *Potato yellow vein virus* (PYVV), *Tomato infectious chlorosis virus* (TICV) and *Tobacco rattle virus* (TRV) in infected potato leaf tissue extracts with a sensitivity of the same order as simplex PCR. Amplification of three target viruses and a plant internal amplification control (PIAC) was optimized by adding bovine serum albumin (BSA) during cDNA synthesis, increasing the PCR extension time, reducing the PCR extension temperature and optimizing the concentration of each primer pair. The multiplex PCR detected these viruses dilutions of 1:2,000, 1:800 and 1: 4,000 respectively for PYVV, TICV

and TRV, when individual cDNA samples were tested using virus-specific and PIAC primers. On the other hand, dilutions of 1:2,000, 1:500 and 1:2,000 respectively were required for PYVV, TICV and TRV, when the three cDNAs were mixed. This multiplex was found to be reliable and sensitive for simultaneous detection of three potato viruses and internal control. This cost-effective procedure may be useful for quarantine and certification programs (Wei et al. 2009).

Pepino mosaic virus (PepMV), a *Potexvirus*, originally described on Pepino (*Solanum muricatum*), seriously affected tomato production in Europe and North America. In order to detect PepMV strains, a single RT-PCR-RFLP assay was developed for simultaneous detection of the five different genotypes CH2, US2, CH1/US1, EU and PE of PepMV. This protocol required two different coding sequences (polymerase and coat protein genes) that were digested by six different restriction enzymes (Hansen et al. 2008). In a later investigation, a multiplex one-step RT-PCR procedure was developed for simultaneous detection and identification of three groups of PepMV: European/Peruvian, Chilean1/US1 and Chilean2/US2 groups, followed by a restriction analysis using a single restriction enzyme *Sac*I for the separation of the European, Peruvian, Chilean2 and US2 isolates. The multiplex RT-PCR-*Sac*I protocol was performed by a mix of six primers that amplified a part of the RNA dependent RNA polymerase (RdRp) gene of PepMV and an internal control. Amplification resulted in a 980-, 703- or 549-bp PCR product for European/Peruvian, Chilean1/US1 or Chilean2/US2 groups respectively. It is likely that simultaneous amplification of several amplicons together with the use of a cocktail of primers could affect the detection limit of the multiplex RT-PCR reaction. Hence, multiplex and single RT-PCR assays were employed for the detection of two isolates of PepMV assigned to the EU/PE or CH2/US2 groups in tomato samples that were serially diluted. The end-point dilution limit for multiplex RT-PCR was 5^{-7} (0.64 μg/ml) and 5^{-6} (3.2 μg/ml) for the EU/PE or CH2/US2 isolates respectively (Fig. 2.21). Comparison with single RT-PCR format indicated that presence of cocktail of six primers did not affect the limit of detection of the virus isolates tested. Multiplex RT-PCR-*Sac*I assay showed 3,125 times higher level of sensitivity compared to DAS-ELISA and molecular hybridization methods. When field samples of tomato were tested, 23, 24 and 26 samples out of 42 tested positive with ELISA, dot blot hybridization and multiplex RT-PCR-*Sac*I assays respectively. All positive samples detected by ELISA and dot blot were also detected by multiplex RT-PCR-*Sac*I assay. Incorporation of two primer targets to a host mRNA as an internal control provided dependable safeguard against false negative results. The multiplex RT-PCR-*Sac*I assay allowed the identification of several double- and tripple-infected tomato plants. Since multiple infections by viruses are common in tomatoes, the protocol developed in this investigation may facilitate determination of the phytosanitary status of tomato crops reliably and rapidly (Alfaro-Fernández et al. 2009; Appendix 15).

Beet necrotic yellow vein virus (BNYVV), causative agent of rhizomania – a major disease of sugar beet, is transmitted by *Polymyxa betae*. *Beet soilborne virus* (BSBV) and *Beet virus Q* (BVQ) are also transmitted by *P. betae*. A sensitive and specific technique for the detection of BNYVV, BSBV, BQV and their vector

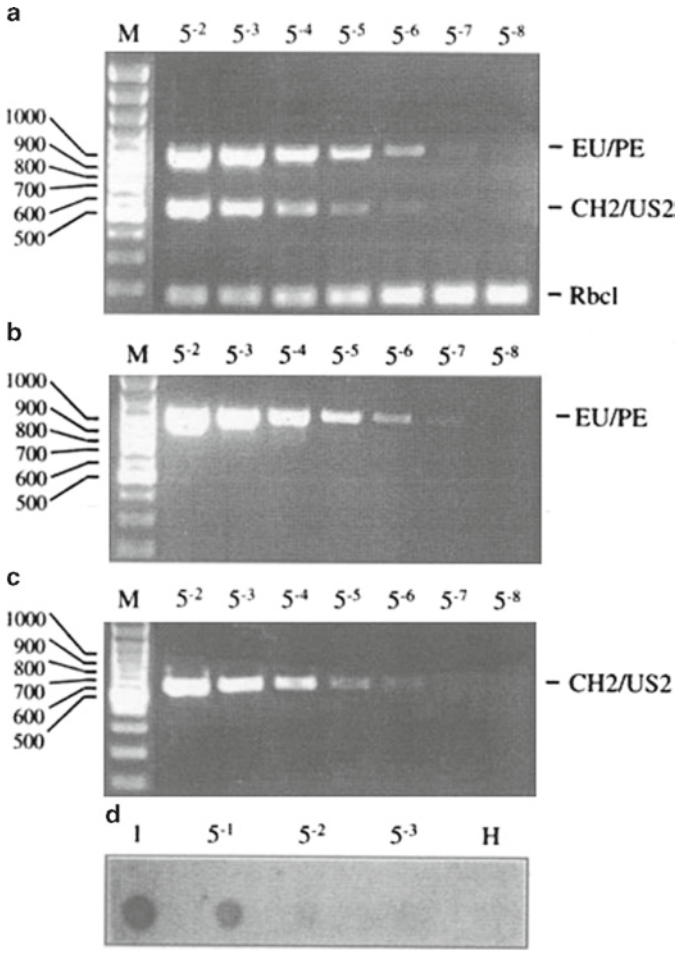

Fig. 2.21 Comparative sensitivity of detection of PepMV by multiplex or single RT-PCR or CAL/US2 or non-radioactive molecular hybridization techniques using different primer pairs. (**a**) Multiplex RT-PCR assay on fivefold serial dilutions; (**b**) single RT-PCR assay with specific primers EU/PE; (**c**) PepMV genotypes; (**d**) non-radioactive molecular hybridization (Courtesy of Alfaro-Fernández et al. 2009 and with kind permission of Springer Science and Business Media, Heidelberg, Germany)

P. betae in plants and soil samples was considered essential. Sugar beets and soils collected from different regions of Belgium, Bulgaria, Hungary and Turkey were tested by a multiplex RT-PCR assay along with DAS-ELISA test. Four pairs of primers specific for individual viruses and their vector were combined in the mRT-PCR assay which detected simultaneously BNYVV, BSBV, BQV and *P. betae* . The mRT-PCR procedure proved to be at least 128 times more sensitive than DAS-ELISA. The major advantage of this protocol is the simultaneous detection of all

three viruses and their vector, with the sensitivity greater than DAS-ELISA test (Meunier et al. 2003). In a later investigation, detection and differentiation of *Beet necrotic yellow vein virus* (BNYVV), *Beet soilborne virus* (BSBV) and *Beet virus Q* (BVQ) was achieved by adopting an RT-PCR format. Primers capable of amplifying a part of coat protein (CP) gene of each virus were employed. Amplicons of expected size were purified, cloned and sequenced using an ABI automatic sequencer. Based on the sequence data two groups of BNYVV were differentiated. On the other hand, CP sequences of BSBV and BQV isolates examined, did not show much variation. The very low level of genetic diversity among Polish isolates studied may be due to their adaptation to different environmental conditions and limited host range (Borodynko et al. 2009)

Tospoviruses infect a wide range of host plants including vegetable, ornamental and leguminous crops. A one-step RT-PCR was developed for the simultaneous detection and identification of five different tospovirus species. The RT-PCR system includes six primers in a single tube. A single universal degenerate primer corresponding to the 3′-noncoding region conserved among tospoviruses is used with five species-specific primers against the different viral SRNA. Specific PCR products, 848-bp for *Watermelon silver mottle virus* (WSMoV), 709-bp for *Tomato spotted wilt virus* (TSWV), 589-bp for *Impatiens necrotic spot virus* (INSV), 511-bp for *Melon yellow spot virus* (MYSV) and a 459-bp for *Iris yellow spot virus* (IYSV) were amplified. This protocol could be applied under field conditions for the detection of not only single infection, but also for multiple infections by five tospoviruses from 18 crops of six different species. This half-day assay can be performed in a single test, yielding useful results for epidemiological investigations (Uga and Tsuda 2005).

Cymbidium mosaic virus (CymMV) and *Odontoglossum ringspot virus* (ORSV), belonging to the genera *Potexvirus* and *Tobamovirus* respectively, are the most commonly prevalent and economically important viruses affecting orchid industry. As these two viruses induce less conspicuous foliar symptoms, it is difficult to recognize infection by them. A multiplex RT-PCR procedure was developed for the detection of CyMV and ORSV in *Phalaenopsis* orchid plants, by employing specific primers based on the sequences of coat protein (CP) gene of the respective viruses. In addition, one primer pair derived from the plant mitochondrial NADH dehydrogenase gene (*nad5*) was included to amplify *nad5* mRNA as the internal control in this assay. The primer pair for internal control provides a useful indicator of the quality of plant extract and the effectiveness of RT-PCR, in addition to its ability to minimize the risk of obtaining false negative results. The fragment of internal control was constantly amplified from total RNA of healthy and infected plant extracts. The presence of CyMV and ORSV in *Phalaenopsis* orchids from flower market in Taiwan was detected by the multiplex RT-PCR protocol. The expected fragments of 666-, 474- and 185-bp respectively specific for CyMV and ORSV and plant *nad5* mRNA were amplified only in the plants infected singly or doubly, indicating specificity of the primer employed. The differences in the concentrations of CyMV and ORSV did not affect the amplification of one virus-specific fragment by the other virus in the multiplex RT-PCR. Detection limits of ORSV and CyMV were

10 pg and 10 ng respectively. The multiplex RT-PCR format may greatly reduce the cost of testing, in addition to providing reliable results by minimizing false negative results which is desirable for routine large scale virus diagnosis required for certification of orchids (Lee and Chang 2006; Appendix 16).

In a later investigation, the involvement of ORSV and CymMV and tospoviruses in leaf-yellowing of *Phalaenopsis* orchid was examined by employing RT-PCR assay. ORSV and CymMV were successfully detected simultaneously by RT-PCR from total RNA extracts using both commercial kits and the simple-direct-tube (SDT) technique and the primers designed in this investigation. ORSV and CymMV were detected respectively in 55% and 34% of plants. Plants coinfected with both viruses tended to show severe symptoms. The SDT was efficient in extracting RNA providing a more cost-effective RT-PCR protocol for screening in commercial orchid nurseries for producing high quality virus-free plants. In addition, another *Orchid fleck virus* (OFV) was also detected in Australian orchids by using virus-specific primers, while none of the samples showed infection by the tospovirus *Capsicum chlorosis virus* (CaCV) reported earlier on *Phalaenopsis* (Yamane et al. 2008; Appendix 17).

Calla lily (*Zandtedeschia* spp.) plants are infected by several viruses of which four potyviruses are considered to be economically important in Taiwan. A multiplex RT-PCR assay for the detection of *Dasheen mosaic virus* (DsMV), *Turnip mosaic virus* (TUMV), *Konjac mosaic virus* (KoMV) and *Zantedeschia mild mosaic virus* (KoMV) was developed to save time and cost of testing the viruses individually. Specific primers for each virus were designed based on the sequence of 3′ terminal region of respective viruses. A primer pair specific to plant mitochondria *nad5* mRNA was used to amplify a specific fragment (185-bp) as an internal control of RT-PCR, in order to prevent false-negative results. The specificities of primers were verified by employing simplex and multiplex PCR formats. Fully expanded leaves of calla lily plant randomly collected from fields were tested by multiplex RT-PCR protocol. The multiplex RT-PCR assay detected virus infection in 72% (36/50) of calla lily samples and about two-thirds (23/36) of infected samples showed mixed infection. On the other hand, indirect-ELISA test could detect infection only in 50% of the samples, using potyvirus-specific antibodies. The results indicated that the multiplex RT-PCR assay might be useful for rapid and reliable detection of potyviruses infecting calla lily especially for certification programs for producing virus-free stocks (Hu et al. 2010).

A new virus was isolated from a mixed infected *Brugmansia* sp. cv. Pride of Hannover plants, with indistinct mosaic patterns on the leaves. An RT-PCR protocol for diagnosis was developed to give results 1 h earlier than conventional procedures. The protocol incorporated increased Mg^{2+} concentration to enhance *Taq* polymerase activity, increased *Taq* polymerase concentration and reduced annealing and elongation times. An amplification product of 550-bp was obtained from total RNA extracts from plants infected by the new virus. No bands were amplified with total RNA extracts of plants infected with *Tobacco mosaic virus* (TMV), *Tomato mosaic virus* (ToMV), *Tobacco mild green mosaic virus* (TMGMV), *Pepper mild mottle virus* (PMMoV), *Streptocarpus flower break virus* (SFBV) or the three subgroup

3 viruses of the genus *Tobamovirus* which includes 22 definitive species. The results suggested that the new virus may belong to subgroup 1 of *Tobamovirus* (Ilmberger et al. 2007).

A survey was taken up to assess the incidence of *Sugarcane mosaic virus* (SCMV), *Sorghum mosaic virus* (SrMV) and *Sugarcane streak mosaic virus* (SCSMV) on cultivated hybrid sugarcane and noble canes. In the one-step RT-PCR assay employing the primer pair SCMV-R3/SCMV-F5 for SCMV, SrMV-F/SrMV-R for SrMV, and SCSMV-F/SCSMC-R for SCSMV generated amplicons of expected size of 900-bp and 850-bp for SCMV and SrMV respectively. The absence of an amplicon specific for SCSMV indicated that this virus was absent in the samples tested. To confirm the RT-PCR data, 33 and 10 RT-PCR products for SCMV and SrMV CPs respectively were subjected to direct sequencing, which confirmed that their nucleotide sequence shared more than 76% homology with CP gene of the corresponding virus. RT-PCR data showed that most of the hybrid and noble canes in southern China were commonly infected by either SCMV or SrMV singly or doubly with both viruses (Xu et al. 2008).

The sensitivity of PCR can be remarkably enhanced by combining antibody binding with virus protein and PCR amplification. Immuno-PCR is a highly sensitive procedure in which a DNA fragment is linked to an antigen–antibody complex, using protein A (linking to antibody) and streptavidin (attached to DNA). Protein A and streptavidin have a strong affinity to each other. This complex is then bound to a biotin-labeled DNA sequence which is subsequently amplified by PCR. Sensitivity of immuno-PCR technique may be 10^5 times more than that of ELISA test (Sano et al. 1992). In immunocapture (IC), the virus is captured (concentrated) by using specific antibody and the specific RNA sequence of the captured virus particles is amplified by PCR.

Availability of antigen-specific antibody is the only requirement for performing immuno-PCR assay, whereas nucleic acid sequence data is also needed for immunocapture (IC)-RT-PCR technique. These techniques have been widely employed for the detection of several plant viruses, because they are highly sensitive. The sensitivity of detection may be 250 times greater than the standard PCR assay as determined for *Plum pox virus* (PPV) (Wetzel et al. 1992). PPV was detected in 31% of ELSA-negative leaf samples and in 23% of ELISA-doubtful samples indicating the effectiveness of IC-RT-PCR format in providing reliable results especially during field surveys to assess the incidence precisely. Additionally, by analyzing PCR products by RFLP technique, the majority of the isolates were found to be M serotype of PPV (Varveri and Boutiska 1998). Although IC-RT-PCR format is generally more sensitive (about 1,000 times) than ELISA, its superiority was not evident when aerial plant tissues were tested for the detection of PPV in plum trees, probably due to irregular distribution of the virus in foliar tissues. However, when root samples were tested, IC-RT-PCR assay detected PPV in 92–100% samples as against 36–65% by ELISA, indicating the bulked samples from fibrous roots were more suitable tissues for the detection of PPV (Adams et al. 1999). PPV was detected in nine wild apricot accessions including eight ELISA-negative and one ELISA-positive, indicating the higher sensitivity of IC-RT-PCR format (Spiegel et al. 2004). *Prunus necrotic ringspot*

virus (PNRSV) was detected by employing avian myeloblastosis virus reverse transcriptase enzyme and by incubating at 46°C for RTase reaction resulting in higher levels of amplification for PCR products compared to incubation at 37°C. Further, improvement in PCR yields was possible by preheating the reaction mixture at 55°C for 5 min. The sensitivity of IC-RT-PCR assay was significantly enhanced by these changes in the protocol (Rosner et al. 1998).

The IC-RT-PCR assay was employed for the detection of PPV, causing Sharka disease in *Prunus domestica* cv. Cacanska, in infected shoots (grown under tissue culture conditions) treated with different concentrations of ribavirin (1-ß-ribofuranosyl-1,2,4-triazole-3-carboxamide) that has antiviral activity. The effectiveness of ribavirin treatment was assessed at 2 years after treatment by the percentage of plants remaining virus-free. IC-RT-PCR and DAS-ELISA tests were evaluated for their efficacy in ensuring the freedom of treated plants from PPV. Percentages of virus-free plants increased progressively with increase in the concentration of ribavirin used for treatment of infected shoots. As the DAS-ELISA test was not able to detect low concentrations of PPV, higher percentages of treated plants were indicated to be virus-free. On the other hand, IC-RT-PCR assay showed that only 15.38% and 16.66% of plants treated with 40 and 60 mg/l of ribavirin respectively to be virus-free, as against 76.9% and 100% of treated plants to be virus-free, as determined by ELISA test. The results showed that IC-RT-PCR assay could be reliably used for testing plants treated with antiviral chemicals applied for obtaining virus-free stocks in vitro (Paunovic et al. 2007).

Citrus tristeza virus (CTV) remains one of the major threats to citrus production all over the world. This virus has been spreading continuously by propagation of infected planting materials. Immunocapture (IC)-RT-PCR assay was applied for the detection of the quick-decline strain of CTV, as this format combines the specificity of PCR and simplicity of ELISA tests, resulting in the enhancement of sensitivity of tests remarkably. The sequence of the CP gene of CTV strains was used for designing the primers and fluorescent probes. After PCR amplification, fluorescence of hybridized probes is determined, instead of performing electrophoretic analysis. This assay detected CTV strains in all ELISA-positive samples and also in additional 20% of the samples that were ELISA-negative, indicating the greater sensitivity of this protocol (Nolasco et al. 1997). In a later investigation, IC-RT-PCR format was employed using primers corresponding to both ends of the CP gene. The extracts prepared from the bark tissues of field-infected citrus plants were tested. IC-RT-PCR results confirmed the presence of CTV in all samples collected from orchards in the coastal regions of Croatia, Montenegro and Albania (east Adriatic region). CP variants, as revealed by single strand conformation polymorphism (SSCP) analysis, coexisted as different genomic variants in all isolates. The results suggested the possibility of mixed infections with variants displaying different biological characteristics. Regardless of absence of visible symptoms in field trees, severe CTV variants appear to be widespread and long-present in the Adriatic region making it a reservoir of severe CTV strains (Cerni et al. 2009).

Apple stem-pitting virus (ASPV) and *Pear vein yellows virus* (PVYV) infecting apple and pears are closely related viruses. As the results of the RT-PCR assay was

not reproducible (10–50%), IC-RT-PCR format was evaluated. Virus detection by IC-RT-PCR was also only partially successful in extracts from woody plant tissues, when the antiserum that successfully trapped and decorated virus particles in immunosorbent electron microscopy (ISEM) was used. Hence, a bacterially-expressed protein employing ASPV CP gene as chimerical protein was used as an immunogen. The antiserum (647) was prepared against this protein for use in IC-RT-PCR assay. This antiserum 647 was suitable as a trapping antibody in IC-RT-PCR protocol. The assay primers amplified 1,548-, 610-, and 264-bp fragments in all samples from *Nicotiana occidentalis* to which all the three ASPV isolates were transmitted. These virus isolates were detected in apple varieties also (Jelkmann and Kein-Konrad 1997).

Grapevine leaf roll-associated virus 1 and *3* (GLRaV-1 and -3) were effectively detected by applying IC-RT-PCR assay employing specific primers. The presence of these two viruses was detected in infected plants as well as in the mealybug vector *Planococcus ficus* (Acheche et al. 1999). Specific primers were designed based on the sequences from conserved heat shock protein (HSP) 70 region of closteroviruses for the detection of GLRaV-1. No amplification occurred in grapevine tissues infected by GLRaV-2, -3 and -4, indicating the specificity of the primers for GLRaV-1. This protocol was about 125 times more sensitive than ELISA test (Sefc et al. 2000). IC-RT-PCR assay was demonstrated to be more sensitive and specific for the detection of *Arabis mosaic virus* (ArMV) and *Grapevine fanleaf virus* (GFLV) simultaneously. A single pair of degenerate primers designed from the sequences coding for movement proteins of the target viruses was used in this protocol which was 10 times more sensitive than ELISA tests (Wetzel et al. 2002). *Grapevine A* (GVA) is the type species for the genus *Vitivirus* in the family *Flexiviridae*. GVA isolates were conventionally detected and differentiated based on the types of symptoms produced on mechanically inoculated *Nicotiana benthamiana.* The presence of GVA in the samples (1,141) of petioles and canes of grapevines from different locations of Jordan was tested by employing DAS-ELISA test which indicated 14.2% infection in the samples. Using RT-PCR or IC-RT-PCR format, a fragment of 430-bp of the coat protein (CP) gene was amplified by employing the primer pair H587/C995. Cloning and sequencing of amplicons showed that GVA isolates existing in Jordan shared 100% nucleotide identity with each other and were identical at molecular level (Misbeh et al. 2007).

Immunocapture (IC)-RT-PCR assay may be performed in the microplate wells also. Virions are captured by the antibodies coated in the microplate wells for enrichment followed by lysis of virions, reverse transcription and amplification of viral genome. *Raspberry bushy dwarf virus* (RBDV) RNA3 was efficiently and reliably detected by this protocol using combinations of four primers (Kokko et al. 1996). Incidence of RBDV in grapevine (a non-*Rubus* natural host) was first observed in Slovenia. Grapevine samples (161) collected from different winegrowing areas were first tested by DAS-ELISA. The ELISA-positive samples (99) were tested by IC-RT-PCR assay for confirmation of the infection by RBDV. In addition, infection of redgrapevine cv. Modri Pinot by RBDV was confirmed by IC-RT-PCR protocol (Pleško et al. 2009).

A semi-automated immunomagnetic capture (IMC)-RT-PCR assay was developed for the detection of three ampeloviruses, *Pineapple mealbug wilt-associated virus 1*, *2* and *3*. The IMC-RT-PCR assay showed sensitivity level similar to that of IC-RT-PCR format, but the results could be obtained earlier. Further, IMC-RT-PCR may be applied either as a one- or two-step RT-PCR for detection of the viruses separately or together in a triplex assay from fresh, frozen or freeze-dried pineapple leaf tissues. The IMC-RT-PCR format can be automated for high throughput screening of pineapple planting propagules and has the potential for detection of other RNA viruses infecting other plant species, if suitable antisera are available (Gambley et al. 2009).

Immunocapture-RT-PCR assay was evaluated for its efficacy for the detection of *Potato virus Y* (PVY). The sensitivities of DAS-ELISA and PCR-ELISA assays were compared with IC-RT-PCR format by estimating dilution end point of purified preparations of PVY^{O} in healthy tobacco extract. Dilution end point of DAS-ELISA corresponded to 10 ng/ml virus concentration with a mean absorbance value of 0.118 at 405 nm, whereas that of healthy control was 0.034. In IC-RT-PCR followed by electrophoresis, an amplification product of the expected 577-bp was visualized up to 10 pg/ml virus on gel. Each IC-PCR mixture was diluted 200-fold into the microplate wells and PCR-ELISA was performed to give positive absorbance value in the IC-PCR product from 100 fg/ml virus. The sensitivity of IC-PCR-ELISA was 100,000 greater than that of DAS-ELISA test for detection of PVY (Varveri 2000).

Immunocapture-RT-PCR procedure was applied for the reliable and efficient detection of *Tomato spotted wilt virus* (TSWV) using monoclonal antibodies (MAbs). With different dilutions of MAbs at 1:22 (v/v), plant saps at 1:10 (w/v) and using 100U of MMLV reverse transcriptase (Promega, USA) in 20 μl reaction volume, a clear single band of approximately 0.6 kb was amplified from four tested TSWV isolates using primers TSWVN-F and TSWV-R. No amplification occurred from unaffected field plant samples (250) of pepper, lettuce, tobacco, tomato and ornamental crop plants, as revealed by TAS-ELISA test conducted earlier. TAS-ELISA test was also found to be equally efficient and sensitive for the detection of TSWV isolates (Wu et al. 2009).

Simultaneous detection of two pepper-infecting tobamoviruses, *Pepper mild mottle virus* (PMMoV) and *Tobacco mild green mosaic virus* (TMGMV) was accomplished by employing a single-tube multiplex IC-RT-PCR procedure. The multiplex IC-RT-PCR assay efficiently detected simultaneously two different tobamoviruses. Two RT-PCR products corresponding to PMMoV (317-bp) and TMGMV (510-bp) were detected and the viruses could be differentiated by the size of their amplified products on the same lane. No band was detected in either buffer or healthy sap negative control. IC-RT-PCR format showed high sensitivity because of the initial immunocapture enrichment followed by PCR amplification. High specificity of the assay was due to the combination of virus-specific antibody capture and primer specificity. The viruses were detected directly in crude plant extracts. DAS-ELISA test could not discriminate between PMMoV and TMGMV, whereas the specificity of primer pairs PM317-F/-R and CPTMG-F/-R for the respective target

viruses ensured the detection and discrimination of these viruses in single-virus and duplex IC-RT-PCR assays. The duplex format was applied for field survey to assess the incidence of these tobamoviruses in pepper crops in Korea (Kim et al. 2006; Appendix 18).

The efficacy of IC-RT-PCR assay for the detection of a novel tobamovirus, *Florida hibiscus virus*, was assessed, along with immunoassays. Partially purified virus preparations or crude extracts of infected hibiscus and *Chenopodium quinoa* were tested by IC-RT-PCR protocol. Amplification products of the expected size of 535-bp were detected in agarose gels. No amplification products were detectable in extracts of uninfected hisbiscus or *C. quinoa* plants. With serial dilutions to determine detection limits, amplified products from purified virus preparations were obtained with as little as 500 pg/ml (50 pg/100 µl reaction). The detection limit for infected *C. quinoa* extracts was a dilution of 1:102,000, while the limit of detection with hibiscus leaf and bark extracts were 1:51,200 and 1:12,800 respectively. IC-RT-PCR assay was found to be more sensitive, when purified virus preparations (62-folds) and plant extracts (32-folds) were tested. ELISA formats and TBIA test with their simplicity and small labor requirements, readily available reagents and applicability to large number of samples are more suitable, convenient and practical for the detection of hibiscus virus. On the other hand, IC-RT-PCR assay with its high sensitivity and requirement of small quantities of samples may be considered as an useful alternative to ELISA and TBIA tests for detection of *Florida hibiscus virus* (Kamenova and Adkins 2004; Appendix 19).

Onion yellow dwarf virus (OYDV) was detected in infected garlic plants by IC-RT-PCR assay. A one-tube single-step procedure was followed. The primers were designed based on the sequences of the central region of the coat protein gene. A single band of the expected size of ca. 601-bp was detected in the ethidium bromide stained agarose gel. Healthy garlic tissue extract did not yield any amplification product indicating the specificity of the assay (Mahmoud et al. 2007). *Soybean mosaic virus* (SMV) was detected by performing IC-RT-PCR procedure using a pair of primers designed to amplify a fragment in the coding region of SMV coat protein (CP). A part of the CP gene of SMV sequences was adsorbed using PAbs on the walls of microfuge tubes followed by RT reaction and PCR amplification. There was no need for extraction of total RNA as in the case of RT-PCR format. Positive reactions were inferred by detecting the 469-bp fragment in the agarose gel after staining with ethidium bromide (Ahangaran et al. 2009).

Black pepper (*Piper nigrum*) is an important spice crop grown in many South East Asian countries including India. A single-tube multiplex RT-PCR assay was developed for the simultaneous detection of *Cucumber mosaic virus* (with RNA genome) and *Piper yellow mottle virus* (PYMoV) (with DNA genome) infecting pepper plants. Total nucleic acid extracted from mixed infected plants was subjected to multiplex RT-PCR. Two PCR products of expected size 650-bp for CMV and 450-bp for PYMoV were amplified. No amplification occurred with nucleic acid extracts from healthy plants. Black pepper samples (49) collected from Kerala and Karnataka States of India were tested. Three samples showed infection by both CMV and PYMoV, whereas 24 and 7 samples were positive for PYMoV and CMV alone.

The protocol developed in this investigation provided amplifiable templates for simultaneous detection of one RNA virus and one DNA virus infecting black pepper (Bhat and Siju 2007).

Although ELISA test remains as the method of choice among immunoassays, the level of sensitivity and specificity is not sufficient to detect certain strains especially in dormant organs like tubers or dormant trees. PCR-ELISA (PCR-microplate hybridization) combination may be useful in such cases. Combination of PCR and ELISA techniques has been shown to be more sensitive and effective. PCR-ELISA procedure was applied for the simultaneous detection and identification of *Prunus necrotic ringspot virus* (PNRSV) and *Apple mosaic virus* (ApMV). Multiple alignments of PNRSV and ApMV CP gene sequences were made to select PCR primers in the regions conserved between two viruses. Virus-specific oligonucleotides were used as capture probes in the PCR-ELISA method. The selected primer pairs were effective for the detection of both PNRSV and ApMV as indicated by the analysis of a range of isolates of both viruses. Simultaneous application of the specific capture probes of both viruses resulted in the improvement of levels of sensitivity of detection of PNRSV and ApMV by PCR-ELISA combination (Candresse et al. 1998). PCR-ELISA procedure was found to be effective for the detection of other viruses such as *Plum pox virus*, *Cherry leaf roll virus*, *Citrus tristeza virus* and *Tomato ringspot virus* (Olmos et al. 1997; Rowhani et al. 1998).

Apple stem grooving virus (ASCV) was detected by applying the Titan One tube RT-PCR system and the primer pair ASGV4F/ASGV4R for amplifying ASGV viral sequences directly from diluted plant sap of either ASGV-infected *Chenopodium quinoa* or sap from leaves or bark of apple trees. The amplification products were visually detected in the ethidium bromide-stained gels by Southern blotting or in the colorimetric assay. The RT-PCR product was hybridized with both a biotin labeled capture probe linked to a streptavidin-coated microtiter plate and a digoxigenin (DIG)-labeled detection probe. The complex was then detected with an anti-DIG conjugate labeled with alkaline phosphatase. It is possible to detect as little as 400 fg of ASGV in plant tissue extracts spiked with purified virus preparation. By using the primer pair ASGV4F/ASGV4R, the target virus was detected in infected apple trees from different origins. No signal was obtained with amplification products obtained with primers targeting the CP region of the ASGV genome or with primers specific for *Apple chlorotic leaf spot virus* (ACLSV) and *Apple stem pitting virus* (ASPV). The protocol developed in this investigation combines the power of PCR to increase the number of copies of the targeted gene sequence, the specificity of DNA hybridization and the ease of colorimetric detection and sample handling in microplates. This protocol may be suitable for routine large scale testing of planting materials for certification programs, since about 100 samples can be processed simultaneously in one microplate, (Marinho et al. 2003).

PCR-ELISA method is used to detect the amplicons produced after their adsorption on a microplate, increasing the sensitivity significantly. PCR-ELISA procedure was applied for the detection of strains of *Potato virus Y* (PVY). The isolates of PVY were maintained in tobacco. Tests with a large number of PVY isolates of the O, N and NTN groups originating from different hosts and localities were run concurrently.

The immunocapture (IC)-PCR mixture was diluted 200-folds into microplate wells and PCR-ELISA was performed to give a positive absorbance value in the IC-PCR product from 100 fg/ml of virus. The purified virus (100 fg/ml) in healthy tobacco extract became readily detectable, giving an increase in sensitivity level by 100-fold over that of IC-PCR assay (Varveri 2000; Appendix 20).

Combination of PCR and RFLP techniques has been useful for the detection and differentiation of plant viruses or their strains or closely related viruses. Specific viral sequences were amplified by PCR using suitable primers, from 25 isolates of *Plum necrotic ringspot virus* (PNRSV). These isolates of PNRSV differed in the type of symptoms induced in six different *Prunus* spp. Three restriction enzymes *Eco*RI, *Taq*1 or *Rsa*I were employed to cleave the amplicons from the isolates of PNRSV. All isolates clustered into three groups based on sequence comparison, RFLP patterns and phylogenetic analyses of RNA4 and coat proteins (CPs). However, no clear relationship could be established between the type of symptoms caused or host specificity and differences in the molecular characteristics (Aparicio et al. 1999). In contrast, relationship between biological and molecular characteristics was seen in the case of isolates of *Barley yellow dwarf virus* (BYDV). The RFLP pattern of the isolate BYDV-PAV-DK determined using the restriction enzyme *Hae*III was distinctly different. Unique restriction profile was obtained after digestion of PCR products from CP region of BYDV-PAV-DK and a laboratory-maintained isolate BYDV-PAV-IL. Rates of transmission of these two isolates by two of three biotypes of *Rhopalosiphum padi* were distinctly different, although symptom type did not show discernible difference. The unique restriction enzyme profile of BYDV-PAV-DK1 isolate appeared to be a dependable characteristic for differentiation in the epidemiological investigations (Moon et al. 2000).

The RT-PCR and RFLP techniques have been applied in tandem for the detection, identification and differentiation of plant viruses. Serologically related *Tobamovirus* species could be detected and differentiated by employing RT-PCR/RFLP procedures successfully (Letschert et al. 2002). *Soybean mosaic virus* (SMV) and its strains were detected by employing a primer pair that amplified a 1,385-bp fragment of cylindrical inclusion (CI) coding region. PCR amplicons were digested with restriction enzymes *Rsa*I, *Eco*RI, *Acc*I and RFLP profiles were distinct for each strain forming the basis for differentiation of five strains, in addition to seed-borne SMV isolates from soybean cultivars. The results of RT-PCR/RFLP analysis correlated well with those obtained using differential soybean cultivars used for differentiation of strains based on symptom phenotypes (Kim et al. 2004). New severe strains of *Melon necrotic spot virus* (MNSV) could be detected and discriminated by employing RT-PCR followed by RFLP procedures (Kubo et al. 2005). For the detection and identification of potyviruses, a combination of RT-PCR assay and reverse blot hybridization was applied. Three degenerate primers located at the NIb and CP regions were designed. The cDNA fragments (1.0–1.2-kb) of the viruses were amplified from infected plant tissues. Sequences located between the 3′ end of the NIb gene and the 5′ end of the CP gene were used for designing species-specific probes which hybridized with DIG-labled RT-PCR products amplified by

degenerate primers. This step was useful for precise identification of potyviruses (Hsu et al. 2005).

Alfalfa mosaic virus (AMV), the type member of the genus *Alfamovirus* in the family *Bromoviridae*, has a wide host range of plant species (150 species in 22 families) including potatoes (Jaspars and Bos 1980). A primer pair AMV-F/AMV-R was designed based on the sequences of AMV-CP gene and they were employed in RT-PCR assay for the detection of AMV in potato plants. The primers were highly specific only to AMV-RNA and they did not generate any amplicon from RNA extracts of healthy potato plants. The expected 351-bp amplicon was detected in all composite samples (up to 1:1,599) in RT-PCR assay employing the primer pair AMV-F/AMV-R. The PCR amplicon (351-bp) was digested with the restriction enzyme *Sac*I and subjected to RFLP analysis, resulting in the formation of two fragments 201- and 150-bp. The RFLP profiles were similar in the tested samples, confirming the specificity of the primers and identity of the AMV isolates. The combination of RT-PCR and RFLP techniques may be an useful approach for screening potato samples on a large scale for detecting AMV in potato plants and tubers as well (Xu and Nie 2006).

Grapevine fanleaf virus (GFLV) transmitted by the nematode *Xiphinema index* causes serious economic losses in grapevines. GFLV was transmitted to *Chenopodium quinoa* from which the virus was purified. The antiserum against the purified preparation reacted specifically with Tunisian grapevine samples in DAS-ELISA format. Positive samples were subjected to oligoprobe-RT-PCR to amplify a 606-bp region of viral CP gene sequence. PCR products amplified by H2042 and C2647 primers were digested with endonculease *Alu*I. RFLP analysis provided three restriction profiles with which two strains of GFLV present in Tunisia could be clearly identified. RT-PCR-RFLP technique may provide data useful for epidemiological and resistance breeding investigations (Fattouch et al. 2005a).

2.1.6.3 Real-Time PCR Assay

Real-time PCR assay has been developed in order to overcome the limitations of conventional PCR assay. PCR products are detected by real-time PCR, as the reaction is progressing without the requirement of post-PCR steps. Real-time PCR using specific instruments and fluorescent probes offers the advantage of combining the amplification, detection and quantification of target molecules in a single-step. Real-time PCR procedures may be divided into two types as non-specific and specific. Non-specific methods involve the use of dyes like SYBR Green I, that emit fluorescent light when intercalated into ds-DNA. Unbound dye, in solution, emits very little fluorescence, but when the dye is bound to DNA, fluorescence is remarkably increased in proportion to the amount of total ds-DNA present in the reaction. As the dyes are incapable of discriminating between different ds-DNA molecules, it is necessary to prevent synthesis of non-specific amplicons and also dimers by adopting accurate primer design and optimization of PCR conditions. Specific methods employ oligonucleotide probes labeled with a donor fluorophore and an acceptor

dye (quencher) that produce a light signal according to the fluorescence resonance energy transfer (FRET) chemistry. The specific methods include TaqMan, molecular beacons and scorpion PCR.

Real-time PCR allows the increase of specific PCR amplicons to be monitored during successive amplification cycle. A pair of PCR primers and an internal hybridization oligonucleotide probe provide dual specificity. TaqMan™ (PE Biosystems) chemistry utilizes the 5′ → 3′ nuclease activity of *Taq* DNA polymerase to cleave specific probe which is labeled with a 5′ reporter (FAM) and 3′ quencher (TAMAR) fluorescent dye during strand elongation of target sequence. A detectable fluorescent signal is generated during amplification.

During PCR, the probe anneals to a complementary strand of an amplified product, while *Taq* polymerase cleaves the probe during the extension of one of the primers and the dye molecules are displaced and separated. After separation, the electronically excited reporter cannot be suppressed by the quencher dye. Hence, variation occurs in the green emission intensity concentration of PCR amplicons in the reaction. The number of PCR cycle at which the fluorescent signal (emission) exceeds a certain background fluorescence level is called threshold cycle (Ct), is directly proportional to the amount of target DNA present in the sample. Fluorogenic probes can be labeled with different distinguishable reporter dyes to amplify and detect two or more distinct sequences in a single PCR reaction tube without the need for melting curve analysis. Real-time PCR systems are fully automated with 'closed tube' product detection providing reduced risk of cross-contamination. Further, up to 96 samples can be analysed at a time without the need for post-PCR steps. Although several advantages over standard PCR assay are provided by real-time PCR, the expensive nature of fluorescent probes and the instruments required, limit the application of real-time PCR in most laboratories. Attempts are being made to address this limitation by developing portable rapid cycling real-time PCR platforms like Smart Cycler (International Laboratory) which can be applied for multiple sample analysis under field conditions. TaqMan system appears to be particularly useful for large scale applications that require high level of sensitivity, reliability, rapidity and quantification as in the case of seed health testing. The advance nucleic acid analyzer (ANAA) with silicon chip based spectrofluorimetric thermocyclers has been developed for field applications (Belgrader et al. 1999). Real-time PCR technique has been applied more frequently for the detection of fungal (volume 1) and bacterial (volume 2) plant pathogens.

Tomato spotted wilt virus (TSWV) exists in the form of several strains infecting a large number of host plant species, including 925 species of monocots and dicots belonging to at least 70 botanical families (Latham and Jones 1997). Real-time RT-PCR assay was employed for the detection of a wide range of isolates of TSWV present in infected plant species (Roberts et al. 2000). The potential for large scale use of a sensitive TaqMan real-time RT-PCR assay was evaluated for the detection of TSWV in single and bulked leaf samples along with DAS-ELISA for their relative sensitivities. Total RNA was extracted by employing RNeasy® or leaf soak methods. Real-time RT-PCR assay detected TSWV in all infected samples collected from 16 horticultural plant species which included tomato, potato, pepper, lettuce and aster crops.

Real-time RT-PCR was effective in detecting TSWV in all ELISA-positive samples of leaf tissues of 22 plant species. Bulked samples required more robust and extensive extraction methods with real-time RT-PCR, but it generally detected one infected leaf in 1,000 uninfected ones. On the other hand, ELISA was less sensitive when used to test bulked samples, detecting 1 in 800 with pepper, 1 in 200 with tomato and lettuce. Further, ELISA test was less reliable for testing leaf tissues with low virus titers. Despite wide variations, real-time RT-PCR detected TSWV in all locations sampled across the higher sensitivity and broad dynamic range covering at least six orders of magnitude (Dietzgen et al. 2005).

Real-time RT-PCR assay was effective for the detection of *Cucumber vein yellowing virus* (CVYV) using specific primers designed from a nucleotide sequence of the RNA polymerase gene (NIb) conserved among all the available CVYV strains. Real-time RT-PCR format provided reproducible results by detecting CVYV at concentrations as low as 10^3 molecules of the target CVYV DNA and this format could be applied for quantitation of CVYV in young leaves of plants inoculated mechanically (Picó et al. 2005). A real-time PCR was shown to be useful for the specific detection of *Beet necrotic yellow vein virus* (BNYVV). RNA2 of all BNYVV types by one assay, whereas the second assay detected types containing RNA5. Real-time RT-PCR assays were 10,000 more sensitive in detecting BNYVV compared to conventional RT-PCR assay (Harju et al. 2005).

Two *Potato virus Y*-(PVY^N and PVY^O) specific fluorescent TaqMan-based real-time RT-PCR assays were developed to detect single nucleotide polymorphism (SNP) (A/G_{2213}) that could be employed as a molecular determinant. The PVY^N isolates causing tobacco leaf necrosis were detected and differentiated by targeting the molecular determinant. Detection, characterization and quantification of a wide range of PVY isolates in the samples containing 10^3–10^8 viral transcripts were performed using these protocols. As these assays had high specificity, they could be applied effectively for simultaneous detection and reliable quantification of PVY^N and PVY^O isolates in mixed solutions regardless of the Y^N/Y^O ratio (Balme-Sinibaldi et al. 2006). Viruses infecting sweet potatoes, *Sweet potato feathery mottle virus* (SPFMV), *Sweet potato virus G* (SPVG) and *Ipomoea vein mosaic virus* (IVMV) and S*weet potato chlorotic stunt virus* (SPCSV) and *Sweet potato leaf curl virus* (SPLCV) were detected and quantified by real-time procedure. These viruses were detected by simplex reactions directly from infected sweet potato plants. The presence of PCR inhibitors apparently did not show any adverse effect on the amplification of virus-specific templates. Real-time PCR estimation of concentrations of SPFMV, IVMV and SPVG was at a lower level in sweet potato plants infected singly compared to virus titers reached in singly infected *Ipomoea setosa* cv. Brazilian Morning Glory and *I. nil* cv. Scarlet O'Hara plants. Real-time PCR detected SPLCV-US in 17 of 20 samples tested as against 5 of 20 samples giving positive results with conventional PCR format. By obtaining the relative titer levels of SPLCV-US in positive samples, the minimum concentration detectable by real-time PCR was 1,000 times less than that could be detected by standard PCR assay. Real-time PCR was not only a faster and safer method (eliminating the use of hazardous materials), but also, most importantly it was more sensitive for detection

and quantification of sweet potato viruses directly from sweet potato plants. However, the requirement of expensive instruments limits the use of real-time PCR in most laboratories (Kokkinos and Clark 2006).

Grapevine viruses have been efficiently detected and precisely identified by employing real-time RT-PCR assay. *Grapevine fanleaf virus* (GFLV) was effectively detected in grapevine plants and also in the nematode vector *Xiphinema index* collected from the rhizosphere of grapevine plants in Italy. A 1,157-bp fragment of the GLFV RNA-2 coat protein gene was amplified. A fluorescent scorpion probe based on the highly conserved CP region was employed for the detection of GFLV (Finetti-Sialer and Ciancio 2005). Specific and efficient detection of different isolates of *Grapevine leaf roll-associated virus 1*, *2*, *3*, *4*, *5* and *9* (GLRaV-1, -2, -3, -4, -5 and -9) from various countries in South Africa, Europe, Australia, Asia and Latin America and the United States was accomplished. TaqMan® primers and probes that targeted the regions with 100% sequence identity were designed. The real-time TaqMan® and conventional RT-PCR assays were compared for detection of viruses using purified total RNA as well as crude extract. The results showed that TaqMan® RT-PCR assay was found to be more sensitive than the conventional one-step RT-PCR for testing different isolates of GLRaV-1, -2, -3, -4, -5 and -9 either using RNA or crude extracts of infected tissues (Osman et al. 2007).

Citrus tristeza virus (CTV) has been disseminated to almost all citrus-growing countries primarily through exchange of infected budwood and the aphid vectors have been responsible for the secondary spread of the virus. TaqMan real-time RT-PCR assay was developed employing purified RNA targets or coupled to tissue-print and squash procedure for the detection and quantitation of CTV. The efficacy of DAS-ELISA, IC-RT-PCR (using purified RNA) was assessed. DAS-ELISA had a dilution end point detection at $1{:}10^3$, whereas IC-RT-nested PCR format detected CTV targets up to 1.5×10^6 and real-time RT-PCR up to 10^9. For the detection of CTV in field grown trees, 235 trees of 432, were found to be CTV-positive by tissue-print ELISA and this analysis was confirmed by the conventional and tissue-print real-time RT-PCR formats. Tissue-print ELISA test revealed differences in the number and intensity of immunoprecipitates localized in the vascular area of imprints. Using real-time RT-PCR, CTV isolates from different hosts and origin could be detected and quantified. This format detected consistently as few as 17 targets of purified CTV transcripts. The sensitivity of real-time RT-PCR was 1,000 times higher than IC-RT-nested PCR and 10^6 times higher than ELISA test. The quantitation limit ranged from 1.7×10^2 to 10.7×10^9 transcript copies. The tissue-print and squash real-time RT-PCR formats exhibited high sensitivity, specificity and feasibility and reliability for detection and quantitation of CTV in immobilized plant materials. This assay can be very useful for epidemiological investigations (Bertolini et al. 2008).

A real-time multiplex PCR technique was applied for the detection and differentiation of *Plum pox virus* (PPV). SYBR Green I employed was inexpensive and the procedure was simple providing more reliable results. In addition, this procedure eliminated the need for electrophoretic analysis of amplicons or RFLP profile determination (Varga and James 2005). The efficiency of four direct

sample preparation methods (dilution, spot, squash and tissue-print) coupled with real-time PCR was evaluated for the detection of PPV. The four sample preparations methods coupled to real-time RT-PCR detected all described PPV strains using either TaqMan or SYBR Green chemistries. The dilution and spot methods had the same detection limits, but they were 10 and 100 times more sensitive than Co-PCR and DASI-ELISA methods respectively. The nylon and Whatman membranes proved adequate for immobilization of target nucleic acids. The spot real-time RT-PCR was demonstrated to be a reliable technique for the detection of PPV in plants during the dormant period, when erroneous diagnosis were reported earlier using other diagnostic methods. Analysis of 405 adult *Prunus* trees using different techniques with samples collected in the winter and the following spring showed that 201 of 405 trees were infected by shaka disease caused by PPV. Conventional real-time RT-PCR and spot real-time RT-PCR had sensitivity of 97.5% and 93.6% respectively, whereas IC-RT-PCR and DASI-ELISA tests were less sensitive with 91.5% and 86.6% respectively. Spot real-time and DASI-ELISA (the validated serological method) were applied for a large scale analysis of 2,919 *Prunus* samples, using the source plant extracts for both tests. Spot real-time RT-PCR assay was 100 times more sensitive than DASI-ELISA test. Spot real-time RT-PCR assay circumventing RNA purification allows high-throughput testing of PPV rapidly and reliably (Capote et al. 2009; Appendix 21).

Dahlia mosaic virus (DMV) causes appreciable reduction in economic values to dahlia crops grown in many countries including the United States. Samples from several states of the US were tested by employing the real-time PCR assay. The viral genome was cloned and sequenced. DMV-specific primers were designed based on the sequence data. PCR-based assay was able to detect DMV in >90% of the samples tested. A real-time PCR assay was adapted for rapid detection of DMV as effective steps were required to contain the rapid spread of the disease that was already widespread. Real-time PCR assay has the potential not only for sensitive detection of DMV in infected plants, but also for facilitating production of virus-free dahlias from propagating stocks (Pappu et al. 2005).

Barley yellow mosaic virus (BaYMV) and *Barley mild mosaic virus* (BaMMV) were more reliably detected by real-time PCR format especially in late season and in mixed infection samples than ELISA test. The possibility of using rapid automation of extraction procedure provided an additional advantage for routine detection of these viruses by real-time PCR format (Mumford et al. 2004). An RT-PCR assay using specific primers was employed for the detection of isolates of *Wheat spindle streak mosaic virus* (WSSMV) from Canada, France, Germany, Italy and United Kingdom. A real-time PCR with SYBR-Green was effective for quantifying WSSMV in wheat samples. In addition, the virus was also detected in soil samples also by this real-time PCR assay. No amplification occurred in extracts from wheat samples infected by other viruses, indicating the specificity of the protocol developed in this investigation (Vaïnopoulos et al. 2006).

2.1.6.4 Molecular Beacon Technique

A novel fluorescence-based nucleic acid pathogen detection technique was introduced by Tyagi and Kramer (1996). The probe, designated, a molecular beacon, consists of a single-stranded DNA (oligonucleotides) with a stem-loop structure. The loop portion encloses a probe sequence that is complementary to a target sequence. The stem portion is formed by the annealing of the 5′ and 3′ arm sequences which are not related to the target sequence. A fluorescent moiety is attached to the 5′ arm terminus, while a quenching moiety is attached to the 3′ arm at the opposite end. The molecular beacon is added to a solution containing the target nucleic acid and the mixture is heated to 80°C, followed by cooling the mixture to 20°C. The arms associate to form a stem-loop conformation, when there is no target molecule. Under this condition, the fluorescence emitted from the fluorophore is quenched by fluorescence resonance energy transfer (FRET) via the quencher due to their close proximity to each other. In contrast, when the target is present, the probe forms a hybrid with the target via its complementary sequence within the loop region, resulting in the displacement of the fluorescent moiety from the quenching moiety leading to emission of fluorescent signal. Thus emission of fluorescent signal is produced by molecular beacons, only when they hybridize with their complementary target nucleic acids. As the unhybridized molecular beacons do not fluoresce, there is no need for removing them from the reaction mixture. By tagging each molecular beacon with fluorescent moieties possessing different emission wave lengths, it is possible to employ multiple molecular beacons for detection of different targets. RT-PCR reactions are performed with primers that amplify specific genome sequences of interest, yielding targets complementary to their respective molecular beacons for subsequent detection. Furthermore, molecular beacon technique can analyze 96 samples simultaneously with equal efficiency as RT-PCR and ELISA tests.

Two orchid viruses, *Cymbidium mosaic virus* (CyMV) and *Odontoglossum ringspot virus* (ORSV) were detected by employing molecular beacon technique. One pair of primers was designed to detect the RNA-dependent RNA polymerase (RdRp) genes and the second pair was employed to detect the coat protein (CP) gene of both CyMV and ORSV simultaneously. RT-PCR was performed using the above primer pairs to amplify the genome targets from total RNA extracted from CyMV- and ORSV-coinfected *Oncidium* orchid leaves. Molecular beacons specific to CyMV were tagged with 6-carboxyfluorescein (FAM), while molecular beacons specific to ORSV were tagged with tetra-chloro-6-carboxy-fluoroscein (TET). The universal quenching moiety 6-carboxy-tetramethyl-rhodomine (TAMRA) was attached to the 3′ ends of all molecular beacons employed. Molecular beacon procedure could successfully detect as little as 0.5 ng of viral RNA of both orchid viruses simultaneously in 100 mg of coinfected *Oncidium* orchid leaves. Only tubes containing total RNA isolated from CyMV and ORSV infected leaves yielded significant increases in fluorescence intensities upon addition of both sets of molecular beacons. Molecular beacon technique can provide a rapid and specific detection method for screening plants for virus-free

certification, quarantine verification, germplasm collection and selection of disease-resistant plants (Eun and Wong 2000). A fluorescent scorpion probe was designed based on the highly conserved CP region of the genome of *Grapevine fan leaf virus* (GFLV). This probe allowed quantitative estimation of GFLV RNA2 in a single nematode vector *Xiphinema index* collected from the rhizosphere of GFLV-infected grapevine plants. This diagnostic procedure was in equivalence with real-time RT-PCR assay in efficiency of virus detection (Finetti-Sialer and Ciancio 2005).

Strawberry vein banding virus (SVBV), a ds-DNA virus belongs to the genus *Caulimovirus* (retroid viruses) and it is listed as a quarantine pathogen by the European Plant Protection Organization (EPPO). In order to improve the sensitivity and to reduce the test time for obtaining results of detection of SVBV, an assay based on nucleic acid-based amplification (NASBA) and real-time detection using molecular beacons (real-time NASBA) was developed. This assay was evaluated along with biological indexing, the commonly used method for certification of SVBV-free plants and a newly optimized PCR-based procedure. NASBA assay was designed to amplify part of the RNA coding the CP of SVBV. The probe Bio1 and the primer set P_1B/P_2B were selected based on the high level of sensitivity, enabling detection of 10 molecules of template/reaction in vitro. When tested on healthy and infected samples, high increase in fluorescence was obtained only with SVBV-infected leaves, but no increase in fluorescence was noted with healthy leaves or leaves from plants infected with *Strawberry crinkle virus* (SCV) or *Strawberry mottle virus* (SMoV) (Fig. 2.22). The presence of SCV or SMoV in those plants was, however, confirmed by SCV- or SMoV-specific NASBA tests respectively. Both PCR and NASBA detected clearly in three of five samples and similar results were obtained by biological indexing also. Real-time NASBA offers several advantages over PCR such as reduction in contamination risks, non-requirement of post-PCR steps, the possibility of automation and high throughput application of NASBA procedure and the need for less expensive and sophisticated instruments. Further, NASBA can be performed in standard ELISA format commonly used in many laboratories (Vašková et al. 2004; Appendix 22).

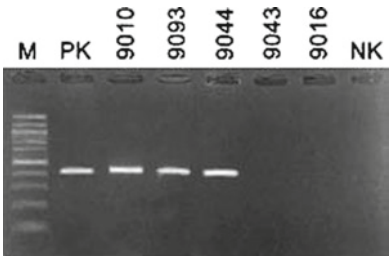

Fig. 2.22 Detection of isolates of *Strawberry vein banding virus* (SVBV) by PCR assay. M, 100 bp DNA ladder; PK, positive control (DNA of full-length SVBV clone); *Lanes 9010*, *9033*, *9044*, *9043* and *9016*, sample extracts; and *Lane NK*, negative control (healthy). (Courtesy of Vašková et al. 2004 and with kind permission of Springer Science, Heidelberg, Germany)

2.1.6.5 Single-Strand Conformation Polymorphism (SSCP) Analysis

A single-strand conformation polymorphism analysis offers a reliable alternative for detection of differences in the genomic DNA (Orita et al. 1989). The + and – strands of ds-DNA, if separated, become metastable sequence-specific folded structures with distinct electrophoretic mobilities in nondenaturing polyacrylamide gels. It is possible to detect even single nucleotide changes, under such conditions (Magome et al. 1999). In the case of strains *Beet necrotic yellows vein virus* (BNYVV), they could be assigned to respective A, B or P groups. Further, SSCP procedure was effective in detecting minor variants, new strains of BNYVV. The SSCP analysis required much less time than RFLP analysis and it was particularly useful for detecting and differentiating serologically indistinguishable strains of the viruses (Koenig et al. 1995).

Detection and differentiation of isolates of *Citrus tristeza virus* (CTV) was accomplished by applying SSCP analysis. The CTV isolates from diverse geographical areas were examined for the differences in their coat protein (CP) genes. The CP genes of CTV isolates (17) were subjected to RT-PCR. The amplicons were cloned and sequenced. Sequences of the clones showed sequence homology between 91.7% and 99.8%. After clone amplification and denaturation of PCR products, they were compared based on the results of SSCP analysis in 8% polyacrylamide gels. The patterns of 16 or 17 clones exhibited variations under two different electrophoretic conditions. The SSCP analysis with combination of two electrophoretic conditions and restriction of eight clones with *Eco*9II allowed discrimination between 21 of 22 CP gene clones selected for comparison. SSCP analysis provides results rapidly, as it is not necessary to have full sequence data for comparison. In addition, SSCP analysis is inexpensive and the results form a sound basis for identification and differentiation of several genes or gene regions (Rubio et al. 1996).

The SSCP analysis was applied for detecting and identifying *Citrus tristez virus* (CTV) isolates with different geographic origins and pathogenicity properties. Further, the SSCP profiles were used as molecular markers to monitor cross-protection involving mild and severe isolates of CTV. The SSCP profiles of mild isolates (forming two DNA bands) were simpler than most virulent isolates (producing more than two bands) of CTV. The set of SSCP profiles of the four genes p13, p18, p20 and p23, allowed identification of individual isolates, but no profile characteristic of a geographic area or a biogroup (based on symptom type) was evident. Sweet orange plants inoculated with a mild or a severe isolate yielded SSCP profile characteristic of each isolate, whereas the SSCP profile of plants successively inoculated with both isolates was a composite of the two individual profiles. The SSCP profile of singly infected plants remained constant, while the profile of doubly infected plants exhibited variations with time. The SSCP analysis may be useful to monitor the changes in the virus population in budwood sources facilitating the detection of potentially dangerous changes in RNA populations of mother plants used for propagation (Sambade et al. 2002; Appendix 23).

2.1.6.6 Double-Stranded RNA Analysis

Detection of ds RNA is useful for the early and rapid recognition of infection of plants by viruses, when the infecting viral genome is ds-RNA or when ds-RNA is produced during the process of replication of ss-RNA viruses. The presence of ds-RNA may be detected by either PAGE analysis or antiserum reaction with ds-RNA. Quantitative estimations indicated variations in ds-RNA due to host–virus combinations (Valverde et al. 1986). Detection of infection by viruses that are difficult to purify or that reach vey low concentrations in infected plants may be detected by ds-RNA analysis. Further latent infections and viruses for which antisera are not available, ds-RNA analysis may be applied successfully as in the case of *Groundnut rosette virus* (GRV) (Breyel et al. 1988) and *Citrus tristeza virus* (CTV) (Habili 1993).

The ds-RNA may be isolated and labeled or cloned as cDNA for preparing nucleic acid probes. The ds-RNA was isolated from leaves of soybean cv. Wayne infected with dwarfing (D) or yellowing (Y) strain of *Soybean dwarf virus* (SbDV). Each strain produced two virus-specific ds-RNAs. SbDV-D ds-RNAs were smaller than SbDV-Y ds-RNA species. Northern blot hybridization analyses showed that the strains were related and the ds-RNAs corresponded to viral genomic-length and 3′ subgenomic-length species. The size-specificity of SbDV-D and SbDV-Y ds-RNAs could be employed as a phenotypic marker for in vitro strain differentiation (Smith et al. 1991). In a further study, virus isolates from forage legumes collected from eight different states of the US, were found to be closely related to SbDV. Gel profiles of ds-RNA preparations of SbDV-D, SbDV-Y (endemic isolates) and *Bean leaf roll virus* (BLRV) exhibited two distinct bands representing genomic and subgenomic ds-RNAs. The genomic and subgenomic BLRV ds-RNAs were barely visible and they were consistently larger than those of endemic isolates. The probe pSbDV-Y13 hybridized strongly to both genomic and subgenomic RNAs of all isolates of SbDV, but not to BLRV, indicating the lack of homology between the 3′ ends of the SbDV-Y and BLRV genomes (Damsteegt et al. 1999).

Two ^{32}P-labeled cDNA clones specific for ds-RNA sequences were designed as probes for detecting *Grapevine leaf roll-associated virus 3* (GLRaV-3). The probes were specific in detecting GLRaV-3 in extracts of leaves, petioles or cortical tissues of infected grapevine plants and they did not hybridize with total RNA from healthy controls or from vines infected by other viruses, indicating the specificity of the probes (Saldarelli et al. 1994). *Tomato chlorosis virus* (ToCV) has two prominent ds-RNAs with several small ds-RNAs. Nonradioactive DIG-11-UTP labeled probes derived from cDNA clones representing portions of RNAs 1 and 2 were employed for the detection of ToCV by dot blot hybridization technique (Wisler et al. 1998). Bing sweet cherry (*Prunus avium*) is affected by a disease inducing stem-pitting symptoms for which the nature of the causal agent(s) is not clear. Several unique ds-RNAs were isolated to determine the etiology of cherry stem-pitting disease (CSP) disease. A total of four ds-RNA species were isolated

from either CSP-diseased or apparently healthy Bing cherry trees. In addition, two related, but biologically and molecularly distinct strains of *Cherry green ring mottle virus* (CGRMV) were recovered from diseased Shirofugen flowering cherry trees. CSP appears to be a disease complex for which the primary causal agent(s) remain unknown (Zhang et al. 1998; Appendix 24).

The feasibility of utilizing ds-RNA-based detection methods for routine large scale application was demonstrated. The ds-RNAs of *Cucumber mosaic virus* (CMV), and *Potato virus X* could be differentially detected in infected plants. Likewise, *Chenopodium quinoa*, when infected individually by different viruses such as CMV, *Tobacco rattle virus* (TRV), *Tobacco ringspot virus* (TRSV), *Arabis mosaic virus* (ArMV), *Grapevine fanleaf virus* (GFLV) and *Carnation mottle virus* (CarMV) could be detected by ds-RNA analysis (Yamashita et al. 1996). CMV causes the banana mosaic disease (BMD), considered as a serious threat to banana industry. The presence of the satellite associated with CMV influences the symptom type on infected banana plants. The ds-RNAs extracted from *Nicotiana glutinosa* inoculated with different isolates of CMV were subjected to agarose gel electrophoresis. Presence of an additional RNA was detected only in plants inoculated with isolate 22. The additional ds-RNA was gel-purified and used as a template for RT-PCR amplification. The ds-RNA analysis was useful to characterize the CMV isolates associated with banana mosaic disease (Chou et al. 2009).

Detection of ds-RNA by immunoassays has been accomplished in the case of some viruses, by preparing antiserum against polyinosinic-polycytidylic acid. The ds-RNA from *Cucumber mosaic virus* (CMV) and *Plum pox virus* (PPV) was extracted from infected plants by heating crude sap at 80°C for 2 min and maintaining a pH of 6.0. Indirect ELISA format was effective in detecting CMV and PPV in the plant tissue extracts. PPV could be detected readily in the extracts of *Nicotiana benthamiana* at 50 days after inoculation (Aramburu and Moreno 1994). Double-stranded RNA analysis is commonly applied for the detection of cryptic viruses which belong to the genus *Cryptovirus* and the family *Partiiviridae*. These viruses have a small, segmented ds-RNA genome in the size range of 1–3 kbp. Cryptic viruses are widespread and their existence in many more plant species has not been detected, because of the absence of visually recognizable symptoms. The cryptic viruses are readily transmitted through seeds and pollen of infected plants. The long term cultivation of in vitro propagated plants is a drastic change of environment compared to the natural life cycle of the plant species. Hence, the effects of in vitro culture conditions on the survival of cryptic viruses were assessed in plants generated through continuous tissue culturing. Immunoblotting procedure was adopted for the detection of *Carnation cryptic virus* (CarCV). The Hungarian *Dianthus* germplasm entries (21) and *Silene vulgaris* were grown aseptically for 16 years. The total nucleic acids of the *Dianthus* sp. and *S. vulgaris* were separated by non-denaturing gel electrophoresis and the ds-RNA pattern was visualized by immunoblotting using ds-RNA-specific monoclonal antibodies. Genomic ds-RNAs of CarCV were detected in all germplasm collections maintained in in vitro cultures.

In addition to CarCV, the *Beet cryptic viruses 1*, *2* and *3* (BCV-1, -2 and -3) could persist under in vitro conditions. The results indicated that cryptic viruses are so well adapted to their hosts that can persist under in vitro conditions even after a long duration of 16 years of culturing, despite the drastic changes in the environments (Szegö et al. 2005).

2.1.6.7 Reverse Transcription-Loop-Mediated Isothermal Amplification (RT-LAMP) Technique

Loop-mediated isothermal amplification (LAMP), a novel nucleic acid amplification method was developed by Eiken Chemical Co. Ltd., Tokyo, Japan, has the potential to replace PCR-based assays, because of its simplicity, rapidity, specificity and cost-effectiveness. RT-LAMP is based on the principle of standard displacement reaction and stem-loop structure that amplifies the target with high specificity, selectivity and rapidity under isothermal conditions, thereby avoiding the need for using an expensive thermal cycler. The amplification efficiency of LAMP procedure is extremely high, due to continuous amplification under isothermal conditions producing a large amount of the precipitate magnesium pyrophosphate as a byproduct leading to turbidity. Using four specially designed primers that include six short sequences matching the target DNA fragment, the DNA is amplified under isothermal conditions within a short period of time. The reactions are easily monitored by detecting the turbidity using an inexpensive turbidimeter. Further, the higher amplification efficiency of the LAMP procedure enables simple visual observation of amplification through the naked eye by using a UV lamp in the presence of an intercalating dye such as SYBR Green I or ethidium bromide (Notomi et al. 2000; Parida et al. 2004).

The RT-LAMP assay is a simple diagnostic tool in which the reaction is carried out in a single tube by mixing of the buffer, primers, reverse transcriptase and DNA polymerase and incubating the mixture at 63°C for 60 min. Compared to RT-PCR and real-time PCR, RT-LAMP has the advantages of reaction simplicity and detection sensitivity. The higher sensitivity and specificity of RT-LAMP is considered to be due to the continuous amplification of DNA under isothermal conditions, employing six primers that recognize eight distinct regions of the target. The increases in the turbidity of the reaction mixture correlates with the amount of DNA synthesized. Hence, real-time monitoring of RT-LAMP reaction can be achieved by real-time measurement of turbidity. Measurement of turbidity is extremely simple, compared to real-time TaqMan RT-PCR and NASBA assays that require fluorogenic primers and probes as well as expensive detection equipments. Furthermore, the time required for obtaining the results by RT-LAMP assay is just 30 min compared to 3 or 4 h needed for RT-PCR-based assays (Notomi et al. 2000; Parida et al. 2004).

A simple and rapid procedure, reverse transcription-loop-mediated isothermal amplification (RT-LAMP) of DNA was developed for the efficient detection of *Japanese yam mosaic virus* (JYMV) in infected plants. This procedure does

not require elaborate RNA extraction steps and precise thermal cycling and gel electrophoresis. JYMV was successfully detected in leaves, propagules and roots of Japanese yam plants. The virus titer reflected by the intensity of turbidity of reaction mixture could be measured by a turbidimeter easily (Fukuta et al. 2003). Detection of *Tomato spotted wilt virus* (TSWV) was accomplished by immunocapture-reverse transcription-loop-mediated isothermal amplification (IC-RT-LAMP) procedure. TSWV genomic RNA was reverse transcribed, followed by amplification under isothermal (65°C) conditions for 60 min. The intensity of turbidity of the reaction mixture was estimated. The turbidity of the reaction mixture was proportional to the concentration of the amplicons. IC-RT-LAMP assay was found to be more sensitive (100 times) than IC-RT-PCR assay in detecting TSWV in infected chrysanthemum plants and providing the results rapidly (Fukuta et al. 2004).

In a later investigation, RT-LAMP of DNA was applied for the detection of *Potato virus Y* (PVY). A set of four primers matching a total of six sequences of the CP gene of PVY was designed in such a way that a loop could be formed and elongated during DNA amplification. By using PVY-CP complementary DNA clones as templates, the LAMP reaction was optimized by adjusting the concentrations of $MgSO_4$, dNTPs and *Bst* DNA polymerase. Insoluble magnesium pyrophosphate is the byproduct of LAMP and a positive reaction was accompanied by a visible precipitate. The concentration of this compound is measured using the turbidimeter to monitor the reaction. Absorbance readings were recorded at 405 and 600 nm using a microplate reader and/or a spectrophotometer respectively. By measuring the turbidity, RT-LAMP could be monitored visually and spectrophotometrically using a microplate reader which can save significant time and cost in analyzing data. The one-step RT-LAMP-turbidity method gave results comparable with the two-step RT-PCR procedure for the detection of PVY from leaf and tuber samples. Of the total 240 samples, 234 were diagnosed similarly by both methods. The relative simplicity in reaction setup and product detection and high efficiency are the advantages of one-step RT-LAMP procedure. Further, the isothermal conditions required by RT-LAMP could be provided easily using a waterbath, whereas the rapid and accurate temperature required by RT-PCR needs an expensive thermocycler. Since no gel electrophoresis is needed for visualization of positive reactions in RT-LAMP assays, use of hazardous compounds such as ethidium bromide for staining can be avoided by applying RT-LAMP procedure (Nie 2005).

Plum pox virus (PPV) was efficiently detected by applying a one-step, accelerated reverse transcription loop-mediated isothermal amplification (RT-LAMP) procedure. The six primers required for accelerated RT-LAMP were designed based on the sequences of a conserved region in the C-terminus of the coat protein (CP) coding region of PPV. RT-LAMP was employed to detect isolates of five strains of PPV including the strains D, M, EA, C and W. PPV could be detected reliably in both herbaceous and woody host plant species infected by the virus. The sensitivity of RT-LAMP technique was compared to real-time RT-PCR with SYBR Green I and melting curve analysis, using serially diluted total RNA extracts. RT-LAMP

assay was found to be equally sensitive, except at lower template concentrations at which real-time RT-PCR gave more consistent results. PPV could be detected after a 30-min incubation at 63°C. However, a longer incubation period was required for lower concentrations of the target. RT-LAMP was shown to be a very sensitive, low-cost diagnostic tool that has potential for large scale application for assessing the disease incidence and preventing its further spread of this devastating virus (Varga and James 2006).

2.1.6.8 Heteroduplex Mobility Analysis

Heteroduplex mobility analysis (HMA) is based on the differences in the rate of migration between a DNA heteroduplex and a DNA homoduplex that can be useful to identify mismatches or deletions in DNA sequences. The HMA technique was first applied to determine the variability of *Human immuno-deficiency virus* (HIV) Type 1. Heteroduplexes are found between PCR-amplified fragments of variable region of different isolates of the virus, after denaturation and reannealing. The difference in migration of heteroduplexes in nondenaturing gel electrophoresis is due to the effects of primary sequence changes forming mismatches which cause bulges in double-stranded DNA fragments (Delwart et al. 1994). HMA analysis was applied for the detection and differentiation of five isolates of *Zucchini yellow mosaic virus* (ZYMV) collected from cucurbit plants in different areas of Taiwan. A cDNA fragment of 760-bp covering the variable region of the N-terminal half of the CP gene was amplified by RT-PCR and subsequently subjected to HMA analysis for sequence variations. Comparison of the CP genes of the five Taiwan isolates indicated that they shared 92.8% to 98% nucleotide identities and 96.4–99.3% amino acid identities. The results of HMA procedure agreed well with those of phylogenetic analysis based on the sequence data of the ZYMV isolates studied (Lin et al. 2000).

Heteroduplex mobility assay (HMA) was employed for testing cassava plants for the presence of begomoviruses. The assay involved amplification of the highly conserved core region of the CP gene of field isolates followed by denaturing and annealing with a number of reference strains. The HMA profiles obtained, were able to differentiate four different viral species and 11 different virus strains. Further, the HMA profiles showed good correlations with sequencing results and phylogenetic comparisons with other sequenced cassava viruses. This procedure was found to be sensitive and rapid, providing the advantage of being able to detect mixture of viruses in field-grown cassava crops (Berry and Rey 2001). The HMA procedure was found to be effective in detecting *Grapevine leafroll associated virus 2* (GLRaV-2) in samples collected from grapevine accessions of different cultivars from vineyards in Italy, Greece, France and Brazil during a survey carried out in 2001–2002 to assess the distribution of GLRaV-2 in these countries. Differences in the sequences in ORF coding the coat protein (CP) of GLRaV-2 were revealed by the HMA procedure (Angelini et al. 2004).

2.1.6.9 DNA Array Technology

Among the multipathogen detection systems, DNA array technology has been demonstrated to be the most promising one. DNA array technology aims to miniaturize traditional bioanalytical detection system so that hundreds of biomolecules with unique identity can be detected simultaneously in one single experiment. In a microarray platform (glass slide) thousands of diagnostic 'probes' can be spotted and hybridized with labeled targets like nucleic acids extracted from the specimens and chemically modified with a fluorescent or other reporter (compound/molecule). DNA microarray provides a medium for hybridization of the known with unknown DNA samples based on base-pairing rules and automating the process of identifying the unknowns. Microplates or standard blotting membranes may be used in the experiments based on the size of the deposited sample spotted and they may be designated macroarrays (>300 μ in diameter) or microarrays (<200 μ in diameter). Specialized robotics and imaging equipment may be used for dispensing samples on the platform. Recent versions of the 'pan-viral array' include oligonucleotides specific to more than 1,000 viruses including plant viruses (Wang et al. 2003; Morais et al. 2006). The results of the experiments can be captured on x-ray film. A positive reaction is indicated by the presence of a light gray to black dot, whereas negative reaction is inferred by a white color. The hybridization intensity is expressed as gray values to indicate reaction strength for quantification of the results. Microarrays for the detection of plant viruses have been applied to a lesser extent compared to fungal or bacterial pathogens (Call et al. 2001; Agindotan and Perry 2007).

DNA microarray technology has been commonly applied for identification/detection of sequences (genes or gene mutations) and determination of expression levels or abundance of genes. Later, the possibility of exploiting microarray technology was examined for its potential for viral diagnostics. Detection and characterization of viruses such as *Hepatitis C virus* (Park et al. 2001) and characterization of *Poliovirus* vaccine (Proudhikov et al. 2000) could be accomplished by microarray technique. Several viruses may be detected in a single generic assay. A single glass microscope slide which forms the microarray, can accommodate DNA probes up to 30,000. The DNA arrays contain gene sequences from each of the viruses that are to be detected in a single assay. The microarray is exposed to fluorescently labeled cDNA from the sample to be tested, followed by scanning with a microarray scanner for detecting the targets in the sample concerned. Commercial kits such as Qiagen can be employed as per the instructions of the manufacturer. Different designations like biochip, DNA chip, gene chip and gene array have been used by various investigators (Shi et al. 2003).

Four viruses infecting potatoes, *Potato virus X* (PVX), *Potato virus Y* (PVY), *Potato virus A* (PVA) and *Potato virus S* (PVS) were detected effectively by employing a microarray procedure in plants infected by individual or mixtures of these viruses. Closely related viruses or strains of these viruses could be detected and discriminated even when the sequence identity was less than 80%. The assay was able to select sequence variants with greater than 90% sequence identity and the results were comparable with those of ELISA in the sensitivity

levels (Boonham et al. 2003). In another investigation, short synthetic single-stranded oligomers (40-nt) were employed as capture probes, instead of PCR products. Potato viruses PVA, PVS, PVX, PVY, *Potato virus M* (PVM) and *Potato leaf roll virus* (PLRV) were efficiently detected in both single and mixed infections by using a microchip. The principal strains of PVY and PVS could be detected and differentiated by employing oligonucleotide-based microarray technique (Bystricka et al. 2005).

A membrane-based macroarray, capable of detecting hundreds of plant pathogens in a single assay was developed for the detection of plant viruses in single and multiple infections. *Cucumber mosaic virus* (CMV) serogroups and subgroups could be detected and differentiated by the macroarray detection system. The coat protein (CP) genes of 14 different isolates were amplified using cy3-labeled generic- and species-specific primers. These amplifications were hybridized against a set of five different serotype- and subgroup-specific 24-mer oligonucleotides bound to an aldehyde-coated glass slide via an aminolinker (Deyong et al. 2005). A nonradioactive macroarray system was employed to test the presence of viruses using 70-mer oligonucleotide probes immobilized on nylon membranes. By using total plant RNA extract, cDNA and second-strand syntheses were carried out, employing an anchor primer sequence with random pentamers coupled at the 3′ end. Subsequent synthesis by PCR using the anchor primer alone led to a relatively unbiased amplification of plant and viral RNAs. After chemically labeling the cDNAs, the product was used as a target in hybridizing analyses. By adopting this protocol, *Cucumber mosaic virus* (CMV), *Potato virus Y* (PVY) and *Potato leaf roll virus* (PLRV) were detected. Viral RNAs were amplified between 100- and 1,000-fold and they could be detected in single and mixed infections. All the three viruses were readily detected in the macroarray with strong signals observed relative to the background on the membranes. The labeled target DNA hybridization was specific to the infecting virus and no cross-hybridization to probes for heterologous viruses was seen. The sensitivity of detection by the macroarray was comparable to DAS-ELISA test. Conventional RT-PCR and real-time RT-PCR assays were 10^3 and 10^5 times more sensitive respectively than macroarray system developed in this investigation (Agindotan and Perry 2007; Appendix 25).

2.1.6.10 Next-Generation Sequencing and Metagenomic Analysis

Metagenomics is an approach for the study of microbial populations in a sample by analyzing the nucleotide sequence content. This method has been applied to a wide range of environmental samples from sea to humans (Sogin et al. 2006; Zhang et al. 2006). Diagnosis through metagenomic procedure offers the possibility of overcoming certain limitations of pathogen detection associated with parallel screening (multiplex) methods and the nonspecificity associated with traditional detection procedures. Sequences produced from an infected plant will

include sequence from any pathogen(s) present in the plant. The extraction of RNA from infected plant, production of cDNA with a random priming method and sequencing will produce data for a large range of potential pathogens. RNA viruses, viroids and the RNA stages of actively replicating DNA viruses can be directly screened.

The metagenomic diagnostic procedure utilizing next-generation sequencing has been applied for the detection of *Pepino mosaic virus* (PepMV) infecting tomato plants and an uncharacterized *Gayfeather mild mottle virus* (GMMV) infecting *Liatris spicata*. A subtractive hybridization method was followed to enrich viral cDNA. From the 71,146 fragments of sequence generated, 29,095 were identified as having similarity to published viruses based on BLAST searching. Cluster analysis from an alignment of 1a replicase protein sequences from the *Bromoviridae* family reliably placed the new virus within the *Cucumovirus* genus and the whole-genome comparisons showed that the most related virus was *Tomato aspermy virus* (TAV). However, for most diagnostic requirements, a full-genome sequence is not a necessity. Although identification of the new virus using this approach is extremely rapid, the analysis costs (approximately £1,000/sample) are highly prohibitive (Adams et al. 2009). Unless the testing cost is brought down to a level affordable to the growers, this approach may be only in the realm of academic interest, than of practical utility.

2.2 Detection of Viroids in Plant Organs

Viroids are the smallest known agents capable of inducing plant diseases, when they gain entry into susceptible host plant tissues. They have small, single-stranded covalently closed circular RNAs with a highly base-paired rather rod-like stiff conformation. Viroid RNAs do not code for any viroid-specific protein. They depend entirely on the host enzyme machinery for the replication of the genomic RNAs. Viroids may be considered as highly gifted, modern "RNA parasites" that have shed off all the dispensable processes of viral pathogens to make use of the host in the most sophisticated manner (Tabler and Tsagris 2004). Viroids replicate independently, when introduced into cells/tissues of susceptible plants and reach concentrations sufficient enough to produce characteristic symptoms or remain latent in some host plant species.

2.2.1 Molecular Characteristics

Viroids have GC-rich genome consisting of 246–401 nucleotides (nts). On the basis of biological, biochemical and structural properties, the viroids have been classified into two different families as indicated below (Góra-Sochacka 2004; Mayo and Brunt 2007):

1. Pospiviroidae –	Genus:	*Pospiviroid* –	Potato spindle tuber viroid
			Chrysanthemum stunt viroid
			Citrus exocortis viroid
			Tomato apical stunt viroid
			Tomato chlorotic dwarf viroid
			Tomato planta macho viroid
		Hostuviroid –	Hop stunt viroid
		Cocaviroid –	Coconut cadang-cadang viroid
			Coconut tinangaja viroid
			Citrus viroid IV
			Hop latent viroid
		Apscaviroid –	Apple scar skin viroid
			Apple dimple fruit viroid
			Australian grapevine viroid
			Citrus bent leaf viroid
			Citrus viroid II
			Citrus viroid III
			Grapevine yellow speckle viroid 1
			Pear blister canker viroid
		Coleviroid –	Coleus blumei viroid
			Coleus blumei viroid 1, 2, and 3
2. Avsunviroidae –	Genus:	*Avsunviroid* –	Avacado sunblotch viroid
			Chrysanthemum chlorotic mottle viroid
		Pelamoviroid –	Peach latent mosaic viroid
		Elaviroid –	Eggplant latent viroid

Viroids assigned to the family *Pospiviroidae* include species with a central conserved region (CCR) and without hammerhead ribozymes. *Avsunviroidae* contains species lacking CCR, but they are able self-cleave through hammerhead ribozymes. Members of *Pospiviroidae* replicate in the nucleus, whereas the viroids included in *Avsunviroidae* replicate in the chloroplasts (Flores et al. 2003, 2005).

The viroids do not show distinct variations in their structure. It may not be possible to differentiate them based on the structural dissimilarities. *Potato spindle tuber viroid* (PSTVd), the first viroid to be characterized, has been studied in some detail. This viroid possesses a serial arrangement of 26 double-stranded segments interrupted by bulge loops of varying size. The extended secondary structure is folded into a more globular tertiary conformation, as the single-stranded loops do not interact. The viroids are both stable and flexible structurally. The native rod-like structure of viroids is converted, through thermal denaturation into a hairpin-containing circle in a highly cooperative fashion (Diener 1971; Henco et al. 1979).

2.2.2 Biological Methods

Viroids induce symptoms that are often similar to those due to viruses. Because of the similarity in symptoms and methods of transmission, viroids could be recognized

as a separate group only in the second half of twentieth century. Diener (1971) showed that potato spindle tuber disease was due to a single-stranded RNA with no protein coat that is present in the case of a conventional virus. Potatoes infected by *Potato spindle tuber viroid* (PSTVd) may exhibit different degrees of stunting with folage turning slate gray with dull laminar surface. However, the characteristic effect of PSTVd infection is seen on the tubers which become abnormally elongated assuming a spindle or cylindrical shape with prominent eyes (Diener 1979). Symptoms caused by *Apple scar skin viroid* (ASSVd) are restricted to apple fruits and the symptom type may vary depending on the apple cultivar (Koganezawa et al. 2003). Citrus plants are the natural hosts of at least five viroid species belonging to the family *Pospiviroidae*: *Citrus excortis viroid* (CEVd), *Citrus bent leaf viroid* (CBLVd), *Hop stunt viroid* (HSVd), *Citrus viroid III* (CVd-III) and *Citrus viroid IV* (CVd-IV). These viroids induce distinguishable symptoms in citrus plants (Duran-Vila et al. 2000).

Few viroids like *Cucumber pale fruit viroid* (CPFVd) produce distinguishable symptoms. Most viroids, however, induce symptoms in their natural hosts that may be confused with the symptoms caused by other diseases. Hence, inoculations to host plant species that reacts with specific symptoms to the viroid under investigation have to be carried out. Diagnostic hosts that have been found to be useful for the detection and identification of some viroids are presented in Table 2.10. Biological indexing usually takes a long time and require large glasshouse space. The titer of viroids could be determined by inoculating assay hosts that react with necrotic local lesions as in the case of PSTVd by inoculating *Scoplia sinensis* that produced countable local lesions (Singh 1971). Hop stunt disease may be more reliably detected by cucumber bioassay. This assay has to be performed in a temperature-controlled greenhouse or plastic house at 30°C. *Hop stunt viroid* (HSVd) could be detected by cucumber bioassay in a sample containing a mixture of one HSVd-infected leaf disc and 200 HSVd-free leaf discs (Sasaki and Shikata 1980). The practical

Table 2.10 Diagnostic host plants for virioid detection

Viroid	Host plant/cultivar	References
Potato spindle tuber Viroid (PSTVd)	Tomato cv. Rutgers	Raymer and O'Brien (1962)
	cv. Allerfrüheste-Freiland	Singh (1970)
	Scopolia sinensis	Singh (1971)
Tomato bunchy top viroid (TBTVd)	Tomato cv. Rutgers	Benson et al. (1965)
Citrus exocortis viroid (CEVd)	*Citrus medica* cv. Etrog	Calavan et al. (1964)
	Gynura aurantica	Weathers and Greer (1972)
Chrysanthemum stunt viroid (CSVd)	*Chrysanthemum morifolium* cv. Mistletoe	Keller (1953)
Chrysanthemum chlorotic mottle viroid (ChCMVd)	*C. morifolium* cv. Deep Ridge	Dimock et al. (1971)
Cucumber pale fruit viroid (CPFVd)	*Cucumis sativa* cv. Sporu	van Dorst and Peters (1974)
Hop stunt viroid (HSVd)	*C. sativus*	Sasaki and Shikata (1977)

utility of the method for detection of HSVd was demonstrated using samples from field-grown infected plants (Sasaki et al. 1981).

Total nucleic acid preparations from grapevine plants were assayed for their infectivity by inoculating onto *Gynura aurantica* plants. Inoculated plants were topped to promote production of new shoots. Development of symptoms of infection was monitored for 30 days after inoculation. The symptoms induced in *G. aurantica* were similar to those caused by *Citrus exocortis viroid* (CEVd). An infectious RNA with same electrophoretic mobility as the CEVd was reisolated from the infected plants, establishing the identity of the viroid infecting grapevine as CEVd (Flores et al. 1985). *Eggplant latent viroid* (ELVd) was purified by sequential polyacrylamide gel electrophoresis (sPAGE) procedure. The RNA with the slow mobility characteristic of viroid circular forms was eluted and inoculated via three incisions into the epicotyl of 5-cm high eggplant seedlings that were grown in the greenhouse. The symptoms of infection were observed about 6 months after inoculation (Fadda et al. 2003). Citrus samples (112) collected during a 3-year survey in southern Italy were tested by graft-inoculating into Arizona 861-S1 Etrog citron plants. Viroid symptoms were produced in the indicator plants by 104 of 112 citrus samples collected in the survey. The viroids detected by biological indexing were *Citrus exocortis viroid* (CEVd), *Hop stunt viroid* (HSVd) and *Citrus viroid III* (CVd-III) (Malfitano et al. 2005).

2.2.3 Physico-Chemical Methods

Biological indexing procedures which require long time to provide the results, have been employed for detecting the viroids in mother plants or asexually propagated planting materials in certification and quarantine programs. But the need for developing more precise, reliable and rapid techniques was realized in order to prevent the introduction and subsequent spread of viroids, particularly viroids infecting perennial horticultural and ornamental crops. Physico-chemical methods have been shown to be useful for the detection of the viroid itself or the specific products of host–viroid interaction.

2.2.3.1 Phloroglucinol Test

Plants infected by viroids, as in the case of other microbial pathogens, may contain compounds that are not present in healthy plants. Detection of such compounds may be useful for identifying viroid-infected plants. For example, in the ray cells in the bark tissue of citrus plants infected by *Citrus exocortis viroid* (CEVd), the presence of certain compounds that can react with aldehyde decoupling reagents such as phloroglucinol-concentrated HCl has been observed. The reaction results in the production of characteristic color which can be recognized under the light microscope. Cross-sections of the bark tissues are immersed in alcoholic phloroglucinol

solution followed by treatment with concentrated HCl for a few seconds. A positive color reaction may be observed well in advance of the development of early visible symptoms of bark scaling in CEVd-infected citrus plants. A strong correlation (98.6%) between the positive color reaction and presence of CEVd was established by Childs et al. (1958). No such color reaction could be seen in citrus plants infected by psorosis, xylopsorosis and tristeza diseases. However, diagnostic hosts were used commonly by researchers in preference to the phloroglucinol test (Diener 1979).

2.2.3.2 Polyacrylamide Gel Electrophoresis Technique

Polyacrylamide gel electrophoresis (PAGE) procedure has been applied to separate the nucleic acid molecules based on their differential mobility in an electrical field. *Potato spindle tuber viroid* (PSTVd) was detected in potato and tomato plants by extracting the cellular nucleic acids from both healthy and infected plants. The nucleic acids are separated by the conventional PAGE procedure, followed by staining to recognize different bands of nucleic acids. The nucleic acids with high and low molecular weights migrate to short or long distances in the gels depending on their sizes, smaller ones moving to longer distances than the larger nucleic acid molecules. The PSTVd, as a nucleic acid, appeared as a distinct band only in the extracts from infected plants. PAGE analysis can be applied to detect the presence of both mild and severe strains of PSTVd. Several elite or basic potato seed stocks have been freed of PSTVd by eliminating infected tubers that were identified by employing PAGE technique in certification programs (Morris and Wright 1975). The presence of viroid and viroid-like RNAs in grapevines was detected by PAGE analysis. Under non-denaturing conditions, a band with mobility faster than that of *Citrus exocortis viroid* (CEVd) was detected. Under fully denaturing conditions, two bands were observed, one co-migrating with circular forms of CEVd and a second one migrating at a faster rate than the linear forms of this viroid. This RNA species did not hybridize with a cDNA probe to the *Citrus exocortis viroid* (CEVd). Using the bioindicator *G. aurantica*, the identity of the viroid was established as CEVd (Flores et al. 1985).

The viroid molecules differ distinctly from normal host RNAs in their electrophoretic mobility in nondenaturating and partially denaturating gels. This property of viroids can be made as the basis for differentiating the viroids from host RNAs. The leaf extracts are run first in nondenaturating gels in one direction and are run either in the reverse direction or at 90° to the first direction under denaturating conditions. By silver-staining the gels, viroids can be detected at 600 pg level. Purified ccRNA1 of *Coconut cadang-cadang viroid* (CCCVd) could be detected at a concentration as low as 0.4 to 1.6 ng (Schumacher et al. 1983). In another investigation, CCCVd was detected by precipitation by non-nucleic acid fraction with sodium sulfate and polyethylene glycol (PEG). The nucleic acid fraction was extracted with phenol-SDS-chloroform followed by precipitation with ethanol and fractionation with lithium chloride. The nucleic acids were separated by PAGE method, followed by staining with toluidene blue. This procedure had a detection

limit of about 1 μg of cadang-cadang RNA (ccRNA) per band. The variations in the electrophoretic mobility of cadang-cadang isolates could be used as the basis of distinguishing the CCCVd isolates (Randles 1985).

Return gel electrophoresis (REG) method was shown to be more effective in detecting PSTVd in potato true seeds. This procedure was found to be comparable with or superior to the nucleic acid hybridization method (Singh et al. 1988). Detection of *Apple scar skin viroid* (ASSVd) in apple leaf tissue, *Hop stunt viroid* (HSVd) in plum bark and *Citrus exocortis viroid* (CEVd) in citrus leaves was accomplished efficiently by employing the return gel electrophoresis technique (Asai et al. 1998). Analytical agarose gel electrophoresis, another variant of electrophoresis, was developed for the separation of nucleic acids more efficiently. This simple procedure was employed for reliable detection of *Coconut tinangaja viroid* (TiVd) in coconut leaf samples. The coconut leaf extract was subjected to two dimensional electrophoresis. The specific band containing circular molecules typical for viroids was detected in the gel. The identity of TiVd was established by applying either diagnostic oligonucleotide probe (DOP) hybridization assay or reverse-transcription-polymerase chain reaction (RT-PCR) procedure (Hodgson et al. 1998).

Citrus viroids were detected by employing sequential polyacrylamide gel electrophoresis (sPAGE) technique in extracts of young bark tissues obtained through phenol extraction and purification by CF-11 cellulose column. *Citrus exocortis viroid* (CEVd), *Citrus viroid II* (CVd-II) and *Citrus viroid III* (CVd-III) were detected during the warm season (June to November). But only CEVd could be detected in other months by applying sPAGE procedure (Tessitori et al. 1996). In a later study, the sPAGE procedure was applied for the analysis of nucleic acid extracts of the samples collected from field-infected citrus plants during a 3-year survey undertaken in the southern Italy. Twelve trees contained a single RNA band with the molecular size of *Hop stunt viroid* (HSVd) (7 samples) or CEVd (2 samples) or *Citrus viroid III* (CVd-III) (3 samples). In addition, two or more viroid-like RNAs with electrophoretic mobility of CEVd and/or *Citrus bent leaf viroid* (CBLVd) and/or HSVd and/or CVd-III and/or CVd-IV were detected, demonstrating the mixed infections by viroids in many citrus trees. The results of sPAGE analysis were consistent with those of Northern and dot blot analyses (Malfitano et al. 2005).

2.2.4 Immunoassays

Viroid genomes do not have any messenger activity, whereas viruses have specific messenger activity that directs the synthetic machinery of the host plant to produce virus-specific proteins. This fact was demonstrated by employing *Xenopus laevis* oocyte system which did not translate the viroid genome to form any protein. This deficiency results in the absence of any viroid-specific protein in the infected plants (Davies et al. 1974; Semancik et al. 1977). However, enhancement of host-specific protein contents was observed in tomato infected by *Potato spindle tuber viroid*

(PSTVd) and *Gynura aurantica* infected by *Citrus exocortis viroid* (CEVd) (Zaitlin and Hariharasubramanian 1972; Conejero et al. 1979). Accumulation of a host protein with a low molecular weight of 70-kDa in tomatoes after inoculation with *Tomato planta macho viroid* (TPMVd) was observed. An antiserum was generated against this host protein fraction designated PM antigen. The antiserum was employed to detect TPMVd infection in tomato using the double-diffusion technique (Diener et al. 1985). However, induction of PM antigen does not seem to be specific to TPMVd infection alone. The presence of PM antigen in tomato plants infected by other viroids such as PSTVd, *Chrysanthemum stunt viroid* (CSVd), *Tomato apical stunt viroid* (TASVd), *Columnea viroid* (CVd) and the virus *Cucumber mosaic virus* (CMV) was also detected, indicating the lack of specificity of production of PM antigen only in TPMVd infection. As such, immunoassays do not seem to have significant utility for the specific detection of viroids.

2.2.5 Nucleic Acid-based Techniques

As the immunoassays are practically not applicable for the detection of viroids which lack a protein component, different nucleic acid-based techniques have been evaluated for their usefulness in detecting viroids in affected crop plants.

2.2.5.1 Nucleic Acid Hybridization Techniques

Potato spindle tuber viroid (PSTVd) was first detected by nucleic acid spot hybridization (NASH) procedure. The viroid RNA is bound to a solid matrix nitrocellulose membrane. ^{32}P-labeled cDNA probes are allowed to hybridize with the immobilized viroid RNA. This procedure was shown to be effective in detecting PSTVd in large number of potato tuber samples (Owens and Diener 1981). Later, it was possible to prepare riboprobes using plasmid transcription vectors containing promoters for SP6, T3 and T7 bacterial polymerases (Melton et al. 1984; McInnes and Symons 1989). The single-stranded cRNA probes were found to be more sensitive than similar cDNA probes and they could be prepared more easily and uniformly labeled (Lakshmanan et al. 1986; Candresse et al. 1990). Spot (dot-blot) hybridization was shown to be useful for early detection and assay of several viroids causing potato spindle tuber, avocado sunblotch and coconut cadang-cadang diseases (Owens and Diener 1981). This procedure was effective in revealing latent infection of avocado by *Potato spindle tuber viroid* (PSTVd) (Querci et al. 1995). Infection of apple by a new viroid causing fruit crinkle in apple was detected and it was possible to differentiate this viroid from already existing *Apple scar skin viroid* (ASSVd) by applying hybridization technique (Ito et al. 1993).

As the need for avoiding hazardous radioactive probes was realized, non-radioactive labels such as digoxigenin and biotin were increasingly used for preparing probes. Digoxigenin (DIG)-labeled cDNA probes were prepared for the full-length cDNA

clone of *Chrysanthemum stunt viroid* (CSVd). These probes detected CSVd specifically in dot blot hybridization. The sensitivity of detection of CSVd in 2 M LiCl solution of nucleic acids corresponding to 17 μg of fresh weight, in dot blot hybridization with DIG-labeled probes, was 100 times greater than that of the return PAGE procedure (Li et al. 1997). Likewise, PSTVd was also detected by employing dot blot hybridization procedure using DIG-labeled probes. This assay protocol was as sensitive as the tests using radioactive probes. PSTVd was detected in composite samples taken for mass indexing program, indicating the applicability of the method for routine indexing (Welnicki and Hiruki 1992). In another study, PSTVd was detected in seven potato cultivars employing DIG-labeled cDNA probes. With this technique PSTVd was detectable directly in the extracts of potato leaves and tubers (Nakahara et al. 1997). The results of dot blot hybridization procedure formed the basis for preparing a calendar for diagnosis of *Apple scar skin viroid* (ASSVd) and *Dapple apple viroid* (DAVd) (Paduch-Cichal et al. 1998).

Peach latent mosaic viroid (PLMVd) was found to be widely distributed in peach germplasm from Europe, Asia, North America and South America. The cherry isolate of PLMVd has 337 nucleotides (nts) in length and is 91–92 % homologous to PLMVd isolates from peach. Dot blot and Northern blot hybridization analyses of total nucleic acids extracted from plum, apricot, cherry and peach samples resulted in hybridization signal only from infected stone fruit samples. PLMVd from peach had an electrophoretic mobility similar to the viroid from plum (Fig. 2.23; Hadidi et al. 1997; Appendix 26). In a later investigation, dot blot hybridization method DIG-labeled viroid-specific probes was employed for the detection of PLMVd and hop stunt viroid (HSVd) infection in peach trees. High incidence of PLMVd (77%) and HSVd (69%) as single or mixed infection in samples from orchards in Czech Republic was indicated by the diagnostic tests (Hassan and Ryšánek 2004).

Citrus-infecting viroids, *Citrus exocortis viroid* (CEVd), *Citrus bent leaf viroid* (CBLVd), *Hop stunt viroid* (HSVd), *Citrus viroid III* (CVd-III) and *Citrus viroid IV* (CVd-IV) were detected by Northern blot hybridization technique, using viroid-specific probes in inoculated Etrog citron (Barbosa et al. 2005). In a later investigation, a total of 123 samples were collected from commercial citrus groves and nurseries. The presence of viruses and viroids was tested by dot blot hybridization. Eleven of 32 Washington Navel and Naveline oranges were found to be infected by *Citrus tristeza virus* (CTV), whereas four Clementine trees were positive for *Cirus infectious variegation virus* (CVV). All these virus-infected trees were found to be coinfected by HSVd and 50% of them were infected also by CEVd. Although widely spread, viroids did not induce conspicuous symptoms in the field, because of the use of sour orange as rootstock which is tolerant to viroids (Barbossa et al. 2007).

Coconut cadang-cadang viroid (CCCVd) was considered to be apparently confined to the Philippines. Occurrence of a number of coconut palm diseases of unknown etiology has been reported from other countries. CCCVd-related sequences are defined as RNAs in the size range expected for CCCVd that hybridize with probes representing part or all the minimal 246 nt CCCVd sequence, but for which sequence information is not available. A dot blot assay was performed to test the

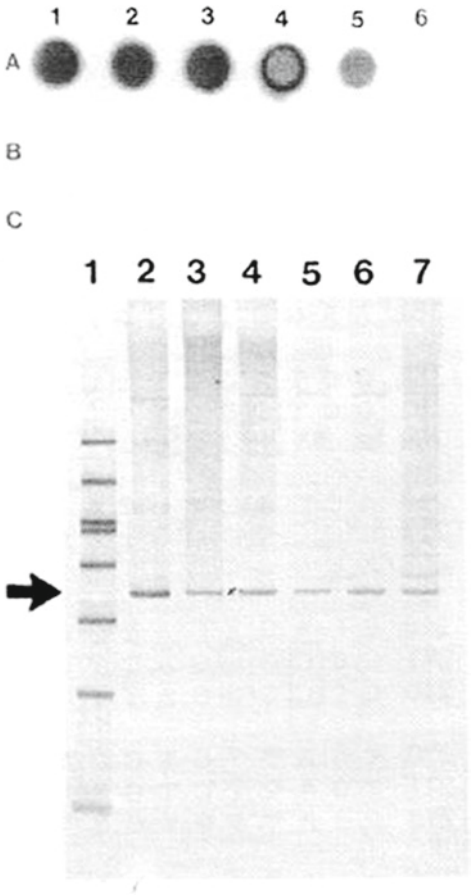

Fig. 2.23 Detection of *Peach latent mosaic viroid* (PLMVd) in plant by dot blot hybridization and northern blot hybridization analyses using cRNA probes. *Top*: dot blot hybridization analysis; A, viroid-infected plum from Romania (*lane 1*), apricot from Japan (*lane 3*) and Nepal (*lane 4*); healthy plum and apricot controls (*lanes 2 and 5* respectively). *Bottom*: northern blot hybridization analysis; viroid-infected plum from Romania, apricot from Japan (*lane 3*) Nepal (*lane 4*), cherry from Romania (*lane 6*) and peach from Italy (*lane 8*); and non-infected plum, apricot and cherry plants (*lanes 2*, *5* and *7* respectively). Note the hybridized upper bands with longer-than-unit-length and double stranded PLMVd and the hybridized lower bands with unit-length PLMVd (Courtesy of Hadidi et al. 1997; The American Phytopathological Society, MN, USA)

presence of CCCVd-related RNA. Positive signals were obtained for two of five palms with cadang-cadang, two of five palms with leaf scorch disease (LSD), three of five palms with coconut rapid decline (CRD) disease and two of eight palms with premature decline (PD) disease. The results of dot blot hybridization did not indicate a consistent association between a positive signal and any of the positive disease syndrome (Vadamalai et al. 2009; Appendix 27).

Detection of viroids or viruses by nucleic acid hybridization or RT-PCR assays require skilled technical expertise for nucleic acid isolation which is time consuming and expensive. Tissue-printing hybridization (TPH) or imprint hybridization has been demonstrated to be an alternative and faster detection procedure for viroids. Further, this method is simple, requiring minimal sample preparation. The sap from cells that have been mechanically disrupted or opened up is printed directly on the nitrocellulose membrane which has high binding affinity for nucleic acids. A TPH protocol was developed for the detection of *Apple scar skin viroid* (ASSVd) in apple plants graft-inoculated with infected scion tissues (Podleckis et al. 1993).

The TPH assay was applied to test the samples collected from stone fruit trees for the presence of *Peach latent mosaic viroid* (PLMVd) and *Hop stunt viroid* (HSVd). Of the 336 samples tested, 30 samples were infected by PLMVd or HSVd (Table 2.11). In addition, the presence of the viroids in infected plants was confirmed by RT-PCR assay also (Michelutti et al. 2005). Field surveys were undertaken to assess the incidence of viroid diseases affecting *Prunus* species in Turkey. Tissue-printing hybridization (TPH) technique was applied to test 352 stone fruit samples of which 88 were found to be infected by viroids. Of the infected ones, 61 peaches and apricots showed infection by *Peach latent mosaic viroid* (PLMVd). The remaining 27 samples of apricot, peach or plum were infected by *Hop stunt viroid* (HSVd). Mixed infections by PLMVd and HSVd were detected in two samples. The TPH test provided a relatively clear picture of the sanitary status of stone fruit crops in this region (Gümüs et al. 2007).

A reliable TPH format was applied for the detection of *Apple scar skin viroid* (ASSVd), *Apple dimple fruit viroid* (ADFVd) and *Pear blister canker viroid* (PBCVd) in field grown plants. Dot blot hybridization (DBH) and TPH were employed using probes with non-radioactive labels to detect ADFVd, ASSVd and PBCVd in experimentally inoculated apple plants. All three viroids were detected effectively by both methods. Each probe hybridized strongly and exclusively with the complementary viroid RNA. The best TPH signals were always obtained from petioles collected from the apical part of the shoots, suggesting that the sensitivity of TPH could be improved by using petioles of young leaves. No nonspecific or cross-hybridization of each probe with other nontarget pome fruit viroids was

Table 2.11 Detection of viroids by tissue-printing hybridization technique (Michelutti et al. 2005)

Host plant species	Number of samples tested	Viroids detected	
		HSVd	PLMVd
Peach and nectarine	116	0	28
Sweet and sour cherry	84	0	0
Plum	54	0	0
Apricot	44	2	0
Other cherry species	38	0	0
Total	336	2	28

observed. TPH assay has been shown to be a useful assay for specific detection and discrimination of viroids infecting pome fruits (Lolic et al. 2007).

Citrus exocortis viroid (CEVd), *Chrysanthemum stunt viroid* (CSVd), *Hop stunt viroid* (HSVd) and *Avocado sunblotch viroid* (ASBVd) were detected by applying the tissue-printing hybridization (TPH) technique in their respective natural host plant species. This procedure, in addition to providing results rapidly, can also be useful to determine the sites of viroid accumulation (Romero-Durban et al. 1995). *Potato spindle tuber viroid* (PSTVd) was detected in stem rachis of two tomato cultivars by employing a ^{35}S-labeled PSTVd-specific RNA probe. PSTVd reached a concentration that was detectable by tissue-printing hybridization (TPH) protocol earlier in the susceptible cv. Rutgers than in the tolerant cv. Gold Kugel which remained asymptomatic. The distribution and localization of PSTVd in different tissues could be studied by TPH format (Stack-Lorenzen et al. 1997). In the case of citrus viroids, the sensitivity and reliability of TPH procedure could be improved by first inoculating *Citrus medica* (Etrog citron) and employing DIG-labeled RNA or DNA probes (Palacio-Bielsa et al. 1999).

Ribonuclease protection assay (RPA) is a liquid hybridization-based RNA diagnostic protocol that uses enzymes resistant to inhibitors. This is a relatively robust, specific and sensitive test useful for detecting and quantifying specific RNAs in a complex mixtures of total RNA, for detecting variations in the nucleotide sequences of RNA populations and for assessing the genetic heterogeneity in population of RNA viruses. The RPA test was applied for the detection of *Coconut cadang-cadang viroid* (CCCVd) sequences in infected coconut (*Cocos nucifera*) and African oil palm (*Elaeis guineensis*). An RNA probe complementary to full-length $CCCVd_{246}$ was employed terminating at nucleotide 65 in the upper conserved region and linked to a non-viroid 5′ sequence which functioned as an internal control for the ribonuclease activity. Extracts from CCCVd-infected palms protected three major fragments of approximately 250, 125 and 50 nt and a variable number of minor fragments. Extracts of healthy coconut palms, *Potato spindle tuber viroid* (PSTVd)-infected tomato and transfer RNA did not protect the probe. RPA test detected CCCVd sequences in 13 of 18 oil palms surveyed in commercial plantations in Malaysia. Variations in signal intensity were observed between positive samples and the intensity was generally lower than in coconut palms infected with CCCVd. The results of RPA showed that sequences closely related to CCCVd were not confined to the Philippines, in contrast to the conclusions of earlier investigations, since CCCVd-related sequences could be detected in infected coconuts in Malaysia and Sri Lanka (Vadamalai et al. 2009).

2.2.5.2 RT-PCR Assay

As in the case of detection of viruses, RT-PCR assay has been demonstrated to be sensitive, specific, rapid and reliable for the detection, identification and differentiation of viroids infecting various plant species. Most of the viroids have been successfully detected by RT-PCR assay using appropriate primers (Narayanasamy 2001, 2008).

The RT-PCR assay was shown to be efficient and reliable for the detection of *Peach latent mosaic viroid* (PLMVd) by using DNA primers for cDNA synthesis and PCR amplification of a full-length viroid DNA product from extracts of PLMVd-infected peach tissues. Amplified viroid cDNA hybridized to ^{32}P-labeled PLMVd cRNA probe. This viroid was detected in the extracts of different tissues such as leaf, bark and fruits. When the peach germplasm entries were tested, the worldwide distribution of PLMVd could be inferred, as the entries from different geographical locations were positive for PLMVd (Shamloul et al. 1995). RT-PCR assay was applied to detect PLMV) in peach germplasm from Europe, Asia and the Americas, the average percentage of infection being 55%. All the 52 apricot samples from Italy tested negative for PLMVd. In contrast, samples of apricot from Japan and Nepal, plum and cherry from Romania and Italy were PLMVd-positive. During the test duration of 3 years, viroid-infected cherry, plum and apricot plants tested positive for PLMVd with RT-PCR assay (Table 2.12). The results of RT-PCR assay were corroborated by the results of molecular hybridization tests performed along with RT-PCR assay (Hadidi et al. 1997).

Stone fruit trees are frequently affected by *Peach latent mosaic viroid* (PLMVd) and *Hop stunt viroid* (HSVd), both belonging to the family *Pospiviroidae*. PLMVd is considered to be a devasting pathogen of peach. Hence, it is listed as a quarantine pathogen by European Plant Protection Organization (EPPO). The viroid strain infecting apple shared similarities in sequences with PLMVd as indicated by RT-PCR and dot blot hybridization analysis of the amplified product with PLMVd

Table 2.12 Detection of *Peach latent mosaic viroid* (PLMVd) in samples from stone fruits by RT-PCR assay (Hadidi et al. 1997)

Country of origin	Stone fruit	No. of infected plants/no. of plants tested	Percentage of infection
Italy	Peach	29/64	45
	Apricot	0/52	0
	Peach	7/13	54
	Cherry	2/3	66
Fr. Yugoslavia	Peach	3/8	37
	Plum	1/3	33
Romania	Peach	5/8	60
	Plum	1/3	33
	Cherry	1/2	50
Nepal	Peach	3/6	50
	Apricot	1/1	100
Pakistan	Peach	1/2	50
Japan	Peach	2/2	100
	Apricot	1/1	100
Brazil	Peach	2/7	29
USA	Peach	3/7	71
France	Apricot	1/1	100

cRNA probe (El-Dougdoug 1998). An optimized RT-PCR protocol was developed for detecting PLMVd and HSVd that is suitable for certification system that is in operation in the Czech Republic. The primers selected were specific and sensitive in detecting the expected PCR products in samples containing nucleic acid equivalent to 10 ng of leaf tissues. The primer pair (R + F1) was more sensitive than the primer pairs employed earlier. This primer pair detected PLMVd in several peach cultivars of different ages (5–12 years). HSVd was also detected in leaves, petioles and bark tissues obtained from field-grown cv. NJC 103 plants. The time of collecting the samples appeared to be a critical factor in the successful detection of HSVd. Samples collected in summer and late autumn were suitable for testing the presence of HSVd (Hassan et al. 2004). In another study, RT-PCR assay was employed for the detection of PLMVd in peach and pear trees, HSVd in pear, peach and almond trees and *Pear blister canker viroid* (PBCVd) in pear trees. Mixed infections of pear with PBCVd + HSVd and PLMVd + HSVd were detected. The identity of the different viroids was established by comparing their nucleotide sequences with characterized isolates. Detection of viroids present in the crude extracts of leaves or bark tissues and also in total RNA preparations could be accomplished by employing the protocol developed in this study. This protocol has the applicability for routine indexing of plants and plant material in certification programs (Hassen et al. 2006; Hassani et al. 2006).

Citrus exocortis viroid (CEVd) was detected by employing DIG-labeled probe prepared by PCR. Total nucleic acids (TNAs) were extracted from citrus leaves and using CEVd-specific primers, specific sequences in TNA were amplified by RT-PCR assay. Presence of CEVd was detected in all infected citrus samples including two samples that were found negative by indexing on Etrog citron Arizona 861-S1 as indicator plants. The bioindexing and electrophoresis method failed to differentiate viroid species in addition to the requirement of long time to provide the results (Saito et al. 1995). RT-PCR assay was effective in detecting CEVd and *Citrus cachexia viroid* (CaCaVd). A single-tube RT-PCR format was developed for the amplification of nucleic acids of these two viroids. Total nucleic acid (TNA) extracts from infected plants or viroidal template released by directly boiling small tissue pieces were used for performing RT-PCR assay. The TNA (0.15 ng) from infected plants spotted on nylon N^+ membrane and released in glycine-NaCl buffer was successfully used in single-tube amplification of these viroids. The amplification efficiency was enhanced by wetting nylon membrane in NaOH-EDTA solution prior to amplification (Turturo et al. 1998).

Occurrence of two viroid species *Hop stunt viroid* (HSVd) and *Hop latent viroid* (HLVd) has been observed in hop gardens in the Czech Republic. Molecular sampling of HSVd in grapevines in the environs of hop gardens was carried out. Specific RT-PCR primers were designed to unambiguously distinguish between HLVd and HSVd infections. These primers were used for detection and analysis of HSVd cDNAs from individual samples. Primers were assigned for HSVd detection with respect to HLVd/HSVd homology. At least ten sequences of HSVd have been described in the subviral RNA database and they form the grapevine-specific (HSVdg) group. The infectivity of the HSVdg sample was high, as 80% of inoculated plants were clearly infected and RT-PCR signal was detected at 3 weeks post-inoculation. The preliminary

screening of hop materials from selected hop gardens revealed no HSVd infection as determined by RT-PCR assay (Matoušek et al. 2003).

Hop stunt viroid (HSVd), *Hop latent viroid* (HLVd) and *Apple fruit crinkle viroid* (AFCVd) infect commercial hop (*Humulus lupulus*) plants. Samples from 12 known and several unidentified cultivars were tested for HSVd, HLVd and AFCVd by RT-PCR assay. A one-tube duplex RT-PCR was developed for detection of HSVd and HLVd using viroid-specific primers and a separate RT-PCR assay was performed for detection of AFCVd using primers that react with a wide range of apscaviroids. HSVd was detected in 10 of 33 hop gardens in 8 of 12 cultivars grown. HLVd was detected in all three hop-growing regions and it was widely distributed in Washington. AFCVd was not detected in any of the hop samples collected. However, the absence of AFCVd remains to be confirmed by testing more number of samples (Eastwell and Nelson 2007).

Apple scar skin viroid (ASSVd) was found to be one of the most destructive diseases of apple in Korean orchards. The RNA molecules were extracted from apples bearing dapple apple symptoms using CF-11 RNA extraction procedure. The cDNAs were synthesized from the purified RNAs employing RT-PCR assay. The PCR products were cloned and sequenced. Nucleotide sequences of viroid molecules isolated from six apple cultivars were similar to that of Korean strain ASSVd-K. This strain was detected by RT-PCR assay in samples from several locations in Gyeonbuk Province in Korea. As the viroid can spread through asexual propagation of apple, it is emphasized that adequate care has to be taken to ensure freedom of plant materials from ASSVd and other viroids. (Kwon et al. 2002). Apple plants with mixed infection of sequence variants of ASSVd were located in Himachal Pradesh State in India. Forty five fruit and bark samples from symptomatic apple plants were tested by RT-PCR assay. Amplicons of the expected size ~330 bp were detected from five samples of apple cv. Starking Delicious plants. Ten randomly selected cDNA clones were sequenced from each sample and identified as ASSVd in a BLAST search. Seven of these clones were found to be new sequence variants of ASSVd (Walia et al. 2009).

A modified RNA extraction procedure was developed for extracting five viroids infecting grapevines – *Hop stunt viroid* (HSVd), *Citrus exocortis viroid* (CEVd), *Grapevine yellow speckle viroid* (GYSVd-2) and *Australian grapevine viroid* (AGVd) – by employing RT-PCR assay. The DNA primers were carefully selected for optimization of viroids in low copy number. This procedure was very effective for the detection of grapevine viroids in vines regenerated by shoot apical meristem culture (SAMC) and fragmented shoot apex culture (FASC) (Wah and Symons 1997). In Brazil, isolates of CEVd and HSVd were detected in both citrus and grapevines. Total RNA from leaves of grapevine was used as template for RT-PCR amplification with specific primers for the five viroids. The amplified products were separated by agarose gel electrophoresis and DNA fragments of the expected sizes were eluted, cloned and sequenced. Grapevine samples were found to be doubly infected with CEVd and HSVd. Though yellow speckle symptoms were observed in grapevine plants, neither the grapevine viroids, GYSVd-1 and GYSVd-2 usually associated with this type of symptoms, nor AGVd remaining

asymptomatic could be detected. The presence of grapevine and citrus viroids was attributed to the introduction of plant materials from other countries. However, a clean stock program offering viroid-free propagation materials is followed only for citrus viroids and such a program is yet to be developed in Brazil for grapevine viroid disease management (Eiras et al. 2006).

A viroid-like RNA was detected in the eggplant (*Solanum melongena*) in the eastern Spain. Denaturing PAGE analysis revealed two bands with the mobilities expected for circular and linear forms and treatment with RNase, but not with DNase, resulted in their disappearance. The RT-PCR assay was performed with a pair of adjacent primers of opposite polarity PIII and PIV. The existence in infected tissues of plus and minus *Eggplant latent viroid* (ELVd) circular forms was inferred from Northern hybridization and from RT-PCR amplification of the complete viroid sequence irrespective of which primer of each pair was used in the RT-reaction (Fadda et al. 2003).

Coconut cadang-cadang viroid (CCCVd) causing the cadang-cadang disease in coconut in Malaysia and *Coconut tinangaja viroid* (CTiVd) in Guam have been responsible for appreciable losses. Two sets of forward and reverse primers were designed from the $CCCVd_{246}$ sequence for the RT-PCR amplification of CCCVd related molecules: GVR4 from nucleotides 191–172, GV4 from nucleotides 192–209, GVR5 from nucleotides 71–41 and GV5 from nucleotides 76–105. Both CCCVd and CTiVd were detectable by ethidium bromide staining after one round of RT-PCR amplification of total nucleic acid extracts from coconut palm. CCCVd was isolated from infected oil palms in the Philippines at levels similar to that in coconut palms, indicating the infection of other palms by CCCVd (Vadamalai et al. 2009). In a later study, RT-PCR assay was found to be less sensitive than the ribonuclease protection assay (RPA) in detecting CCCVd sequences. The CCCVd sequences could be detected by RPA in oil palms which were negative for RT-PCR assay. Probably RT-PCR assay was affected to a greater level by factors such as enzyme inhibitors and variability of both target concentration and nucleotide sequences (Vadamalai et al. 2006, 2009; Appendix 28).

Tissue-printing hybridization (TPH) procedure involving immobilization of viroid nucleic acids onto filter paper, followed by RT-PCR amplification using specific primers was adopted for the detection of *Potato spindle tuber viroid* (PSTVd) in primarily and secondarily infected potato plants, particularly in in vitro plants and tubers. The print-PCR format simplified the sample processing, in addition to providing the possibility of testing large number of potato plants. The dot-PCR based on dotting plant sap on the filter paper can be used for testing in vitro potato plantlets and tuber tissues for indexing. Reliable results can be obtained in samples consisting of one infected plant and nine healthy plants for detecting the secondary infection. The dotted or printed filter paper squares can be stored for up to 2 weeks in Triton X-100 at 4°C under dry conditions (Weidemann and Buchta 1998).

Polymerase chain reaction (PCR), preceded by reverse-transcription (RT), has been employed for the detection of *Potato spindle tuber viroid* (PSTVd) and other viroids. Although the PCR-based assays offer several advantages, they have certain practical limitations for routine diagnostic applications, since they tend to be labor-intensive,

in addition to functioning as 'open tube' with the risk of carry-over contamination resulting in false positive results. On the other hand, real-time PCR assay systems such as TaqMan combine a probe (labeled at each end with a reporter and a quencher dye and which is designed to anneal to sequence internal to the PCR primers) with the 5′ exonuclease activity of *Taq* polymerase. When the probe is intact, fluorescence emitted by the reporter is absorbed by the quencher (fluorescent resonance energy transfer). During amplification the probe is cleaved separating the dyes, resulting in the release of fluorescence related to the amount of product amplified. The increase in reporter fluorescence is monitored in real-time during amplification using a combined thermal cycler and fluorescence reader like the ABI PRISM™ 7700. The TaqMan assays are 'closed tubes' eliminating the risk of cross-contamination between samples and no post-PCR manipulation like gel electrophoresis is required. Thus, the problems associated with conventional PCR-based assays may be avoided by using real-time PCR assay.

Potato spindle tuber viroid (PSTVd), a pathogen of quarantine importance, has been reported to cause serious losses in many solanaceous crops. Under the European Union Plant Health Directive, EU countries should have the ability to detect and identify accurately and rapidly the introduction of harmful organisms or plant products. A one-tube real-time RT-PCR assay based on TaqMan™ chemistry was developed for the efficient and reliable detection of PSTVd. A total of 13 isolates of PSTVd from a broad range of geographical locations was tested using TaqMan assay. In addition, three other pospiviroids, *Tomato chlorotic dwarf viroid* (TCDVd), *Citrus exocortis viroid* (CEVd) and *Chrysanthemum stunt viroid* (CSVd) were also examined. The presence of TCDVd could be detected, while the other two viroids gave negative results. The real-time RT-PCR format developed in this investigation was 1,000 times more sensitive, in detecting PSTVd than hybridization test. The real time RT-PCR format was found to be highly sensitive and well-suited for large scale application, because this format is amenable for automation using laboratory liquid handling robotics and the results obtained, could be easily interpreted (Boonham et al. 2004).

PCR-based techniques have been demonstrated to be rapid, sensitive and specific in detecting viroids, causing different crop diseases. However, it is essential to prevent false-positive reactions due to cross-contamination between samples by including negative control, whereas positive controls are useful to monitor the efficiency of nucleic acid extraction and performance of the reactions. For the detection of *Potato spindle tuber viroid* (PSTVd), an exogenous internal standard i.e., in vitro RNA transcribed from a plasmid containing a modified PSTVd (isolate Howell) was used. To this exogenous sequence, a specific probe was designed with a fluorescent label different from that of the PSTVd-specific probe. By utilizing the same primers as the target organism PSTVd, this internal standard provides a tool to measure the performance of the specific reaction. In addition, by using another fluorescent probe, this standard can be readily discriminated from the target organism. In order to obtain reliable PCR results, it is essential to monitor false-positive and false-negative controls like cytochrome oxidase (COX) primers (probes) to monitor the efficiency of the nucleic acid extraction and an internal control to monitor inhibition of the PCR targets (Kox et al. 2005).

Biolistic method has been employed for inoculation of plants with various viral genomes either in the form of RNA or cDNA (Kekarainen et al. 2002; Merits et al. 2002). Attempt was made, taking advantage of the small size of the viroid genome and infectivity of its cDNA and transcript RNA, using a Helios Gene Gun device. The biolistic method allows for efficient inoculation with monomeric linear viroid constructs derived from the wild type. In addition, biolistic transfer of native RNA significantly enhanced the pathogenic potential of *Potato spindle tuber viroid* (PSTVd) on tomato. With the optimized conditions for biolistic inoculations, lower limits of infection, as detected 3 weeks post-inoculation, were 50 ng and 200 pg per plant for efficient inoculation with monomeric cDNA fragments and native viroid RNA respectively. The biolistic inoculation of plants with *Hop stunt viroid* (HSVd) and *Hop latent viroid* (HLVd) was also successfully performed. The presence of the viroids was detected by employing RT-PCR assay in their respective host plants. Using the biolistic approach, it is possible to infect specific tissues, such as hairy roots culture that cannot be inoculated by conventional methods (Matoušek et al. 2004).

Diagnostic methods that allow the simultaneous detection and/or identification of multiple pathogens are preferable for routine diagnosis of microbial pathogens affecting a crop plant species, because they can save time, labor and cost of testing. In addition, inclusion of specific primers to host mRNA as internal controls in the RT-PCR to ensure safe interpretation of negative results, is desirable for the detection of viroids affecting stone fruit trees. A total of 645 samples from collections of different cultivars and orchards in the Czech Republic was tested by employing RT-PCR assay. This assay efficiently amplified all targets under the same RT-PCR conditions, using a cocktail containing 0.7 μM of *Hop stunt viroid* (HSVd)-specific primers plus 0.25 μM of the *nad5* primers as the amplification control. The viroid-specific amplification products plus the host mRNA in peach trees naturally infected by *Peach latent mosaic viroid* (PLMVd) and/or HSVd were detected. Three amplified products of expected sizes, 339-bp for PLMVd, 300-bp for HSVd and 181-bp for host mRNA were detected (Fig. 2.24). The simultaneous RT-PCR

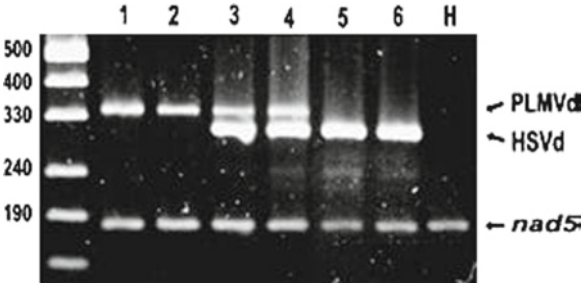

Fig. 2.24 Detection of *Plum latent mosaic viroid* (PLMVd) and *Hop stunt viroid* (HSVd) simultaneously in field peach trees by one-step multiplex RT-PCR assay. *Lanes 1* and *2*, PLMVd-infected samples; *Lanes 3* and *4*, mixed infection of samples with PLMVd and HSVd; *Lanes 5* and *6*, HSVd-infected samples; H, healthy sample (Courtesy of Hassan et al. 2009 and with kind permission of Springer Science and Business Media, Heidelberg, Germany)

assay was shown to be highly applicable for diagnosis of stone fruit viroids. Further, the co-amplification of host mRNA as an internal control increased the potential of the RT-PCR protocol as a reliable tool to avoid false negative results (Hassan et al. 2009).

The effectiveness of PCR-based procedures has been assessed for the detection of viroids infecting citrus. Nucleic acids were phenol-extracted and purified by CF-11 cellulose chromatography or extracted with SDS-potassium acetate and analyzed by sequential PAGE (sPAGE), RT-PCR or multiplex RT-PCR procedures. The simplex and multiplex RT-PCR assays were more sensitive than sPAGE method, partially overcoming the problem due to low viroid concentrations in winter and spring seasons. Multiplex RT-PCR format, using two sets of primers, was as sensitive as simplex RT-PCR format, facilitating simultaneous detection of *Citrus exocortis viroid* (CEVd), *Citrus viroid IIa* (CVd-IIa) and *Citrus viroid IIb* (CVd-IIb) in mixed infections. However, the amplified DNA yield was usually lower compared with simplex RT-PCR assay. Greenhouse-grown plants gave better DNA yield than plants from the fields. Differential seasonal influence on the viroid detection was noted. CEVd could be detected in all seasons, whereas CVd-II and CVd-III could be detected only in specific seasons (Tessitori et al. 1996).

Citrus plants are infected by five viroid species and a large number of sequence variants in China. A simple and sensitive one-step multiplex RT-PCR procedure was developed for simultaneous detection and differentiation of *Citrus exocortis viroid* (CEVd), *Citrus bent leaf viroid* (CBLVd), *Hop stunt viroid* (HSVd), *Citrus viroid III* (CVd-III) and *Citrus viroid IV* (CVd-IV). The *ubiquitin* gene which has a higher expression stability and expression level than the most house-keeping genes, was employed as an internal control. A micro and rapid procedure for extracting total nucleic acids from healthy and infected citrus plants was developed for use in this investigation. The six expected amplified products were detected, when the assay with the mixture of five single infection extracts was performed. Most of the infected citrus plants harbored more than one viroid species and a few plants were infected up to four citrus viroids. The sensitivity of uniplex and multiplex RT-PCR formats was at the same level. Interference between primer pairs did not occur in naturally infected samples from field-grown citrus plants. The expected PCR amplicons were detected up to a dilution of 10^{-4} of the original total nucleic acid extracts. Samples from representative 40 viroid-infected citrus trees comprising of sweet orange, lemon, mandarin and mandarin hybrid were tested by one-step multiplex RT-PCR assay. The results obtained from multiplex RT-PCR assay corresponded exactly to the results from sPAGE and single RT-PCR assays for amplifying full-length sequence of each viroid. The assay revealed that 25 of 40 samples from cultivars imported from other countries were RT-PCR positive. The results clearly indicated the need for monitoring the health status of mother trees periodically, since the viroids from a single mother tree may be transmitted to numerous asexually propagated progenies. This rapid and specific one-step multiplex RT-PCR procedure has the potential to effectively check the spread of viroids, as a reliable diagnostic tool (Wang et al. 2009; Appendix 29).

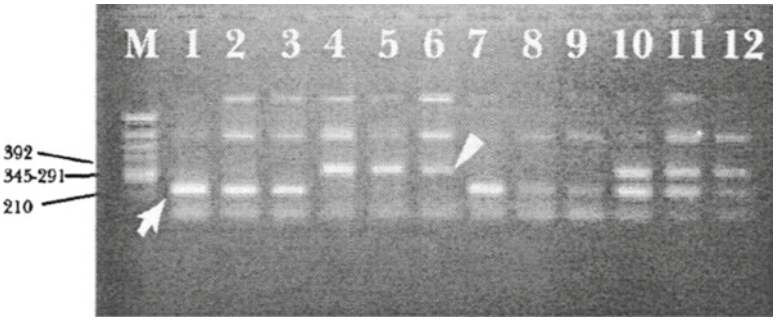

Fig. 2.25 Detection of *Chrysanthemum stunt viroid* (CSVd) and *Chrysanthemum chlorotic mottle viroid* (CChMVd) by multiplex RT-PCR assay using direct templates from infected or uninfected plants. M, DNA size (bp) markers; *Lanes 1–3*, low-titer CSVd-infected plants; *Lanes 7–9*, high-titer-CChMVd-infected plants; *Lanes 4–6*, high-titer-CSVd- and low-titer-CChMVd-infected plants; *arrow head* indicates CSVd band and arrow indicates CChMVd bands; *Lanes 10–12*, high-titer-CSVd and CChMVd-infected plants; *Lanes 1*, *4*, *7 and 10*, direct templates from shoot tips; *Lanes 2*, *5*, *8 and 11*, direct templates from stems; *Lanes 3*, *6*, *9 and 12*, direct templates from leaves (Courtesy of Hosokawa et al. 2007; Japan Society for Horticultural Science, Kyoto, Japan)

Chrysanthemum (*Dendranthema grandiflorum*) crops are infected by *Chrysanthemum stunt viroid* (CSVd) and *Chrysanthemum chlorotic mottle viroid* (CChMVd), causing considerable economic losses. These viroids were detected by a multiplex RT-PCR assay. The antisense hexamer AAAGGA (5′–3′) was designed. Using the CSVd- and CChMVd-cDNA templates which were transcribed simultaneously by the hexamer, the multiplex PCR assay could detect both viroids from doubly-infected plants without any nonspecific amplification. The sequences of 354- and 208-bp bands on agarose gel amplified by multiplex RT-PCR agreed with the CSVd and CChMVd sequences respectively. The detection sensitivity of multiplex RT-PCR format was almost the same as that of standard RT-PCR assay (Fig. 2.25). Furthermore, multiplex RT-PCR successfully detected both CSVd and CChMVd in the sap containing templates obtained directly by inserting a syringe needle into the stem, leaf and shoot tips of infected plants. The multiplex RT-PCR format has the potential to appreciably reduce the diagnostic cost, labor and time by reducing the number of assays needed for detection of both viroids (Hosokawa et al. 2007).

2.3 Detection of Viral Pathogens in Seeds and Planting Materials

Viruses are disseminated by different methods to short and long distances through propagative materials. Infected seeds and asexually propagated planting materials such as tubers, bulbs, suckers, setts or cuttings or budwood form the primary sources of infection, as they either introduce the new pathogens into areas where

they are nonexistent or provide additional inoculum facilitating rapid spread of the disease. Further, spread of viruses to subsequent generations, occurs through the multiplication of the infected seeds and planting materials which may or may not exhibit symptoms of infection. Hence, use of virus-free seeds and planting materials lays a sound foundation for an effective disease management system leading to profitable crop production. Certification of seeds and planting materials to ensure supply of clean seeds and planting material is being followed in several countries to lessen the incidence and subsequent spread of the virus diseases. Various methods have been developed for detecting the presence of viruses in the seeds and planting materials rapidly and reliably. The usefulness and applicability of different diagnostic procedures available are discussed hereunder.

2.3.1 Detection of Viruses in Seeds

Many seedborne viruses have been recognized as pathogens of quarantine importance. The use of virus-free seeds as a model of control is emphasized for virus diseases caused by *Barley stripe mosaic virus*, *Lettuce mosaic virus*, *Bean common mosaic virus*, *Cowpea mosaic virus* and *Soybean mosaic virus* (Allen 1983). However, only a few programs are being continued for routine testing of virus infection of seeds (Lange and Heide 1986).

2.3.1.1 Biological Methods

Viruses, in most cases, do not cause distinct symptoms on the seeds as in the case of fungal and bacterial pathogens. However, viruses cause general reduction in seed size and poor filling of grains resulting in significant lowering of 1,000-grain weight which is one of the yield attributes considered for determination of the yield potential of cultivars or breeding lines. As it is not possible to isolate viral pathogens in any nutrient medium, the extracts of seeds to be tested are inoculated onto the healthy plants of the same species or a diagnostic plant species. These diagnostic/indicator plants develop characteristic symptoms more rapidly than the original host plant species which take long time as in the case of woody plants. Growing-on test, seed indexing, is adopted for the seed-transmitted viruses like *Lettuce mosaic virus* (LMV). Seedlots to be tested are sown in pots in insect-proof greenhouses. The plants growing from these seeds are carefully examined for the development of symptoms induced by the virus concerned. The adverse effect of virus infection on seed germination can also be determined (Grogan 1983). In the case of *Turnip yellow mosaic virus* (TYMV) and *Tobacco mosaic virus* (TMV) infecting *Arabidopsis thaliana*, seeds collected from infected plants were tested by grow-out test and protein A sandwich (PAS)-ELISA procedure. Grow-out tests were carried out by sowing seeds from infected plants in disposable plastic pots in insect-proof greenhouse. Estimation of seed transmission TYMV by grow-out tests was more

accurate than PAS-ELISA tests. TMV was not transmissible through the seeds of *A. thaliana* (de Assis Filho and Sherwood 2000). The requirement of large greenhouse space and labor are the limitations of biological methods available for detection of plant viruses. Hence, the lower levels of sensitivity and long time to obtain the results from biological methods necessitated the development of more sensitive, reliable and rapid techniques for the detection of viruses in the seeds.

2.3.1.2 Immunoassays

As the biological methods are laborious, labor-intensive and time-consuming, more rapid and sensitive diagnostic methods to obtain reliable results have to be developed. Various immunoassays have been shown to meet this requirement for detecting viruses in the seeds of several crops. The immunodiagnostic techniques initially developed for the detection of viral pathogens were later adapted for the detection of seedborne fungal and bacterial pathogens. Extracts of seeds for detection of viruses may be prepared by grinding the seed samples and suspending in a suitable buffer, followed by centrifugation to sediment the heavier fragments. The supernatant containing the viruses is tested for the presence of the target virus(es). Alternatively, the test seeds are germinated and the extracts are prepared from the seedlings alone or both seedlings and seed remnants using appropriate buffer. The latter procedure allows virus multiplication during seed germination to reach higher concentrations, thus improving the sensitivity of the immunoassay.

The immunoassays have to be sensitive enough to satisfy the standards prescribed for each seed-transmitted virus. For example, presence of even a single lettuce seed infected by *Lettuce mosaic virus* (LMV) in a lot of 5,000 seeds, is not allowed in the United Kingdom (Maude 1996). The standards are determined based on the potential of the virus concerned for rapid spread and the economic importance of the crop and the extent of damage that can be caused, if infected seeds are not eliminated. Hence, the diagnostic technique should be able to detect the presence of infected seeds in seedlots precisely and reliably to facilitate the rejection/acceptance of seed consignments.

Enyzme-Linked Immunosorbent Assay

Immunoassays based on the formation of precipitin lines such as Ouchterlony double diffusion test for the detection of *Pea seedborne mosaic virus* (PSbMV) (Zimmer 1979) and radial immunodiffusion test for the detection of *Barley stripe mosaic virus* (BSMV) (Slack and Shepherd 1975) in seeds were successfully employed. But these assays require large amounts of antiserum and long time to provide the results. The tests are less frequently used now and immunoassays involving the use of labeled antibodies are currently employed for the detection of viruses in the seeds of various crops. Enzyme-linked immunosorbent assay (ELISA) and its variants are most widely applied for the detection of viruses in

seeds. The protocols applied for detection of viruses present in whole plants may be employed for detection of viruses in the seed extracts also, making some modifications, if necessary.

Squash mosaic virus (SMV) was detected in extracts from seed coats, papery layers and distal halves of embryos from individual cucurbit seeds (Nolan and Campbell 1984). Transmission of *Lettuce mosaic virus* (LMV) in lettuce seeds has been studied in detail for developing an effective disease management system based on the use of virus-free seeds. The standard double antibody sandwich (DAS)-ELISA format was effective in detecting one LMV-infected seed in a lot of 7,000 healthy seeds. In a later investigation, ELISA test showed an improvement in sensitivity of LMV detection. The ELISA format was sensitive enough to satisfy the tolerance level of zero infected seed out of 30,000 seeds, a standard prescribed for LMV in lettuce seeds in the United States (Dinant and Lot 1992).

High Plains virus (HPV) is transmitted through sweet corn seeds. Seedlings emerging from 46,000 seeds were tested by ELISA test for the presence of HPV. Three sweet corn plants tested positive for the ELISA test (Forster et al. 2001). The sensitivity of ELISA was shown to be equal to the reverse transcription-polymerase chain reaction (RT-PCR) in the case of *Cowpea aphidborne mosaic virus* (CAMV) transmitted through the seeds of peanut. ELISA test was suitable for testing larger seed lots of peanut to assess the extent of seed infection by CAMV (Gillaspie Jr. et al. 2001). Indirect ELISA format has been shown to be effective for the detection of *Papaya lethal yellowing virus* (PLYV) in seeds, soil and also in water from the rhizosphere of infected plants (Cammarco et al. 1988). Use of monoclonal antibodies (MAbs) may increase the sensitivity and specificity of virus detection. *Bean common mosaic virus* (BCMV) was detected in individual bean (*Phaseolus vulgaris*) seeds by indirect ELISA format using MAbs. Furthermore, the presence of BCMV in flour samples from seeds could be detected and it was possible to predict the incidence of BCMV in germplasm accessions (Klein et al. 1992). ELISA formats have been applied for the detection and identification of viruses present in the seeds of a wide range of crop plants and other plant species that may serve as sources of primary infection for crops planted in subsequent seasons (Table 2.13).

Cowpea aphidborne mosaic virus (CAMV) transmitted through the seeds of peanut appeared to become a serious problem, because of distribution of peanut germplasm to other countries. Presence of CAMV was tested in 1,553 peanut seeds by direct antigen coating (DAC)-ELISA format and eight seeds tested positive for this diagnostic test. Seeds were more difficult to process than leaf tissues for the immunoassays. Seed slices (1 mm) were found to be suitable for DAC-ELISA test. This format detected CAMV in about 1–2% of seeds which seemed to be greater than the percentage of seeds that normally could be expected to produce infected plants (approximately 0.15%) (Gillaspie et al. 2001). In another study, DAS-ELISA format along with IC-RT-PCR assay was evaluated for detecting *Peanut mottle virus* (PeMV) and *Peanut stripe virus* (PStV). Peanut seeds and leaves of seedling from infected seeds were tested. DAS-ELISA test produced positive results only with samples that had high virus concentration. The sensitivity of DAS-ELISA was less than IC-RT-PCR assay, when composite samples consisting of different proportions of infected and healthy tissues was tested (Gillaspie et al. 2007).

Table 2.13 Detection of viruses in seeds of various plant species by ELISA formats

Host plant species	Virus	References
Alfalfa	*Alfalfa mosaic virus*	Pesic and Hiruki (1986)
Barley	*Barley stripe mosaic virus*	Lister et al. (1981); Klein et al. (1992)
Bean	*Bean common mosaic virus*	Jafarpour et al. (1979)
Broad bean	*Broad bean stain virus*	Makkouk et al. (1987)
	Cucumber mosaic virus	Jones and Proudlove (1991)
Lettuce	*Lettuce mosaic virus*	Maury-Chovelon (1984); Dinant and Lot (1992)
Lupin	*Cucumber green mottle virus*	Faris-Mukhayyish and Makkouk (1983)
Melon	*Melon necrotic spot virus*	Avgelis and Barba (1986)
Papaya	*Papaya lethal yellowing virus*	Cammarco et al. (1988)
Pea	*Pea early browning virus*	Van Vuurde and Maat (1985)
	Pea seedborne mosaic virus	Hamilton and Nicholas (1978); Ding et al. (1992)
Peanut	*Indian peanut clump virus*	Dalfosse et al. (1999)
	Peanut mottle virus	Bharathan et al. (1984)
	Peanut stripe virus	Culver and Sherwood (1988)
Prune	*Prune dwarf virus*	Mink and Aichele (1984)
	Prune necrotic ringspot virus	
Soybean	*Soybean mosaic virus*	Maury et al. (1985)
Squash	*Squash mosaic virus*	Nolan and Campbell (1984)
Sweet corn	*High Plains virus*	Forster et al. (2001)
Tobacco	*Tobacco ringspot virus*	Lister (1978)

Tobacco rattle virus (TRV) and *Broad bean wilt virus* (BBWV) are transmitted through the seeds of sugar beets. Protein A coated (PAC)-ELISA procedure was applied for the detection of TRV and BBWV in racemes of 2-year beet plants, seedlings grown from the seeds of these plants and seedlings from commercially available sugar beet seeds. Of the 13 flowering plants, eight were diagnosed with TRV and two plants showed the presence of BBWV. The concentrations of TRV were greater (0.3–0.5 optical units) than that of BBWV. Artificial vegetative propagation of beet enhanced the transmission of TRV reaching high level of seed infection of 62%. The commercial seeds showed 15% infection by TRV in one batch, while the other batch of seeds had 20% infection by TRV (Dikova 2005).

Arabidopsis thaliana has been used to study several host–pathogen interactions, because of its short lifecycle and capacity to produce larger number of seeds/plant. Seed transmission of *Turnip yellow mosaic virus* (TYMV) and *Tobacco mosaic virus* (TMV) in *Arabidopsis thaliana* was investigated. *A. thaliana* has been used to study several host–pathogen interactions, because of its short lifecycle and capacity to produce larger number of seeds/plant. TYMV and TMV attain high concentrations in this host plant rapidly. Protein A sandwich (PAS)-ELISA format was employed to detect these viruses. Both TYMV and TMV were detected in seeds of *A. thaliana*, but only TYMV was transmitted to seedlings from infected seeds. The presence of the virus in the seed coat did not result in seed infection, unless the embryo was also infected as in TYMV (Table 2.14). The antigen of TYMV was detected in single seed

Table 2.14 Detection of antigen of TYMV and TMV in different parts of seeds of ecotypes of *Arabidopsis thaliana* by ELISA test (de Assis Filho and Sherwood 2000)

	Ecotypes of *A. thaliana*			
	Dijon	La-O	Dijon	La-O
Seed parts	TYMV (%)	TYMV (%)	TMV (%)[a]	TMV (%)
Embryo only	5.4 (184)	24.5 (184)	0 (180)	0 (180)
Seed coat only	58.2 (184)	12.6 (184)	100 (180)	100 (180)
Seed coat and embryo	10.9 (184)	13.0 (184)	0 (180)	0 (180)
Whole seed	72.6 (274)	59.5 (274)	100 (180)	100 (180)
Seedling[b]	26.0 (423)	20.6 (471)	0 (846)	0 (760)

[a] Sixty seeds were tested as groups of 10 and 120 seeds as groups of 20 seeds

[b] Assessment by grow-out tests

or part of single seed, whereas 10 seeds or parts of 10 seeds were required for the detection of TMV by PAS-ELISA format. Pollination of flowers on healthy *A. thaliana* with pollen from TYMV-infected plants did not result in systemic infection of healthy plants, although pollen carried TYMV to the seed (de Assis Filho and Sherwood 2000).

Rice yellow mottle virus (RYMV), belonging to the genus *Sobemovirus*, causes an important rice disease in Africa. The presence, infectivity and transmissibility of RYMV in the seeds of six wild host species were investigated by applying DAS-ELISA procedure. Each of four isolates of RYMV was detected by ELISA test in individual seeds of the wild rice species *Oryza barthii* and *O. longistaminata* and the standard susceptible rice BG90-2. RYMV could not be detected in individual seeds of other wild species *Dactyloctenium aegyptium*, *Eragrostis ciliaris*, *E. tenella* and *E. tremula*. But the extracts from several seeds of these wild species contained concentrations of virus detectable by ELISA test. Infectious RYMV virions were present in extracts of freshly-harvested seeds, whereas most infectivity was lost in dried seeds, possibly due to inactivation of RYMV by desiccation occurring during seed maturation. Despite the presence of RYMV in dry seeds of BG90-2 or wild host species, no evidence of transmission of the virus from the seeds to seedling was obtained, irrespective of host plant–virus combinations. Hence, the presence of RYMV in seeds does not appear to have epidemiological importance, since infected seedlings will not be produced from such seeds (Allarangaye et al. 2006).

Dot Immunobinding Assay

Dot immunobinding assay (DIBA), also designated dot-ELISA (Banttari and Goodwin 1985) or enzyme-linked immunoblot assay (Wang et al. 1985) is analogous to ELISA in principle. In this procedure, nitrocellulose or nylon-based

membrane is used in place of microtiter plates for immobilizing the antigen (virus) present in the seed extracts. Seedborne inoculum of *Barley stripe mosaic virus* (BSMV) in barley and *Bean common mosaic virus* (BCMV) in bean (*Phaseolus vulgaris*) was detected by applying DIBA. This protocol was efficient in detecting the viruses in single seeds, seed samples and seed flours of known infection levels. High correlation was observed between the results of DIBA, immunosorbent electron microscopy (ISEM) and symptom development in plants growing from infected seeds under greenhouse conditions. Seed extracts in phosphate-buffered saline are blotted in premarked positions on nitrocellulose membrane, followed by reaction with antibodies and enzyme conjugate and staining with nitroblue-tetrazolium substrate solution for visualizing the development of color, indicating positive reaction. DIBA procedure was found to be highly suitable for routine seed health testing by certification personnel (Lange and Heide 1986).

Detection of *Pea seedborne mosaic virus* (PSbMV) by DIBA test was found to be more sensitive than with ELISA procedure. Use of MAbs was necessary for ELISA test to be as sensitive as DIBA test using PAbs (Lange 1986). In contrast, DIBA test required MAbs specific to *Peanut mottle virus* (PeMV) to provide sensitive detection as the ELISA test. But ELISA provided the needed level of sensitivity of detection of PeMV with both MAbs and PAbs (Sherwood et al. 1987). Several viruses such as PSbMV, BSMV, BCMV, *Pea early browning virus* and *Squash mosaic virus* could be detected using plain paper in place of nitrocellulose membrane in the DIBA test, making the test simpler without compromising the sensitivity of the test (Lange et al. 1991). In spite of the advantages of DIBA test, such as ease of sample processing and possibility of storing and transport of blotted membranes, ELISA formats have been employed extensively for the detection of seedborne viruses than any other immunoassay.

Fluorescent Antibody Technique

Enzyme-linked fluorescent assay (ELFA) was shown to be efficient for the routine detection of seedborne viruses, *Lettuce mosaic virus* (LMV) and *Soybean moasaic virus* (SMV) in seeds. ELFA test detected LMV in a single seed present in a lot of 500 seeds and it was found to be 10–25 times more sensitive than DAS-ELISA format (Dolares-Talens et al. 1989). Likewise, SMV was also more efficiently detected in soybean seeds compared to the standard ELISA test (Hill and Durand 1986). In another investigation, radio-immunoassay (RIA) was developed for the sensitive detection of viruses in seeds using radioactive gamma for labeling immunoglobulins. *Lettuce mosaic virus* (LMV) could be detected in a seedlot of 30,000 seeds containing only three infected seeds of lettuce. The need for handling potentially hazardous ^{125}I isotopes for labeling globulins specific to the target virus(es), has been the limiting factor for its wider application (Ghabrial and Shepherd 1982; Dinant and Lot 1992).

Electron Microscopy-Based Immunoassays

Seedborne viruses can be detected by employing immunosorbent electron microscopy (ISEM) method, if the number of seed samples is limited. Virion particles in the seed extracts or purified preparations are adsorbed onto electron microscope grids that have been made specific for the target virus, followed by application of staining or metal shadowing procedure. The adsorbed virus particles can be visualized under the electron microscope. *Lettuce mosaic virus* (LMV) was detected in the pooled seed extract containing one part of infected seed extract mixed with 99 parts of healthy seed extract. ISEM was successfully employed for the detection of *Barley stripe mosaic virus* (BSMV), *Tobacco ringspot virus* (TRSV) and *Soybean mosaic virus* (SMV), when the disease incidence was low (Brlansky and Derrick 1979).

Detection of viruses in situ in different plant tissues has been accomplished by using labeled antibodies specific to target virus(es). The presence of *Alfalfa mosaic virus* antigen could be detected in the cytoplasm and vacuoles of ovule integuments, microspores, mature pollen grains and anther tapetum cells of infected alfalfa plants. Raft-like aggregates of virus particles and large crystalline bodies were also seen in the cytoplasm of pollen grains and anther tapetum cells (Pesic et al. 1988). *Cucumber mosaic virus* (CMV) transmitted through spinach seeds, was detected by employing immunogold labeling method. Presence of CMV virions was detected in the cytoplasm of ovary wall cells, ovule integuments and nucellus, anther, and seed coat cells as well as in the fine fibril-containing vesicles and electron dense inclusions of amorphous aggregates in the central vacuoles of these cells in the infected seeds of spinach (Yang et al. 1997). It is not feasible to apply electron microscopic techniques for routine diagnosis of virus diseases. But they may be useful as confirmatory procedures for the results obtained from other diagnostic tests or biological methods.

2.3.1.3 Nucleic Acid-Based Techniques

Nucleic acid-based techniques have been employed for the detection and identification of viruses present in the seeds as well as in the asexually propagated planting materials. RNA viral genomes have to be subjected to reverse transcriptase action to obtain cDNA of the fragment specific to the target virus, followed by PCR amplification. Modifications of RNA extraction procedure may be necessary to avoid the coextraction of PCR inhibitors which may be present in higher concentrations in the seeds compared to other plant tissues.

Nucleic Acid Hybridization Technique

Transmission through seeds is an important way of survival and perpetuation of viruses in plants. Further, the virus spreads through seeds to long distances very rapidly to other areas/countries, unless the viruses are intercepted by plant quarantines. *Prunus necrotic ringspot virus* (PNRSV) is transmitted at varying rates through seeds

at as high as 76.9–88.8% in cherry (Vértesy 1976; Kryczynski et al. 1992) and at a low of 9% in almond (Barba et al. 1986). However, the routes by which seeds get infection, are not clearly known. Progress of infection of PNRSV in apricot reproductive organs during their development up to seedling stage was studied by employing hybridization procedures. Dot blot hybridization of PNRSV RNA showed that the least amount of PNRSV was present in the embryos, while the endosperm and testa had the highest concentrations of the virus (Fig. 2.26). Invasion of apricot embryo in

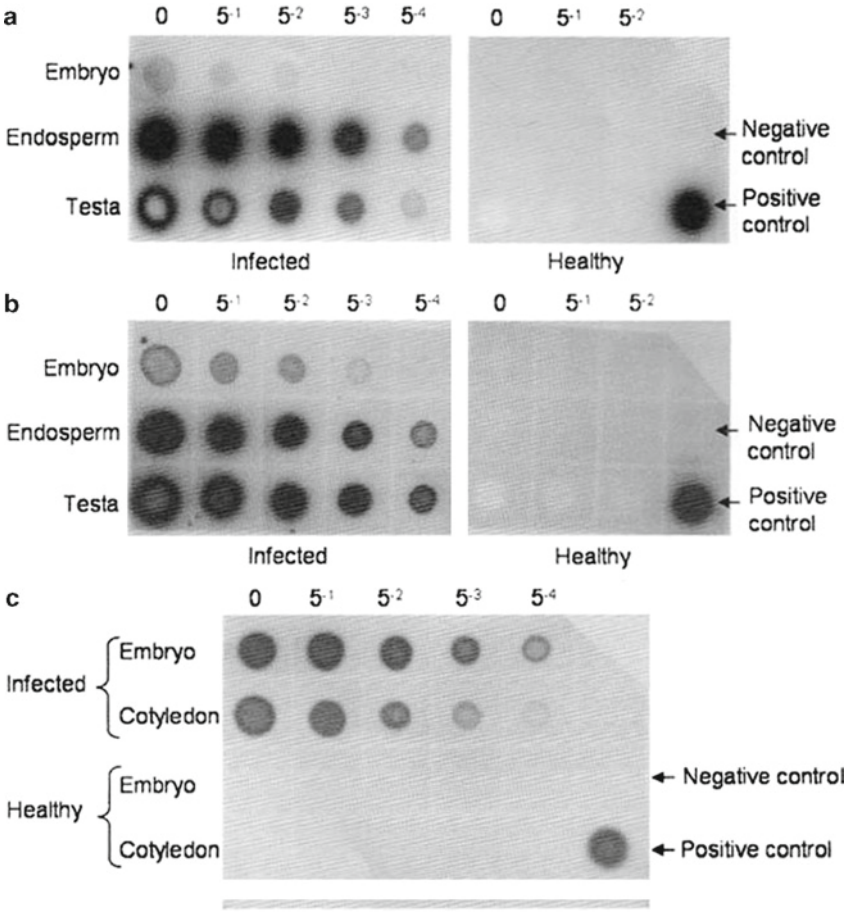

Fig. 2.26 Detection of *Prunus necrotic ringspot virus* (PNRSV) RNAs in seeds by dot blot hybridization technique. (**a, b**) Serial dilutions of total RNA extracted from embryo, endosperm and testa at torpedo (**a**) and bent cotyledon (**b**) developmental stages blotted and hybridized against viral RNA; (**c**) total RNA from mature embryos and cotyledons analyzed separately showing slightly higher accumulation of PNRSV in the embryos; RNAs extracted from PNRSV-infected cucumber (positive control) and uninfected cucumber (negative control) (Courtesy of Amari et al. 2009; Society for General Microbiology, Reading, UK)

the early developmental stages was revealed by in situ hybridization analysis of immature infected seeds. The viral RNA could be detected in embryos in the globular developmental stage obtained by hand pollination of virus-free flowers with infected pollen grains. Viral infection of seed integuments, nucellus and testa was visualized in immature seeds by in situ hybridization procedure. Tissue-printing hybridization analysis of apricot fruits resulting from hand-pollination of infected plants with infected pollen showed uniform distribution of PNRSV in the fruit. Seedlings from infected seeds showed PNRSV infection in 10% of seedlings tested by dot blot hybridization. This investigation provides the details of virus movement from gametes to apricot seedlings (Amari et al. 2009).

Reverse Transcription-Polymerase Chain Reaction Assay

Pea seedborne mosaic virus (PSbMV) in pea seeds (Kohnen et al 1992) and *Cucumber mosaic virus* in lupin seeds (Wylie et al. 1993) were efficiently detected by RT-PCR assay. Anthers of stone fruit plants are exchanged between countries for breeding purposes. Application of RT-PCR assay revealed that anthers might be a source of *Plum pox virus* (PPV), when desiccated anthers stored at 0°C for 1–2 years were assayed. The results indicated the need for monitoring international movement of *Prunus* germplasm to prevent the introduction and spread of the inoculum of PPV to other countries (Levy et al. 1995). In a later investigation, the presence of PPV in both seed coat and cotyledons of apricots could be detected by RT-PCR assay. On the other hand, ELISA test was able to detect PPV only in the seed coat, indicating the greater sensitivity of RT-PCR assay in detecting PPV in the tissues containing virus concentrations not detectable by ELISA test (Pasquini et al. 1998).

Results of growing-on test and RT-PCR assay to detect the presence of *Cowpea aphidborne mosaic virus* (CAMV) in peanut seeds were compared. CAMV was detected by RT-PCR assay in greater number of seeds than the number of seedlings growing from seeds that exhibited symptoms of CAMV infection. This revealed that presence of the virus, as detected by RT-PCR assay may not result in the seedling infection which has epidemiological importance. In 1,553 peanut seeds pretested by DAC-ELISA format, eight seeds tested positive by ELISA and this was confirmed by RT-PCR assay. Another set of 57 seed samples comprising of 5–24 seeds (in a total of 761 seeds) were ELISA-negative. But RT-PCR assay detected CAMV in seven of the 57 seed samples. Seed slices of 2–4 mm containing the seed coat and cotyledons produced more reproducible results by RT-PCR assay. In contrast, *Peanut stripe virus* (PStV) and *Peanut mottle virus* (PeMV) could be reproducibly detected by RT-PCR from a single seed from 100-seed composites using smaller slices (approximately 1 mm). The results indicated the higher level of sensitivity of RT-PCR assay compared to ELISA test (Gillaspie Jr. 2001).

Comparative efficacy of RT-PCR assay along with DAS-ELISA test for the detection of *Peanut mottle virus* (PeMV) and *Peanut stripe virus* (PStV) was tested. Samples were arranged in a 96-well format with four rows of 12 wells each containing

extract from a slice from a seed of one peanut accession. The other four rows contained slices from another accession. The RNA extracts of the pooled seed samples coupled with RT-PCR could be used for the detection of both PeMV and PStV in peanut seedlots. DAS-ELISA test was suitable only for samples containing high concentrations of the viruses. Hence, pooled samples with low virus titers failed to test positive by DAS-ELISA test. On the other hand, pooling of RNA from samples by row and column demonstrated that the sensitivity of RT-PCR was adequate to detect PStV and PeMV in peanut samples of seed slices (Gillaspie et al. 2007).

Three viruses *Peanut clump virus* (PCV), *White clover mosaic virus* (WClMV) and *Carrot red leaf virus* (CaRLV) infecting peanut, broad bean and carrot respectively are transmitted through seeds of their natural host plant species. RT-PCR assay was developed for the detection of these viruses using specific primer pairs. The primers PCVuni5/PCVuni3 amplified a fragment of 631-bp from PCV, whereas WClMV-CP5/WClMV-CP3 produced an amplicon of 559-bp from WClMV. The primer pair CL1-UP/CL1-DN produced a product of 626-bp from CaRLV. Total nucleic acids were extracted from infected seeds and healthy plants. RT-PCR assays were performed using the extracts. RT-PCR assays were highly specific for detection of PCV, WClMV and CaRLV in seed extracts and they have been demonstrated to have the potential as alternative to the ELISA tests which do not provide consistent results, particularly for quarantine programs (Lee et al. 2004).

Pepper (chilli) plants are infected by several viruses. *Pepper mild mottle virus* (PMMoV) (designated earlier as *Tobacco mosaic virus*-pepper strain) is transmitted through seeds. The efficacy of treatment with trisodium phosphate (Na_3PO_4) and dry sterilization at 70°C for 3 h in eliminating the virus from pepper seeds was assessed. The extent of seed infection was checked by tissue-printing immunoassay with a PMMoV-specific antiserum and RT-PCR assay. The effect of disinfestation of PMMoV-infected seeds by chemical and heat sterilization was determined by RT-PCR assay using extracts from individual treated seeds. RT-PCR amplification of extracts of seeds harvested from systemically infected plants prior to treatment indicated that the incidence of PMMoV infection was almost 100%. Post-treatment RT-PCR analysis showed that the DNA fragments specific to PMMoV were detectable in seeds treated with chemical and heat. Neither treatment individually was able to eliminate the virus entirely from seedlots. However, combination of both treatments (chemical followed by heat) resulted in the absence of a band specific for PMMoV in the gel, indicating the elimination of the virus completely. This was confirmed by the absence of local lesions on *Nicotiana glutinosa* plants inoculated with extracts of treated seeds (Toyoda et al. 2004).

Onion crops are infected by *Artichoke yellow ringspot virus* (AYRSV) belonging to the genus *Nepovirus*, the members of which are known to be transmitted through seeds of plant species susceptible to the respective virus(es). AYRSV was found to be seed transmissible in broad bean (Avgelis et al. 1992) and vetch (*Vicia sativa*) (Terzakis et al. 2002). Seed transmission of AYRSV was tested by biological and RT-PCR assays. All symptomatic onion plants originating from infected seeds were tested by RT-PCR assay. All AYRSV isolates were specifically detected by RT-PCR using virus-specific primers that amplified a unique 530-bp fragment from the extracts of onion

Table 2.15 Detection of *Cocoa swollen shoot virus* (CSSV) in cocoa seedlings (Quainoo et al. 2008)

	PCR/Capillary electrophoresis	
Experimental trees	No. of seedlings tested	No. of seedlings infected
T2	102	40 (39%)
T4	98	53 (54%)
H (CSSV-free)	135	0 (0%)
Total	335	–

plants developed from infected seeds. AYRSV was transmitted through seeds of onion cultivar, used in this study, to relatively high percentage of 20%. The results indicated that transmission of AYRSV through pollen and seeds might be helpful for survival and perpetuation of the virus in nature (Maliogka et al. 2006).

Cocoa swollen shoot virus (CSSV), belonging to the genus *Badnavirus*, causes the devasting disease of cocoa that threatens the economies of cocoa producing nations. Cocoa is propagated largely by seeds and hence, the perils of using infected seeds are well realized. In order to ascertain the seedborne nature of CSSV, in the light of conflicting reports on seed transmission of CSSV in cocoa, PCR assay was applied to find out, if this virus was transmitted through seeds. Primers for CSSV were designed based on the conserved regions of six published sequences of the CSSV genome. An internal control targeting a nuclear cocoa microsatellite was used in a multiplex PCR with the CSSV assay. All pollen grains isolated from anthers of infected Amelonado cocoa trees (T2 and T4) tested 100% positive for the presence of CSSV by PCR/agarose gel electrophoresis (AGE) and PCR/capillary elelctrophoresis (CE). The component parts of cocoa seeds (testa, cotyledon and embryo) from T2 and T4 revealed varying levels of CSSV infection by PCR/AGE, while PCR/CE detected CSSV in all component seed parts (100%). PCR/CE revealed CSSV infection in seedlings raised from seeds obtained from CSSV-infected trees (Table 2.15). The results suggested that CSSV was transmitted through seeds as revealed by PCR assays (Quainoo et al. 2008).

2.3.2 Detection of Viruses in Asexually Propagated Planting Materials

Asexually propagated plant materials have to be free from viruses to prevent the build up of virus inoculum by planting infected materials repeatedly. Once diseased plants are established in commercial orchards, there is no effective way of curing the infected plants by applying any therapeutic control measures. Probably the most effective disease management option is removal of infected plants. The principal method proven to be most efficient in managing virus and virus-like diseases in perennial crops involves production of nuclear stocks free of diseases by efficient certification/clean stock programs. Effective and reliable methods of detecting and

identifying the viruses in the mother plants rapid are the basic requirement for raising crops successfully and profitably.

Vegetatively propagated materials such as tubers, bulbs, corms, suckers, setts, cuttings or budwood for grafting and the source plants have to be tested by sensitive and reliable methods to ascertain their health status. Latent infection by viruses without expression of any visible symptoms is frequently observed in many crops. Adequate care and proper sampling of plant materials will be required to avoid escape of infected plant materials, because of application of inappropriate method of detection of viral pathogens. Hence, application of efficient diagnostic technique(s) is the most important method of preventing unlimited and uncontrolled spread of viruses through propagative materials. Viruses present in the propagative materials can be detected by biological, immunological and nucleic acid-based assays.

2.3.2.1 Bioindexing Methods

Biological indexing involves inoculation of the same natural host plant species or assay/diagnostic host plants that can react with specific distinguishable symptoms rapidly. Assay host plants frequently used for detecting viruses are *Chenopodium amaranticolor*, *C. quinoa*, *Cucumis sativus*, *Nicotiana benthamiana*, *N. clevelandii* and *N. occidentalis. Prunus* spp. and varieties have been found to be useful for detection of graft-transmissible viruses infecting stone fruit trees. *Tobacco rattle virus* (TRV) with nematodes as natural vectors, infects potato plants causing corky ringspot (CRS)-like symptoms in the tubers. *N. tabacum* cv. Samsun, *N. clevelandii* and *Petunia hybrid* were used as bait plants by planting them in the soil naturally infested with the nematode *Paratrichodorus minor*. Nine plants from each plant species were placed in growth chambers at 20°C and 30°C for 21 days. Plants showing virus-like symptoms were scored. Visual observations were confirmed by performing antigen-trapped indirect ELISA procedure (Pérez et al. 2000). Generally, the procedures followed for the detection of viruses in plant organs may be adopted. These methods require long time and large greenhouse space and they are not practicable to test large number of samples. Immunoassays and nucleic acid-based methods, on the other hand, offer many advantages over biological methods.

2.3.2.2 Immunoassays

Polyclonal antisera can be prepared easily in the case of sap transmissible viruses. But purification of antigens for non-sap transmissible viruses is difficult, because of low titers and irregular distribution of viruses in the infected plant tissues. Among the serodiagnostic methods, DAS-ELISA and indirect DAS-ELISA formats have been applied more frequently for detection of viruses in asexually propagated plant materials. In the Netherlands, it was estimated that about 11×10^6 ELISA tests were performed annually on propagative materials including about 5×10^6 tests for indexing seed potatoes and about 6×10^6 tests for indexing ornamental plant materials,

because of ease of performing these tests by relatively less trained personnel at a lower cost (Clark and Adams 1977; Huttinga 1996).

Successful detection of viruses in propagative plant material may be possible only after some treatments to break dormancy or activating dormant tissues of the propagative materials. *Potato virus Y* (PVY) and *Potato virus A* (PVA) could be detected by ELISA only after breaking dormancy of potato tubers (Vetten et al. 1983; De Bokx 1987). Detection of *Potato moptop virus* (PMTV) by ELISA is facilitated by storing potato tubers at 20°C for 3 weeks (Sokmen et al. 1998). Use of inorganic pyrophosphatase (PPase) from *Escherichia coli* conjugated with antibodies and tetrazolium phosphatase as substrate enhanced the sensitivity of detection of *Potato viruses X*, *Y*, *M*, *S* and *leaf roll virus* by ELISA tests (Mizenina et al. 1991). In the case of *Iris severe mosaic virus*, just after lifting the bulbs, a small piece of bulb tissue was removed. Then the wounded bulbs were stored at 17°C or 20°C for 3 weeks prior to testing by ELISA test (van der Vlugt et al. 1993). The scales of lily had to be stored at 20°C for 2–3 weeks under fluorescent light (12–16 h/day) prior to detection of *Lily mottle virus* (LMoV) by ELISA. In contrast, *Lily symptomless virus* (LSV), *Cucumber mosaic virus* (CMV) and *Lily X virus* did not require any prior treatment for their detection in infected lilies (Derks et al. 1997). Likewise, storage appeared to have some influence on detection of *Tomato spotted wilt virus* (TSWV) in dahlia. The tubers of dahlia had to be stored for a minimum period of 4 weeks followed by taking mixed samples of three roots per tuber for reliable detection of TSWV in dahlia by ELISA test, since the virus was not uniformly distributed in the tubers (van Schadewijk 1996).

ELISA formats have been effective for the detection of viruses infecting several horticultural crops. ELISA tests were efficient in detecting *Citrus psorosis-associated virus* in citrus plants. This assay could detect the viruses in greater number of infected citrus accessions in which biological indexing did not detect virus infection, indicating the possibility of ELISA test providing more reliable results (D'Onghia et al. 1998). The *Dioscorea bacilliform virus* (DaBV) infecting yams was detected rapidly and identified precisely by using ELISA tests. Further, serological relationship of DaBV with *Sugarcane bacilliform virus* and *Banana streak virus* was also established by employing ELISA test (Phillips et al. 1999).

Incidence of new virus diseases may be observed now and then. Detection and identification of the causal virus(es) has to be carried out rapidly and reliably. Application of ELISA established the occurrence of a new mutant strain of *Potato virus M*, designated *Potato virus M*-ID (Cavileer et al. 1998). A new strain of *Potato virus Y* (PVY) infecting *Dioscorea alata* was identified by employing ELISA test (Odu et al. 1999). A strain of *Citrus tristeza virus* (CTV) caused severe symptoms on sweet oranges. By employing different combinations of MAbs and PAbs in indirect ELISA format, the strain causing stem-pitting was identified and differentiated from other CTV strains (Nikolaeva et al. 1998). Specific detection of CTV was possible by using an MAb which reacted only with extracts of tissues infected by CTV. No positive reaction was noted with non-infected citrus plant extracts (Öztürk and Cirakolu 2003). Resolution of the cause of a disease that was defying attempts to determine the nature of the pathogen, was achieved by employing ELISA test. Strawberry pallidosis

was recorded several decades ago before the viral origin could be proved. PAbs were used to detect the putative causal agent in the petiole tissue blots. Further evidence to identify the causal agent was provided by RT-PCR assay. The virus causing strawberry pallidosis was identified as a member of *Closteroviridae* family, *Crinivirus* genus and it was found to be most closely related to *Cucumber yellows virus* and *Cucurbit yellow stunt disorder virus* (Tzanetakis and Halgren 2004).

Isolates of *Potato virus Y* (PVY) inducing systemic necrosis in tobacco are designated PVY^N strain group. ELISA test using strain-specific monoclonal antibodies (MAbs) is employed for detection, identification and differentiation of PVY^O from PVY^N. Certified potato seedlots of 960 and 286 from different locations across North America were planted in trials in Washington and Oregon respectively. The incidence of PVY^O-infected lots averaged 16.4% and 25.9% in Washington and Oregon trials respectively. PVY^O infection was detected in 31 cultivars originating from 25 states/provinces and 132 seed growers. A total of 240 seedlots were positive for PVY. Incidence of PVY^N remained relatively constant during the 2 years (2001 and 2003) of testing. Another strain $PVY^{N:O}$ infection was approximately doubled during 2002 and 2003. Certification agencies used only tests to differentiate PVY^O from PVY^N. The prevalence of PVY in potato seedlots at high levels poses a serious threat to potato production in the US, indicating current measures to reduce incidence of this virus were inadequate (Crosslin et al. 2006).

Sweet potatoes are infected by *Sweet potato feathery mottle virus* (SPFMV, *Potyvirus* genus), *Sweet potato virus 2* (SPV2, *Potyvirus* genus) and *Sweet potato chlorotic fleck virus* (SPCFV, *Carlavirus* genus). Virus titers are often low in sweet potato plants and direct detection using immunoassays from plant tissues is unreliable. Scions from *Ipomoea setosa*, a universal indicator host for sweet potato viruses were used for top-grafting on test plants. Tuberous roots and leaves of grafted *I. setosa* scions were tested for the presence of sweet potato viruses. SPFMV, SPV2 and SPCFV were detected by ELISA tests. SPCFV was present in mixed infection with the other two viruses. The results of ELISA were confirmed by RT-PCR assay (Jones and Dwyer 2007). DAS-ELISA test was applied to test the sweet potato plants for the presence of SPFMV prior to subjecting them to thermotherapy for eliminating the virus. The sweet potato plants were treated at 42°C/day and 39°C/night for 3 weeks, followed by meristem-tip culturing and allowing them to develop in vitro. Tissue culture generated plants were tested routinely for successive 2 years using dot-ELISA format. The results showed that the test plants were entirely free from SPFMV infection, indicating the effectiveness of chemotherapy in eliminating SPFMV (El Far and Ashoub 2009).

Fruit propagation plant materials, root stocks and seedlings of varieties of fruit trees were tested by ELISA formats for the presence of viruses. *Prunus necrotic ringspot virus* (PNRSV) was detected in plum rootstock sample; *Prune dwarf virus* (PDV) in cherry seedling samples and *Plum pox virus* (PPV) in peach root stock and cherry rootstock and seedlings. Mixed infection by PDV + PPV was noted in cherry seedling sample. Apple and pear rootstock and seedling samples did not show the presence of any virus against which tests were conducted. Over all, 6.13% of propagative plant materials meant to be imported into Turkey were found to be infected by one or two viruses (Table 2.16) (Özdemir and Kaya 2008).

Table 2.16 Detection of viruses in propagative plant materials by ELISA formats (Özdemir and Kaya 2008)

Propagative materials	No. samples	Viruses detected
Cherry rootstocks	8	PNRSV, PDV, PPV, CLRV, ToRSV
Cherry seedlings	25	PRMV, APLPV
Peach rootstocks	8	PNRSV, PDV, PPV, CLRV, ToRSV
Peach seedlings	11	PRMV, APLPV
Plum rootstocks	6	PNRSV, PDV, PPV, CLRV, TORSV
Plum seedlings	2	PRMV, APLPV
Apple rootstocks	26	APMV, ACLV
Apple seedlings	17	
Pear rootstocks	5	ACLSV
Pear seedlings	6	

ACLSV: *Apple chlorotic leaf spot virus*, APLPV: *American plum line pattern virus*, ApMV: *Apple mosaic virus*, CLRV: *Cherry leaf roll virus*, PDV: *Prune dwarf virus*, PNRSV: *Prunus necrotic ringspot virus*, PPV: *Plum pox virus*, PRMV: *Peach rosette mosaic virus*, ToRSV: *Tomato ringspot virus*

The International Council for the Study of Virus and Virus-like Diseases of the Grapevine (ICVG) has recognized over 70 infectious agents (virus, viroids and phytoplasmas) affecting grapevines (ICVG 2003). Virus diseases have significant impact on plant growth, yield and fruit quality and they spread to long distances through infected propagative materials (buds/cuttings/rooted cuttings from infected canes). Hence, the effective control of the disease depend on the effectiveness of clean stock programs. In Bulgaria, the propagated grapevine materials are checked in AgroBioInstitute, Sofia by DAS-ELISA test. In accordance with the European Legislation and the national system for production of certified vine growing materials operating in Bulgaria, 1,295 samples from grapevine varieties and rootstocks were checked for their virus status during 2004–2006. A total of 30.5% of plant materials tested were ELISA positive by one (86.08%) or more viruses (13.92%). *Grapevine fleck virus* (23.3%), *Grapevine fanleaf virus* (5.05%) and *Grapevine leafroll-associated viruses 1* and *3* (3.34% and 2.78% respectively) were more frequently detected. *Grapevine Bulgarian latent virus* (0.37%) was detected occasionally. The results emphasized the need for constant monitoring of virus disease incidence in planting materials to avoid appreciable losses that are likely to be caused by virus diseases in future (Kamenova et al. 2007). In order to detect *Grapevine leaf roll-associated virus 2* (GLRaV-2) and *Grapevine virus B* (GVB) in grapevine propagative materials, an ELISA protocol was optimized by employing extraction buffer in BIOREBA Kit. Both GLRaV-2 and GVB were detected in planting materials and this ELISA protocol was recommended for certification program in Slovenia. The results were confirmed by Western blotting and electron microscopy coupled with decoration procedure (Tomažič et al. 2008).

Source plants of sweet orange (*Citrus sinensis*) cv. Musambi collected from orchards at Sahiwal and Faisalabad, Pakistan were analyzed by ELISA test for the presence of viruses infecting citrus. Microbudding technique was applied for early propagation of virus-free plants using thermotherapy. Microbudding technique

and thermotherapy combined with indexing based on ELISA reduced the time required for developing virus-free Musambi plants (Abbas et al. 2006). A survey was undertaken in commercial groves for selection of elite mother plants of citrus for producing clean stocks in Cyprus. All mother plants were tested by biological indexing and ELISA test and they were found to be free of *Citrus trizteza virus* (CTV) and *Citrus variegation virus* (CVV). Some mother plants, however, were infected with *Citrus psorosis virus* (CPsV). A standard procedure of shoot-tip grafting technique was applied for eliminating CPsV in selected citrus isolates. Mirco-grafted plants, regrafted on sour orange seedlings in vivo and successfully established were transferred to the glasshouse. These plants were tested 6–9 months later. Virus-free plants were used as primary source material for the basic citrus plantation (Kaparia-Isaia et al. 2007). *Banana streak virus* (BSV) with DNA genome and *Cucumber mosaic virus* (CMV) with RNA genome cause losses in proportion to the extent of infection of suckers (propagation units of banana). Suckers, pseudostem and leaf tissues were tested by DAC-ELISA and dot blot ELISA formats for the presence of BSV and CMV. Detection limit of DAC-ELISA was 10^{-2} dilution of tissue extracts, while dot blot ELISA was able to detect the viruses at 10^{-3} dilution of tissue extracts. The results indicated that dot blot ELISA procedure was slightly more sensitive than DAC-ELISA format in detecting the banana viruses (Rajasulochana et al. 2008).

2.3.2.3 Nucleic Acid-Based Techniques

Detection of viruses in propagative plant materials has been accomplished effectively by applying different nucleic acid-based techniques. Although the nucleic acid-based techniques are generally more sensitive and specific, the cost-effectiveness of these techniques and expertise required may be limiting factors for their large scale applications.

Nucleic Acid Hybridization Techniques

Hybridization techniques for the detection of viruses in propagative plant materials have been employed to a limited extent. *Potato leaf roll virus* (PLRV) could be readily detected in dormant potato tuber tissues by using nonradioactive digoxigenin (DIG)-labled cRNA probe of approximately 2,100-bp. No cross-reaction with *Potato viruses X* and *Y* was observed, indicating the specificity of the hybridization technique which was 2,000-folds more sensitive than ELISA test (Loebenstein et al. 1997). Non-radioactive tissue blot hybridization procedure was applied for the detection of *Tobacco rattle virus* (TRV), the type member of the genus *Tobravirus* with nematodes as natural vectors, infecting potato tubers causing corky ringspot (CRS) symptoms. Necrotic potato tuber tissue was extracted with a cork borer and blotted on a BioBlot N-plus nylon membrane for 15 s. The cRNA probes specific for the 16-kDa ORF of the Florida isolate of TRV were prepared from gel-purified

DNA fragment amplified by PCR from the cloned PCR product. The 463-bp fragment was labeled with non-radioactive biotin and employed for hybridization with the immobilized targets in the nylon membrane. A positive signal was detected when the membranes with four samples of each of the potato cvs Red Lasoda, and Siebago and three samples of the cv. Atlantic with typical symptoms of TRV infection were tested. No signal was detectable in membranes containing healthy tissue samples (Pérez et al. 2000).

Potatoes are known to be infected by many viruses belonging the genera *Alfamovirus*, *Carlavirus*, *Luteovirus*, *Potexvirus*, *Potyvirus*, *Tobravirus*, *Tospovirus* and *Tymovirus.* Hence, potato plantlets generated by tissue culture technique have to be tested for multiple viruses prior to propagation for seed potato production. As protocols differ with viruses infecting potatoes, it would be advantageous to develop a multiplex assay that could detect as many viruses as possible, to save time, labor and cost of testing by many individual tests. A non-radioactive nucleic acid spot hybridization (NASH) assay was developed for simultaneous detection of the carlavirus *Potato virus S* (PVS), potexvirus *Potato virus X* (PVX) and potyvirus *Potato virus Y* (PVY) in potato tissue cultured plantlets. The sensitivity of NASH procedure was assessed by hybridization of PVS-, PVX- and PVY-specific probes with membranes spotted with a dilution series of tissue cultured plantlet extracts. The viruses could be individually detected with specific probes at a dilution of 1:40 of plantlet extracts. It is significant that the PVY probe which was amplified from plantlets infected with PVY^{O}, could also detect other strains of PVY in plantlets infected with PVY^{N}, PVY^{NTN} and $PVY^{N:O}$. The multiplex hybridization test, using a cocktail of different probes, successfully detected PVS, PVX and PVY in a single hybridization assay (Janczur et al. 2006).

Reverse Transcription (RT)-PCR Assay

The reverse transcription-polymerase chain reaction (RT-PCR) assay has been applied for the detection, identification and quantification of a wide range of plant viruses present in clonal planting materials. Single and mixed infections of potato tubers by *Potato virus Y* (PVY) could be accomplished by digesting the PCR products with appropriate endonuclease restriction enzymes (Rosner and Maslenin 1999). A common electric drill was employed for sampling large number of dormant tubers by simultaneously removing and macerating tuber eye samples. This method of sampling was found to be rapid, simple and inexpensive. The dormant tubers from plants with primary infection were tested by both PCR and ELISA and the presence of *Potato leafroll virus* (PLRV) was detected by both procedures (Souza-Dias et al. 1999). The greater sensitivity and reliability of RT-PCR assay compared with ELISA test was revealed for the detection of PLRV in dormant tubers from field-grown plants and in vitro propagated microtubers. ELISA test was able to detect PLRV only in the microtubers, whereas RT-PCR assay detected PLRV in both types of tubers (Spiegel and Martin 1993). Assessment of incidence of PLRV by field inspection and RT-PCR assay was compared. Higher rates of incidence of

PLRV were detected by RT-PCR assay than by visual examination. Infection levels of sprouts from potatoes planted in the greenhouse were determined by ELISA test and RT-PCR assay. Positive detection of PLRV in 85% of the sprouts was in agreement with the diagnostic techniques (Russo et al. 1999).

Specific detection of PVY strains was achieved by applying another technique designated 'detection of immobilized amplified product in a one-phase system' (DIAPOPS). Four strains of PVY (O, N, NTN, C) present in dormant tubers could be specifically detected by DIAPOPS procedure. However, this procedure failed to detect PVY strains in some samples, while ELISA test gave positive results (Nielson et al. 1998). A PCR-based assay was developed by exploiting a recombination site in the coat protein (CP) gene of the *Potato virus Y* strain (PVY^{NTN}), causing the potato tuber necrotic ringspot disease (PTNRD). This format allowed more reliable diagnosis of the PVY strains, PVY^{O}, PVY^{C} and PVY^{NTN}. This assay was found to be more reliable for the detection of PVY strains than most commonly employed RT-PCR procedures. The protocol developed in this investigation was particularly efficient in detecting PVY^{NTN} strain in symptomless tubers during field surveys and useful for seed health programs (Boonham et al. 2002).

Tobacco rattle virus (TRV), type member of the genus *Tobravirus*, is naturally transmitted by nematodes *Trichodorus* spp. and *Paratrichodorus* spp. The bipartite RNA genome of TRV produces two classes of isolates: M type (multiplying type) is composed of a larger particle (containing RNA-1) and shorter particle (containing RNA-2) that encodes for the coat protein; NM type (nonmultiplying type) lacks RNA-2 and hence, contains no nucleoprotein. NM type isolates cause infection in plants and they are common in nature, although they lack a coat protein (Harrison and Robinson 1982). TRV was detected in potato tubers by RT-PCR of total RNA extracted from a core of tuber tissue. The primer A complimentary to residues 6,555–6,575 and primer B identical to residues 6,113–6,132 of TRV-RSYM RNA-1 were employed for amplification after reverse transcriptase action on viral RNA. After amplification, the product of 463-bp fragment was cloned, sequenced and compared to sequences of TRV isolates from Europe and Canada. Nucleotide sequence homology varied from 90% to 94% between Florida isolate and other isolates. RT-PCR was sufficiently sensitive to detect TRV RNA from 1 μg of total nucleic acids from potato tubers. Since the protocol developed in this study amplified a segment from RNA-1, it could detect TRV in tubers infected with TRV-NM type and -M type in Florida with greater sensitivity and accuracy than ELISA tests (Pérez et al. 2000). By employing the RT-PCR assay targeting the CP gene in RNA-3 of *Potato moptop virus* (PMTV), the virus could be detected in potato seedlots and ware potato during surveillance in the United States and Canada. Of the 3,221 samples, PMTV was detected in 4.3% of the samples. Confirmatory tests using two primer sets targeting gene segments in RNA-2 and RNA-3 were carried out. Amplicons generated from RNA-2 and RNA-3 were identified by RFLP analysis. ELISA, ISEM and infectivity tests on *Nicotiana debneyi* also provided supporting evidences for establishing the identity of PMTV (Xu et al. 2004).

A one-step reverse transcription loop-mediated isothermal amplification (RT-LAMP) of DNA was employed for detecting *Potato virus Y* (PVY) in potato

Table 2.17 Comparative efficiency of RT-LAMP and RT-PCR techniques for detection of PVY in potato tubers and leaf samples (Nie 2005)

Type of sample	Quantity (number)	Positive reaction		Consistency (%)[a]
		RT-LAMP	RT-PCR	
Tuber	120	83	84	116/120 (96.7)
Leaf	120	91	89	118/120 (98.3)
Total	240	174	173	234/240 (97.5)

[a]Comparison of results of individual samples diagnosed by RT-LAMP and RT-PCR assays

tuber samples. Potato tubers collected from research field plots were tested by both RT-PCR and one-step RT-LAMP turbidity analysis. Of the 120 tubers tested, PVY was detected in 83 and 84 samples by RT-LAMP and RT-PCR assays respectively. Among the individual tuber tests, the results for 116 tests were similar and a positive correlation of 97.5% between these two tests was observed. The principal advantage of one-step RT-LAMP protocol was shown to be its simplicity of measuring the turbidity by a spectrophotometer, instead of using an expensive and sophisticated thermocycler in RT-PCR assays (Table 2.17; Nie 2005).

Globalization, following GATT agreement between countries, led to the relaxation of importation regulations resulting in free movement of plants and propagative plant materials carrying microbial pathogens including viruses inadvertently. *Alfalfa mosaic virus* (AMV) occurring in potato fields in several provinces in Canada was detected by RT-PCR assay, in addition to bioassay and ELISA test. RT-PCR assay using specific primer set AMV-F/R amplified a fragment of 351-bp. All PCR amplicons after digestion with restriction enzyme *Sac*I, specifically yielded two fragments of 150-bp and 201-bp. AMV RNAs were readily detected in composite samples of 200–400 tubers or 400–800 potato leaves. The results suggested that RT-PCR followed by RFLP analysis might be a useful approach for screening potato tubers on a large scale to assess the infection by AMV (Xu and Nie 2006). Presence of *Potato virus Y* (PVY) and its strains in potato tubers and leaves has been detected by ELISA and RT-PCR assays in different countries, with different levels of reliability and accuracy. A statistical analysis of PVY detection performance of a two PCR methods was taken up. PVY detection performance of one PCR-based procedure was statistically different from the performance of ELISA, while no difference between another PCR format and ELISA test was evident. A positive PVY result may be more likely, if leaves rather than tubers are tested, indicating that leaves are better sources for detecting PVY in potato (Bolotova et al. 2009).

Ipomoea setosa, a universal indicator host plant for sweet potato viruses, is graft-inoculated to test plants for biological indexing. This method requires up to 8 weeks to provide results. Hence, an efficient PCR procedure was developed to detect *Sweet potato leaf curl virus* (SPLCV) in in vitro plants using a reliable and simple method of extraction of nucleic acids. The degenerate primer pair SPG1/SPG2 was more sensitive than the other two primer pairs tested. A major amplicon of 514-bp was detected only in samples containing templates from infected plant

materials. End-point dilution of 10^{-9} (2 pg) for degenerate primer pair was sufficient for detection of SPLCV. The high sensitivity and broad detection of these primers make them the best choice for detection of this virus. Several in vitro clones that were negative by PCR and grafting assays were propagated and released as virus-free materials to growers (Li et al. 2004). In Australia, the healthy sweet potato stock scheme relies on the propagation from selected mother plants for graft-inoculation with *I. setosa* scions. Since transient symptoms or latent infections may be difficult to recognize, RT-PCR assay was developed for detection of *Sweet potato chlorotic fleck virus* (SPCFV) and *Sweet potato feathery mottle virus* (SPFMV). This assay could be reliably applied to detect them in mother plants from which nuclear stocks might be built up for multiplication and distribution (Jones and Dwyer 2007).

Applicability of ELISA test for the detection of *Onion yellow dwarf virus* (OYDV) infecting onion and garlic crops may be difficult, because of the problems associated with OYDV-specific antibody production. RT-PCR was developed to provide rapid and reliable detection of OYDV in the propagation materials generated by meristem tip culture employed to obtain virus-free stocks. Specific primers from conserved region of RNA-dependent RNA polymerase (RdRp) gene and 3′ UTR region of RNA of OYDV isolates were designed and employed in the RT-PCR assay. The primers successfully amplified virus-specific fragment (approximately 1.1 kb) in naturally infected garlic bulb. All samples tested positive by RT-PCR, indicating infection of onion and garlic. But ELISA test failed to detect OYDV in onion samples, revealing the superiority and suitability of RT-PCR assay for reliable detection of OYDV in both onion and garlic crops (Arya et al. 2006).

Prunus necrotic ringspot virus (PNRSV) present in dormant bark tissues was detected by applying a modified RT-PCR procedure. As this protocol was found to be effective and reliable for PNRSV detection, it was recommended for screening imported budwood materials in post-entry quarantine programs and for production of virus-free propagation materials (Spiegel et al. 1996). *Plum pox virus* (PPV) causes serious losses in many *Prunus* spp. necessitating the development of a rapid, reliable and sensitive diagnostic procedure for its detection, to facilitate eradication of infected plants. As the available ELISA test was not sensitive to provide reliable results, a real-time fluorescent RT-PCR assay was evaluated. This assay was more sensitive than ELISA or conventional PCR methods and the results were highly reproducible and obtained rapidly. PPV could be detected in propagative plant materials by real-time RT-PCR assay which could be applied for detecting PPV in multiple hosts and in various tissues of the test plants (Schneider et al. 2004).

Apple stem-pitting virus (ASPV), *Cherry rasp leaf virus* (CRLV) and *Cherry necrotic rusty mottle virus* (CNRMV) cause diseases of quarantine importance. They spread to other countries or geographical locations primarily through import of infected plants or planting materials. Development of rapid and reliable diagnostic techniques was considered important to prevent the introduction of these viruses to other countries. A total of 14 primers was designed and tested for their suitability for detecting these viruses. The prime set prASCP-N/prASCP-C amplified ASPV-specific fragments of 578- and 306-bp fragments, while prCR4/prCR5-JQ3D3

primer set efficiently amplified fragments 697- and 429-bp specific to CRLV. The primer set prCN4/prCN6-NEG produced CNRMV-specific fragments of 370- and 257-bp. Total RNAs extracted from healthy controls or from other virus-infected woody tissues gave no detectable amplified product. Homogenization of even woody tissues followed by RNA extraction through silica column and RT-PCR allowed specific detection of the target virus(es) consistently, although leaves were more suitable source for total RNA extraction. The protocol developed in this investigation, is relatively simple and it can be easily applied for the detection of viruses infecting woody trees (Park and Kim 2004).

In order to determine the sanitary status of mother plants of stone fruit crops, RT-PCR assay was employed. Of the 120 apricot samples tested, the unique fragment of 200-bp specific to *Apricot latetnt virus* (ApLV) was amplified from the extracts of 19 samples indicating the presence of ApLV. A total of 35 stone fruit samples consisting of 10 peach, 12 plum and 13 cherry samples was tested by nested RT-PCR format. The extracts from two cherries yielded an amplicon of 190-bp specific for *Plum bark necrosis stem pitting-associated virus* (PBNSPaV). The incidence of PBNSPaV was detected in stone fruit trees for the first time in western Anatolia, Turkey. This report indicates the need for constant monitoring to detect the incidence of new viruses for checking its further spread (Gümüs et al. 2007). *Indian citrus ringspot virus* (ICRSV) is a serious problem affecting citrus production in India. In order to obtain virus-free plants, the effect of hot water and moist hot air treatment on the elimination of ICRSV was assessed. Nodal segments from infected mother plants were treated at different temperature (40–50°C for 30 min). The percentages of virus elimination achieved varied from 40.74% to 60.0% and 48.15% to 73.33% as assessed by RT-PCR and ELISA procedures respectively. The results indicated that ELISA test was less efficient in detecting low virus concentrations while RT-PCR assay could detect the virus in such ELISA-negative plant materials, resulting in lower percentages of virus elimination by the treatments (Sharma et al. 2008).

In the attempts to eliminate *Grapevine leafroll-associated virus 1* (GLRaV-1) and *Grapevine rupestris stem-pitting-associated virus* (GRSPaV) from the grapevine cv. Agiorgitiko, in vitro thermotherapy and tissue culture methods were combined. A micropropagation protocol for mass production of virus-free plants was adopted. The effectiveness of meristem- and shoot-tip culture in combination with thermotherapy was assessed by a nested RT-PCR format. Meristem tip culture combined with thermotherapy was the most effective in eliminating both GLRaV-1 and GRSPaV as revealed by nested RT-PCR assay. Elimination rate of GLRaV-1 was greater (91.2%) than that of GRSPaV (67.6%) by the treatments. The ratio of virus elimination to survival was higher for meristem-tip culture than for shoot-tip culture (Skiada et al. 2009). Successful elimination of *Lily symptomless virus* (LSV) from lily was achieved by repeated shoot meristem excision before and during in vitro culture and thermotherapy (at 35°C for 42 days) to bulblets. Leaf tissues from bulblets formed before or post-heat treatments were analyzed by RT-PCR or ELISA tests. LSV was eliminated from line 499 without thermotherapy, while line 599 became free of LSV only after heat treatment. The virus-free lily bulblets showed vigorous growth and acclimatized promptly (Nesi et al. 2009).

Assessment of virus infection of potato tubers by immunocapture (IC)-RT-PCR format has been demonstrated to be more sensitive than by visual examination. *Potato leaf roll virus* (PLRV) antigen was captured by employing paramagnetic beads coated with antibodies specific to target virus followed by addition of cell wall degrading enzymes, cellulases and macrozyme to the tuber extract to improve the sensitivity of the assay performed in microtiter plates. The protocol used in this investigation was rapid, reproducible and semiquantitative and amenable for automation for large scale application. Furthermore, the potato tuber inspection requiring 5 days by visual examination was dramatically reduced to only 1 day for IC-RT-PCR assay, thus saving enormous time for certification personnel (Shoen et al. 1996). Potato tuber necrotic ringspot disease (PNRD) caused by a PVY strain, PVY^{NTN} could be detected and also differentiated by employing IC-RT-PCR assay (Tomessoli et al. 1998). In another study, the suitability of IC-RT-PCR format for detection of *Potato moptop virus* (PMTV) was examined. This technique was found to be 100 times more sensitive than ELISA test for detection of PMTV (Rantanen et al. 1999).

An immunocapture (IC) step prior to RT-PCR concentrates virion particles in dilute plant tissue extracts resulting in enhancement of sensitivity of detection. Difficulty in detection of *Tobacco rattle virus* (TRV) and *Potato moptop virus* (PMTV) in potato tubers could be overcome by the IC-RT-PCR format. A multiplex assay for the detection of TRV and PMTV directly in potato tubers was developed by combining PCR with a tube fluorescent product detection (TaqMan®). The new protocol could be used in place of two separate tests – a TRV-RT-PCR and a PMTV-ELISA – currently used in single tube multiplex format. Forty isolates of TRV and PMTV could be reliably detected with high sensitivity (100–10,000-fold). Furthermore, reduction in contamination risk, elimination of electrophoresis step and saving time and costs of post-PCR amplification processes are the advantages of the TaqMan® assay (Mumford et al. 2000).

The genome of PVY^{NTN} strain has three major recombinant junctions (RJs) at the sites HC/Pro.P3, NIa and the C-terminal region of CP gene. In order that the sense and antisense primers completely match the nucleotide sequences at either side of the RJ, specific primer pairs were designed to target the RJs. When all three primer pairs were employed simultaneously in a multiplex RT-PCR assay, three fragments (641-, 448- and 290-bp) were amplified exclusively from the recombinant PVY^{NTN}. On the other hand, only a single fragment of 641-bp was amplified from PVY^{O} isolate. Likewise, no amplification occurred from the non-recombinant PVY including PVY^{O} and North American (NA)-PVY^{NTN} strains, indicating the specificity of the primers developed in this investigation (Nie and Singh 2003).

Emergence of new strains of *Potato virus Y* (PVY) and strain variants in Europe and North America has been observed in the last few decades. Several RT-PCR assays available were limited by their inability to detect all combinations of mixed strain infections. Hence, a single multiplex RT-PCR assay that can assign PVY strain type and detect mixed infections with respect to the major strain types was developed. All 11 PVY^{O} isolates produced amplicons of 269- and 689-bp, while 13 PVY^{N} isolates gave PCR products of 328- and 398-bp. Five isolates produced a single 328-bp product representing NA-$PVY^{N/NTN}$. A separate complementary PCR

assay was employed to verify the NA-PVY$^{NTN.}$ The multiplex assay identified 17 samples with strain mixtures from 119 previously characterized isolates that were considered to be single-strain samples. The multiplex assay is robust and inexpensive and suitable for seed production specialists interested in determining PVY infection type using a single assay (Lorenzen et al. 2006).

A multiplex RT-PCR assay was developed for the detection of *Potato virus Y* (PVY), *Potato virus X* (PVX) and *Potato leafroll virus* (PLRV) that are more frequently found in the potato crops. Specific primer pairs designed based on CP gene sequences of respective viruses, amplified products of 810-bp for PVY, 700-bp for PVX and 548-bp for PLRV. This multiplex RT-PCR assay had higher sensitivity compared with standard RT-PCR format and its application can result in early and sensitive detection of PVY, PVX and PLRV and it may be useful for quarantine, certification and breeding programs (Schoen et al. 1996; Shalaby et al. 2002). Likewise, the multiplex RT-PCR procedure was applied for the simultaneous detection of *Potato yellow vein virus* (PYVV), *Tobacco rattle virus* (TRV) and *Tomato infectious chlorosis virus* (TICV) infecting potatoes. This protocol was found to be sensitive and practical for the detection of these three viruses in a small volume of 10 µl with the same sensitivity as obtained with single PCR format. This multiplex PCR procedure capable of detecting PYVV, TRV and TICV is cost-effective and will be useful for quarantine and certification personnel, as it can save considerable time and labor (Wei et al. 2009).

Cucumber mosaic virus (CMV) and *Piper yellow mottle virus* (PYMoV) are associated with the stunt disease accounting for considerable loss in black pepper crops. A single-tube multiplex (m)-RT-PCR was developed for the simultaneous detection of CMV and PYMoV in the mother plants. The total nucleic acids from infected plants, when analyzed by mRT-PCR assay, produced two amplicons of expected sizes 650-bp for CMV and 450-bp for PYMoV. No amplification from nucleic acids extracted from healthy plants was observed. The mRT-PCR procedure was effective in detecting these viruses in the nursery and field-grown plants and it could be applied for screening large number of samples rapidly (Bhat and Siju 2007).

2.4 Detection of Viroids in Seeds and Planting Materials

Viroids have been demonstrated to be transmitted through seeds and vegetatively propagated plant materials, in addition to their transmission through contaminated tools and propagation practices such as grafting and budding.

2.4.1 Detection of Viroids in Seeds

2.4.1.1 Biological Methods

Detection of viroids in seeds has been carried out by conventional seed testing method, growing-on test under greenhouse conditions. Visual examination is based

on symptom development in the seedlings growing from the specific number of infected seeds sown and percentages of infected seeds are determined. *Potato spindle tuber viroid* (PSTVd) was found to be transmitted through pollen and true seeds of potato (Fernow et al. 1970) and through seeds of tomato (Benson and Singh 1964). *Prunus necrotic ringspot virus* (PNRSV) and *Prune dwarf virus* (PDV) were reported to be transmissible through pollen and seeds of PDV-infected sour cherry plants (Gilmer and Way 1961). Transmission of *Grapevine yellow speckle viroid 1* (GYSVd-1) and *Hop stunt viroid* (HSVd) through seeds of eight grapevines cultivars was demonstrated by using a combination of dot blot hybridization, Northern hybridization and RT-PCR procedures (Wah and Symons 1997, 1999). *Peach latent mosaic viroid* (PLMVd) was found to be transmitted through pollen by pollinating healthy plants with pollen from infected plants. Two peach cvs. Velvet C and Vivian C were infected when they were pollinated with pollen from cv. Nectaross (Barba et al. 2007). Seed transmission of *Citrus exocortis viroid* (CEVd) through seeds of *Impatiens walleriana* and *Verbena* × *hybrida* was assessed by collecting seeds from commercial sources. All 19 samples contained viroid infection with a seedling transmission rate of 66% in *I. walleriana* and 28% in *V.* × *hybridia*. After 2 years of seed storage, the percentage of seed transmission was reduced considerably in these host plant species (Singh et al. 2009).

2.4.1.2 Nucleic Acid-Based Techniques

Potato spindle tuber viroid (PSTVd) was detected by applying RT-PCR assay. Total nucleic acid (TNA) extracts or Gene Releaser™-treated extracts of tree potato pollen and seeds were subjected to PCR amplification. An amplified full-length PSTVd cDNA could be obtained by treating as few as five pollen grains with Gene Releaser™ (Shamloul et al. 1997). A tissue-printing RT-PCR procedure was simplified by using just filter paper in place of nylon membrane and this format was used to detect PSTVd in potato tubers and in vitro generated potato plantlets. The printed filter paper squares can be stored for about 2 weeks facilitating transport to different locations (Weidemann and Buchta 1998). Dot blot hybridization and RT-PCR procedures were applied to detect the presence of *Peach latent mosaic viroid* (PLMVd) in pollen and seeds of infected plants. PLMVd was present in 100% of analyzed pollen samples collected from nine peach trees used as donors. Specifically, positive signals from hybridization and RT-PCR were obtained in the supernatants and in washed pollen of PLMVd-infected donor trees, indicating that the viroid was located both externally and internally. In PLMVd-infected seeds, the viroid was detected in the integuments of 242 of 500 seeds (49%) and in 224 of 500 seeds of peeled kernels (45%). However, none of the seedlings grown from infected seeds was found to be infected by PLMVd, indicating lack of seed transmission of this viroid (Barba et al. 2007).

Transmission of *Hop latent viroid* (HLVd) through pollen of hop plant was studied during different stages of pollen and seed development. HLVd was eliminated during maturation and germination of hop pollen. HLVd degeneration in hop pollen is a

selective process that efficiently eliminated HLVd during pollen maturation and germination. HLVd was detected by RT-PCR assay only in very low concentrations in mature pollen after anthesis and no viroid could be detected in vitro in germinating pollen, suggesting complete degradation of circular and linear forms of HLVd. Elimination of HLVd from pollen correlated with developmental expression of pollen nuclease and specific RNAses. The developmentally activated HBN1 mRNA and pollen ribonuclease could participate in the mechanism of HLVd recognition and degradation (Matoušek et al. 2008).

The RT-PCR assay was applied to detect *Citrus exocortis viroid* (CEVd) in the seeds of *Impatiens walleriana* and *Verbina* × *hybridia*. CEVd was detected in the non-germinated seeds, as well as in the seedlings that developed from infected seeds. The viroid detection rate in seeds was lower than in seedlings due to smaller amount of seed tissue available, compared with seedlings which might allow viral replication during germination, resulting in the increase in virus concentration to a detectable level. These seedlings were grown in separate plots. In three RT-PCR tests, CEVd was detected in leaves and petals of *Impatiens* and *Verbena* from all CEVd-positive plants, but not from healthy plants. This investigation provided strong evidence for the presence of CEVd in commercially distributed seeds and its seed transmission in these ornamental plant species (Singh et al. 2009).

2.4.2 Detection of Viroids in Propagative Planting Materials

Viroids are primarily disseminated through seeds and vegetatively propagated planting materials. Spread of viroids through contaminated tools may occur in certain cases. Involvement of a natural vector responsible for the spread for any viroid is yet to be demonstrated conclusively. Detection of viroids in asexually propagated planting materials may be achieved by applying the same or similar methods applicable for detection of viroids in different plant organs. But reliable specific and successful diagnostic methods have to be selected by quarantine and certification personnel who need results rapidly. Conventional biological methods depending on inoculation on indicator/assay plants require large greenhouse space and long time to provide results which are often inconsistent. Modern molecular techniques developed in one country, on the other hand, are more precise, reliable and rapid, but need validation by testing the protocols in several locations for the reliability of results obtained and proof of cost-effectiveness.

Potato spindle tuber viroid (PSTVd) could be detected in seven potato cultivars by employing DIG-labeled cDNA. This procedure was effective in detecting PSTVd directly in the extracts of potato tubers and leaves (Nakahara et al. 1997). It was possible to detect *Peach latent mosaic viroid* (PLMVd) by employing labeled cRNA probes for hybridization. PLMVd was efficiently detected in stem pieces and dormant buds of peach (Skrzeczkowski et al. 1996). Similar approach was followed for detection of PLMVd in addition to bioindexing on GF305 seedlings to confirm the results obtained from hybridization technique (Turturo et al. 1998). In a later

investigation, a protocol for efficient extraction of stone fruit viroids was developed for use in RT-PCR assay for the detection of PLMVd in planting materials. Total RNA was extracted from leaves, petioles and bark of test plants. Two primer pairs which amplified the expected PCR products of 208- and 114-bp were detected in the agarose gels after electrophoresis and staining with ethidium bromide (Hassan et al. 2004).

Chrysanthemum stunt viroid (CSVd) and *Chrysanthemum chlorotic mottle viroid* (CCLMVd) were detected by employing a microtissue direct RT-PCR protocol. The presence of these two viroids could be detected in very small amounts of tissues of chrysanthemum plants, facilitating the identification of viroid-free plants. Tissue samples are taken at a depth of 0.1–0.2 mm using a razor or syringe and they are transferred directly to reverse transcription (RT) mixtures. Both viroids were detected in plants with varying concentration of viroids. This protocol has the potential for application for viroid detection in plants generated from apical meristem (Hosokawa et al. 2006).

Evaluation of the sanitary status of the Clonal Genebank of stone fruit was taken up by employing molecular methods in Canada. A total of 336 trees consisting of 116 peach and nectarine, 84 sweet and sour cherries, 54 plums, 44 apricots and 38 other cherries was tested by tissue-printing hybridization (TPH) procedure, for the presence of *Peach latent mosaic viroid* (PLMVd) and *Hop stunt viroid* (HSVd). Infection of thirty samples constituting 28 peaches and nectarines infected by PLMVd and two apricots infected by HSVd was revealed by TPH method. The results of this investigation formed the basis for selecting mother plants for vegetative propagation (Michelutti et al. 2005).

Sanitary status of citrus varieties and/or clones with respect to viroid infection was evaluated. Commercial groves were surveyed to select elite mother plants of citrus. All mother plants tested for viroids by RT-PCR assay were free of *Citrus tristeza virus* (CTV), and *Citrus variegation virus* (CVV). But they were found to be infected by *Citrus exocortis viroid* (CEVd) and *Citrus bent leaf viroid* (CBLVd). Micrografting in vitro technique was successful in eliminating the viruses and viroids in three lemon, six mandarin and twenty ‘Jaffa’ orange plants which formed the primary source materials for further propagation to government mother stock plantations, private nurseries and growers in Cyprus (Kaparia-Isaia et al. 2007).

Citrus samples collected from eight provinces of China and maintained in a screenhouse of National Citrus Virus Exclusion Center (NCVEC) were tested for the presence of various viroids infecting citrus. A simple and one-step multiplex RT-PCR procedure was developed for detecting five viroids, *Citrus exocortis viroid* (CEVd), *Citrus bent leaf viroid* (CBLVd), *Hop stunt viroid* (HSVd), *Citrus viroid III* and *IV* (CVd-III and IV). A micro and rapid total nucleic acid (TNA) extraction procedure was employed for extracting TNAs from citrus samples. The assay was performed with a cocktail of primer pairs specific to all five viroids for their simultaneous detection in one-step multiplex RT-PCR. Most of the plants tested, harbored more than one viroid species and a few plants were infected up to four citrus viroids. This investigation highlights the fact that contamination of a single mother tree may result in the spread of viroids to several locations through propagative

planting materials obtained from that single mother tree. Hence, it is imperative that all source plants should be subjected to rigorous testing prior to multiplication of planting materials in the nurseries by employing reliable and effective diagnostic techniques (Wang et al. 2009).

Appendix 1: Detection of Plant Viruses by Different Formats of Enzyme-Linked Immunosorbent Assay (ELISA) (Clark and Adams 1977)

Double-Antibody Sandwich (DAS)-ELISA

(i) Precipitate globulins from the antiserum using 36% sodium sulfate; wash the precipitate with 18% sodium sulfate and store at −70°C; conjugate a portion of globulin with alkaline phosphatase using glutaraldehyde as the coupling agent.
(ii) Dilute the globulins fraction (unlabeled) with 0.05 M carbonate buffer at pH 9.6 to yield a concentration of 10 μg protein/ml; add 200 μl of antibody solution to each well in polystyrene ELISA plates and incubate at 37°C for 3–5 h; empty the wells, wash thrice with 0.15 mM phosphate buffered saline solution pH 7.2 containing 0.05% Tween 20 (PBS-Tween) and dry.
(iii) Add samples (purified antigen or extracts of infected tissues) in 200 μl quantities in PBS-Tween; incubate at 4°C overnight or for 18 h and wash the wells as before.
(iv) Add aliquots of 200 μl of enzyme-labeled antibody conjugate to each well; incubate for 4 h at 37°C and wash the wells as before.
(v) Add enzyme substrate *p*-nitrophenyl phosphate at a concentration of 1 mg/ml in diethanolamine buffer at pH 9.8 at room temperature; stop the reaction after 30 min by adding 3 M NaOH at 50μl/well.
(vi) Determine the color intensity (OD) at 405 nm in an ELISA reader.

Direct Antigen Coating (DAC)-ELISA

(i) Add samples at 200 μl to each well in the ELISA plate, incubate at 37°C for 1 h and wash the wells with PBS-Tween.
(ii) Add antiserum at suitable dilution at 200 μl/well; incubate for 1 h at 37°C and wash the wells with PBS-Tween.
(iii) Add enzyme-labeled antirabbit IgG at 200 μl to each well; incubate for 1 h at 37°C and wash the wells with PBS-Tween.
(iv) Follow steps (v) and (vi) as in DAS-ELISA.

Protein A-Coating ELISA

(i) Dissolve protein A (1–10 mg/ml) in carbonate buffer; dispense 200 µl/well in ELISA plate; incubate for1 h at 37°C and wash in PBS-Tween.
(ii) Dispense antiserum (at suitable dilution) at 200 µl/well; incubate for 1 h at 37°C and wash with PBS-Tween.
(iii) Dispense 200 µl of samples (purified antigen/extracts of tissues at suitable dilutions); incubate at 37°C for 1 h and wash the wells with PBS-Tween.
(iv) Dispense 200 µl of antiserum and proceed as in step (ii).
(v) Dispense enzyme-labeled antirabbit IgG or Fc at 200 µl/well; incubate for 1 h at 37°C and wash with PBS-Tween.
(vi) Follow steps (v) and (vi) as in DAS-ELISA.

Indirect ELISA

(i) Dispense goat or chicken antivirus globulins (1–10 µl/ml) at 200 µl/well; incubate at 37°C for 1 h and wash with PBS-Tween.
(ii) Dispense 200 µl of suitably diluted samples in each well; incubate at 37°C for 1–3 h and wash with PBS-Tween.
(iii) Dispense 200 µl of antivirus rabbit globulin/well; incubate at 37°C for 1–3 h and wash with PBS-Tween.
(iv) Dispense 200 µl of antirabbit globulin conjugate/well; incubate for 1 h at 37°C.
(v) Follow steps (v) and (vi) as in DAS-ELISA.

Appendix 2: Detection of *Grapevine leaf roll-associated virus-3* Using a Single-Chain Fragment Variable Antibody in ELISA (Cogotzi et al. 2009)

(i) Coat the microtiter plates with anti-GLRaV-3 PAb (IgGLR3, 0.5 mg/ml) (Agritest, Italy) or purified scFvLR3 or C_L-LR3 at different concentrations (starting from 0.33 mg/ml) and incubate for 2 h at 37°C.
(ii) Wash the plates three times with PBST containing 0.05% Tween-20 and incubate overnight at 4°C after adding extracts from infected grapevine petioles or phloem tissues that are crushed in ten volumes of extraction buffer [PBS containing 0.05% Tween-20, 20% polyvinylpyrrolidone (PVP), pH 7.4] and centrifuge at 300 g for 3 min.
(iii) Detect the captured antigens with an alkaline phosphatase (AP)-conjugated anti-GLRaV-3 IgG (IgGLR3AP, Agritest s.r.1), scFvLR3 (scFvLR3AP) or C_L-LR3 (C_L-LR3AP), diluted in conjugate buffer (PBS containing 0.05%

Tween-20, 20% PVP, 2% bovine serum albumin, pH 7.4) and incubate for 3 h at 37°C and wash the plate as done before [step (ii) above].

(iv) Incubate the plates with 1 mg/ml of *p*-nitrophenylphosphate in substrate buffer (0.1 M Tris–HCl, pH 9.5).

(v) Record the absorption values at 405 nm after 1 h using ELISA reader.

Appendix 3: Detection of *Iris yellow spot virus* (IYSV) by ELISA Incorporating a Blocking Agent (Smith et al. 2006)

(i) Extract the virus from plant tissues in phosphate buffered saline (1g/20 ml) consisting of 10 mM potassium phosphate, 150 mM sodium chloride, pH 7.2, Tween 20 (5 ml/l) and polyvinylpyrrolidone (20g/l) (PBST) using a leaf press (Pollahne, Hannover, Germany).

(ii) Follow double antibody-sandwich ELISA protocol (Appendix 1).

(iii) Incorporate a blocking step by adding skim milk powder (5% w/v) to the conjugate buffer.

(iv) Perform the tests with and without the blocking step to determine the test reliability for both sets of tests.

Appendix 4: Detection of *Cucumber mosaic virus* (CMV) by Dot Immunobinding Assay (DIBA) (Zein and Miyatake 2009)

(i) Collect young upper leaves from healthy and CMV-infected plants; roll them longitudinally to a form a tight scroll; cut with a sharp razor blade at the center and hold the cut surface immediately on the surface of a PVDF membrane (Bio-Rad) for 6–8 s.

(ii) Homogenize the sample tissues with extraction buffer; use the extract for diluting the purified preparations (1:100) and gently transfer 2 µl of the mixture onto a PVDF membrane (0.45 µm pore size, Bio-Rad).

(iii) Air-dry the membranes; block for 30 min with TBS buffer consisting of 10 mM Tris–HCl, pH 7.4, 0.15 M NaCl) containing defatted milk powder (50 g/l) (TBS-milk buffer) or in PBS buffer consisting of 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, 3 mM NaN_3, pH 7.4) containing 20 g/l Triton X-100 and defatted milk powder (PBS-milk buffer); wash the membranes three times in distilled water, if PBS-milk buffer is used.

(iv) Incubate the membrane for 60 min in 1 µg/ml MAbs diluted with TBS-milk buffer or PBS-milk buffer; wash times of 5 min each with TBST or PBST (TBS or PBS + Tween-20, 3g/l) followed by two washes with distilled water.

(v) Incubate the membranes for 60 min in 1/5,000 dilution in TBS-milk or PBS-milk buffer with appropriate goat anti-mouse alkaline phosphatase (AP)-conjugated antibody (Amersham Pharmacia Biotech).
(vi) Wash the membranes as before and equilibrate in substrate buffer (0.1 M Tris–HCl, pH 9.5) for 5 min and the chromogenic substrate 5-bromo-4-chloro-3-indolyl-phosphate/4-nitroblue tetrazolium chloride (BCIP/NBT).
(vii) Observe the development of purple color indicating positive reaction.

Appendix 5: Detection of *Tomato spotted wilt virus* (TSWV) in Different Tissues of Infected Plants by Tissue Blot Immunoassay (TBIA) (Whitefield et al. 2003)

(i) Roll the healthy and infected leaves of herbaceous plants into a tight bundle; cut with a razor blade; gently blot the cut surface onto 0.45 μm Nitro ME nitrocellulose (NC) membranes (Micron Separations, Inc., USA) for 3–5 s.
(ii) Incubate the rootlets sampled from dried test plant tubers in deionized water of 16–20 h at room temperature (RT) in 24-well microtiter plates (Costar Corp., USA); cut a cross section of each rootlet with the razor blade and press the cut surface onto the NC membrane.
(iii) Air-dry the blotted membranes at RT; place them in glass hybridization tubes; allow reaction with reagents as detailed below: perform all incubations and washing steps at RT in a hybridization oven (Lab-Line, USA) at 16 rpm and expose the membranes sequentially to reagents and pour the reagents in and out of the tube without removing the membrane.
(iv) Block membranes with 10 ml of 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) consisting of 0.14 M NaCl, 1.0 mM potassium phosphate, 8.0 mM sodium phosphate and 2.5 mM KCl, pH 7.5 for 1 h.
(v) Treat the membranes with the appropriate dilution of anti-NSs MAb from stock solution (1 mg/ml) based on preliminary experiments, as working dilutions may vary from 1:100 to 1: 200; dilute the antibody (Abs) in 4 ml of Ab dilution buffer consisting of PBS, 0.1% Empigen BB (Calbiochem, USA) and 0.1% BSA and incubate membranes with Ab for 2 h.
(vi) Wash the membranes three times in PBS with 0.5% Tween (PBST) for 5 min for each wash and incubate in 4 ml of a 1:2,000 dilution of rabbit anitmouse alkaline phosphatase (AP) conjugate (Bio-Rad) for 1 h.
(vii) Wash the membranes again in PBST three times for 5 min/wash; add AP substrate (Sigma Fast BCIP/NBT tablets) and incubate the membranes for 5–15 min.
(viii) Rinse the membranes in water and examine after air-drying at RT.
(ix) Look for the development of purple precipitate on the membrane, indicating the positive reaction.
(x) Under field conditions, perform the entire procedure in disposable 50 ml polypropylene tubes (Sarstedt, USA); incubate the tubes in pocket or backpack and agitate periodically.

(xi) Actively growing plant samples do not require presoaking step followed for testing dried tubers and perform all other steps without any change as mentioned above.

Appendix 6: Detection of *Citrus psorosis virus* (CPsV) by Direct Tissue Blot Immunoassay (DTBIA) (Martin et al. 2002)

(i) Select young tender leaves or shoots; cut transversely; roll the leaf blade tightly to form a cylinder and gently press the freshly cut surface onto nitrocellulose membrane of 0.45 μm pore size (Bio-Rad, Spain) or nylon membrane (Amersham, Spain).
(ii) Air-dry the prints; block with TBS buffer (10 mM Tris–HCl, pH 7.4, 0.5 M NaCl) containing 50 g/l defatted milk powder (TBS-milk powder) or in PBS buffer (8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl , 0.14 M NaCl, 3 mM NaN_3, pH 7.4) containing 20 g/l Triton X-100 and 50 g/l defatted milk powder (PBS-milk buffer) and wash the membranes in the latter case with distilled water before incubation with antibodies.
(iii) For indirect detection, incubate the membranes in 1/10,000–1/50,000 dilution of ascites fluid containing MAb 13C5 or 2A3 or a 1:1 (v/v) mixture of the two MAbs in TBS-milk buffer for 90 min.
(iv) Wash three times with TBST or PBST [TBS or PBS plus Tween 20 (3g/l)] and two washes (first and last) with distilled water and incubate in 1/20,000 dilution of TBS-milk or PBS-milk buffer with appropriate alkaline phosphatase (AP) conjugated antibody.
(v) Wash the membranes as done earlier and equilibrate in substrate buffer (0.1 M Tris–HCl, pH 9.5) for 5 min and add the substrate.
(vi) For direct detection, block the membrane as described earlier; incubate in a 1/10,000 dilution of the A322 antibodies conjugated with AP in TBS-milk buffer for 3 h and wash.
(vii) Equilibrated in substrate buffer as indicated earlier; add the substrate and incubate at room temperature; use the chromogenic substrate 6-bromo-4-chloro-3-indolylphosphate (BCIP) or nitroblue tetrazolium (NBT) or chemiluminescent substrates [SPD or CPD- star (Roche Diagnostics)] as per manufacturer's instructions.

Appendix 7: Detection of *Citrus psorosis virus* (CPsV) by Western Blotting Technique (Loconsole et al. 2006)

(i) Grind the infected leaf samples (100 mg) from citrus or *Chenopodium quinoa* in 2.5 volumes of extraction buffer consisting of 0.5 M Tris–HCl, pH 8.8, 2% sodium dodecyl sulfate (SDS), 40% sucrose, 4% 2 mercaptoethanol; boil the extracts for 3 min and centrifuge at 4,000 rpm for 4 min.
(ii) Analyze the aliquots of 5 μl of the supernatant by electrophoresis in 10% SDS slab gels together with recombinant virus coat protein (CP) (5 μg/μl) and 1 μg PC of purified virus preparation from *C. quinoa*.

(iii) Electroblot the gels on polyvinyl difluoride membranes (PVDF, Immobilon-P, Millipore, USA); block with 1% bovine serum albumin (BSA), 5% non-fat dry milk, 0.05% Tween-20 in PBS buffer 1× for 2 h at room temperature and incubate the membranes with crude As.Ps.Rc1 serum diluted 1:2,000, 1:1,000 or 1:500 for 1 h.
(iv) Wash three times of 10 min each in 1 × TBS, 0.1% Tween-20, incubate the membranes for 30 min with a 1:12,000 dilution in blocking solution of a goat antirabbit IgG-AP conjugate (Sigma, USA).
(v) Wash three times of 15 min each; add 1 ml of CDP-star substrate (1:100) (Roche Applied Science, Switzerland) and detect the protein bands by exposure to x-ray films for 30 min.

Appendix 8: Detection of an *Ampelovirus* by Western Blot Analysis (Valverde et al. 1990; Maliogka et al. 2009)

Extraction of ds-RNA from Virus-Infected Plants

(i) Grind 80 g cortical scrappings of grapevine in liquid nitrogen; transfer to a flask along with two volumes of 2 × STE (0.1 M NaCl, 0.05 M Tris-base, 0.001 M EDTA) also containing 2% ß-mercaptoethanol, 1% SDS and 0.7% Na_2SO_3 and shake well for 30 min.
(ii) Centrifuge at 8,000 g for 15 min; transfer 10 ml of the upper aqueous phase to a 50-ml centrifuge tube (if 10-ml tube is not available, adjust the volume by adding 1 × STE); add 2.1 ml 95% ethanol to each tube and mix well.
(iii) For chromatography, add the sample to cellulose (CF-11, Sigma-Aldrich); agitate for 1 h at room temperature and centrifuge at 16,000 g for 5 min.
(iv) Discard the upper phase; dilute the pellet in 200 ml STE 16 (STE 10 × + 16% ethanol) and repeat centrifugation three times with the last one performed at 23,000 g for 7 min.
(v) Dissolve the pellet in STE 16 and transfer to a column and repeat chromatography cycle before the final ds-RNA precipitation.
(vi) Treat with RNase, DNAse I and proteinase K; analyze in 1.3% low-melting-point agarose (Applichem, Germany) for 3.5 h at 100 v and visualize using SYBR gold (Molecular probes, Eugene, USA).

Production of Virus-Specific Antiserum with Recombinant Coat Protein (CP)

(i) Amplify the full-length CP gene using the primer set M3CpF/M3CpR which includes the *Sph*I and *Pst*I restriction sites respectively at the 5′ end; ligate the amplified DNA fragment directionally in the pQE31 protein expression vector (Qiagen, Germany) bearing 6 His residues at the N-terminus and introduce the

plasmid by transformation into *Escherichia coli* strain M15 (Qiagen, Germany).

(ii) Add 1 mM isopropylthiogalactopyranoside (IPTG) to the bacterial culture for induction of protein expression and incubate for 4 h.

(iii) Purify the expressed CP protein under denaturing conditions using affinity chromatography with Ni-NTA resin (Qiagen) as per the manufacturer's recommendations.

(iv) Immunize a New Zealand white rabbit with three intramuscular injections of 150, 272 and 300 mg of purified recombinant protein respectively in an equal volume of incomplete Freund's adjuvant at 2-week intervals.

(v) Collect the blood at 10 days after the last injection and purify the antibodies using protein A affinity chromatography.

(vi) Perform Western blotting analysis using the antiserum at a dilution of 1/2,000 and extracts from mature leaf petioles or cortical scrappings prepared by grinding in five volumes of loading buffer 2 × 100 mM Tris–HCl, pH 6.8, 2% ß-mercaptoethanol, 4% SDS, 0.2% bromophenol blue and 20% glycerol.

Appendix 9: Detection of *Phalaenopsis* Orchid Viruses by Immunoblot Technique (Jan and Yeh 1995; Zheng et al. 2008a,b)

(i) Grind the leaves from mock-inoculated and virus-infected plants with three volumes (v/w) of dissociation buffer containing 100 mM Tris–HCl, pH 7.2, 2% ß-mercaptoethanol, 10% sucrose, 0.005% bromophenol blue and 10 mM EDTA.

(ii) Boil the crude antigen extract and separate electrophoretically on a 12% sodium dodecyl sulphate (SDS)-polyacrylamide gel.

(iii) Transfer to a 0.45 mm nitrocellulose membrane (Bio-Rad, USA) and pre-incubate with TSW buffer containing 10 mM Tris–HCl, pH 7.4, 0.9% NaCl, 0.25% gelatin, 0.1% Triton X-100 and 0.2% SDS.

(iv) Incubate with primary antiserum against target virus (CP or NP) at required dilution (1:4,000) in TSW buffer for 1 h and then with alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG (Jackson Immuno Research Labs, USA) for 1 h (1:5,000 dilution in TSW buffer).

(v) Develop color by incubating with chromogenic substrates-nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolylphosphate paratoluidine salt in 100 mM NaCl, 5 mM $MgCl_2$ and 100 mM Tris–HCl, pH 9.5, at room temperature for 10–30 min.

(vi) Stop the reaction by rinsing the membrane in distilled water.

(vii) Use extracts from healthy leaves and normal serum as controls.

Appendix 10: Detection of *Passion fruit woodines virus* (PWV) by Light Microscopic Staining Technique (Jan and Yeh 1995)

(i) Prepare fresh epidermal strips from healthy and virus-infected leaves of golden passionfruit and treat them with 5% Triton X-100 in distilled water for 5 min.
(ii) Float the epidermal strips on a solution of 10% pectinase and 4% cellulose in 20 mM phosphate-buffered saline (PBS, pH 7.2) for 30–60 min.
(iii) Incubate the strips first with antisera against cylindrical inclusion protein (CIP) or the 51K protein (amorphous inclusion) at 1:400 dilution for 30 min at 37°C and incubate with protein A-gold complex (Sigma, P-1039 diluted at 1:6) for 30 min at 37°C.
(iv) Rinse the strips extensively with PBS after each treatment; mount the slides in 10% glycerine and view the strips under a light microscope with a blue filter.

Appendix 11: Detection of *Potato virus Y* (PVY) by Immunohistochemistry Method (Ryang et al. 2004)

(i) Immerse the sampled plant tissues immediately in fixative consisting of 50% ethanol, 5% acetic acid and 3.7% formalin.
(ii) Dehydrate and infiltrate the tissues in a graded series of ethanol solutions – 50%, 70%, 90% and 100% each for 30 min and embed them in paraffin (Paraplast-plus, Sigma).
(iii) Cut sections of 12 μm thick of stem tissue using a rotary microtome and place them on glass slides.
(iv) Dewax the sections in xylene and wash in 100% ethanol.
(v) Dehydrate in a graded series of ethanol (70%, 50% and 30%) and distilled water for 10 min each.
(vi) Incubate the sections in PBST and 1% BSA for 1 h and then incubate with PVY-CP-specific antibody diluted to 1:200 in PBST/BSA for 2 h at 37°C.
(vii) Incubate the sections with alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma) diluted to 1:200 in PBST/BSA for 2 h at 37°C.
(viii) Wash the sections and stain with BCIP/NBT liquid substrate system.
(ix) Wash the stained sections in distilled water and observe under the microscope.

Appendix 12: Detection of *Grapevine leaf roll-associated virus 3* (GLRaV-3) by Electron Microscopic Decoration Technique (Cogotzi et al. 2009)

(i) Crush the tissues infected by target virus in 0.1 M phosphate buffer, pH 7.2 containing 2% nicotine.

(ii) Sensitize the carbon grids by floating on drops of rabbit IgG capable of recognizing GLRaV-1, GLRaV-3 and GLRaV-7 or purified C_L-LR3 diluted in 0.1 M phosphate buffer, pH 7.2 for 1 h at 37°C.
(iii) Wash the grids with buffer; float the grids on drops of plant tissue extracts at 4°C for 48 h to allow trapping of target virus particles.
(iv) For virus decoration, rinse pre-coated grids exposed to the antibodies described above or C_L-LR3 diluted in PBS.
(v) Allow reaction of CL-LR3 with antibodies conjugated with alkaline phosphatase (C_LAP) diluted to1:50 and wash thrice with PBS.
(vi) Negatively stain the trapped and decorated virus particles with 2% uranyl acetate and examine under transmission electron microscope (TEM).

Appendix 13: Detection of *Grapevine fan leaf virus* (GFLV) by RT-PCR Assay (Bashir and Hajizadeh 2007)

Extraction of Total RNA

(i) Incubate 200 mg leaf tissue sample in 1 ml cold extraction buffer consisting of 91 mM K_2HPO_4, 30 mM KH_2PO_4, 292 mM sucrose, 0.22 mM bovine serum albumin (BSA) fraction II, 0.8 mM PVP 25, 30 mM ascorbic acid, pH 7.6 in a mortar for 20 min; grind well; add 1 ml more of extraction buffer and grind to get a homogenous extract.
(ii) Centrifuge at 1,050 g for 4 min; transfer the supernatant to a fresh 1.5 ml tube; centrifuge at 16,800 g for 10 min; discard the supernatant and dissolve the pellet in 50 mM Tris, pH 8.0 containing 10 mM EDTA, 0.1% ß-mercaptoethanol and 1.25% SDS and incubate for 10 min at 60°C.
(iii) Add 80 µl 5 M potassium acetate; place the tube on ice for 30 min and centrifuge for 15 min at 16,800 g.
(iv) Transfer the supernatant to a fresh tube; add 0.1 volume of 3 M sodium acetate and 1 volume of ice-cold isopropanol; keep the tube for 1 h at −20°C and centrifuge at 16,800 g for 30 min.
(v) Wash the pellet with 200 µl 80% ethanol; dry and suspend in 30 µl sterile distilled water.

Synthesis of First Strand cDNA

(i) Incubate 2.5 µl aliquot containing 50 pmol oligo $d(T)_{16,}$ 1.0 µl RNA and 1.1 µl RNAase-free water at 70°C for 5 min and chill immediately on ice.
(ii) Add 7.5 µl of reverse transcription mix containing 1 × reverse transcription buffer (Fermentas, Lithuania), 10 mM of each dNTP, 10 U RNAase inhibitor

and 100 U M-MuLV reverse transcriptase (Fermentas) and incubate at room temperature for 15–20 min, followed by incubation at 42°C for 60 min.
(iii) Stop the reaction by heating at 70°C for 10 min.

Polymerase Chain Reaction (PCR)

(i) Use appropriate pairs of primers (S2515/A3300 and CP433V/912C).
(ii) Dispense 12.5 µl PCR mix containing 2 mM magnesium chloride, 0.5 pmol each of the primer, 1 × *Taq* DNA polymerase buffer, 0.3 U *Taq* DNA polymerase (Fermentas) and 2.5 µl of cDNA.
(iii) Provide the following thermocycling conditions:
For S2515/A3300 primer set: one cycle at 94°C for 1 min, followed by 35 cycles of 94°C for 30 s, 40 or 50°C for 30 s, 72°C for 60 s and finally one cycle of 72°C for 10 min.
For CP433V/912C primer set: same as for S2515/A3300 primer set, except performing annealing at 40°C or 48°C for 45 s and extension at 72°C for 45 s.

Appendix 14: Detection of Strawberry Viruses by Multiplex RT-PCR Assay (Chang et al. 2007)

Total Nucleic Acid Extraction

(i) Grind 0.05 g leaves in a mortar with liquid nitrogen; transfer the powdered tissue to a microfuge tube containing 500 µl CTAB buffer consisting of 2% CTAB, 100 mM Tris, pH 8.0, 20 mM EDTA, 1.4 M NaCl; add 2% ß-mercaptoethanol just before use and incubate for 20 min at 65°C.
(ii) Extract the suspensions with chloroform/isoamyl alcohol (24:1) twice and separate the phases by spinning.
(iii) Add 1/10 volume NaAc, pH 5.2 and 2.5 volume ethanol to water phase; mix well; remove the pellet; wash with 70% ethanol and dry on a clean bench.
(iv) Resuspend in 50 µl of DEPC (diethylpyrocarbonate) water.

Extraction of Total RNA

(i) Follow the steps A(i) and A(ii) above.
(ii) Add ¼ volume 10 M LiCl to water phase; precipitate RNA at −20°C for 60 min; redissolve the precipitate in TE buffer and extract with phenol/chloroform/isoamylalcohol and chloroform/isoamylalcohol successively.
(iii) Precipitate RNA; wash twice; dry on a clean bench and resuspend in 30 µl DEPC water.

Reverse Transcription (RT) Reaction

(i) Incubate 30–50 ng (1 µl) of total RNA or 90–130 ng (1 µl) of total nucleic acid extract in 11.5 µl of sterile distilled water plus 1 µl of dNTPs (each 2.5 mM), 0.5 µl random primer (9-mer) (50 µM) and 0.5 µl oligo d(T)18 (50 µM) (TaKaRa, Japan) at 65°C for 5 min.
(ii) Add 4 µl buffer 5 × (TaKaRa, Japan), 0.5µl RNasin and 1 µl AMV (5 U/µl) TaKaRa, Japan) and incubate at 37°C for 2.5 h.
(iii) Inactivate the enzyme by incubating at 70°C for 15 min.

Polymerase Chain Reaction (PCR)

(i) Use primer pairs Y1/Y2 for *Strawberry mild yellow edge virus* (SMYEV), D1/D3 for *Strawberry mottle virus* (SMoV) and I2/SM2 for *Strawberry vein banding virus* (SVBV).
(ii) Use single PCR reaction mixture containing 1 µl of first strand cDNA, 1 × PCR buffer, 0.2 µM dNTPs, 0.2 µM each primer, 1 U *Taq* polymerase in a total volume of 20 µl.
(iii) Provide the following conditions for amplification:
For Y1/Y2 or D1/D3 primer pair: initial denaturation step at 94°C for 2 min, followed by 35 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 30 s and final elongation at 72°C for 5 min.
For I2/SM2 primer pair set: initial denaturation step at 94°C for 3 min, followed by 35 cycles at 94°C for 30 s, 55°C for 60 s, and 72°C for 2 min and final elongation at 72°C for 5 min.

Duplelx PCR Assay

(i) For SMYEV and SMoV use reaction mixture containing 1µl of first strand cDNA , 2 × PCR buffer, 0.2 mM dNTPs, 0.27 µM D1/D3 primers, 1 U $_{Taq}$ polymerase in a total volume of 20 µl.
(ii) Provide the following cycling conditions: denaturation step at 94°C for 2 min, followed by 35 cycles at 94°C for 30 s, 57°C for 40 s and 66°C for 2 min and final elongation at 72°C for 5 min.

Multiplex PCR Assay

(i) For SMYEV, SMoV and SVBV, use reaction mixture containing 1µl of first strand cDNA, 2 × PCR buffer, 0.2 mM dNTPs, 1 U Taq polymerase and use primer concentration 0.17 µM D1/D3, 0.25 µM I2/SM2 and 0.15 µM Y1/Y2 in a total volume of 20 µl.

(ii) Provide the following cycling conditions: initial denaturation step at 94°C for 2 min, followed by 35 cycles at 94°C for 30 s, 55°C for 40 s and 68°C for 2.5 min and final elongation step at 72°C for 5 min.
(iii) For duplex and multiplex assays, resolve the PCR products by electrophoresis in 1.5% agarose gels in 1 × TBE buffer and after staining with ethidium bromide visualize under UV light.

Appendix 15: Detection of *Pepino mosaic virus* (PepMV) by Multiplex One-Step RT-PCR Assay (Alfaro-Fernández et al. 2009)

Extraction of Total Nucleic Acids

(i) Place 0.5–0.6 g of leaf tissue in a sample bag (Agadia, USA) suitable for use on a Homex grinder (Bioreba, USA) and homogenize with 5 ml of lysis buffer consisting of 4 M guanidine isothiocyanate, 0.2 M sodium acetate, pH 5.0, 25 mM EDTA, 2.5% PVP-40 (w/v) and 1% 2-mercaptoethanol (v/v) (added just before use).
(ii) Transfer the homogenate (1 ml) to a graduated microfuge tube using a disposable plastic transfer pipette; mix with 100 µl 20% sarkosyl (w/v) and incubate at 70°C for 10 min with agitation (1,200 rpm) using an Eppendorf thermometer.
(iii) Transfer approximately 600–650 µl homogenate to QIA Shredder (Qiagen) column using a new disposable plastic pipette and centrifuge at 14,000 × g for 2 min in an Eppendorf microcentrifuge.
(iv) Transfer 25 µl of column flowthrough to a new 1.5 ml microfuge tube; mix with 225 µl 95% ethanol; load onto an RNeasy (Qiagen) column and centrifuge at 8,000 × g for 45 s.
(v) Discard the flowthrough; apply 700 µl RW1 buffer (Qiagen) to the column and wash thoroughly by centrifugation at 8,000 × g for 15 s.
(vi) Transfer the column to a clean 2 ml collection tube; apply 500 µl RPE buffer (Qiagen) to the column and wash through by centrifugation at 8,000 × g for 15 s.
(vii) Discard the flowthrough; apply additional 0.5 ml RPE buffer to the column and centrifuge at 14,000 × g for 5 min to remove all traces of ethanol.
(viii) Transfer the column to a new 1.5 ml microfuge tube and elute RNA by applyi ng 100 ml RNA-free water and centrifuge at 8,000 × g for 1 min.
(ix) Label the collection tubes appropriately and store the RNA samples at −80°C till use for RT-PCR assay.

Primers Employed

(i) Select specific region of the RNA polymerase gene for identification of PepMV isolates; three specific primers – PepMV-DEP, PepMV-D1 and PepMV-D2 – and a common antisense primer (PepMV-R) for differentiation of EU/PE, CH1/US1 and CH2/US2 groups of PepMV respectively.

RT-PCR Assay

(i) Perform RT-PCR reaction using the SuperScript III one-step RT-PCR system with Platinum *Taq* DNA polymerase kit (Invitrogen Life Technologies, Spain); use a mixture of all primers at a final concentration of 0.25 pmol/µl and the primers of internal control *Rbc1* gene at 0.05 pmol/µl corresponding to the partial sequence of ribulose 1,5-biphosphate carboxylase chloroplast gene.
(ii) Provide the following conditions for amplification: initial incubation at 50°C for 30 min, followed by 94°C for 2 min and 40 cycles of 94°C for 15 s, 50°C for 30 s and 68°C for 1 min and a final incubation at 68°C for 10 min.
(iii) Resolve the amplified PCR products by electrophoresis, using 1.2% agarose/TAE gels stained with ethidium bromide.
(iv) To confirm the viral origin of the amplified fragment, purify them with High Pure PCR Product Purification Kit (Roche Diagnostics, Germany) and directly sequence the amplicons.
(v) Digest 10 µl of PCR amplicon directly with *Sac*I enzyme (MBI Fermentas, Lithuania) in a total volume of 20 µl as per the manufacturer's recommendations.
(vi) Analyze the digestion products in a 5% TAE polyacrylamide gel followed by staining with ethidium bromide.

Appendix 16: Detection of Orchid Viruses by Multiplex RT-PCR Assay (Lee and Chang 2006)

Simplex RT-PCR Assay

(i) Extract total RNA and viral RNA from plant tissues using Plant Total RNA Extraction Miniprep System (Viogene, USA) as per the manufacturer's instructions.
(ii) Use primer pair CymMV CP-F1/CymMV CP-R1, ORSV CP-F1/ORSV CP-R1 and mt-F2/mt-R1 for the amplification of *Cymbidium mosaic virus* , *Odontoglossum ringspot virus* and the internal control respectively.
(iii) For RT reaction with a total volume of 12 µl, mix 2 µl extracted plant total RNA (200 ng) with/without viral RNA, with 0.5 µl of 5 µM reverse primer, CymMV CP-R1, ORSV CP-R1 or mt-R1; add 5µl double distilled (dd) water; heat the solution for 10 min at 70°C and cool immediately on ice for 5 min.
(iv) Add RT mixture containing 3.25 µl dd water, 2.5 µl 5 × first strand buffer (Promega, USA), 1.2 µl dNTPs (10 mM), 0.25 µl rRNasin (40 U/µl) (Promega) and 0.25 µl AMV reverse transcriptase (10 U/µl, Promega) and incubate at 42°C for 60 min.
(v) For PCR use 20 µl reaction volume containing 2 µl RT product, 2 µl 10 × DyNAzyme™ II DNA polymerase buffer (Finnzymes Inc., Finland), 2 µl forward

and reverse primers 5 μM CymMV CP-F1/CymMV CP-R1, ORSV CP-F1/ORSV CP-R1 or mt-F2/mt-R1, 2 μl dNTPs (2 mM), 0.4 μl DyNAzyme™ II DNA polymerase (2 U/μl, Finnzymes Inc.) and 11.6 μl dd water.

(vi) Perform amplification providing the following conditions: initial denaturation at 96°C for 5 min, followed by 30 cycles of 96°C for 30 s, 50°C for 30 s, 72°C for 30 s, and a final extension step at 72°C for 7 min.

(vii) Analyze the PCR products on agarose gels by electrophoresis, as described in subdivision C below.

Multiplex RT-PCR Assay

(i) Perform RT reaction in the same manner as described above except that the three reverse primers- CymMV CP-R1, ORSV CP-R1 and mt-R1 are to be added simultaneously.

(ii) Carry out PCR with 20 μl volume comprising of 11.6 μl dd water, 2 μl of 10 × DyNAzyme™ II polymerase buffer, 2 μl multiplex primer set (including 2.5 μM mt-F2/mt-R1, 1.25 μM CymMV CP-F1/CymMV CP-R1, 1.25 μM ORSV CP-F1/ORSV CP-R1), 2 μl dNTPs (2 mM); mix 0.4 μl DyNAzymeTM DNA polymerase and assay in the same PCR conditions.

(iii) Analyze the PCR products on agarose gels after electrophoresis as in subdivision C below.

Agarose Gel Electrophoresis

(i) Resolve aliquots of 10 μl of RT-PCR products in 2% agarose gel in TAE buffer consisting of 40 mM Trisacetate and 1 mM EDTA.

(ii) Stain the gel with ethidium bromide (05 μg/ml) and illuminate with UV light.

(iii) Determine the fragment sizes with Kodak Digital Science™ ID image analysis software (Eastman Kodak Co., USA) by comparison with DNA molecular weight markers (Invitrogen, USA).

Appendix 17: Detection of Orchid Viruses by Multiplex RT-PCR Assay Using Simple-Direct-Tube (SDT) Method for RNA Extraction (Suehiro et al. 2005; Yamane et al. 2008)

Extraction of Total RNA by SDT Method

(i) Grind 0.1 g infected leaves in 0.3 ml of phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBST); transfer 50 μl crude sap to a polypropylene

PCR tube (0.5 ml) using a truncated tip (cut 5 mm of the top portion) and incubate for 15 min at room temperature.

(ii) Remove the crude sap using a truncated tip; wash twice with 50 µl PBST; add 30 µl diethylpyrocarbonate water to the tube and incubate at 95°C for 1 min and cool on ice.

(iii) Use the RNA solution for RT-PCR assay.

Multiplex RT-PCR Assay

(i) Use the primer pair CymMV-5034F/CymMV-5189R for CymMV and primer pair ORS CP-F/ORS CP-R for ORSV.

(ii) Use the reaction mixture containing a total 5 µl consisting of 2.375 µl RNA solution, 0.25 µl of 5 µM reverse primer, 1 µl 5 mM MgCl2, 0.5 µl 10 × RT buffer, 0.5 µl dNTP mixture, 0.125 µl RNase inhibitor and 0.25 µl AMV reverse transcriptase; incubate for 30 min at 50°C, 5 min at 99°C and 5 min at 5°C.

(iii) Perform PCR amplification with 5 µl 5 × PCR buffer, 0.25 µl of 5 µM forward primer, 0.125 µl TaKaRa Taq HS and 14.5 µl sterile distilled water added to the cDNA solution.

(iv) Heat the tubes at 94°C for 2 min; provide 35 cycles of amplification-denaturation at 94°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 30 s.

(v) Mix aliquots of 8 µl amplicon with 2 µl 5 × loading buffer; electrophorese in 4% agarose gel in Tris-acetate-EDTA (TAE) buffer at 100 V for 40 min; include a DNA ladder as molecular weight markers.

(vi) Stain the gels with ethidium bromide and photograph on UV transilluminator.

Appendix 18: Detection of Pepper Tobamoviruses by Immunocapture (IC)-RT-PCR Assay (Kim et al. 2006)

Double Antibody Sandwich (DAS)-ELISA

(i) Coat the microtiter plates (Polystyrene plates, Costar) with 100 µl/well of IgG diluted to 1 ng/ml in coating buffer, pH 9.6 at 37°C for 3 h and wash the wells with PBS-T three times for 3 min each.

(ii) Add 150 µl plant extract to each well; incubate overnight at 4°C and wash for three times as done earlier with PBS-T.

(iii) Add 100 µl IgG-enzyme conjugate; incubate for 3 h at 37°C and wash as done before.

(iv) Add 100 µl of substrate (1 µg/ml of *p*-nitrophenylphosphate in 10% diethanolamine, pH 9.8) to each well.

(v) Record absorbance values at 405 nm using microplate reader.

Immunocapture RT-PCR Assay

(i) Coat microtubes (200 µl) with 50 µl antibodies to both viruses [*Pepper mild mottle virus* (PMMoV) and *Tobacco mild green mosaic virus* (TMGMV)] at 1 µg/ml for each tube; incubate at 37°C for 2 h and wash the tubes three times with PBS-T.
(ii) Add 50 µl leaf extract or purified virus preparation to each tube; incubate for 2–3 h at 37°C or overnight at 4°C and remove the solution by pipetting; add 80 µl of PBS-T/tube; centrifuge and remove the wash solution by pipetting.
(iii) Wash with PBS-T and finally with deoinized water.
(iv) Perform RT-PCR procedure using 'AccessQuick RT-PCR Kit (Promega); add reaction mix to microtubes after washing them; reaction mix contains: 12.5 µl mastermixture, 0.5 µl AMV reverse transcriptase; 1 µl (10 pmole) of each primer and nuclease-free distilled water up to a final volume of 25 µl.
(v) Use appropriate primers (PM317-F/PM317-R for PMMoV; CPTMG-F/CPTMG-R for TMGMV) and centrifuge briefly.
(vi) Carry out the RT reaction with one cycle at 42°C for 45 min and 35 cycles of PCR amplification using the step program: 95°C for 45 s; 50°C for 50 s; 72°C for 60 s, followed by final extension at 72°C for 10 min.

Appendix 19: Detection of *Florida hibiscus virus* by Immunocapture (IC)-RT-PCR Assay (Kamenova and Adkins 2004)

(i) Coat sterile 0.6 ml polypropylene microcentrifuge tubes with 100 µl of virus specific IgG (1 mg/ml diluted in 0.05 sodium carbonate buffer, pH 9.6); incubate for 3 h at 37°C and wash three times with PBST in 200 µl/tube.
(ii) Homogenize healthy and virus-infected plant tissues in ELISA sample buffer and centrifuge at 10,000 × g for 10 min at 4°C.
(iii) Prepare suitable dilutions of supernatants and virion preparations; dispense 100 µl aliquots to the coated tubes; incubate overnight at 4°C and wash three times with PBST as done before.
(iv) Design suitable specific primer pairs; synthesize first strand cDNA by Moloney murine leukemia virus reverse transcriptase using 200 U/µl (Promega, USA) at 47°C for 45 min in the buffer provided by the manufacturer.
(v) Provide the following conditions: 30 cylces of PCR amplification with *Taq* polymerase (50 U/µl; Promega) at 94°C for 1 min, 59°C for 1 min and 72°C for 1 and 30 s in the manufacturer's buffer.
(vi) Analyze the amplified products by electrophoresis on native 2% agarose gels and detect the bands by ethidium bromide staining.

Appendix 20: Detection of *Potato virus Y* (PVY) by PCR-ELISA Test (Hataya et al. 1994; Varveri 2000)

(i) Precipitate the PCR product using ethanol; dissolve in TNE buffer (10 mM Tris–HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, pH 8.0) and denature by heating.
(ii) Dilute the denatured product by 200-fold in 10 × SSC (1 × SSC: 15 mM NaCl, 0.015 M sodium citrate, pH 7.0) – 10 mM EDTA, pH 7.0 directly into the microplate wells; incubate for 2 h at 37°C and wash three times with PBS + Tween-20.
(iii) Fill each well with 100 µl hybridization solution consisting of 5 × SSC, 10 mM EDTA, pH 7.0,).1% Tween-20; 50% formamide containing heat-denatured DIG-labeled probe at a dilution of 1:500; incubate at 42°C for 12 h and wash three times.
(iv) Fill the wells with 100 µl alkaline-phosphatase-conjugated anti-DIG antibody (Boehringer Mannheim); incubate for 1 h at 37°C and wash three times.
(v) Add *p*-nitrophenyl phosphate substrate at 1 mg/ml diethanolamine and incubate for 2 h.
(vi) Record the absorbance values at 405 nm with a colorimeter (Dynatech MR 5000).

Appendix 21: Detection of *Plum pox virus* (PPV) by Spot Real-Time PCR Assay (Capote et al. 2009)

Preparation of Crude Extracts

(i) Collect five to eight spurts or dards with dormant or swelling buds/tree in the winter and five to eight spurs or shoots with fully expanded leaves/tree in the following spring; grind the winter samples in plastic bags containing a heavy net (Plant Print Diagnostics, Valencia) using a hammer in the presence of 1:20 (w/v) sodium diethyldithiocarbamate and process the spring samples also in the same buffer in plastic bags containing a soft net (Bioreba), using the Homex-6 machine (Bioreba).
(ii) Prepare serial dilutions of the extract from PPV-infected peach seedling with the extract of healthy seedling.

Spot Real-Time PCR Assay

(i) Load 5 µl of crude extract onto 0.45 µm positively charged nylon membrane (Roche) or on Whatman 3 MM paper filter.
(ii) Provide the reaction cocktail for SYBR Green chemistry: 1 × Power SYBR Green Master Mix (Applied Biosystems), 6.25 units of MultiScribe reverse transcriptase (Applied Biosystems), 10 U of RNase inhibitor, 0.3 µM primer P1, 0.05 µM primer P2.
(iii) Provide RT-PCR variables as follows: 48°C for 30 min, 95°C for 10 min, 45 cycles of amplification at 95°C for 15 s and 60°C for 1 min.

(iv) Prepare a melting point curve for temperatures between 60°C and 95°C.
(v) Perform analysis using an ABI Prism 7000 and StepOnePlus software packages (Applied Biosystems).

Appendix 22: Detection of *Strawberry vein banding virus* (SVBV) by Real-Time NASBA Technique (Vašková et al. 2004)

Extraction of Nucleic Acids

(i) Place leaves in extraction bags containing sample extraction buffer (1:10 ratio, w/v) consisting of 0.14 M NaCl, 2 mM KCl, 2 mM KH2PO4, 8 mM $Na_2HPO_4.2H_2O$, pH 7.4), 0.05% Tween-20 (v/v), 2% polyvinylpyrrolidone 40, 0.2% ovalbumin (w/v), 0.5% BSA (w/v) and 0.05% sodium azide and homogenize with the Bioreba HOMEX 6 homogenizer
(ii) Transfer 100 µl of the homogenized solution for extraction of nucleic acids by the RNeasy Plant Mini Kit or DNeasy Plant Mini Kit (Qiagen, Germany)
(iii) Elute the RNA in 100 µl RNase-free water and store at −60°C.
(iv) Elute DNA in 200 µl low salt DNeasy dilution buffer and store at −20°C.

Real-Time Nucleic Acid Sequence-Based Amplification (NASBA)

(i) Use appropriate primer pair and probe for amplification of template present in the purified preparations or plant tissue extracts containing nucleic acids.
(ii) Add 3 µl sample to 12 µl NASBA premixture (final concentration in 20 µl reaction mixture: 40 mM Tris–HCl, pH 8.5, 0.5 mM DTT, 12 mM KCl, 15% DMSO (v/v), 1 mM dNTPs, 2 mM each of ATP, UTP and CTP, 1.5 mMGTP and 0.5 mM ITP, 0.2 µM of each primer).
(iii) Add 9 ng molecular beacons for each reaction; carry out denaturation of the mixture at 65°C for 5 min and adjust the temperature to 41°C for 5 min.
(iv) Add pre-mixed enzymes, 375 mM sorbitol, 21 µg BSA, 0.08 U RNase H, 32 U T7 RNA polymerase and 6.4 U AMV-RT (BioMerieux BV, the Netherlands) and incubate at 41°C for 90 min.
(v) Perform real-time measurement using the iCycler iQ™ (Real-time PCR Detection System, Bio-Rad, USA) and excite the fluorophore at 575 nm.
(vi) Determine fluorescence emission at 620 nm at 2 min interval.
(vii) Separate 3 µl NASBA amplification product using a nondenaturing gel electrophoresis; and blot on Z-probe nylon membrane.
(viii) Hybridize to SVBV-specific biotinylated probe (Bio1) and visualize using enhanced chemi-luminescent (ECL) detection (Amersham Pharmacia Biotech).
(ix) Expose the membrane to x-ray film.

Appendix 23: Detection and Differentiation of *Citrus tristeza virus* (CTV) Isolates by Single-Strand Conformation Polymorphism Analysis (Sambade et al. 2002)

cDNA Synthesis

(i) Use ds-RNA enriched preparations for cDNA synthesis by RT-PCR as the template and specific primers for genes p18, p13 p20 and p23 to be designed using the sequences conserved in CTV isolates T36, VT and T385.
(ii) Perform RT-PCR in an A20 air thermal cycler (Idaho Technologies, USA) using a reaction mixture (25 µl) containing 20 mM Tris–HCl, pH 8.4, 50 mM KCl, 3 mM MgCl2, 4 mM each of dATP, dCTP, dGTP, and dTTP, 500 µg/ml BSA, 1 µM primers, 4 U RNase OUT, 20 U SuperScript II reverse transcriptase (RT) and 1 U *Taq* DNA polymerase (Life Technologies, USA).
(iii) Provide the following cycling conditions: reverse transcription for 30 min at 46°C, RT inactivation at 94°C for 2 min, 40 cycles of 94°C for 5 s, 55°C for 5 s, 72°C for 30 s and final extension at 72°C for 2 min.
(iv) Analyze the cDNA by electrophoresis on 2% agarose gel.

Single-Strand Conformation Polymorphism (SSCP) Analysis

(i) Mix 1 µl PCR amplicon with 9 µl of denaturing solution consisting of 95% formamide, 20 mM EDTA, pH 8.0, 0.05% bromophenol blue and 0.05% xylene-cyanol; heat at 90°C for 10 min and immediately chill the mixture on ice.
(ii) Separate the DNA strands by electrophoresis in a nondenaturing polyacrylamide minigel (8% acrylamide) using TBE containing 89 mM Tris–borate, pH 8.2, 2 mM EDTA as electrophoresis buffer and a constant voltage of 300 V for 1.5 h or 200 V for 3 h at 4°C.
(iii) Stain the gels with silver nitrate.

Appendix 24: Isolation of ds RNA of Plant Viruses (Zhang et al. 1998)

(i) Freeze plant tissues (leaf, petiole or root) in liquid nitrogen; grind to a fine powder using a pestle and mortar and extract with a mixture of 14 ml 1 × STE (containing 0.05 M Tris pH 6.8), M NaCl and 1 mM EDTA, 2 ml 10% sodium dodecyl sulfate (SDS), 1 ml 2% bentonite and 18 ml 2 × STE-saturated phenol.
(ii) Shake the extract well for 30 min; centrifuge for 15 min at 8,000 rpm and add ethanol to have 16.5% concentration.

(iii) Pass through a cellulose column (Whatman CF-11) that binds ds-RNA; wash thoroughly with 1 × STE buffer at 16.5% ethanol and elute from the column with 1 × STE.
(iv) Precipitate from the eluate with 1/10 volume of 3 M sodium acetate and 2. 5 volumes of ethanol and resuspend the precipitate in 50 µl water.
(v) Electrophorese half of the above preparation through a 6% polyacrylamide gel and stain with ethidium bromide (1 µg/ml).
(vi) Visualize the bands under UV light.

Appendix 25: Detection of Plant RNA Viruses by Macroarray Technique (Agindotan and Perry 2007)

Extraction of Total RNA from Plants

(i) Extract total RNA from samples of 200 mg of leaf tissues using RNeasy Plant Mini Kit (Qiagen) as per the manufacturer's instructions.
(ii) Estimate the RNA concentration at 260 nm using a spectrophotometer.

Probes for Target cDNAs

(i) Design probes (ca. 70 nts) based on the sequences of target viruses (CMV, PVY and PLRV).
(ii) Evaluate virus specificity of each oligonucleotides using the Basic Local Alignment Search Tool (BLAST).
(iii) Use commercially synthesized oligonucleotides (Integrated DNA Technologies, USA).

Printing of Oligonucleotide Probes on Membranes

(i) Dilute the ribosomal and virus-specific DNA oligonucleotide probes to 10 and 20 µM respectively in 1 × spotting buffer containing 4 mM sodium carbonate buffer, pH 8.3, 3 × SSC (consisting of 0.15 M NaCl and 0.15 M sodium citrate), 50% dimethyl sulfoxide (DMSO), 0.01% N-laroylsarcosine and 1 mg bromophenol blue.
(ii) Transfer the probes in 30 µl aliquots into the wells of a polypropylene 384-well microtiter plate (Nalge Nune International, NY) and a clean pin replicator (V & P Scientific Inc., USA) for 384-well plates as per manufacturer's recommendations.
(iii) Cut the Hybond-N Plus membrane (GE Healthcare Biosciences Corp, USA) to the size (12 × 10 mm pieces) of a multiprint device (V & P Scientific) and copier unit (V & P Scientific) aligned over the 384-well plate.

(iv) Print the oligonucleotide probes on a membrane on the same spots twice (pin replicator delivers ca. 0.2 µl per print); allow 5 min for air-drying the membrane before the second printing and fix the printed oligonucleotides onto the membrane by UV-cross-linking for 2 min at 120 mJ/s.

Labeling and Hybridization

(i) Use the labeling kit (AlkPhos Direct labeling kit, GE Healthcare Bio-Sciences Corp) as per the manufacturer's recommendations.
(ii) Wet the membrane by incubating in 0.5% sodium dodecylsulfate (SDS) (w/v) at 55°C for 1 h followed by a rinse in 0.1 M Tris–HCl, pH 8.4 for 5 min.
(iii) Perform prehybridization and hybridization at 55°C using the protocol suggested by AlkPhos Direct kits in 50-ml polypropylene tubes.
(iv) Use 10 ml of hybridization buffer for every 20–25 cm^2 with 200 ng labeled target DNA for prehybridization; use only 5 ml of the buffer for hybridization with 200 ng labeled target DNA and perform hybridization at 55°C overnight.
(v) Wash the membranes twice in a primary wash buffer containing 2 M urea, 0.1% SDS, 50 mM sodium phosphate, 150 mM NaCl and 0.2% blocking agent supplied with the kit at 55°C for 15 min each, followed by washing in a secondary wash buffer containing 50 mM Tris–HCl, 100 mM NaCl, pH 10 for 10 min at room temperature on a shaker.
(vi) Incubate the membranes with CDP-Star chemiluminescent (GE-Healthcare Bio-Sciences Corp) reagent for 5 min; drain and expose to chemiluminescence BioMax film (Kodak Co, NY) for 1 h to overnight.

Appendix 26: Detection of *Peach latent mosaic viroid* (PLMVd) by Hybridization Techniques (Hadidi et al. 1997)

Extraction of Total Nucleic Acids

(i) Powder the leaf tissue sample (0.2 g) with liquid nitrogen; extract in TE buffer consisting of 10 mM Tris–HCl, 1 mM Na^2 EDTA, pH 8.0; treat with an equal volume of phenol/chloroform (1:1) for 15–20 min and centrifuge at 3,000 rpm for 5 min at 4°C.
(ii) Precipitate the nucleic acids from the supernatant by adding 0.1 volume of 3 M sodium acetate, pH 5.3 and 2.5 volumes of absolute ethanol; keep the mixture at −70°C for at least 2 h and centrifuge at 12,000 rpm for 10 min at 4°C.
(iii) Resuspend the pellet in TE buffer; concentrate the total nucleic acids with 20% polyethylene glycol (PEG) 6,000 and 2.5 M NaCl solution; add distilled water

to make up the volume to 100 µl; incubate in ice water for 1 h and centrifuge at 12,000 rpm for 20 min in a microcentrifuge.

(iv) Wash the pellet once in 70% ethanol; dry in vacuo and dissolve in 20 µl deionized water.

Dot Blot Hybridization

(i) Mix 10 µl total nucleic acids of each sample with 10 µl 20 × SSC/formaldehyde solution (3:2, v/v) [1 × SSC = 0.015 M sodium citrate and 0.15 M NaCl, pH 7.0]; keep the mixture at 65°C for 30 min and chill the mixture on ice for 2 min.

(ii) Blot 20 µl of each mixture on a wet 0.45 µM Nytran membrane (Schleicher and Schuell) that have been soaked in a 6 × SSC solution; place the membrane on a minifold apparatus under vacuum and wash with 200 µl 6 × SSC solution.

(iii) Cross-link to the membrane by irradiation in a UV cross-linker.

Northern (RNA) Blot Hybridization

(i) Mix 50 µl of total nucleic acids of each sample with 50 µl 20% PEG 6,000 and 100 µl water; keep on the ice for 1 h and centrifuge at 12,000 rpm for 10 min at 4°C in microcentrifuge.

(ii) Wash the pellet with 70% ethanol; dry under vacuum; resuspend in 10 µl TE buffer.

(iii) Load 10 µl of PEG-precipitated total nucleic acids on a 6% polyacrylamide gel and electrophoresce for 1.5 h.

(iv) Wash the gel with 1 × TAE (40 mM Tris–HCl, pH 8.0, 20 mM sodium acetate and 2 mM EDTA) for 10 min; electrotransfer to a TAE-equilibrated 0.2 µm Nytran membrane at 0.5 A for 14 h at 4°C.

(v) Cross-link to the membrane by irradiation in UV cross-linker.

Appendix 27: Detection of *Coconut cadang-cadang viroid* (CCCVd) by Dot Blot Hybridization Technique (Vadamalai et al. 2009)

Extraction of Nucleic Acids Enriched with the Viroid

(i) Blend the chopped leaf samples (10–20 g) in 120 ml chilled 100 mM $NaSO_3$; filter through cotton muslin; shake vigorously for 30 min at 4°C with polyvinyl

pyrrolidone (20 g/l); mix vigorously with 50 ml chloroform for 5 min and centrifuge at 10,000 × g for 4 min.

(ii) Add polyethylene glycol (PEG) (80 g/l) to the supernatant; dissolve by stirring at 4°C and collect the precipitate after centrifugation as done earlier.

(iii) Extract the nucleic acids by resuspending the precipitate in 2 ml of SDS (10 g/l); add 2 ml aqueous phenol (900 g/l) containing 8-hydroxyquinoline (1 g/l) and shake vigorously.

(iv) Collect the upper phase after centrifugation at 10,000 g for 10 min; reextract with 1 ml phenol and 1 ml chloroform for 5 min; add NaCl to 0.1 M; add cetyltrimethyl ammonium bromide (CTAB) (3.3 g/l) and incubate for 30 min on ice.

(v) Centrifuge at 10,000 × g for 10 min; wash the pellet with 0.1 M sodium acetate in 75% ethanol and once with 100% ethanol and air-dry.

(vi) Resuspend the pellet in 500 µl sterile double distilled water (SDDW) ; add an equal volume of 4 M LiCl; incubate the mixture at 4°C for 15–18 h and centrifuge at 12,000 × g for 15 min.

(vii) Recover LiCl-soluble component; add 2.5 volumes of ethanol; allow the mixture to stand for 2–3 h; collect the precipitate by centrifugation at 12,000 g for 15 min; air-dry the pellet; resuspend the precipitate in 500 µl SDDW and store at −20°C.

Dot Blot Hybridization Assay

(i) Cut leaf samples (1 g) into 50 mm lengths; crush in a plastic bag with 2 ml buffer (2 M NaCl, 100 mM sodium acetate, 10 mM EDTA, 50 mM Tris–HCl, pH 7.5 and 0.1% monothioglycerol) and extract 0.5 ml aliquots with 1% SDS and phenol-chloroform-isoamyl alcohol.

(ii) Precipitate nucleic acids with isopropanol; wash the pellets with 95% ethanol; air-dry and redissolve in sterile water.

(iii) Apply 1 µl samples (at different dilutions 1, 1/10 and 1/100) to nylon membrane (Zeta-Probe, Bio-Rad, USA) and cross-link by UV irradiation.

(iv) Prehybridize the membranes at 65°C for 1 h in buffer containing 5 × SSC (750 µM NaCl, 75 mM sodium citrate, pH 7), 2.5% SDS, 0.3% PVP 40 and 100 µg/ml autoclaved herring sperm DNA.

(v) Exchange the buffer for the same buffer containing 160 pmoles/ml of an oligonucleotide probe labeled with digoxingenin at the 5′ terminus; perform the hybridization at 65°C for 3 h; then at 40°C for 45 h.

(vi) Wash the membrane sequentially in 3 × SSC, 2.5% SDS for 15 min at 38°C; 0.5 × SSC, 2.5% SDS for 15 min at 35°C; then 0.2 × SSC, 1% SDS for 15 min at 60°C.

(vii) Block the membranes and detect the DIG-labeled probe with commercial monoclonal antibodies-phosphatase-conjugated antibody and substrate.

Appendix 28: Detection of *Coconut cadang-cadang viroid* (CCCVd) by Ribonuclease Protection Assay (Vadamalai et al. 2009)

(i) Use a complementary sense RNA (cRNA) probe labeled with ^{32}P transcribed from a pGEM®-T Easy vector (Promega Corp, USA) containing a monomeric insert of the 246-nt form of CCCVd.
(ii) Precipitate the nucleic acids from leaf samples (5 g from oil palm or 1 g from coconut) with three volumes of ethanol in the presence of 0.1 M sodium acetate; air-dry the pellets; resuspend in 30 µl hybridization buffer containing 80% formamide, 40 mM PIPES (1,4-piperazine-diethanesulphonic acid), pH 6.5, 400 mM NaCl, 1 mM EDTA, pH 8.0, probe (2×10^5 c.p.m); heat for 10 min at 95°C and incubate at 55°C for 12–18 h.
(iii) Include a tube containing 100 µg cRNA from wheat germ (Sigma, Australia) as negative control.
(iv) Add to each tube, 350 µl digestion buffer consisting of 300 mM NaCl, 10 mM Tris, pH 7.5 and 5 mM EDTA, pH 8.0 containing 10 µg/ml of RNAse A and 10 U/ml RNAse T1 (Roche) and incubate at 30°C for 1 h.
(v) Stop the RNAse digestion by adding 10 µl of 20% SDS and 2.5 µl of proteinase K (10 mg/l) (Amresco, USA) to each tube and incubate at 37°C for 20 min.
(vi) Add tRNA to a final concentration of 0.025 µg/µl and 400 µl of phenol-chloroform-isoamyl alcohol mix (1:1) followed by final extraction.
(vii) Transfer 300 µl from the supernatant to a new tube; precipitate the nucleic acid with three volumes of ethanol at −70°C for 30 min and collect the pellet by centrifugation.
(viii) Resuspend the pellets in 10 µl of denaturing loading buffer consisting of 0.25% bromophenol blue, 0.25% xylene cyanol, 10 mM sodium EDTA, pH 8.0 and 80% formamide; heat for 5 min at 95°C and chill on ice.
(ix) Fractionate by 5% denaturing PAGE (8 M urea) at 40 mA for 1 h; fix the gel for 10 min in 0.5% acetic acid and 10% ethanol and wash once with double-distilled water.
(x) Transfer to a wet sheet of 3-mm Whatman filter paper; dry for 2 h at 80°C in a gel dryer (Bio-Rad) and autoradiograph at −70°C for 1–72 h with an intensifying screen.

Appendix 29: Extraction of Citrus Viroids for Multiplex RT-PCR Assay (Wang et al. 2009)

Extraction of Total Nucleic Acids

(i) Place the 5–10 mg samples (leaf, bark, fruit skin or root tissues) in 1.5 ml Eppendorf tubes immersed in liquid nitrogen ; grind the tissues with sterile

plastic pestle (Bio-Rad); homogenize with 60 µl TES buffer [100 mM Tris–HCl, 2 mM EDTA and 2% SDS (w/v)] and 60 µl of a mixture composed of water saturated phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v); incubate in a water bath at 70°C for 5–10 min; centrifuge at 12,000 × g for 5–10 min.

(ii) Make a hole at the bottom of a 0.5 ml Eppendorf tube using a hot 25 gauge needle; add a small quantity of glass beads (425–600 µM, Sigma) maintained in TNE buffer (10 mM Tris–HCl, 100 mM NaCl, 1 mM EDTA) for covering the hole in the tube; fill this minicolumn with a slurry of Sephadex G50-80) (Amersham Biosciences) pre-equilibrated with the above TNE buffer; place inside a 2.2-ml centrifuge tube and centrifuge at 5,000 × g for 3 min to pack the matrix.

(iii) Place this packed minicolumn into a sterile 1.5-µl Eppendorf tube; transfer 40 ml of the aqueous phase of the extract (from step (i) above) into the matrix, discarding the pellet and centrifuge the mini-column at 5,000 × g for 4 min to retrieve the eluate (about 20–80 µl).

(iv) Use this total nucleic acid extract directly for RT-PCR or store at −20°C for periods up to 1 year.

(v) Assess the purity of RNA extracted using a spectrophotometer and determine the OD_{260}/OD_{280} ratio (ranging from 1.6 to 1.8).

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Chapter 3
Detection of Viral and Viroid Plant Pathogens in the Environment

Abstract Plant viruses and viroids with obligate parasitism as the mode of existence, have to be in association with living cells of plant hosts always. However, some highly stable viruses have been detected in a free state in soil, water or air. Other viruses depend on the vectors such as insects, mites, nematodes or fungi present in the environment for their spread from infected plants to healthy plants which may be available in the same field or far away. The role of alternative/additional host plant species functioning as sources of virus infection has been demonstrated by applying various diagnostic techniques. The presence of viruses has been detected by employing biological, immunological and nucleic acid-based techniques. The effectiveness and reliability of detection varies significantly. However, molecular techniques have been shown to be more sensitive, specific and fast in providing the results, although they are unable to indicate the level of pathogenic potential of the viruses detected in the environmental samples. Viroids lacking natural vectors and stability outside living cells have been detected in several additional/alternative plant species which may function as reservoir of viroid infection.

Viruses and viroids constitute a group of pathogenic entities with obligate parasitic mode of existence, requiring the presence of living cells for their replication and survival. Most of the plant viruses cannot live in a free state outside the living cells of their hosts. But some of them, because of their highly stable structure, may be present in the soil, water or air in a free state. The vectors of viruses such as insects, mites, nematodes and fungi may either retain infectious virions in their bodies for different periods of time. Some of the viruses are propagative in nature with the ability to multiply in their vectors as in the plant hosts. The vector bodies provide different and alternative biological environment in which viruses can survive in the absence of their natural host plant species as in the case of *Rice dwarf virus.* Viruses and viroids may be able to infect only a few plant species or infect a wide range of plant species which facilitate their survival in the absence of crop plants. Hence, these alternative host plant species provide an important support system for the perpetuation of viruses and viroids in nature. It is important to detect the presence of viruses and/or viroids which are found in single or mixed infections in the host plants species to determine the extent of availability of sources of infection that have a vital role in the incidence and subsequent spread of the diseases.

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3.1 Detection of Plant Viruses in the Environment

3.1.1 Detection of Plant Viruses in Soil

Plant viruses may be present in the natural ecosystems including soil, water, air and forests. Some of the highly stable viruses may be able to remain in the soil in a free state and infect the susceptible plants when they are available and no known biological vectors are involved in their transmission from plant to plant. But the majority of plant viruses have either nematodes or fungi as vectors that remain as a component of soilflora. Hence, these viruses can be grouped into two classes: (i) viruses with abiotic transmission and (ii) viruses with biotic transmission.

3.1.1.1 Detection of Viruses with Abiotic Transmission

Many viruses occurring in forest and some in agricultural ecosystems lack biotic vectors. Soilborne viruses with abiotic transmission belong to the genera *Tobamovirus*, *Potexvirus* and *Tombusvirus* and very little is known about their mechanism of abiotic soil transmission. Different methods have been applied for their detection in the soils.

Biological Methods

Soilborne transmission of *Tomato mosaic virus* (ToMV) and *Tobacco mosaic virus* (TMV) in agricultural or greenhouse settings has been demonstrated (Broadbent et al. 1965; Lanter et al. 1982). Tomato plants became infected, when they were grown in soil that contained ToMV-infected plant debris from previous crop. Root infection of tomato by ToMV carried the virus to young shoots where it could be detected (Broadbent 1965). No causal relationship could be established between the microflora and microfauna of glasshouse soils and the persistence of ToMV in the soils (Broadbent et al. 1965). High level of abiotic soil transmission of ToMV was observed in glasshouse experiments. Almost all tomato plants were infected when the seedlings were raised in soil with infective debris (Pares et al. 1996).

Elution and bait plant methods were adopted to detect infectious tobamoviruses in forest soils of New York State. Soils from two forest sites, Whiteface Mountain (WF) and Heiberg Forest (HF) were tested using *Chenopodium quinoa* as bait plants. Both *Tobacco mosaic virus* (TMV) and *Tomato mosaic virus* (ToMV) were transmitted to *C. quinoa* by elution from one of two HF soil samples, but not from WF soil samples. Abiotic soil transmission of tobamoviruses to trees may result in localized spread and persistence of these viruses in forest ecosystems (Filhart et al. 1998). The extracts of soils infested with *Pepper mild mottle virus* (PMMoV) produced local lesions on *Chenopodium* spp. The number of local lesions in this infectivity assay indicative of virus concentration in the sample, was consistent with the absorbance values in indirect-ELISA tests (Takeuchi et al. 2000).

Immunoassays

Enzyme-linked immunosorbent assay (ELISA) was employed to detect *Tomato mosaic virus* (ToMV) in tomato plants grown on soils with infective plant debris. Based on visual examination, the apparent infection level was only 0.10%. But the presence of ToMV was detected in up to 70% of the tomato plants by ELISA tests. Immunoelectron microscopy revealed the presence of ToMV virions in roots and leaves of tomato plants grown on soils containing infective plant debris (Pares et al. 1996). The bait plants *Chenopodium quinoa* were planted in soils amended with ToMV eluted from organic and mineral fractions of soils. DAS-ELISA test was applied to detect the presence of ToMV in the roots of bait plants. The virus was detected in the roots of *C. quinoa* grown on soil fractions from two locations in the USA. Both ToMV and *Tobacco mosaic virus* (TMV) were transmitted from only 1 of 13 concentrated soil eluates and the viruses were detected by ELISA in 12 of 73 (16%) of bait plant root extracts representing 5 of 13 soil samples (38%). Apparently the bait plant method was more sensitive than elution for detection of tobamoviruses in forest soils (Filhart et al. 1998).

The non-precoated indirect (Id)-ELISA format was developed for detecting *Pepper mild mottle virus* (PMMoV) in field soils. Absorbance values obtained using Id-ELISA were relatively low after directly applying PMMoV-infested soil extract. But heat treating the soil extract prior to testing significantly enhanced the absorbance values. The heat treatment was found to be an essential requirement for efficient detection of PMMoV in the infested soil samples, although the efficiency of virus recovery differed depending on the soil properties. The results of infectivity assays based on number of local lesions on *Chenopodium* spp. corroborated the results obtained from Id-ELISA tests. Id-ELISA format combined with heat treatment of soil extract may provide reliable results for detection of tobamoviruses in field soils (Takeuchi et al. 2000).

The ELISA formats have been successfully employed for the detection of viruses not only in the plants, but also in soil and other environmental samples. DAS-ELISA format was applied to check the presence of *Pepper mild mottle virus* in the extracts of soil from fields cropped to green pepper (*Capsicum annuum)* and it was optimized for the detection of PMMoV. Positive results obtained with ELISA format were confirmed by inhibition testing using specific anti-PMMoV antibody, immuno-electron microscopy (IEM), RT-PCR assay and infectivity tests on diagnostic/assay host plant species. Soils with infestation of PMMoV could be detected and identified reliably by employing the DAS-ELISA procedure resulting in avoidance of possible damage to green pepper crops (Ikegashira et al. 2004). Sugar beet crops suffer considerably due to *Beet necrotic yellow vein virus* (BNYVV) associated with rhizomania disease. Infection of beets by BNYVV could be detected by planting sugar beet bait plants, followed by ELISA test to confirm the presence of the virus in bait plants. Breakdown of resistance of sugar beet varieties could be recognized by ELISA test. Sugar beet cultivars showing resistance, when grown in BNYVV-infested soil from Salinas, California, became susceptible, when planted on BNYVV-infested soil from Imperial Valley, California, as revealed by ELISA assessment of viral infection. The differential reactions of sugar beet cultivars might be due the presence of BNYVV isolates with varying pathogenic potential in different areas (Liu et al. 2005).

3.1.1.2 Detection of Viruses with Biotic Soil Transmission

Majority of soilborne viruses has been shown to be transmitted by organisms such as nematodes and fungi living in the soils. Vectors of viruses are primarily parasitic on the plant species to which viruses are transmitted, while feeding on their plant hosts. Nematodes have been reported to be vectors of several viruses infecting horticultural crops such as grapevine, strawberry and tomato, whereas fungal vectors transmit the viruses infecting agricultural crops such as wheat, barley and peanut. Immunoassays and nuleic acid-based techniques have been successfully applied for the detection of plant viruses in the soilborne vectors.

Immunoassays

An indirect immunofluorescence procedure was developed to localize the nepovirus *Tobacco ringspot virus* (TRSV) in the nematode *Xiphinema americanum.* Treatment of fragments of viruliferous nematodes with PAbs specific to TRSV, followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin G resulted in virus-specific bright fluorescence only in the lumen of the stylet extension and esophagus. TRSV-specific signals were noted in the virus-retention region of 44% of the nematode fragments examined. With longer acquisition access period (duration allowed for the nematodes to be in contact with the roots of TRSV-infected tobacco plants), the percentage of nematodes labeled with virus-specific fluorescence and virus transmission percentage increased proportionally (Table 3.1). This protocol provided a rapid and simple means of monitoring specific attachment of plant viruses in the nematode vector tissues facilitating them to become viruliferous (Wang and Gergerich 1998) (Appendix 1).

The presence of *Tobacco rattle virus* (TRV) in the nematode vector *Paratrichodorus anemones* was detected by employing immunogold labeling technique. TRV particles were found attached to the cuticle lining of the posterior tract of pharyngeal lumen of *P. anemones* (Karanastasi et al. 2001). TRSV and *Tomato ringspot virus* (TomRSV) transmitted by *Xiphinema americanum* were detected in the nematodes by applying immunofluorescent labeling and electron microscopy techniques. TRSV and TomRSV were localized in different regions of the food canal of *X. americanum*. TRSV was primarily localized to the lining of the lumen of the stylet extension and esophagus, but only rarely in the triadiate lumen, whereas TomRSV was localized only in the triadiate lumen. This investigation indicated the differential tissue preference for virus retention probably determined by the nematode-virus combination (Wang et al. 2002).

Raspberry bushy dwarf virus (RBDV) infecting both raspberry and grapevine, is transmitted by the nematode *Longidorus juvenilis.* The grapevine isolates of RBDV were characterized by serological properties. Three monoclonal antibodies (MAbs) D1, R2 and R5 were employed to differentiate between isolates of raspberry and grapevine by employing triple antibody sandwich (TAS)-ELISA format. An isolate

Table 3.1 Detection of *Tobacco ringspot virus* (TRSV) by immunofluorescence and bait techniques (Wang and Gergerich 1998)

Virus acquisition access period (days)	Virus-specific labeling		Virus transmission	
	Frequency[a]	Percentage	Frequency[b]	Percentage
0	0/52	0	0/20	0
1	1/56	2	1/20	5
3	4/51	8	2/20	10
5	18/50	36	11/20	55
7	20/50	40	11/20	55
10	26/50	52	12/20	60
15	33/61	54	10/20	50
22	28/51	55	10/20	50

[a]Ratio of nematode fragments showing fluorescence in the virus retention regions labeled with FITC to total numbers of fragments examined

[b]Number of bait plants infected by TRSV/total number of plants inoculated with nematodes at the rate of 10/plant

from red raspberry cv. Golden Bliss reacted with all three MAbs, whereas grapevine isolates could react with only two MAbs R2 and R5, but not with D1, indicating differences in the antigenic determinants between the raspberry and grapevine isolates of RBDV (Pleško et al. 2009).

Immunoassays have been employed for the detection of plant viruses transmitted by fungal vectors. *Polymyxa graminis* and *P. betae* have been reported to be the vectors of 12 different viruses belonging to the genera *Benyvirus*, *Bymovirus*, *Furovirus* and *Pecluvirus*. *P. graminis*, the vector *Barley mild mosaic virus* (BMMV), was tested for the presence of the virus in the fungal cells. Labled bundles of virus-like particles were detected in zoospores of *P. graminis* released from barley roots infected by BMMV (Jianping et al. 1991). Presence of labeled virions of *Beet necrotic yellow vein virus* (BNYVV) was observed inside the zoosporangia and zoospores of *P. betae* (Peng et al. 1998). *Peanut clump virus* (PCV) and *Indian peanut clump virus* (IPCV), members of *Pecluvirus* were transmitted by *P. graminis*. A direct antigen coating (DAC)-ELISA format was developed for the detection of the resting spores of *P. graminis* from root materials of sorghum which was affected by IPCV in India and West Africa. DAC-ELISA procedure was successful in detecting *P. graminis* in the different stages of its life cycle in the naturally and artificially infected sorghum plants (Delfosse et al. 2000).

Nucleic Acid-Based Techniques

Rose and lily crops are seriously affected by *Strawberry latent ringspot virus* (SLRSV) and *Arabis mosaic virus* (ArMV) that are transmitted by *Xiphinema diversicaudatum* and *Longidorus macrosoma* respectively under natural conditions. The nematodes were isolated from the soil around the roots of rose and lily plants. *X. diversicaudatum* and *L. macrosoma* were placed separately in RNAlaterTM solution. RT-PCR amplification was performed using virus-specific primers which were

designed to amplify a part of the coat protein gene, about 200-bp for SLRSV and 520-bp for ArMV. Total RNA from nematodes gave specific amplification of ~520-bp and ~200-bp for ArMV and SLRSV respectively. The protocol developed in this investigation was found to be easy and rapid and it could be applied for the detection of these two viruses in two different nematode species (Kulshrestha et al. 2005).

Grapevine fanleaf virus (GFLV) has the nematode *Xiphinema index* as the natural vector transmitting the virus from infected grapevine to healthy plants. The nematodes remain viruliferous for long time and they are capable of acquiring GFLV not only from infected plants, but also from root debris scattered in the soil after removal of plants (Raski et al. 1973). Hence, the need for assessing the nematode populations that may function as potent source of GFLV was realized. A real-time RT-PCR was performed on *X. index* collected from the rhizosphere of GFLV-infected grapevines at Palagiano, Italy. A 1,157-bp fragment of GFLV RNA-2 coat protein (CP) gene was amplified and sequenced. A fluorescent Scorpion probe was designed to detect a highly conserved CP region. A second region with isolate-specific multiple nucleotide polymorphisms was used to detect GFLV isolates using molecular beacons (MB). The Scorpion probe permitted quantitative estimation of GFLV RNA-2 in single nematodes, using a dilution series of a 692-nucleotide transcript of the CP gene. This real-time PCR format allowed detection of GFLV RNA-2 in individual *X. index* with minimum template threshold of 800 fg or 2.8×10^6 RNA-2 molecules/nematode. This technique appears to be suitable for detecting the virus and studying nematode transmission biology (Finetti-Sialer and Ciancio 2005).

Tomato ringspot virus (ToRSV), *Tobacco ringspot virus* (TRSV) and *Tobacco rattle virus* (TRV) are transmitted by nematode vectors present in the soil. Collagenase, a commercially available enzyme was used for effective extraction of viral RNA from nematodes. A sensitive nested-PCR protocol for the detection of ToRSV and TRSV in *Xiphinema americanum* and TRV in *Paratrichodorus allius* was developed. Detection of TRSV using nested RT-PCR assay in groups of 10 and 50 nematodes was accomplished. The intensity of bands (257-bp) was comparable with that obtained from TRSV-infected cucumber leaves. Likewise, the amplicon 707-bp from viruliferous nematodes carrying TRV, was comparable to the one present in TRV-infected tobacco leaves. Virulifierous nematodes carrying ToRSV produced an amplicon of 435-bp after nested RT-PCR amplification. The nested RT-PCR required considerably less time compared with bioassay method, although the molecular method needed intensive labor for hand-picking nematodes (Martin et al. 2009) (Appendix 2).

A real-time fluorogenic 5′ nuclease PCR (TaqMan) was developed to study the relationship of *Tobacco rattle virus* (TRV) with nematode vectors *Paratrichodorus pachydermus* and *Trichodorus similis.* Two independent primer probe sets were designed targeting the 18S gene of the ribosomal cistron of the vector species. Relative quantification of target DNA present in unknown samples was performed by comparison of the fluorescence signals of the samples to those obtained from plasmid standard dilutions. Another three primer/probe sets were also employed to target TRV: one set for RNA-1 and two other sets for RNA-2 of specific isolates. By employing the protocol developed in this investigation, both vector species and

TRV RNA-1 and RNA-2 could be detected in the field soil samples. This assay has the potential to provide rapid, accurate and sensitive molecular information for risk assessment in the field (Holeva et al. 2006).

Raspberry bushy dwarf virus (RBDV) infecting grapevine is transmitted by the nematode *Longidorus juvenilis*. The nematodes were extracted from fresh soil samples by the whirling-motion method and the total RNA was extracted using RNeasy Plant Mini Kit (Qiagen). Nested RT-PCR format was applied for the detection of RBDV in the nematode extracts. Specific amplification products representing RBDV were detected in the nematode *L. juvenilis.* The virus could be detected in the fresh soil samples and also after storage for 4–8 months in the refrigerator (Fig. 3.1). The protocol developed in this investigation may be useful in epidemiological studies (Pleško et al. 2009) (Appendix 3).

Beet necrotic yellow vein virus (BNYVV) is transmitted by the fungal vector *Polymyxa betae*. In addition, *Beet soilborne virus* (BSBV) and *Beet virus Q* (BVQ) have also been reported to be transmitted by *P. betae.* BNYVV is considered to be the causal agent of the economically important rhizomania disease of sugar beet. BSBV and BVQ are also associated with this disease. A multiplex RT-PCR assay was developed to simultaneously detect all the three viruses in *P. betae*. The primers were designed by using the PRIME program of the Genetics Group (Devereux et al. 1984). The selected primer pairs amplified the specific fragments of the expected sizes viz., 545-bp for BNYVV, 399-bp for BSBV, 291-bp for BVQ and 170-bp for *P. betae*. The four primers pairs were combined in the multiplex (m)-RT-PCR assay. The sensitivity of mRT-PCR assay was compared with that of DAS-ELISA test. The detection threshold of mRT-PCR assay was up to 128 times greater than that of DAS-ELISA test. Soil samples (25) from Bulgaria, France, Germany, Hungary, Italy, Sweden,

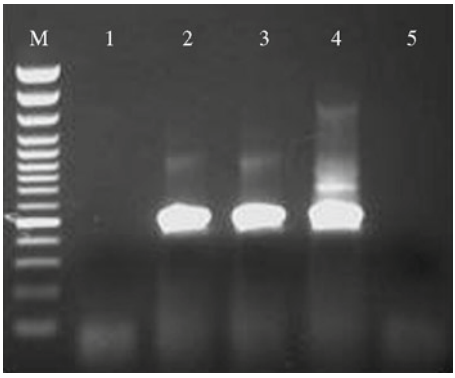

Fig. 3.1 Detection of *Raspberrry bushy dwarf virus* (RBDV) by nested PCR assay in the nematode vectors stored in soil samples for different periods. M: 100 bp DNA ladder; *Lane 1*: *Xiphinema wuittenezi* (five individuals) in soil stored for 8 months; *Lane 2*: *Longidorus juvenilis* (six individuals) in soil stored for 8 months; *Lane 3*: *L. juvenilis* (44 individuals) in soil stored for 4 months; *Lane 4*: positive control (cDNA from infected raspberry plants); *Lane 5*: water control (Courtesy of Pleško et al. 2009 and with kind permission of Springer Science and Business Media, Heidelberg, Germany)

the Netherlands and Turkey were analyzed by mRT-PCR assay. BNYVV occurred with BSBV and often with BVQ as well. The major advantage of this technique is the simultaneous detection of three viruses, facilitating epidemiological and resistance breeding investigations to gather reliable data (Meunier et al. 2003).

Polymyxa spp. is the vector of *Wheat soilborne mosaic virus* (WSBMV), *Barley yellow mosaic virus* (BaYMV) and *Beet necrotic yellow vein virus* (BNYVV) which cause serious losses in the respective crops. WSBMV is transmitted by *Polymyxa graminis* and the virus persists in the fungal vector in the infested soils for several years. A technique to detect the virus and vectors directly in the soil was developed. Among the three buffers tested, EDTA lysis buffer gave the highest recovery of DNA consistently from all the six soils tested. Hence, DNA extracted from three soils infested with *Polymyxa betae* and three soils infested with *P. graminis* was analyzed using an EDTA lysis buffer in combination with a MagneSil™ DNA extraction kit and Kingfisher™ magnetic particle processor. By using an automated approach to nucleic acid extraction and purification, clean DNA extracts could be obtained. Primers and probes corresponding to sequences within the ITS region 2 (ITS2) of ribosomal DNA were designed. The primers and probes enabled recovery and amplification of *P. betae* and *P. graminis* DNA using real-time PCR and TaqMan chemistry. For *P. graminis*-infested soils, the purity of DNA obtained was sufficient to allow *Polymyxa* DNA to be amplified without dilution to remove inhibitors. On the other hand, for *P. betae*-infested soils, the DNA extracts had to be diluted (1:10) for successful amplification of fungal DNA. Using TaqMan PCR format, a standard curve was constructed from uninfected soil spiked with known numbers of cystosori of *P. betae.* Then the quantity of *P. betae* inoculum from naturally infested soils was extrapolated from the standard curve. The real-time PCR was rapid and reliable for direct detection and quantification of *Polymyxa* DNA in less than 1 day. Quantification may be required for epidemiological investigations to determine the number of viruliferous *Polymyxa* cysts in the soil and the protocol developed in this investigation may be suitable also for collecting reliable data for studying cultivar resistance to the soilborne virus diseases (Ward et al. 2004) (Appendix 4).

A broad-spectrum RT-PCR procedure was developed for the detection of *Soilborne cereal mosaic virus* (SBCMV) isolates inducing mosaic diseases in European countries. Primers were designed based on the sequences of conserved 3′-untranslated region (UTR) of RNA-1 and RNA-2 of SBCMV. The 3′ end region was a privileged target for the detection of a wide range of isolates, because of the sequence conservation of the tRNA-like structure. In addition, primers were also designed for virus quantitation using real-time RT-PCR with SYBR chemistry. No cross-reaction could be seen with *Wheat spindle streak mosaic virus* that frequently occurred along with SBCMV. The procedure adopted was more sensitive than ELISA test in detecting and quantitating SBCMV. Further, it was effective in detecting the isolates of SBCMV from Belgium, France, Germany, Italy and United Kingdom. The real-time RT-PCR protocol showed promise for reliable comparison of soil inoculum potential as well as for determining levels of cultivar resistance to SBCMV (Vaïanopoulos et al. 2009).

3.1.2 Detection of Plant Viruses in Water

Microbial plant pathogens especially fungal and bacterial pathogens are known to spread through rain, irrigation and river waters. But the presence of plant pathogens in environmental water sources has been monitored rarely and only few attempts have been made to detect the plant viruses in water (Koenig 1986; Gosalves et al. 2003).

3.1.2.1 Biological Methods

Water samples from some of the rivers of southern Italy whose water is normally used for irrigating crops were tested. After concentrating by centrifugation at 5,000 rpm and resuspending the sediments in phosphate buffer, the suspension was inoculated onto *Chenopodium quinoa*, the assay host for several plant viruses. The viruses detected in the river waters were *Tobacco mosaic virus* (TMV), *Cucumber mosaic virus* (CMV) and two other unidentified viruses (Piazzolla et al. 1985). A survey was undertaken to test water samples from small ponds and creeks in a forested area near Bad Münstereifel, near Bonn. The presence of *Carnation Italian ringspot virus*, among several viruses, was detected in the water samples. The virus had been previously isolated twice from carnation plants originating from Italy and USA. The significance and role of this virus in the forest ecosystem is not known (Büttner et al. 1987). Plant viruses present in water used for irrigation can infect plants through roots, inducing symptoms in due course in aerial plant parts. Viruses may be released from infected plants into drainage water and then spread to other plants in the same or nearby fields (Koenig 1986). In the 47 environmental water samples collected in Hungary, 26 plant viruses were detected (Horváth et al. 1999). *Melon necrotic spot virus* (MNSV) present in hydroponic cultures was considered to be responsible for different adverse effects on crops observed (Horváth et al. 1999).

3.1.2.2 Nucleic Acid-Based Techniques

As the plant viruses are likely to be present in very low concentrations in the environmental samples, they have to be concentrated to a level that may be detectable by diagnostic tests. *Tomato mosaic virus* (ToMV), a highly stable, rod shaped RNA virus was used as the model virus for testing environmental water samples and chromatographic fractions after using a Convective Interaction Media® (CIM) chromatographic columns as the concentrating procedure. A quantitative ToMV-specific real-time RT-PCR was developed to monitor the health status of environmental waters from rivers in Slovenia. ToMV was reliably detected at as low as 12 virions per real-time PCR reaction which corresponded to the initial concentration of approximately 4.2×10^{-10} mg (6,300 virions) of ToMV/ml of sample. Seven out of nine water sources from different locations tested positive for ToMV by real-time RT-PCR assay. In addition, ISEM was used to visualize ToMV particles in the concentrated samples. The real-time RT-PCR assay was more sensitive by three to

five orders than DAS-ELISA. The real-time RT-PCR proved to be efficient in detecting ToMV in two samples which tested negative for DAS-ELISA. This protocol represents a reliable and rapid method for screening environmental water samples for the presence of plant viruses (Boben et al. 2007).

With high stability and a wide host range of plant hosts, *Tomato mosaic virus* (ToMV) has been detected in plants, soil, water and clouds. Fog and cloud samples were collected and they were concentrated by centrifugation. The presence of ToMV in the fog and cloud samples was tested by bioindexing on *Chenopodium quinoa*, DAS-ELISA and RT-PCR-blot hybridization (BH) tests. Local lesions (about 400) typical of ToMV were produced on *C. quinoa* inoculated with the composite cloud/fog concentrates. Of the eight samples tested, only two concentrates from Whiteface Mountain and Mount Desert Rock had absorbance values that approximated the positive/negative threshold values of 0.186 and 0.177 respectively. RT-PCR-BH assay detected ToMV in 25 of 44 cloud and fog concentrates, as the products of PCR appeared as distinct bands on 1% agarose gels after elelctrophoresis and staining with ethidium bromide. Nucleotide sequencing results within the coat protein gene confirmed the identity of the virus isolated from clouds and fog as ToMV. This investigation provided evidence for the possible atmospheric spread of infectious viruses, representing a potentially significant long-distance transmission mechanism for stable viruses like ToMV (Castello et al. 1995).

As *Tomato mosaic virus* (ToMV) is highly stable, the possibility of the presence of plant viruses in ancient ice was explored. ToMV was detected by RT-PCR assay in glacial ice subcores <500 to approximately 140,000 years old from drill sites in Greenland. Subcores that contained multiple ToMV genotypes suggested diverse atmospheric origins of the virus. Detection of ToMV in ice suggested the possibility that the stable viruses of humans and other hosts may be present there and entrapped ancient viruses may be continually or intermittently released into the modern environment (Castello et al. 1999).

3.1.3 Detection of Plant Viruses in Air

As in the case of soils, plant viruses present in the air may be transmitted abiotically or biotically. Abiotic transmission of highly stable viruses may occur. But airborne organisms such as insects and mites are predominantly involved in the transmission of a large number of plant viruses. Delicate vectors like thrips and mites are carried by wind and these vectors may transmit the viruses even the highly unstable viruses like *Tomato spotted wilt virus*, when they feed on healthy plants.

3.1.3.1 Detection of Viruses with Abiotic Transmission

The possibility of airborne transmission of plant viruses such as *Tobacco mosaic virus* (TMV) and *Tomato mosaic virus* (ToMV) which are highly stable in vivo was explored. ToMV-spruce (*Picea rubens*) pathosystem was examined in this respect.

Virus-free spruce seedlings were planted in raised plywood boxes lined with plastic and filled with a Promix sand and the boxes were placed at two elevations 1,015 and 960 m on Whiteface Mountain. ToMV was detected by ELISA test in seedling roots in both sites after about 1 year. Mean virus concentration within seedling roots was significantly greater in seedlings placed at higher elevation. The results seem to support the likelihood that clouds and/or precipitation may be the source of virus that may result in infection of red spruce, since infectious ToMV had been detected in the clouds and fog in an earlier study (Castello et al. 1995). However, the manner in which ToMV becomes airborne is not clear (Filhart et al. 1997).

3.1.3.2 Detection of Viruses with Biotic Transmission

Biotic transmission occurs in a majority of plant viruses through the agency of insects and mites present on the aerial plant parts as pests, after acquiring the virus(es), while feeding on the infected plants and subsequently feeding on healthy plants. The presence of viruses has been detected by employing immunoassays and nucleic acid-based techniques, after providing different periods of acquisition access on the infected plants.

Immuoassays

Immunochemical studies have provided the possibility of visualizing virus particles in the vector tissues. The presence of *Tomato spotted wilt virus* (TSWV) was not detected by immuno-electron microscopy (IEM) in the adults of *Frankliniella occidentalis* that had access to the infected source plants, as TSWV was not retained by them, though the virus was ingested by the thrips. In contrast, the larvae of *F. occidentalis* showed the presence of TSWV in the midgut epithelium and hemocoel after feeding on infected plants. The results indicated that passage of TSWV through the midgut was a critical factor determining the vector competence and transmission could occur only if the midgut allowed the passage of the virus to reach the salivary glands of the thrips (Ullman et al. 1992). Antibodies generated against nonstructural proteins (NSs) of TSWV were employed to demonstrate the presence of NSs in thrips cells. Electron microscopic observations indicated that TSWV could replicate in the vector tissues (Ullman et al. 1993). In situ immunolabeling of salivary glands and other tissues of adult *F. occidentalis* revealed the accumulation of large amounts of nucleocapsid (N) and NSs and presence of several vesicles containing virus particles in the salivary glands. The results suggested that salivary glands might be a major site of virus replication (Wijkamp et al. 1993).

Tomato spotted wilt virus (TSWV) was detected in 210 individuals of 340 *F. occidentalis* and in 24 of 120 *F. schultzi* laboratory-grown adult thrips which were allowed access to tomato plants infected by TSWV as larvae (Cho et al. 1988; Marchoux et al. 1991). Production and accumulation of two TSWV-specific proteins [nucleocapsid (N) and nonstructural (NSs) proteins] were detected by

ELISA in the viruliferous larvae and adults of *F. occidentalis* after ingestion of the virus from infected plants. The concentrations of both proteins increased significantly within 2 days above the levels ingested, indicating the possible multiplication of TSWV in the thrips (Wijkamp et al. 1993). Use of monoclonal antibodies (MAbs) specific to NSs proteins improved the detection sensitivity. The changes in the accumulation of TSWV N protein were monitored at different developmental stages of *Thrips setosus* by employing ELISA test. There was a progressive increase in the N protein concentration commencing from the first to second instar larval stage following acquisition feeding on infected plants and the N protein content reached its maximum concentration on the fifth day. Then a progressive decline in N protein content from the second instar larval to pupal stage occurred. Although adult thrips had low N protein concentration, their vector efficiency was not affected (Tsuda et al 1996). Immuno-electron microscopy (IEM) procedure was adopted for localization of TSWV in tissues of *F. occidentalis* that transmitted the virus efficiently (Nagata et al. 1999).

Differences in vector efficiency of transmission of *Chrysanthemum stem necrosis virus* (CSNV) by different species of thrips were determined by combining a leaf disc assay and DAS-ELISA test. *Frankliniella schultzii* transmitted CSNV more efficiently than *F. occidentalis. Thrips tabaci* was unable to transmit CSNV, although low amount of CSNV could be detected in 75% of the population of *T. tabaci* test by ELISA method. This indicated that the mere presence of a virus in the insect body would not result in the successful transmission of the virus carried by the insect species (Nagata and de Avila 2000).

Successful virus transmission appears to depend on heavy infection of salivary glands during thrips development (Nagata et al. 1999, 2002). The significance of the pattern of TSWV N protein accumulation during thrips development as a determining factor for vector competence was examined. The accumulation of TSWV-protein and transmission of TSWV by the second instar larvae and adults of five thrips species were studied. A triple antigen sandwich (TAS)-ELISA format showed that large amounts of TSWV nucleocapsid (N) protein were present in the ELISA-positive larvae of *Frankliniella occidentalis*, *F. intosa*, *Thrips tabaci* and *T. setosus*, but not in *T. palmi*, a nonvector species. The ELISA titre of and the proportion of virus-infected individuals of two *F. occidentalis* and *F. intosa* increased or did not significantly change from the larval to the adult stages. On the other hand, a decline in the three species of *Thrips* was observed in these two parameters. Thus higher concentrations of TSWV N protein in the adults of *F. occidentalis* and *F. intosa* appeared to contribute to a higher frequency of virus transmission by these vector species. The results indicated that the patterns of TSWV N protein accumulation from larva to adult differed between species of *Frankliniella* and *Thrips* and this factor may affect TSWV transmission by adults of these species (Inoue et al. 2004).

In a similar study using five thrips species known to be vectors of tospoviruses in Japan, the vector competence of the thrips species with respect to *Impatiens necrotic spot virus* (INSV) was examined. Using the petunia leaf disk, the inability of *Thrips setosus*, *T. palmi* and *T. tabaci* to transmit INSV was demonstrated. The INSV N protein was not detected by DAS-ELISA in these species of thrips.

In contrast, *Frankliniella occidentalis* and *F. intosa* were able to transmit INSV. Most *F. occidentalis* adults showing ELISA-positive absorbance values, transmitted INSV. In this case, only one third of ELISA-positive *F. intosa* adults could transmit the virus, as determined by petunia leaf disk assay. The amounts of INSV N protein as detected by ELISA test and number of INSV-transmitters were greater in *F. occidentalis* than in *F. intosa.* The results indicated that *F. occidentalis* was the most efficient vector of INSV in Japan (Sakurai et al. 2004) (Appendix 5).

Aphids form one of the major groups of insects involved in the transmission of plant viruses which show three distinct types of relationships [nonpersistent (stylet-borne), persistent (circulative) and propagative] with their vector species. The presence of *Potato leaf roll virus* (PLRV) in the tissues of the aphid vector *Myzus persicae* was detected by employing immunogold labeling and immunofluorescence techniques. PLRV particles were observed in the intestinal epithelium indicating that intestinal cells may form the pathway for transport of PLRV from gut lumen into hemocoel (Garret et al. 1993). Indexing of aphid populations collected from the fields, for the presence of plant viruses, has been carried out by employing immunoassays and nucleic acid-based techniques. Vector indexing of the aphid *Myzus persicae* which is able to transmit more number of plant viruses than any other aphid species, revealed that *Beet mild yellowing virus* (BMYV) could be detected by ELISA test in *M. persicae* effectively. A greater percentage of the aphids trapped in sugar beet crop was viruliferous than the aphids trapped in oilseed rape crop. However, there was no significant difference in the percentages of *M. persicae* carrying *Beet western yellows virus* in both sugar beet and oilseed rape crops (Stevens et al. 1995). Standard DAS-ELISA and enzyme-amplified ELISA formats were evaluated for their efficacy in detecting BWYV. When the aphids were tested after a 48-h acquisition feeding on infected source plants, the reliability and efficiency of detection of BMYV in the aphids was enhanced, although it was not possible to detect the virus in individual aphid (Polák 1998). However, *Faba bean necrotic yellows virus* (FBNYV) could be detected in individual aphids of *Aphis craccivora* and *Acyrthosiphon pisum* by employing triple antibody sandwich (TAS)-ELISA format (Franz et al. 1998).

Leafhoppers constitute another major group of insect vectors which predominantly transmit circulative (persistent) or propagative viruses requiring a definite incubation (latent) period in the vector, after virus acquisition from infected plants. The leafhoppers become viruliferous after the completion of incubation period (extending from a few days to several weeks). Propagative viruses are able to multiply in the vectors and some of them are transmitted to the subsequent generations through the eggs of female viruliferous leafhoppers as in the case of *Rice dwarf virus* (RDV). Thus viruliferous leafhoppers by themselves may serve as a source of virus infection. Indexing the leafhoppers for the presence of plant viruses will be essential for studying the epidemiology of virus diseases and for the effective disease management. The presence of *Chickpea chlorotic dwarf virus* (CCDV) was detected in individual leafhoppers *Orosisus occidentalis* by applying DAS-ELISA test. The titer of CCDV declined progressively when the leafhoppers were fed on a nonhost for the virus, indicating that the virus was unable to multiply in the leafhopper vector (Horn et al. 1993).

The efficacy of different enzyme labels and corresponding substrates employed in ELISA tests, was evaluated for the detection of *Maize streak virus* (MSV) in maize plants and the leafhopper vector *Cicadulina mbila* based on dilution end point of virus detection. The horseradish peroxidase (HRP) and penicillinase-based ELISA tests were able to detect MSV in three and crushed leafhoppers respectively. Avidin–biotin-penicillinase amplification technique was able to detect MSV in single leafhopper. The alkaline phosphatase (ALP) system was not useful, because of high background of the leafhopper contents. Protein A coating (PAC)-penicillinase format gave nonspecific reactions, making it unsuitable for MSV detection in the leafhopper vectors (Afolabi and Thottappilly 2008). The pineapple mealybug wilt-associated viruses cause appreciable losses in pineapple crops. Mealybugs (*Dysmicoccus brevipes*) are considered to be the vectors of the viruses associated with wilt disease. The presence of the causal virus could be detected in the mealybugs collected from wilted pineapple plants by indirect ELISA format (Hu et al. 1996).

Nucleic Acid-Based Techniques

Hybridization- and amplification-based techniques have been successfully applied for the detection of plant viruses in their vectors and for studying the virus-vector relationships. Southern blot analysis was shown to be effective for the detection of *Tomato yellow leafcurl virus* (TYLCV) in whiteflies (*Bemisia tabaci*) at different periods after the acquisition access period on infected source plants. TYLCV-DNA could be detected in 15% of whiteflies after a period of 30 min and in all whiteflies after an 8-h access period. When the whiteflies were fed on young infected leaves containing higher concentration of TYLCV, it was possible to detect the virus earlier (within 2 h) in the insects. Quantitative estimation showed that a single whitefly could acquire no more than 600 million viral genomes (1 mg viral DNA) (Zeidan and Czosnek 1991). Detection and quantification of TYLCV in *B. tabaci* was accomplished by applying DIG-labeled probes. The whiteflies were tested after different acquisition access periods on TYLCV-infected tomato plants. The whiteflies acquired the maximum amount of TYLCV-DNA (0.5–1.6 ng/insect) and the viral DNA could be detected up to 20 days after the termination of acquisition access period. It seemed that the ability of the whitefly to transmit TYLCV was lost earlier than the period for which the virus was detectable in the insect body (Caciagli and Bosco 1997).

Potato virus Y (PVY) is transmitted by *Myzus persicae* in a nonpersistent (stylet-borne) manner. PVY can be acquired from infected plants after short acquisition feeding period (a few minutes) and it can be transmitted to healthy plants immediately without an incubation (latent) period in the aphid, which is required in the case of persistent and propagative viruses. Sensitive techniques are needed to detect the target viruses found in extremely low concentration in the vector aphids. Hence, the suitability of employing print-capture (PC)-PCR-ELISA procedure was examined for the detection of PVY in single aphid. The simple PC-PCR protocol involving a single aphid imprint was made on 3 MM paper. PVY targets were detected by PC-PCR-ELISA protocol in 38 of 97 individual aphids tested (39.2%). The percentage of positive

aphids varied between replications from 25% to 50%. The relative acquisition rate of PVY by *M. persicae* (39.2%) determined by PC-PCR-ELISA was in agreement with aphid transmission efficiency from inoculativity tests conducted in parallel. The PCR-ELISA protocol was more sensitive (100 folds) than ELISA and it was more suitable for processing large number of samples (Varveri 2000).

Plum pox virus (PPV) is transmitted by several aphid species in a non-persistent (noncirculative) manner. (Ng and Perry 2004). A mere feeding of viruliferous aphids on a healthy tree for a few minutes may lead to viral infection. A reliable estimate of the number of virions or RNA targets that a single aphid might carry and transmit successfully to a healthy plant will be useful. A real-time quantitative RT-PCR assay, based on the sensitive TaqMan chemistry was developed for the detection and quantification of RNA targets from non-persistently transmitted PPV in individual fresh or aphids captured previously and squashed on paper. Reliable quantitation ranged from 40 up to 4×10^8 copies of control transcripts. The detection technique was able to detect PPV in both plant materials and individual PPV vector (*M. persicae*) and non-vector (*Aphis nerii*) revealing that viral targets were acquired by both vector and non-vector aphids. The number of aphids carrying PPV detected by real-time RT-PCR and nested RT-PCR in a single closed tube was similar in parallel assays, although the sensitivity of real-time RT-PCR was 100 times higher than nested RT-PCR and 1,000 times greater than DASI-ELISA and conventional RT-PCR assays. The combination of squash capture of targets on paper and real-time RT-PCR allowed reliable quantitation of PPV targets acquired by individual aphids with potential for providing data for PPV epidemiology (Olmos et al. 2005).

The real-time RT-PCR (TaqMan) assay was applied for the detection and quantification of *Citrus tristeza virus* (CTV) RNA targets in aphid vector and citrus plant tissues. Single individuals of *Aphis gossypii* were squashed on paper or nylon membranes (tissue-print) and the squashed samples were inserted into Eppendorf tubes or placed inside ELISA plate wells for large scale assays. The RNA targets were detected by real-time RT-PCR protocol. Immunocapture (IC)-nested PCR procedure in a single closed tube detected CTV-targets in 12 of 54 *A. gossypii* (24%) individually squashed on paper, whereas squash real-time RT-PCR assay detected CTV-targets in 20 of 50 single aphids (40%) tested. In addition, the presence of CTV was detected in 50 of 280 field-collected aphids (19.3%) by squash real-time PCR assay. The percentage of aphids carrying CTV-targets increased with increasing acquisition access period on infected sources (Table 3.2). Detection and quantification of CTV-targets in aphids could establish the relationship between the

Table 3.2 Detection and quantitation of *Citrus tristeza virus* (CTV)-RNA targets in *Aphis gossypii* after different periods of acquisition access on infected plants (Bertolini et al. 2008)

Acquisition periods allowed (h)	No. of aphids tested	No. of aphids tested positive	No. of CTV targets	
			Range	Mean
1	40	4	4,728–16,748	8,379
24	40	8	11,285–101,753	35,922
48	49	17	3,028–123,791	38,280

aphid species and spread of virus in the field. This investigation provided a sensitive, specific and reliable tissue-print and squash real-time RT-PCR format for detection and quantification of CTV in immobilized plant and vector tissues without the need for immunocapture phase. Immobilized material can be used without risk by quarantine and certification programs and in epidemiological investigations (Bertolini et al. 2008) (Appendix 6).

Reliable evidences for the pathway of virus passage inside the insect body from acquisition to transmission of plant viruses by whiteflies were obtained by employing a membrane feeding system and PCR procedure. *Bemisia tabaci* vector of *Squash leafcurl* virus (SLCV, genus *Geminivirus*) and a nonvector species *Trialeurodes vaporariorum* were allowed access to infected plants for 0.5 to 96 h. The viral DNA was detected in the honeydew of both vector and nonvector species, indicating that SLCV was able to pass through the digestive system of both species. However, SLCV-DNA could be detected in the saliva and hemolymph of *B. tabaci* alone, but not in the case of *T. vaporariorum*. The results indicated that SLCV could pass through the gut barrier of *B. tabaci* and reach the hemolymph from which the virus diffused into the salivary glands and ejected into the healthy plants along with the saliva. SLCV was apparently not able to penetrate the digestive epithelia of nonvector species resulting in its inability to transmit the virus (Rosell et al. 1999).

The print capture (PC)-PCR assay was employed for the detection of *Tomato yellow leafcurl virus* (TYLCV) in tomato plants and the vector *Bemsia tabaci*. Squashes of insect and plant tissues were applied onto nylon membrane strips (1 × 2 mm) that were then introduced into PCR reaction mixture. The PCR products were electrophoresed in the gel, blotted and hybridized with radio-labeled virus-specific DNA probe. TYLCV could be detected in individual whitefly and at sites of inoculation at 5 min after inoculation feeding by viruliferous whiteflies in some plants and in all inoculated plants at 30 min after inoculation feeding. This indicated that whiteflies were efficient in transmitting sufficient virus concentration that reaches detectable levels after 5–30 min after inoculation feeding. TYLCV could be detected in different tissues of whitefly such as head, thorax and abdomen of *B. tabaci* at 5, 10 and 25 min after acquisition feeding in infected tomato plants (Atzmon et al. 1998).

Tomato yellow leafcurl Sardinia virus (TYLCSV) and the strains Israel and Mild of *Tomato yellow leafcurl virus* (TYLCV-IL, TYLCV-Mld) were found to infect cucurbit crops for the first time by applying the nested PCR assay. In addition, a semi-quantitative PCR (sqPCR) protocol was developed for the detection of TYLCV-Mld strain in the whitefly vector *Bemisia tabaci.* In order to find out the possibility of the spread of TYLCV-Mld from cucumber plants to tomato by *B. tabaci*, the whiteflies were fed on cucumber plants infected by TYLCV-Mld. Results of sqPCR assay revealed that TYLCV-Mld DNA could be detected in the whiteflies fed on infected cucumber plants. This investigation provided evidence that cucurbit plants could serve as reservoirs for viruses that cause TYLCV and *B. tabaci* could transmit TYLCV-Mld from infected cucumber to some of the tomato and also jimson weed (*Datura stramonium*) plants. Since jimson weed species is present in the Jordan Valley, it may turn out to be potential virus reservoir in the locations where tomato crops are raised (Anfoka et al. 2009).

3.1.4 Detection of Plant Viruses in Alternative Host Plant Species

The role of alternative host plant species (crops, wild host and weed species) is more important for the survival of viruses in the absence of their natural crop plant hosts, compared to other microbial pathogens that can exist as saprophytes on dead organic matter or as spore forms that are resistant to adverse environmental conditions. Plant viruses may have a wide host range or may be restricted to a few plant species. Wider the host range, greater will be the difficulty to manage the diseases caused by such viruses, especially if the virus can infect crop plants that may be grown side by side in the same farm. For example, *Tomato yellow leafcurl virus* (TYLCV) has been demonstrated to spread from cucumber to tomato crops through whitefly *Bemisia tabaci*. In addition, the jimson weed (*Datura stramonium*) may be able to serve as potential source of infection, aiding the survival and perpetuation of TYLCV in the absence of principal host plant species (Anfoka et al. 2009). Likewise, *Hop stunt viroid* (HSVd) and *Citrus exocortis viroid* (CEVd) have been reported to infect grapevines frequently as mixed infections along with *Grapevine yellow speckle viroid 2* (GYSVd-2), indicating the possibility of persistence of the viroids in the perennial crops like grapevines (Eastwell and Nelson 2007).

Detection of plant viruses in all plant sources is important to plan for an effective disease management system primarily involving elimination of all sources of inoculum thoroughly as early as possible. As in the case of primary natural host plant species, detection methods based on the biological, immunological and nucleic acid characteristics have to be employed depending on their sensitivity, rapidity, reliability and cost-effectiveness. Bioindexing has been largely replaced by immunoassays and nucleic acid-based techniques for the detection of viruses in the alternative host plant species, because of their greater sensitivity, specificity and reliability and the results of these techniques will be available much earlier compared with the time required by the biological methods.

3.1.4.1 Immunoassays

Immunoassays have been demonstrated to provide improved detection of plant viruses compared to biological methods of detection of plant viruses in the weed plant species. Presence of virus reservoirs in weed plant species is one of the important epidemiological factors determining the extent of virus disease incidence and subsequent spread of the diseases. *Tomato spotted wilt virus* (TSWV) has an extremely wide host range encompassing over 650 plant species including both dicots and monocots (Goldbach and Peters 1994). After analyzing more than 9,000 samples by ELISA test, 44 plant species in 16 families were found to be infected by TSWV. Among plant species that were ELISA-positive, 25 plant species were considered as important reservoirs of TSWV and 24 species were shown to be new host plant species susceptible to TSWV (Cho et al. 1989). Transmission of *Sowbane*

mosaic virus (SoMV) through seeds of *Chenopodium album* and *C. quinoa* was detected by DAS-ELISA format. These weed species carrying the virus through seeds may introduce the virus to new areas and also facilitate carryover of TSWV to other generations, in the absence of natural host plant species (Kazinczi and Horváth 1998). A tissue blot immunoassay (TBIA) procedure was developed to detect TSWV in *Ranunculus asiaticus* and other plant species. In addition to *R. asiaticus*, *Begonia* × *hiemalis*, *Chrysanthemum* sp., *Eustoma* sp., *Impatiens* sp., *Datura stramonium*, *E. fosbergii*, *Malva parviflora* and *Senecio cruentus* showed the presence of TSWV in tissue blots. This investigation revealed the presence of additional host plant species that could be potential sources of TSWV infection for several valuable crop plants (Whitefield et al. 2003).

Rice yellow mottle disease caused by *Rice yellow mottle virus* (RYMV) is widespread in Africa. The virus is able to infect several wild species of Oryza and weed species. Apart from cultivated *Oryza sativa*, *O. glaberrima*, *O. barthii* and *O. longistaminata* and other members of *Poaceae*, *Echinochloa colona*, *Ischaemum nigrosum* and *Panicum repens* are also infected by RYMV (Konaté et al. 1997). Enzyme-linked immunosorbent assay (ELISA) was applied to detect RYMV in rice and other additional host plant species. RYMV was detected in *O. barthii*, *O. longistaminata*, *Dactyloctenium aegyptium*, *Eragrostis ciliaris*, *E. tenella* and *E. tremula* plants. The weed grasses were reported to be new hosts of RYMV as indicated by the results of ELISA tests (Allarangaye et al. 2006).

Many weed plant species exhibit virus-like symptoms and the identity of the virus(es) involved has to be established rapidly in order to prevent the spread of the viruses from weeds to crop plants. This approach was made to identity the viruses infecting weeds by inoculating the isolates to 10 different diagnostic plant species, examining the purified viruses under electron microscope and analyzing by RT-PCR and sequencing. Three viruses *Turnip mosaic virus* (TuMV), *Broad bean wilt virus* (BBMV) and *Cucumber mosaic virus* (CMV) were identified by the above mentioned tests. RT-PCR assay was performed using virus-specific oligonucleotide primers followed by cloning. The nucleotide sequences of coat protein genes of the three viruses were determined and the amino acid sequences of CPs were deduced. The amino acid sequence homologies between the weed isolates of TuMV, BBWV and CMV and the isolates reported earlier were 92.7–99.7%, 96.2–97.7% and 93.9–98.6% respectively. The results indicated that the weed species susceptible to these viruses may be the potential sources of infection for the crops grown in the adjacent fields (Kwon et al. 2000).

Potato virus Y has a worldwide distribution and causes economically important diseases in potato, tomato, pepper and tobacco. Several solanaceous weeds such as *Physalis floridana*, *Solanum nigrum* and *S. dulcamara* harbor PVY and act as potential sources of infection (Beemster and de Bokx 1987). In order to determine the weed species that might be infected by PVY, 21 weed species were aphid- and/or sap-inoculated with PVY. The inoculated and control plants were tested by DAS-ELISA, using a cocktail of MAbs. Plants of *Erodium cicutarium*, *Geranium pusillum*, *Lactuca serriola* and *Lamium purpureum* tested positive by ELISA. Infection of these weed species by PVY was confirmed by back-inoculating to tobacco, and also by performing IC-RT-PCR assay (Fig. 3.2). This investigation showed for the first time that infection *of*

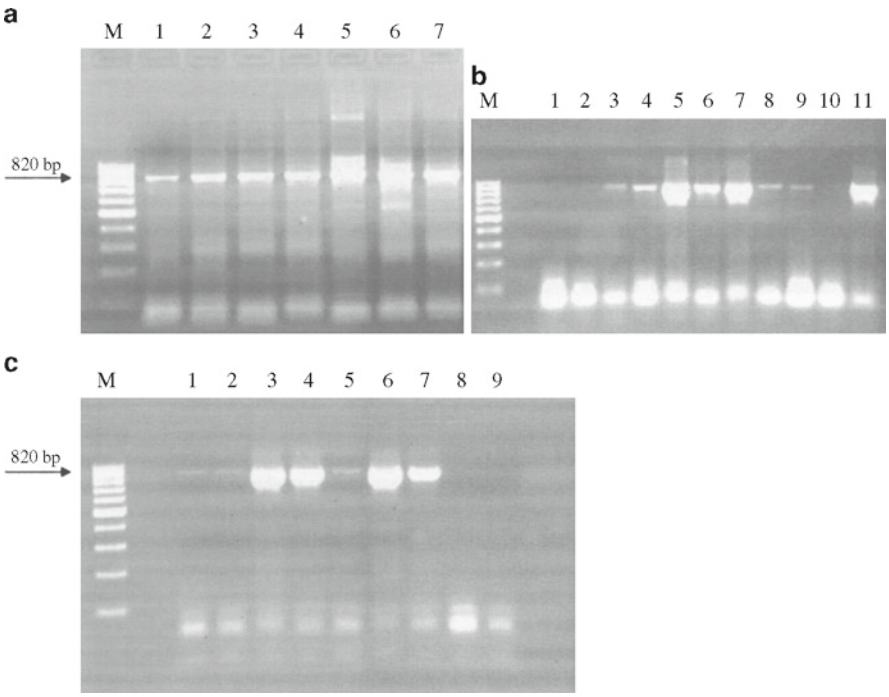

Fig. 3.2 Detection *of Potato virus Y* from different wild plant species by IC-RT-PCR assay. (**a**) M: 100 bp DNA ladder; *Lanes 1–5*: *Lactuca seriola*; *Lanes 6* and *7*: PVY positive control. (**b**) M: 100 bp DNA ladder; *Lanes 1–6*: *Nicotiana tabacum* inoculated with sap from *Erodium cicutarium*; *Lanes 7–9*: *Nicotiana tabacum* inoculated with sap from *Geranium pusillum*; *Lane 10*: negative control; 11: positive control. (**c**) M: 100 bp DNA ladder; *Lanes 1* and *2*: *E. cicutarium*; *Lanes 3* and *4*: *N. tabacum* inoculated with sap from *Lamium purpureum*; *Lanes 5* and *6*: *N. tabacum* inoculated with sap from *E. cicutarium* (5) and *G. pusillum* (6); *Lane 7*: positive control; *Lane 8*: negative control; *Lane 9*: RT (reverse transcriptase) control (Courtesy of Kaliciak and Syller (2009) and with kind permission of Springer Science and Business Media, Heidelberg, Germany)

E. cicutarium, *G. pusillum* and *L. purpureum* could occur and that they could be important sources of inoculum for the aphid vectors to acquire and spread the viruses to crop plants (Kaliciak and Syller 2009).

Turnip mosaic virus (TuMV) belonging to the genus *Potyvirus* in the family *Potyviridae* is the most predominant among viral pathogens infecting Brassica crops. During field surveys in eight provinces of Iran, 532 weed samples were collected from plants exhibiting virus-like symptoms and they were tested by DAS-ELISA format for the presence of TuMV using specific PAbs. TuMV was detected in 340 of 532 samples (64%) and the infected plants belonged to *Rapistrum nigosum*, *Sisumberium loselli*, *S. irio* and *Hirschfeldia incana* in the family *Brassicaceae.* TuMV isolates from weed species IRN TRab and IRN SS5 fell into an ancestral basal-Brassica group. This investigation showed for the first time the wide distribution and phylogenetic relationships of TuMV from weeds (Farzadfar et al. 2009).

PVY from infected weed plant species *Erodium cicutarium*, *Geranium pusillum*, *Lactuca serriola* and *Lamium purpureum* was inoculated onto tobacco. IC-RT-PCR assay was employed to detect and identify PVY isolates from the above mentioned host plant species (Kaliciak and Syller 2009).

3.1.4.2 Nucleic Acid-Based Techniques

Curtoviruses cause curlytop diseases in many vegetable crops and other 300 plant species including chile pepper (*Capsicum annuum*), sugarbeet and tomato. These viruses are vectored by leafhopper species *Circulifer tenellus* exhibiting circulative, persistent and nonpropagative relationship with the viruses transmitted by them. Over 4,950 aysmptomatic weed samples were collected from ten pepper fields in southern New Mexico State during 2003–2005. Primers based on the sequences of a conserved region of the curtoviruses were designed for use in the PCR assay. The infected plants were further tested for specific curtoviruses using the primers designed to detect a portion of the replication-associated protein (*rep*) gene. Amplification of the *cp* gene was accomplished from the extracts of 3.7%, 1.17% and 1.9% of weed samples collected in the 3-year period. Analysis of the *rep* nucleotide sequences revealed that 32.9% of weed isolates were closely related to *Beet mild curly top virus* (BMCTV). The 576-bp fragments of the CP region were amplified from 114 weed samples using the CP primer set cp4f and cp6r. From the PCR-positive samples, 92 PCR amplicons of the CP region from these 114 weed samples were sequenced. Ninety two isolates shared at 98% of the nucleotide identity with BMCTV-W4 or *Beet severe curly top virus* (BSCTV). The rep 1f/reprr set of primers were successful in amplifying a 488-bp fragment of the *rep* region of 18 samples. The 2repf1/2repr1 set of primers amplified a 723-bp fragment of the *rep* region for 62 samples. Approximately 12.4% of the isolates were closely related to BSCTV. The remaining isolates (54.7%) which shared a very high level of nucleotide sequence identity to each other, represent a new curtovirus. The new virus was identified as *Pepper yellow dwarf virus* (PeYDV) which might have resulted due to mutations in the genome and recombination between BMCTV-W4 and BSCTV (Lam et al. 2009).

The possibility of cucurbit crops grown in or adjacent to tomato fields serving as reservoirs for viruses causing tomato yellow leafcurl disease, was examined. *Tomato yellow leafcurl Sardinia virus* (TYLCSV) and the strains Israel and Mild of TYLCV (TYLCV-IL and TYLCV-Mld) were investigated. Cucumber samples (31%) were infected with viruses causing TYLCD, TYLCV-Mld and TYLCV-IL. Nested (n) PCR results showed that cucumber, squash, melon and watermelon plants, although did not exhibit any visible symptoms, were susceptible to TYLCV-Mld, TYLCSV and TYLCV (Jordan)JV infections. Further, the expected DNA fragments of TYLCD-associated viruses were detected in the uppermost leaves of symptomless plants by nPCR format. The jimsonweed plants (*Datura stramonium*) growing in the tomato fields were also found to be infected by TYLCD-associated viruses, as indicated by nPCR procedure (Anfoka et al. 2009).

3.2 Detection of Viroids in the Environment

Viroids, the simplest of the plant pathogenic agents, have not been demonstrated to exist in a free state outside the living cells of their respective host plant species. The presence of viroids in the soil, water or air either alone or in the organisms present in these substrates has not been reported and they differ from other groups of microbial plant pathogens in this respect. Transmission of any viroid through a natural vector, possibly other than man, is not known. Contaminated tools like cutting knives, as in the case of *Potato spindle tuber viroid* (PSTVd) may mechanically transfer the viroids to tissues of healthy plants. As such, presence of viroids in alternative or additional host plant species that are susceptible to the viroid concerned has been reported. However, it is not known how the viroid present in another host plant species can reach the crop plant species is unclear and the role of the alternative host plant species in the viroid survival and spread of viroid diseases is yet to be understood. It has been observed that one viroid may infect different crop plant and weed species and one plant species may be infected by two or more viroids. Various diagnostic techniques have been demonstrated to be effective for detection and differentiation of viroids. Various methods of detection of viroids and their applicability for large-scale use have been indicated already (Volume 3, Chapter 2).

Citrus trees are infected by many viroids of which five belong to the family *Pospiviroidae.* They are *Citrus exocortis viroid* (CEVd), *Citrus bent leaf viroid* (CBLVd), *Hop stunt viroid* (HSVd), *Citrus viroid* III (CVd-III) and *Citrus viroid* IV (CVd-IV) (Duran-Vila et al. 1993). Later two more viroids, *Citrus viroid OS* (CVd-OS) from Japan and *Citrus viroid V* (CVd-V) isolated from *Atlantia citrioides* from Spain were detected and identified (Ito et al 2001; Serra et al. 2008). A one-step multiplex RT-PCR assay was able to detect these five viroids, CEVd, CBLVd, HSVd, CVd-III and CVd-IV. In the absence of natural and efficient vector, the viroids can spread only through seeds and vegetatively propagated planting materials. Selection of suitable viroid-free seeds and planting materials seems to be the practicable method of containing the viroid diseases. As these viroids are transmitted through propagation materials strict enforcement of certification regulations would be of great importance for containing the spread of these viroids (Wang et al. 2009).

Weed species growing in and around crop plants have been shown to be the possible sources of infection for fungal, bacterial and viral plant pathogens. Possibly these pathogens with natural biotic vectors, can spread also through soil, water or air from weeds to crop plants. In contrast, the viroids, without any known biotic vectors and inability to spread through other agencies, should have some mechanism (s) for their spread from infected plants to healthy plants. An attempt has been made to explore the feasibility of weed species serving as reservoirs of viroid infection. A biolistic procedure was adopted to inoculate weed plants species growing in potato and hop fields with *Potato spindle tuber viroid* (PSTVd) and *Hop stunt viroid* (HSVd) populations as either RNA or cDNA. The RT-PCR assay was applied for the detection and quantification of PSTVd and HSVd in the inoculated weed plants. Sequence analysis of PSTVd progenies replicated in *Chamomilla reculita* and *Anthemis arvensis* showed a wide

spectrum of variants related to various strains from mild to lethal variants. The sequence variants isolated from *Amarathus retroflexus* and *Veronica argensis* exhibited similarity or identity to the super lethal AS1 viroid variant. All HSVd clones from *Galinsoga ciliata* corresponded to an HSVdg variant which was found to be strongly pathogenic for European hops (Matoušek et al. 2007).

Appendix 1: Detection of *Tobacco ringspot virus* (TRSV) in the Nematode Vector by Immunofluorescence Technique (Wang and Gergerich 1998)

(i) Select individually active nematodes (about 200) after giving adequate acquisition periods of feeding on virus-infected plants; transfer them to 1.5-ml centrifuge tube containing tap water; centrifuge at 14,000 × g for 4 min and remove the water from the tube.
(ii) Incubate for fixation in 1 ml of 2% formaldehyde for 1 h at 4°C; remove the fixative solution after centrifugation; place the nematodes on a clean glass slide in a small amount of 2% formaldehyde and cut the nematodes into pieces, using a razor blade.
(iii) Suspend the nematode fragments in two or five drops of blocking buffer consisting of 0.14 M NaCl, 0.01 M phosphate buffer, 3% bovine serum albumin (BSA) and 0.2% Triton X-100, pH 7.2, for 15 min at 4°C; gently pipette out the solution into a clean microcentrifuge tube; centrifuge for pelleting the nematode fragments and remove the supernatant.
(iv) Dilute the purified primary antibody specific to the target virus to 1:50 in blocking buffer; transfer 200 µl to the microcentrifuge tube containing nematode fragments; incubate in an orbital shaker at 28°C for 18 h and wash the fragments four times for 10 min each with blocking buffer at room temperature.
(v) Incubate the fragments in 200 µl of a 1: 50 dilution of fluoroscein isothiocyanate (FITC), conjugated goat anti-rabbit immunoglobulin G (Sigma Chemical Co., USA) in blocking buffer in an orbital shaker for 20 h at 28°C and wash the fragments four times for 10 min each in blocking buffer and dry in a vacuum dryer for 15 min.
(vi) Mount the dried pellet on a glass slide in 10 µl of 50% glycerol in phosphate-buffered saline and gently tease the nematodes for their dispersal in the buffer and examine under an epifulorescent microscope.

Appendix 2: Detection of Plant Viruses in Vector Nematodes by Nested PCR Assay (Martin et al. 2009)

Extraction of Viral RNA from Nematodes

(i) Hand-pick nematodes into 100 µl sterile water in 1.5-ml microfuge tubes; add 100 µl collagenase solution containing 10 mg/ml in 50 mM Tris, pH 7.4, 1 mM $CaCl_2$ and incubate the tubes for 1 h at 37°C.

(ii) Add 50 mg glass beads (acid-washed 425–600 μm, Sigma); then add 200 μl 2 × extraction buffer consisting of 400 mM Tris, pH 8.5, 600 mM LiCl, 200 mM EDTA, 3% lithium dodecyl sulfate, 2% deoxycholic acid and 2% tergitol and add 2% ß-mercaptoethanol just before use.
(iii) Vortex the closed tubes at full speed for 1 min; add 400 μl of 6 M potassium acetate, pH 6.5, vortex again and chill on ice for 30 min.
(iv) Spin the tubes in a microfuge at the maximum speed for 5 min; transfer the supernatant to a new tube; add 1 μl glycogen; mix well; add an equal volume of isopropanol; mix well by inverting the tubes four or five times and chill for 30 min at −20°C.
(v) Centrifuge the tubes for 10 min at maximum speed; dry the pellet after decanting the supernatant; wash the pellet with 500 μl 70% EtOH and dry the final pellet under vacuum for 5 min.
(vi) Dissolve the pellet in 20 μl Molecular Biology Grade (MBG) water (Invitrogen); chill on ice for immediate use or store at −20°C.

Nested PCR Assay

(i) For DNA preparation, transfer 2 μl RNA preparation to a 200-μl tube for 20 μl RT reaction as follows: combine 10.2 μl MBG water, 4 μl 5 × first strand buffer, 1 μl 0.1 MDTT, 1 μl 10 mM each dNTP mix, 0.7 μl RNase OUT (Invitrogen), 0.4 μl of 10 μM primer and 0.7 μl Superscript III (Invitrogen) and incubate at room temperature for 1 min and then at 50°C for 60 min.
(ii) Use 1 μl of RT as a template in a 50 μl PCR using the first primer pair specific for target virus and *Taq* polymerase (Invitrogen) as per the manufacturer's instructions (or alter concentration, if required).
(iii) Provide the following conditions for amplification: 2 min at 94°C, followed by 40 cycles each at 94°C for 40 s, at 56°C for 40 s, at 72°C for 1 min and finally at 72°C for 4 min.
(iv) For nested PCR, use 1 μl of the first PCR amplicon as template in a 50 μl reaction with the same parameters and conditions as in the first round PCR except for replacement with nested primer pair.
(v) Analyze 15 μl of nested PCR amplicon by agarose gel electrophoresis and staining with ethidium bromide.

Appendix 3: Detection of *Raspberry bushy dwarf virus* (RBDV) in the Nematode Vector *Longidorus juvenilis* by Nested PCR Assay (Pleško et al. 2009)

(i) Extract the nematodes from soil samples; store them at 4°C; extract the total RNA from the nematodes using RNeasy Plant Mini Kit (Qiagen).
(ii) Use RBDV-specific primers U1, L3 and L4.

(iii) For cDNA synthesis, add 10 µl of extracted total RNA to 15 µl of reaction mix containing 50 pmol of primer L4, 5 µl × 5M-MLV RT buffer (Promega), reverse transcriptase (Promega), 200 U RNasin (Promega) and incubate for 1 h at 50°C.
(iv) Add 10 µl of RT reaction to 40 µl of reaction mix consisting of 75 mM Tris–HCl, pH 8.8, 20 mM ammonium sulfate, 0.01% Tween-20, 2 mM $MgCl_2$, 0.2 µM dNTPs, 50 µmol of each of the primers U1 and L4 and 2.5 U *Taq* DNA polymerase (Fermentas) for the first round PCR.
(v) Provide the following conditions for PCR: initial denaturation for 5 min at 94°C, followed by 40 cycles of 1 min at 95°C, 1.5 min at 55°C and 1.5 min at 72°C and final elongation for 10 min at 72°C.
(vi) Use 1 µl of the amplicon for second amplification using the primers U1 and L3 in place of the primer L4 and provide all conditions as mentioned above.
(vii) Analyze the amplified products on 1% agarose gels, after staining with ethidium bromide.

Appendix 4: Detection of *Polymyxa* DNA in the Soil by Real-Time PCR Assay (Ward et al. 2004)

Extraction of DNA from Soils

(i) Collect soils with *Polymyxa betae* from sugar beet fields and soils with *P. graminis* from cereal fields; mix 5 g soil samples with 10 ml of EDTA-based lysis buffer (containing 50 mM Tris–HCl, pH 7.2, 50 mM EDTA, 3% SDS, 1% 2-mercaptoethanol) to form a slurry; transfer 1-ml aliquots to 2-ml tubes (five replicates) containing five 3-mm tungsten carbide balls (Qiagen) and centrifuge at 13,000 g for 2 min.
(ii) Transfer the clarified soil extract to a fresh 2-ml Eppendorf tube; extract the DNA using a Wizard ManeSil DNA Purification System for food kit (Promega) and a Kingfisher Magnetic Particle Processor as per the manufacturer's recommendations; determine DNA purity using a spectrophotometer at 260 nm (DNA), 280 nm (protein) and 320 nm (background turbidity) and calculate absorbance ratio = $(A_{260} - A_{320})/(A_{280} - A_{320})$ (the ratio of pure DNA is 1.8).

TaqMan Real-Time PCR

(i) Design primers to correspond to unique areas of sequence using Primer Express software (Applied Biosystems, USA); use primer pair and probe Pgraminis 690F/758R/713T specific to *P. graminis* and Pbetae689F/760R/718T specific to *P. betae*.
(ii) Set up TaqMan reactions in 96-well reaction plates using a PCR core-reagent kit (Applied Systems) as per the manufacturer's recommendations; for each

reaction add 1 µl DNA to 24 µl mastermix in the appropriate well, giving a final reaction volume of 25 µl.

(iii) Provide the following conditions: 50°C for 2 min, 95°C for 10 min and 40 cycles of 60°C for 1 min, 95°C for 15 s within the 7,700 Sequence Detection System (Applied Biosystems) using real-time data collection.

Appendix 5: Detection of *Impatiens necrotic spot virus* (INSV) by DAS-ELISA in Thrips Vectors (Sakurai et al. 2004)

(i) Coat each well of microtiter plate with 100 µl coating buffer (containing 0.05 M sodium carbonate, pH 9.6) with 1.5 µg/ml IgG of PAb (raised against N protein of INSV-J) and incubate for 3 h at 37°C.

(ii) Block nonspecific binding sites with 100 µl 1% BSA in PBS consisting of 0.14 M NaCl, 1 mM KH_2PO_4, 8 mM Na_2HPO_4, 2.5 mM KCl, pH 7.4; load the wells with 100 µl thrips extracts homogenized with a micropestle in a sample buffer containing 2% polyvinyl pyrrolidone(MW 40,000), 0.2% BSA in PBS including 0.05% Tween-20 (PBST) and incubate overnight at 4°C.

(iii) Maintain controls with comparable thrips without access to infected source plants.

(iv) Add 100 µl IgG alkaline phosphatase conjugate (1.5 µg/ml) in a sample buffer to each well; incubate for 3 h at 37°C and rinse the wells with PBST three times between each step above.

(v) Add substrate 100 µl of 1 µg/ml *p*-nitrophenyl phosphate in 0.01 M diethanolamine buffer, pH 9.8 to each well and record absorbance values at 405 nm after 1 h reaction using an ELISA plate reader.

(vi) Absorbance values greater than the mean plus four times the SD of controls are considered as positive reaction.

Appendix 6: Detection of *Citrus tristeza virus* RNA Targets by Tissue Squash Real-Time RT-PCR Assay in Aphids (Bertolini et al. 2008)

Preparation of Single Aphid Squash Samples

(i) Squash single aphid species (*Aphis gossypii*) on 3 MM paper or nylon membranes with the rounded end of an Eppendorf tube and insert the pieces of paper or membrane harboring/squashed sample into Eppendorf tubes.

(ii) Add 100 µl of 0.5% Triton X-100 or 100 µl buffer containing 0.1 M glycine, 0.05 NaCl and 1 mM EDTA and incubate at 95°C for 10 min.

(iii) Vortex the contents in the tube; place the tubes on ice and use 5 µl of this extract as template for real-time RT-PCR.

Real-Time RT-PCR Assay

(i) Select nucleotide sequence flanked by universal primers Pin1 and Pin2 to design the primers and probes and use Primer Express software (Applied Biosystems) to obtain the optimal oligonucleotide probe sequences.
(ii) Perform TaqMan assays for real-time RT-PCR in ABI Prism 7,000 Sequence Detection System software (Applied Biosystems) using the reaction cocktail containing IX TaqMan Universal PCR Master Mix (Applied Biosystems) consisting of 1 µM 3′ UTR, 150 nM TaqMan probe 18IT and 5 µl of purified RNA or extracted RNA targets from immobilized samples.
(iii) Provide the following conditions: one-step at 48°C for 30 min and 95°C for 1 min followed by 45 cycles of amplification at 95°C for 15 s and 60°C for 1 min.
(iv) Perform data acquisition and analysis using ABI Prism 7,000 software and repeat the assays six times for determining the sensitivity and reliability of the assay.

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Chapter 4
Assessment of Variability in Plant Viral and Viroid Pathogens

Abstract Assessment of variability in different characteristics of viral and viroid pathogens affecting various crops has provided information on the differences in the pathogenic potential, host range and distribution of different strains/variants of a virus or viroid. Biological, immunological and nucleic acid-based techniques have been shown to be efficient and effective in differentiating the strains of viruses to varying degrees. Variations in plant virus strains may occur due to mutation or selection from a mixture of strains, because of the incorporation of resistance gene(s) into the cultivars that were susceptible earlier. Changes in the population structure of viruses have to be constantly monitored by applying sensitive techniques that can provide results rapidly to recognize the occurrence of new more virulent virus strain(s) to facilitate formulation of effective disease management strategies applicable to different ecosystems. Viroids, the simplest plant pathogenic entities containing exclusively short circular RNA molecules, also vary distinctively when their environment is significantly altered. The viroids have high potential for producing variants that can infect different plant species that may be available along with crop plants. In addition, perennial fruit trees with long span of life, may allow accumulation of several sequence variants in the same tree without the need for passage through other plant species. Nucleic acid-based techniques have been demonstrated to be very effective in detecting and differentiating variants even with a change in a single nucleotide in the genome of the viroids.

Viral and viroid pathogens with obligate mode of parasitism on the plants have to establish compatible relationships with different plant species to a greater degree than the other microbial plant pathogens that can survive outside their plant/vector hosts for short or long time as saprophytes. The viruses and viroids have to depend on the plant hosts entirely for their replication and survival. Viruses and vrioids differ in their structure and genetic constitution, although they have similar mode of transmission to healthy plants through grafts. The absence of a protein coat and the lack natural vectors primarily differentiate the viroids from the viral pathogens.

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4.1 Assessment of Variability in Plant Viral Pathogens

Plant viruses may have a restricted or wide host range and some of them are able to multiply in their natural vector species. As the internal cellular conditions in various plants and insects are widely different, the viruses have to adapt themselves to establish infection successfully. Latent infections without inducing any visible symptoms in the plant species represent the most compatible relationship with such host plant species, while development of necrotic lesions indicates high level of virulence of the virus in those host plant species. As the viruses are not known to have any physiological functions such as respiration, direct interaction between the viral genome and products of host genes may necessitate suitable changes in the viral genome, resulting in variations in the pathogenic potential (virulence) of the viruses. Such interactions may lead to variations in the biological, biochemical, immunological and genetic characteristics of viruses and their strains. Information on the existence of different strains of a virus and their virulence may be useful for preparing effective disease management strategies such cross-protection using mild strains and breeding for resistance to virus disease(s). The effectiveness of the techniques that are useful for assessing the variability in viruses and their strains is discussed below.

4.1.1 Assessment of Variations in Biological Characteristics

Variations in the type and intensity of symptoms induced by strains of some plant viruses have been observed. The term 'virulence' has frequently been applied to indicate the degree of symptom severity induced by viruses. Virulent strains *Tobacco mosaic virus* (TMV) cause severe symptoms, while mild (less virulent) strains induce mild or no visible symptoms. Cultivars of a crop plant species possess different levels of resistance/susceptibility to viral pathogens resulting in development of different intensity of symptoms varying from complete absence of visible symptoms or masking of symptom development. In certain host–virus combinations as in the case of TMV and tobacco cv. Xanthi nc., the systemic spread of the virus is arrested and only necrotic local lesions are produced on inoculated leaves. Such hypersensitive reaction (HR) has been practically exploited for the management of TMV which has no natural vector for spreading the virus. This type of field resistance has practical utility in restricting the virus spread (Narayanasamy 2001).

Variations in the pathogenic potential may occur because of selection of a strain from a mixture of strains of a virus by a host plant species which may provide suitable environment for the development of a particular strain of the virus. Introduction of a resistance gene from wild or immune host plant species into the cultivar, or exposure to adverse environmental conditions such as high temperature may result in mutation in the viral genome. A new strain with ability to overcome the newly incorporated resistance gene or adverse environmental conditions, has to be produced, if the virus has to survive. Production of new mutants may be possible by

application of chemicals like nitrous acid. The mild strains produced artificially or selected from naturally occurring mixture of strains have been successfully employed to offer protection against severe strains of the same virus (Narayanasamy 2008).

For the differentiation of strains of viruses by biological methods, the local lesion/assay host plant species or a set of plant species that react with differential responses may be used. It is considered that a local lesion is initiated by a single virus particle. Hence, by single-local lesion inoculation (analogous to single spore isolation for fungal pathogens) onto appropriate plant species, different strains of the virus may isolated/differentiated. The procedure, however, is labor-intensive and requires large greenhouse space, in addition to long time. Another biological characteristic that has been used for differentiation the plant viruses is based on the nature of relationship of viruses with their vectors belonging to different species of mites, insects, nematodes and fungi (Narayanasamy and Doraiswamy 2003). The nature of relationships between viruses and their vectors may show distinct differences in the acquisition and inoculation access periods, presence and length of latent periods required to become viruliferous and the length of time for which the virus is retained by the vector species. The aphid *Myzus persicae* is known to transmit the maximum number of viruses which determine the nature of relationship (non-persistent, persistent and propagative) with their vectors. However, differences between strains of a virus in this characteristic have not been consistently observed (Volume 3, Chapter 2).

Potato virus Y (PVY) strains have been differentiated based on the type of symptoms induced on potato cultivars and other plant species. The common strain PVY^{O} causes mosaic symptoms on most potato cultivars. The necrotic strain PVY^{N} causes a lethal veinal necrosis in tobacco, while necrotic ringspot symptoms in potato tubers and tobacco are due to the strain PVY^{NTN}. Some isolates of PVY^{NTN} are recombinants between PVY^{O} and PVY^{N} strains, whereas others might have arisen by point mutations. Recombinant strains PVY-Wilga or $PVY^{N:O}$ induce veinal necrosis in tobacco, like PVY^{N} strain, but they are serologically related to PVY^{O}. The indicator plant *Solanum brachycarpum* exhibits a necrotic response to inoculation with PVY^{N} and mosaic symptom to inoculation with PVY^{O} . The potato cv. Allegany has the HR gene *Ny* and responds with local lesion formation to PVY^{O} and remains asymptomatic, when inoculated with PVY^{N}. The potato cv. Yukon Gold produce tuber necrosis when inoculated with PVY^{N} isolates (Fig. 4.1; Chrzanowska 1994; Boonham et al. 2002; Nie and Singh 2002; Baldauf et al. 2006).

A novel *Angelonia flower break virus* (AnFBV) causing flower break and mild foliar symptoms was biologically characterized by mechanically inoculating 45 different plant species including tomato, tobacco and cucumber and weed plants. Obvious visible symptoms appeared at about 12–14 days after inoculation. *Nicotiana benthamiana* showed local and systemic symptoms, while *N. rustica* and *N. sylvestris* developed local lesions. AnFBV infection of these plants was confirmed by ELISA tests (Adkins et al. 2006). Variations in symptomatology, host range and pathogenicity of strains/isolates of *Zucchini yellow mosaic virus* (ZYMV) have been reported (Antignus et al. 1989; Lecoq and Purcifull 1992). In a later investigation, variations in the biological characteristics of isolates of ZYMV were examined

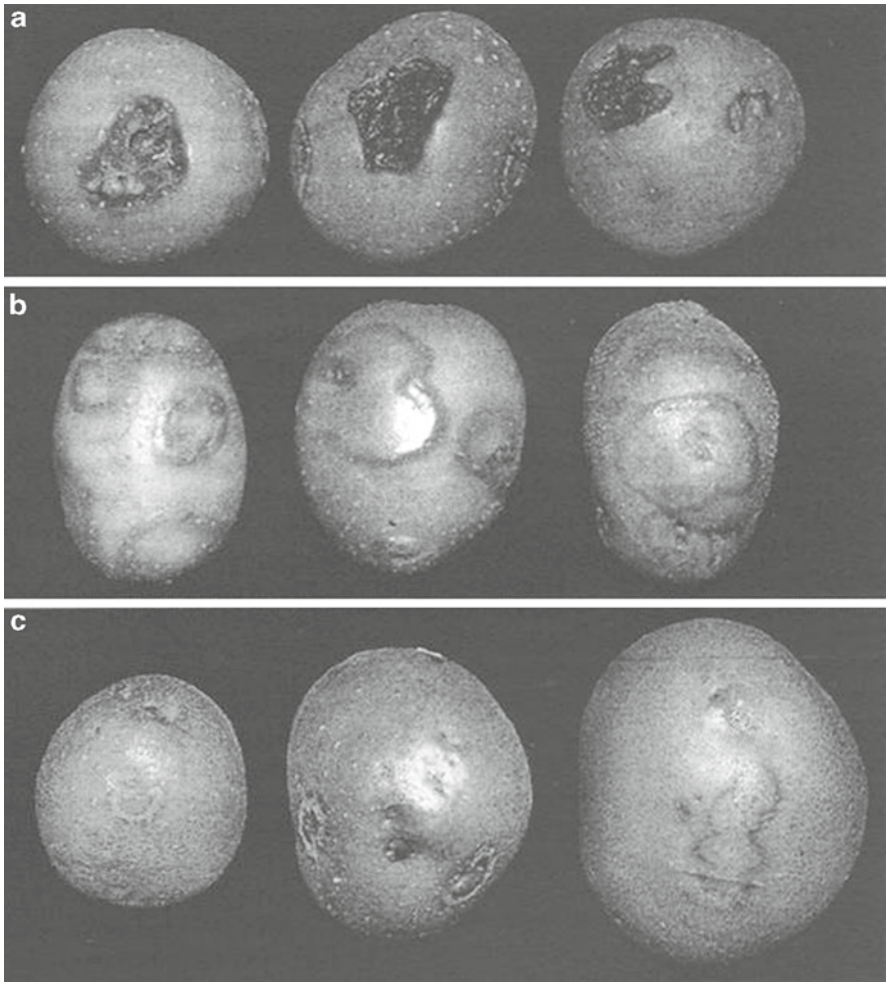

Fig. 4.1 Variations in symptoms induced by *Potato virus Y* isolates in greenhouse-grown potato cv. Yukon Gold tubers. (**a**) PVY^{NTN} reference isolate FL40 inducing advanced necrosis at the stem-end of tubers; (**b**) PVY^{NTN} symptoms induced by the isolate PB312 with raised necrotic rings around the tuber eyes; (**c**) atypical tuber necrosis induced by $PVY^{N:O}$ reference isolate AL1 (Courtesy of Baldauf et al. 2006; The American Phytopathological Society, MN, USA)

by inoculating different plant species and by sequencing of genomic portion of the C-terminal part of Nib and N-terminal part of the CP gene. All 12 Iranian isolates induced necrotic local lesions in *Chenopodium amaranticolor*, while only four isolates were able to produce necrotic local lesions in *Gomphrena globosa* and other isolates were not able to infect this host plant species. The absence of these isolates of ZYMV in *G. globosa* was confirmed by DAS-ELISA tests (Bananej et al. 2008).

Tomato crops grown in large plastic houses in Sapopena-PR, Brazil are infected by *Tobacco mosaic vi*rus (TMV) and other members of the genus *Tobamovirus*. The strain TMV-Sapopena was unable to infect *Petunia hybrida* described as a host for TMV and *Tomato mosaic virus* (ToMV). But severe mosaic symptoms developed in tomato cv. Santa Clara and Avansus with strong leaf narrowing, while the cv. Carmen developed only mild symptoms following inoculation with TMV-Sapopena strain. This strain was differentiated based on these differences in the type of symptoms induced in plants (da Silva et al. 2008). *Cucumber mosaic virus* (CMV) isolates (10) associated with banana mosaic disease (BMD) in Taiwan were compared for their biological characteristics. The CMV isolates were inoculated onto banana (Cavendish) plantlets using the cotton aphid *Aphis gossypii*. The isolates were differentiated into four pathotypes on the basis of symptoms induced on banana, *Nicotiana glutinosa* and *Vigna unguiculata* (cowpea) which were mechanically inoculated. Three different symptom types (A, B and C) were recognized using *N. glutinosa* as a differential host plant. Two isolates became systemic inducing mosaic symptoms on newly expanded leaves, while other isolates produced local lesions on inoculated leaves. The satellite RNA associated with CMV may affect symptom expression. The possibility of satellite RNA having a role on the symptom severity was investigated. Both banana and *N. glutinosa* did not show any change in the symptom severity due to the presence or absence of satellite RNA (Chou et al. 2009).

4.1.2 Assessment of Variations in Physico-chemical Characteristics

The stability of viruses has been determined both in plant sap and after their purification from the sap extracted from infected plants. The physical properties of viruses in extracted sap such as longevity *in vitro* (LIV), dilution end point (DEP) and thermal inactivation point (TIP) were determined by early workers, in the case of sap transmissible viruses. These properties were used to differentiate highly stable viruses like *Tobacco mosaic virus* (TMV) and highly unstable viruses like *Tomato spotted wilt virus* infecting the same plant species. However, these properties are rarely determined for putative identification of a new virus occurring in a location. The properties of purified viruses such as molecular weight of virus particles, coat protein and different nucleic acid species present in the monopartite/multipartite viruses, sedimentation coefficient of viral components, strandedness (single or double), electrophoretic mobility, viral genome organization, amino acid sequence of viral coat protein and virus-associated proteins and nucleotide sequences of viral genome, presence of satellite virus and satellite RNA have been determined. These properties, in addition to the virus particle morphology have been used for classification of viruses into different groups.

Electrophoretic properties of *Cucumber mosaic virus* (CMV)-NB strain causing tomato fruit necrosis disease were studied. The elelctrophoretic mobility of CMV-NB

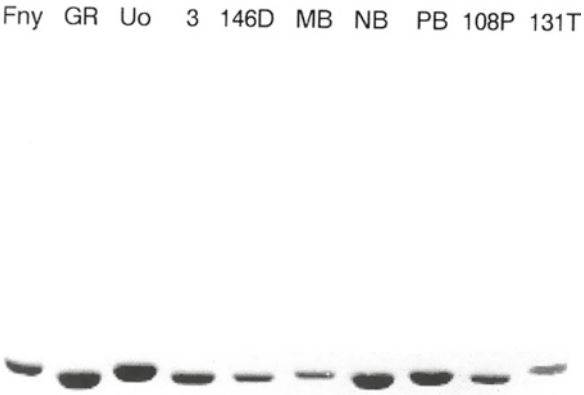

Fig. 4.2 Comparative electrophoretic mobilities of the capsid proteins of NB and other strains of *Cucumber mosaic virus* (CMV). SDS-PAGE analysis of the capsid protein of CMV strains. (Courtesy of Yordanova et al. 2002; J Culture Collections, CRIA, Brazil)

strain coat protein was compared with other CMV strains. The differences observed, were rather strain-specific. The NB strain showed higher mobility of the CP and it was similar to the strains GR and 3 (subgroup II) and strains PB and Uo (subgroup I) (Fig. 4.2). The electrophoretic analysis indicated that there might be single amino acid substitutions with acidic residues in the CP of NB strain which are situated on the surface of virus particles. Hence, negative charges may accumulate on the intact virion surface resulting in significant changes in their electrophoretic mobility in the alkaline medium (Yordanova et al. 2002). *Tomato black ring virus* (TBRV), belonging to the genus *Nepovirus*, has two genomic RNA species. RNA1 contains the genes involved in viral replication and polyprotein processing, while RNA2 has genes governing synthesis of viral CP and viral movement in the plants. Electrophoretic analysis showed the presence of two RNA bands of size typical for TBRV in all isolates from cucumber (TBRV-Cuc), tomato (TBRV-Tom), black locust (TBRV-MJ, -N1, -L10 and -Pn) and potato (TBRV-Pot). Majority of the isolates had some additional bands of small non-genomic RNAs also (Jończyk et al. 2004).

4.1.3 Assessment of Variations in Immunological Characteristics

Identification and differentiation of plant viruses and their strains with varying pathogenic potential has been accomplished by employing various immunoassays. Both polyclonal and monoclonal antibodies (PAbs and MAbs) have been shown to be effective. The specificity and sensitivity of immunoassays have been significantly improved by the development of MAbs. Among the immunoassays, enzyme-linked immunosorbent assay (ELISA) has been more frequently applied for determination

of variations in plant viruses and their strains. The viral coat protein, structural and nonstructural proteins have been employed for the production of antisera for use in differentiation of strains of plant viruses.

4.1.3.1 Immunoblot Analysis

Viral proteins, after separation by electrophoresis are transferred from the gel matrix to a membrane and then they are probed with specific immunoprobes. The immunoblot analysis may be used to establish relationship between viruses and their strains. By using bacterially-expressed coat protein of *Sweet potato sunken vein virus*, the Kenya isolate (SPSVV-Ke) was shown to be closely related serologically and to be similar to Closterovirus isolates infecting sweet potato in Israel, Nigeria and the USA (Hoyer et al. 1996). The strains of *Wheat streak mosaic virus* (WSMV) were found to be serologically distinct by employing western blot analysis and such information was necessary for breeding wheat cultivars resistant to different strains of WSMV occurring in different countries (Montana et al. 1996). Mixed infections by *Grapevine leaf roll-associated viruses* (GLRaVs) and *Grapevine corky bark-associated virus* are common in grapevine crops. Immunoblot procedure was shown to provide reliable detection and differentiation of these viruses and their strains present in infected grapevine plants (Monis and Bestwick 1997). Soybean plants were found to be infected by *Soybean dwarf virus*-dwarfing (SbDV) and *Soybean dwarf virus*-yellowing (SbDV-Y) in Japan. These two viruses were shown to be serologically related by preparing immunoblots probed with SbDV-D antiserum containing PAbs. A single 26-kDa coat protein (CP) band was recognized in the blots, confirming the close serological relatedness of the virus isolates present in the USA. Specific MAbs reacted differentially with endemic isolates (Damsteegt et al. 1999). *Papaya leaf distortion mosaic virus* (PLDMV) occurs in severe forms in Okinawa Island, Japan. A virus that reacted with a PLDMV antiserum was found for the first time in a cucurbitaceous weed [PLDMV-C(T)]. The C biotype of PLDMV (PLDMV-C) was unable to infect papaya and indistinguishable from PLDMV-P in serological tests. The serological relationships among strains of PLDMV were examined by employing western blotting and SDS-immunodiffusion tests. Positive reactions between all antigens and antisera were observed. The antisera against PLMDV-P (LDM), -P(YM) and -C(T) reacted well with all antigens in both serological tests (Mayoka and Hataya 2005).

Angelonia flower break virus (AnFBV), a new carmovirus infecting Angelonia plants, has a 38-kDa protein which appeared as a major polypeptide band in the gel after performing SDS-PAGE analysis of disrupted preparations. Immunoblots with AnFBV antisera gave a clear and strong reaction with the 38-kDa band. This protein was identified as the AnFBV coat protein. In the reciprocal tests with the antisera against *Pelaragonium flower break virus* (PFBV). *Carnation mottle virus* (CarMV) and *Saguaro cactus virus* (SgCV), positive reactions with homologous viruses alone were observed, indicating the capacity of immunoblotting technique to differentiate AnFBV from other viruses (Adkins et al. 2006). Infection of *Phalaenopsis* orchids

by a new potyvirus causing chlorotic spots on the leaves was observed in Taiwan. This virus isolate 7–2 reacted positively with the MAb POTY reactive to the members of potyvirus group. The SDS-PAGE analysis revealed the presence of one structural protein of 33–34 kDa present in the purified virus preparation. Western blotting of the leaf extracts from virus-infected *Chenopodium quinoa* using a 4,000-fold dilution of rabbit PAb showed similar band of 34-kDa. Back-inoculation of the virus from *C. quinoa* to *Phalaenopsis* resulted in the production of chlorotic spot symptoms, confirming that *Phalaenopsis chlorotic spot virus* (PhCSV) as the causal agent of the newly observed disease in Taiwan (Zheng et al. 2008).

4.1.3.2 Enzyme-Linked Immunosorbent Assay

The usefulness of ELISA tests for differentiation of strains of several viruses has been reported: *Tomato spotted wilt virus* (Sherwood et al. 1989; de Avila et al. 1990), *Beet mild yellowing virus* (Stevens et al. 1994; Smith et al. 1996), *Soybean mosaic virus* (Hill et al. 1994), *Potato virus Y* (Canto et al. 1995), *Apple chlorotic leaf spot virus* (Malinowski et al. 1997) and *Beet necrotic yellow vein virus* (Mahamood and Rush 1999). In addition to the coat protein (CP) of *Potato virus Y* (PVY), a helper component protein (HC-Pro) that helps in the aphid transmission is also synthesized in the infected plants. MAbs and PAbs generated against the HC-Pro could be used to differentiate the strains of PVY (Canto et al.1995; Blanco-Urgoiti et al. 1998). An MAb was employed to differentiate *Beet mild yellowing virus* (BMYV) with a differing host range from other strains of BMYV commonly observed under field conditions (Smith et al. 1996).

Monoclonal antibodies (MAbs) have greater discriminating capacity as they can react with different specific epitopes present on the viral coat protein or other virus-associated protein. Plant viruses and their strains have been assigned to different serotypes based on their reactivity to different MAbs. The serotypes may have similar biological or structural characteristics in common. By employing DAS-ELISA format using a combination of the universal MAb5B, and MAbs specific for serotypes, the West *African Rice yellow mottle virus* (RYMV) isolates (73) were grouped into three distinct serogroups. These serogroups were correlated to two RYMV pathotypes that were differentiated based on their reaction on a set of differential rice cultivars (Konaté et al. 1997). Likewise, a panel of 16 MAbs raised against *Bean yellow mosaic virus* (BYMV) was shown to be effective in differentiating strains of BYMV by performing TAS-ELISA tests. Variations in serological characteristics showed relationship with four pathotypes (I–IV) recognized based on pathogenic potential of the isolates (Sasaya et al. 1998). Two serotypes of *Sweet potato feathery mottle virus* (SPFMV) prevalent in Uganda were differentiated by a specific MAb generated against the coat protein of SPFMV. The geographical distribution and ability to infect some sweet potato cultivars of these two serotypes showed distinct variations (Kareyeija et al. 2000).

Isolates of *Soilborne wheat mosaic virus* (SBWMV) from USA, France and Japan showed variations in their reactivity to three MAbs. It was suggested that

three amino acids of the viral coat protein surface differed and they may determine the antigenic reactivity of the isolates of SBWMV (Chen et al. 1997). The MAbs and PAbs prepared against *Chinese wheat mosaic virus* (CWMV) and *Soilborne wheat mosaic virus* Oklahoma isolate (SWMV-OKI) were employed for the differentiation of isolates of *Wheat mosaic virus* (WMV), SBWMV, *Oat golden stripe virus* (OGSV) and *European wheat mosaic virus* (EWMV). The dominant epitopes of CWMV shared partially with OGSV, while isolates of SBWMV exhibited similarity with CWMV, OGSV and EWMV to varying degrees (Ye et al. 2000). Incidence and distribution of the serotypes of *Barley yellow dwarf virus* (BYDV) was monitored by employing specific MAbs. The extent of incidence of PAV serotype transmitted by *Rhopalosiphum padiavenae* and MAV serotype transmitted by *Macrosiphum avenae* was shown to be directly correlated to their respective aphid vector populations (Quillec et al. 2000).

Members of the genus *Tospovirus* have similar virus particle morphology, genome organization and similar vector relationship with thrips. A threshold of 90% amino acid sequence identity of tospoviral nucleocapsid protein (NP) that encapsidates viral RNAs is one of the important criteria for species designation (Goldbach and Kuo 1996). Based on the serological relationships and phylogenetical analysis of NPs, the genus *Tospovirus* has been assigned with 16 official and tentative virus species that can be clustered into three major serogroups and four monospecies serotypes (Jan et al. 2003). *Tomato spotted wilt virus* (TSWV) and *Watermelon silver mottle virus* (WSMoV) are the representatives of TSWV and WSMoV serogroups respectively. *Calla lily chlorotic spot virus* (CCSV) isolated from Taiwan was identified as a tospovirus serologically but distantly related to WSMoV based on serological relationship established by employing PAbs and MAbs to WSMoV NP and CCSV NP in indirect ELISA format and low-intensity banding in immunoblotting. The MAbs produced against CCSV NP or WSMoV NP reacted specifically with homologous antigens, but not with heterologous antigens in both ELISA and immunoblot analyses (Lin et al. 2005).

Citrus tristeza virus (CTV) strains occurring in Israel were differentiated into two serogroups based on their reactivity with MAbs. These serogroups were correlated with groups differentiated by sequencing of their CP genes (Shalitin et al. 1994). Differentiation of strains of CTV inducing stem pitting symptoms in citrus is important, because of the great damage caused by this strain to citrus production. By employing different combinations of PAbs and MAbs in indirect DAS-ELISA format, stem pitting inducing strains of CTV were differentiated, facilitating the eradication of infected citrus trees effectively (Nikolaeva et al. 1998). *Citrus psorosis virus* (CPsV) from 53 field sources was analyzed for the variability of CP gene of the virus. By employing 23 MAbs, nine serogroups and at least 10 different epitopes were recognized. The reaction patterns in TAS-ELISA format group the field sources into nine patterns designated A to I, the most frequent pattern being A with 39.6% of the sources. Different citrus species and cultivars growing in the same plot usually exhibited distinct reaction patterns. When 40 isolates from different countries were tested using 24 MAbs, 14 reaction patterns and at least 17 different epitopes were differentiated (Alioto et al. 2003).

Monoclonal antibodies (MAbs) and polyclonal antibodies (PAbs) have been employed for screening fruit trees for the presence of viruses commonly infecting them. MAbs generated against *Apple chlorotic leaf spot virus* (APCLSV) were used in ELISA tests for routine screening of apple trees. Whereas MAb5 could detect most of the strains of APCLSV, MAb1, MAb2 and MAb9 were required for differentiating non-typical APCLSV strains (Malinowski et al. 1997). *Prunus ring-spot virus* (PNRSV) infects rose and *Prunus* trees. The reactivity of PNRSV isolates to MAbs generated against PNRSV strain infecting rose was determined using ELISA test. The most common PNRSV serotype in rose was different from the most prevalent serotype in *Prunus* spp. Pathogenicity tests showed that all rose isolates (17) were able to infect *Prunus persica* seedlings, but only three of the four *Prunus* isolates were weakly virulent to *Rosa indica* (Moury et al. 2001).

Plum pox virus (PPV), with potential to cause serious losses, exists in the form of several strains. Serotypes of PPV designated PPV-D and PPV-M could be differentiated by employing specific MAbs in DAS-ELISA format. Confirmation of the results was obtained by performing PCR or RFLP analysis of PCR products (Candresse et al 1998). In another investigation, PPV isolates were classified into four serotypes M, D, C and El Amar (Myrta et al. 1998). In a later study, in order to assess the variability in the isolates of PPV (43) collected from five different orchards in Romania, triple antigen sandwich (TAS)-ELISA format was applied using PPV-D and PPV-M specific MAbs. All isolates reacted positively to at least one of the two MAbs, as well as to PPV-D or/and PPV-M specific primers in PCR assays. Using TAS-ELISA procedure, 21 of 43 isolates (48.8%) were identified as PPV-D, 17 isolates (39.6%) as PPV-M and five isolates (11.6%) were identified as a mixture of D and M strains (Table 4.1). The results of TAS-ELISA tests were similar to those of immunocapture (IC)-RT-PCR assay (Zagrai et al. 2008).

Cucumber mosaic virus (CMV) is known to occur in the form of several strains. MAbs were generated using a mixture of CMV isolates belonging to subgroups I and II. The MAbs were employed to differentiate 12 weed-characterized strains of CMV. The presence of virus- and subgroup-specific epitopes on CMV-CP was revealed by these MAbs (Hsu et al. 2000). In another investigation 51 isolates and strains of CMV viruses prevalent in Bulgaria were serologically differentiated into

Table 4.1 Serological detection and differentiation of *Plum pox virus* (PPV) isolates (Zagrai et al. 2008)

	DAS/TAS-ELISA			
Orchard no.	PPV poly	PPV-D	PPV-D	PPV D+M
1	10	6	2	2
2	10	3	7	0
3	10	4	5	1
4	10	7	3	0
5	3	1	0	2
Total	43	21	17	5
Percentage	100	48.8	39.6	11.6

subgroups I and II using different ELISA formats with PAbs and MAbs and immunodiffusion tests. The results were corroborated by PCR and RFLP data (Hristova et al. 2002). *Cucumber mosaic virus* NB strain (CMV-NB) causes tomato fruit necrosis disease. Antigen-coated plate (ACP)-ELISA format was applied using PAbs and MAbs for the detection and differentiation of this strain from eight other CMV stains. When PAbs were employed, CMV-NB strain behaved as a strain of subgroup I. However, application of MAb CF11 revealed higher extinction values for CMV-NB compared to other CMV strains, but CF11 was not serotype specific (Yordanova et al. 2002). Tomato crops are commonly infected by *Tobacco mosaic virus* (TMV) and *Tomato mosaic virus* (ToMV) and they may cause single or mixed infections. ToMV could be efficiently detected and differentiated by employing a specific MAb (19.H1) generated against ToMV in plate-trapped antigen (PTA)-ELISA format. There was no cross-reaction with TMV, since only the band corresponding to 17.5 kDa protein of ToMV was recognized by the MAb. The PTA-ELISA employing the specific MAb provided reliable differentiation of the virus in addition to the diagnosis of ToMV infection effectively (Duarte et al. 2002).

Monoclonal antibodies (MAbs) were generated against coat protein (CP) of *Grapevine leaf-roll-associated virus 1* (GLRaV-1). The MAbs differed in their levels of specificity of reaction with different isolates of GLRaV-1, as revealed by DAS-ELISA and western blotting tests. Two MAbs IC4 and IB7 positively reacted respectively with 25 and 32 isolates of GLRaV-1 infection in grapevine plants. The MAb IG10, on the other hand, reacted with both GLRaV-1 and GLRaV-3 coat proteins, indicating that these two viruses might share some antigenic determinants (epitopes) (Seddas et al. 2000). Grapevine virus D (GVD) was shown to be differentially detected by employing the specific MAb in ELISA format (Boscia et al. 2001).

Rice yellow mottle virus (RYMV) accounts for heavy yield losses (97%) in Africa. Strains of RYMV have been differentiated based on variation in symptoms induced in rice cultivars (Konate et al. 1997). Antigen-coated plate (ACP)-ELISA format using PAbs was applied for the differentiation of the strains of RYMV. Serological Differentiation Indices (SDI) data were generated. Each virus isolate is tested against a twofold serial dilution of each antiserum and the test is repeated three times. The SDI represents the average number of twofold dilution steps between homologous and heterologous viruses at standard absorption value of 0.5. The SDI values are read directly from the graph and the final value is expressed as a mean of the replicates. The RYMV isolates from different locations in Cote d'Ivoire were classified into three main serogroups, Sg1, Sg2 and Sg3 and six subgroups Sg1a, Sg1b, Sg2a, Sg2b, Sg3a and Sg3b. The existence and levels of serodiversity of the RYMV isolates were reliably determined by using SDI values. Construction of phylogeny for plant viruses using SDI data generated from ACP-ELISA allows rapid evaluation of serological diversity of many isolates and strain. The results provided evidence for a possible relationship between serological property, host plant and ecological origin of RYMV isolates. The phylogenetic classification of each RYMV isolate defined by SDI data using ACP-ELISA test can be potentially useful for epidemiological investigations. As this procedure is simple, cheap and accurate and it can be applied even in moderately equipped laboratories (Sere et al. 2007).

4.1.4 Assessment of Variations in Genomic Characteristics

Various approaches have been made for assessing variability in biological, physico-chemical and immunological characteristics of plant viruses, which may indirectly reflect the variations in genomic characteristics. Hence, assessment of variations in genomic characteristics directly may be expected to be more reliable and reproducible. Among the various nucleic acid-based techniques, PCR-dependent methods have been more frequently applied for detection and differentiation of plant viruses and their strains. Perennial fruit crops and vegetable crops are often infected by several viruses or their strains individually or in combination producing variable symptoms. It is difficult to resolve the components of the disease complex induced by combined infection by two or more viruses. Hence, it is essential to rapidly detect and differentiate the viruses or strains precisely to estimate the possible losses and to initiate measures to minimize the spread of the disease(s) and consequent yield losses.

4.1.4.1 Double-Stranded RNA Analysis

The presence of double-stranded (ds) RNAs in the infected plants may be observed as a replicative form of single-stranded (ss) RNA viruses or as the infecting viral genomes of the dsRNA viruses (*Phytoreovirus*, genus). The dsRNA profiles generated through Northern blot hybridization may be useful for identifying or classification of an unknown virus or strain. The dsRNA profiles of 26 *Cucumber mosaic virus* (CMV) isolates were prepared and these isolates were grouped into seven distinct dsRNA profile types. As the differences between the profiles were stable, the CMV isolates were classified based on the dsRNA profiles which reflected a greater range of biological characteristics than other methods available (Pares et al. 1992). The presence of four distinct viral dsRNA in chrysanthemum plants infected by *Tomato aspermy virus* was detected. In contrast, plants infected by *Chrysanthemum virus B* (CVB) had only one dsRNA. Thus the dsRNA profiles may be used as the basis for identification and differentiation of viruses infecting the same host plant species (Chung et al. 1999).

Polymorphism of heterologous duplexes of RNA transcripts was analyzed in order to assess the variation that can be used for differentiation of virus strains. By using the PCR products of *Prunus necrotic ringspot virus* (PNRSV), complementary RNA (cRNA) transcripts were prepared. The electrophoretic mobilities of the heterologous duplexes made from cRNA transcripts originating from different isolates of PNRSV showed significant variations. Conformational variations among the transcript duplexes seem to confer this polymorphism termed as double-stranded (ds)-transcript polymorphism (ds-TCP). Identification and differentiation of strains of PNRSV could be accomplished by using ds-TCP as an additional tool (Rosner et al. 1998, 1999). Virus isolates from forage legumes collected from eight different states of USA were identified as luteoviruses closely related to *Soybean dwarf virus*-dwarfing

(SbDV-D) and *Soybean dwarf virus*-yellowing (SbDV-Y). Gel profiles of dsRNA preparations of SbDV-D and SbDV-Y endemic isolates exhibited two distinct bands representing the genomic and subgenomic bands for SbDV-Y were slightly larger than those of SbDV-D. The dsRNA and Northern blot analysis confirmed genomic relatedness of the isolates to SbDV (Damsteegt et al. 1999).

Double-stranded RNA (dsRNA) analysis was performed to establish the relationship between the new virus *Angelonia flower break virus* (AnFBV) with *Carnation mottle virus* (CarMV). The dsRNA isolated from AnFBV-infected *Nicotiana benthamiana* plants was fractionated on agar gels. Four major bands were detected and the largest band of about 4.0 kbp comigrated with the equivalent dsRNA of CarMV. Hence, this band was considered to represent the putative dsRNA form of the AnFBV genomic RNA. In addition, three additional dsRNA species of about 1.75-, 1.60- and 1.31-kbp were also present in the gels. Northern blot analysis of total RNA from AnFBV-infected *N. benthamiana and Angelonia* showed the presence of a 3.96-kb RNA, following probing with an AnFBV CP-specific DIG-labeled probe. Likewise, an RNA band was observed in northern blots prepared with AnFBV viral RNA, confirming the identity and similarity of dsRNA of AnFBV to that of CarMV (Adkins et al. 2006).

Cassava frogskin disease (CFSD) of unknown aetiology occurring in Colombia was investigated. The presence of virus-like particles, inclusion-like bodies and dsRNA viral genomic segments that were associated with CFSD-affected cassava plants was observed. Nine species of dsRNA were separated by a series of PAGE employing dsRNA standard markers. The putative proteins predicted from the sequences of the cassava virus cDNA clones showed similarity with P1, P2, P3, P4, P5 and P10 proteins of *Rice ragged stunt virus* (RRSV). Hybridization analyses of dsRNA identified S1, S2, S3, S4, S5 and S10 genomic segments in the extracts from CSFD-infected plants. They were not detectable in healthy control plants. Phylogenetic analyses confirmed that the virus causing CFSD is a member of the family *Reoviridae* and it is most closely related to RRSV. The presence of the virus in 26 isolates obtained from different locations was detected by real-time PCR assay (Calvert et al. 2008).

4.1.4.2 Hybridization Assays

Hybridization with cDNA or cRNA probes is an easy and powerful method for detecting differences located in any region of the genome (Rosner and Bar-Joseph 1984); Rosner et al. 1986). However, the need for RNA purification and for radioactive probes which have short life and safety hazards, limit the wider use of hybridization methods. Availability of non-radioactive probes like digoxigenin (DIG)-labeled DNA and RNA probes in the recent years, has enlarged the scope for their wider applicability. A non-isotopic hybridization procedure was developed to differentiate isolates of *Citrus tristeza virus* (CTV) using DIG-labeled cDNA probes and different kinds of target RNA. Hybridization of DIG-labeled probes with purified dsRNA or concentrated total RNA extracts spotted on nylon membranes allowed detection of

CTV nucleic acid equivalent to as little as 0.1–1.0 mg infected tissues. Comparatively the sensitivity level was similar or slightly better than that was obtained by hybridization with a ^{32}P-labeled probe. Hybridization of tissue prints with DIG- labeled probes under stringent conditions (60°C and 50% formamide) could differentiate CTV isolates in citrus plants grown either in the greenhouse or in the field (Narváez et al. 2000).

Cucumber fruit mottle mosaic virus (CFMMV) causing severe mosaic symptoms on cucumber fruits, was identified based on the sequence data. The complete sequencing of the viral genome indicated that the genome of CFMMV was distinct from other viruses already known to infect cucurbits. The riboprobe was generated from the CP gene sequence of *Cucumber green mottle mosaic virus*-Israel (CGMMV-Is) and this riboprobe was found to react strongly with CGMMV-Is, but not with the total RNA extracts from plants infected with CFMMV or with virion RNA of this virus. Similar differential reactions of the riboprobes with homologous and heterologous RNA of CGMMV-Is and CFMMV were observed. Two subgroups were recognized among cucurbit-infecting viruses based on the sequence data, phylogenetic analysis and biological properties. The subgroup I included strains and isolates referred to as CV3, CV4, CGMMV-W, CGMMV-SH and CGMMV-Is, whereas subgroup II comprised of CGMMV, CGMMV-Y and CFMMV (Antignus et al. 2001).

A system of microarrays has been developed for detecting and differentiating plant viruses and their strains. The amplicons from plant viral RNA are used for their differentiation by hybridization to synthetic oligonucleotide probes arranged in a two dimensional array on a glass slide. *Cucumber mosaic virus* (CMV) known to be highly heterologous in its coat protein (CP) was used as the model pathogen. The CP genes of 14 different isolates were amplified using cy3-labeled generic, but species-specific primers. These amplicons were hybridized against a set of five different serotype and subgroup-specific 24-mer oligonucleotides (probes) bound to an aldehyde-coated glass slide via an aminolinker. The probes targeted regions optimal for the differentiation between subgroups 1 and 2 or between subgroups 1a and 1b. This microarray procedure allowed a clear differentiation of the 14 different CMV isolates into serogroup 1 and 2 and it was also able to assign nine out of ten different serogroups/isolates correctly into subgroup 1a and 1b. Such a clear differentiation was not attainable using RFLP analysis using the restriction enzyme *Msp*I. The differentiation hybridization against five specifically selected nucleotides clearly demonstrates the high potential of oligonucleotide-based microarray technology for the virus isolate level detection and differentiation. This report presents the development of a diagnostic chip for plant viruses for the first time (Deyong et al. 2005).

4.1.4.3 Polymerase Chain Reaction-Based Techniques

RNA viruses have comparatively greater potential for rapid genetic changes due to frequent point mutations and genome rearrangement/recombination that are considered to be the principal mechanism of evolution of RNA viruses (Roossinck 1997).

RNA recombination might account for viral sequence variability, repair of some defective RNA molecules as well as production of shortened form of viral RNA known as interfering RNAs (Bruyere et al. 2000). The variability of RNA viruses may be related to specific regions of their genomes. The most conservative region is the one encoding viral polymerase enzyme. The coat protein (CP) gene is generally variable and virus species-specific. The epitope characteristics determine serotype and species of the virus and it is used frequently in phylogenetic analyses. The knowledge of genetic diversity of virus population and the distribution of the variable and conserved region within the viral genomes may be useful in the sequence-specific detection of viruses and the prediction of occurrence of resistance-breaking viral phenotypes, in addition to development of new strategies for viral disease management.

Polymerase chain reaction (PCR)-based techniques have been demonstrated to be highly effective and sensitive for detection and differentiation of strains of several plant viruses infecting a wide range of crops. *Citrus tristeza virus* (CTV) isolates from Florida T3 and T30 were examined to determine the extent of similarity between them. The isolatesT3 and T30 showed a relatively consistent or symmetrical distribution of nucleotide sequence identity in both the 5′ and 3′ regions of the 19.2-kb genome. In contrast, comparison of the sequence of isolate T36 with other isolates showed a dramatic decrease in the sequence identity in the 5′ proximal 11-kb region of the genome. A cDNA probe derived from this region of T36 genome hybridized differentially to the seven isolates not selected by theT36 probe. Primers designed from cDNA sequence for PCR, selectively amplified these 10 isolates, allowing them to be classified as similar to T3, T30 or T36. In contrast, individual cDNA probes derived from the 3′ terminal open reading frames (ORFs) of the T3, T30 and T36 genomes hybridized to dsRNA from all Florida CTV isolates tested. PCR primers designed from the T36 capsid protein gene sequence amplified successfully from all isolates. The isolates were classified into two groups based on these data. The VT group included VT, T3 and T30 isolates. The T36 group could be differentiated from VT group by the presence of a highly divergent 5′ genomic sequence (Hilf et al. 1999).

Pineapple mealybug wilt-associated virus 1–5 (PMWaV-1–5) have been reported to be associated with the disease in different combinations. In order to detect and differentiate the PMWaV, cDNAs amplified by random RT-PCR and RT-PCR of the heat shock protein (HSp70h) region of the viral genome using degenerate primers were used. *Ampelovirus*-like sequences (13) of ~540–2,500-bp in size were prepared. All the sequences had a significant similarity to the helicase, RNA-dependent RNA polymerase (RdRp) or HSp70h regions of *Grapevine leafroll-associated virus 3* genome. Phylogenetic analyses of selected regions of these sequences indicated that PMWaV-5 was a distinct species and most closely related to PMWaV-1. The amino acid sequence variation observed in the RdRp region of PMWaV-1 isolates was 95.8–98.4% and of PMWaV-3 isolates was 92.2–99.5% (Gambley et al. 2008).

The genetic variability of the Polish isolates of *Tomato black ring virus* (TBRV) was studied based on the restriction analysis of almost full-length cDNA derived from viral RNA1 and RNA2. RT-PCR amplification of each TBRV isolate was

performed and four products were obtained for each isolate. Two of these amplicons corresponded to RNA1 sequence and the other two products corresponded to RNA2 sequence. Restriction analyses of the RT-PCR products revealed differences among the TBRV isolates. A high diversity in the restriction patterns for RNA1 was discernible. The restriction patterns were more diverse for the RT-PCR products corresponding to the genes in the 5′ end proximity of both RNAs coding for a putative protease cofactor and 2a protein on RNA1 and RNA2 respectively. They appeared to have more variable restriction patterns in comparison with sequences in the 3′ end proximity of the RNAs. The results indicated the existence of the genetic diversity of the Polish TBRV isolates and also which of their genomes probably gathered or/and maintained the diversity (Jończyk et al. 2004).

Tomato spotted wilt virus (TSWV) belonging to the genus *Tospovirus* is known to infect a wide range of crops and weeds. TSWV has a tripartite single-stranded genome composed of L, M and S RNA. L RNA is of negative sense, while M and S RNAs have an ambisense coding strategy (Goldbach and Peters 1996; Moyer 1999). TSWV can adapt rapidly by reassortment of genome segments to be able to infect new plant species (Moyer 1999). In order to differentiate four tospoviruses TSWV, *Impatiens necrotic spot virus* (INSV), *Tomato chlorotic spot virus* (TCSV) and *Groundnut ringspot virus* (GRSV), the RT-PCR procedure was applied. Amplification of the genomes of Australian isolates of TSWV was performed, followed by sequencing of the amplimers. Nucleotide sequences of a 587-bp region of the N (nucleocapsid protein) gene (SRNA) were determined and compared. The sequences of the isolates showed 95.7–100% identity in pair-wise comparison. Phylogenetic analysis confirmed the close relationship among the isolates. However, diversity of population within a single TSWV isolate was discernible. They were compared based on the sequences of independent RT-PCR reactions. No significant difference could be observed in the N gene region of the TSWV isolates in resistance-breaking genes in tomato or pepper, when compared with other isolates (Dietzgen et al. 2005).

For the identification and differentiation of potyviruses, the method involving reverse transcription of total RNA from a potyvirus-infected plant, followed by PCR with universal primers Sprimer/M4, was applied. The expected fragment, purified from an agarose gel was used in semi-nested PCR with three degenerated primer sets: Sprimer/D1000, Sprimer/RYAFDY-C and U34/M4. Three amplified fragments were excised from agarose gels, purified and then used as templates for direct sequencing. The nucleotide sequences of CP gene were analyzed and compared using the GENETYX-Mac software. The CP gene sequences were obtained for 11 potyviruses including *Chinese artichoke mosaic vi*rus (ChAMV), *Clover yellow vein virus* (CYVV) *Soybean mosaic virus* (SMV), *Watermelon mosaic virus* (WMV) and *Zucchini yellow mosaic virus* (ZYMV). The new isolates of ChAMV and WMV showed maximum identities of 95% and 99% respectively with isolates reported earlier. The protocol developed in this investigation has the potential for use in the determination of the complete CP sequences and the information may be applicable to differentiate potyviruses having few differences at the consensus sequences (Yamamoto and Fuji 2008).

Investigations on the genetic diversity of *Banana streak virus* (BSV) showed that a large diversity existed among virus sources inducing BSV-symptoms, which are currently subsumed under the species BSV. Five serologically and genetically distinct naturally occurring isolates of BSV were earlier identified. Such large genomic diversity poses appreciable problem in the development of a virus indexing and general diagnostic protocol. Application of MAbs and PAbs generated against a Nigerian isolate of BSV was not effective in detecting the virus by TAS-ELISA in several *Musa* leaf samples indicating the presence of more than one strain of BSV in Nigeria. Of the 51 leaf samples tested, 48 were positive by immunoelectron microscopy (IEM), 25 by IC-PCR and only 19 by TAS-ELISA tests. In the IC-PCR procedure developed at the International Institute of Tropical Agriculture (IITA), an antiserum for immunocapture and the PCR primers designed for BSV Onne (Nigeria) strain were employed. The first group of 17 samples clearly tested as BSV in TAS-ELISA and IC-PCR methods, whereas of the 19 samples of the second group, only four samples were positive for IC-PCR and two samples weakly reacted in TAS-ELISA tests. The results suggested that the currently available tools neither of serology nor of sequence-specific IC-PCR were effective in offering specific diagnosis and differentiation of the members of the genus *Badnavirus* including *Banana streak virus* (Agindotan et al. 2006).

Sharka disease caused by *Plum pox virus* (PPV) causes serious losses in several European countries. Hence, it is important to assess the extent of genetic variability of PPV isolates to know their distribution and pathogenic potential to develop resistant cultivars suitable for different ecosystems. Existence of two major serologically distinguishable strains PPV-D and PPV-M using strain-specific MAbs has been recognized (Boscia et al. 1997). Both strains could be discriminated by *Rsa*I polymorphism in the 243-bp DNA fragment amplified by P1 and P2 primers located at the C-terminus of PPV CP gene or by direct IC-RT-PCR typing using PD- and PM-specific oligonucleotides (Olmos et al. 1997). RT-PCR assay was performed using primers that amplified a 243-bp fragment in the C-terminus of the CP coding region. Restriction fragment length polymorphism (RFLP) analysis applied to the amplified 243-bp fragment showed that restriction sites of *Alu*I and *Rsa*I were present in the plum and apricot samples. An amplified 836-bp cDNA fragment derived from the P3-6K, coding region of both isolates had restriction profiles typical of strain D. Nucleotide identities of 99–100% were observed for the 243-bp fragments of the Kazakhstan isolates when compared with the corresponding regions of strain D and 94 to 95% identity with strain M (Fig. 4.3). Nucleotide sequence analysis of the entire CP coding region of the plum and apricot isolates resulted in the identification of a unique deletion of six nucleotides (two deduced proline amino acid residues) in the N-terminal region in the plum isolate. The biological significance of this deletion is unclear at present. Several nucleotide substitutions in the CP coding region were found to be common to plum and apricot isolates and they appear to be unique to the Kazakhstan isolates (Spiegel et al. 2004).

Isolates of *Plum pox virus* (PPV) obtained from a small focus of infection in apricot orchard in Apulia, Italy, were characterized by their reactivity with strain-specific MAbs, RFLP and sequence analyses. The virus isolates were serologically

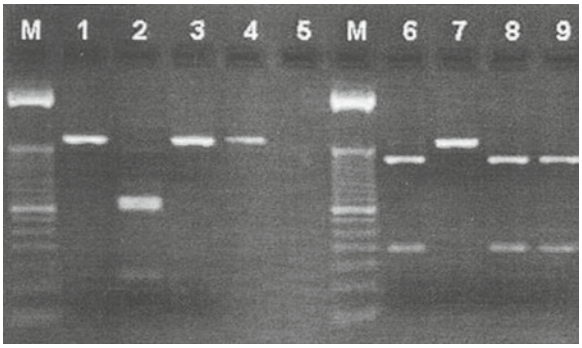

Fig. 4.3 Differentiation of isolates of *Plum pox virus* (PPV) by RT-PCR assay and RFLP analysis. PPV-specific P3-6K fragment (836-bp) is digested with restriction enzymes *Asu*I or *Dde*I. M, 50 bp DNA ladder; *Lanes 1–4*, digests of *Asu*I; *Lanes 6–9*, digests of *Dde*I; *Lanes 1* and *6*, isolate D in *Nicotiana benthamiana*; *Lanes 2* and *7*, isolate M in *N. benthamiana*; *Lanes 3* and *8*, plum P5R8; *Lanes 4* and *9*, apricot A8R2; *Lane 5*, blank. (Courtesy of Spiegel et al. 2004; The American Phytopathological Society, MN, USA)

typed by DASI-ELISA format. One isolate PPV-BR was analyzed by RFLP procedure, using the PCR products derived from two genome regions, CP and PS-6K1. PPV-BR was found to be a recombinant, as it typed as PPV-M by strain-specific MAbs and RFLP analysis of the CP gene, but as PPV-D by analysis of the P3-6K1 gene. Sequence analysis of the (Cter)NIb-(Nter)CP region of this recombinant showed that the recombination breakpoint was located in the C-terminus of the NIb gene. PPV isolates have been grouped into two main clusters, one comprising all PPV-D isolates and another all PPV-M isolates. The PPV-M cluster split into two subclusters comprising typical PPV-M isolates or all recombinant isolates (PPV-Rec). The recombinant isolates clustered in a branch clearly distinct from PPV-M isolates. The Italian PPV recombinant was genetically very similar to recombinants reported earlier from other countries, suggesting that the recombinants might have a common origin (Myrta et al. 2005).

In a later investigation, molecular strain typing was performed by IC-RT-PCR targeting three genomic regions corresponding to (Cter)CP, (Cter)Nib/(Nter)CP and CI. RFLP analysis was applied for distinguishing D and M strains based on *Rsa*I polymorphism located in (Cter)CP. All PCR products targeting (Cter)CP and 8 PCR products spanning the (Cter)Nib/(Nter)CP cistrons were sequenced. All PPV isolates typed as PPV-M by serological analysis and by molecular differentiation in the genomic region corresponding to (Cter)CP were confirmed by nucleotide sequencing to be homologous to PPV recombinant (PPV-Rec) reported earlier. The IC-RT-PCR analysis confirmed that 20 isolates were of PPV-D type and 15 isolates were of PPV-M type. The assay also detected three more mixed infections. RFLP analyses corroborated these results based on the presence of the *Rsa*I polymorphism in PPV-D strain (Table 4.2) (Zagrai et al. 2008).

Citrus tristeza virus (CTV), a member of the genus *Closterovirus* occurs in the form of several strains. Some isolates of CTV are essentially symptomless, but

Table 4.2 Molecular detection and differentiation of *Plum pox virus* (PPV) isolates from orchards in Romania (Zagrai et al. 2008)

Orchard no./assay	PPV poly	PPV-D	PPV-M	PPV D+M
IC-RT-PCR (P1/P2 and P1/PD or PM)				
1	10	6	2	2
2	10	3	7	0
3	10	4	4	2
4	10	7	2	1
5	3	0	0	3
Total	43	20	15	8
Percentage	100	46.5	34.9	18.6
RFLP (*Rsa*I)				
1		6	2	2
2		3	7	0
3		4	4	2
4		7	2	1
5		0	0	3
Total		20	15	8
Percentage		46.5	34.9	18.6

most of the isolates induce observable symptoms. The genome is a single-stranded, positive sense RNA molecule of 19,226–19,296 nt, organized in 12 ORFs and capable of coding at least 17 protein products. Sequences of the 5′ terminal region of the genomic RNA of eight isolates of CTV were grouped into three types (I, II and III) with intragroup sequence identity higher than 88% and intergroup sequence identity as low as 44%. Sequencing of an additional 58 cDNA clones from 15 virus isolates showed that all sequences could be unequivocally assigned to one of the three types identified earlier. The relative frequency of each sequence type was assessed in 57 CTV isolates of different geographic origin and pathogenic characteristics by RT-PCR with sets of type-specific primers using CTV dsRNA as the template. Isolates containing type III sequences caused only mild to moderate symptoms in Mexican lime, used as an indicator plant for most CTV isolates. The isolates causing stem pitting in sweet orange and/or grapefruit, generally contained type II sequences. There was no distinct association of any of the sequence type with geographical area, as well as types present in isolates obtained from 12 different countries (Ayllón et al. 2001; Sambade et al. 2002). The decline inducing strains of CTV could be differentiated from non-decline inducing strains by employing the RT-PCR assay. Two internal primers, one (CP_4) specific for non-decline inducing (T30) and CP_3 specific for decline inducing strains of CTV and two terminal primers HCP_1 and HCP_2 for the ends of the capsid protein gene were employed. This RT-PCR protocol was able to detect and also differentiate decline inducing and non-decline inducing strains of CTV accurately, since the 320-bp fragments were amplified only from the decline inducing strains of CTV (Huang et al. 2004).

Pathogenic potential of CTV isolates, serological activity of the CTV isolates determined by MAbs and dsRNA analysis have been used as the basis for differentiating

CTV isolates. Genetic variation in two groups of CTV isolates, one from a Spanish source obtained by successive host passages and another Japanese source obtained by successive aphid transmission were investigated. Single-strand conformation polymorphism (SSCP) analysis of genes p18 and p20 was performed. The estimation of genetic diversity within and between isolates and evaluation of genetic differentiation between populations was performed based on nucleotide sequences of representative haplotypes of each isolate and gene. In some isolates, within-isolate diversity was greater than diversity with other isolates, because their population contained distinctly related sequence variants. Genetic variation of different genes, repeated inoculation in the field, homologous RNA recombination between sequence variants and the presence of different defective RNAs are some of the factors that may contribute to biological variability of CTV isolates (Ayllón et al. 2006).

Differentiation of a panel of 12 isolates of CTV of different geographical origins with different biological properties was attempted based on the polymorphism in the CP gene of CTV by cleavase fragment length polymorphism (CFLP) and single-strand conformation polymorphism (SSCP) analyses. Biologically characterized sources of CTV were analyzed by IC-RT-PCR followed by CFLP analysis targeted to CP gene. All CTV isolates, except one examined, were placed in different clusters, frequently grouped with isolates or standards with which they shared some kind of geographical or biological relationship. On the other hand, most of the clones were placed in clusters in a different way based on SSCP analysis. The CP gene of CTV isolates (12) was obtained after RT-PCR amplification and the products were analyzed by CFLP procedure. Most of the isolates were accurately grouped according to the sequences of the variants constituting the isolates. In plants artificially inoculated with a mixture of mild and severe isolates, the patterns were more closely related to the severe isolates. This may possibly be due to the higher concentrations reached by severe isolates in plants with mixed infections. The CFLP method may be effective in identifying and differentiating CTV isolates more precisely (Marques et al. 2006).

Genetic diversity of isolates of *Citrus tristeza virus* (CTV) from 20 citrus-growing countries around the world was investigated (Garnsey et al. 1995). The CP gene of these isolates was amplified by RT-PCR, cloned and characterized by single-strand conformation (SSCP) analysis. Haplotypes that produced different patterns within each geographic region were sequenced and database of 153 accessions of CTV was assembled. Phylogenetic analysis indicated the existence of seven well-defined clusters, coefficient of differentiation being 0.78. An asymmetric PCR-ELISA typing (PET) assay was developed in the frame of this clustering pattern, using a set of eight hybridization probes. Inclusion of any unknown haplotype in a group was determined by comparing its pattern of reaction against the whole set of probes, but not, as previously done in hybridization assays, in an all-or-nothing basis. The results could be interpreted objectively through a visual basic application that is comparable to the rates of hydrolysis of the ELISA substrate of an assayed isolate to a matrix of rates of hydrolysis obtained from standard haplotypes. This assay can be automated to the same extent as an ELISA format (Nolasco et al. 2009).

Seedling yellows (SY) and/or stem pitting (SP) symptoms are induced by severe isolates of *Citrus tristeza virus* (CTV) in grapefruit and sweet orange which face serious threat from these isolates. Identification and differentiation of these isolates could be achieved by employing real-time RT-PCR format employing a general primer set and three TaqMan locked nucleic acid (LNA) probes targeting sequences characteristic of severe, mild (non-Sy, non-SP) and T 36-like isolates. Successful amplification was obtained from fresh or silica-desiccated CTV-infected samples and all isolates, except one, reacted with one or more probes. RT-PCR assays with homologous and heterologous transcript RNA mixes demonstrated that each probe reacted only with its cognate sequence which was detected even at ratios below 2.5%. Pathogenically distinct CTV isolates (56) from different countries were analyzed by real-time RT-PCR protocol. The mild isolates reacted only with the mild probe, whereas sever SP and SY isolates reacted with the severe-SP or the T 36-like probes respectively. Reliable identification of potentially dangerous CTV isolates rapidly may be useful for developing appropriate management systems to minimize the production losses (Ruiz-Ruiz et al. 2009).

Assessment of variability of the CP gene of *Citrus psorosis virus* (CPsV) was taken up based on serological characteristics and sequence analysis of two genomic regions located in the 3′ (region C) and 5′ (region V) halves of the gene. Sequence analysis of 19 isolates of the sources from Campania, Italy, exhibited limited nucleotide diversity of the CP gene in the population. Diversity was slightly higher in the region V than in region C, as revealed by genetic distance between isolates that ranged from 0.000 to 0.028 for region C and from 0.000 to 0.038 for region V. The CPsV isolates from Campania were more distantly related to CPsV-4 isolate from Florida, USA in both the C and V regions of the CP gene. Significant nucleotide differences between this isolate and group of Argentinean CPsV isolates were also detected in a 313-nt segment of RNA1. Phylogenetic analysis showed that the CPsV-4 isolate was clearly separated from Campania sources. No correlation was discernible between serogroups, specific amino acid sequences, field location or citrus cultivar (Alioto et al. 2003).

Peach mosaic virus (PcMV) is serologically related to *Cherry mottle leaf virus* (CMLV), but they cause distinct diseases in peach and cherry respectively. Both are transmitted by different eriophyid mite species. PcMV is classified as a member of the genus *Tichovirus*, family *Flexiviridae*, based on its relationship with CMLV and the available sequence data. The complete nucleotide sequence showed that PcMV shared 73% identity with CMLV. The CP amino acid sequence identity between isolates of PcMV ranged from 97% to 99% versus 83% identity when compared with the CP of CMLV. Further, phylogenetic analysis supported the classification of PcMV and CMLV as members of the genus *Trichovirus*. PcMV and CMLV appear to be products of a recombination event between members of the genus *Trichovirus* and members of an NB protein-containing group of virus likely within the family *Flexiviridae*. Based on the sequences, PcMV-specific primers PM16AFF and PM16AFR were designed for the detection of PcMV by RT-PCR assay which showed no cross-reaction with a broad range of viruses belonging to the family *Flexiviridae* (James et al. 2006).

During a survey to assess the incidence of *Prunus necrotic ringspot virus* (PNRSV) in an orchard of *Prunus cerasus* cv. Montmorency and *P. avium* cv. Medelfingen in New York, prevalence of high percentage of latent infection (87%, 33 of 38 trees) was observed. Sequence analysis of the CP gene (675-bp) indicated a population structure consisting of one predominant molecular variant of 10 isolates and six minor molecular variants for seven isolates. A high sequence identity was found between the CP gene of PNRSV isolates from cherry trees and other isolates from diverse hosts and various geographic origins at the nucleotide and amino acid levels (88–100%). Clustering of PNRSV isolates from cherry trees in New York in the predominant group PV-96 was indicated by phylogenetic analyses (Oliver et al. 2009).

Cherry leaf roll virus (CLRV) isolates were differentiated by applying the RFLP procedure. Restriction patterns from the partial 3′ non-coding region (NCR) genomic fragments (approximately 420-bp) of 43 CLRV isolates belonging to different phylogenetic groups were compared following digestion with endonucleases *Bsp*143I, *Alu*I, *Rsa*I, *Eco*RI and *Eco*130I. Another group of 23 isolates were analyzed by computer assisted restriction analysis. The restriction enzymes *Bsp*143I, *Alu*I and *Rsa*I enabled the differentiation of isolates from group B and all but two isolates from group A. A major portion of group E isolates could be distinguished from isolates belonging to phylogenetic group C or D2. It was possible to differentiate isolates in group D1 from two isolates of group A. In addition, a combination of IC-RT-PCR-RFLP techniques was able to successfully differentiate samples from CLRV-infected walnut, black elderberry and birch and to determine their phylogenetic relationships. The protocol developed, in this investigation, may facilitate rapid phylogenetic classification of CLRV isolates detected in different host plant species by universal IC-RT-PCR and studies on population diversity including genetic drift within virus populations (Buchhop et al. 2009).

Grapevine fan leaf virus (GFLV) is responsible for considerable production losses in the European and Mediterranean countries. Genetic diversity of 20 isolates of GFLV occurring naturally in grapevine gardens was assessed. By employing RT-PCR assay, a 605-bp fragment containing a part of viral coat protein (CP) gene was amplified. The RFLP analysis was applied to determine sequence variations among the isolates of GFLV and the results were confirmed by sequence analysis. The presence of a complex mixture of closely related genomes in infected source plants was observed. RFLP analysis following digestion of the amplicons with the endonuclease enzyme *Alu*I showed that GFLV populations in Tunisian vineyards consisted of two restriction profiles corresponding to the distinct subpopulations SP1 and SP2. Incidence of SP2 population was observed to be at a higher level compared with the SP1 populations. Variations to a magnitude of up to 11% between the isolates were recorded (Fattouch et al. 2005). Genetic diversity of GFLV isolates was assessed by performing RT-PCR assay followed by digestion of the amplicon with the restriction enzyme *Eco*RI. Two restriction profiles were obtained, when the digests were subjected to RFLP analysis. The Iranian isolates were 84–95% identical to each other and 84–91% identical to isolates from other countries (Bashir and Hajizadeh 2007).

Grapevine virus A (GVA) is the type virus of the genus *Vitivirus* in the family *Flexiviridae*. The isolates of GVA were distinguished biologically into three groups (I, II and III) based on the type of symptoms induced in mechanically inoculated *Nicotiana benthamiana.* Molecular analysis showed that CP gene of GVA had a certain degree of variability. The primer pair H587/C995 could be used to study the variability of the coat protein (CP) gene among GVA groups I and II (Goszczynski and Jooste 2002). The primer pair H587/C995 was variant-specific and it could be employed for the detection of GVA isolates belonging to group I and II. Most of the GVA isolates shared 100% nucleotide similarity. Another GVA-5R isolate shared 90%, 83% and 76% nucleotide identities and 98%, 92% and 80% amino acid similarities with GVA groups I, II and III respectively (Misbeh et al. 2007).

Blackcurrant reversion virus (BRV) induces blackcurrant reversion disease (BRD) and full blossom disease (FBD). The genetic diversity of 15 isolates occurring in Czech Republic was assessed. A substantial part of the 3′ non-translated region (3′-NTR) of the RNA2 was amplified using the RT-PCR assay and the amplimers were sequenced and compared with sequences available in the GenBank. The isolates did not show significant variations in the sequences of the targeted genome fragment. The isolates from Canada and New Zealand showed 96.6% and 92.2% sequence identity respectively with two isolates occurring in Poland. Sequence analyses of BRV isolates resulted in a phylogenetic tree with four branches, each consisting of six to nine isolates. No relationship with geographic origin of BRV isolates could be established (Přibylová et al. 2008).

Variability in the biotypes of *Papaya leaf distortion mosaic virus* (PLDMV) was studied. Based on the host range studies, two biotypes – P biotype capable of infecting papaya and C biotype unable to infect papaya were recognized. PLDMV, exhibiting 44–50% identities in the polyproteins, was identified as a distinct species in the genus *Potyvirus.* Like the W biotype of *Papaya ringspot virus* (PRSV), the non-papaya-infecting papaya biotype of PLDMV was able to infect cucurbitaceous host plants. The CP sequence of PLDMV-C in naturally infected-*Trichosanthes bracteata* was compared with those of three strains of the P biotype (PLDMV-P), LDM and two additional strains, M (mosaic) and yellow mosaic (YM), which were biologically different from each other. The CP sequences of three strains of PLDMV-P share high identities of 95–97%, while they share identities of 88–89% with that of PLDMV-C biotype. Significant changes in hydorphobicity and a deletion of two amino acids at the N-terminal region of the CP of PLDMV-C were observed. The phylogenetic analysis based on the CP amino acid sequences grouped PLDMV strains in one cluster, distinct from clusters of other potyviruses. In the PLDMV cluster, P biotype strains (LDM, YM or M) were found to be closely related to each other, but slightly separated from strain T of biotype C. PLDMV may be primarily a pathogen of *Cucurbitaceae* and PLDMV-P might have adapted to infect papaya by mutation from PLDMV-C biotype (Mayoka and Hataya 2005).

Potato virus Y (PVY) strains cause distinctive symptoms in potato plants and tubers. Induction of veinal necrosis in indicator plants is the basis of biological differentiation of PVY isolates. In order to relate molecular characteristics with the biological properties, a one-step fluorescent RT-PCR assay on a single nucleotide

polymorphism (SNP) was developed. By employing this approach, 42 PVY isolates were assigned to their respective groups, in addition to codetection of mixed infection involving PVY^N and PVY^O strains. This procedure has the potential for use in reliable classification of PVY isolates without the need for inoculation on tobacco cv. Xanthi plants in the greenhouse (Glais et al. 2002; Jacquot et al. 2005). Potato tuber necrotic ringspot disease (PTNRD) is due to the strain PVY^{NTN}. This strain was differentiated by employing strain-specific endonucleases that cleave their respective PCR products. Single cleavages of PCR products derived from the 5′ end of PVY^{NTN} genome by *Nco*I and that of the PVY^N by *Bgl*II were accomplished. Then the digests were resolved by PAGE analysis. Differentiation of these two PVY strains based on the restriction profiles was shown to be reliable and reproducible. PCR-based techniques have been effectively applied to precisely detect and differentiate the components of mixed infections (Rosner and Maslenin 1999; Lorenzen et al. 2006). Four different viral species infecting *Ranunculus asiaticus* were detected and differentiated. A new viral species belonging to the genus *Potyvirus* was detected and identified. The viral genome fragments were amplified through RT-PCR and the amplicons were cloned and sequenced. Sequence and phylogenetic analyses suggested that three of the isolates belonged to the genus *Potyvirus* (Turino et al. 2006).

Members of *Potyvirus*, PVY, *Tobacco etch virus* (TEV), *Pepper mottle virus* (PMV), *Pepper veinal mottle virus* (PVMV) and *Chilli veinal mottle virus* (ChVMV) infect pepper (chilli) in different countries. The genetic diversity within PVMV and its relationship with other potyviruses was investigated. The nucleotide sequences of the 3′-proximal part of the NIb gene, the entire CP gene and the 3′ non-translated region (NTR) of PVMV isolates were determined. The CP gene sequence diversity revealed three clades that corresponded to the geographic locations. These clades included closely related isolates that potentially belong to two viral species. The PVMV isolates were differentiated into two species by employing RT-PCR for the amplification of the targeted fragments, based on large indel in the CP gene (Fig. 4.4). The isolates of PVMV and ChVMV were grouped into three and two pathotype based on their pathogenicity to pepper genotypes carrying different resistance factors. Specificity of resistance only partially corresponded to molecular diversity of the isolates (Moury et al. 2005).

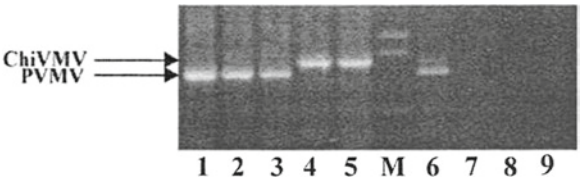

Fig. 4.4 Differentiation of isolates of *Pepper vein mottle virus* (PVMV) and *Chilli veinal mottle virus* (ChiVMV) using RT-PCR assay. *Lanes 1–3*, PVMV isolates; *Lanes 4* and *5*, ChiVMV isolates; *Lane 6*, DNA size marker; *Lane 7*, PVMV and ChiVMV mixed infection; *Lane 8*, healthy control; *Lane 9*, *Potato virus Y.* (Courtesy of Moury et al. 2005; The American Phytopathological Society, MN, USA)

Genetic variability of isolates of *Tomato black ring virus* (TBRV) occurring in Poland, was assessed on the basis of restriction analysis of almost full-length cDNA derived from both viral RNA1 and RNA2. Restriction analysis of the RT-PCR products from viral RNA1 and RNA2 showed difference among TBRV isolates. The restriction patterns were more diverse for the RT-PCR products corresponding to genes in the 5′ end proximity of both genomic RNAs. A dendrogram was formulated based on the restriction patterns obtained for TBRV isolates examined. The sequences in the 5′ proximity of RNAs code for a putative protease cofactor and 2a protein on RNA1 and RNA2 respectively. A single nucleotide substitution may facilitate virus adaptation to host translation machinery due to the correlation of the viral amino acid codons with ones used by the host plant (Jończyk et al. 2004).

Pepino mosaic virus (PepMV) belonging to the genus *Potexvirus*, infects tomato crops in Spain and other European countries. The population structure of PepMV was analyzed by comparing the nucleotide sequences of the RNA-dependent RNA polymerase (RdRp), triple gene block (TGB) and CP genes of the isolates. The Spanish isolates of PepMV mostly comprised of highly similar isolates belonging to the strain reported in Europe ie., European tomato strain. Genetic distance among isolates within the European tomato strain was unrelated to the location or year of collection. Molecular characterization based on the analysis of 547-nt fragment from RNA polymerase gene or an 844-nt fragment of CP gene of tomato-infecting PepMV isolates from Europe and North America showed a high nucleotide sequence identity (above 99%) among all isolates. The results suggested that PepMV might have been introduced into Spain from different sources (Pagán et al. 2006). *Tobacco mosaic virus* (TMV) infecting tomato in Sapopnea, Brazil showed variation in its pathogenicity to *Petunia hybrida*. RT-PCR assay was applied to discriminate TMV-Sapopena from other tobamoviruses. The TMV-1/-2 primer pair was very effective in amplifying a 409-bp product located in the TMV movement protein. Sequence analysis of deduced amino acids showed 100% identity with part of the P30 protein of TMV isolates from China and Japan, 90% for TMV from Australia and Canada and less than 80% with other tobamoviruses (da Silva et al. 2008).

Phalaenopsis chlorotic spot virus (PhCSV) infecting *Phalaenopsis* orchid plants was tentatively identified as a potyvirus based on biological and immunological characteristics, in addition to determination of particle morphology using electron microscopy. The degenerate primers Pot1 and Pot2 designed for amplifying the conserved regions including the 3′-end of NIb and 5′- end of CP genes of the viral RNA, were employed in the RT-PCR assay. Based on the BLAST (Basic local alignment search tool) analysis, the 1,189-bp DNA fragment of PhCSV was found to share 60.6%, 63.1%, 58.9% and 56.6% nucleotide identities and 65.6%, 65.1%, 52.5% and 44.8% amino acid identities with those of *Bean yellow mosaic virus* (BYMV), *Beta mosaic virus* (BtMV), *Bean com*mon *mosaic virus* (BCMV) and *Turnip mosaic virus* (TuMV) which were closely homologous. The sequence comparison of the conserved region indicate the identity of PhCSV as a potyvirus. The phylogenetic analysis revealed that the CP of PhCSV sequence was more closely related to *Basella rugose mosaic virus* (BaRMV) (Zheng et al. 2008).

Sugar beet (*Beta vulgaris*) crops are infected by the soilborne viruses, *Beet necrotic yellow vein virus* (BNYVV), *Beet soilborne virus* (BSBV) and *Beet virus* Q (BVQ). In order to determine the extent of variability of the isolates of the soilborne viruses occurring in Poland, the CP gene sequences of three isolates of each of these viruses were determined, after amplifying the targeted gene fragments. Sequences of the CP gene of BNYVV isolates were 567-nt long coding for 189 amino acids. The identity of these sequences at nucleotide and amino acid levels ranged from 96% to 100%. Two groups of isolates (A and B) could be identified by phylogenetic analysis. The CP gene of BSBV isolates had 495 nucleotides (165 amino acids). They showed high nucleotide identity (98–100%). The amino acid residue aspargine at position 65 in the Polish isolates varied from the German isolate which had threonine in its place. The CP gene of BVQ isolates with 501-nt showed 97% identity at nucleotide level. The Polish isolates contained three additional nucleotides, resulting in an extra amino acid residue (arginine) at position 86, when compared to BVQ isolates from other countries (Borodynko et al. 2009).

Genetic diversity of *Sugarcane mosaic virus* (SCMV) isolates prevalent in southern China was assessed by sequence analysis of the CP genes of 33 SCMV isolates and 10 isolates of *Sorghum mosaic virus* (SrMV) which is also able to infect sugarcane. The near full-length CP gene sequences were analyzed using DNAMAN 4.0 software (Lynnon BioSoft, Quebec) and the DNA Star 5.01 package (DNA Star Inc., USA), along with the sequence data of all available isolates in GenBank. The SCMV isolates (173) with the exception of MDB and Abaca strains could be classified into five groups which include three groups known earlier, the sugarcane (SCE), maize (NZ) and Thailand groups and two newly identified noble sugarcane (NSCE) and Brazil groups (Table 4.3). The SrMV isolates (22) were divided into two groups, HS (hybrid sugarcane) and NS (noble sugarcane) groups. The HS group included five of eight SrMV hybrid isolates, while the NS group contained two SrMV noble isolates and three hybrid isolates. The results of this investigation indicated that SCMV or SrMV isolates, regardless of which phylogenetic group they belong, had rather wide host ranges and they could be transmitted nonpersistently by several species of aphids among maize, hybrid and noble sugarcanes and other plants within the family *Gramineae* (Xu et al. 2008).

Table 4.3 CP gene nucleotide identities (average %) between *Sugarcane mosaic virus* (SCMV) phylogenetic groups and distinct strains (Xu et al. 2008)

Group/strain	SEC	MZ	NSCE	Thailand	Brazil	MDB	Abaca
SEC	95	78	78	78	82	78	78
MZ		88	84	80	79	83	78
NSCE			87	76	79	80	77
Thailand				94	80	82	79
Brazil					92	86	80
MDB						–	80

The family *Geminiviridae* includes four genera, *Begomovirus, Curtovirus, Mastrevirus* and *Topocuvirus*. The genera are differentiated and classified based on biological properties, including host range and vector transmission and on viral genome organization (Fauquet et al. 2005). Begomoviruses transmitted by different species of whiteflies are mostly bipartite with the viral genome being divided into two components designated DNA-A and DNA-B with a size range of 2,500–2,800 nucleotides (nts). DNA-B is absent in the monopartite begomoviruses. The grossest kind of variation among the genomes of begomoviruses is the absence of DNA-B in certain begomoviruses like *Tomato yellow leafcurl virus* (TYLCV) from Israel (Is) and from Sardinia (Sa). Despite the lack of the DNA-B encoded BV1 and BC1 movement proteins, TYLCV-Is and TYLCV-Sa are able to spread efficiently through the infected plants. Comparison of genome nucleotide sequences among bipartite begomoviruses, show that the sequences of DNA-B are more diverse than those of DNA-A. Consequently, nucleic acid probes specific for DNA-A hybridize to a greater or lesser extent with the DNA of many heterologous begomoviruses. The greatest variation among begomovirus genomes is the *AV1* gene coding viral coat protein (CP). The relationships among begomoviruses based on the amino acid sequences of CP exhibit the close relatedness of the viruses occurring in a geographical location (Harrison and Robinson 1999). The International Committee of Taxonomy of Viruses (ICTV) has provided guidelines to define an isolate of the begomovirus as a distinct species. A rule-of-thumb value of <89% nucleotide sequence identity threshold for DNA-A has to be considered as the basis of differentiation. The nucleotide sequence identity of DNA-B is not to be reckoned as a reliable indicator of species demarcation due to the possibility of component reassortment or recombination in bipartite begomoviruses (Fauquet et al. 2003; Idris and Brown 2004).

Bean golden mosaic virus (BGMV) is a bipartite virus containing the genomic components DNA-A and DNA-B. Major difference in DNA sequence and biological properties separated BGMV into at least two distinct types. Type I is represented by BGMV-Bz (Brazil) and type II is represented by BGMV-PR (Puerto Rico), BGMV-GA (Guatemala) and BGMV-DR (Dominican Republic). Viral DNA fragments of BGMV-Bz collected from bean-growing areas in Brazil, comprising of part of the *rep* gene, the common region and part of the *cp* gene were amplified by PCR, cloned and sequenced. The geminivirus in bean samples had sequences nearly identical to that of BGMV-Bz. The sample from lima bean contained a new species of geminivirus which was designated *Lima bean golden mosaic virus* (LBGMV-BR). While all sequences from bean samples clustered with BGMV-Bz, the sequence from the lima bean isolate remained separately. DNA sequence comparison of the virus isolate from the weed species *Lebonurus sibiricus* showed that the virus infecting *L. sibericus* was a new geminivirus species and hence, it was named as *Lebonurus mosaic virus* (Faria and Maxwell 1999). *Tomato yellow leaf curl virus* (TYLCV) and its strains were investigated to determine the extent of genetic diversity. PCR-amplified products of viral RNA were cloned and sequenced. Sequence comparison revealed that PCR fragments of TYLCV isolates from Mersin, Turkey exhibited 98% nucleotide sequence identity to a TYLCV isolate from Egypt and 97% identity to many isolates from other geographical locations.

The completely sequenced isolate of TYLCV showed its close relationship with other isolates from the TYLCV 'prototype' strain. The high sequence identity to TYLCV throughout the genome showed that isolate tested, was not of recombinant origin (Köklü et al. 2006).

Several distinct whitefly-borne geminiviruses are associated with the cassava mosaic disease (CMD) prevalent in African countries. Whereas *African cassava mosaic virus* (ACMV) occurs in all cassava-growing countries, *East Africa cassava mosaic virus* (EACMV), *East Africa cassava Cameroon virus* (EACMV), *East Africa cassava mosaic Zanzibar virus* (EACMZV) and *South African cassava mosaic virus* (SACMV) have been reported from different geographical locations. Variations in the sequences of DNA-A IR regions were verified. Further, the sequences of PCR products comprising of the DNA-A IR were analyzed. Close relationship between isolates of EACMCV from Nigeria and EACMCV from Cameroon (EACMCV-CM) was observed (Pita et al. 2001). Comparison of complete DNA-A genome sequences showed 98% sequence identities of the EACMCV isolates from Nigeria. The IR sequences of ACMV isolates from East Africa were closely related to the IR sequences from ACMV from Nigeria. In addition, there was no significant variation among the EACMV isolates that had been sequenced, indicating the extent of sequence similarity with EACMCV-CM (Ariyo et al. 2005).

The nucleotide sequence identity among *Pepper golden mosaic virus* (PepGMV) components ranged from 91% to 96% DNA-A and from 84% to 99% for DNA-B, with each PepGMV component most closely related to the corresponding component of *Cabbage leafcurl virus* (CaLCV). The nucleotide sequence identity of the isolates of PepGMV was above the range suggested by ICTV for species demarcation threshold (<89%). Hence, the three virus isolates were considered as distinct strains of PepGMV with the potential for genetic material exchange (Brown et al. 2005). The presence of at least two begomoviruses in tomato in Venezuela was reported. Whitefly-transmitted *Tomato yellow mosaic virus* (ToYMV) and *Potato yellow mosaic virus* (PYMV-VE) were differentiated based on partial sequence data. It was proposed that PYMV-VE should not considered as a distinct species, but rather synonymous with ToYMV (Morales et al. 2001). In a later investigation, evidence for the incidence of four distinct begomoviruses in tomato in the Andean States was obtained. Analysis of the partial sequence of the A components suggested the presence of four groups of distinct begomovirus sequences. All four groups appeared to be New World in origin. Two groups of sequences were closely related to *Potato yellow mosaic virus*-Venezuela strain tomato and *Tomato Venezuela virus* respectively. The other two groups of sequences appear to belong to two new begomovirus species (Nava et al. 2006).

Euphorbia mosaic virus (Eu MV), a tentative species within the genus *Begomovirus* infects *Euphorbia heterophylla* in Mexico. The complete bipartite genome was cloned from total DNA extracts and the nucleotide sequence was determined. The DNA-A sequence of EuMV-Yucatan Penninsula (EuMV-YP) isolate shared 95% identity with the partially characterized type EuMV isolate from Puerto Rico. The DNA-A and DNA-B components have 2,613 and 2,602 nucleotides respectively. The 165-nt common region (CR) sequence for the DNA-A and DNA-B

components shared a lower than expected nucleotide identity of 86%. The phylogenetic analysis of the DNA-A and DNA-B components indicated that EuMV-YP is a New World begomovirus and it is a new member of the *Squash leaf curl virus* (SLCV) clade (Hernández-Zepeda et al. 2007b). In another investigation, the sequence analysis of the genomes of *Sida yellow mosaic Yucatan virus* (SiYMYuV) infecting *Sida acuta* and *Corchorus yellow vein Yucatan virus* (CoYVYuV) infecting *Corchorus siliquosus* were carried out. The SiYMYuV DNA-A shared the highest nucleotide identity (86%) with *Okra yellow mosaic Mexico virus* (OkYMMV). The complete DNA-B component shared the highest nucleotide identity (80%) with CoYVYuV. Likewise, COYVYuV DNA-A and DNA-B exhibited nucleotide identities with respective viruses SiYMYuV and CoYVYuV in the clade containing the *Abutilon mosaic virus* (AbMV) (Hernández-Zepeda et al. 2007a).

Cucurbit leaf crumple virus (CuLCrV) has become economically important, because of its destructive potential, as observed in the Imperial Valley of southern California. By employing overlapping primers in the PCR assay, full-length DNA-A and DNA-B clones of the isolate of CuLCrV collected from the Imperial Valley were obtained. These clones proved to be pathogenic to various cucurbit species and common bean (*Phaseolus viulgaris*) cv. Topcrop. Variations in the susceptibility of squash, watermelon, cantaloupe and honeydew melon to CuLCrV were demonstrated by agroinoculation. Similarity in genome organization of CuLCrV to other bipartite begomoviruses was also observed. The phylogenetic analysis revealed that CuLCrV belonged to the *Squash leaf curl virus* (SLCV) cluster of New World bipartite begomoviruses. By using specific primers in the PCR assay, CuLCrV could be detected and differentiated from other begomoviruses, including SLCV (Hagan et al. 2008).

Rice tungro disease (RTD) occurring in severe forms in the South East Asian countries, is caused by *Rice tungro bacilliform virus* (RTBV) with a DNA genome and *Rice tungro spherical virus* (RTSV) with an RNA genome. RTBV, capable of inducing most of the symptoms of RTD, depends on RTSV for transmission by the leafhopper vectors. RTSV by itself causes mild discoloration of the leaves of infected rice plants. Four strains of RTBV G1, G2, Ic and L could be differentiated by RFLP analysis of viral DNA, following digestion with restriction enzymes *Pst*I, *Bams*HI, *Eco*RI and *Eco*RV of viral DNA. Identical restriction profiles were produced by strains G1 and Ic. Digestion of viral DNA with *Eco*RI and *Eco*RV, yielded restriction patterns that could differentiate G2 and L strains and also from G1 and Ic. This protocol was effective in differentiating strains occurring under field conditions (Cabauatan et al. 1998). Likewise, the RFLP profiles of *Barley yellow dwarf virus* (BYDV) formed the basis of their differentiation. The isolate BYDV-PAV-DK1 which occurred infrequently could be differentiated based on the unique restriction enzyme pattern generated following digestion of the PCR products with *Hae*III from the CP region. Occurrence of this strain could be tracked by the presence of this unique RFLP profile in the samples, facilitating epidemiological investigations (Moon et al. 2000).

Barley yellow mosaic disease (BYMD) has been reported to be economically important, as it causes appreciable losses in winter barley crops in north-western

Europe and East Asia. *Barley mild mosaic virus* (BaMMV) and *Barley yellow mosaic virus* (BaYMV), belonging to the genus *Bymovirus* in the family *Potyviridae* are associated with BYMD. Both viruses are transmitted by the fungal vector *Polymyxa graminis*. The occurrence of a BaMMV pathotype capable of infecting barley cultivars carrying *rym5* resistance gene, indicated the variation in the pathogenic potential of the BaMMV isolates. Sequence analyses revealed variations in the VPg cistron of RNA1 of BaMMV. The ability of a variant of BaMMV-2 to overcome the *rym4*-mediated resistance correlated with a codon change in the VPg cistron of RNA1. Analysis of the new BaMMV isolates may be useful to identify variations in the VPg region of RNA1. The variants showed differences in their amino acid sequences of the VPg cistron both from each other and from the French pathotype. Pathogenicity of BaMMV towards barley cultivars carrying *rym* gene may be affected by substitution of single amino acids at different position in the molecule (Habekuss et al. 2008).

4.1.4.4 Heteroduplex Mobility Analysis

Heteroduplex mobility analysis (HMA) is useful for determining genetic differences between viral sequences. Fragments of variable regions of different isolates of a virus are amplified by PCR and after denaturation and reannealing heteroduplexes are formed between the amplified products. The heteroduplexes show differences in their migration in non-denaturing gel electrophoresis.

The HMA was applied to analyze the variability among five isolates of *Zucchini yellow mosaic virus* (ZYMV) collected from cucurbit fields in Taiwan. A cDNA fragment of 760-bp covering the variable region of the N-terminal half of the CP gene was amplified by RT-PCR and the amplicons were subjected to HMA analysis for determining sequence variations in the isolates. When the isolate TW-NT1 was combined with any of the other four isolates, the heteroduplexes formed migrated much more slowly than did the heteroduplexes obtained in combinations among other four Taiwan isolates, indicating that the four isolates shared a high degree of sequence homology, while the fifth isolate (TW-NT1) was more distinct. Comparison of the CP genes of the five Taiwan isolates indicated that they shared 92.8–98.7% nucleotide identities and 96.4–99.3% amino acid identities. The results of HMA agreed well with those of phylogenetic analysis based on the sequence data of the five Taiwan isolates of ZYMV (Lin et al. 2000).

The HMA procedure was adopted for the differentiation of begomoviruses infecting cassava. The assay involved the amplification of highly conserved core region of the CP region of field isolates, followed by denaturing and annealing with a number of reference strains. The HMA profiles were able to differentiate four different viral species involved in the development of cassava mosaic disease (CMD) and 11 different virus strains. Further, the results of HMA showed good correlation with those of sequencing and phylogenetic analyses. This technique was found to be sensitive and rapid in providing the results and also an additional advantage of detection of mixtures of viruses in field-grown cassava (Berry and Rey 2001).

4.1.4.5 Single-Strand Conformation Polymorphism Analysis

Single-strand conformation polymorphism analysis (SSCP) has been demonstrated to be a powerful alternative to determine differences in the viral genomes. When the + and – strands of a dsDNA, usually a PCR product, are separated by heat treatment, they attain metastable sequence-specific folded structures conferring on them particularly electrophoretic mobilities in non-denaturing polyacrylamide gels. Even single nucleotide exchanges may become detectable. A non-radioisotopic variant of SSCP analysis, preceded by IC-RT-PCR assay was applied to compare the genomic characteristics of *Beet necrotic yellow vein virus* (BNYVV) from more than 100 sugar beet samples from Europe and other countries. The existence of two major strain groups of BNYVV designated type-A and type-B has been identified. Prevalence of B-type in Germany and France and the A-type in most other countries was confirmed by SSCP analysis. Minor variants with a very restricted distribution were detected occasionally. New rhizomania (disease complex associated with infection by BNYVV) outbreaks in England caused either by the A- or the B-type or mixtures of both types were recorded, suggesting introduction of BNYVV from several sites abroad. An entirely different BNYVV P-type was identified in France and other strains groups in samples from China were also determined (Koenig et al. 1995).

Apple stem grooving virus (ASGV) with worldwide distribution, is the type species of the genus *Capillovirus.* The isolates of ASGV from fruit trees generally consist of two to four sequence variants that differ appreciably from each other in nucleotide sequences. With a view to characterizing the populations of sequence variants present within a single apple tree, a combination of IC-RT-PCR assay and SSCP analysis of a nested asymmetric PCR was applied. In the SSCP analysis of the PCR products from ASGV-infected apples, Japanese pear or European pear trees, two to four bands were recognized in samples from all trees, indicating that ASGV exists as a mixture of sequence variants. The composition of sequence variants (the number of bands and their relative quantity) differed among leaf samples from different branches, showing that each variant is distributed unevenly within an individual tree. The composition of sequence variants contained in ASGV isolates was altered following serial passage of the isolates in *Chenopodium quinoa* plants, as revealed by SSCP analyses, suggesting that dominance of variants may depend on the ability of the variants to adapt in woody plants or herbaceous plants (Magome et al. 1999).

Grapevine virus A (GVA) included in the genus *Vitivirus* is associated with grapevine Shiraz disease (SD) transmitted by the mealy bug *Planococcus ficus*. GVA can be transmitted also by mechanical inoculation. The virus is extensively heterogenic. Divergent variants of GVA clustered into three molecular groups I, II and III that could be differentiated based on nucleotide similarity in the 941–942 nucleotide 3′ terminal part of the virus genome. The variants share 91.0–99.8% nucleotide similarity within groups and 78.0–89.3% nucleotide similarity between groups in that genomic region (Goszczynski and Jooste 2002). In a later study, comparative molecular analysis of the movement protein (MP) gene (ORF3) of

single GVA variants of the three molecular groups of variants transmitted from various grapevines to *Nicotiana benthamiana* was taken up. The SSCP analysis of a 234-nt sequence showed that group II variants could be differentiated from group I and group III. The SSCP analysis of the 234-bp RT-PCR products revealed the presence of slowly migrating bands that were diffuse in appearance indicative of GVA variants of molecular group II associated with the majority of grapevines affected by SD. The SSCP analysis has the potential for rapid identification of variants of group II consistently associated with various grapevines with different SD status (Goszczynski 2007).

4.2 Assessment of Variability in Viroid Pathogens

Viroids are the smallest known infectious agents of plants, causing diseases of economic importance in several crops. The viroid pathogens have been classified based on biological and structural properties into two families, *Pospiviroidae* and *Avsunviroidae,* members of which replicate in the nucleus and chloroplast of host plants respectively (Flores et al. 2005). Assessment of variability in the molecular characteristics has been shown to be reliable for differentiation of viroids and their strains/variants.

4.2.1 Assessment of Variations in Nucleic Acid Characteristics

4.2.1.1 Hybridization-Based Techniques

Citrus spp. grown in Epirus, Greece is known to be infected simultaneously by viruses and viroids. Differentiation of the components of the mixed infections was attempted by applying dot blot hybridization procedure, using specific DIG-labeled probes. The riboprobes to *Citrus exocortis viroid* (CEVd) and *Hop stunt viroid* (HSVd) were transcribed using the plasmids. Hybridization was performed overnight at 56°C with different amounts of probes for the viruses and viroids infecting citrus. All citrus plants infected by *Citrus trizteza virus* (CTV), or *Citrus psorosis virus* (CPsV) or *Citrus infectious variegation virus* (CVV) were found to be co-infected by HSVd and 50% of them by CEVd. HSVd was detected up to 85% of the samples collected from two different locations, whereas CEVd was present only in 32–39% of the samples. Although there was high incidence of viroids, conspicuous symptoms of viroid infection were not observed, due to the use of sour orange rootstocks which are tolerant to these viroids (Barbarossa et al. 2007).

Potato spindle tuber viroid (PSTVd), a quarantine pathogen included in European Plant Protection Organization (EPPO) A2 list, infects a wide range of host plant species (over 150 species in 12 families), including several species of *Solanum*. Infection of *S. jasminoides* and *S. rantonnetti* by PSTVd was observed in Italy.

Molecular variability of the isolates of PSTVd from these plant hosts was assessed by applying dot blot hybridization in conjunction with polymerase chain reaction (PCR) amplification. By using the primers PSTVd-32 and PSTVd-33, a cDNA of about 360-bp corresponding to the full-length PSTVd RNA could be amplified from infected samples. RT-PCR analyses showed that all samples testing positive by dot blot hybridization, generated an RT-PCR amplicon of the 360-bp fragment. Cloning and sequencing of the amplified product generated from the isolates of *S. jasminoides* confirmed the identity of PSTVd in this plant species. The isolates of PSTVd from *S. jasminoides* and *S. rantonnetti* showed limited sequence variability (Di Serio 2007).

4.2.1.2 PCR-Based Techniques

Apple dimple fruit viroid (ADFVd) induces a serious fruit disorder that reduces the market quality of the apple fruits drastically. An RT-PCR procedure was developed with differently labeled primers in conjunction with a universal primer for detection and differentiation of isolates of ADFVd and *Apple scar skin viroid* (ASSVd) which also induces similar (dapple) symptoms on the apple fruits as ADFVd. The sequence variability of two ADFVd field isolates from two commercial apple cultivars was investigated. Sequencing of 18 full-length cDNA clones revealed five new sequence variants. Sequence comparison showed nine polymorphic positions distributed in different regions of the ADFVd molecule. As ADFVd and ASSVd cause similar symptoms, it is essential to ascertain the extent of infection by these two viroids. Simultaneous detection of both viroids in a multiplex format was accomplished. Reverse transcription with primer ADA-36 was followed by a single PCR reaction with this primer and two viroid specific primers AD-38 rd and AS-3ft. The resulting products were analyzed by agarose gel electrophoresis and examined under UV. The sequence conservation existing between certain regions of the ADFVd and ASSVd, as well as their sequence divergence in other regions were used to design viroid-specific primers, each labeled with a different fluorescent dye for rapid detection and discrimination of the two viroids by RT-PCR amplification. A cDNA of the expected size and fluorescence (254-bp and red, and 330-bp and green) was amplified from tissues infected by ADFVd and ASSVd respectively. This protocol could also differentiate these two viroids in double infections in which one of them was present in higher concentration than the other viroid (Di Serio et al. 2002).

The incidence of *Apple scar skin viroid* (ASSVd) was recorded for the first time in Himachal Pradesh, India. The genetic diversity of the isolates of ASSVd was assessed by performing RT-PCR assay. The amplicons (~330-bp) were cloned and sequenced. Ten clones so generated showed significant variability (94–100%) with each other. Further, variability was found to be more common in the pathogenic domain of the viroid genome. Four clones had 330-nt, while the other six clones had an additional nucleotide. Seven clones were considered as new sequence variants of ASSVd. Two clones showed 100% identity to a Chinese isolate, whereas six

clones exhibited 99% nucleotide similarities to a Korean isolate. The remaining two clones were more similar to the Chinese and Japanese isolates of ASSVd. The RT-PCR protocol developed in this investigation was useful for detection and discrimination of isolates of ASSVd (Walia et al. 2009).

Peach latent mosaic viroid (PLMVd) belonging to the genus *Pelamoviroid* in the family *Avsunviroidae* and *Hop stunt viroid* (HSVd) belonging to the genus *Hostuviroid* in the family *Pospiviroidae* cause important diseases in *Prunus* spp. PLMVd isolates (348–351 nt) causing peach calico (PC) disease were reported to have an additional sequence that contains the PC pathogenicity determinant (Malfitano et al. 2003). Phylogenetic studies indicated that PLMVd isolates could be divided into three main groups (Ambrós et al. 1998). Sequencing of the PCR products showed that nine new PLMVd variants were present in the 177 peach samples examined. The length of the sequenced PLMVd isolates ranged between 335- and 338-nt. All the obtained sequences clustered together in group III. HSVd isolates have been divided into three groups primarily based on overall homology, as plum-type, hop-type and citrus-type. The length of the isolates sequenced was 297- and 298-nt. Two HSVd isolates from apricot cv. Saturn were not clustered in one of the previously proposed groups, suggesting a novel putative group of HSVd isolates. Both HSVd isolates were found to be derived from recombination (Mandic et al. 2007).

Hop stunt viroid (HSVd) infects a large number of woody plant hosts such as *Prunus* spp., *Citrus* spp., and *Vitis* spp. HSVd along with *Citrus exocortis viroid* (CEVd) has been detected in both citrus and grapevines. Hence, in order to differentiate these two viroids, total RNA from leaves of grapevine *Vitis vinifera* 'Cabernet Sauvignon' and *V. labrusca* 'Niagara Rosada' was used as template for RT-PCR assay. Primers specific for HSVd, CEVd, *Grapevine speckle viroid 1* (GYSVd-1), *Grapevine speckle viroid 2* (GYSVd-2) and *Australian grapevine viroid* (AGVd) were employed. The PCR amplicons were cloned and sequenced. The grapevine samples analyzed showed the presence of both HSVd and CEVd. Phylogenetic analysis showed that Brazilian grapevine HSVd variants clustered with other grapevine HSVd variants, forming a specific group separated from citrus variants. On the other hand, the Brazilian CEVd variants clustered with other citrus and grapevine variants (Eiras et al. 2006).

Molecular characterization of *Hop stunt viroid* (HSVd) isolates from different naturally infected *Prunus* sources including apricot, plum and peach was performed by determining the nucleotide sequences of eleven isolates. Five new sequence variants of 296-nt (3 variants) or 297-nt (2 variants) comparable to the known HSVd isolates were identified. Phylogenetic analyses indicated that one apricot isolate clustered with a recombinant group, whereas all others (one apricot, two plum and one peach isolate) clustered with the hop-group, confirming the genetic diversity of HSVd isolates. The sequence variability appeared to be more related to the geographical origin of the isolates than to their hosts (Gazel et al. 2008).

The genetic diversity of *Cirtrus exocortis viroid* (CEVd) present in a single field tree of the citrus cv. Clementinier (*Citrus reticulata*) was investigated by sequencing cloned cDNA copies of natural isolates. Nucleotide sequence analysis showed that

CEVd had a genome of 370- or 371-nt in length. Differences were detected between four sequences at 14 positions out of 371-nt, giving a variability of 3.8%. This investigation indicated that viroids are quasi-species and sequence variation may occur naturally without passage in an indicator plant species. The selection pressures introduced by different host plant species and further intensified by environmental factors may lead to a drift in the population of the quasi-species status and emergence of a new master sequence. Further, the long potential life span of citrus trees in the fields (over 60 years) may provide adequate opportunities for the accumulation of several sequence variants in each tree (Elleuch et al. 2003, 2006).

Association of *Hop stunt viroid* (HSVd) with yellow corky vein disease of citrus (CYCVD) occurring in India was investigated to establish its causal relationship with the disease. In silico analysis showed that HSVd has 295 nucleotides and that the isolates exhibited nearly 100% nucleotide identity with six citrus cachexia isolates of HSVd. This variant was tentatively designated *Hop stunt viroid*-ycv (Roy and Ramachandran 2003). Later *Citrus exocortis viroid* (CEVd) was also found to be associated with CYCVD. BLAST analysis revealed the alignment of the sequences with a different CEVd. The isolates from CYCVD-infected plants was tentatively named as a variant of CEVd-ycv. This variant showed close relationship with CEVd *Gynura* variants reported from Australia (Roy and Ramachandran 2006).

Coconut cadang-cadang viroid (CCCVd), causative agent of lethal cadang-cadang disease of coconut palms, belongs to the genus *Cocaviroid* in the family *Pospiviroidae*. CCCVd comprises two monomeric 'fast' ($CCCVd_{246}$ and $CCCVd_{247}$) and also 'slow' ($CCCVd_{296}$ and $CCCVd_{299}$) electrophoretic forms as well as dimeric forms of each monomer. Variants of CCCVd were identified in oil palm (*Elaies guineensis*) growing in Malaysia. Three size classes containing 297-, 293- and 270-nt were identified. Comparison of 296-nt form of CCCVd with variants showed that all variants found in oil palm plants substitute $C^{31} \rightarrow U$ in the pathogenicity domain and $A^{175} \rightarrow C$ in the right-hand terminus. Different sizes of the viroid RNA observed, might be due to mutation and deletion. Mutations in the P and C domain were associated with a severe form of cadang-cadang disease known as 'brooming'. This investigation showed that the viroid molecules closely related to the lethal CCCVd were responsible for the orange spotting disorder affecting oil palm plantations in Malaysia, a region outside the Philippines where the viroid was considered earlier to be confined (Vadamalai et al. 2006).

4.2.1.3 Heteroduplex Mobility Analysis

Very high variability, plasticity and wide sequence distribution of *Hop stunt viroid* (HSVd) compare well with other extremely variable viroids such as *Peach latent mosaic viroid* (PLMVd). As HSVd has the potential to infect a wide range of crop plants such as cucumber, grapevine, citrus, plum, peach, pear, apricot and almond, the need for assessing the extent of variability and monitoring of HSVd in connection with hop growing along with other fruit crops was realized. Molecular sampling of

HSVd in grapevines in the environs of hop gardens was carried out. As *Hop latent viroid* (HLVd) also occurred along with HSVd, specific RT-PCR primers were employed to differentiate these two viroids infections. The cDNAs of HSVd from individual samples were analyzed by temperature gradient gel electrophoresis (TGGE) and heteroduplex analysis. These analyses revealed that HSVd formed dominant populations in all samples examined. In some of the samples, codominant heteroduplexes were found, but mainly minor duplexes were observed, suggesting the presence of quasi-species. A potential danger of hop cultivation near vineyards was underscored by the observation that about 70% incidence of HSVd populations in grapevines was found in the environs of hop gardens in the Czech Republic (Matoušek et al. 2003).

4.2.1.4 Single-Strand Conformation Polymorphism Analysis

Single-strand conformation polymorphism (SSCP) analysis has been demonstrated to be useful for differentiating strains that are indistinguishable by serological or other methods. Furthermore, SSCP analysis is more rapid than RFLP procedure. Genetic diversity in the field isolates of *Citrus exocortis viroid* (CEVd) was assessed by applying SSCP analysis. Seven different groups of variants exhibiting one to six changes that did not indicate overall variability among the CEVd clones were distinguished. In addition, SSCP analysis was effective in recognizing additional single nucleotide variations among clones that initially clustered together (Palacio and Duran-Vila 1999).

Peach latent mosaic viroid (PLMVd) induces three different symptoms of leaf yellowing, discoloration along leaf sides and leaf mosaic. In order to gain an insight into the possible molecular basis of induction of different symptoms by different field isolates, six isolates of PLMVd were examined by applying SSCP and nucleotide sequence analyses. The RT-PCR products of these six isolates were cloned. Twenty two to 24 clones per isolate were amplified by PCR and the corresponding PCR products were subjected to SSCP analysis. A variety of electrophoretic profiles were recognized and each SSCP pattern was then considered as a haplotype, reflecting the existing heterogeneity in all the isolates. The results showed that each PLMVd was composed of a population of genetically related variants (haplotypes), one being predominant with frequencies from 32% to 57% and most others having a low frequency of 4–5%. Comparison after sequence alignment demonstrated that the predominant haplotype had the least variation with others among them and its sequence was identical to the consensus sequence. The predominant haplotype displayed a wide representative of sequence for others in a PLMVd isolate. The similarities and genetic distance between the predominant sequences from peach showing the same symptom were higher and smaller respectively than that with different symptoms; they were more than 98.8% and <1% respectively, between the predominant sequences with different symptomatic source (Xu et al. 2008) (Appendix).

Appendix: Differentiation of *Peach latent mosaic viroid* (PLMVd) Isolates by SSCP Analysis (Xu et al. 2008)

(i) Mix aliquots of 0.2 μl of the RT-PCR products amplified from nucleic acid preparations or PCR products amplified from plasmids with 0.8 μl denaturing loading buffer (containing 95% formamide, 10 mmol/l Na_2OH, 0.05% bromophenol blue, 0.05% xylene cyanol); heat the mixture at 100°C for 8 min and chill on ice for 3 min.

(ii) Load the mixture into prerun (300V for 10 min) and precooled (at 4°C for 30 min) 8% non-denaturing polyacrylamide subjected to three conditions:
Condition I – acrylamide: bisaccarylamide 49:1
Condition II – 29:1
Condition III – two equal sections – the upper section is 29:1 and the bottom section is 49:1 gel (20 × 12.5 × 0.1 cm)

(iii) Perform electrophoresis at 200V in a refrigerator at 4°C in 1 × TBE buffer (89 mmol/l Tris, 89 mmol/l boric acid, 2.5 mmol/l EDTA, pH 8.3) for 16–20 h.

(iv) Stain the gel with silver.

(v) Analyze the clones from different isolates first in condition I; identify haplotypes with more than one clone; analyze further in condition II and finally verify the clones representing the predominant haplotype screened in the first two conditions, in condition III.

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Chapter 5
Diagnosis of Viral and Viroid Diseases of Plants

Abstract Plant viruses and viroids infect a large number of crops and other plant species, in spite of their extremely small size and elementary structure. The viruses are strictly obligate, intracellular parasites and adopt themselves for the varying conditions existing in their plant and natural vectors. For the diagnosis of virus diseases, it may not be possible to fulfill all steps of the Koch's postulates. But the consistent and constant association of the virus(es) with the newly observed disease(s) can be demonstrated by detecting the viruses by applying various techniques depending on their biological, immunological and nucleic acid characteristics. Use of local lesion host/assay host plant species has been very useful to prove the pathogenicity of the viruses that require long time for symptom expression, as in the case of viruses infecting fruit trees. Immunological methods have greater sensitivity and reliability compared to biological methods for diagnosing virus infections, particularly latent infections. In the recent years, polymerase chain reaction-based and hybridization based-methods are being recommended as diagnostic tool because of the accuracy, rapidity, sensitivity and specificity of the results. Nucleic acid-based techniques are primarily applied for the diagnosis of viroid diseases. The disease diagnostic centers (DDCs), plant quarantines and certification programs have a vital role in the prevention of introduction of viral and viroid diseases from other countries or locations within the country. The need for upgrading and providing required facilities to these centers and programs is emphasized.

Crops cultivated in different ecosystems are exposed to various microbial plant pathogens, in addition to different kinds of other biotic and abiotic agents that can adversely affect their growth and yield potential to varying magnitudes, depending on the duration for which the disease-inducing agents have access to the susceptible plant species. Any deviation from the normal functions and development leads to disease in the affected plant species. The nature of the agent(s) involved in observed diseased condition, has to be ascertained immediately to facilitate application of necessary preventive measures to minimize the further spread of the disease(s). Microbial plant pathogens are able to cause distinct macroscopic symptoms that can be visually seen in most cases and hence, these diseases can be separated with some experience from the problems caused by insect pests or adverse environmental

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conditions or industrial pollutants injected into the atmosphere. Disease diagnosis pertains to the identification of the nature and cause of the disease, while detection relates to establishment of the identity of the causative agent present in the infected plants. Plant Pathogen detection and disease diagnosis are inseparable processes, the results of one process complimenting the other resulting in reliable identification of the disease-causing agent precisely for initiating follow up action for the measures to minimize disease spread and the consequent yield losses to the growers.

Disease diagnosis is based on the postulates enunciated by Robert Koch and it is needed essentially to recognize the primary viral/viroid pathogen causing the disease. In a disease complex, two or more viral pathogens may be involved, making it difficult to identify the viruses present in the infected plants with complex disease symptoms. It is possible for masking symptoms of one virus by the presence of another more virulent virus. Symptom expression may not be clear or may take long time for the development of symptoms as in the case of virus diseases infecting fruit trees like citrus and grapevine with long span of life. In such cases the infection may take place in the mother plants and the planting materials may not exhibit any visible symptoms. By the time symptoms become observable, the pathogen might have well established in the internal tissues of the plants, making it difficult, if not impossible, to apply any effective measures to reduce disease spread. However, reliability on the development of external symptoms of infection alone may lead to erroneous identification of the virus(es) and consequently the control methods that are suggested may prove to be ineffective. Hence, accurate identification of the disease-causing virus(es) is the basic requirement for planning effective virus disease management strategies. In addition, precise identification and differentiation of the viruses or virus strains enables more informed decision to be made about the choice of cultivar (showing resistance, if available) and other virus disease management strategies.

Viruses and viroids are structurally primitive compared to fungal and bacterial pathogens. Yet they can infect almost all crops as the other kinds of plant pathogens. The viruses possess several characteristics with which they can be distinguished. The entire group of viruses and viroids are obligate parasites and so far they have not been cultured in cell-free media. Structurally they are extremely small in size and resemble cellular contents (nucleoproteins) rather than the cells of the fungal and bacterial pathogens. The presence of protein and nucleic acids as the only two major components, absence of any physiologic functions and dependence on a vector, in most cases, for their spread from infected to healthy plants are the characteristics that differentiate the viruses from fungal and bacterial plant pathogens. The major types of symptoms such as mosaic, vein clearing, chlorosis and leaf curl are quite distinctive for virus diseases. However, nutritional disorders may induce symptoms similar to some symptoms of virus infections. The involvement of a virus as the causal agent of the newly observed disease may be proved by transmission through grafting, mechanical inoculation or insect vectors. In contrast, the nutritional disorders cannot be transmitted by any means and the affected plant may recover, if the deficient nutrient is supplied to the affected plants. But no such recovery occurs in the case of virus diseases. Diagnosis of virus infections in different crops can be accomplished by applying suitable diagnostic tests. The relative effectiveness and adoptability of the diagnostic tests is discussed below.

5.1 Choice of Diagnostic Tests for Viral Diseases

5.1.1 Biological Tests

Viruses are obligate, intracellular parasites, deriving all requirements for their replication (multiplication) from the susceptible host cells. In the process of obtaining necessary amino acids and nucleotides for the synthesis of virus protein and genomic nucleic acids, the host cell synthetic machinery is diverted to the production of virus-related compounds. Hence, the pattern of synthesis of compounds is drastically altered to the advantage of the invading virus.

5.1.1.1 Symptomatology

The macroscopic symptoms expressed externally are considered to be due to the deranged metabolism of the host plants following infection by viruses. No toxic metabolites, hormones or enzymes are produced by viruses to account for the necrosis and abnormal growth patterns induced by the viruses in the infected plants. Characteristic mosaic patterns are caused by several viruses on a variety of host plants providing a clue for the tentative identification of the virus inducing the newly observed disease. Infection of pearl millet (*Pennisetum glaucum*) and sorghum (*Sorghum bicolor*) by *Wheat streak mosaic virus* (WSMV) was observed for the first time. The identity of the virus was tentatively established based on the similarity of symptoms, ability of the virus to be transmitted by mechanical inoculation and by wheat curl mites (*Aceria tosichella*) and confirmed later by applying the enzyme-linked immunosorbent assay (ELISA) (Seifers et al. 1996). Another new virus disease of wheat causing yellow head was investigated. The virus was experimentally transmitted by punch inoculation from wheat to maize and by rub-inoculation to *Nicotiana benthamiana* to record the symptoms for comparison with those of other viruses infecting wheat. As the symptoms induced by the new virus were different and the results of other tests also indicated the differences between the virus causing yellow head disease and other viruses infecting wheat, it was designated *Wheat yellow head virus* (Seifers et al. 2005).

In certification programs, biological testing appears to be the most reliable and practical method for diagnosing the virus infections in propagative plant materials. The woody indicator peach seedling GF305 is recommended for indexing for the *Plum pox virus* (PPV). The symptoms are expressed in the root stock at 3–4 weeks after grafting (Gentit 2005). Local lesion/assay host plants have been used for rapid detection and identification of some plant viruses/strains. Production of systemic symptoms and/local lesions on certain plant species have been used for the identification of *Potato virus Y* (PVY) strains (Baldauf et al. 2006). The isolates of *Zucchini yellow mosaic virus* (ZYMV) could be identified by their ability to produce local lesions in *Chenopodium amaranticolor* or *Gomphrena globosa* (Bananej et al. 2008). Based on the symptoms induced by different isolates of *Cucumber mosaic virus* (CMV) on banana, *Nicotiana glutinosa* and cowpea, four different pathotypes

of CMV were differentiated. Using *N. glutinosa* which responded with local lesions or systemic infection, three symptoms types (A, B and C) among the isolates of CMV were recognized (Chou et al. 2009).

The internal symptoms typical of virus infection may be observed. Formation of inclusion bodies in the infected host cells or tissues is a characteristic feature of virus infection. Characteristics of inclusion bodies and their intracellular locations are listed among the criteria for the classification of plant viruses. Some of the virus groups may be tentatively identified based on the characteristics of virus inclusions. The presence of cylindrical or pinwheel inclusions in host plant cells is indicative of infection by *Potato virus Y* and other viruses belonging to the genus *Potyvirus* (Martelli and Russo 1984).

5.1.1.2 Transmission Tests

Differences in the mode of transmission have been used for grouping the viruses as mechanically transmitted viruses and vector-transmitted viruses. Viruses infecting strawberries have been grouped as aphid-transmitted viruses (7), whitefly-transmitted viruses (3), nematode-transmitted viruses (5), oomycete-transmitted virus and viruses with unknown vectors (Martin and Tzanetakis 2006). Viruses like *Tobacco mosaic virus* (TMV) and *Potato virus X* (PVX) are highly infectious and stable and they can be easily transmitted by mechanical (sap) transmission. These viruses do not have any natural vector. Majority of the viruses are transmitted by one or more species of insects, mites, nematodes or fungi. The type of relationship between the virus and vector is determined by the virus and hence, this characteristic is used for different viruses and the diagnosis of diseases induced by them. Some viruses like *Rice dwarf virus* (RDV) are able to multiply in the vector insect species and also show transovarial transmission to subsequent generations through the eggs of infective leafhoppers (Fukushi 1940). Determination of the virus-vector relationship [styletborne (nonpersistent), circulative (persistent) or propagative] may be useful in putative identification of the virus causing newly observed virus.

5.1.2 Physico-chemical Tests

The stability of the viruses in the sap extracted from infected plant tissues may provide a clue to the identity of the viruses. *Tomato mosaic virus* (ToMV) and *Tomato spotted wilt virus* (TSWV) infecting tomatoes, show distinct differences in their physical properties such as longevity in vitro (LIV) and thermal inactivation point (TIP). ToMV is highly stable and remains infective for long time, whereas TSWV is highly unstable, lose its infectivity within a short time even at low temperatures (Narayanasamy and Doraiswamy 2003). As the viruses are obligate parasites, they have not been cultured in cell-free media. The morphology of virus particles has been determined by taking ultrathin sections of the infected tissues or after

purification followed by negative staining and observing under the electron microscope. The viruses have been grouped into rigid rod-shaped, flexible elongate, spherical, bullet-shaped and geminate viruses, based on the virus particle morphology (Shikata 1981; Milne 1993). Electron microscopic observations on the purified virus preparations negatively stained with 2% phosphotungstate revealed the presence of *Turnip mosaic virus* (TuMV), *Broad bean wilt virus* (BBWV) and *Cucumber mosaic virus* (CMV) in several weed plant species in the areas adjacent to commercial crops in Korea (Kwon et al. 2000). Electron microscopy has been employed for spot-checking the presence of viruses in the plant infected by newly observed tissues to establish the identity of the virus tentatively.

The virus nucleoprotein separated from the extract of tissues of infected plants is characterized by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) technique. The electrophoretic mobility of the virus-related protein determined by SDS-PAGE technique may be useful for virus identification. The presence of a unique 32–34 kDa protein in the extracts of wheat plants infected by *Wheat yellow head virus* (WYHV) was revealed by SDS-PAGE procedure. This protein was absent in the healthy plants. A similar protein was detected in the extracts of maize and *Nicotiana benthamiana* plants inoculated with WYHV, indicating the diagnostic value of the virus protein for specific diagnosis of the disease induced by WYHV (Seifers et al. 2005). The characteristics of the nucleoprotein of *Calla lily chlorotic spot virus* (CCSV) were the basis for differentiation of this virus from other tospoviruses and the specific diagnosis of the disease caused by CCSV in Calla lily plants (Lin et al. 2005). Likewise, the presence of a unique protein band representing the virus coat protein (CP) was revealed by SDS-PAGE analysis of the extracts of *Angelonia* plants infected by *Angelonia flower break virus* (AnFBV) (Adkins et al. 2006) and extracts of *Phalaenopsis* plants infected by a new potyvirus, *Phalaenopsis chlorotic spot virus* (PhCSV) (Zheng et al. 2008).

5.1.3 Immunoassays

Immunodiagnosis of virus diseases has been demonstrated to be efficient and reliable, as immunoassays are based on the immunoreactivity of virus-related proteins. Antisera have been raised against virus coat protein (CP) and inclusion body proteins induced in infected plants and these antisera have been employed for diagnosis of the diseases caused by the respective viruses. Application of antisera containing monoclonal antibodies (MAbs) and bacterially-expressed (BE)-virus proteins has substantially enhanced the sensitivity and specificity of the immunoassays (Cerovska et al. 2010). Different variants of enzyme-linked immunosorbent assay (ELISA) have been extensively employed for virus detection and diagnosis of grapevines leaf roll and rugose wood complex disease (Martin et al. 2000), *Tomato spotted wilt virus* in different crop plant species (Hassani-Mehrban et al. 2005; Lin et al. 2005) and wheat virus diseases (Seifers et al. 2005). The presence of single and multiple viruses in stone fruit trees was detected by applying ELISA tests. Infection by *Plum pox virus* (PPV),

Prunus necrotic ringspot virus (PNRSV) and *Prune dwarf virus* (PDV) singly or in combination was revealed by the ELISA tests (Michelutti et al. 2005).

Universal potyvirus group-specific MAbs generated against *Bean common mosaic necrosis virus* (BCMNV) and *Bean common mosaic virus* (BCMV) were employed in indirect ELISA format for ascertaining the extent of infection of bean germplasm lines by these viruses (Larsen et al. 2005). Likewise, specific MAbs were employed in indirect ELISA test to assess the infection of citrus by severe strain of *Citrus tristeza virus* (CTV) to facilitate the eradication of citrus plants infected by the severe strain of this virus (Yokomi and De Borde 2005)**.** The virulence (pathogenic potential) of strains may vary with different isolates of viruses, the severe strains being responsible for greater magnitude of losses. *Plum pox virus* (PPV) exists in the form several strains with varying levels of virulence. By using specific MAbs in DAS-ELISA format, four serotypes D, M, C and El Amar were differentiated (Myrta et al. 1998). In a later investigation, using triple antibody sandwich (TAS)-ELISA format, 48% of the isolates were identified as PPV-D, 39.6% as PPV-M and 11.6% as mixtures of D and M strains. Differentiation of the PPV strains was shown to be essential to precisely identify the strain causing the disease in a given geographical location to facilitate risk assessment (Zagrai et al. 2008).

5.1.4 Nucleic Acid-based Techniques

Molecular methods are more essential for reliable and sensitive diagnosis of virus diseases, since the sequences of the coat protein (CP), movement protein (MP) or polymerase genes exhibit variations between virus species, genera and families making the precise identification of the target virus and its strains possible. Enzymatic amplification of the signal probe or target nucleic acid sequences enhances the sensitivity and specificity of the diagnostic techniques. Amplification of the target primer using polymerase chain reaction (PCR) has spectacularly increased the effectiveness and reliability of diagnostic assays. Hybridization-based and PCR-based techniques have been widely employed for reliable and rapid diagnosis of virus diseases affecting various crops.

Hybridization methods were successfully employed for the diagnosis of several diseases caused by viruses such as *Cucumber mosaic virus* (Takeshita et al. 1999), *Prunus necrotic ringspot virus* (Heuss et al. 1999; Kulshrestha et al. 2005) and *Apricot latent virus* (Ghanen-Sabanadzovic et al. 2005). Hybridization procedures have been found to be effective for simultaneous detection of two or more viruses by employing a cocktail of specific single probes against viruses infecting vegetable crops (Saito et al. 1997) and stone fruit crops (Saade et al. 2000). By employing unique riboprobes designated ‘polyprobes’ up to six different viruses viz., *Apple mosaic virus*, *Apple chlorotic spot virus*, *American plum line pattern virus*, *Plum pox virus*, *Prune dwarf virus* and *Prunus necrtotic ringspot virus* present in the same sample together or in different samples individually were detected and differentiated with equal efficiency. As the fruit trees are likely to be infected by

multiple viruses, it may be advantageous to employ these methods especially for indexing mother plants to ascertain their sanitary status before the materials are taken for propagation (Heranz et al. 2005).

Polymerase chain reaction (PCR)-based techniques have been demonstrated to be highly sensitive, specific, rapid and reliable for detection and identification of viruses causing diseases individually or associated with other viruses causing complexes as in grapevines. Specific primers and kits are available commercially. Reverse transcription (RT)-PCR for RNA viruses either alone or preceded by immunocapture (IC) step has been demonstrated to be very effective for diagnosing latent infections by viruses like *Potato virus Y* (Agindotan et al. 2006), *Plum pox virus* (Myrta et al. 2005; Zagrai et al. 2008), *Citrus tristeza virus* (Huang et al. 2004). A combination of IC-RT-PCR-FRLP techniques was shown to be efficient in diagnosing *Cherry leaf roll virus* (CLRV) infection in walnut, black elderberry and birch and in establishing the relationship between the isolates and other viruses (Buchhop et al. 2009). Real-time RT-PCR format has the potential to overcome some of the limitations of standard PCR procedure and provide more precise identification of the virus(es) involved in the causation of diseases in various crops as in the case of *Citrus* spp. infected by *Citrus tristeza virus* (CTV) (Ruiz-Ruiz et al. 2009). Efforts are being made to simplify to facilitate the mobility of the techniques and applicability under field conditions and to reduce the cost of testing.

Reverse transcription (RT)-PCR assay followed by restriction digestion has been shown to be simple, rapid and specific for the detection and identification of strains of viruses. This approach was made for the diagnosis of infection by *Alfalfa mosaic virus* (AMV) in potato plants and tubers. A primer pair AMV-F and AMV-R specific to AMV CP gene was found to be specific and sensitive for detecting AMV in potato leaves and tubers. AMV RNAs were readily detectable in composite samples of 400–800 potato leaves or 200–400 tubers. The RT-PCR procedure followed by restriction fragment length polymorphism (RFLP) analysis may be effective in screening potato samples on a large scale for the presence of AMV (Xu and Nie 2006).

The RT-PCR assays described earlier could characterize *Potato virus Y* (PVY) isolates as to the strain type. However, they were limited in their ability to detect only some combinations of mixed strains infections. Hence, a single multiplex RT-PCR assay that could assign PVY strain type and detect mixed infection with respect to major strain types, was developed. The effectiveness of the multiplex RT-PCR protocol was demonstrated by using 119 archived PVY isolates. Assays with strain isolates gave results comparable to the results obtained earlier in most cases. It was possible to diagnose 16 mixed infections that were not detected by earlier investigations. The protocol developed in this investigation may be useful for seed potato tuber production specialists interested in diagnosing the infection of tubers by different combination of PVY strains (Lorenzen et al. 2006).

Potato crops are affected by several viruses and viroids all over the world and their transmission through tubers to subsequent generations is of serious economic concern. Detection of multiple pathogens simultaneously in plants and planting materials has been successfully accomplished. A macroarray procedure was applied for the detection of eleven potato viruses and the viroid *Potato spindle tuber viroid*

(PSTVd). Infections diagnosed by the macroarray were due to *Alfalfa mosaic virus, Cucumber mosaic virus, Potato mop top virus, Potato leaf roll virus, Potato latent virus, Potato virus A, Potato virus M, Potato virus S, Potato virus X, Potato virus Y* and *Tobacco rattle virus* for which tests are commonly performed by personnel of North American potato seed certification programs. The results obtained from macroarray method were entirely consistent with those obtained by using the ELISA format. Macroarray technique has been demonstrated to be a cost-effective approach to the simultaneous diagnostic detection of multiple pathogens from infected plants (Agindotan and Perry 2008).

Multiplex diagnostic techniques offer the advantages of rapid results and reduction in cost of testing. Four potyviruses infecting calla lily viz., *Dasheen mosaic virus* (DsMV), *Turnip mosaic virus* (TuMV), *Konjac mosaic virus* (KoMV) and *Zantedeschia mild mosaic virus* (ZaMMV) could be detected simultaneously by a multiplex RT-PCR assay. Primer pairs specific to each of the four viruses and a primer pair specific to host plant mitochondrial *nad5* mRNA as internal reaction control were employed for amplification of the target DNA fragments of the viruses concerned. Inclusion of the reaction control guarded against false-negative results thus ensuring reliable diagnosis of the virus infection especially when the disease incidence might be low. During field surveys the multiplex RT-PCR assay proved to be more efficient in diagnosing more single as well as mixed infections compared to indirect ELISA tests frequently employed for diagnosing virus diseases of Calla lily (Hu et al. 2010).

The need for rigorous testing of mother plants for their freedom from virus diseases that affect production drastically, has been underscored by many studies. Spread of *Citrus tristeza virus* (CTV) through infected propagation materials is known. Due to the traditional use of trifoliate orange (*Poncirus trifoliata*) rootstock, tolerant for decline symptoms, CTV was found to be continuously spreading by the propagation of infected materials, making the East Adriatic a reservoir of diverse CTV genotypes. The presence of CTV was detected by ELISA tests and the results were corroborated by IC-RT-PCR assay confirming the presence of CTV in all tested samples. This finding reveals the danger of obtaining budwood and other propagation materials from such plants that may not exhibit clear symptoms of CTV (Cerni et al. 2009). The role of crop plants and weed plants serving as sources of inoculum for a virus has to be determined for possible elimination of all suspected sources of the virus concerned to reduce the disease incidence and subsequent spread. Infection of several weed species inoculated artificially with *Potato virus Y* (PVY) was diagnosed by employing ELISA test and confirmed by IC-RT-PCR assay. Natural PVY infection in four wild species collected in the potato-growing fields was detected by these tests. The results indicated the need for thorough elimination of sources to reduce the incidence of PVY in potato crops (Kaliciak and Syller 2009).

The necessity of evaluating diagnostic procedures developed by different researchers for the efficiency and reliability by a group of selected laboratories was realized. The operational capacity of a duplex RT-PCR assay for simultaneous detection of *Prune dwarf virus* (PDV) and *Prunus necrotic ringspot virus* (PNRSV) was evaluated by nine European laboratories. A total of 576 samples from *Prunus*

trees with known sanitary status, corresponding to 32 samples in two replications for each laboratory were tested. The level of sensitivity achieved by the method was 98.3% for PDV and 90.4% for PNRSV. The specificity attained was 87.4% for PDV and 94.3% for PNRSV. A strong agreement between data was indicated by the statistical analysis. The results of the evaluation of duplex RT-PCR assay showed that the protocol could be integrated into certification programs (Massart et al. 2008).

Molecular assays have been demonstrated to be invaluable for the rapid detection and identification of viral pathogens which have limited biological characteristics to be used for reliable detection and differentiation. Yet the biological assays have to be adopted as the fundamental assay for the continued success of clean stock/certification programs. This status of diagnostic approach, as one of the bases of crop disease management, has to be recognized, because uncharacterized graft transmissible agents (GTAs) (viruses, viroids and phytoplasmas) and unknown pathogens are present in large numbers. In addition to using biological indicators, necessity for officially recognizing and integrating laboratory-based techniques (immunoassays and nucleic acid-based techniques) into the certification programs has to be realized. The need can be understood by the fact that the modern techniques can provide more rapid and sensitive detection and identification of known agents, especially of regulated and exotic pathogens within the confines of a laboratory, rather than as live pathogen-infected specimen in grafted indicator plants in greenhouses or in the open fields, providing opportunity for the new pathogen to escape from the testing centers (Rowhani et al. 2005).

5.2 Choice of Diagnostic Tests for Viroid Diseases

5.2.1 Biological Tests

Viroids are the simplest plant pathogenic entities containing small single-stranded circular RNA molecule as the genome and they lack the protein component that is present in viral pathogens. They cause distinct symptoms that may form a basis for differentiation of some of the viroids. Bioindicator plants that produce symptoms rapidly and differentially react to different viroids, may be useful for detection and identification of viroids. In order to hasten the indexing process for the presence of citrus viroids, a laboratory technique combining shoot-tip grafting in vitro and biological indexing on indicator plants was developed. The classical in vitro method, diagnosis based on expression of symptoms on indicators requires 11–14 weeks after inoculation. In contrast, microindexing on cuttings by grafting on citron in vitro needs only 12 days after inoculation to provide the results. Furthermore, microindexing on cuttings by grafting is easier and more reliable than microindexing on seedlings (Kapari-Isaia et al. 2008). As the bioindexing is the feasible method in most laboratories in the developing countries, this in vitro indexing

procedure holds promise for reliable and rapid diagnosis of citrus viroid diseases. Based on the biological and structural characteristics, viroids are classified into two families: *Pospiviroidae* and *Avsunviroidae*. The members of these two families multiply respectively in the nucleus and chloroplast of host plants (Volume 3, Chapter 2).

5.2.2 Nucleic Acid-based Techniques

As the viroids are devoid of protein, nucleic acid-based techniques are primarily employed for improved detection of viroids and diagnosis of the diseases caused by them.

5.2.2.1 Hybridization-Based Techniques

Infection by single or multiple viroids or in combination with viruses, have been frequently observed, making the diagnosis and resolving the components of the disease complexes considerably difficult. Dot blot hybridization technique using digoxigenin (DIG)-labeled viroid-specific probes has been demonstrated to be effective for the diagnosis of infection by single viroid as in the case of *Potato spindle tuber viroid* (PSTVd). The protocol was found to be suitable for mass indexing programs (Welnicki and Hiruki 1992). Likewise, dot blot hybridization using DIG-labeled viroid-specific probes was effective for diagnosing infection by *Peach latent mosaic viroid* (PLMVd) and *Hop stunt viroid* (HSVd) in peach trees, when they were present singly or in combination (Hassan and Ryšánek 2004). Viroid-specific probes were employed in northern hybridization procedure, for the detection of CEVd, *Citrus bent leaf viroid* (CBLVd), HSVd, and *Citrus viroid* III and IV (CVd-III and IV) in the inoculated Etrog citron that was used as indicator plant species (Barbosa et al. 2005). Coinfection of CEVd and HSVd with *Citrus tristeza virus* (CTV), *Citrus psorosis virus* (CPsV) or *Citrus infectious variegation virus* (CVV) was diagnosed by hybridization procedure using specific probes (Barbarossa et al. 2007).

5.2.2.2 Polymerase Chain Reaction-Based Methods

The usefulness of polymerase chain reaction (PCR)-based procedures for diagnosing viroid infections in different crops has been demonstrated. The tissue printing procedure involving immobilization of *Potato spindle tuber viroid* (PSTVd) in the plant extract onto a nitrocellulose membrane, followed by application of RT-PCR protocol was effective in diagnosing primary and secondary infection in potato plants (Weidemann and Buchta 1998). The RT-PCR assay has been reported to be effective for the diagnosis of *Citrus exocortis viroid* (CEVd) (Turturo et al. 1998), *Hop stunt viroid* (HSVd) (Nakahara et al. 1999) and *Citrus viroids* (Barbosa et al. 2005).

Real-time RT-PCR assay has distinct advantages over the standard PCR method. Fluorescence RT-PCR using TaqMan™ technique was able to detect *Potato spindle tuber viroid* (PSTVd) with high level of accuracy and sensitivity (Boonham et al. 2004). Four methods viz., DIG-probe, return (R)-PAGE, RT-PCR and real-time TaqMan, were tested for selecting a diagnostic protocol for testing the presence of PSTVd in in vitro and glasshouse-grown potato plants for the purposes of post-entry quarantine and production of pathogen-free nuclear stock. The detection limits were 10–20 mg of PSTVd-infected tissue for R-PAGE, 0.25–0.5 mg for DIG-probe, 0.062 mg for RT-PCR and 0.0155 mg for real-time TaqMan techniques. The DIG-probe and R-PAGE protocols were recommended as primary detection methods, with confirmation of viroid presence by any of the four validate detection protocol. As none of the detection methods could specifically identify PSTVd, sequencing of the viriod nucleic acid has to be accomplished. It has been suggested that sequencing may be performed by sending PCR amplicons to a commercial sequencing laboratory for analysis (Jeffries and James 2005). Sequencing of PCR amplified products has been performed for specific identification of the viroids and also their variants which may be present in the same or different plants in the same location. The length and sequences of the viroid genome were determined for *Peach latent mosaic viroid* (PLMVd) and *Hop stunt viroid* (HSVd) infecting peach and apricot respectively (Mandic et al. 2007). The sequences of the isolates of HSVd from apricot, plum and peach were compared, following cloning of PCR products. The observed variations in their sequences appeared to be more due to the geographical origin of the isolates than to their host plant species (Gazel et al. 2008).

5.3 Agencies Involved in Disease Diagnosis

Disease diagnosis may be the primary responsibility of the personnel of diagnostic centers, plant quarantines and certification programs. In addition, diagnosis of virus diseases may be required for epidemiological and breeding for disease resistance programs to some extent. Precision, sensitivity, rapidity, reproducibility, simplicity and cost-effectiveness of diagnostic methods may determine the quality and utility of diagnosis. The speed of providing reliable results may have high priority, while choosing the method suitable for epidemiological investigations, since large number of samples may have to be analyzed during field surveys. As the conventional methods, although simple, are labor-intensive and require large greenhouse space and longer time to give the results. The conventional methods are being largely replaced by more sensitive methods involving the use of antibody-dependent immunological and nucleic acid-based techniques which can provide results rapidly.

Disease diagnostic centers (DDCs) have the responsibility of analyzing the samples received from the growers and communicating the results with necessary advice for follow up action by the growers. Plant Pathology departments attached to the Universities/Research stations are entrusted with this function of offering advisory service to the clientale group. Plant quarantines, both domestic and

international, have the primary to responsibility of preventing the introduction of new pathogens/pests into the country by enforcing specific regulations for the import of plants and plant materials. They are able to employ serodiagnostic methods more frequently compared to nucleic acid-based techniques. Certification personnel have the responsibility of certifying seeds and other propagative plant materials for freedom viral and viroid pathogens. It may suffice to employ generic diagnostic methods for pathogen detection for plant quarantine and certification personnel, whereas the epidemiologists may need more specific and sensitive diagnostic methods that can identify the viral pathogens up to strains causing epidemics in one area. Such specific information may be needed to verify whether the same virus strain already exists in other areas of the country and to take up necessary measures to restrict the spread of the disease to other parts of the country. The higher level of sensitivity and specificity of detection methods will be useful to assess the distribution of different strains of a virus or viroid strains/variants with varying levels of pathogenic potential (virulence). In addition, evolution of new strains/variants of indigenous viruses has to be monitored by epidemiologist by employing discriminative methods. Information on the population structure of the virus or viroid gathered will be useful for developing disease forewarning system to alert the farmers on the possible occurrence of a more aggressive virus/viroid strain/variant, if necessary (Narayanasamy 2002, 2008).

5.3.1 Disease Diagnostic Centers

Accurate and reliable identification and differentiation of viral/viroid pathogens and their strains with different pathogenic potential is the basic requirement of the development of effective disease management systems. Plant disease diagnostic centers (DDCs) are expected to provide organized, systematic and professional service for rapid detection and precise identification of viral pathogens as the primary cause(s) of the disease problems observed in the region where DDC is located. Any disease management system will be ineffective, if it is not based on the proper diagnosis of the disease to be controlled. Hence, DDCs have the responsibility of making accurate identification of the disease problems and suggesting suitable changes in the disease management strategies with the aim of reducing both qualitative and quantitative losses that have to be otherwise incurred by the cultivator. The credibility of the DDCs depends on the availability of adequate facilities to analyze large number of samples within short time and to offer corrective measures without any loss of time, based on the results obtained.

Adoption of molecular techniques for the detection of viruses/viroid and diagnosis of diseases caused by them, will enhance the sensitivity and reliability of results and reduce the false-positive or -negative results. Among the immunodiagnostic methods, enzyme-linked immunosorbent assay (ELISA) formats have been extensively employed to obtain results with greater reliability and reduction in time required for analysis compared with biological methods. Polymerase chain reaction

(PCR)-based techniques have been demonstrated to provide improved detection and differentiation of the viral pathogen isolates which exhibit differences in their virulence and other characteristics. The results from PCR-based techniques are more precise and can be obtained much earlier compared to the immunodiagnostic tests. Many DDCs in North America and Europe are capable of applying highly technical diagnostic procedures. Access to computers for maintenance and retrieval of information has significantly enhanced the quality of diagnostic services offered by these DDCs. The disease diagnostic centers in the developing countries, on the other hand, have to be upgraded and they should be established in as many locations as possible to cater to the needs of more areas, so as to bring larger number of farmers under the protective umbrella. Thus the aim of establishing the DDCs to record the extent of disease incidence, diagnose the disease problems accurately and to offer effective suggestions to keep the pathogens at bay, can be satisfactorily realized. The DDCs have also been involved in field surveys on regional or national basis to assess the distribution of crop diseases and monitor the 'pathogen path' to indicate the vulnerability of adjacent states/regions for possible attack by the disease(s).

Many viruses are known to exist as strains that differ distinctly in their virulence (pathogenic potential), some capable causing drastic reduction in crop yields. Hence, it is essentially required to detect, identify and differentiate the severe strains from mild strains of the same virus. *Sugarcane mosaic virus* (SCMV) and *Sorghum mosaic virus* (SrMV) occur in the form of strains. Sequence variability within SCMV populations around the world is significant and the identification of strains might be best achieved by direct analysis of sequence data. However, it may be impractical to characterize a large number of isolates, using standard sample preparation and processing procedures. Hence, a simple protocol that yields quality sequence information, requiring neither viral RNA purification nor cloning of RT-PCR products was applied for the rapid diagnosis of infection by different strains of SCMV and SrMV. This rapid, cost-effective protocol has been shown to have the potential for large scale virus surveys (Gómez et al. 2009).

A plant biosecurity system was visualized for early detection, precise diagnosis and rapid follow up action to minimize disease spread. The National Plant Diagnostic Network (NPDN) has been established in the USA, with specific the objective of (i) establishing a national communications system linking plant diagnostic centers; (ii) upgrading infrastructure for effective diagnosis; and (iii) providing training to facilitate rapid reporting of outbreaks of pathogens/diseases. A nationwide network aims to function as a cohesive system to rapidly detect and diagnose high consequence pathogens in the agricultural and natural ecosystems. The network ensures that all participating diagnostic facilities are alerted to possible outbreaks and are technologically equipped to rapidly detect and identify high risk pathogens. NPDN has established an effective communication network and developed standardized diagnostic and reporting protocols. Cataloguing of diseases and pests occurrence has been taken up to widen the national database (Stack et al. 2006). The concept of establishing similar national network with suitable modifications may be considered by other countries for exchange of information on the occurrence of new pathogens in the respective countries.

5.3.2 Plant Quarantines

Exclusion of plant pathogens by adopting different approaches is one of the principles of crop disease management. Plant quarantines play a vital role in excluding plant pathogens by preventing their introduction through infected plants and plant materials. Domestic and international plant quarantines have been established by most countries to exercise statutory control of plant health through legislation under which appropriate actions can be taken by the country concerned to prevent introduction or spread of the notified plant pathogen(s) that are not present or insignificant in that country concerned. The principles of establishing plant quarantines recognize the sovereignty of the country which has the right to impose the conditions and phytosanitary measures to be taken by the exporting country. The need for establishing modern quarantine laboratories has been well realized by several nations, after the implementation of the General Agreement on Tariffs and Trade (GATT), since there is a spectacular increase in the movement of people and plant materials that are likely to carry and introduce the plant and human pathogens into another country where they may be absent. This situation necessitated the enforcement of sanitary and phytosanitary measures at global level.

The International Plant Protection Convention (IPPC) was established in 1991, following the acceptance of GATT by majority countries. Basic principles required for formulating standards for plant quarantine procedures in relation to the international trade have been formulated by an expert committee (FAO 1991). Accordingly, an organism is considered to be of quarantine significance (QS), if its exclusion is perceived as important enough to protect agriculture and natural vegetation of the importing country. Sixteen basic principles have been suggested by the expert committee with the expectation that judicious application of these principles might lead to reduction or elimination of the use of unjustified phytosanitary measures as a barrier to trade. Eight regional plant protection organizations have been set up to advise and assist member governments on the technical, administrative and legislative measures concerned with plant quarantine activities. The QS organisms have been divided into two groups designated List A and List B organisms. List A includes dangerous organisms and List B encloses organisms of importance for individual countries. List A is subdivided into List A1 containing pathogens absent in all member countries (exotic pathogens) and List A2 enclosing pathogens present only in some countries in the region (Narayanasamy 2002). Some of the representative viral pathogens included in the List A1 and List A2 by European and Mediterranean Plant Protection Organization (EPPO) are as follows: *American plum line pattern virus, Andean potato mottle virus, Bean golden mosaic virus, Citrus leprosis virus, Citrus mosaic virus, Eggplant mosaic virus, Lettuce infectious yellows virus, Peach mosaic virus, Peach rosette mosaic virus, Potato black ringspot virus, Potato yellow dwarf virus, Raspberry leaf curl virus, Strawberry latent C virus, Tomato mottle virus* and *Watermelon silver mottle virus.*

The other approach of excluding viral pathogens is through certification of seeds and asexually propagated plant materials such as tubers, suckers, corms, setts and

budwood materials. The asexually propagated plant materials play a vital role in spreading the viral and viroid diseases to long distance and also to several subsequent generations especially in perennial fruit tree crops (Narayanasamy 2006). The imported plants and plant materials are checked carefully in post-entry quarantine (PEQ) by the plant quarantine personnel. Disease-free materials are released after testing the plants and plant materials using appropriate techniques. In order to check the spread of the viral and viroid pathogens within the country, the certification personnel inspect the seed lots and mother plants from which propagative materials are taken. Necessary tests are performed before certifying them for freedom from these pathogens. Various kinds of detection techniques based on the biological, immunological and genomic nucleic acid characteristics have been employed for detection and differentiation of viruses and viroids present in test materials. The relative usefulness and applicability for large scale use of the detection and identification methods have been indicated in Volume 3, Chapter 2 to enable the technical personnel to choose the ones that suit their requirements.

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Index

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