Discussion
Chapter 5

DISCUSSION

Potato (*Solanum tuberosum* L.) is world’s single most important tuber crop with a vital role in the global food system. It is the fourth major food crop after wheat maize and rice in the world. It is a wholesome food and can play a major role in eradicating hunger and malnutrition, which is one of the important millennium development goals of United Nations Organisation. India has special significance with respect to potato since it is grown under the sub-tropical conditions where crop duration is 80-90 days only. As such, potato produces more food and edible protein per unit area and time as compared to any other food crop. The high content of fiber, vitamins, minerals, carbohydrates and protein per hectare per day make potato one of the major world food crops, especially in developing countries (Woolfe, 1987; Horton and Sawyer, 1985).

Potato cultivated in different regions of the world is severely affected by a number of diseases incited by fungi, bacteria, viruses, nematodes and phytoplasmas which reduce the production in both quality and quantity through their adverse effects (Qamar and Khan, 2003). Potato is host to a large number of viruses. At least 37 viruses naturally infect cultivated potatoes (Beemster and de Bokx, 1987; Salazar 1996; Jeffries 1998). The viruses reported to infect potatoes in India include PVA, PVM, PVS, PVX, PVY, *potato leaf roll virus* (PLRV), *groundnut bud necrosis virus* (GBNV) and *tomato leaf curl New Delhi virus* (ToLCNDV) (Khurana and Garg, 2003). PVA, PVM, PVS and PVY are transmitted non-persistently by aphids, PLRV is persistently transmitted by aphids, GBNV which causes potato stem necrosis disease is persistently transmitted by thrips and ToLCNDV which causes potato apical leaf curl disease is transmitted persistently by the white fly *Bemisia tabaci*. PVA and PVX are very well known to cause severe economic losses particularly in combination with other viruses.

*Potato virus A* (PVA), a member of the genus *Potyvirus*, occurs worldwide, infects many potato cultivars, and can cause yield losses of about 40% (Bartels, 1971). It causes mild mosaic symptoms in most potato cultivars and in combination with PVX, it can cause a severe disease known as “potato crinkle” (Maclachlan *et al.*,
1954). But depending upon cultivar and virus strain, there are many cases without symptoms. Combination of PVY and PVA also results in severe mosaic symptoms. PVA is spread in the field by various species of aphids the most potent being *Myzus persicae*.

*Potato virus X* (PVX) was first described by Smith (1931). It, the type member of the genus *Potexvirus*, has flexuous rod-shaped virions and is the most common virus infecting potatoes (Schutz and Bonde, 1944). It is highly contagious, spreading upon cutting of tubers or mechanically and also upon plant-to-plant contact or through farm machinery or contaminated implements and even contaminated hands and clothes of farm workers. Combined infection with other viruses, particularly PVA and PVY causes severe diseases and higher yield losses.

In developing countries with tropical and sub-tropical climates, virus diseases are of major concern since weather favours the survival of both the virus hosts and their vectors round the year. Potato being a vegetatively propagated crop, once infected, carries the pathogen(s), particularly viruses, through subsequent generations thereby reducing tuber yield in each generation. Control of potato viruses is difficult because, unlike pathogenic microorganisms, viruses cannot be controlled through chemical means and are best managed through preventive measures. For potato industry to be viable in these countries, regular supply of healthy seed stocks in sufficient quantity and at reasonable rates is very essential. The first requirement for this is the availability of healthy (pathogen-free, particularly the viruses) nuclear seed stocks and for the selection of healthy nuclear seed stocks, availability of efficient, sensitive and reliable virus detection techniques is absolutely essential. The aim of the work presented in the thesis was to develop sensitive and reliable techniques for detection of PVA and PVX for their better management.

5.1 Development of molecular diagnostics for PVA and PVX

5.1.1 Confirmation of the presence of PVA and PVX virus culture

Cultures of PVA and PVX used in the study were those maintained at CPRI, Shimla. However, purity of the culture was reconfirmed with immuno electron microscopy which is one of the most authentic methods of virus identification (Garg *et al.*, 2003). Symptoms of PVA infection appeared after about 15 days of sap inoculation in *N. toboaccom* cv. Samsun. Leaf symptoms comprised mosaic, faint mottling and sometimes leaf distortion. Only mosaic was observed in potato cv. Green Mountain.
particularly under mild temperature conditions (15 to 25°C). The severity of symptoms depends on virus isolate, potato cultivar and environmental conditions (Dedic, 1975; Hooker, 1981). PVX symptoms appeared after about two weeks of sap inoculation in *N. glutinosa* and *D. stramonium*. Leaf symptoms comprised vein clearing followed by mosaic and mottling in *N. glutinosa*. Only mosaic was observed in *D. stramonium*.

### 5.1.2 Standardization of RNA extraction for PVA and PVX

High quality RNA in sufficient quantities is required for many important studies such as RT-PCR for conversion of RNA to cDNA for PCR detection of RNA viruses, cDNA library construction, labelling probes for gene chip, etc. RNA extraction from plants is influenced due to the presence of phenolic compounds and other secondary metabolites. Different researchers have tried RNA extraction from different type of plant tissues ranging from potato leaves, tubers and sprouts (Singh and Singh, 1995; Singh and Sing, 1998; Bright *et al.*, 2007; Peiman and Xie, 2006); from recalcitrant plant tissues like pelagonium and pine needles (Schultz *et al.*, 1994); from soil (Borneman and Triplett, 1997); tissues like fruits having a very high water content like watermelon (Davis *et al.*, 2006) and woody plants. Many plant species contain inhibitors which affect the extraction of nucleic acids (New berry and Possingham, 1979), and reverse transcription or PCR amplification, e.g. grapevine, banana and peanut (Rowhani *et al.*, 1993; Thomson and Dietzen, 1995). In other cases underground organs like gladioli corms, banana roots and corms and potato tubers have been shown to contain PCR inhibitors (Vunsh *et al.*, 1990; Thomson and Dietzen, 1995; Singh and Singh, 1996). In order to remove these inhibitor, the RNA extraction methods have been modified by using either the simple step of passing the extract through a Sephadex G-50 (Vunsh *et al.*, 1991) to multi-step method, involving sucrose, bovine serum albumin, polyvinyl pyrrolidone (PVP10), ascorbic acid, 2-mercaptoethanol, sodium dodecyl sulfate and potassium acetate (Rowhani *et al.*, 1993). For elimination of PCR inhibitors from potato tubers, the following methods have been tried (Singh and Singh, 1996): dilution of nucleic acid extracts, inclusion of 1% soluble PVP (PVP40) in the extraction buffer (John, 1992), precipitation of nucleic acid with equal volume of isopropanol (Hanni *et al.*, 1995), and the use of phosphate-buffered saline containing 0.5% Tween -20 (PBS-Tween) (Barbara *et al.*, 1995).
In present study the method for RNA extraction was standardized by using three different methods viz. kit (Qiagen), immunocapture and conventional. Four μl each of the extracted RNA was further used for RT-PCR amplification of PVA and PVX CP gene. In case of PVA; RNA extracted by Qiagen kit and immunocapture, almost equal amplifications were observed whereas only faint amplification was observed in case of conventional method. Maximum amplification was observed when immunocapture method was used. In case of PVX; RNA extracted by kit and immunocapture, almost equal amplifications were observed whereas only slightly less amplification was observed in case of conventional. Thus, all three methods were equally sensitive and any of them could be applied for detection of PVX. Immunocapture was effective in RNA template preparation both in PVA and PVX but require virus specific antisera. The immunocapture technique has been of particular to overcome enzyme inhibiting components such as polysaccharides and oxidizing compounds hampering PCR (Wetzel et al., 1992; Nolasco et al., 1993; Chevalier et al., 1995; Rowhani et al., 1995; Craig et al., 2004; Webster et al., 2004; Mulholland, 2005; Albrechtsen, 2006; Vincelli and Tisserat, 2008; Araby et al., 2009). Immunocapture is a highly sensitive technique that allows the detection of viruses especially in where virus titer is low.

5.1.3 Standardization of RT-PCR conditions for PVA and PVX CP gene amplification

The polymerase chain reaction (PCR), as detection method, is rapid, versatile, specific, and sensitive. PCR has been broadly used in plant pathology for the detection and diagnosis of such pathogens as viroids, viruses, bacteria, phytoplasma, fungi, and nematodes (Henson and French, 1993). RT-PCR is known to be at least 1000 times more powerful than ELISA in terms of detection sensitivity for potato viruses (Jeon et al., 1996). Large scale testing of both pre harvest and post harvest samples is possible through NASH and RT-PCR (Singh, 1999).

RT-PCR for the detection of potato viruses viz. PVY, PLRV, PVX, BWYV, ScYLV, PVA, PVS, PVM and PALCV has been reported by various workers (Shalaby et al., 2002; Suluja et al., 2005; Prill et al., 1988; Maia et al., 2000; Singh and Singh, 1997; Kaushal et al., 2007; Xianzhou et al., 2008 and Nagata et al., 2004). Although unquestionable in advantages, PCR is very expensive requiring costly equipment (thermocycler) and molecular biology grade consumables. In addition,
PCR-based techniques are prone to render false positives due to its extremely high sensitivity coupled with the ease of contamination by aerosols, hair, skin, gloves, contaminated reagents, commercial preparations of Taq DNA polymerase, or even autoclaved material containing target sequences (Dwyer et al., 1992; Henson and French, 1993). Moreover, only a limited number of samples can be tested in one run. Therefore it might not be a 'first choice' for large scale indexing. Nonetheless, high sensitivity and rapid response make PCR a convenient approach for testing 'mother' seed stocks/plants. Keeping this fact of high sensitivity, RT-PCR was standardised for cloning, molecular detection and sequence analysis of CP genes of PVA and PVX in the present study.

In present investigation, total RNA from infected leaf tissue of PVA and PVX was extracted by using commercial kit (RNeasy Plant Mini kit, Qiagen) and used for cDNA synthesis. Complete cDNA encoding coat protein gene was PCR amplified using forward and reverse primers of PVA and PVX CP genes. The amplification of coat protein gene of PVA was best observed in 25 μL reaction mixture comprising 0.8 μM of each primer, 150 μM dNTPs, 2.5 U Taq DNA polymerase, and 5 μL of cDNA. PCR cycles were optimized and comprised initial denaturation at 94°C for 5 min followed by 40 cycles each of 1 min at 94°C, 1 min at 45°C, and 1 min at 72°C and then final extension at 72°C for 10 min. The expected size of 1100 bp of the CP gene of PVA was obtained. RT-PCR has been used for detection of Potyvirus in general (Langeveld et al., 1991; Korschineck et al., 1991; Dekker et al., 1993; Colinet et al., 1994; Thomson & Dietzgen, 1995). An RT-PCR protocol was developed for detection of PVA using a primer pair from P1 gene of the PVA genome and in PVY using two 20-mer primers located in nuclear inclusion genes NIa and NIb (Singh and Singh, 1996, 1997). A simple and reliable procedure for sample preparation and reverse transcription-polymerase chain reaction (RT-PCR) for detection of PVA was reported by Cerovska et al. (1998) and Qunan et al. (2004).

Amplification of coat protein gene of PVX was best observed in 25 μL reaction mixture containing 0.4 μM of each primer, 100 μM dNTPs, 2.5 U Taq DNA polymerase, 1.0 mM MgCl₂ and 5 μL of cDNA. Amplification cycles comprised initial denaturation at 94°C for 5 min and subsequent 30 cycles each of 1 min at 92°C, 1 min at 57°C, 1 min at 72°C followed by final extension at 72°C for 10 min. The expected size of CP gene of 714 bp of PVX was observed. A rapid and sensitive assay
for the specific detection of plant viruses using reverse RT-PCR-ELISA was applied successfully for detection and characterization of an isolate of PVX from infected potato tissues collected in Egypt (Soliman, et al., 2000). Specific oligonucleotide primers designed to amplify a 360 bp from the coat protein gene of the PVX genome was used in RT-PCR assays. Vlugt et al. (2002) developed a method for the detection of viruses from the genus *Potexvirus.*

### 5.2 Cloning and sequencing of coat protein gene of PVA

PVA genome is composed of a messenger-polarity ssRNA of 9565 to 9572 nucleotides including a 3′-poly (A) tail and a virus-encoded genome-linked protein (VPg) covalently attached to the 5′-end of the viral RNA. HC-pro (helper component proteinase) is a multifunctional protein. Potyviral coat protein (CP), in addition to encapsidation of genomic RNA, performs at least two more functions. The conserved tripeptide DAG located at the N-terminus is a determinant of aphid transmission, and mutations of CP affect both the internal regions and the terminal regions resulting in significant inhibition of the virus cell to cell spread which suggest role of CP in virus transport function (Atreya et al., 1995, Dolja et al., 1995).

In the present investigation, PCR amplified product of coat protein gene of PVA was cloned into pDrive vector of size 3.85 kbp with T overhang. Transformation of *E. coli* DH5α strain with ligation mixture of PVA resulted in several white colonies on the IPTG/X-gal medium supplemented with ampicillin. White colonies were used for screening the recombinant clones of PVA. The confirmation of clones of PVA-CP gene was done by PCR amplification and restricting the plasmid with *EcoRI* restriction enzyme. An amplicon of 1100 bp was generated from recombinant plasmid during PCR. Restricted product resulted in two fragments of size 3.85 kbp and 1100 bp which confirmed the successful cloning of PVA CP gene. Sequencing of PVA revealed that the CP gene of Indian isolate of PVA consisted of 810 bp. The CP gene nucleotide sequence of PVA isolate (India) was compared to thirteen PVA isolates from Finland, one from Sweden and one from Estonia. The Indian isolate showed 87.2 to 99.0% homology with different isolates at nucleotide and amino acid levels.

The compiled molecular data on the potyviruses have shown that the coat protein gene and 3′-terminal region are the most useful regions of potyviral genomes in sequence variation analysis. These two regions are commonly used as markers of genetic relatedness of potyviruses (Frenkel et al., 1991; Shukla et al., 1994).
number of studies have been carried out to analyze the phylogenetic relationship between the strains of potyviruses based on the CP gene sequences. Complete CP amino acid residue sequences are available for more than 40 distinct potyviruses and they vary considerably in size (260-330 amino acids) because of their N termini. Some of the studies on the strainal variation among potyviruses based on their CP gene sequence variability are on PVY (Cerovska et al., 2001), peanut stripe virus (Mckern et al., 1991; Higgins et al., 1998), banana bract mosaic virus (Rodoni et al., 1999), zucchini yellow mosaic virus (Kundu et al., 1998; Desbiez et al., 2002), plum pox virus (Cervera et al., 1993), sugarcane mosaic virus (Frenkel et al., 1991), onion yellow dwarf virus (Kobayashi et al., 1996) and pepper vein mottling virus (Gorsane et al., 2001). Rajamaki et al. (1998) analysed coat protein and 3'-end non translated region (3’NTR ) nucleotide sequences of 13 isolates, and helper component proteinase (HC) of 9 isolates of PVA and compared them with the 8 previously determined PVA CP, 3’NTR and HC sequences. The sequence similarity was 92.9, 93.4 and 94.8% respectively. Kekarainen et al. (1999) compared the complete nucleotide and deduced amino acid sequences of CP gene of different isolates of PVA viz. Ali, U, Her (from potato, Solanum tuberosum) and tamarillo mosaic virus (TamMV from tamarillo, Solanum betacea) with the previously reported sequence of PVA isolate B11. Most parts of the polyprotein showed over 95% amino acid residues sequence similarity. The cylindrical inclusion (CI) protein and the 6K1 protein were the most conserved proteins among the five isolates. TamMV was the most different isolate. Sequence similarity between TamMV and the other isolates was the lowest in regions close to the 5'-end [5'-non-translated region (NTR) and P1 region] and 3'-end of the NTR (N-terminus of coat protein) of the genome. Quan et al. (2003, 2004) amplified 0.8 kb CP gene fragment of PVA by using specific primers designed for the fragment. The gene was cloned into E. coli DH5α and sequenced. By comparison with the sequence of CP gene of other isolates of PVA, it was found that it had high homology with other isolates, with the highest homology of nucleotides up to 99%. Based on CP amino acid sequence, the phylogenetic tree of PVA was introduced and the isolates were clustered into many groups.

5.3 Cloning and sequencing of coat protein gene of PVX
PVX genome has RNA of 6.0-6.4 kb which encodes five open reading frames (ORFs). ORFI encodes a 166 kDa protein thought to be an RNA-dependent RNA
polymerase (RdRp) (Forster et al., 1988, Huisman et al., 1988). The central overlapping ORFs (2, 3, and 4) are called a triple gene block (TGB), and the respective ORFs encode 25, 12, and 8 kDa polypeptides that have been suggested to be associated with cell-to-cell movement of the viral genome in infected plants. Proximal to the 3' terminal end, ORF5 encodes a 25 kDa viral coat protein. Like the CPs of other members of the potexvirus, it contains highly variable N-terminal segment which is not required for virion assembly. The coat protein of PVX is essential for TGB-encoded movement proteins potentiated cell to cell movement (Forster et al., 1992., Lough et al., 1998).

In the present studies, the coat protein gene of PVX was cloned into pDrive vector of size 3.85 kbp with T overhang. Transformation of E. coli DH5α strain with ligation mixture of PVX resulted in several white colonies on the IPTG/X-gal medium supplemented with ampicillin. White colonies were used for screening the recombinant clones of PVX. The confirmation of clones of PVX-CP gene was done by PCR amplification and restricting the plasmid with BamH1 and NotI restriction enzyme. The amplicon of 714 bp was observed in recombinant plasmid and restricted product resulted in two fragments of size 3.85 kbp and 714 bp which confirmed the successful cloning. Sequencing analysis revealed that the CP of Indian isolate of PVA consists of 714 bp. The CP gene nucleotide sequence of PVX isolate (India) was compared to twenty PVX isolates from different geographical regions. The Indian PVX isolate shared 79-99.0% similarity to different isolates at nucleotide and amino acid level.

Komatsu et al. (2005) compared the complete genomic RNA of four Japanese PVX isolates PVX-BS, -BH, -OG, -TO and the previously sequenced PVX ordinary strains. These strains differ in their pathogenicity in the wild potato (Solanum demissum) and tobacco (Nicotiana tobaccum cv. Samsun). The sequence similarity of genomic sequences of these five PVX strains at nucleotide level was 95.4-98.5%. Phylogenetic analysis indicated that the Japanese PVX strains originated from an ancestral PVX strain in the European group.

The complete nucleotide sequence of PVX_{HB} strain was studied by Querci in (1993). The genomic sequence of PVX_{HB} contains 6432 nucleotides, exactly the length reported for PVX_{CP} (Orman et al., 1990), whereas the RNAs of two European PVX strains, PVX_{H3} and PVX_{S}, both from resistance group 3 were 6435 nucleotide
long (Huisman et al.; 1988, Skryabin et al., 1988). Three nucleotides deletion in the CP at position 5732 to 5734, determining the replacement of threonine (T) and alanine (A) in PVX<sub>3</sub> and PVX<sub>S</sub> with asparagine (N) in PVX<sub>HB</sub> and PVX<sub>CP</sub>. The coat protein sequences of two South American strains, PVX<sub>HB</sub> and PVX<sub>CP</sub> contains 236 amino acids and was one residue shorter than those of PVX<sub>3</sub> and PVX<sub>S</sub> (237 amino acids). Computer analysis and translation of the sequences confirmed that general structure was conserved among all PVX strains. The sequence contains five open reading frames. Soliman et al. (2000) and Jung et al. (2000) reported partial nucleotide sequence analysis of Egyptian and Korean isolates of PVX CP gene. The CP gene was amplified using virus specific primers and cloned. The nucleotide sequence homology was 82-96% in Egyptian isolate and 88-99% in Korean isolate.

The complete genome sequence of a Japanese isolate PVX-OS, Korean isolate PVX-Kr and Chinese isolate PVX-FX21 has been determined (Kagiwada et al., 2000; Choi & Ryu, 2008; Yu et al., 2008). Total nucleotide sequences of the three isolates were 6,435 excluding the poly(A) tail and having five open reading frames similar to other reported PVX strains. Comparison of the nucleotide sequence of all the three with those of European and South American PVX strains revealed 95-96% homology with European strains and 77-78% with South American strains. Advances in the molecular biology of potexviruses have been intensively reviewed recently (Verchot Lubicz et al., 2007). Until now, the complete genomic sequence of 13 isolates has been determined (Huisman et al., 1988; Skryabin et al., 1988; Orman et al., 1990; Kavanagh et al., 1992; Malcuit et al., 2000; Kagiwada et al., 2002).

5.4 Comparison between conventional RT-PCR and IC-RT-PCR for PVA and PVX detection

Serological and molecular techniques differ not only in the target viral component to be detected but also in their specificity, sensitivity, and facility of automation. Recently, specificity of serological methods was combined with the sensitivity of the PCR technique in a single assay in which viral particles were initially antibody-captured and then their nucleic acid amplified by PCR (Jansen et al., 1990; Wetzel et al., 1992; Nolasco et al., 1993). This attractive technique, called immunocapture-PCR (IC-PCR) was 250 times more sensitive than conventional PCR. IC-PCR avoids using the level of RNA purification of the extract usually required to eliminate the interfering compounds that affect the PCR-based methods. Wetzel et al., 1991
compared RT-PCR and IC-RT-PCR for the detection of plum pox virus (PPV). An amplified fragment of expected size was detected after RT-PCR of infected sap diluted only up to 100 fold while IC-RT-PCR proved able to detect PPV sequences in the same sample even after a 10000 fold dilution, indicating an at least 100 fold higher sensitivity. A similar sensitivity was obtained using immunoglobulin-coated eppendorf tubes for the same virus (Jansen et al., 1990).

We have determined the detection of PVA and PVX in 10 fold dilution series of infected plant sap with RT-PCR and RT-IC-PCR. It was found that PVA and PVX could be detected in plant sap diluted 1000 fold in case of RT-PCR and 100,000 fold diluted sap in case of IC-RT-PCR. Hence detection of PVA and PVX in crude infected sap with IC-RT-PCR was 1000 folds more sensitive than that with conventional RT-PCR.

5.5 NASH based diagnostics
Molecular hybridisation as a diagnostic tool in plant virology was first used to detect viroids (Owens and Diener, 1981) and later applied to plant viruses (Maule et al., 1983; Garger et al., 1983; Salazar and Querci, 1992). Molecular hybridization, based on specific interaction between complementary purine and pyrimidine bases forming A=T and G=C base pairs, results in the formation of a stable hybrid between the target sequences and those of the probe. The stability of the hybrid depends on the number of hydrogen bonds formed and on both electrostatic and hydrophobic forces. Electrostatic forces rely on the phosphate molecules of the nucleic acid backbone whereas hydrophobic interactions are maintained between the staggered bases. The most common method for molecular hybridization, the dot-blot hybridization technique, involves the direct application of the target nucleic acid preparation solution to a solid support, such as nitrocellulose or nylon membranes, and subsequent detection with appropriate specific probes (Hull, 1993; Pallas et al., 1998).

Sensitivity for the PVA and PVX detection with NASH was determined. Various dilutions viz. 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256 and 1:512 of the PVA and PVX infected plant sap were prepared by mixing with the healthy potato sap and spotted onto nitrocellulose membrane. The blot was probed with $^{32}$P-labeled and DIG-labelled probes. The detection limit for PVA and PVX in radioactive $^{32}$P labelled probe was 1:512 and non-radioactive DIG labeled probe was up to 1:128 in both cases. Twenty one potato leaf samples suspected to be infected with PVA were tested
with both the techniques. Out of 21 samples, 16 tested positive for PVA with both the techniques; signal being stronger with radio-labelled probe. Twenty one potato leaf samples suspected to be infected with PVX were tested with both the techniques. Out of 21 samples, seventeen samples were found positive for PVX with both the techniques. NASH technique has been reported for the detection of viruses using radioactively labelled complementary DNA (cDNA) probes (Owens and Diener, 1981; Salazar et al., 1983; Baulcombe et al., 1984, Wetzel et al., 1990; Querci et al., 1993), as well as for potato spindle tuber viroid (PSTVd) in potato, using radioactively labelled RNA probes (Salazar et al., 1988). The use of radioactive probes has several disadvantages, such as short lifetime of radioactive probe (14.2 days for $^{32}$P), problems associated with storage and handling of radioactive waste due to potential health and environmental hazards. These difficulties can be overcome by using non-radioactive probes. Recently, several non-radioactive probes have been developed and put to practical use. RNA or DNA probes prepared with biotinylated nucleotides have sensitivity similar to radioactive probes. The results obtained in sensitivity, the advantage of using non-toxic materials and the possibility of long storage of biotinylated probes, greatly increased their application. The detection of viruses and viriod using biotinylated RNA and DNA probes for PVX and PVS in crude leaf extracts has been reported (Hopp et al., 1988; Roy et al., 1988, 1989; Eweida et al., 1989; 1990; Soliman et al., 2000). Non-radioactive NASH detection method was developed by Quan et al. (2004) using DIG labelled PVA CP gene as a probe. Other nonradioactive systems of nucleic acid labelling and detection, including luminescent DNA probes and luminography, were compared in the detection of purified PVX, PVY, PVS, and PSTVd by other researchers (Audy et al., 1991, Verma et al., 2006).

5.6 Serological diagnostics

Serological detection, also known as immuno chemical techniques, involves the use of antibodies (monoclonal or polyclonal) raised against specific antigens (Albrechtsen, 2006; Bos, 1999; Maroon-Lango, 2004). It was originally developed for the detection of viruses which, unlike bacteria and fungi, cannot be cultured (Schaad et al., 2003). Earlier reports of these methods date back to the 1970s. Accurate detection of plant viruses is essential for preventing their spread to new areas. Although enzymelinked immunosorbent assay (ELISA) is used routinely to detect
viruses and in resistance breeding programs, a major limitation appears to be the availability of high quality antisera. In order to produce antibodies to viral proteins by conventional methods, purification of the virus is required. It is often difficult to obtain purified preparations not contaminated with healthy plant proteins (Brunt and Lawson, 1995), so, polyclonal antisera often contain nonspecific antibodies which react with healthy plant extracts (Van Regenmortel, 1982). Additionally, elaborate facilities are required for virus purification (Brunt and Lawson, 1995). It has been generally described that the purification of filamentous viruses belonging to potyviruses are rather difficult because of their properties of aggregation and instability during the purification process (Choi et al., 1977; Damirdagh et al., 1970; Lister, 1971; Stace-Smith, 1970; Uyeda et al., 1975). Application of high molarity buffers as extracting solutions Triton X-100 (alkylenoxypolyethoxyethanol) for clarification of viruses and polyethylene glycol (PEG) for concentration of virus solved many serious problems in purification process (Choi et al., 1977; Tomlinson, 1996). Most of the procedures used for the purification of potyviruses involve differential centrifugation and differ in the process of clarification of the extracts. Fribourg, (1970) reported partial purification of potato virus A by differential centrifugation, employing three different buffer systems (borate, citrate, and phosphate). Potato virus A was purified from Nicandra physaloides by a simple method (Singh and McDonald, 1981) that omitted organic solvent clarification and consisted of differential centrifugation followed by equilibrium centrifugation in CsCl. Khurana et al., 1987; Singh, 1990 used New Zealand white rabbits (9-12 months) for production antiserum for potato virus Y. Rabbits were immunized by four intramuscular injections by emulsifying antigen with 1:1 Freund’s adjuvant. The rabbits were bled through marginal ear vein 10 days after the last injection.

In present studies, PVA was purified from infected leaves of Nicotiana tabaccum cv. Samsun as per the procedure described by (Singh and McDonald, 1981). Virus concentration was 7 mg/mL and total yield of the virus was 21 mg. Purity of the virus was checked with Transmission electron microscope (TEM). Long flexuous rods, typical for a potyvirus, without any host debris were observed. Rabbits were immunized by using 2 mg/mL of the virus. The antiserum titre was checked with microprecipitin test and was found to be 1:512 ELISA kit was standardized by checker board with the dilutions 1:200, 1:500 and 1:1000 both for IgG and EC.
Infected and healthy plant sap dilution used was 1:10 and 1:50. The optimal dilution was found to be 1:1000 both for IgG and EC.

5.6.1 Enzyme-linked Immunosorbent Assay (ELISA)
Clark and Adams (1977) were the pioneers of ELISA. Since its introduction, ELISA has been recognized as the most widely used serological method in plant virology (Albrechtsen, 2006; Bos, 1999; Schaad et al., 2003; Webster et al., 2004). High sensitivity, ease of use, speed, cost effectiveness and the ability to quantify pathogen are the different characteristics that contributed to the successful use of the technique (Clark, 1981; Maroon-Lango, 2004; Miller and Martin, 1988; Webster et al., 2004). ELISAs, in the form of multiwell ELISA, dot-blot ELISA and tissue print ELISA, have been developed and used for diagnosis of viruses in the cucumo, luteo, potex, poty, and tospovirus groups. ELISA have been used for diagnosis of viruses on large scale especially in vegetatively propagated crops such as tuber, bulbs and perennial fruit trees (Hansen and Wick, 1993) and to detect viruses in insect vectors and seeds (Martin, 1998). Dot ELISA have been used to detect PVS, PVX and PVY (Banttari and Goodwin, 1985) and PLRV (Smith and Banttari, 1987). DIBA has great potential for mass screening (Singh et al., 1993) with the introduction of monoclonal antibodies (Mabs), serological differentiation of strains improved greatly (Fernandez Northcote and Gugarli, 1987; Ellis et al., 1996). Potyviruses and Potexviruses can be easily detected in leaves and tubers through various forms of ELISA of which DAS-ELISA (Gugerli, 1979; Gugerli and Fries. 1983; Singh and Somerville, 1992), dot-ELISA (Weidemann, 1988) and direct tissue blotting (Samson et al., 1993) are commonly used.

In present studies, DAS-ELISA was performed for the detection of PVA and PVX and results compared with those of IEM. Forty samples of Kufri Chipsona-III were taken for detection. ELISA kit used for PVX was already developed by CPRI. Out of 40 samples tested with DAS-ELISA, 24 were found positive for PVA and 29 were found positive for PVX.

5.7 Validation of the RT-PCR protocol developed for the detection of PVA and PVX infected leaves
Four lots comprising 250 potato leaf samples received from field were tested with DAS-ELISA for various potato viruses including PVA. Nine samples tested positive for PVA and these were cross-checked with RT-PCR and immunoelectron
microscopy. Interestingly, only five tested positive to PVA both in RT-PCR and IEM. These studies highlighted the sensitivity of RT-PCR to be as high as IEM and there are chances of false positives in ELISA. Seven samples tested positive for PVX with DAS-ELISA. They were cross-checked with RT-PCR and IEM. Out of these 7 samples, five tested positive in both RT-PCR and IEM and the remaining two negative. These studies further confirmed that RT-PCR is as reliable as IEM and chances of false positives remain in ELISA. Further, 8 mericlones were tested through RT-PCR and IC-RT-PCR for the detection of PVA. Out of the eight samples, 5 tested positive to PVA in RT-PCR but 6 tested positive to PVA in IC-RT-PCR. IC-RT-PCR is thus a more sensitive technique. It allows the detection of viruses especially where virus titer is low, or where PCR inhibiting compounds are present (Wetzel et al., 1992; Nolasco et al., 1993; Rowhani et al., 1995; Craig et al., 2004). Same eight samples were also tested for PVX by both the techniques. Only two samples tested positive to PVX with both RT-PCR and IC-RT-PCR. No amplification was observed in healthy control.