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DIFFERENT TECHNIQUES FOR DIAGNOSTIC OF POTATO VIRUSES: A BRIEF REVIEW

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ABSTRACT

Potato is ranked fourth among the food crops and fifth for human consumption. It provides more yield as compared to cereals and gives more calories. Fungal, viral, thyroid, bacteria, nematode, phytoplasmas and abiotic factors play a pivotal role in yield reduction of potato crop. 38 different potato viruses naturally infect potato crops and PVA, PVM, PVS, PVX, PVY, PLRV and PMTV are reported in three consecutive potato crop of Pakistan. Increasing incidence of PVX and PVY in main potato growing areas of is getting an alarming position and PLRV has caused significant yield losses. The present review article demonstrates different techniques for identification and detection of these viruses..

Key word: Potato, PMTV, PLRV, PVY, PVX, PVX, PVM, PVA.

INTRODUCTION

The cultivated potato (*Solanum tuberosum* L) is the world's leading staple food and vegetable crop and ranked fourth in production after rice, wheat and maize (Mumford *et al.*, 2000; Rauscher *et al.*, 2006). It provides 15 times more yield and calorie production per unit area is higher than cereals (McGillivray, 1953). It has a potential to serve as a valuable food for the ever increasing world population as it contains higher dry matter, higher protein production and its adaptability to a wide range of climates (Jamal *et al.*, 2012). It also provides proteins, minerals, carbohydrates, vitamins (B & C) and high quality dietary fibers (Swaminathan, 1962). Potato crop was introduced in sub-continent in the early 17th century by the British as a short duration crop. More than three dozen plant viruses, a viroid and phytoplasmas, fungal, bacterial and nematode can infect the potato crop (Jeffries, 1998). A lower incidence (5-10%) of the viruses (singly or combined) infections in the current or previous crop hardly reduces crop yield (Khurana, 1998). A higher virus incidence coupled with early or severe infection causes serious depressions in the tuber yield (Garg, 1987). Accurate estimates about the economic losses caused by viruses are lacking in potato crop. Viruses are obligate parasites utilizing plant metabolites and the cell biosynthetic processes to support their propagation in host tissues (Antignus, 2000). At least 38 different plant viruses have been reported to naturally infect the potato crop. Among these, Potato Virus A (PVA), Potato Virus B (PVB), Potato Virus C (PVC), Potato Virus D (PVD), Potato Virus E (PVE), Potato Virus F (PVF), Potato Virus G (PVG), Potato Virus K (PVK), Potato Leaf Roll Virus (PLRV), Potato Virus M (PVM), Potato Mot Top Virus (PMTV), Potato Virus S (PVS), Potato Virus X (PVX) and Potato Virus Y (PVY) are very significant and widespread (Singh *et al.*, 2008). Sixteen viruses have been found only in Latin America and most of them from the Andes. Fourteen viruses are important only in some geographical areas or have been found only occasionally in potato crop (Abba, 2011). It was rough estimated that potato viruses may cause yield losses up to 50% in tuber yield (Sharma, 2008). In Pakistan, climatic and soil conditions are highly conducive for autumn, spring and summer potato crops(Zanoni, 1991). Bacteria (Anwar *et al.*, 2013), nematode (Gondal *et al.*, 2012; Parveen *et al.*, 2013), fungus (Ashraf *et al.*, 2012; Mehboob *et al.*, 2013) and virus (Abbas *et al.*, 2012; Abbas *et al.*, 2013; Abbas *et al.*, 2014) along with a biotic factor are the main problems in the country. Among viral diseases, PVX, PVY, PLRV, PVA, PVM, PVS and PMTV have been observed and caused up to 83% yield loss in potato crop (Mughal and Khalid, 1985; Abbas *et al.*, 2012). PVX, PVS, PVA, PVM and PVY are more common viruses, while PLRV is prevalent in northern areas where insect vector pressure is high.

Enzyme linked Immunosorbent Assay (ELISA) has been extensively used in plant diagnostic laboratories of due to its automation, easy in use, sensitivity and a large number of samples can be tested (Abbas *et al.*, 2012). Significant nucleic acid research has revolutionized molecular diagnostics of plant viruses and molecular hybridization moreover PCR played significant role in plant virology (Miller and Martin, 1988). The combination of serology and PCR (Immunocapture-PCR) became more sensitive and economical than conventional PCR because viral RNA purification was eliminated in this technique. Direct binding PCR (DB-PCR) and print-capture PCR (PC-PCR) are also important in the detection of potato viruses (Rowhani *et al.*, 1995). Complementary DNA (cDNA) libraries of coat protein gene have been prepared by using the genomic RNAs of PLRV, PVX and PVY by coat protein (CP) gene specific sense and antisense primers. Jamal *et al.* (2012) has cloned and sequenced CP gene (613 bp) of PVX from

Pakistani isolate (HE577130) and nucleotide evidence showed its maximum genetic similarity with USSR isolates of PVX while this was the first report of PVX from Pakistan. (Abbas *et al.*, 2014) reported recombinant strain of PVY on the basis of biological, serological and nucleotide evidence of the CP gene from two Pakistani isolates (JQ425622, JQ518266 and JQ518267).

Identification and Detection of Viruses in Potato: Bacterial, fungal and nematode disease can be effectively controlled by chemical methods, but no chemicals exist that could be used as a direct field control of viral disease in potato crop. Sensitive and reliable confirmation of virus in seed stock and eradication of viral infected seed tubers is more effective, efficient and reliable method to prevent the viral infection in natural condition (Pallás *et al.*, 1998). Nucleic acid of plant virus is surrounded by a protective protein coat and detection methods were evolved from these two components. Previously, plant viruses were detected on the bases of the protein component through serological methods (ELISA) because these methods were found effective at early stage of detection (Clark and Adams, 1977). The protective protein coat contains only 2-6% of the genetic information of viral genome so these methods are not effective for complete genome information (Pallás *et al.*, 1998). Viroids are not enveloped in protective protein coat, thus serological techniques cannot be applied for viroids confirmation. Viroids can be detected from bioassays and reverse gel electrophoresis technique, but these methods are not suitable for a large number of samples. Nucleic acid base research and application of recombinant DNA technology allow researcher to diagnose plant virus and viroids more effectively. Molecular hybridization along with polymerase chain reaction (PCR) assay is also being used in scientific laboratories of plant virology (Mumford *et al.*, 2000).

Susceptible Indicator Host Plants: Symptom expression is an initial step to disease diagnosis, but it is not a reliable criterion for confirmation of the virus (Abbas *et al.*, 2012). Symptom interpretation should be treated with caution because many factors are involved in symptom development, such as growth stage, nutrition deficiency, insect sucking, environmental conditions, host genotype, time of infection, virus strain, etc (Batool *et al.*, 2011). Although symptom development on a range of susceptibilities indicator host plants is a considerable diagnostic value, but it is insufficient for identification of viruses. Symptom development study of host range has significant value in studying the new, unknown virus or its strains because these host plants produced characteristic symptoms in response of a particular virus. A large number of herbaceous plants are reported as susceptible indicator hosts to several viruses and produced systemic infection or local lesions on mechanical transmission. *Chenopodium amaranticolor, C. annuum, Physalis floridana, C. album, Nicotiana glutinosa, N. rustica, N. tabacum cv. Samsun, N. benthamiana, Datura stramonium, D. metel, D. stramonium, Solanum demissum x,* S. *tuberosum Gomphrena globosa* and *Lycopersicon esculentum* were used as a test plant for potato viruses (Abbas *et al.*, 2012). Indicator plants rose in glass houses for experimental purposes, but it is time consuming to test a large number of samples. The main drawback of this diagnostic method is that these host plants exhibit different symptoms in response of same virus strains (Khurana, 2004).

Serology: Antibodies produced by a hybrid cell (hybridoma) formed by fusion of a B lymphocyte cell with a mouse myeloma cell are known as monoclonal antibodies (Mabs) and they were produced first time against Tobacco mosaic virus (TMV). Mabs have been successfully prepared against more than 50 different plant viruses, including PLRV, PVA, PVM, PVS, PVX and PVY (Mumford *et al.*, 2000). Old immunological test procedures included chloroplast agglutination, micro-precipitation tests and gel immunodiffusion (Hampton *et al.*, 1990). In order to increase the sensitivity of serological tests, the use of solid phase to adsorb an antibody or antigen with the subsequent attachment of an antigen or antibody and the use of an antibody-enzymes conjugate to detect the antigen (ELISA) has become an important procedure in plant virus detection. Clark and Adams (1977) developed an ELISA technique for successful detection of plant viruses and this technique became more popular within a short period of time due to its simplicity, adaptability, rapidity, sensitivity and accuracy. The double antibody sandwich (DAS-ELISA) test on a solid phase (usually plastic) has been used most commonly (Gul *et al.*, 2013). Specific antibody was used for trapping the plant viruses and a specific enzyme-labeled antibody (conjugate) is being added (Abbas *et al.*, 2012). At the end, enzyme substrate was added and the reaction of viruses was observed visually (development of color) or spectrophotometrically (Abbas and Hameed, 2012). A variation of the above method is the indirect ELISA, in which plates are coated with antigen, and the primary antiviral antibody of one animal species (e.g. rabbit) is added. Commercial kits for PVA, PVM, PVS, PVX, PVY, PLRV and other viruses are available and showed valuable results when potato leaves are tested (Abbas *et al.*, 2012). Positive, healthy and buffer or blank controls should be added in ELISA for more reliable results (Mughal and Khalid, 1985). In the certification laboratories of Swiss, ELISA is used for testing more than 20.000 seed potato tubers per day against PVY and PLRV (Torrance and Robinson, 1989). Thousands of potato plant samples are tested for potato virus (PVA, PVM, PVS, PVX, PVY, PLRV) with ELISA in Pakistan at Crop Disease Research Institute (CDRI) Islamabad under the National Agricultural Research Center. The minimum level of virus detection by ELISA is about 2ng/ml and these techniques are unable to detect the virus at the initial stage of infection (low titer). This is one of the main reasons that serologically negative samples produced symptoms at the lateral stage of infection (Abbas *et al.*, 2013). Low titer (theoretically one viron) will multiply (billions of copies) within a short period (few days) and healthy potato plants can be infected in the open field by mechanical inoculation along with the insect vector through viral infected potato

plants (Betancourt *et al.*, 2008).

In Dot-ELISA, antibodies or antigens bound to nitrocellulose or nylon membranes have been used to detect PVX, PVS, and PVY and PLRV but this method is readily adaptable to field samples (Smith and Banttari, 1987). Suspected potato sample's crude saps were spotted onto a membrane and allow it to dry at room temperature. Bovine serum albumin (BSA) is used for saturation the surface of the membrane and primary specific viral antibody are introduced on the surface. Secondary antibody-enzyme conjugate and substrate are added and the reaction was observed from an insoluble colored product at reaction sire. Tissue blotting is also a serological technique which has more resemblance with ELISA (Lin *et al.*, 1990) and this method is more reliable for the confirmation of PVX and PVY from tubers in the field (Bravo-Almonacid *et al.*, 1992). Blots are made by pressing the freshly cut tissue surface gently but firmly on a nitrocellulose membrane and antigens in tissue blots are detected by enzyme-labeled probes. This technique has much more important because it provides specific, rapid and simple tool for large-scale diagnosis of plant viruses.

Immunosorbent Electron Microscopy (ISEM): It is a highly sensitive technique which was developed by combining the electron microscopy and serology and it was introduced by Debrick (1973) for the detection of plant viruses. ISEM is more sensitive than ELISA for some viruses (Gars and Khurana, 1991) and a thousand times more sensitive than conventional electron microscopy (Garg *et al.,* 1989). Electron microscopy (EM) is used for the detection of size and shape of a particular virus and these basic steps play a pivotal role towards virus identification. EM also provides information on virus morphology to be obtained within minutes after sampling a diseased plant and it is used to examine viruses in crude extracts of infected plants (Gera and Marco, 2001). Among potato viruses, PLRV is phloem-restricted with low titer and create hindrance in detection with conventional electron microscopic and ELISA.

Polymerase Chain Reaction Technology: A detection procedure of virus in infected samples must be sensitive, specific, rapid, easy to use, reliable and cost-effective. PCR offers sensitivity and specificity to detect plant viruses and its strains and PCR has already revolutionized research in molecular biology (Mughal and Khalid, 1985). New applications of PCR are being published at an increasing rate and it is evident that it is being used in many fields of basic and applied research (Nosheen *et al.*, 2013). RT-PCR (reverse transcriptase-PCR) methods are more popularized now a days and the main advantage of these methods is that viruses can be detected from total RNA using sap from dormant potato tubers (Singh *et al.*, 2008). Billions of nucleic acid copies were achieved through multiple cycles of three steps at different temperature. These steps include, denature the DNA, anneal two oligonucleotide primers to the denatured (opened) DNA strands and primer extension by thermostable DNA polymerase to synthesize the target sequence whose ends is defined by the primers (Nasir *et al.*, 2010). The PCR products can be visualized through gel electrophoresis analysis (Awan *et al.*, 2010). PCR requires DNA fragment for amplification, but the important plant virus exhibiting RNA genome. RNA was converted to complementary DNA (cDNA) through reverse transcription (RT) and RT-PCR technology is 1000 times more sensitive than ELISA in term of detection sensitivity for potato viruses (Jeon *et al.*, 1996). The success of PCR depends upon specific sense and antisense primers for the amplification of the viral genome. The nucleotide sequence of the viral genome is available in GenBank (NCBI). Oligonucleotide primers must be 18-25 nucleotide residues in length, with no annealing 3 end, 50 % G+C content, no secondary structures and high G+C content at the 3' ends. Primer can also be designed for conserved or variable regions (Abbas and Hameed, 2012). Specificity of PCR is largely affected by annealing temperature and successful amplification depends upon G+C content and primer length. Primers having 20 nucleotides require increase of 2°C for every addition of A or T and 4°C for G or C. RT-PCR was applied in detecting PVY, PLRV, PVX, PVA, PVS and PVM (Nie *et al.*, 2008).

PCR techniques are prone to render false positives due to its extreme sensitivity coupled with the ease of contamination by contaminated reagents, gloves, hair, skin, aerosols, commercial preparations of *Taq DNA* polymerase, or even autoclaved material containing target sequences (Dwyer and Saksena, 1992). Only a limited number of samples can be tested in one run and it is the main reason that this technique is limited to scientific labs rather than plant diagnostic labs. Rapid response and high sensitivity enable PCR a more reliable method for testing the limited number of 'mother' seed stocks/plants (Arif *et al.*, 2014). RT-PCR was optimized for Alfalfa mosaic virus (AMV), Impatiens necrotic spot virus (INSV), Tobacco rattle virus (TRV), Tomato spotted wilt virus (TSWV), PLRV, PMTV, PVA, PVM, PVS, PVX, PVY and Potato spindle tuber viroid (PSTVd). The ordinary (PVY^{O}), Common (PVY^{C}), necrotic (PVY^{N}) and recombinant (PVY^{NTN}, $PVY^{N:O}$ and $PVY^{N\text{-}wi}$) strains of PVY could be differentiated using multiplex primers (Crosslin and Hamlin, 2011).

Immunocapture-PCR: Potato viruses consist of two components (protective protein coat and nucleic acid) and molecular and serological tools differ in the target viral component, sensitivity, specificity and facility of automation. The serological and molecular tools were combined in a single technique termed as Immunocapture-PCR (IC-PCR) in which virus particle was captured (protective protein coat) and then was amplified (nucleic acid) with PCR (Nolasco *et al.*, 1993). This method removed the virus purification step which is necessary to eliminate the interfering plant cell components that affect the PCR-based methods. Immunocapture-PCR is 250 times more sensitive than normal PCR. This technique was successfully applied to the PLRV detection (Leone *et al.*, 1997).

Molecular Hybridization: This technique is used for confirmation of viroids and viruses (Salazar *et al.*, 1992).

These methods are based on interaction between purines (A=T) and pyrimidine (G≡C) and results in the formation of a stable hybrid between the target sequences and those of the probe. These hybrids depend on the number of hydrogen bonds, electrostatic and hydrophobic forces (Salazar *et al.*, 1992). Electrostatic forces rely on the phosphate molecules of the nucleic acid backbone whereas hydrophobic interactions are maintained between the staggered basis. The dot-blot hybridization is the most common technique which involves the target nucleic acid and appropriate specific probes (Pallás *et al.*, 1998). Nonradioactive riboprobes were used for successful confirmation for PVS, PVX (Eweida *et al.*, 1990), PVY and PLRV (Hopp *et al.*, 1988). No universal protocols depend available for molecular hybridization analysis and the choice of protocol on the virus, host and probe (Pallás *et al.*, 1998).

Genetic Diversity: It has numerous advantages, including the availability of large numbers of markers that cover the entire genome and their expression are not affected by the environment (Gepts, 1993). Genetic diversity in sorghum has been estimated using several types of molecular markers such as, RFLP (Aldrich and Doebley, 1992; Deu *et al.*, 2006), RAPD (Vierling *et al.*, 1994; Uptmoor *et al.*, 2003), AFLP (Uptmoor *et al.*, 2003; Menz *et al.*, 2004) and SSR (Brown *et al.*, 1996; Menz *et al.*, 2004; Casa *et al.*, 2005). In each of these studies, the authors studied a precise subset of sorghum germplasm.

Microsatellites or SSR markers are particularly attractive for studying genetic differentiation because they are co-dominant (Akkaya *et al.*, 1992) and abundant in the genome (Lagercrantz *et al.*, 1993). However, there are some limitations, previous genetic information is needed, huge upfront work required, problems associated with PCR (Kubik *et al.*, 2001).

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