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Techniques for Detection and Diagnosis of Plant Viruses: A Review

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ABSTRACT

Plant viruses are famed for serious economic losses to most of the agricultural and horticultural crops all around the world. The losses caused by plant viruses are estimated to be several billion bucks per year and are therefore a major threat to global crop production and food security. Traditional diagnostic methods based on biological and physical properties of virus such as appearance of symptoms, bioassay on indicator plants, vector borne and virus particle morphology by electron microscopy are cumbersome, time-consuming and expensive. Recognition of disease based on the symptoms indicated by virus affected plants, can invariably be the first process in several cases, however it's confusing additionally as a result of the symptoms are changeable and depends on several factors like abiotic conditions. There are several sensitive and effective diagnostic methods developed during the last four decades based on virus protein *e.g.*, immunosorbent electron microscopy (ISEM), enzyme-linked immunosorbent assay (ELISA) and nucleic acid *e.g.*, nucleic acid hybridization, isothermal and thermostable PCRs, for plant virus detection, each having its own advantage and disadvantage. This review provides an update on the progress made on the techniques that are useful for early, sensitive, accurate and reliable detection and diagnosis of plant viruses.

Key words: Assay, Diagnostic methods, Nucleic acid, PCR, Plant virus.

The term virus diagnosis and virus detection appear to be synonyms but diagnosis means the distinctive characterization in precise terms of a genus, species, or phenomenon. It is also the examination of the signs or symptoms, assessment of the research or test results and also the investigation of the assumed or probable causes. Effective cure is not possible without effective diagnosis. However, meaning of detection is to find out the virus. Accurate diagnosis is a prerequisite for the development of satisfactory measures to control a virus disease. Transfer of viruses, their hosts and vectors across borders has been knowingly or unknowingly facilitated by globalization of trade by free trade agreement (FTA) and the rapid climate change.

Yield losses of cultivated crops have been estimated to be 20%-40% due to biotic stresses Savary *et al*. (2012). Diseases are induced in crops mainly during their growth period, harvesting and post-harvesting time period. These damages can be decreased by applying detection and diagnosis techniques. To manage the crop loss by plant pathogen, diagnosis plays the key role (van der Want and Dijkstra, 2006). This is the very first step in the crop management system Aboul Ata *et al*. (2011).

Like other pathogens, plant viruses cause a huge loss to economy (Agrios, 2005; Strange, 2005). Viruses hold second rank after fungi in the list of most important plant pathogens. The damage caused by viruses depends on virus strain, region, host plant variety and also the time of infection so it is very difficult to predict and assess the actual loss (Strange, 2005). Major symptoms of viral diseases include stunting, mosaic, streaking, necrosis, mottling, wilting, leaf curling and distortion *etc*. Sometimes viruses may not produce any visible symptoms as many virus infections are latent and symptomless. In addition to this, when plants respond to un-favourable weather, nutritional imbalances, damage caused by abiotic factors and other pathogenic infections, viral symptoms may not be readily observed (van der Want and Dijkstra, 2006). It is very difficult to diagnose the viral disease only by studying the symptoms as compare to other pathogens (Lievens *et al*. 2005).

The plant viruses are very small as compared to fungi and bacteria and generally cannot be observed under light microscope. Transmission electron microscope (TEM) is used to observe them. Viruses are composed of some coat proteins and either DNA or RNA as genetic material. *Tobacco mosaic virus* (TMV) was the very first virus that was recognized over a century ago, after that 1000s of plant viruses came into know (King, 2011).

The management of viral diseases based on direct methods for their control, such as the use of virucides, has not been developed to date unlike other pathogens. Therefore, viral diseases can be fought with indirect

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strategies, such as killing insect vector, eliminating diseased plants, or avoiding the planting of infected seeds. Because of this, the methods to detect and identify viruses are crucial for handling viral diseases. So the detection methods must be convenient, effective, specific and quick as possible Joo-Jin *et al*. (2014). The methods for detecting and identifying viruses are challenging in viral disease management Aboul Ata *et al*. (2011).

Previously the research methods were usually based on symptom advancement infected plants or biological indexing. However the diagnosis based on symptom is not well-founded because symptoms may vary depending on the virus strain, presence of any mixed viral infections, the variety and growth stage, growing climate and sometimes, the viral symptoms are similar to those induced by the environmental injury. However the biological methods are widely used assays among other diagnostic techniques for plant viruses. It is because of their simplicity and they do not require special knowledge or mastership (Jones, 1993; Naidua and Hughes, 2001).

To detect plant viruses enough methods have been developed, such as microscopically observation, serological techniques and molecular methods (Webster *et al*., 2004; Makkouk and Kumari, 2006, Lopez *et al*., 2009). The methods of assay, detection and diagnosis were divided into four groups, which depends upon the virus properties *i.e*. biological activities of virus particle, physical properties of the virus particle, properties of viral proteins and properties of the viral nucleic acid (Koenig and Lesemann, 2008). Among them a number of methods for the diagnosis of plant viral diseases are briefly introduced in this review.

Methods based on biological activities

The biological methods of diagnosis are very time consuming than other applicable methods even though, they are still useful because infective analysis can give us ideas of the application of viable virus particle. Even in most cases only inoculation into an appropriate host can resolve that the virus can cause mild disease or severe disease. Although this group have many limitations also, specifically when used in new viruses. Here we are summarizing some of the biological methods used for plant virus diagnosis.

Indicator host

Symptoms of disease on plants in the field are not sufficient to give positive identifications of their own. Now-a-days in plant virology searches are there for the suitable species of the host plant that can give the clear-cut identification and characteristics for the virus and virus being studied. These plants acts as indicator host and provide basic tool for the virus diagnosis. There are very good indicator host varieties found in genera *Nicotiana*, *Solanum*, *Chenopodium*, *Cucumis*, *Phaseolus*, *Vicia* and *Brassica*. Approximately 200 species were tested and accession of tobacco to find the useful indicator plants van Dijk *et al*. (1987). These methods are important for epidemiological studies.

Host range

In earlier research on plant viruses, host range was the important criteria in diagnosis. This method still has importance because of its information used in certain circumstances. Sometimes host range is more important than other tests. R*aspberry bushy dwarf virus (*RBDV) is a very good example of this method (Murant *et al*. 1986). Ali *et al*. (2009) mechanically inoculated the extracts from *Tobacco steak virus* (TSV) infected bean leaves on to *Nicotiana benthamiana* and *Chenopodium quinoa* which found an appropriate host for TSV.

Cytological studies

Cytological effects detectable by light microscopy can sometimes be used to supplement macroscopic symptoms in diagnosis. Researchers (Christie and Edwardson, 1986) provided an elaborated catalog of virus-induced inclusions and dis-cussed the problems involved and also Edwardson *et al*. (1993) reviewed this approach for the diagnosis of viruses.

However electron microscopy of thin sections is necessary for some types of inclusion to provide informa-tion for use in diagnosis. There were nine virus groups that induce inclusions which are diagnostic for the group (Hamilton *et al*. 1981). Presence of characteristic inclusions may be diagnostic for a particular virus when a specific host plant is involved, for example, the *Citrus tristeza virus* (CTV) in citrus trees (Brlansky, 1987). Individual strains of a virus may produce distinctive cytological effects, as occurs with *Cauliflower mosaic virus* (CaMV); Shalla *et al*. (1980).

Methods based on physical properties of the virus

Density of virus, dilution end point and electrophoretic mobility are the physical properties of a virus which were taken to be a measure of infectivity of the virus in sap extract was previously used to detect plant viruses. Earlier the viruses within a group, for the *Cucumovirus* group and for *Pea enation mosaic virus* (PEMV) were distinguished through electrophoretic mobility (Hanada, 1984; Hull, 1977). However, these properties are unreliable and no longer used to detect the virus.

Methods based on properties of viral protein

There are some advanced serological techniques which made the diagnosis process easier and more sensitive with low cost and also target at viral proteins. These serological methods of diagnosis, detection and identification of viruses in plants play a vital role so here we are explaining some of them.

Enzyme Linked Immunosorbent assay (ELISA)

ELISA has greatly improved the accuracy of plant virus detection (Clark and Adams, 1977). This technique was used first time during 1970s and is still the most widely used serological technique due to its high efficiency. Very minute amount of antibody is required for the detection of disease and the process can be semi-automated. Common ELISAs

are performed in polystyrene plate capable of binding antibodies or proteins with association of the enzyme-substrate reaction. Level of infection is measured based on the optical density of ELISA reaction Webster *et al*. (2004). Lots of viruses has been detected using this technique including *Cucumber mosaic virus* (CMV), CTV, *Potato leaf roll virus* (PLRV), *Potatovirus* × (PV×) and *Potato virus Y* (PVY) (Sun *et al*., 2001; El-Araby *et al*., 2009).

Tissue blot immunobinding assay (TBIA)

TBIA is another serological method for the diagnosis of plant viruses. Lin was first who described the assay and performed for the useful detection of virus over whole plant (La *et al*., 1999, Lin *et al*., 1990, Abad and Moyer, 1992). In this technique the freshly cut tissue surface on nitrocellulose membranes and then the tissue blots were made by pressing with a gentle force. Present antigen was then detected by enzyme labeled immunological probes (Fegla *et al*. 2001). The major advantage of this test was the elimination of sap extraction, which is the most time consuming step in all previous techniques. In addition, once the plant tissue is blotted on the nitrocellulose membrane, the test can be completed either few days or few months later. A number of viral diseases are diagnosed by this technique which were caused by *Bamboo mosaic virus* (BoMV), *Bean yellow mosaic virus* (BYMV), CTV, *Cymbidium mosaic virus* (CyMV), *Papaya ringspot virus* (PRSV), *Sweet potato feathery mottle virus* (SPFMV) and *Tomato spotted wilt virus* (TSWV) (Bove *et al*., 1988, Eid *et al*., 2008, Hancevic *et al*., 2012, Lin *et al*., 1990, Makkouk and Kumari, 2006, Shang *et al*., 2011, Webster *et al*., 2004).

Quartz crystal microbalance immunosensors (QCMI)

The QCMI measures the mass based on vibrations and frequency change in real time and is a noveltechnique for plant virus detection. The detection instrument for QCM is portable and QCM coated with virus-specific antibodies which detect plant viruses has long life span, hence can be used for *in situ* detection of plant viruses (Eun *et al*., 2002, Becker and Cooper, 2011). This technique was reported successful for the detection of CyMV, TMV and *Turnip yellow mosaic virus* (TYMV) (Eun *et al*., 2002; Becker and cooper, 2011, Dickert *et al*., 2004; Zan *et al*., 2012).

Methods based on viral nucleic acid

The properties of a viral nucleic acid, such as whether it is DNA or RNA, double stranded or single stranded, or consists of one or more pieces, are main points for assigning an unknown virus to a particular family or group. Diagnosis based on viral nucleic acids is more sensitive and specific than serological and any other methods. Here we are summarizing some frequently and recently used methods.

Polymerase chain reaction (PCR)

PCR method was developed in 1990 for virus detection and offered the user exquisite levels of specificity and sensitivity (Vunsh, 1990). PCR is processed by the specificity of the primers proceeded with three steps, denaturation at 94°C, an-nealing of primers at 50-75°C (depend on primers) and elonga-tion at 72°C (McCartney *et al*., 2003, Makkouk and Kumari, 2006). The amplified DNA fragments separated by agarose gel electrophoresis and the bands are visualized by staining with ethidium bromide and irradiation with ultraviolet light.

The above procedure work well for DNA viruses but for RNA viruses Reverse Transcription PCR (RT-PCR) is used for the detection, which requires reverse transcriptase enzyme before the regular PCR step (Webster *et al*., 2004; Lopez *et al*., 2009). It has been developed and employed to detect many viruses such as PVX, PLRV and *Potato virus S* (PVS) in stem or seeds of potato (Ham, 2003; Peiman and Xie, 2006; Peter *et al*., 2009, Drygin *et al*., 2012). In addition, RT-PCR was used for quarantine purpose to detect plant RNA viruses such as *Cucumber vein yellowing virus* (CVYV), *Cucurbit yel-low stunting disorder virus* (CYSDV), *Potato aucuba mosaic virus* (PAMV), *Potato yellow dwarf virus* (PYDV) and *Tomato chlorosis virus* (ToCV) (Lee *et al*., 2011).

Real time PCR

Research workers welcomed the idea of the ability to visualize the progress of amplification in a quantitative manner (Lomeli *et al*., 1989). This approach leads to the foundation of "real-time" PCR. Real-time PCR was developed as one of the technical methods to detect the amplification products of PCR in real-time and also authorize accurate quantification of PCR products (McCartney *et al*., 2003; Ruiz Ruiz *et al*., 2009). Real-time PCR can be previously reduced detection time and can also be used for small concentration of target gene making possible to diagnose (Lopez *et al*., 2009; Heid *et al*., 2011) because there is no need of gel electrophoresis for the confirmation.

Real-time PCR has been increasingly used because this method has been showed valuable detection for plant viruses McCartney *et al*. (2003). Hasiow *et al*. (2008) detected and diagnosed *Pepino mosaic virus* (PepMV) member of *Potex virus* genus in tomato by using real time PCR.

Multiplex PCR

Multiplex PCR used multiple gene-specific primer sets within a single PCR mixture and can simultaneously detect two or more products in a single reaction. Therefore, the method is cost effective when two or more viruses are present in a single host plant (Lopez *et al*. 2009). The compatibility of the designed primers must be visualized experimentally. Multiplex PCR was proposed to enable simultaneous detection of different DNA or RNA by running a single reaction (James, 1999; Williams *et al*., 1999). This method required several specific primers to detect over two viruses (Singh *et al*., 2000; Menzel *et al*., 2002; Li *et al*., 2011; Qu *et al*., 2011). There are several examples reported of simultaneous detection of viruses and also other plant pathogens in one host Singh *et al*. (2000). A multiplex PCR had been developed for instantaneous and sensitive detection of the viruses like CMV, *Cherry leaf roll virus*

(CLRV), *Strawberry latent ringspot virus* (SLRSV) and *Arabis mosaic virus* (ArMV) in a single compartmentalized tube. The many major characterized viruses were simultaneously detected at diseased apple trees through multiplex-PCR (Menzel *et al*. 2002).

Loop mediated isothermal amplification (LAMP)

The LAMP technique is one of the most sensitive novel gene detection methods that amplify nucleic acids with high specificity, efficiency and rapidity under isothermal conditions (Notomi *et al*. 2000). It is cost effective and user-friendly which can carry out in a simple laboratory water bath or heat block. LAMP detects the viral amplicons using photometry for solution turbidity (Mori *et al*. 2001). By adding SYBR Green; a colour change can be seen without equipment. This technique was performed at a constant temperature for one hour using the four primers (Notomi *et al*. 2000). The specificity of LAMP is due to the recognition of six distinct sequences by four specifically designed primers.

Plant viruses such as *Plum pox virus* (PPV) can be detected by LAMP (Varga and James, 2006). The RT-LAMP has been developed for simple monitoring of RNA viruses including PVY and PLRV (Nie, 2005; Ju, 2011) and many other RNA viruses (Liu *et al*., 2010; Almasi *et al*., 2013; Shen *et al*., 2014; Keizerweerd *et al*., 2015).

Nucleic acid sequence based amplification (NASBA)

NASBA is commonly used to amplify RNA sequences. In the early 1990s this technique was developed for continuous amplification of nucleic acids in a single mixture at a single temperature (Compton, 1991). As compared to conventional PCR it works at isothermal condition instead of thermal cycling (Vaskova *et al*. 2004). The entire process was carried out at 41°C for 60 min and visualized a real-time assay using molecular beacons Lopez *et al*. (2009).

Real-time NASBA has been applied to detect plant viruses including *Strawberry vein banding virus* (SVBV), *Apple stem pitting virus* (ASPV) and PPV (Vaskova *et al*., 2004; Klerks *et al*., 2001; Olmos *et al*., 2007; Leone *et al*., 1997).

Rolling circle amplification (RCA)

The DNA viruses with small single stranded circular genomes the RCA can be used Haible *et al*. (2006). This technique uses the bacteriophage Ö29 DNA polymerase. RCA is based on the rolling replication of short single-stranded DNA (ssDNA) circular molecules (Fire and Xu, 1995; Lizardi *et al*., 1998; Najafzadeh *et al*., 2013) by adding DNA polymerases at a constant temperature, which only requires a simple platform, such as heating blocks or a water bath (Tsui *et al*. 2011).

This technique has been used to detect various geminiviruses some of them are listed here: *Abutilon mosaic virus* (AbMV), *Tomato yellow leaf curl Thailand virus* (ToYLCTV), *Chilli leaf curl virus* (ChiLCV), *Alfaalfa leaf curl virus* (ALCV), *Wheat dwarf virus* (WDV) (Fischer *et al*., 2015; Knierim *et al*., 2007; Nehra and Gaur, 2015; Parizipour *et al*., 2017).

Microarray

Microarray is the advanced form of the southern blotting technique. In oligonucleotide array oligochips are used which are composed of thousands of specific probes spotted onto a solid surface like a glass plate (Lopez *et al*. 2009). Synthesized ssDNA probes with about 25 bp to 70 bp nucleotides are hybridized with the virus extracted from plant (Lee *et al*., 2003; Wang *et al*., 2002; Wang *et al*., 2003). Main drawback of this technique is cost since it requires the highly sophisticated processing machine for spotting probes and reading reactions and also needs dust-free room (Wang *et al*. 2002). The other problem is construction of oligonucleotide to be hybridized to target DNAs in terms of specificity and sensitivity (Dugat Bony *et al*. 2012). Because of these problems, it has not been widely used and is still under research phase.

Some trials to use this method can be found because it is able to detect both known and unknown sequences in environmental samples, resulted in identifying unknown viruses by Oligo-chip (Boonham *et al*., 2002; Dugat Bony *et al*., 2012; Schena *et al*., 1995; Nam *et al*., 2014). This method was used for detection of potato viruses such as *Potato virus A* (PVA), *Potato virus M* (PVM), PVS, PVX, PVY and PLRV and cucurbit-infecting plant viruses (Lee *et al*., 2003; Bystricka *et al*., 2005).

CONCLUSION

In modern agriculture, plant virus diseases are enormously increasing and resulting in dangerous outbreaks leading to huge economic losses because there are no commercialized chemical to manage them. To control the increased spread of plant virus diseases and to avoid threats of epidemics caused by known and/or novel/emerging/re-emerging/ invasive viruses, it is very necessary to diagnose the viruses on time. Till now there is no an ideal detection method to fulfil all requirements, it is important to choose an appropriate diagnostic test that can fit the purpose. Every method of pathogen detection and diagnosis has their own advantages and limitations but recent developments in molecular detection technology led to the development of more convenient, effective and specific assays. Those will help researchers for early detection of disease infection. These new techniques are effective when used parallel with ancient methods. Many kinds of techniques are available now and are developing for the diagnostic purpose. Since there is no an ideal detection method to fulfill all requirement to detect, it is very important to develop appropriate and effective techniques which can be applied for management of viral diseases in worldwide level. Using this sustainable agriculture will be achieved.

Conflict of interest: None.

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