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Detection of virus through molecular tools

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Introduction:

Accurate diagnosis of virus diseases and diseases in general, is a first important step for any crop management system. With virus diseases, plant treatment after infection often do not lead to an effective control. Accordingly, virus diseases are managed most effectively if control measures are applied before infection occurs. The use of healthy (virus-free) plant propagation material is among the most effective approaches to adopt by farmers. One of the elements essential for successful certification programs to produce such propagation material is the availability of sensitive diagnostic methods. Few decades ago, virus detection was based mainly on biological techniques which are too slow and not amendable to large-scale application. Advances in molecular biology and biotechnology over the last three decades were applied to develop rapid, specific and sensitive techniques for the detection of plant viruses. This topic of assignment will summarize the development and use of the main immunological and nucleic acid-based methods for virus detection.

1. Immunological-Protein Based Methods:

The use of serology for the detection of plant viruses was in use for more than half a century (Torrence *et al*., 1981). However, the use of advanced immunodiagnostic methods for the identification and detection of viruses made the detection, easier, more sensitive and with reasonable cost (Lankow *et al*., 1987). In this paper we will summarize the important features of the most commonly used techniques in recent years.

1.1. ELISA

During the last three decades, enzyme linked immunosorbent assay (ELISA) was widely used method for the detection of viruses that is highly sensitive, simple, fast and most importantly has the ability to quantify virus content in plant tissue. The binding of the virus and specific antibody is made visible through an antibody tagged with an enzyme which can react with a substrate to produce a colored, water-soluble product.

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The first reported method was the double antibody sandwich ELISA (DAS-ELISA) where the antibody is bound to the solid phase (e.g., polystyrene microtiter plate), then the test samples, enzyme labeled antibody and the substrate are added sequentially, with unbound material removed by washing between steps (Clark and Adams, 1977).

In a positive test, the substrate solution turns colored, whereas a negative test remains colorless. The color intensity, which is proportional to virus contents, can be measured spectrophotometrically. Since the report of **Clark and Adams** in 1977 (Clark and Adams, 1977), many ELISA variants were reported, by using different enzymes or universal conjugates. In this later case the test is known as triple antibody sandwich ELISA (TAS-ELISA). In other variants, the first step of coating the solid phase with antibodies is deleted, and consequently virus particles are adsorbed directly on the solid phase, and the test is known as direct antigen coating ELISA (DAC-ELISA). In addition, immunoassay sensitivity can be enhanced by the use of different amplification systems, with avidin-biotin being the most common.

In addition to the polystyrene plates, a number of solid phase supports were found adequate. Assays in which antibodies or virus particles are bound to nitrocellulose membrane filters were used and known as immunoblots or dot-blots. Dot blot ELISA tends to be rapid, easy to perform and conservative of reagents and often more sensitive than ELISA carried out in a microtiter plate (Banttari and Goodwin, 1985). Immunoblot assays use the same reagents used in microtiter plate ELISAs, except that the substrate produces an insoluble product which precipitates onto the membrane. Positive reactions can be determined visually.

An interesting development was the printing of plant parts cut surfaces on nitrocellulose membranes and then the test continues in a way similar to dot-blot assays. The procedure is known as the tissue-blot immunoassay (TBIA) (Lin *et al*., 1990; Makkouk and Comeau, 1994). 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 NC membrane, the test can be completed either few days or few months later. This is a big advantage in remote places, where facilities for processing NC membranes do not exist. In such locations, samples can be printed on NC membranes and then sent/mailed to distant locations for processing.

As a result of the progress made in the last two decades in the medical diagnostic industry, a number of procedures and devices have been developed that increase speed, sensitivity and ease of use of immunoassays in

the field. One of these approaches for the detection of plant viruses is the "dipstick", developed earlier for the physicians' office and home use, is now being used for the detection of various pathogens, including viruses, in the field (Baker et al., 2003; Rowland et al., 2005).

Small plant tissue is placed in an extraction bag which contains an extraction buffer, then the bag is rubbed with a pen or blunt object to crush the samples, the tip of a strip (e.g., Immuno Strips form Agdia) is inserted in a vertical position into the extract and the result will appear as a colored line within 3-5 minutes.

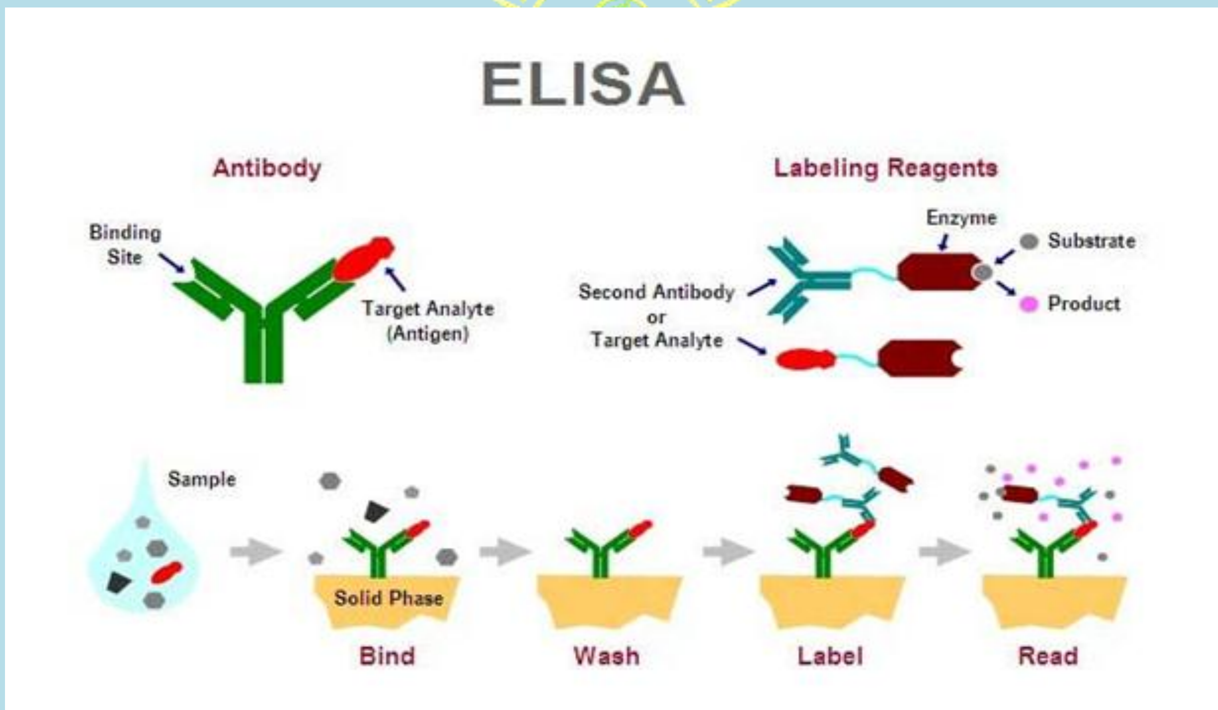

Fig.1: Enzyme Linked Immunosorbent Assay (ELISA)

2. Molecular-nucleic acid-based methods

Nucleic acid-based virus detection systems make use of cloned DNA probes in a dot-blot assay or specifically designed primers in a polymerase chain reaction (PCR) test. Both approaches have the potential to detect single nucleotide differences. These two approaches will be summarized in the following paragraphs.

2.1. Dot-blot assay:

This development in nucleic acid hybridization technology offers a good potential for virus detection (Meinkoth et al., 1985). The target viral nucleic acid from a plant sample is spotted onto a solid matrix, commonly nylon or nitrocellulose membranes, and bound by baking. Free binding sites on the membrane are blocked with a non-homologous DNA and a protein source. Thereafter, hybridization with a labeled probe is carried out. The label is then detected by autoradiography (for radioactive probes), or by

a colorimetric reaction if an enzyme label is used. The sensitivity of dot-blot hybridization is about the same as ELISA. A modification of the dot-blot assay, squash blotting, has been used to detect some viruses (Boulton *et al*., 1987).

2.2. PCR (polymerase chain reaction):

The polymerase chain reaction (PCR) has been used as the new standard for detecting a wide variety of templates across a range of scientific disciplines, including virology. The method employs a pair of synthetic oligonucleotides or primers, each hybridizing to one strand of a double stranded DNA target, with the pair spanning a region that will exponentially reproduced. The hybridized primer acts as a substrate for a DNA polymerase, which creates a complementary strand via sequential addition of deoxyncleotides. The process can be summarized in three steps: (i) dsDNA separation at temperatures above 90ºC, (ii) primers annealing at 50-75C, and (iii) optimal extension at 72-78ºC. The rate of temperature change, the length of the incubation at each temperature and the number of times each cycle is repeated are controlled by a programmable thermal cycle. The amplified DNA fragments will then be separated by agarose gel electrophoresis and the bands are visualized by staining the resulting bands with ethedium bromide and irradiation with ultraviolet light. The specificity of PCR testing is dependent on the primer sets used. There are virus species specific primers and genus specific primers. Figure 1 illustrates the use of primers that can detect all species of the genus Nanovirus and other primer sets that can detect an individual virus species within that genus. The above procedure work well for DNA viruses (e.g., viruses of the genera Geminivirus, Nanovirus and Caulimovirus).

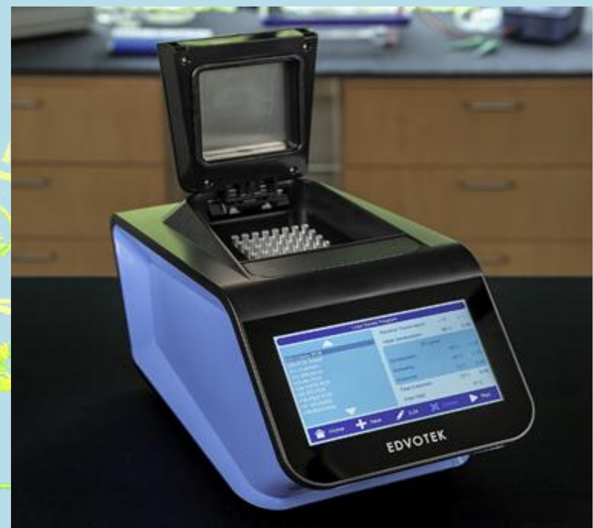

Fig.2: The polymerase chain reaction (PCR)

2.3. Real time PCR:

The ability to visualize the progress of amplification in a quantitative manner was welcomed by research workers. This approach has provided insight into the kinetics of the PCR reaction and it is the foundation of “real time” PCR.

The monitoring of accumulating amplicon in real time PCR has been possible by the labeling of primers, probes or amplicon with fluorogenic molecules. The increased

speed of real time PCR is largely due to reduced cycle times, removal of post-PCR detection procedures and the use of fluorogenic labels and sensitive methods of detecting their emissions. The reduction in amplicon size generally recommended by the inventors of commercial real-time assays may also play a role in this speed, but decreased product size does not necessarily improve PCR efficiency.

As long as fluorochromes are on the probe, the quencher molecule stops all fluorescence by the reporter. However, as Taq polymerase extends the primer, the intrinsic 5’ to 3’ nuclease activity of Taq degrades the probe, releasing the reporter fluorochrome. The amount of fluorescence released during the amplification cycle is proportional to the amount of product generated in each cycle. Similar to the conventional PCR, in case of

Table-1: The most frequently utilized molecular techniques for detection of viruses and their most important features

Technique	Sensitivity[a]	Specificity[b]	Feasibility[c]	Rapidness	Cost
Molecular hybridisation	+[d]	++++	++	+	+++
FISH	++	++	+++	+	++
Conventional PCR	+++	++++	+++	+++	+++
Nested PCR in a single tube	++++	++++	+++	++	+++
Cooperational-PCR[e]	++++	++++	+++	+++	+++
Multiplex PCR	+++	++++	+++	+++	+++++
Multiplex nested PCR	++++	++++	++	+++	++++
Real-time PCR[f]	+++++	+++++	++++	+++++	+++
NASBA[g]	+++++	++++	++++	++++	++
LAMP	++++	++++	+++	++++	++
Microarrays	+	+++++	+	++	+

Quantitative real-time PCR is based on detection of a fluorescent signal produced proportionally during the amplification of a PCR product. A probe (e.g., TaqMan) is designed to anneal to the target sequence between the traditional forward and reverse primers. The probe is labeled at the 5’ end with a reporter fluorochrome and a quencher fluorochrome added at the 3’ end. The probe is designed to have a higher Tm than the primers, and during the extension phase, the probe must be 100% hybridized for success of the assay.

RNA viruses, amplification can be measured after extraction of total RNA and preparation of a cDNA by a reverse transcription (RT) step. Real time PCR has proven increasingly valuable diagnostic tool for plant viruses. However, it requires an initial high capital investment to acquire the needed equipment, as compared to other techniques.

2.4. Multiplex PCR

The simultaneous detection of two or more DNA or/and RNA targets can be afforded by duplex or multiplex PCR in a

single reaction with several specific primers included in the PCR cocktail. Multiplex PCR is very useful in plant pathology because different bacteria or viruses frequently infect a single crop or host. This methodology has demonstrated to be a valuable tool for detection and identification purposes (López et al, 2006).

There are several examples of simultaneous detection of viruses and also bacteria and fungi at the same time (Atallah and Stevenson, 2006). Nevertheless, there are still very few examples in which more than three plant viruses are amplified in a single PCR-based assay, probably due to the technical difficulties of a reaction involving so many compatible primers.

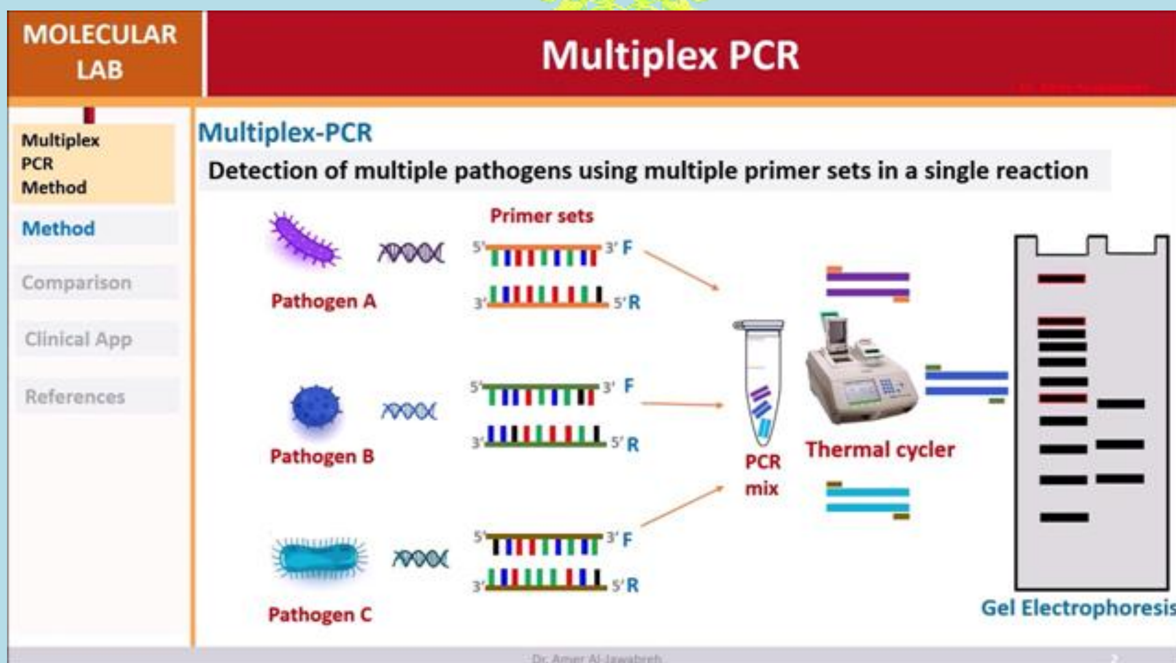

Fig.3: The **Multiplex** (PCR)

Two successful examples are the simultaneous detection of the six major characterised viruses affecting olive trees: CMV, CLRV, SLRSV, Arabis mosaic virus (ArMV), Olive latent virus-1 and Olive latent virus-2 and the simultaneous detection of nine grapevine viruses (ArMV, grapevine fanleaf virus, grapevine virus A, grapevine virus B, rupestris stem pitting-associated virus, grapevine fleck virus, grapevine leafroll-associated virus-1, -2 and -3).

2.5. Molecular hybridization

Molecular hybridization-based assays were first utilized in plant pathology to detect Potato spindle tuber viroid (Owens and Diener, 1981) and adapted to virus detection (Hull, 1993). However, certain problems associated with the use of radioactive probes, relatively low sensitivity and complexity of these techniques and the development of amplification-based assays have minimized

new improvements and applications. Today, the most common molecular hybridization format for the detection of viruses is non-isotopic dot-blot hybridization using digoxigenin-labelled probes. This technique has been employed for Apple mosaic virus (ApMV), Prunus necrotic ringspot virus (PNRSV), Prune dwarf virus (PDV), PPV, and Apple chlorotic leaf spot virus (ACLSV). Furthermore, multiple RNA riboprobes or polyprobes have been used to detect different viruses and they can be associated with tissue printed or squashed material in addition to the spotted extracts.

2.6. Nested PCR

Sensitivity and specificity problems associated with conventional PCR and RT-PCR can be reduced by using nested PCR-based methods, based on two consecutive rounds of amplification (Simmonds et al., 1990; PorterJordan et al., 1990).

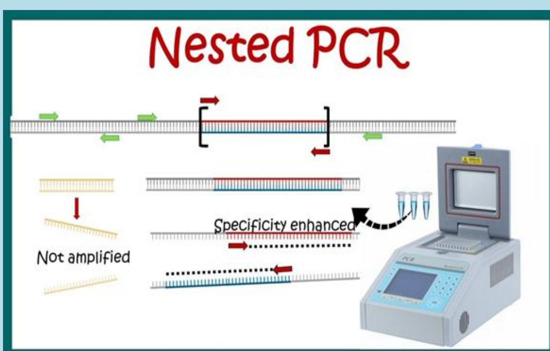

Fig.4: The **Nested PCR**

Usually, the products of the first amplification are transferred to another tube before the nested PCR is carried out using one or two internal primers (heminested or nested amplification respectively).

2.7. Real-time PCR

Real-time PCR allows the monitoring of the reaction while it is in course, thus avoiding the need to manipulate amplicons that implies high risk of contamination. At the same time the method requires 12 fewer reagents and less time, and also allows additional studies to be performed during detection (quantification of original target population, detection of several variants of a pathogen or point mutations in a gene). Among the different variants of PCR, real-time PCR represents a quantum leap and is a tool that has proven indispensable in a wide range of molecular biology protocols. In the detection field, this high throughput technique has improved the systems in use, achieving very accurate speed, specificity and reliability, with many protocols having been developed in the last years. The basis of conventional PCR and the majority of the primers designed for detection can be adapted to the peculiarities of real time PCR, adding another important reason for such transfer. When setting up a real-time PCR protocol for detection, it is necessary to adapt it to the specific conditions of the detection system and instrumentation, and to the characteristics of the concentration of reagents and cycling, which differ to those of conventional PCR. Among them, the most

important are primer design, reaction components and conditions.

2.8. Nucleic Acid Sequence Based Amplification (NASBA)

NASBA is an isothermal amplification method that can be used to detect RNA targets. The reaction requires the use of three enzymes, AMV-RT for reverse transcription and to obtain double stranded cDNA, RNase H to hydrolyzed the RNA fragment of the hybrid molecule DNARNA and T7 RNA polymerase to produce a large amount of anti-sense, single strand RNA transcripts corresponding to the original RNA target. It can be achieved by using two specific primers, one of them including at 5' end the T7 promoter, NTPs and also dNTPs. The entire NASBA process is performed at 41ºC for 60 min and the typical level of amplification is at least a factor of 10^9.

This technology has been applied for detecting plant viruses such as Apple stem pitting virus (Klerks et al., 2001), PPV (Olmos et al., 2007a), Potato virus Y, ArMV and the bacteria *C. michiganensis* subsp. *sepedonicus* and *R. solanacearum*.

2.9. Loop-mediated isothermal amplification (LAMP)

Loop-mediated isothermal amplification (LAMP) is another type of isothermal amplification that it is being increasingly used in the diagnostic field offering sensitivity and economic costs (Notomi et al., 2000). The method requires a set of four specifically designed primers that recognize six distinct sequences of the target and a DNA polymerase with strand displacement activity. The amplification products are stem-loop DNA structures with several inverted repeats of the target and cauliflower-like structures with multiple loops, yielding >500 g/ml. The LAMP reaction was enhanced by the addition of loop primers (Nagamine et al., 2002), reducing time and increasing sensitivity. The amplification takes place at 60-65ºC for 60 min. Although it was initially developed for DNA it can be adapted to amplify RNA (RT-LAMP) The method has only been applied to the detection of some plant viruses such as PPV, with a sensitivity level similar to that obtained by real-time PCR (Varga and James, 2006).

Fig.4: Loop-mediated isothermal amplification (LAMP)

2.10. Microarray technology

Since the development of microarray technology for gene expression studies (Schena et al., 1995), new approaches are extending their application to the detection of pathogens. Microarrays are generally 26 López et al. 14 composed of thousands of specific probes spotted onto a solid surface (usually nylon or glass). Each probe is complementary to a specific DNA sequence (genes, ITS, ribosomal DNA) and hybridisation with the labelled complementary sequence provides a signal that can be detected and analysed. Although there is great potential for microarray technology in the diagnosis of plant diseases, the practical development of this application is still in progress.

Conclusions

Recent developments in molecular detection technology led to the development of more convenient, effective, and specific assays and permitted the use of these tests for detecting plant pathogens, including viruses. Such assays will help growers, crop agronomists, and plant-health professionals not to rely exclusively on symptomatology and/or time-consuming diagnostic procedures, and permit early detection of viral infection. These new techniques are effective management tools to be used in parallel with knowledge of the crop, understanding the biology of the pathogen and the ecology of the disease. Thus, these tools can be excellent tool to determine the point in time at which control measures should be implemented. In addition, such diagnostic assays are essential tools for programs devised to produce virus-free plant propagation materials. Viral genome sequence data available made it very easy to design primers for different uses, for broad or specific detection of viral pathogens. Similarly, the production of monoclonal antibodies gave immunological tests increased capacity in terms of specificity, not provided earlier by polyclonal antibodies.

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