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Research article

IMPACT OF MOLECULARLY DIAGNOSE OF DISEASES IN SOME IMPORTANT FRUIT CROPS

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ABSTRACT: The social and economic consequences of the failure to recognize contain or control threatening pathogens require that every effort can be made to engage in efficient and effective programs of surveillance, diagnosis and detection. The fact that threats are many and resources few points to the increasing importance of diagnostic networks that can rapidly and precisely identify causal organisms of disease as a crucial first step towards the deployment of control and mitigation strategies. This technology continues to evolve with pull-through from human and veterinary disease diagnostics and it is hoped that more sensitive and specific devices are developed for a range of pathogens that can be deployed in the field. Where as detection refers to the identification of microorganisms or their products, e.g., toxins in any number of substrates including plant tissues, soil and water.
Key Words: Molecular diagnose, Fruit crops, Polymerase chain reaction (PCR), DNA, RNA, Bacteria, Fungi and Virus.

INTRODUCTION

At the moment unlike for bacterial and fungal diseases no chemicals exist that could be used as a direct field control of viral or viroid diseases and therefore the early detection by means of sensitive molecular diagnostic methods is the main way to control them [40, 27]. Plant viruses composition relies on both traditionally called informative molecules (nucleic acids) and functional molecules (proteins). Instant identification of causal organisms of diseases depends on the diverse range of technologies from traditional taxonomy to the latest developed advanced molecular methods. Molecular hybridization as a diagnostic tool in plant virology was first used to detect viroids [47] and later, applied to plant viruses [41, 21]. Molecular hybridization based on specific interaction between complementary purine and pyrimidine bases forming A-T and G=C base pairs, result in a stable hybrid formed by part (or the totality) of the nucleic acid sequence of the pathogen to be detected (target molecule) and the labelled complementary sequence (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. DNA-based assays, particularly PCR and real-time/quantitative PCR (qPCR) are being adopted in diagnostic laboratories; ordinary PCR testing for many pathogens is now routine and affordable in the developed world.

For routine analysis in scheme certification programs, sample manipulation must be reduced to a minimum. This was achieved by using the tissue-imprinting technique that avoids sample extraction and body require the direct transfer of the plant material (stem, cutting, leaf) on to a nylon or nitrocellulose membrane. This technique was first described to detect proteins by immunocytolocalization [10] later applied to RNA detection [43] and then, adapted for detection and localization of plant viruses [39, 11].

Among them, molecular hybridization and polymerase chain reaction (PCR) have received great interest lately and have been incorporated in the diagnostic field of plant pathology. The objective of this chapter is to provide basic ideas lie behind the molecular diagnostic and the current status of molecular diagnostics in fruit crops.

PRINCIPLES

Molecular diagnostics deals with the detection of different pathogens such as bacteria, fungi and viruses followed by the detection of disease-relevant mutations using molecular techniques. Most techniques in molecular diagnostics are based on the identification of proteins or nucleic acids. Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) can be amplified and detected with diverse methods such as PCR, real-time PCR, hybridization and DNA microarrays.

NEED FOR MOLECULAR DIAGNOSTICS IN FRUIT CROPS

Plant microorganisms including bacteria, fungi and viruses are responsible for increasing economic losses worldwide. They can cause a large range of symptoms in most cultivated plants which can be affected in their different parts with various agronomic impacts. Microbes that cause plant diseases are difficult to control because of the lack of efficient products for chemical treatment under field conditions.

Molecular techniques can provide the basis for the efficient, accurate and rapid detection of pathogen inoculums are already being used to supplement critical conventional methods. Developing countries which are the major producer of tropical and subtropical fruits are facing the critical problem of endemic disease. To meet the nutritional demands of ever increasing population, molecular diagnostic techniques can play a crucial role as these techniques are very effective, accurate and less time consuming. Traditional diagnostic methods such as isolation on agar plate and pathogenecity tests are time consuming and cannot fulfill the urgent need of disease diagnosis in case of accidentally released pathogen. Day by day advancement in technology resulted in to the development of new diagnostic tool such as RT-PCR that can diagnose the disease in one day or less. Diagnostic tools are advancing with highly reliable classical agar plating, serology and molecular-based assays already available to detect and identify key plant pathogens and many more in the pipeline. Routine diagnosis of many crop diseases can now be made in one day or less by real-time PCR.

METHODS FOR MOLECULAR DIAGNOSTIC

There are several methods developed for diagnose of disease. Which can be classified in to three broad categories.

DNA based diagnostics

Polymerase chain reaction (PCR) and DNA/RNA probe technology for the detection, identification and quantification of microbes. Real time PCR has been successfully used to provide rapid, quantitative data on fungal species. The PCR method utilizes an enzymatic and an exponential amplification of specific DNA sequences. This goal may be achieved through multiple cycles of three steps performed at different temperatures to: (i) Denaturate the DNA (ii) Anneal two oligonucleotide primers to the denatured DNA strands and (iii) Primer extension by thermostable DNA polymerases to amplify the target sequence whose ends are defined by the primers. The presence of amplified product can be determined by gel electrophoresis analysis. In the case of RNA pathogens viroids and most plant viruses a previous reverse transcription step (RT) must be included to copy the target RNA into cDNA prior to being amplified (Fig.1).

In this chapter, some of the molecular approaches to fungal diagnostics based on polymerase chain reaction (PCR) and DNA/RNA probe technology are discussed. This includes several technological advances in PCR-based methods for the detection, identification and quantification of fungi including real-time PCR which has been successfully used to provide rapid, quantitative data on fungal species from environmental samples.

Dot blot technique, consisting of PCR amplification using pathogen specific primers, followed by probing of the PCR product with a specific-specific oligonucleotide probe in a dot blot hybridization test. A dot blot assay can analyzed many samples for a single organisms, as the PCR products from the samples are blotted on the membrane and hybridized to a single probe specific for the particular organism.

Dot blot hybridization assay would allow rapid identification of fruit crop pathogens and thus would help disease management to reduce economic losses. Southern blot hybridization and analysis of the resulting RFLP patterns provided more information on genetic relationships than dot blot assays, which are rarely used now. Strains that were indistinguishable by dot hybridization could be differentiated by RFLP analysis [6].

The Southern blot is a technique employed to ascertain information about the molecular weight and relative amount of a DNA sequence of interest. The assay was first developed by Edwin Southern and is a combination of gel electrophoresis of DNA (often first fragmented by restriction enzyme digestion) transfer of the same to a charged membrane and hybridization of a labeled DNA probe. Following hybridization, the membrane is washed to remove unbound probe and an image obtained via autoradiography or using equipment such as a phosphorimager. The image will indicate the location(s) to which the probe hybridized, with the intensity of the signal observed serving as a measure of relative abundance.

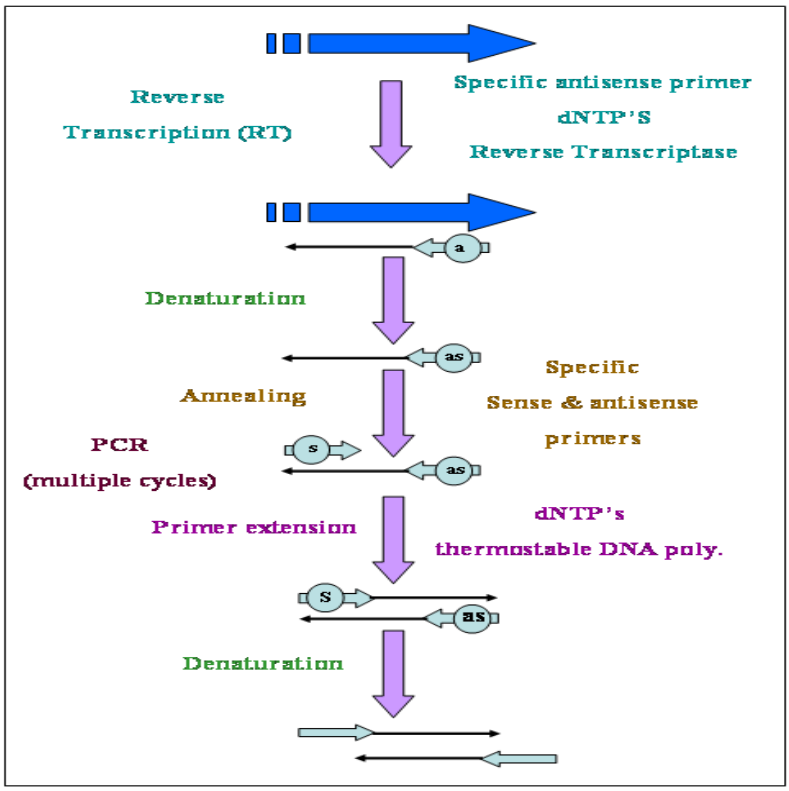

Fig. 1: Schematic diagram of the RT-PCR procedure used for the detection of plant and viroids.

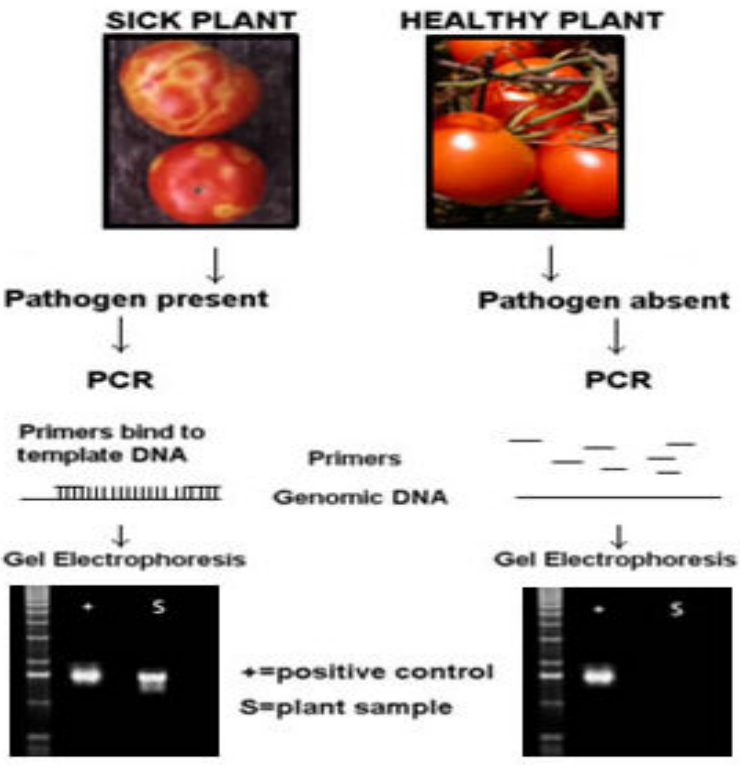

Fig. 2: Systematic diagram of PCR based diagnostic method (Source: [1])

Table. 1: Molecular diagnostic has done in some important fruit crops

S. No.	Crop	Disease	Pathogen	Detection	Reference
1	Mango	Malformation	*Fusarium*	AFLP analysis (sequences of *tef1* were used to design species-specific primers)	Lima [34]
2	Mango	Anthracnose	*Colletotrichum gloeosporioides*	Cloning of a carbendazim-resistant gene	lin and sheng [35]
3	Almond & avocado	Anthracnose	*Colletotrichum gloeosporioides*	PCR amplification of genomic DNA with four primers	Freeman [20]
4	Guava	Guava rust	*Puccinia psidii*.	Develop a species-specific, nested polymerase chain reaction (PCR)-based detection assay	Langrell [32]
5	Guava	Guava rust	*Puccinia psidii*	Development of SSR markers	Zhong [59]
6	Guava	Guava wilt	*Nalanthamala psidii* and *N. diospyri*	Analyses of the internal transcribed spacer regions and 5.8S rDNA (ITS rDNA), LSU rDNA and partial b-tubulin gene	Schroers [52]
7	Banana	Sigatoka leaf spot	M. *fi jiensis*, *M. musicola* and *M. eumusae*	Species-specific molecular-based diagnostic tools	Arzanlou [3]
8	Grapefruit & lime	Bacterial cankar	Bacteria	Competitive PCR method is based on the accumulation of two PCR products	Cubero [14] and Hartung [26]
9	Citrus	Leprosies	Virus	Develop the first specific, molecular diagnostic tool	Locali [37]
10	Papaya	Meleria	Meleira virus (PMeV)	Primers specific for PMeV were designed based on nucleotide sequences of the viral dsRNA	Araujo [2]
11	Papaya	PRSV (Papaya ring spot virus)	Virus	Develop a polyclonal antiserum for PRSV by using Enzyme-linked immunosorbent assay (ELISA) for skin	Kunkalikar [30]
12	Papaya	Dieback	*Phytoplasma*	developed the DNA detection using the polymerase chain reaction (PCR) with primers specific	Guthrie [25]
13	Papaya	Papaya ring spot virus-type P (PRSV-P)	Virus	Comparison of random amplified polymorphic DNA (RAPD) and DNA amplification fingerprinting (DAF) for sex determination	Magdalita [38]

Restriction Fragment Length Polymorphism (RFLP) markers are codominant markers that are more or less specific depending on the probe used (cDNA, genomic DNA, etc). They are difficult and quite expensive to use. They require the extraction of a large quantity of good quality DNA and operations are lengthy. It takes around two weeks after DNA extraction to read the bands. DNA was digested by a restriction enzyme that cut the DNA at a particular site. A large number of fragments were obtained in this way. These fragments were then separated according to their size by migration in agar gel under the influence of an electric field. After migration, the DNA was transferred to nylon membrane, then exposed to a probe (small DNA primer specific to the locus studied), which was either radioactive-labelled or non-radioactive-labelled (antibody-antigen complex, e.g. Digoxygenine). Irrespective of the number of probes used or their origin the results as regards diversity structuring were comparable. However, this technique is laborious to use. In addition, with the discovery of the PCR technique new types of markers came into being.

The technique called reverse dot-blot, involves the use of multiplex PCR to amplify and label simultaneously the regions of the DNA that contain the mutations. The PCR products are then used as probes for hybridization with a membrane that contains an array of oligonucleotides representing several different mutations and their corresponding normal sequences. The hybridization results are read like a checklist, where the mutations and normal alleles in a patient are determined from the positive reactions in the test (Fig. 2).

RNA based diagnostics

Viroids and most of the plant viruses, including the totality of viruses affecting stone fruit trees have RNA genomes. RNA-RNA hybrids are more stable than RNA-DNA hybrids; therefore more stringent hybridization conditions can be selected in the case of RNA-RNA hybrids that will help to increase specificity and lower nonspecific background. Hence, RNA probes are preferred over DNA ones to detect stone fruit viruses.

The Northern blot is used to study the expression patterns a specific type of RNA molecule as relative comparison among of a set of different samples of RNA. It is essentially a combination of denaturing RNA gel electrophoresis and a blot. In this process RNA is separated based on size and is then transferred to a membrane that is then probed with a labeled complement of a sequence of interest.

The results may be visualized through a variety of ways depending on the label used however, most result in the revelation of bands representing the sized of the RNA detected in sample. The intensity of these bands is related to the amount of the target RNA in the samples analyzed. The procedure is commonly used to study when and how much gene expressing is occurring by measuring how much of that RNA is present in different samples. It is one of the most basic tools for determing at what time certain genes are expressed in living tissues.

Most methods used for (viroid) RNA extraction require use of phenol or other toxic organic solvents making them undesirable for diagnostic laboratories that process large number of samples. Recently, an extraction method that avoids the use of phenolics previously described for obtaining plant genomic DNA [18] to enrich partially purified extracts in viroid-like RNAs [48] or in the purification of double stranded viral RNAs [19] was adapted for viroid detection [4, 8].

Protein based diagnostics

Specific monoclonal and/or recombinant antibodies are available for many plant pathogens and have contributed to the specificity of serological detection. ELISA kits are being developed for virus disease diagnosis in high-value crops including ornamentals, fruits and spices.

These are based on the monoclonal and/or recombinant antibodies against pathogens. Accurate routine disease detection requires high levels of specificity, sensitivity and speed combination of ELISA and PCR technologies can be used to improve sensitivity of detection and to avoid problems with inhibitors or PCR often found in plants. Protein extraction was performed using adult insect tissues. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed for total storage proteins according to the method early described in [31].

The resulting amplified DNAs are added directly to *in vitro* coupled transcription/translation reactions and the products are analyzed by SDS-polyacrylamide gel electrophoresis for the appearance of truncated proteins. While PTT has gained widespread use in clinical and diagnostic laboratories the technique can also be applied to other research applications such as producing intact or truncated proteins for protein interaction studies or biochemical analysis.

Hybridized filters can be either processed immediately or stored dry. The labelled hybrids are detected by an ELISA reaction using conjugates composed of high-affinity DIG-specific antibodies coupled to alkaline phosphatase (AP). The diagnosis of plant diseases and detection of pathogens rely on a diverse range of technologies from traditional taxonomy to advanced molecular methods. Laboratory ELISA are widely adopted, but these and other advanced diagnostic technologies are often inaccessible to laboratories in the developing world because of their relatively high cost.

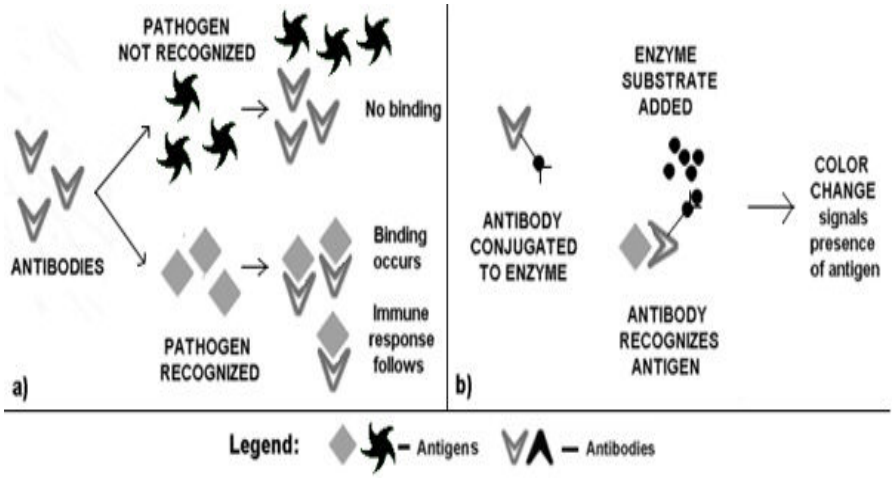

Fig. 3: Systematic diagram of Antibody-Antigen Interaction (Source: [1])

In Western blotting, proteins are first separated by size, in a thin gel sandwiched between two glass plates. The proteins in the gel are then transferred to a PVDF, nitrocellulose, nylon or other support membrane. This membrane can then be probed with solutions of antibodies. Antibodies that specifically bind to the protein of interest can then be visualized by a variety of techniques, including chemoluminescence or radioactivity.

Antibodies can also be used to purify proteins. Antibodies to a protein are generated and are often then coupled to "beads". After the antibody has bound to the protein of interest, this antibody-protein complex can be separated from all other proteins by centrifugation. During centrifugation the beads to which the antibody is coupled will pellet (bringing the protein of interest down with it) whereas all other proteins will remain in the solution. Alternatively, antibodies coupled to a solid support matrix like Sephadex or Sepharose beads, for example, can be used to remove a protein of interest from a complex solution. After washing unbound and non-specifically bound materials away from the "beads" the protein of interest is then eluted from the matrix usually by adding a solution with a high salt concentration, or by varying the pH of the solution in which the matrix is contained. The beads can either be suspended in solution (batch processing) or packed into a tube (column processing) (Fig. 3).

Combinations of ELISA and PCR technologies are used to improve sensitivity of detection and to avoid problems with inhibitors or PCR often found in plants. The application of these technologies in plant pathology has greatly improved our ability to detect plant pathogens and is increasing our understanding of their ecology and epidemiology. ELISA test although reliable and very appropriate for routine diagnosis, lacks of the required sensitivity when pathogens are present at very low titer.

For a wide range of disease management applications, there is a need for comprehensive diagnostic kits that can detect the presence of numerous pathogens in a single test. One could have a kit that could identify all the pathogenic species present in a given sample. Kits could also be host-based by having the capability of concurrently testing for all the key pathogens of a given host. Medical laboratories working on cystic fibrosis have designed a method that can detect several disease-related mutations in a single assay [28].

MOLECULAR DIAGNOSTICS IS DONE IN SOME SUBTROPICAL FRUIT CROPS

Mango

Characterization of *Fusarium* population associated with mango malformation disease in Brazil through AFLP analysis, sequencing of portions of the *tef1* and *tub2* genes crossing of strains on carrot agar and pathogenicity tests has been reported by Costa [13]. Additionally, sequences of *tef1* were used to design species-specific primers for PCR detection of the causal agents in plant tissue. Three clusters of isolates were observed in the AFLP analysis. The majority of the isolates felt in the first cluster, while the second contained isolates of *F. sterilihyphosum* from Brazil and South Africa. The third cluster grouped isolates of *F. mangiferae* from Egypt, India, South Africa and USA. A unique Brazilian clade in the *Gibberella fujikuroi* species complex was observed in the phylogenetic analysis. Its isolates were also cross-fertile, producing a typical *Gibberella* teleomorph. All the crosses between isolates of the Brazilian clade and isolates of *F. sterilihyphosum* and *F. mangiferae* were not fertile. Isolates of the Brazilian clade and *F. sterilihyphosum* were pathogenic to inoculated mango plants. A primer set that amplifies a 380 bp fragment from isolates of the Brazilian clade and *F. sterilihyphosum* was selected. Another fragment of 217 bp in size was obtained with a specific primer set to *F. mangiferae*. Specificity and sensitivity of primer sets were evaluated. The first primer set detected the presence of the causal agents of malformation in infected trees and in greenhouse plants artificially inoculated. Lee [33] analyzed random amplified polymorphic DNA (RAPD), internal transcribed spacer (ITS) of rDNA and partial β-tubulin gene DNA sequence and culture characteristics on PDA and PDA Benomyl. Results were obtained for the molecular analyses of 31 strains belonged to *Colletotrichum gloeosporioides,* ribosomal DNA group (RG) 4 of Moriwaki [46], 8 strains belonged to *C. acutatum,* A2 group of Talhinhas [54] and 5 strains to *C. acutatum*, A3 group of Talhinhas [54]. Most isolates of *C. gloeosporioides* RG4 grew faster on PDA than strains of *C. acutatum*, A2 and A3 groups and most RG4 strains were sensitive to Benomyl. However, a few strains of RG4 grew slower and were resistant to Benomyl. On the basis of molecular characteristics, apple isolates of *C. acutatum* were clearly differentiated from red pepper isolates of the species, but apple isolates of *C. gloeosporioides* were not.

Lin and Sheng [35] reported cloning of a carbendazim-resistant gene from *C. gloeosporioides* of mango in South China, preliminary results observed that the amino acids were altered at residues 181, 198, 237 and 363. All of the mutant positions were detected by allele-specific PCR. The allele-specific fragments were amplified in MBC-resistant strains by the positive primers but not in wild type strains. The allele-specific fragments were amplified in wild-type strains by the negative primers but not in MBC-resistant strains and preliminary findings proved that the point mutation occurred at amino acid codon 198 causing a change from glutamic acid (GAG) to alanine (GCG), which is closely associated with conferring MBC-resistance in the field.

An enzyme assay was employed to further test the above results. It involved an *Acc* restriction site (CGCG) at the positions of the amino acid residues at 197 and 198 (GACGAG_GACGCG) in MBC-resistant strains in which *Acc* digested a 329 bp fragment into 107 and 222 bp, while the fragments from wild-type strains remained undigested. Based on the above assays, all of the MBC-resistant and wild-type strains were detected successfully. It strongly suggested that the altered amino acid residue at position 198 played the leading role in conferring MBC-resistance in mango anthracnose in south China. Moalemiyan [45] collected volatile organic compounds from the headspace of mango cv. Keitt inoculated with *Lasiodiplodia theobromae* (stem-end rot), *C. gloeosporioides* (anthracnose), mock (as the first control) and non-wounded-non-inoculated mango (as the second control) were analyzed using GC/MS to investigate the feasibility of automatic detection and diagnosis of diseases of mango in stores. A total of 37 metabolites, relatively consistent in 8 replicates were identified based on mass spectral match using NIST library. 1-Pentanol was specific to *Lasiodiplodia*-inoculated mangoes while thujol was detected only in *Colletotrichum*-inoculated mangoes. Discriminant analysis models based on normalized abundances of 35 consistent metabolites and normalized abundances of 150 mass ions correctly classified 67 and 75 per cent of the observations, respectively, based on cross validation. They reported for the first time to detect and differentiate between anthracnose and stem-end rot diseases of mangoes (cv.Keitt) based on their volatile production patterns using GC/MS. The methods developed have the potential applications to mango industry to detect and diagnose diseases of mango fruits, at relatively early stages of the disease progress, after validation under commercial conditions.

Freeman [20] reported PCR amplification of genomic DNA with four primers produced uniform banding patterns for all the Israeli almond isolates from different geographic locations in Israel. DNAs from the U.S. almond isolates were distinct from DNAs of the Israeli isolates. The avocado isolates from Israel and the United States were more diverse, with numerous arbitrarily primed-PCR phenotypes being observed. *Hae*III digestion patterns of A1T-rich DNA distinguished between the almond and avocado isolates. Southern hybridization of the repetitive nuclear-DNA element GcpR1 to *Pst*I-digested genomic DNA of almond and avocado isolates revealed no polymorphic fragments among the almond isolates, whereas polymorphic fragments were observed among the avocado isolates. Amplification and subsequent restriction enzyme digestion of the internal transcribed spacer 4 and 5 regions between the small and large nuclear subunits of DNA encoding rRNA failed to distinguish between *C. gloeosporioides* isolates from a diverse host range. In artificial inoculations, avocado isolates produced various lesions on avocado and almond fruits, whereas the almond isolates infected both fruits at a lower rate.

Guava

Genetic variability of six isolate of *Fusarium solani,* pathogen collected from different places of India, the incident of wilt disease of guava in India was carried out by Gupta [24] based on Carboxylesterases isozyme pattern and DNA polymorphism using RAPD-PCR. Pattern of Carboxylesterase revealed a similar isozyme cluster in the isolate namely, Allahabad (isolate-3), Faizabad, (isolate-4), Unnao (isolate-5) and Lucknow (isolate-6). Similar results were obtained when 10 randomly amplified polymorphic DNA markers (OPA1-OPA10) tested in the genome of F*usarium solani* and grouped on basis of obtained allelic data. This pattern of genetic variability in the isolate was also supported by the analysis of the similarity indices and UPGMA dendrogram. Zhong [59] developed and characterized 15 polymorphic microsatellite markers present in the genome of the guava rust fungus, *Puccinia psidii*. The primers for these microsatellite markers were designed by sequencing clones from a genomic DNA library enriched for a simple sequence repeat (SSR) motif of (AG). All these 15 primer pairs successfully amplified DNA fragments from a sample of 22 *P. psidii* isolates, revealing a total of 71 alleles. Heterozygosity at the 15 loci ranged from 0.05 to 1.00 was obtained. Later, the SSR markers developed would be useful for population genetics study of the rust fungus. Langrell [32] reported to develop a species-specific, nested polymerase chain reaction (PCR)-based detection assay, using two primer sets designed from the rRNA ITS region, was developed for Puccinia psidii. Keith [29] reported the first molecular and traditional methods to identify four *Pestalotiopsis* taxa (*P. clavispora*, *P. microspora*, *P*. sp. GJ-1 and *P. disseminata*) on guava for germplasm characterization studies in Hawaii. Schroers [52] reported the molecular analysis of *Nalanthamala psidii* and *N. diospyri* based on analyses of the internal transcribed spacer regions and 5.8S rDNA (ITS rDNA), LSU rDNA and partial b-tubulin gene form a monophyletic clade. Few polymorphic sites in the ITS rDNA and b-tubulin gene indicate that *Nalanthamala psidii* comprises two lineages one of which has been detected only in South Africa.

Banana

The rapid and robust species-specific molecular-based diagnostic tools for detection and quantifi cation of Sigatoka leaf spot disease complex of banana ascomycetous fungi M. *fi jiensis*, *M. musicola* and *M. eumusae* have been developed. Conventional species-specific PCR primers were developed based on the actin gene that detected as little as 100, 1 and 10 pg/µl DNA from *M. fi jiensis*, *M. musicola* and *M. eumusae*, respectively. Later, TaqMan real-time quantitative PCR assays were developed based on the β-tubulin gene and detected quantities as low as 1 pg/µl DNA for each *Mycosphaerella* species from pure cultures and 1.6 pg/µl DNA/mg of dry leaf tissue for *M. fijiensis.* [3].

Citrus

Among fungal diseases, the soil borne diseases of citrus are widespread whereas other foliar diseases are climate dependent. Once the soil-borne pathogen enters in a given locality, in presence of susceptible hosts and favorable environmental conditions for its growth and multiplication, it becomes the endemic problem of the area. Correct diagnosis and regular monitoring of the diseases are required for their quick and economically feasible management. Bove [7] reported Huanglongbing (HLB), a destructive disease of citrus that represents a major threat to the world citrus industry and is slowly invading new citrus growing areas. HLB, whose name in Chinese means "yellow dragon disease" was first reported from southern China in 1919. Dot blot hybridization with a DNA probe and various PCR formats (one-step, nested, multiplex) using species-specific primers based on 16S rRNA or an rplKAJL-rpoBC operon sequence is required for its diagnosis. Graham [23] isolated *X. axonopodis* pv. *citri* from young lesions on fruit, leaves and stems, but bacterial populations decrease in older tissues, increasing the difficulty of recovery. KCB (kasugamycin-cephalexin-Bravo) semi-selective medium is most useful for isolating the *X. axonopodis* pv. *citri* from plant material [22]. *X. axonopodis* pv. *aurantifolii* grows best on a modified nutrient agar medium for isolation and culture from citrus tissues [9]. For disease diagnosis, bacterial isolates from plant material should be confirmed by inoculations of leaves in grapefruit and Mexican lime for A strains, lemon for B strains and Mexican lime for C strains, to reproduce the canker lesion phenotype. Polymerase chain reaction (PCR) methods have been developed for the rapid and accurate identification of the bacterium isolated in culture and from extracts of lesions on leaves and fruits [14, 26]. The primers first used to detect and identify citrus canker strains for competitive PCR method is based on the accumulation of two PCR products from the target sequence and the internal control after a fixed number of cycles. The ratio of the amplified product generated from the target sequence to the product of the internal control is related to the bacterial concentration in the sample [14]. Real time PCR also applied for quantitative PCR and for the rapid, on-site identification of bacteria in plant material [42, 51]. Real time PCR is not based in the accumulation of amplicons at the end of the reaction, but fluorescence detection of the point in time when amplification of the target is initiated. Approaches using of SYBR green dye and Taqman probes have been evaluated in conjunction with primers based on sequences from the pth and ribosomal genes [15], as well as on a gene for the leucine responsive regulatory protein (lrp) [16]. Leprosis, a disease caused by *Citrus leprosies virus* (CiLV) is transmitted by the false spider mite *Brevipalpus* spp. (Acari: Tenuipalpidae) is considered as an unassigned member of the *Rhabdoviridae* family because of its bacilliform, rhabdo virus like morphology. Locali [37] reported to develop the first specific, molecular diagnostic tool for the detection of cytoplasmic-type CiLV *(Citrus leprosies virus)*. The RT-PCR assay was developed especially using the MP primers has proven to be an efficient, rapid and reproducible method for the detection of CiLV. After cloning and sequencing, specific primers were designed to amplify putative CiLV genome regions with similarity to genes encoding the movement protein and replicase of other plant viruses. RNA from infected citrus plants corresponding to different varieties and locations were amplified by reverse transcription-polymerase chain reaction (RT-PCR) using the two pairs of primers. Amplified products were purified cloned in pGEM-T and sequenced. The sequences confirmed the genomic regions previously associated with CiLV.

Papaya

Disease management strategies involve different practices that include plant resistance and prophylactic and curative measures. The papaya diseases related have diverse etiologies, divided into those with biotic (infectious) and abiotic (noninfectious) etiologies that affect the fruit and those affecting the plant. Anthracnose causes significant economic losses in papaya (*Carica papaya* L.) fruits and *C. gloeosporioides* is the primary causal agent of this disease which is characterized by the typical lesions shown results in severe post-harvest losses.

Results were observed in development of DNA based diagnostics system in papaya unequivocally the existence of two *Colletotrichum* species causing anthracnose lesions on papaya fruits: *C. gloeosporioides* and *C. capsici.* PCR-RFLP using the restriction endonuclease MspI reliably reproduced restriction patterns specific for *C. capsici* or *C. gloeosporioides.* Araujo [2] developed a RT-PCR assay for early and accurate detection of *Papaya meleira virus* (PMeV) in the latex from infected papayas. Primers specific for PMeV were designed based on nucleotide sequences of the viral dsRNA obtained using a RT-RAPD approach. When tested for RT-PCR amplification one primers (C05-3) amplified a 669-nucleotide fragment using dsRNA obtained from purified virus particles as a template. The translated sequence of this DNA fragment showed a certain degree of similarity to the amino acid sequence of RNA-dependent RNA polymerases from other dsRNA viruses. When used as the single primer in two RT-PCR kits available commercially, primer C05-3 also amplified the DNA fragment from papaya latex of infected, but not from healthy plants.

Kunkalikar [30] has developed a polyclonal antiserum for PRSV (Papaya ring spot virus) produced by immunizing rabbit. Immunocapture RT-PCR amplified 550 bp coat protein gene of PRSV. Results shows that Enzyme-linked immunosorbent assay for skin of diseased papaya, fruit and stem was mild positive but negative for flowers, latex, seeds and roots from infected plants. DIBA detected virus in infected plant samples and tissue imprinting of PRSV infected squash leaves revealed more localization of virus in interveinal regions as compared to veins. Urasaki [56], identify the Genomic analysis of papaya enabling the molecular breeding and the DNA marker assisted selection. From the collection of the genes cloned, a full length of small GTP-binding protein gene, pgp1 was cloned for molecular breeding of dwarf papaya suitable for greenhouse cultivation was later developed. *Phytoplasmas* are cell-wall-less bacteria belonging to the class *Mollicutes* and are the proposed causative agents of diseases in several hundred plant species [44]. The association of phytoplasmas with dieback and mosaic in papaya has been based solely on PCR amplification of the 16s rRNA gene and adjacent regions using phytoplasma specific primers [17, 36]. The phytoplasmal origin of PCR amplimers from dieback- mosaic and yellow-crinkle affected papaya has been confirmed by restriction endonuclease and DNA sequence analyses [57]. Urasaki [55] reported RAPD technique to determine the sex of a dioecious species, *Carica papaya* L., with three sex types, male, female and hermaphrodite. A 450 bp marker fragment, named PSDM (Papaya Sex Determination Marker) exists in all male and hermaphrodite plants but not in the female plants. Sequence-characterized amplified region (SCAR) markers SCARps were developed from PSDM for sex determation of papaya. Southern hybridization using PSDM as a probe showed that PSDM exists in the male and hermaphrodite genomes but not in the female genome. Results strongly suggest that PSDM is located on the chromosome region that is specific to the male and the hermaphrodite. SCARps are a suitable marker for the precise and rapid diagnosis of sex in papaya. Parasnis [49] reported PCR-based Seedling Sex Diagnostic Assay (SSDA) especially designed for early sexing of papaya seedlings. A male-specific SCAR marker in papaya by cloning a male-specific RAPD (831 bp) fragment and designing longer primers were developed. The potential of this SCAR marker is exploited to develop a simplified and highly accurate sex diagnostic assay for early identification of female seedlings of dioecious species.

Guthrie [25] developed the *Phytoplasma* DNA detection using the polymerase chain reaction (PCR) with primers specific for *phytoplasmas* and for the stolbur group of *phytoplasmas*. The dieback-associated *phytoplasma* was detected one week prior to (four cases) or the same week (nine cases) as symptom expression, while phytoplasma DNA was detected between 3 and 11 weeks prior to expression of mosaic symptom (six cases). Lateral shoot regrowth on the lower stem of plants had suffered dieback disease failed to generate stolbur-specific PCR products in 15 cases. A dual infection with dieback and yellow crinkle or mosaic was diagnosed in a further two cases using restriction fragment length polymorphism digests and both cases were interpreted as secondary infections by the dieback-associated *phytoplasma*. Regrowth in three of seven cases of yellow crinkle and three of nine cases of mosaic-affected plants tested positive for *phytoplasma*-specific DNA. Ratooning of dieback-affected plants and removal of yellow crinkle or mosaic-affected plants is suggested for the management of these diseases. White [58] reported three papaya plants, individually affected by dieback, yellow crinkle or mosaic diseases were subjected to PCR using phytoplasma-specific primers to amplify the 16S rRNA gene plus 16S-23S rRNA intergenic spacer region. Near-complete DNA sequences obtained for the three PCR amplimers subjected to phylogenetic analyses and direct sequence comparison with other phytoplasma 16S rDNA and 16S-23S spacer region DNA sequences. The papaya yellow crinkle (PpYC) and papaya mosaic (PpM) sequences were identical to each other but distinctly different from the papaya dieback (PpDB) sequence showing 90.3% identity in the 16S rDNA and 87.8% identity in the 16S-23S spacer region DNA sequences.

A phylogenetic tree based on 16S rDNA sequences was calculated in which PpYC and PpM are most closely related to the tomato big bud phytoplasma (TBB; 99.7% 165 rDNA sequence identity) from Australia, within subclade iii. This subclade consists of strains only reported occurring in the Southern Asian region and Australia which indicates an Asian/Australasian origin. PpDB is most closely related to the *Phormium* yellow leaf phytoplasma from New Zealand (PYL; 99.9% identity) and the Australian grapevine yellows phytoplasma. These three phytoplasma strains form a distinct clade within subclade xii, which also includes the European strains STOL and VK as another distinct clade. The origin of the closely related but geographically separated AGY-like strains and STOL-like strains of subclade xii is unclear. It is proposed that phytoplasma strains PpDB, PYL and AGY be included in the previously described taxon Candidatus Phytoplasma australiense *'8* and that PpYc, PpM and TBB be assigned to a new taxon, *Candidatus* Phytoplasma Australasia. Magdalita [38] have developed hybrids of *Carica papaya, C. cauliflora* and interspecific species and screened for resistance to two Australian isolates (338, 445) of papaya ring spot virus-type P (PRSV-P) and results obtained using plate-trapped antigen-enzyme linked immunosorbent assay (PTA-ELISA) that the interspecific hybrid and *C. cauliflora* plants were resistant to the Australian PRSV-P isolates.

Comparison of random amplified polymorphic DNA (RAPD) and DNA amplification fingerprinting (DAF) in papaya for sex determination and results shows that DAF reactions produced five times more bands than equivalent RAPD reactions, permitting more efficient screening as DAF reactions were more reliable. Using bulk segregant analysis it is possible easily to define a large number of DAF markers present in only male or hermaphrodite pooled DNAs. Preliminary analyses for linkage associations indicated that these markers were reasonably closely linked to the sex- determining alleles [53].

Apple and Pear

Polymerase chain reaction (PCR) method was used for differentiating among the North American internal apple-feeding pests codling moth, *Cydia pomonella* (L.), oriental fruit moth, *Grapholita molesta* (Busck), lesser appleworm, *Grapholita prunivora* (Walsh) and cherry fruitworm, *Grapholita packardi* Zeller. They reported that 470-bp fragment of mitochondrial cytochrome oxidase subunit I (COI) was sequenced in three to six specimens of each species. Consistent and diagnostic differences were observed among the species in two regions of COI from which forward and reverse primers were designed to amplify a 112 KD 116-bp segment of the gene. The primer sets were used to selectively amplify DNA from specimens of diverse geographic origin for each corresponding target species. Boben [5] reported the analysis with real-time PCR, results concluded that the automated procedure is less time consuming and advantageous over the CTAB method.

Stone fruit

Stone fruit trees are affected by a large number of viruses that cause important economic losses. At the moment, unlike bacterial and fungal diseases no chemical exists to be applied directly to control viral or viroid diseases. So, the early detection by means of sensitive diagnostic methods is the main way to control them. The extraordinary progress made in the nucleic acid research recently and the application of recombinat DNA technology in plant virology have permitted the use of diagnostic methods based on the genomic component of viruses and viroids. Among them molecular hybridization and polymerase chain reaction (PCR) have received great interest lately and have been incorporated in the diagnostic field of plant virology. Sanchez-Navarro [50] reported the comparative analyses of multiplex RT-PCR containing the eight virus pair primers was even more sensitive than the ELISA or molecular hybridization assays. Multiplex RT-PCR technology is being used in routine diagnosis of stone fruit tree viruses.

Strawberry and Raspberry

Phytophthora fragariae varieties *fragariae* and *rubi* are amongst thirteen species of *Phytophthora* the most damaging to strawberry and raspberry crops respectively. In the absence of fully effective host resistance, control depends on cultural measures and agrochemicals. *Phytophthora* infection is difficult to detect and there is no doubt that contaminated plant material has been the main means of disease dissemination. Provision of disease-free planting material would provide the ultimate control measure as land remains free of contamination. Cook [12] has developed DNA-based diagnostic method for detecting *Phytophthora* in strawberry. This technique is very rapid and sensitive that it detects very low levels of *Phytophthora* contamination in short period of time.

CONCLUSION

Among horticultural crops, fruits are of prime importance for an adequate and balanced human diet. In certain parts of the world, fruits are the major dietary staple. Apart from being a rich source of vitamins and minerals, the production of fruits also contributes significantly to regional and national economies through national and international trade.

However, the cultivation of these crops for optimum yield and quality is highly technical and needs improved technological support. Management of perennial fruit crops requires further close monitoring especially for the management of diseases which affect production and subsequent post-harvest losses significantly. The latest diagnostic tools and management strategies of almost all the important temperate, tropical and subtropical fruits. New and exciting diagnostic technology for plant pathogens will continue to develop, as there is sufficient demand for diagnostics in the field of subtropical fruit crops to drive innovation. The number of organizations developing diagnostic technology for plant-based agriculture is small, but nonetheless, selective adaptation of medical technology will take place-the driving factors being applicability and cost (including intellectual property considerations). Many laboratories have adopted ELISA and ordinary PCR for routine use, whereas the highest tier laboratories, including centers of excellence and those responsible for identification of regulated pathogens, are currently using qPCR on a routine basis, usually under appropriate SOPs. Inexpensive field tests such as LFDs should be developed that can precisely diagnose new disease outbreaks to rapidly stimulate decision-making processes for disease management. The next phase in technology adoption is likely to be a move towards generic platform technology such as microarray. Microarray technology is currently for wide utilization for plant pathogen diagnosis, for development of diagnostic chips reduces and demand increases, adoption rates will also increase. Local or regional development and manufacture of diagnostic assays in poorer regions of the world will be necessary to keep costs low enough for adoption of the technology. Supply chain improvements that increase the ease of access to and cost of basic diagnostic supplies and reagents are critical to technology adoption as well. The developing world is leading the way in adaptation of mobile phone technology for information exchange and innovations in this area to enhance disease diagnosis, surveillance and management may find their way into agricultural production systems in developed countries. Whatever the platform, diagnostic technologies will continue to advance and the extent of their applications will be driven by ease of use, cost and the implications of the results they produce. Tests that directly inform a decision, whether it is the implementation of phytosanitary measures, change in cultural practices or application of a particular fungicide for example are the most likely to be adopted.

CONCLUDING REMARKS AND FUTURE PROSPECTS

Diagnostic methods for plant viruses are being continuously improved. In recent years, considerable progress on nucleic acid research had advanced newer methodologies in detecting the genomic components of plant viruses. Although molecular hybridization and PCR have gained new levels of sensitivity compared to serological ones, an acceptable level of automation is lacking. For a stone fruit certification programme, a compromise between simplicity of automation and sensitivity must be chosen. As a general rule, certified or certifiable material may be assayed by serological or non radioactive molecular hybridization
methods whereas more sensitive techniques but also less affordable such as those derived from the PCR approach could be used for primary sources or pre-basic material as well as for
imported, dormant bud wood during post entry quarantine or sanitation purposes.

The simplicity and sensitivity of new molecular methods have been sufficiently improved to detect most of plant viruses at levels below economic thresholds. The goal for the coming years will rely on making these methods more accessible to non specialized laboratories.

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