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Molecular Diagnostics of Fungal Pathogens

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Abstract

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The recent advent of molecular biology has contributed to the diagnosis of plant pathogenic fungi by offering new revolutionary methods for quicker and more accurate detection, identification and quantification. Although molecular diagnostics have been applied to fungi of diverse ecology, their application to pathogens of terrestrial ecosystems has been more striking due to the complexity of their environment over those of airborne nature. Several classical approaches differing in effectiveness had been developed over the years to detect and enumerate soil borne fungal pathogens. Selective growth on specially devised media aiming to exclude the vast majority of soil organisms and allow development of targeted fungi was among the approaches most widely applied. However, dealing with soil fungi has always been a challenging issue because of the complex environment where these pathogens grow. In most cases, recovery on selective media was proved to be method dependent, the target organism was often outgrown by better competitors, morphological characters could be common for several species and bias of the researcher were factors that influenced the outcome of the results. Therefore, the application of molecular techniques to diagnostics of soilborne fungi was rather inevitable. Furthermore, DNA technology was expanded to the detection of fungal pathogens living in various environments i.e. inside plant tissues, on leaf surfaces, in seeds, in irrigation water but also bearing special features such as toxigenicity or resistance to chemicals. Additionally, protocols for molecular detection of quarantine fungi were devised and evaluated for various fungal pathogens. As initial molecular markers, isoenzymes and DNA probes were used to detect and differentiate various fungal species. Subsequently, molecular diagnostics based on the PCR technique have further accelerated the process and facilitate a more sensitive method of detection. Fungi can be identified at the species level by primers designed on selected conserved sequences like the rRNA gene cluster followed by further characterization of the amplified fragment. The rRNA gene cluster became very popular for a number of reasons; it has several hundred copies per genome and it carries highly conserved and variable regions. Sequences of the rRNA subunits have been used for taxonomic and genetic studies, while conserved regions of the internal transcribed spacers (ITS) and the intergenic spacers (IGS) have been targeted for fungal detection. PCR-based fingerprinting techniques (RAPDs, SSRs, AFLPs.) offering higher sensitivity and better resolution, have also been developed. Recently, DNA array technology (also known as biochip or DNA chip) aiming to monitor the whole genome on a single chip, has become available and applied to the molecular diagnosis of fungi. DNA chips are fabricated by high-speed robotics, generally on glass, on which probes with known specificity are hybridized to targeted complementary sequences. In this way, massively parallel gene detection is accomplished and many different microorganisms are identified. Experiments with a single DNA chip can result in a dramatic increase in throughput by providing information on thousands of genes simultaneously. This review presentation focuses on the application of various techniques for the molecular diagnostics of fungal pathogens. It is based on information found in the literature combined with personal research data of the author.

Key words: Detection, identification, fingerprinting, PCR, RAPD, AFLP.

Introduction

Fungi outnumber all other types of pathogens that attack plants and cause a very serious economic impact on agricultural production due to their ability to induce diseases of cultivated crops that result in important yield losses. Among fungi, those that are soil-borne are more difficult to handle for a number of reasons. Soil is a very complex environment that bears difficulties in the identification, isolation and quantification of pathogens. In this respect, detection and enumeration of soil fungal inhabitants have always been challenging issues through the years (49). On the other hand, there has always been a need for fast and accurate recognition of fungal pathogens of airborne nature and especially of those that can not be cultivated in synthetic culture media. This demand has been driven by the need for research on various aspects of plant diseases (e.g. epidemiology, dispersal, population dynamics) with the final goal to develop strategies for effective control.

Over the years, several classical approaches have been developed to detect and identify fungi that cause diseases of plants. Selective media have been devised to exclude the large number of contaminant organisms (mostly saprophytes) and allow growth of target fungi. However, in most cases (especially for soil-borne micro-organisms) recovery has been associated with the method of choice, contaminants as better competitors often outgrow the target pathogen, more than one species share common morphological characters while the bias of the researcher who uses the method is an unavoidable piece in the microbial detection puzzle (96). Obviously, for unculturable pathogens researchers confront even more striking difficulties.

The trend for globalization in agriculture created even higher demands in various aspects of plant protection. With the advancement of international commerce that practically cancelled the geographic boarders between countries (viz. EU), legislation set most strict rules concerning movement of plants and plant material. Phytosanitary inspections and quarantine regulations became more stringent demanding increased control measures not only between countries but also within the territories of a certain country. The use of healthy propagation material, the inspection of planting seed for quarantine pathogens, the prevention of spread of a pathogen to another country, the screening of mother plants for certain pathogens, the monitoring of resistance phenotypes of a fungus to certain agrochemincals are among challenges encountered on a routine base. Furthermore, research frontiers have been widened to more detailed and in-depth studies of host-parasite interactions, disease resistance, pathogens population structure. Thus, the development of methods capable to detect and identify pathogens in plant

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materials in a fast, accurate and sensitive manner, has been necessitated more that ever.

During the last decades, the advent of molecular biology promised to offer radical alternatives in the detection and enumeration of fungal pathogens. At the same time, the acquisition of DNA sequences provided information that led to the identification of new and unknown species. These data, together with classical characterization of fungi in the field, opened new insights into the range of fungal functions and interactions mostly within terrestrial communities (3) but also for fungal plant pathogens as a whole.

The present review paper focuses on the application of various molecular techniques in the detection, identification, characterization and quantification of fungal species that cause diseases of plants. The use of various molecular biological methods i.e. nucleic acid probes, molecular markers, PCR, microarrays and their application in plant pathology are discussed. Examples of molecular applications in the detection, identification, quantification, characterization of pathogens as well as in health testing of propagation material, detection of fungicide resistance, study of fungal population structure, identification of mating types of fungi, production of mycotoxins, determination of early stages of infection in symptomless hosts, recognition of gene flow and inoculum movement, discovery of virulence genes and dissection in host-parasite interactions are cited. In this review, the most common techniques used to develop molecular diagnostic tools for plant pathology are outlined, their applications in disease diagnose are described and the future potential of those methods in developing strategies for more efficient disease control is discussed. The paper is based on information found in the literature supplemented by personal research findings of the author (74).

Molecular Techniques

Isozymes

The first molecular markers that initially were used extensively to study systematics of plants, animals and insects were isozymes (or isoenzymes) (72). As such are defined multiple forms of enzymes capable of catalyzing essentially the same reaction but which may differ in various chemical, physical, or immunological characteristics and arising from genetic control of primary protein structure. For isozyme analysis, choosing the appropriate enzymatic system to study is of grate importance for drawing genetic inferences (58). Isozymes that represent multiple loci usually generate complex banding patterns that are difficult to explain. In general, regulatory enzymes analysis has been applied for the identification or fingerprinting of subspecies taxa like races of formae speciales (58).

Isozyme analysis was initially utilized to study the taxonomy of plant pathogenic fungi (59, 97). The method involves extraction of crude proteins and separation by electrophoresis on starch, non-denaturing polyacrylamide gels (PAGE) or isoelectric focusing. Isozyme zones are visualized after supplying the appropriate substrate necessary for the specific activity of each enzyme. The resulting zymograms (isozyme banding patterns) are used to infer genetic relationships based on interpretations of banding polymorphisms assuming that isozyme zones correspond to equivalent loci.

Analysis of isozyme variation found application in distinguishing fungi bearing overlapping morphological or cultural characters, such as species of the genus *Phytophthora*. In several of these species, morphometric data (dimensions of sporangia) or sexual characteristics were not adequate to separate isolates (15). By comparing 18 isozyme loci, three species of *Phytophthora* (*P. cambivora*, *P. cinnamomi*, *P. cactorum*) could be separated and subsequently the systematics of twelve papillate *Phytophthora* species were re-evaluated (72, 73). Kaufmann and Weidemann (42) subjected thirty three isolates of the fungus *Colletotrichum gloeosporioides* isolated from five plant genera to isozyme analysis. Cluster analysis of the 148 phenotypes generated for 11 different isozymes grouped isolates on the basis of host origin (with only one exception). This study pointed out that isozyme profiles could be a useful tool to characterize intraspecific population diversity of fungal pathogens. In a similar study, Julian and Lucas (41) carried out a population analysis of 101 isolates of the causal agent of the eyespot disease of cereals, *Pseudocercosporella herpotrichoides*, belonging to two pathogenic types W or R on the basis of their differential pathogenicity on wheat of rye, respectively. Of 16 isozymes tested, zone polymorphisms for five enzymes could clearly differentiate W-type from R-type isolates. These results were further confirmed by pathogenicity tests suggesting the value of isozyme analysis population genetics of fungi. We carried out an isozyme analysis to differentiate isolates of *Pyricularia oryzae* from ctenanthe from fungal isolates that originated from rice plants (76). Applying UPGMA analysis on the banding patterns generated by five different isozymes (α-estrase, and lactate, malate, isocitrate, sorbitol dehydrogenases) ctenanthe isolates were found to form a separate group distinctly different from that of the rice isolates. These results were further supported by RAPD analysis of the isolates from both hosts using five different random primers.

Because isozymes are post transcriptional markers, their expression is influenced by environmental changes leading to polymorphisms that might not reflect real differences at the molecular lever. This was the basic reason that application of isozymes in studying fungal plant pathogen variation was rather limited although in general they would provide satisfactory levels of polymorphic loci.

DNA probing

The development of protocols for extracting DNA from various sources i.e. pure fungal cultures, infected plant tissues, infested soil or air samples, spun-off the possibilities of developing molecular diagnostics initially through nucleic acids hybridization. DNA probes were among the first molecular techniques applied in the detection, identification and phylogenetic analysis of fungal pathogen (55, 87). As a general approach when first designing a random genomic DNA probe, the target organism is grown in liquid culture and subsequently fungal biomass is collected, freeze dried and pulverized in liquid nitrogen. Total DNA is then purified following a well worked out protocol to ensure good quality and sufficient quantity of nucleic acids and then digested with selected restriction enzyme following by random cloning of restriction fragments. Subsequently, clones are evaluated in Southern hybridization assays as putative probes (35, 83). Nowadays, generation of DNA probes is based on sequences amplified by PCR (105). For unculturable organisms (such as powdery or downy mildew pathogens), DNA is isolated from spores directly collected from infected plant tissues (36, 91). If DNA of soil-borne pathogens is extracted directly from soil samples, attention must be drawn to co-extraction of humic acids or other inhibitory substances. In this case, appropriate modifications

of DNA isolation protocols (e.g. use of resin columns) have been developed (14, 98).

DNA probes have been widely applied in molecular fungal diagnostics especially before the development of the PCR technique. Probes bearing complementary to the target organism DNA sequences and tagged with reporter molecules (initially radioactive that have mostly been substituted by non-radioactive tagging elements) are hybridized to DNA of the sample usually immobilized on an inert surface (nitrocellulose of nylon membranes). Species-specific DNA probes obtained by cloning random DNA fragments generated from restriction endonuclease digestions of genomic DNA had a number of advantages over classical approaches. Because of their high specificity, a pure culture of the target organism was not necessary, once the DNA probe had been developed. The need that the fungus produces characteristic structures in culture e.g. spores, fruiting bodies, sclerotia etc. to facilitate identification was eliminated by the fact that DNA could be extracted essentially from any form of living mycelium. Cloning of repetitive sequences spread all along the fungal genomes improved the speed, sensitivity and objectiveness of detection and identification of fungi by using DNA hybridization compared with the traditional methods.

Restriction fragment length polymorphisms (RFLPs) are DNA markers coupled with probes to generate patterns after cleavage of a DNA sequence with restriction enzymes. Specific differences occurring in the sequence of DNA lead to alteration of fragment sizes when usually genomic DNA has been subjected to digestion with restriction enzymes (4, 5). Restriction fragments are size separated by electrophoresis, transferred to nylon membranes by capillary forces and immobilized by UV cross-linking. Specific DNA probes are then hybridized to the membrane bound DNA fragments and bands are visualized by appropriate methods. Similarities and differences observed in the banding patterns can be used to differentiate species, races, and strains from each other. RFLPs have found wide application in the detection and characterization of fungi (27, 83, 103, 108).

Among the numerous examples about the use of DNA probes in plant pathogens is the detection of several species of the genus *Phytophthora* both in soil and in host tissues (*P. parasitica*) (26). Judelson and Messener-Routh (40) using DNA hybridization technology, made an initial attempt to quantify pathogen growth in infected plant roots. Mitochondrial sequences became progressively more appealing than genomic DNA fragments because of a number of advantages harbored in the mitochondrial genome. Besides of the presence of the mtDNA in high copy numbers, these sequences have been found to produce less complicated restriction fragment patterns when digested with endonucleases (50). Mitocondrial DNA probes were proved useful tools to differentiate between species that displayed continuous overlapping variability of morphological characters as this has been evident between *P. cryptogea* and *P. drechsleri* (60).

Fusarium oxysporum, the causal agent of vascular wilt of a large number of plant families forms morphologically indistinguishable isolates that are differentiated only by their capability of infecting different plant species (formae speciales) (9). Using a 3.38 kb mitochondrial probe cloned from a forma specialis of *Fusarium oxysporum* that infects cucurbits (f. sp. *cubense*), Bentley *et al.* (6) were able to determine RFLPs in restriction digests of total DNA from 28 isolates of *F. oxysporum* from a variety of hosts and geographic origin. The probe showed mtDNA polymorphisms within and between different special forms allowing the specific detection of isolates that could only be differentiated by only the time consuming and labour intense pathogenicity tests. Another application of mtDNA probe technology is that used for the causal agent of take-all disease of wheat (*Gaeumannomyces graminis* var. *tritici*). The developed probe specifically hybridized to all three varieties of the pathogen while it showed little homology with DNA from other soil-borne fungi (35). In this respect it was used as a diagnostic tool for the isolates recovered from infected wheat roots.

Polymerase chain reaction

The polymerase chain reaction (widely referred to as PCR), a technique introduced in the mid-1980's by Kary Mullis (68), involves the exponential amplification of specific DNA sequences by synthesizing DNA *in vitro*. The procedure takes place in three essential steps: the initial melting of the double strands of the DNA follows the annealing (hybridization) of two synthetic oligonucleotides (primers) with sequences complimentary to the ends of the target fragment and the cycle is completed by the final primer extension (polymerization) by the DNA polymerase.

PCR is undoubtfully the most important technique in diagnostics and has found wide application as a powerful molecular tool mostly due to the development of thermo tolerant DNA polymerases and automated thermocyclers. PCR is preferred over classical or other molecular techniques in the diagnosis of plant pathogens for a number of advantages that makes it very popular (34). Since high quality of DNA is not generally required, there is no need for culturing the target pathogen. PCR cycles are completed in much shorter time than other molecular techniques, thus allowing a very fast screening of a large number of samples. Because of its high sensitivity, (RCR has the theoretical potential to detect a single DNA molecule), minute amounts of the target DNA are required. In conjunction with computer technology, designing of primers permits detection of either a sole pathogen or a group of related microorganisms. Due to its wide application and technological development, PCR becomes less and less inexpensive. Among the uses of PCR in plant pathology are the detection and identification of fungal pathogens in infected plant tissue, the determination of mixed infections, the monitoring of pathogenesis and pathogen ramification in the host, the phylogeny of pathogens, the *in planta* quantification of fungal biomass and the estimation of inocululm density in terrestrial environments.

The ribosomal DNA gene cluster (rDNAs) is an extensively used target sequence for PCR detection of fungal plant pathogens because of a number of useful features. rDNAs bear common sequences found in the nucleus and the mitochondria of eukaryotes. The nuclear rDNA cluster is present as tandem repeats of several hundred copies in cell, which allows high sensitivity of detection. The rDNA gene is consisted of three subunits: a large (LSU) of 28S and a small (SSU) of 18S that are separated by a much smaller gene of 5.8S. The three subunits are connected together with two internal transcribed spacers (ITS1 and ITS2). This whole gene cluster is repeated in the genome many times thus being an appealing target for PCR amplification. Highly conserved regions located in the LSU make feasible to investigate relationships of distantly related genera. On the other hand, the presence of variable sequences like the ITS regions between the subunits and the IGS (intergenic spacers) between the gene clusters permit discrimination of

closely related species of a certain fungal genus (8). Among all the above targets, ITS sequences have gained popularity for being more variable regions and therefore allow selective detection of closely related organisms. Universal primers designed on conserved sequences found on the small and large subunits, have been extensively used for the amplification of ITS regions. The amplified sequences are between 500-800 bp, a relatively small amount of target DNA is required for PCR, while the PCR products have been used as species-specific probes (10, 21, 102). Moreover, determination of ITS sequences after amplification by universal primers, has allowed the detection, identification and taxonomy of unculturable or unknown fugal species.

To differentiate between two closely related species of the soilborne fungal genus *Verticillium* i.e. *V. dahliae* and *V. albo-atrum*, Nazar *et al.* (69) showed that as little as five nucleotide differences at the ITS region were sufficient enough to design species specific primers. The study was extended to a third species, *V. tricorpus* also a pathogen of potatoes (64) revealing 17 bp differences between *V. dahliae* and *V. tricorpus* and 12 between *V. albo-atrum* and *V. tricorpus* at the internal transcribed spacers. Based on that, Robb *et al.* (86) designed primer sets for ITS-targeted PCR assays able to detect and differentiate *Verticillium* pathogens in potato.

PCR-based diagnosis has found wide application in fungal genera where morphological or biochemical characters lead to difficult or even confusing conclusions. Among the numerous examples that appear in the literature are the designing of species specific primers that could amplify *F. oxysporum* f. sp. *vasinfectum* DNA in cotton tissue but not from other cotton mycoflora (62), to differentiate *F. culmorum* and *F. graminearum* from *F. avenaceum* (88) or for the early detection and diagnose of *Tilletia tritici*, the causal agent of common bunt in wheat (39).

The strong potential of PCR technique can be extended beyond diagnostics of plant diseases. PCR offers the sensitivity, speed and accuracy required in other applications as in the case of inspection of propagation plant material for quarantine pathogens or the detection of early or latent infections. We have exploited a variant of PCR technique to develop a methodology to detect and identify pathogens of esca (also called young grapevine decline) in propagation material (13). The decline has been reported in all the major wine-producing regions of the world and its dramatic upsurge the last few years has been associated with the extensive replanting with phylloxera resistant rootstocks. The main pathogens involved in the disease are the ascomycetes *Phaeomoniella chlamydospora* and *Phaeacremonium aleophilum* (65). The symptoms are almost identical with those produced by black-foot disease, a recently identified, significant problem of viticulture that is attributed to *Cylindrocarpon destructans* (2, 85, 93). *Phaeomoniella chlamydospora* and *Phaeacremonium aleophilum* can occur as endophytes in asymptomatic canes of mother vines (19). A major means of the disease spread is believed to be via infected propagation material (19, 65). However classical diagnostic methods based on symptom recording and isolations indicate low incidences of these pathogens in rootstock cuttings (29, 30).

To detect pathogens related to young esca in grapevine rootstocks we developed a two step procedure based on PCR, known as nested PCR (18). This technique is applied when a single pair of primers is not able to give sufficient specificity or sensitivity. In a first step, we used a pair of universal ribosomal primers (102) to amplify any fungal DNA present in grapevine tissue. Consecutively, the product of the initial PCR was subjected to a second round RCR using species-specific primers (31, 95) that would hybridize within the region amplified by the first primer pair and detect the presence of *P. chlamydospora*, *P. aleophilum* and *C. destructans* in grapevine rootstocks. We carried out a survey of grapevine propagation material at different stages of production comparing the classical approach with the molecular methodology we developed. Classical methods of detection were proven insufficient to explain the wide spread of young grapevine decline. The main reason is that pathogens responsible for young esca are slow-growing and difficult to isolate. Applying our nested-PCR protocol, high percentages of infection were detected at very early stages of rootstocks production, contrary to what has been known so far (29, 30). Both pathogens were detected even in asymptomatic cuttings, while the results reinforced the hypothesis that infected mother vines are indeed the major means of disease spread (19). The time required for diagnosis has been reduced to two days. This novel approach could be used as an efficient method to certify the production of healthy vine propagation material and control the quality of rootstock cuttings that are imported from other European countries.

Intergenic spacer region-based PCR (IGS-PCR), although much less popular than ITS sequences, has been exploited for its capability to determine variability of closely related species. In conjunction with subsequent restriction digestions, PCR-RFLP analysis of IGS amplification products revealed species variation in populations of the wilt fungus *F. oxysporum* (1). We have developed an IGS-PCR based diagnostic tool (110) to detect and differentiate the two new vegetative compatibility groups of the cotton wilt pathogen *F. oxysporum* f. sp. *vasinfectum* that have been found in the mid-90's in Australia (47). Since these new races are not present in Greece and the pathogen is also of a seed-borne nature, it is of imperative importance to have available an appropriate methodology to test cotton planting seed imported to the country. It should be noted, however, that IGS region-assisted PCR should be used with additional knowledge of these sequences, since there are cases where heterogeneity in the number of subrepeat units has been found either within (intra-specific) e.g. *Pythium ultimum* (46) or between species (inter-specific) e.g. *V. dahliae* and *V. albo-atrum* (45, 63).

Based on gene sequences other than the rDNA gene cluster, PCR has expanded its use to other aspects of plant pathology. PCR technology has found application to epidemiological studies, to population structure and dynamics of fungal pathogens, to monitoring development of fungicide resistance, to researching phylogenies, to detecting toxigenic fungi.

As an example, epidemiological and population genetics studies have been conducted for the obligate parasite *Plasmopara viticola*, the causal agent of downy mildew of grapevine (23). PCR has contributed in expanding population studies of this pathogen with the development of species-specific, codominant microsatellite markers that permit molecular genotyping of the fungus (25).

When disease management includes the use of chemical compounds as a means of pathogen control, there is always the risk that the fungicide might loose its effectiveness due to development of resistance by the target organism. Thus, it is most desirable to have a tool to monitor the population of

the pathogen in order to achieve the best use of fungicides. Risk for fungicide resistance development has been assessed with conventional screening methods that bear a number of limitations. They usually demand a lot of time and if the target organism is an obligate pathogen are rather expensive. PCR coupled with probes that are allele-specific, PCR-RFLP analysis and allele-specific PCR (AS-PCR) are methods used for the molecular detection of fungicide resistance in several pathogens (57) i.e. benomyl resistance in *Botrytis cinerea* (54) and *Venturia inaequalis* (48) or resistance to strobilurins in *Pyricularia grisea* (44). AS-PCR is the selective PCR amplification of one of the alleles to detect single nucleotide polymorphisms (SNPs) (57). Selective amplification is usually achieved by designing a primer such that the primer will match/mismatch one of the alleles at the 3'-end of the primer. Fraaije *et al.*, (20) designed allele-specific primers capable to detect a single base pair change in the mitochondrial cytochrome b gene that was identified in isolates of *Blumeria graminis* f. sp. *tritici* that had developed resistance to strobilurin fungicides. Applying AS real-time PCR, they could recognize strobilurin-resistant alleles at a frequency of at least 1:10.000 sensitive alleles. This assay had a practical application in field samples following the population dynamics of resistance alleles before and after the spray of fungicides. This methodology exemplifies the possibilities harbored in biotechnological development to design dynamic tools for plant pathology aspects such as the early detection of fungicide resistance and therefore contribute significantly to the assessment and evaluation of the risk that a fungicide develops resistance in field populations of a given pathogen.

We have applied molecular analysis to screen fungal plant pathogenic isolates for their resistance to fungicides. Specifically, we used cleavable amplified polymorphic sequences (CAPS) analysis to characterize *Pyricularia* isolates from ctenanthe and rice as for their sensitivity to strobilurins (76). Strobilurins (QoI) are a recently developed group of fungicides with a novel site-specific mode of action. They interfere with respiration by blocking electron transport at the bc1 complex that follows their binding in the mitochondrial membranes of the sensitive fungal species, at a site known as QoI of cytochrome b (CYTB). Using a set of primers developed by Kim *et al.* (44), we amplified polymorphic sequences of the mitochondrial cytochrome b gene. The PCR products were digested separately with the restriction endonucleases *Fnu*4HI or *Sty*I The resulted patterns supported the absence of G143A (detectable by *Fnu*4HI) or F129L (detectable by *Sty*I) mutations in all tested isolates of *Pyricularia* from both hosts (ctenenthe or rice), suggesting a wild-type sensitivity reaction of the isolates to strobilurins.

The advancement of molecular biological techniques has attributed to the development of diagnostic tools for critical toxigenic fungi that contaminate food and feed. For the toxigenic *Fusarium* species it is now possible to apply molecular methodologies to specifically isolate mating genes of the *Gibberella* complex, to detect *Fusarium*-producing fumonisins and *Fusarium*-producing trichothecenes and enniatins (67). Using multiplex PCR assays, Kerényi *et al.* (43) have been able to determine in a single reaction the mating type of a large number of pathogen samples of *G. fujikuroi* and within this complex the anamorph *F. proliferatum* with significant economic importance, since this pathogen has been implicated with a large number of diseased plants. Intraspecific variation in the IGS region of the fumonisin-producing isolates of *F. verticillioides* from cereals and fumonisin non-producing isolates form banana revealed by PCR-RFLP analysis facilitated to discriminate between toxin producers and non-producers isolates (79). For direct PCR detection, Mirete *et al.* (61) designed two sets of primers at the IGS region that could specifically amplify sequences of both *F. vericillioides* species and fumonicin-producing *F. verticillioides* isolates. Because of the continuous demand to obtain more reliable and simple methods for toxigenic fungi, other target sequences have been utilized i.e. ITS region and a portion of the calmodulin gene (66). Several generic primer sets have been devised to *tir5* and *tir6* genes involved in the production of trichothecene and tested successfully against toxigenic *Fusarium* species (67). A primer set designed at the non-coding region upstream the *tri5* gene was screened against toxigenic *Fusarium* species (70). Although the assay could detect *F. sporotrichioides* could not differentiate it from the newly described species of *F. langsethiae* (70), stressing the need for further development of the detection protocol. Wilson *et al.* (106) finally designed primers capable to differentiate the above species from each other and also from other *Fusarium* species.

Another serious mycotoxin, ochratoxin A (OTA) is produced by *Penicillium nordicum* and *P. verrucosum* but also by various species within the sections Nigri and Circumdati of the genus *Aspergillus*, with predominant producers the species *A. ochraceus* and *A. carbonarius* (71). For the detection of OTA producing fungi, a number of diagnostic PCR primers have been designed by utilizing sequences obtained from AFLPs (89), RAPDs (80), the calmodulin gene (82) and the ochrtoxin polyketide synthase gene *otapks*PN (22).

Fingerprinting techniques

The continuous improvements of the PCR technique led to its linking with various approaches for DNA fingerprinting. Random amplified polymorphic DNA (RAPDs) analysis has attracted a lot of attention after its advent during the 90's (104). Among the reasons for that were, the simplicity of the method (usually only one random synthetic oligonucleotide decamer primer is required) and the necessity of small amounts of genomic DNA. The most serious drawback of RAPDs was the inherent irreproducibility of amplified patterns mostly due to the annealing difficulties of the random primers. To minimize variation and increase reproducibility of the method, whenever we conducted a RAPD analysis, we always replicated the PCR reactions using the same DNA stock for each isolate under test and the same thermocycler and batch of reagents for all the different primers tested throughout the whole analysis (75, 76). Manulis *et al.* (56), applied RAPDs to the carnation wilt fungal pathogen *Fusarium oxysporum* f. sp. *dianthi* and they were able to identify specific banding patterns that were subsequently used as probes to distinguish between races of the pathogen. In another study, genetic relationships could be inferred among the wheat bunt fungi using RAPD markers (94), *Alternaria* species pathogenic to crucifers could be differentiated on the basis of RAPD profiles (92). Progressively, other variants of PCR fingerprinting were developed that would generate more reproducible and robust reactions i.e. universally primed PCR (UP-PCR) (109), amplified fragment length polymorphisms (AFLPs) (38), and simple sequence repeats (SSRs), also known as microsatellites (7).

Aiming to design primers with higher specificity, PCR-based fingerprinting technology was coupled with sequence

analysis of specific amplified bands utilized to identify regions unique to the DNA of the organism in test. This approach known as SCARs (sequence characterized amplified regions) refers to DNA fragments amplified by the PCR using specific 15-30 bp primers, designed from nucleotide sequences established in cloned RAPD fragments linked to a trait of interest (51). By using longer PCR primers, SCARs do not face the problem of low reproducibility generally encountered with RAPDs. Obtaining a codominant marker may be an additional advantage of converting RAPDs into SCARs. Cloning and characterization of pathotype-associated RAPD markers was employed by Pérez-Artés *et al.*, (81) to design primers that would allow differentiation between pathotypes of *V. dahliae* defoliating and non-defoliating to cotton and olive.

Molecular quantification of fungal biomass

Estimation of pathogen inoculum density is a major component associated with disease prediction but also assessment of effectiveness of measures to control disease. Quantification of inoculum is essential mostly for soil-borne pathogens although it is well established that quantity of initial inoculum has a critical importance in the subsequent epidemic caused by pathogens disseminated by the wind. However, there are quite a number of cases where a large variation has been observed between traditional methods attempting to measure inoculum of a pathogen. This problem is exemplified by the methodology that has been used to quantify microsclerotia of the soil-borne pathogen *Verticillium dahliae* in field soils, as this has been documented by an interlaboratory study we participated in, which was carried out to compare the methods of quantification in the same soil samples with known concentrations of microsclerotia (96). The outcome of this survey pointed out that inoculum density estimations for *V. dahliae* found in the literature present only a vague picture of reality (28).

The aforementioned situation stresses the necessity for developing methods for quantification of plant pathogens that would be accurate, robust and reproducible by various researchers who would apply them. The advent of molecular biology appeared able to offer the tools for the development of such methods. Indeed, species-specific PCR primers were designed based mostly on ITS sequences of the target organism and used for molecular quantification of the pathogen using a variant of PCR, known as quantitative or competitive PCR. This type of PCR was developed by the inability to relate the final amount of amplified DNA to the amount of sample DNA initially present at the beginning of the reaction. The main restriction in obtaining quantitative results is inherent in the PCR amplification process. Due to at least at the beginning exponential nature of the amplification process, small differences in any of the variables that control the reaction rate will dramatically affect the amount of the final PCR product (24). Among the variables that influence the rate of PCR are not only the reagents (e.g. concentrations of polymerase, dNTPs, Mg^{++}, DNA, primers) or the amplification conditions (e.g. annealing, extension, amplification temperatures) but also a tube-to-tube variation has been observed to occur, especially when samples are prepared as a pool and subsequently aliquoted into separate tubes, albeit amplified in the same run. As the PCR reaction leaves the log phase, a plateau is reached due to exhaustion of reagents resulting in amounts of product that are no longer proportional to the initial concentration of the template DNA (57). Competitive PCR is a devised variant of the original PCR able to obviate the above mentioned problems and allow precise quantification of unknown amounts of DNA samples.

In competitive PCR, target DNA sequences are co-amplified with a usually smaller DNA fragment (know as internal standard) using the same pair of primers. The amplified competitor is distinguished from the target DNA fragment by size (it is usually smaller). A possible change in any of the variables previously listed will affect the yield of both target DNA and competitive template equally. However, the relative rations of both co-amplified fragments will be preserved with amplification (24). Since the starting concentration of the internal standard is known, the initial concentration of the target DNA can be estimated. For quantification, a standard curve is generated by co-amplifying various amounts of sample DNA against a fixed concentration of competitor molecule and then plotting the ratio of sample/internal standard DNA versus the sample DNA added to the initial mixture. The point of equivalence (where ratio is 1:1) determines the concentration of the unknown sample. Using this methodology, Robb *et al.* (86) quantified the biomass of three species of *Verticillium* pathogenic to potato with the aid of a heterologous competitor (*Verticillium*-specific primer borders were adapted to a DNA fragment from an unrelated fungus). Later on, improved protocols to extract DNA from soil were developed, allowing quantification of *V. dahliae* DNA sequences on a large-scale basis (33, 98).

Aiming to molecularly quantify *V. dahliae* DNA and relate it to microsclerotia (the resting structures of the pathogen responsible for disease development) in field soils, we utilized the above technology but improved PCR co-amplification efficiency by designing a homologous competitor (bearing *V. dahliae* sequences) that would increase sensitivity. Following the method of Förster (17), we designed a linker primer within the ITS region of the rDNA gene cluster of *V. dahliae.* The linker was subsequently used to amplify a homologous internal standard (IS) fragment carrying the same borders with the target sequences but of a smaller size (237 bp) than the *V. dahliae* target sequences (347 bp) (78). IS was tested on DNA extracted from different microsclerotia concentrations of *V. dahliae* produced in pure culture of the pathogen and was able to be co-amplified with the target sequences. Subsequently, a standard curve was generated based on the co-amplification of DNA extracted from known numbers (series of 10 up to 100) of microsclerotia against a standard concentration of IS (40 pg DNA). The concentration of microsclerotia was plotted over the ratio of the PCR products (microsclerotia DNA/IS DNA). To evaluate the efficiency of the method, DNA was isolated from soil artificially infested with known amounts of microsclerotia and subjected to competitive PCR. Amplification products could be related quite well to inoculum densities. At present, the usefulness of the method in field soils is being tested by applying competitive PCR to soil samples naturally infested by *V. dahliae* and comparing the data with those obtained from the conventional quantification methods.

Real-time PCR

The introduction of the real-time PCR technique has revolutionized the molecular quantification methods relying on PCR. Real-time PCR is a sophisticated development of conventional PCR that utilized the advancements of technology to offer the unique possibilities to monitor the amplification process and follow accumulation of the product

as it happens (101). The ammount of PCR product is measured in the test tube during amplification using two different technologies. Either exploiting the 5′ nuclease activity of the *Taq* DNA polymerase by coupling it with specific fluorescent probes (37), or by using fluorescent DNA binding dyes (known as kinetic PCR) (101). In either case, the need for agarose gel electrophoresis of the amplified final product has been eliminated (107). The most known and widely used detection system of real-time PCR is the "Taqman" technology (100) which is based on a FRET (fluorescent resonance energy transfer) probe (101). This reporter system is composed by a short oligonucleotide probe coupled with two fluorescent molecules; a reporter at the 5′ and a quencher at the 3′ of the probe and bearing sequences complementary to one of the amplified strands. Before amplification, fluorescence by the reporter fluorochrome is quenched by the quencher molecule because the two of them are very close to each other. During amplification, the probe that has hybridized on the template DNA strand is degraded by the 5′ nuclease activity of the *Taq* DNA polymerase resulting in the separation of the reporter from the quencher fluorochrome, therefore the former becomes able to fluoresce. The product accumulation is monitored indirectly by using an appropriate optical sensing system rather than measuring the formed DNA directly. When a fluorescent intercalary dye (e.g. SYBR green) that non-specifically binds to double-stranded DNA is used, a rather direct detection is followed, since the fluorescence of the dye attached to the amplified target DNA is measured. Finally, a third detection technology first found in the so called "molecular beacon" is that based on a FRET probe in which the two fluorochromes are kept in close proximity via a stem-loop formation. When the target sequences are amplified, the probe hybridizes to the complimentary sequences of the produced DNA resulting in the separation of the hairpin configuration and thus allowing the generation of fluorescence. A number of reporter systems based on a hairpin structure have been devised afterwards (100). The final aim, which is the quantification of the unknown DNA, comes from the fact that the initial amount of DNA in the sample is related to the number of cycles needed for the fluorescence to reach a specific cycle threshold (the C_T value), defined as that cycle number at which a statistically significant increase in fluorescence is detected (57). After generating a calibration curve by plotting C_T against known amounts of template DNA, target DNA can be quantified (32).

Due to advantages inherent in real-time PCR, its applications to fungal molecular detection, identification and enumeration is continuously expanding in spite of its still high cost over conventional PCR. Amplification of gene copies is accurate and reproducible, post-PCR handling of samples is not required thus risks of possible product carry-over contaminations are restricted and data are obtained in much faster assays with high throughput. Being much more sensitive than conventional PCR, real-time PCR permits multiplex reactions (allowing simultaneous detection of more than one target organisms), limited at the moment only by the number of fluorescent dyes and energizing light sources. As mentioned before, the most serious drawback of the technique is the necessity of specialized, high cost equipment. However, compared with the early stages of the real-time PCR appearance, both size and cost of instrumentation has been reduced forecasting that it is only a matter of time when real-time PCR machines will be within the standard equipment of a general laboratory, as this was seen to happen when conventional PCR thermocyclers replaced the large-tube units (101).

As real-time PCR is expanding its use from human genetics to various biological aspects, an increasing number of examples of its application to plant pathology can be found. Real-time PCR use for diagnostics of soil-borne pathogens has been an inevitable development. Detection and identification of *Rosellinia necatrix* by using a Scorpion primer reporter system, was shown to be very specific (over a large number of various fungal species) and highly sensitive (10 times more) compared with conventional PCR (90). *Fusarium solani* f. sp. *phaseoli* could be detected and quantified directly in soil by Filion *et al.* (16), using as target sequences the translation elongation factor 1 alpha gene of the pathogen in real-time PCR assays monitored by the fluorescent SYBR Green I dye. Besides the speed of DNA extraction protocol from soil (less than 2 h) and the possibility of its adaptation to other microorganisms, the most important feature of this assay was its high specificity, since there wan no cross-reactivity with other DNA from the soil microflora.

Diagnostics in the post PCR era

While the unrevealed potential of the original PCR technique and its variants is still unfolding, the era beyond PCR has already started with the evaluation of several even more sophisticated molecular approaches in fungal diagnostics. The utilization of a novel biomarker, a gene coding for a green fluorescent protein (GFP) isolated from *Aequorea victoria*, a jelly fish that leaves in the Pacific Ocean, has offered new possibilities in the area of host-pathogen interactions, permitting histopathological studies, pathogen monitoring during pathogenesis and ramification and quantification of pathogen in the host tissues like never before. The discovery that heterologous expression of the cloned *gfp* gene can lead to striking green fluorescence production in the recipient organism (11, 84), has made GFP-labeling a very attractive biomarker. The usual methodology used involves transformation of the target organism to achieve constitutive gene expression for GFP. Subsequently, fluorescence is being monitored via UV-microscopy or if available optical sectioning with the more advanced confocal microscope. Moreover, quantification by fluorometry of fluorescence extracted from plant tissues infected with GFP-tagged pathogens can be directly correlated to fungal biomass. Such an approach has been used to quantify the growth of *Colletotrichum*, a foliar pathogen of tobacco during infection of the leaves (12).

We have applied a similar methodology in our Laboratory to study pathogenesis and ramification of the soilborne vascular wilt pathogen *V. dahliae*. Using *Agrobacterium*-mediated transformation, we transferred the enhanced green and yellow fluorescent variant genes (*egfp* and *eyfp*, respectively) to several strains of the pathogen. After an initial screening to select transformants showing pathogenicity equivalent to that of the wild-type parental strains, we monitored the progress of pathogen invasion and host-plant infection by UV-microscopy. Additionally, we could observe fungal development inside the vascular tissues of the plant and quantify fungal biomass by measuring fluorescence of extracted protein with a fluorometer. To our knowledge, this is the first application of histopathology and quantification of a vascular wilt pathogen using GFP-labeling technology (77). Recently, we gained access to the first coral-derived red fluorescent protein gene (*DsRed*) isolated from *Discosoma striata*. We have transformed with

this gene different strains of the vascular pathogens *V. dahliae* and *Fusarium oxysporum*. We are currently using the differentially tagged strains to monitor infection and estimate biomass in plants inoculated with more than one different strains at the same time and to study the interaction *in planta* between a pathogen and a potential antagonist as it would happen on the plant root, since the method does not require any processing of the samples prior to observations.

Another revolutionary invention aiming to overcome the limitations of the multiplex real-time PCR related to the number of different target organisms that can be detected in one reaction, is the development of a technology known as DNA arrays or DNA chip. A set of unlabeled oligonucleotide detectors specific for the taxa under study are immobilized on a solid support (usually a nylon membrane or a glass slide). DNA is subsequently prepared from the sample (e.g. soil, irrigation water or plant tissue) and labeled during or after PCR amplification. The resulting complex labeled mixture representing an array of different microorganisms is then hybridized to the membrane under stringent conditions (that is why the method is also known as reverse dot blot hybridization). According to the hybridization signals that will appear on the membrane, conclusions are drawn about the identity of the microorganisms present in the unknown sample based on the reference set of detectors. Thus, a large number of organisms is potentially detected and identified in a single experiment. The method allows the analysis of complex pathogen populations or other microbial communities and has been adapted from studies of human genetic disorders to the discrimination and identification of DNA from oomycetes, nematodes and bacteria (52, 99). A DNA array approach was applied by Lievens *et al.* (53) to detect and identify to the species level the tomato vascular wilt pathogens *F. oxysporum* f. sp. *lycopersici*, *V. albo-atrum* and *V. dahliae* in infected plants, contaminated water and complex soils substrates. Hybridization signals indicating the presence of the pathogens in tomato roots and stems could be seen even for the pre-symptomatic stage of infection. The pathogens could also be detected in potting mix and irrigation water at a high level of sensitivity reaching 0.5 spores ml^{-1} water. Obviously, there is a great potential in DNA arrays, because using the appropriate detectors detection could be expanded to many other pathogens of tomato including additional fungi but also bacteria and viruses. Although a DNA array membrane implies a highly sophisticated technology, the methodology can have an important practical significance in contemporary agriculture for the detection of pathogens from soil or irrigation water, for the identification of latent infections that would allow in time decision making for sound and effective control strategies but also equip growers with a very powerful tool to correctly diagnose the causal agents of their diseased plants.

Conclusions and Future Developments

It is beyond any doubt that the advancement of molecular biology has offered novel dynamic tools to the diagnosis of fungi that cause diseases to plants. Although the number of applications is less compared with plant pathogenic bacteria or viruses, the use of molecular technology in the diagnosis of fungal pathogens has been expanded considerably since the onset of DNA technology in biology resulting in the development of molecular diagnostic assays for many important crop pathogens. Along these lines, in the near future DNA-based techniques are expected to contribute towards the in-depth elucidation of various aspects of plant pathology i.e. the understanding of the competition of soil-borne pathogens in their complex terrestrial environment, the study of host-pathogens interactions at the molecular level, the discovery of pathogen genes responsible for virulence and the respective pant genes that provide resistance to plants. All these new sequences will be added to the DNA databases and thus offer even more precise targets for designing more sensitive and specific assays with the final goal to quickly and accurately achieve early and correct diagnosis of the pathogen involved in a plant disease, thus planning and implementing control strategies with less chemical inputs, more effective and less costly for the grower and more sound for the environment. The yet unexplored possibilities of PCR-technique will undoubtfully march to new scientific frontiers even more elaborate and sophisticated than real-time PCR. The continuous development of biomarkers (i.e. GFP or DsRed) is expected to offer radical innovations in pathogenesis and ramification of pathogens in plant tissues and the study of biology of microorganisms as a whole. Finally, the so far limited use of robotics to DNA technology will become economically feasible and thus accessible to farmers and will offer the possibility of using a single DNA chip as a practical tool for the diagnosis of hundreds of plant pathogens.

الملخص

بابلوماتس، إ.إ. 2006. التشخيص الجزيئي للممرضات الفطرية. مجلة وقاية النبات العربية. 24: 147-158.

ساهمت التطورات الحديثة في البيولوجيا الجزيئية في تشخيص الممرضات الفطرية في النباتات من خلال إيجاد طرائق جديدة متقدمة تسمح بالكشف السريع وبالتحديد الكمي والنوعي للكائنات الموجودة. وعلى الرغم من تطبيق التشخيص الجزيئي واستخدامه على فطور من بيئات متنوعة إلا أن تطبيقه على الممرضات من الأنظمة البيئية الأرضية كان محدوداً وذلك بسبب تعقيد الظروف البيئية المحيطة بهم وخاصة بالنسبة للممرضات المنقولة بالهواء. لقد تم خلال السنوات الماضية تطوير العديد من الآليات (الطرق) التقليدية المتباينة في درجة كفاءتها وذلك بهدف كشف للممرضات الفطرية التي تنتقل عن طريق التربة وتعريفها وتوصيفها. تعتبر طريقة تنمية الكائنات على بيئات انتخابية متخصصة من أكثر الطرق استخداماً في هذا المجال فهي تهدف لاستبعاد أغلب كائنات التربة والاحتفاظ بالفطور المرغوبة فقط. ولكن على الرغم من ذلك، فقد كان التعامل مع فطور التربة يمثل دائماً تحدياً كبيراً بسبب تعقيد الظروف البيئية التي توجد فيها هذه الكائنات. لقد ثبت في كثير في من الحالات بأن الحصول على الكائنات (الفطور) من أوساط انتخابية ليست مستقلة أي أنها تتأثر بعوامل متعددة، فعلى سبيل المثال قد يستبعد الكائن المستهدف أو يمنع من النمو بسبب وجود منافس أفضل منه على البيئة الإنتقائية، كما أن المواصفات الشكلية للكائنات يمكن أن تكون مشتركة ما بين عدة أنواع بالإضافة إلى أن تحيز الباحث هو عامل مهم في هذا النوع من التجارب وبالتالي فإن مجموع هذه العوامل تؤثر في النتائج المستخلصة من هذه الدراسة. بناءً على ما تقدم، كان لابد من اللجوء للتقنيات الجزيئية بهدف تشخيص فطور التربة. لقد انتشرت تقنيات الـــDNA لتطال الكشف (التعرف) عن الممرضات الفطرية التي تعيش في بيئات متنوعة مثل: داخل (ضمن) الأنسجة النباتية، على سطح الأوراق، في البذور، في مياه الري وقد تم دعمها بدراسة بعض الخصائص النوعية مثل السمومية والمقاومة للمبيدات، بالإضافة إلى أنه قد تم استخدام تقنيات التشخيص الجزيئي في كشف أنواع عديدة من الفطور في الحجر الفطري. كانت الايزوزيمات ومسابر الـــDNA من أوائل المؤشرات الجزيئية التي استخدمت في الكشف والتمييز ما بين الأنواع الفطرية المختلفة، ومن ثم أتى التشخيص الجزيئي المعتمد على التفاعل التسلسلي

للبوليميراز ليسرع عملية التمييز من خلال إيجاد طرائق للكشف أكثر سرعة وأكثر حساسية. يمكن التمييز ما بين الفطريات على مستوى الأنواع من خلال تصميم بادئات تتعرف على مناطق مختارة تتصف بأنها مقاطع من الـــDNA (محفوظة–متشابهة) ما بين الأنواع مثل وحدات المورثات المسؤولة عن الـــrRNA ومن ثم تستكمل العملية بتوصيف قطعة الـــDNA التي تتم مكاثرتها بتلك البادئات. لقد أصبحت وحدات المورثات المسؤولة عن الـــrRNA معروفة ومستخدمة جداً وذلك لعدة أسباب، فهي توجد بعدد من النسخ يصل لعدة مئات في المجين (الجينوم)، كما أنها مكونة من مناطق محفوظة جداً ومناطق مختلفة. لقد تم استخدام المقاطع المأخوذة من تحت الـــrRNA في عمليات التصنيف وفي الدراسات الوراثية، في حين استخدمت المناطق المحفوظة سواء من المنطقة الداخلية المنسوخة (ITS) أو المنطقة الفاصلة بين المورثات (IGS) كهدف للكشف عن الفطور. لقد تم تطوير تقنيات البصمة الوراثية المعتمدة على الـــPCR مثل (AFLP، SSR، RAPD) ذات الحساسية والدقة العالية في عملية التشخيص. حديثاً تم تطوير تقنية مصفوفات الـــDNA المعروفة أيضاً بالشريحة (الرقيقة) أو رقيقة الـــDNA وهي تهدف لمسح كامل المجين والتعرف عليه على رقيقة أو شريحة واحدة، وقد أصبحت هذه التقنية متاحة وقابلة للتطبيق في مجال التشخيص الجزيئي للفطور. يتم تصنيع رقائق الـــDNA بطريقة آلية سريعة جداً، وعادة تكون من الزجاج، وتتم عليها عملية تهجين جزيئي ما بين مسابر متخصصة ومقاطع الـــDNA الهدف المكملة لها. بهذه الطريقة، يتم بشكل متواز كشف وتحديد عدد كبير من المورثات في عدة أنواع من الكائنات الدقيقة. إن التجارب مع شريحة أو رقيقة واحدة من الـــDNA يمكن أن يزودنا بمعطيات ومعلومات هائلة عن عدد كبير من المورثات بشكل متزامن. تسلط هذه المحاضرة المرجعية الضوء على تطبيق عدة تقنيات بهدف التشخيص الجزيئي للممرضات الفطرية، وهي تعتمد أساساً على المعلومات التي وجدت في المقالات بالإضافة إلى معطيات البحث الشخصي للكاتب.

كلمات مفتاحية: تعريف، تشخيص، البصمة الوراثية، PCR، RAPD، AFLP.

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