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Molecular diagnostics for soilborne fungal pathogens

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Summary. Several classical approaches have been developed to detect and identify soil fungal inhabitants through the years. Selective media have been devised to exclude the large number of soil organisms and allow growth of target fungi. However the advent of molecular biology has offered a number of revolutionary insights into the detection and enumeration of soilborne fungal pathogens and also has started to provide information on the identification of unknown species from DNA sequences. This review paper focuses on the application of various molecular techniques in the detection, identification, characterization and quantification of soilborne fungal plant pathogens. This is based on information from the literature and is combined with personal research findings of the author.

Key words: DNA probes, identification, isozymes, molecular fingerprinting, micro-arrays.

Introduction

A large number of fungi that are found in terrestrial ecosystems have a serious economic impact on agricultural production because they cause diseases of cultivated crops that result in important losses of yield. The soil is a very complex environment that creates numerous barriers to the identification, isolation and quantification of soilborne fungi. For this reason the detection and identification of soil borne fungi has been an important goal of research over the years (Kowalchuk, 1999).

To tackle this goal, several classical approaches exist. Selective media exclude the large number of unwanted soil organisms and allow growth of target fungi. However, in most cases fungal recovery is method dependent, the target organism is often outgrown by better competitors, morphological characters may be common for several species while the inevitable bias of the researcher who is applying a given method makes things even more complex (Termorshuizen *et al.*, 1998).

The advent of molecular biology has offered a number of revolutionary new insights into the detection and enumeration of soilborne fungal pathogens and also has started to provide information on identifying unknown species from their DNA sequences. These data, combined with classical characterization of fungi in the field, provide new aspects about fungal functions and interactions within terrestrial communities (Bahnweg, 1998).

This review paper focuses on how molecular techniques are applied in the detection, identification, characterization and quantification of soilborne plant pathogenic fungi. The information is based on the literature and combined with personal research findings of the author.

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Molecular techniques

Isozymes

Isozymes are considered as the early molecular markers that initially found extensive application in plant, animal and insect systematics (Oudemans and Coffey, 1991a). Isozyme technology was later adapted to the taxonomy of phytopathogenic fungi (Vallejos, 1983; Michelmore and Hulbert, 1987). Crude protein extracts were separated by electrophoresis on starch or non-denaturing polyacrylamide gels where the isozymes zones based on specific enzyme activity were visualized by providing an appropriate substrate. Different isozyme patterns were formed by changes in amino acid composition, which affected the charge or conformation of the protein. Genetic interpretations of the banding polymorphisms can be inferred by extrapolations to loci that would correspond to isozyme zones.

Isozyme variation analysis was initially applied to soilborne fungi whose morphological characters exhibited high levels of variation, usually with overlaps between species such as *Phytophthora* spp. In this genus, morphological criteria, such as sporangial dimension and sexual characteristics, often produce confusing and doubtful designations (Erwin, 1983). Oudemans and Coffey (1991a) separated three *Phytophthora* species (*P. cambivora*, *P. cinnamomi*, *P. cactorum*) by comparison of 18 isozyme loci, while in a further study (1991b) they revised the systematics of twelve papillate *Phytophthora* species on the basis of isozyme analysis.

Although isozymes could provide adequate levels of polymorphic loci in a number of cases, their use to study fungal plant pathogens has been limited because of their post transcriptional nature. As such markers, they are subyected to environmental influences that would cause polymorphisms that did not reflect evolutionary events.

DNA probes

With the advent of techniques for the isolation, purification, cloning and hybridization of DNA from various microorganisms, DNA probes were among the first molecular markers applied in the detection, identification and phylogenetic analysis of fungal pathogens (Manicon *et al.*, 1987; Rollo *et al.*, 1987). Species-specific DNA probes generated from cloned random DNA fragments derived from genomic DNA that was digested with various restriction endonucleases had a number of advantages over classical approaches. Because of their high specificity, a pure culture of the target organism was not necessary. Furthermore, DNA could be extracted from any form of living mycelium without the fungus having to produce characteristic structures in culture e.g. spores, fruiting bodies, sclerotia. Since repetitive sequences found in fungal genomes were usually preferred, DNA probes improved the speed, sensitivity and objectiveness of the detection and identification of fungi as compared with traditional methods.

Analysis of restriction fragment length polymorphisms (RFLPs) has been extensively used for the detection and characterization of soilborne fungi (Goodwin *et al.*, 1990; Whitehead *et al.*, 1992; Woo *et al.*, 1996). RFLPs are based on the specific differences in the sequence of DNA that results in fragments of different sizes when genomic DNA is digested by restriction enzymes (Beckmann and Soller, 1983, 1986). Restriction fragments are separated according to their size by electrophoresis and subsequently transferred to nylon membranes by capillary forces and immobilized by UV crosslinking. Labeled DNA probes (initially by radioactive isotopes and later increasingly by non-radioactive means) are hybridized to the membrane bound DNA fragments and specific bands are visualized by appropriate methods.

Cloned chromosomal DNA probes were applied to detect various *Phytophthora* species in soil and host tissue (*P. parasitica*) (Goodwin *et al.*, 1989), and in initial efforts to quantify pathogen growth in infected plant roots (Judelson and Messener-Routh, 1996). Mitochondrial DNA (mtDNA) probes were subsequently developed because of the nature of the mitochondrial genome, which is found in high copy numbers and produces more simple restriction fragment patterns. Such probes were used to differentiate between *Phytophthora* species that display overlapping variability of morphological characters like *P. cryptogea* and *P. drechsleri* (Mills *et al.*, 1991). This technology has also been applied to distinguish among special forms of the soilborne fungal pathogen *Fusarium oxysporum*, the causal agent of vascular wilt of a large number of plant species (Bridge *et al.*, 1995). In one study Bentley *et al.* (1995) used a probe consisting of a 3.38 kb mitochondrial fragment from an isolate of *Fusarium oxysporum* f. sp. *cubense* to determine RFLPs in restriction digests of total DNA from 28 isolates

of *F. oxysporum* from a variety of hosts and locations. This probe showed mtDNA polymorphisms within and between different special forms allowing the specific detection of morphologically indistinguishable isolates. An mtDNA fragment cloned from the causal agent of take-all disease of wheat (*Gaeumannomyces graminis* var. *tritici*) was able to specifically hybridize with all three varieties of the fungus while it showed little homology with DNA from other soilborne fungi (Henson, 1989). In this respect it was useful to identify the pathogen recovered from infected wheat roots.

Polymerase chain reaction

Specific amplification of target DNA sequences using the polymerase chain reaction (PCR) was envisaged by Kary Mullis about twenty years ago (Mullis, 1990). The technique has found wide application as a powerful molecular tool, mostly because thermo tolerant DNA polymerases and automated thermocyclers are now available. PCR is based on the exponential amplification of specific DNA sequences by synthesizing DNA *in vitro* through three essential steps: an initial melting of target DNA, followed by annealing (hybridization) two synthetic oligonucleotide primers to the target ends of the denatured DNA strands, with a final primer extension (polymerization) accomplished by the DNA polymerase.

The wide application of PCR to plant disease diagnosis is due to the advantages that the method offers over the traditional or other molecular techniques (Henson and French, 1993). It does not require a pure culture of the target pathogen (high quality DNA is not necessary), it is highly sensitive (minute amounts are required with the theoretical potential of a single molecule detection level), and it is rapid (fast screening of a large number of samples). It is also highly specific (computer-assisted primer designing permits detection of a single pathogen or a group of related microorganisms) and relatively inexpensive.

PCR-based diagnostics have been used to detect fungal pathogens in infected plant tissues, to recognize mixed infections, to monitor fungal development in the host, to carry out the molecular quantification of fungal biomass *in planta*, and to estimate inoculum density in the soil.

One of the most common targets for PCR detection of plant pathogenic fungi is the ribosomal RNA gene cluster (rRNA) and this for a number of reasons. These clusters are common both in nuclei and in mitochondria of eukaryotic cells, while the nuclear rRNA gene forms tandem repeats with several hundred copies per genome, thus increasing the sensitivity of detection. It includes highly conserved regions such as the large subunit (LSU), the 5.8S and the small subunit (SSU), which permit the study of the relationships of distantly related genera. The RNA gene also includes variable regions such as the internal transcribed spacers (ITS) that are formed between the subunits, and the intergenic spacers (IGS) that are formed between the cluster repeats that facilitate discrimination between closely related species of a fungal genus (Bridge and Arora, 1998). The ITS sequences have been preferred over the subunits because they are more variable and thus permit selective detection of closely related fungi. ITS sequences represent a relatively short region (500–800 bp), are easily amplified by universal primers designed on the conserved subunit sequences, require only small amounts of sample DNA, and have been used for the quick generation of species-specific probes (White *et al.*, 1990; Bruns *et al.*, 1992; Gardes and Bruns, 1993).

Using ITS-targeted PCR, Nazar *et al.* (1991) found that the five nucleotide differences observed in this region between *V. dahliae* and *V. albo-atrum* were sufficient enough to differentiate between these two soilborne fungal pathogens. Furthermore, Moukhamedov *et al.* (1994) showed that there were 17 base-pair differences between *V. dahliae* and *V. tricorpus* and 12 between *V. albo-atrum* and *V. tricorpus*. Based on those differences, primer sets were designed for PCR-based assays for the detection of *Verticillium* species in potato (Robb *et al.*, 1994). Many other applications have also been described in the literature. In the heterogeneous genus *Fusarium*, in which species differentiation is based mainly on morphological or biochemical criteria that in some cases are sometimes difficult or even confusing, PCR technology has improved diagnosis. Specific primers have been designed to amplify *F. oxysporum* f. sp. *vasinfectum* DNA in cotton, but not the isolates of any other mycoflora associated with cotton (Moricca *et al.*, 1998), or to separate *F. culmorum* and *F. graminearum* from *F. avenaceum* (Schilling *et al.*, 1996). Li and Hartman (2003) detected *Fusarium solani*

f. sp. *glycines* in soybean roots and soil using a mt SSU rRNA gene primer set. Also, the soilborne potato pathogens *Spongospora subterranea* f. sp. *subterannea* (Bell *et al.*, 1999), *Helminthosporium solani* (Cullen *et al.*, 2001) and *Rhizoctonia solani* AG-3 (Lee *et al.*, 2002) could be detected and identified in potato and soil using ITS analysis. PCR-based identification and detection of various root rot fungal pathogens exploiting ITS region specificity has been reported on *Rosellinia necatrix* (Schena *et al.*, 2002), *Cylindrocarpon destructans* and *Cylindrocladium floridanum* (Hamelin *et al.*, 1996), *Gaeumannomyces graminis* var. *tritici* (Keller *et al.*, 1995).

In contrast to the ITS sequences, IGS has been less popular as a region for primer designing. The intergenic sequences have been exploited to determine variation within closely related species. RFLP analysis of the PCR amplified IGS region was carried out in populations of *F. oxysporum* (Appel, 1995) revealing interspecific variations in this pathogen, while heterogeneity in subrepeat numbers has been found to exist both within (*Pythium ultimum*) (Klassen and Buchko, 1990) and between species as in *V. dahliae* and *V. albo-atrum* (Kim *et al.*, 1992; Morton *et al.*, 1995).

Fingerprinting techniques

Various methods have been devised for DNA fingerprinting based on the polymerase chain reaction. Among those, random amplified polymorphic DNA (RAPDs) (Williams *et al.*, 1990) has been the most popular because it is simple and requires only small amounts of genomic DNA. However, because of the irreproducibility of RAPD patterns due to the annealing difficulties of the random primers used with this method, other PCR fingerprinting variants have also been developed: UP-PCR (Yli-Matila *et al.*, 1997), AFLPs (Janssen *et al.*, 1996), and simple sequence repeats (SSRs), also known as microsatellites (Bornet, 2001).

While PCR-based fingerprinting methods have been widely used for the molecular characterization and phylogenetic inferences of fungi, some of them (such as RAPDs) have been combined with designing molecular markers for detection and identification. Manulis *et al.* (1994) applied RAPDs to the carnation wilt pathogen *Fusarium oxysporum* f. sp. *dianthi* and they were able to identify specific band patterns that were subsequently used as probes to distinguish between races of the pathogen. Cloning and characterization of pathotype-associated RAPD markers was employed by a Spanish group (Pérez-Artés *et al.*, 2000) to design primers that allowed differentiation between the defoliating and non-defoliating pathotypes of *V. dahliae* on cotton and olive.

Molecular quantification of soilborne fungi

One of the most important aspects of disease prediction and assessment of control strategies in soilborne diseases is the quantification of inoculum density of a pathogen. In many cases, there are large variations between traditional methods as was shown in an interlaboratory comparison to quantify microsclerotia of *V. dahliae* in soil (Termorchuizen, 1998) suggesting that inoculum density data for this soilborne fungal wilt pathogen found in literature present only a vague estimate of reality (Goud and Termorshuizen, 2003).

A felt need for molecular approaches to quantify plant pathogens in soil environments led to the development of specific PCR-based methods. Initially, the molecular quantification of soilborne fungi was based on quantitative PCR. First, a standard curve was generated using a different size (usually smaller) DNA fragment (the internal standard) that would co-amplify with the target sequences using the same pair of primers. Then the pathogen was quantified in unknown samples (Heinz and Platt, 2002). Using this approach, Robb *et al.* (1994) quantified *Verticillium* species fungal biomass in potato with the aid of a heterologous internal sequence (the *Verticillium*-specific primer borders a DNA fragment from an unrelated fungus). Volossiouk *et al.* (1995) developped a protocol for DNA extraction from the soil and for specific amplification of *V. dahliae* DNA gaining the opportunity to quantify the pathogen on large-scale basis.

In our laboratory, we developed a protocol by combining the above approaches to molecularly quantified microsclerotia of *V. dahliae* in the soil. We first produced an internal standard (IS) following the method of Förster (1994). More specifically, we designed a linker primer within the ITS 1 and ITS 2 region of the rRNA gene cluster of *V. dahliae* that was then used to amplify a homologous IS having the same borders with the target fragment but with a smaller size (237 bp) than the *V. dahliae* PCR product (347 bp). The internal

standard was tested on DNA extracted from various concentrations of *V. dahliae* microsclerotia produced in culture. By competitive PCR, both the target and the IS fragments were amplified. Subsequently, a standard curve was generated by competitive amplification of known concentrations of microsclerotia (aliquots of 10 to 100 microsclerotia) and plotting the concentration of microsclerotia over the ratio of the PCR products (microsclerotia DNA/IS DNA concentration). This method was then applied to DNA isolated from soil that was artificially inoculated with known amounts of microsclerotia produced in culture. The method is currently being evaluated on naturally infested soils with the vascular wilt pathogen *V. dahliae* and compared with traditional quantification methods.

An advanced version of conventional PCR that exploits its unique ability to monitor the complete DNA amplification process is real-time PCR (Walker, 2002). After completion of the traditional PCR reaction, amplified products are characterized by size fractionation using agarose gel electrophoresis. In real-time PCR, the level of DNA amplification is measured indirectly with specific fluorescent probes that are activated through the 5'- nuclease activity of the *Taq* DNA polymerase (Holland *et al.*, 1991), eliminating the need for gel electrophoresis. At the same time, kinetic PCR allows real-time monitoring of the DNA product within the PCR tube during the course of the reaction, which is visualized with fluorescent DNA binding dyes (Eurogentec, 2002). Real-time PCR generally uses a fluorogenic probe. One of the PCR primers bears a fluorophore with a configuration that renders fluorescence quenching capability, which is dequenched when the primer becomes incorporated into the dsDNA PCR product. Marrying the above two processes resulted in the fluorescence detection real-time PCR (Gibson *et al.*, 1996). DNA in a sample can be quantified by interpolating a threshold (C_T) value versus a linear standard curve of C_T values obtained from a serially diluted standard solution (Heid *et al.*, 1996).

Use of real-time PCR is continuously expanding in the area of detection and identification because of the numerous advantages it has over conventional PCR. It ensures very accurate and reproducible quantification of gene copies, it does not require post-PCR handling of samples preventing potential PCR product carry-over contamination, and it enables much faster and higher throughput assays. It is also much more sensitive than conventional PCR and allows simultaneous detection of several organisms, limited only by the number of fluorescent dyes and the energizing light source. At present, the most important limiting factor of the method is that it requires specialized, high-cost equipment. However, both the size and cost of real-time PCR instrumentation is being dramatically reduced, suggesting that in the near future real-time technology will become the standard PCR method equipment in the general laboratory, in much the same way that new age PCR machines have superseded the initial large-tube units (Walker, 2002).

Real-time PCR has inevitably found application in soilborne fungal diagnostics. Schena *et al.* (2002) detected and identified *Rosellinia necatrix* from among a large number of fungal species. Using a Scorpion primer they increased the sensitivity of detection 10 times above that of conventional PCR. In another study using real-time PCR, Filion *et al.* (2003) reported the detection and direct quantification of genomic DNA of the plant pathogen *Fusarium solani* f. sp. *phaseoli* from soil. They targeted the translation elongation factor 1 alpha gene of the fungus using real-time PCR assays conjugated with the fluorescent SYBR® Green I dye (Molecular Probes, Eugene, Oregon, USA). Their DNA extraction protocol required less than 2 h, and could be adapted to other soilborne fungi, but most importantly there was no cross-reactivity with soil microflora DNA.

Diagnostics beyond PCR

Although the full potential of PCR has not been reached, other even more sophisticated approaches to fungal molecular biology are being investigated. Among them, the green fluorescent protein (GFP), originally found in the pacific jellyfish *Aequorea victoria*, is gaining much attention. After it was shown that heterologous expression of the cloned gene generated a striking green fluorescence (Prasher *et al.*, 1992; Chalfie *et al.*, 1994), GFP has increasingly been used as a biomarker. In general, the strategy used in GFP-labeling involves fungus transformation to ensure constitutive gene expression of the GFP. Green fluorescence is then monitored by UV-microscopy and quantified by fluorometry indicating the fungal biomass.

This method has been shown presently for foliar fungal pathogens (Chen *et al.,* 2002).

In our laboratory, we transformed the soilborne vascular wilt pathogen *V. dahliae* with enhanced green and yellow fluorescent variants (EGPF and EYFP, respectively) using *Agrobacterium*-mediated transinfection. After selection of transformants with a pathogenicity comparable to that of wild-type strains, the progress of pathogen invasion and host-plant infection was monitored by UV-microscopy. Fungal development was also followed visually inside the plant vascular tissue while the progress of infection was quantified by fluorometric measurement of the protein fluorescence. Thus, quantification of the pathogen by GFP was possible also during its invasion of the host vascular tissue.

A recently devised molecular method that overcomes the problem of the small number target sequences that can be detected simultaneously with multiplex real-time PCR is DNA array technology. This approach, also known as DNA chips (or reverse blot), uses oligonucleotide detectors that are covalently bound to a solid support (i.e. a nylon membrane or glass slide). In the first step, target DNA is simultaneously amplified and labeled and is then hybridized to the membrane under stringent conditions. This method has been adapted from studies of human genetic disorders for use in the discrimination and identification of DNA from oomycetes, nematodes and bacteria. Recently, a DNA array approach was used to detect and identify the tomato vascular wilt pathogens *F. oxysporum* f. sp. *lycopersici*, *V. albo-atrum* and *V. dahliae* to the species level in infected plants, contaminated water and complex soil substrates (Lievens *et al*., 2003). With the DNA array, the presence of the pathogens could be demonstrated in tomato root and stems even at the pre-symptomatic stage of infection. Moreover, the pathogens were also detected in potting mix and in water samples with very high sensitivity (0.5 spores ml^{-1} water). It seems clear that DNA array technology can be developed further to include other tomato pathogens of fungal, bacterial or viral origin or even vascular wilt pathogens of other crops, furnishing a universal vascular wilt detection array (Lievens *et al*., 2003). Such a membrane with DNA arrays could be usefully applied in current horticultural practice for the detection of pathogens from complex substrates such as soil or irrigation water, and also could be a powerful tool to growers for diagnostics of diseased plants.

Conclusions and future perspectives

There is no doubt that molecular techniques have been extensively adapted to the diagnostics of soilborne fungi. However, uses of this techniques are still less than on other plant pathogens. In this perspective, DNA technology is expected to continue to improve understanding of the complex competition of soilborne micro-organisms for secure ecological niches. The soil is a complex environment that requires multiplex assays. The wide diversity in soil chemical composition and texture constitute a challenge for DNA extraction and enzyme-driven reactions. These methods monitor both pathogens and also beneficial organisms in soils. The in-depth exploitation of PCR potential will lead to more sophisticated variants of the technique (improved even from the currently expanding real-time PCR) that will increase the speed and sensitivity of soilborne fungal diagnostics. Biomarkers like GFP will be better adapted and will also be adopted from molecular biology applications for use against plant pathogens giving a better understanding of the soil-microflora interactions. Application of robotics to DNA technology will further improve DNA micro-arrays enabling them to identify PCR products (e.g. soilborne fungi) from hundreds of different primer pairs by a single hybridization on a DNA chip.

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