European Journal of Plant Pathology **110:** 893–908, 2004.

Real-time quantitative PCR: a new technology to detect and study phytopathogenic and antagonistic fungi

Leonardo Schena, Franco Nigro, Antonio Ippolito and Donato Gallitelli
Department of Plant Protection and Applied Microbiology, University of Bari, Via Amendola 165/A, 70126 Bari, Italy (Phone: +39-0805443055; Fax: +39-0805442911; E-mail: leonardo.schena@agr.uniba.it)

Accepted 16 March 2004

Key words: detection, gene expression, molecular beacons, scorpion PCR, SYBR Green I, TaqMan

Abstract

Real-time PCR technologies open increasing opportunities to detect and study phytopathogenic and antagonistic fungi. They combine the sensitivity of conventional PCR with the generation of a specific fluorescent signal providing real-time analysis of the reaction kinetics and allowing quantification of specific DNA targets. Four main chemistries are currently used for the application of this technique in plant pathology. These chemistries can be grouped into amplicon sequence non-specific (SYBR Green I) and sequence specific (TaqMan, Molecular beacons, and Scorpion-PCR) methods. Amplicon sequence non-specific methods are based on the use of a dye that emits fluorescent light when intercalated into double-stranded DNA. Amplicon sequence specific methods are based on the use of oligonucleotide probes labelled with a donor fluorophore and an acceptor dye (quencher). The fluorescent signal eliminates the requirement for post-amplification processing steps, such as gel electrophoresis and ethidium bromide staining. This significantly reduces time and labour required for the analysis and greatly increases the throughput of PCR testing as an automated diagnostic system, making it suitable for large-scale analyses. Furthermore, the use of different fluorescent dyes facilitates the detection of several target microrganisms in a single reaction (multiplex-PCR). Real-time PCR makes possible an accurate, reliable and high throughput quantification of target fungal DNA in various environmental samples, including hosts tissues, soil, water and air, thus opening new research opportunities for the study of diagnosis, inoculum threshold levels, epidemiology and host–pathogen interactions. Moreover, the quantification of specific mRNA transcription by real-time PCR is being increasingly applied to the study of changes in gene expression in response to phytopathogenic and antagonistic fungi.

Introduction

Conventional-PCR has emerged as a major tool for the diagnosis and study of phytopathogenic fungi and has contributed to the alleviation of some of the problems associated with the detection, control and containment of plant pathogens (Henson and French, 1993; Martin et al., 2000). PCR is a sensitive technology that offers several advantages over the traditional methods of diagnosis: micro-organisms do not need to be cultured, the technique possesses the potential to detect a single target molecule in a complex mixture, and it is rapid and versatile. Depending on the design of the primers, both narrow and broad selectivities are possible, thus enabling the detection of a single pathogen at the species or strain level. However, despite these advantages, the adoption of PCR for routine detection of plant pathogens has been slow due to technical limitations related to the post-amplification procedures, which are necessary to detect amplicons. In fact, although PCR

techniques can considerably reduce the time needed for diagnosis compared to conventional culturing methods, they still require additional work when Southern blot or sequencing are required to identify the PCR products. Furthermore, conventional PCR is unreliable for quantitative analysis (Ginzinger, 2002).

Several reviews on the use of real-time PCR in fields other than plant pathology have been published recently (Bustin, 2000, 2002; Didenko, 2001; Lyon, 2001; Schmittgen, 2001; Snider et al., 2001; Ahmed, 2002; Broude, 2002; Ginzinger, 2002). In plant pathology, reviews of interest have focused on crop biosecurity (Schaad et al., 2003) and on the detection of seed-borne fungal pathogens (Taylor et al., 2001a) and phytopathogenic bacteria (Schaad and Frederick, 2002). However, real-time PCR detection systems are available for viruses (Schoen et al., 1996; Eun et al., 2000; Finetti Sialer et al., 2000a, b; Roberts et al., 2000; Boonham et al., 2002; Korimbocus et al., 2002), nematodes (Ciancio et al., 2000; Bates et al., 2002), bacteria (Weller et al., 2000; Hermansson and Lindgren, 2001) and fungi (Table 1).

Real-time technologies

Four main chemistries are utilised to detect and study phytopathogenic and antagonistic fungi (Table 1). These chemistries can be grouped into amplicon sequence non-specific and sequence specific methods (Mackay et al., 2002).

Amplicon sequence non-specific methods

Included in this group are detection methods based on the use of dyes that emit fluorescent light when intercalated into double-stranded DNA (dsDNA). The most utilised intercalating dye is SYBR Green I (Morrison et al., 1998) although other intercalating dyes, such as ethidium bromide (Higuchi et al., 1992) and YO-PRO-1 (Ishiguro et al., 1995) have been used. In solution, the unbound dye exhibits very little fluorescence, but this is enhanced upon DNA-binding. As a consequence, the fluorescence is proportional to the amount of total dsDNA in the reaction. Since these dyes do not discriminate between the different dsDNA molecules, in a PCR reaction, the formation of non-specific amplicons, as well as of dimers, must be prevented by accurate primer design and condition optimisation. At the end of the reaction, products of different length and/or sequence can be observed as distinct fluorescent peaks by heating the reaction from 30–40 to 95 °C whilst continuously monitoring the fluorescence (melting curve analysis). In fact, plotting the first negative derivative of fluorescence vs. temperature, the point at which dsDNA melts will be observed as a drop (peak) in fluorescence (many of the machines will do this calculation in the analysis software). If a single peak representing the specific products is observed, SYBR Green I is a simple and reliable low-cost method for monitoring PCR amplicons and for quantifying template DNA. However, since different amplicons will melt at different temperatures, melting curve analyses also enable the use of SYBR Green I in multiplex detection.

Amplicon sequence specific methods

There are several amplicon sequence specific detection methods based on the use of oligonucleotide probes labelled with a donor fluorophore and an acceptor dye (quencher). A fluorophore is a molecule that absorbs light energy and is promoted to an excited state. In the absence of a quencher the fluorophore falls back to the ground state and releases the excess of energy as light at a longer wavelength (fluorescence). Quenchers are molecules that can accept energy from a fluorophore and dissipate the energy by proximal quenching or by fluorescence resonance energy transfer (FRET) (Didenko, 2001). In proximal quenching, the fluorophore is in close proximity with a quencher and the energy is transferred from the fluorophore to the quencher and dissipated as heat (collisional quenching). As a result, no fluorescence is observed. In FRET quenching, the fluorophore transfers its energy to the quencher (which may be another fluorophore), and the energy is released as light of a longer wavelength (Didenko, 2001).

There are a number of different fluorophore/quencher combinations that can be used, providing that spectral overlap between the fluorescent dye and the quencher molecule is ensured. Fluorophores such as FAM, TET, TAMRA, HEX, JOE, ROX, CY5 and Texas Red and quenchers such as TAMRA, DABCYL and Methyl Red are the most utilised. Furthermore, a new class of

Table 1. Example of real time-PCR applications for the study and detection of phytopathogenic or antagonistic fungi

Pathogen/antagonist	Crop	Chemistry	Reference
Aphanomices euteiches	Alfalfa	TaqMan	Vandemark et al. (2002)
Aspergillus flavus	Maize, pepper and paprika	TaqMan	Mayer et al. (2003)
Aspergillus fumigatus, Geotricum candidum, Candida albicans, Stachybotrys chartarum	Air, water and dust	TaqMan	Haugland et al. (2002)
Aureobasidium pullulans	Table grape and sweet cherries	Scorpion-PCR	Schena et al. (2000), Finetti Sialer et al. (2000a), Schena et al. (2002a)
Blumeria graminis f. sp *tritici*	Wheat	SYBR Green I	Fraaije et al. (2002)
Cladosporium sp., *Ramularia* sp., and *Microsphaera alphitoides*	Peduncolate oak	SYBR Green I	Heuser and Zimmer, (2002)
Colletotrichum coccodes	Potato and soil	TaqMan	Cullen et al. (2002)
Diaporthe phaseolorum and *Phomopsis longicolla*	Soybeen	TaqMan	Zhang et al. (1999)
Fusarium solani f.sp *phaseoli* and *Glomus intraradices*	Bean and soil	SYBR Green I	Filion et al. (2003a), Filion et al. (2003b)
Fusarium species	Wheat	SYBR Green I	Schnerr et al. (2001)
Glomus mosseae, Phytophthora infestans, and *P. citricola*	Various hosts	TaqMan	Böhm et al. (1999)
Helmintosporium solani	Potato and soil	TaqMan	Cullen et al. (2001)
Heterobasidion annosum	Norway spruce	TaqMan	Hietala et al. (2003)
Magnaporthe grisea	Rice	SYBR Green I	Qi and Yang (2002)
Phaeocryptopus gaeumannii	Douglas-fir	SYBR Green I; TaqMan	Winton et al. (2002), Winton et al. (2003)
Phakopsora pachyrhizi and *P. meibomiae*	Soybean	TaqMan	Frederick et al. (2002)
Phytophthora infestans	Potato	SYBR Green I	Avroa et al. (2003)
Phytophthora medicaginis	Alfalfa	TaqMan	Vandemark and Barker (2003)
Phytophthora nicotianae	Citrus roots and soils	Scorpion-PCR	Ippolito et al. (2000), Ippolito et al. (2004)
Pyrenophora spp. and *P. graminea*	Barley seeds	SYBR Green I	Bates et al. (2001), Taylor et al. (2001b)
Pyrenophora teres	Barley	Scorpion-PCR	Bates and Taylor (2001)
Rizoctonia solani	Potato and soil	TaqMan	Lees et al. (2002)
Rosellinia necatrix	Various hosts and soils	Scorpion-PCR	Schena et al. (2002b), Schena and Ippolito (2003)
Septoria tritici, Stagonospora nodorum, Puccinia striiformis and *P. recondita*	Wheat	SYBR Green I	Fraaije et al. (2001)
Spongospora subterranea	Potato, soil and water	TaqMan	Van de Graaf et al. (2003)
Stachybotrys elegans		SYBR Green I	Morissette et al. (2003)
Suillus bovines and *Paxillus involutus*	Soil	Two fluorogenic probes	Landeweert et al. (2003)
Tilletia indica and *T. walkeri*	Wheat	TaqMan	Frederick et al. (2000)
Various pathogens	*Arabidopsis thaliana*	SYBR Green I	Brouwer et al. (2003)
Verticillium dahliae	Olive and soil	Scorpion-PCR	Nigro et al. (2002)

quencher that has no native fluorescence has become available recently as traditional quenchers can suffer from a number of drawbacks including intrinsic fluorescence (i.e. TAMRA) or poor spectral overlap with the fluorescent dye (i.e. DABCYL and Methyl Red). The new quenchers are defined as dark quenchers or black hole quenchers (BHQ) and are suitable for a wide range of dyes since they can quench fluorescence over the entire visible spectrum and into the infrared (Bustin, 2002).

The advantage of fluorogenic probes over DNA binding dyes is that specific hybridisation between probe and target DNA sequence is required to generate a fluorescent signal. Furthermore, fluorogenic probes can be labelled with different distinguishable reporter dyes to amplify and detect two or more distinct sequences in a single PCR

reaction tube, without melting curve analysis (multiplex PCR). The high specificity of these methods enables the discrimination of single base pair mismatches. Specific methods are thus ideal for genotyping single nucleotide polymorphisms (SNPs) and mutation analyses (Thelwell et al., 2000; Lyon, 2001).

Amplicon sequence specific methods include TaqMan (Livak et al., 1995), Molecular beacons (Tyagi and Kramer, 1996), and Scorpion PCR (Whitcombe et al., 1999). The probes used to monitor DNA amplification in a PCR are cleaved in the reaction (TaqMan) or undergo a conformation change in the presence of a complementary DNA target (Molecular beacons and Scorpion-PCR) that separate fluorophore and quencher. In TaqMan, the fluorophore is quenched by FRET, whereas in Molecular beacons and Scorpion-PCR, fluorescence quenching is proximal, due to the close contact of fluorophore and quencher.

TaqMan

In the TaqMan system (Holland et al., 1991; Livak et al., 1995), the probe is a sequence of 25–30 nucleotides in length labelled with a reported dye at the 5′ end, and a quencher at the 3′ end (Figure 1). During amplification, the probe binds to its target sequence and is cleaved by the 5′ nuclease activity of Taq DNA polymerase when the enzyme extends from an upstream primer into the region of the probe. The cleavage separates the quencher from the reporter dye and restores fluorescence. This dependence on polymerisation ensures that cleavage of the probe occurs only if the target sequence is being amplified. While the probe is intact, the proximity of the quencher greatly reduces the fluorescence emitted by the reporter dye by FRET through space. Additional reporter dye molecules are cleaved from their respective probes in each cycle thus causing an increase in fluorescence intensity proportional to the amount of amplicons produced.

Molecular beacons

Molecular beacons (Tyagi and Kramer, 1996) are stem-and-loop shaped hybridisation probes with a fluorescent dye covalently attached on one end and a quencher covalently attached on the opposite end (Figure 2). The loop fragment of the probe is complementary to a sequence of the template and the two ends are complementary to each other

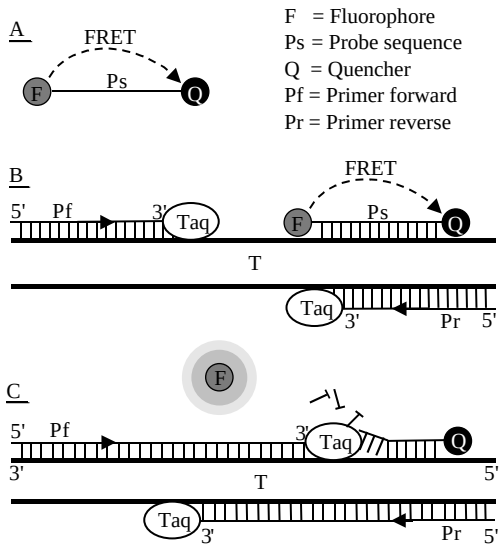

Figure 1. TaqMan. This system consists of a single stranded sequence (probe sequence) with a fluorophore and a quencher attached respectively to the 5′ and the 3′ end (A). The probe sequence binds to the amplicon during each annealing step of the PCR (B) and generates a signal through the 5′–3′ exonuclease activity of the Taq DNA polymerase that degrades the probe sequence and releases both fluorophore and quencher into the solution (C). Once cleaved from the rest of the probe sequence, the 5′ dye is freed from the quenching effect (via FRET).

forming a hairpin-like structure. Due to the stem structure, when the probe is not hybridised, fluorophore and quencher are kept in close proximity and the energy is dissipated as heat (Didenko, 2001). When a Molecular beacon encounters the target DNA a probe-hybrid longer and more stable than the stem-hybrid is formed. Consequently, a conformational change occurs; arm sequences are forced apart and the quenching effect drops down resulting in detectable fluorescence. Because the hairpin shape is very thermostable molecular beacons must have a high specificity to hybridise to a target. This makes the chemistry appropriate for the detection of single nucleotide differences in mutation and SNP analyses. Molecular beacons have been utilised frequently to detect plant viruses in combination with Nucleic Acid Sequence-Based Amplification (NASBA) (Gonçalves et al., 2002).

Scorpion-PCR

Two different formats of Scorpion primer are available: (i) the ‘stem-loop format’ (Whitcombe et al., 1999; Thelwell et al., 2000) and (ii) the

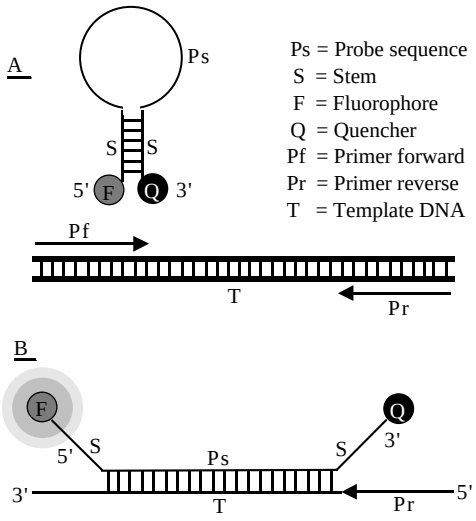

Figure 2. Molecular beacon. This system consists of a hairpin loop structure (probe sequence), a 6-base-long stem that hold the probe sequence in the hairpin configuration and a fluorophore and a quencher linked, respectively, to the 5′ and the 3′ end of the stem (A). Molecular beacons generate signals when hybridise to the amplified template DNA and distance of the 5′ dye molecule from the quenching molecule is great enough to have a specific increase of fluorescence (B). This hybridisation is favoured over the intramolecular binding of the complementary target strand, however, if there is no template DNA the hairpin configuration is restored and the fluorescence quenched.

'duplex format' (Solinas et al., 2001). In both formats the probe element is covalently linked to one of the two primers and quenching is due to the close proximity between fluorophore and quencher as in molecular beacons.

The stem loop format is composed of a PCR primer and a Molecular beacons tail, linked to the 5′ end via a 'PCR stopper' that prevents polymerase read-through (Figure 3). The probe element (loop) is complementary to a sequence newly synthesised by the DNA polymerase as a continuation of the linked primer. During amplification, Scorpion primer is incorporated into the PCR product and in the annealing phase the probe sequence in the Scorpion tail curls back to hybridise to the target sequence in the PCR product. This hybridisation event opens the hairpin loop, eliminates the quenching of the donor fluorophore and an increase in signal is observed. Thus, the Scorpion-primer approach uses a unimolecular mechanism in which the hybridisation reaction occurs within the same strand. The benefits of a unimolecular rearrangement seem significant, since the reaction is effectively instantaneous and occurs prior to any competing or side reactions such as target amplicon re-annealing or inappropriate target folding. This leads to stronger signals, more reliable probe design, shorter reaction times and better discrimination (Thelwell et al., 2000). Comparison among Scorpion primers and alternative chemistries showed that TaqMan probes have high backgrounds and moderate signal strength, molecular beacons have low background fluorescence and low signal strength, Scorpion primers have low backgrounds and high signal strength (Thelwell et al., 2000).

A further improvement of the 'stem-loop' format has been achieved with the 'duplex format'. In this format the probe element has a fluorophore attached at its 5′-end and is annealed to a complementary oligonucleotide bearing a quencher at the 3′-end (Figure 4). The mechanism of action is quite similar to the stem-loop format, since the intramolecular probe-target interaction, which is the most important feature of the Scorpion system, is maintained in both formats. However, in standard Scorpions the quencher and fluorophore remain within the same strand of DNA and some quenching can occur even in the open form. In duplex format, the quencher is on a different oligonucleotide and separation between the quencher and fluorophore is greatly increased, thus decreasing the quenching when the probe is bound to the target (Solinas et al., 2001).

Instrumentation and primer design

In last few years several real-time thermal cyclers with multiplexing and high throughput applications have been proposed on the market. Some of them are suitable for small batches of samples but are fast, thus allowing the user to run different parameters each time. Other instruments are suitable for large batches but are less flexible (Bustin, 2002). All instruments are true 'real-time system' in that they collect data (monitoring fluorescence) during each PCR cycle. Recently, a portable thermocycler has been developed (Schaad and Frederich, 2002).

Detailed information on how to design TaqMan, Molecular beacons, and Scorpion primers can be found on the Eurogentec documentation catalogue (http://www.eurogentec.com/code/en/page_07.asp?Page=409). Briefly, the simplest way

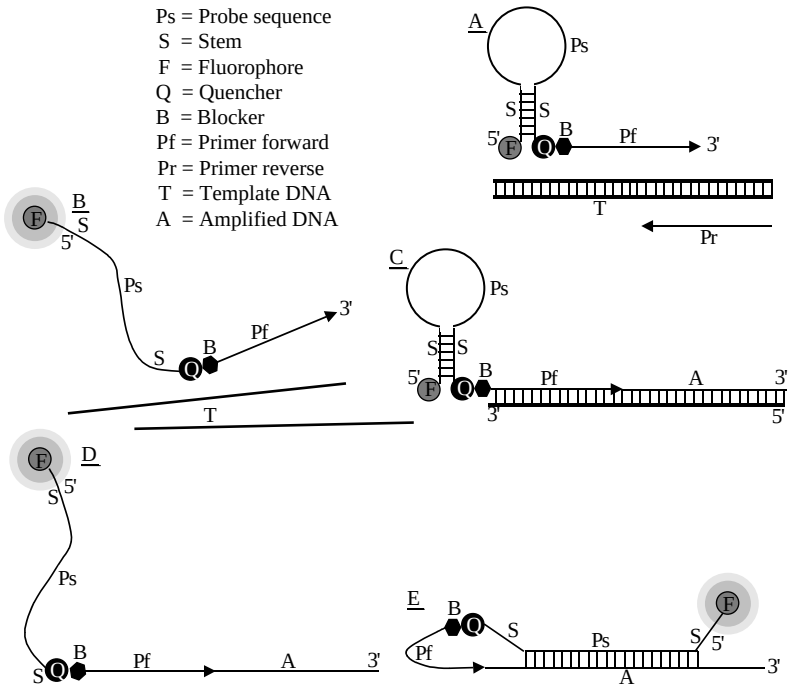

Figure 3. Scorpion PCR (stem loop format). This system is similar to Molecular beacons (Figure 2), however the hairpin loop is linked to the 5′ end of a primer via a PCR blocker (A). Step B: Initial denaturation of target and scorpion sequence. Step C: Annealing of the primer element of the Scorpion to the target DNA and extension of a new DNA fragment to which Scorpion remains attached. Step D: Denaturation of DNA. Step E: During annealing extension the Scorpion probe binds to its complement within the same strand of DNA. This hybridisation event opens the hairpin loop so that fluorescence is no longer quenched and a specific increase of fluorescence is achieved.

to design TaqMan probes is to use the Primer Express™ software, which contains all the necessary parameters (http://www.appliedbiosystems.com/support/tutorials/#). A commercial program to assist with the design of Molecular Beacon is also available (http://www.premierbiosoft.com/molecularbeacons.html). No specific software is available at present to design Scorpion primers. However, since for Scorpion the folding of the primer and probe is the crucial feature, the use of a folding program available on the website (http://www.bioinfo.rpi.edu/applications/mfold/old/rna/form1.cgi) can greatly help in the design. To design primers and/or probes, it is useful to choose regions that are unlikely to cause secondary structures and that have a GC content comprised between 20% and 80%. Primers should have a similar Tm, a length comprised between 17 and 27 bases and not able to form dimers. It is important to keep the amplicons short (70–250 bases), to allow the probe sequences to successfully compete with the complementary strand of the amplicon and make the reaction fast and efficient.

Identification of fungal specific target sequences

In real-time PCR, as well as in conventional PCR, two main strategies can be utilised to identify the specific target sequences needed for the detection and identification of fungal species and/or strains: (i) amplifying and sequencing conserved genes, common to all fungi, with universal primers, (ii) amplifying unknown genomic regions with non-specific random primers (McCartney et al., 2003). The nuclear-encoded ribosomal RNA genes (rDNA) provide attractive targets since they are highly stable, possess conserved as well as variable sequences, and can be amplified and sequenced with universal primers (White et al., 1990; Henson and French, 1993). They also occur in multiple copies (up to 200 copies per haploid genome) arranged in tandem repeats with each repeat

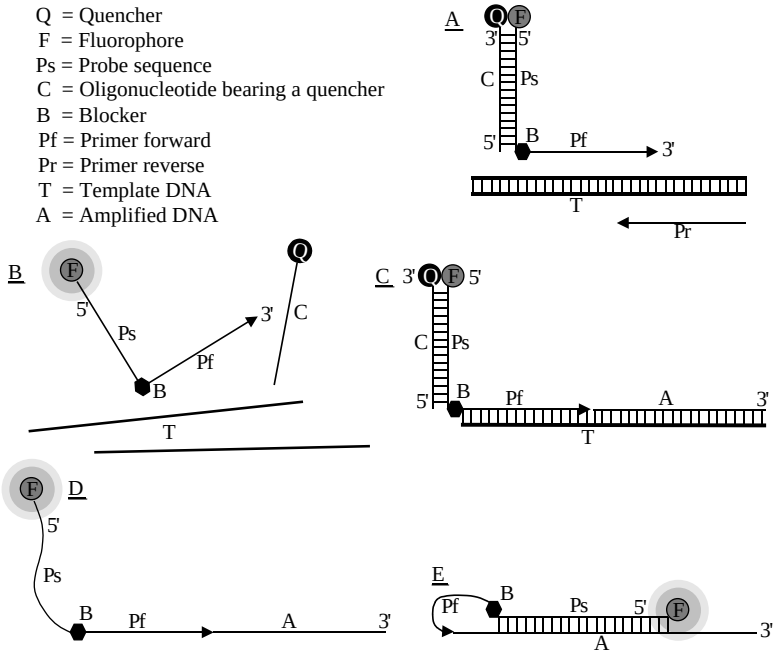

Figure 4. Scorpion PCR (duplex format). The unimolecular probing mechanism is basically the same as in the stem loop-format (Figure 3), however the quencher is on a different oligonucleotide chain (A). Step B: Initial denaturation. Step C: Annealing of the primer element of the Scorpion to the target DNA and extension of a new DNA fragment to which Scorpion remains attached. Step D: Denaturation of DNA. Step E: During annealing extension the Scorpion probe binds to its complement within the same strand of DNA. This hybridisation event prevents annealing of the probe sequence with the complementary oligonucleotide bearing the quencher so that fluorescence is no longer quenched and a specific increase of fluorescence is achieved.

consisting of the 18S small subunit (SSU), the 5.8S, and the 28S large subunit (LSU) genes separated by internal transcribed spacer (ITS) regions (ITS1 and ITS2) (Bruns et al., 1991; Liew et al., 1998). Another part of rDNA is the spacer between the LSU and SSU genes, known as intergenic spacer (IGS) or the non-transcribed spacer (NTS). In some species of *Phytophthora* and *Pythium* the IGS contains the 5S gene (Howlett et al., 1992) which is not present in fungi such as *Verticillium dahliae* and *V. albo-atrum* (Morton et al., 1995). The rDNA-conserved regions, allow probes or primers from one species to be used to detect the equivalent ribosomal genes from other species, genera, families, or even kingdoms (White et al., 1990). However, the equivalent DNA fragments detected in different organisms have many sequence differences that can be exploited in the identification. Among the variable regions, ITS are the most widely sequenced in fungi (Henson and French 1993; Cullen et al., 2001, 2002; Ippolito et al., 2002; Lees et al., 2002; Schena and Ippolito, 2003). The greatest amount of sequence variation in rDNA exists within the IGS regions. Compared to ITS regions, IGS pose more difficulties for amplification and sequencing, however, they can be useful when there are not enough differences available across the ITS. As an example, ITS regions are very similar in *V. dahliae* and *V. albo-atrum*, therefore it is difficult to design primers to differentiate these two species (Nazar et al., 1991). More differences are available across the IGS regions (Pramateftaki et al., 2000) enabling the development of conventional and Scorpion primers to identify and detect *V. dahliae* and *V. albo-atrum* (Nigro et al., 2002).

Among conserved genes, the β-tubulin has been used frequently to develop diagnostics for fungi (McKay et al., 1999; Fraaije et al., 2001). This gene can be amplified quite easily with universal primers (Glass and Donaldson, 1995) and have sequences that can be useful when ITS sequence variation is not suitable for production of a taxon-specific diagnostic. Other genes used as diagnostic targets include ascomycete mating-type genes (Dyer et al., 2001; Foster et al., 2002), the elictin gene (Lacourt

and Duncan, 1997) and the laccase gene (Hietala et al., 2003).

Specific target sequences can also be identified by amplifying random regions of the fungal genome with PCR-based techniques such as random amplified polymorphic DNA (RAPD) and arbitrarily primed PCR (AP-PCR) (Welsh and McClelland, 1990; Williams et al., 1990). PCR amplified fragments are separated by gel electrophoresis to identify unique PCR bands that can be purified, cloned, sequenced and used as probes and/or as a target to design specific sequence characterised amplified region (SCAR) primers. This approach requires more time and experience compared to the amplification of conserved genes, since the analysis of a large number of isolates belonging to both the species of interest and the related species is necessary. As no information is available on the localisation of the amplified fragments in the genome, extensive analyses are required to assess reproducibility and specificity over time. However, SCAR primers with different levels of specificity have been used for a large number of fungi and shown to be particularly useful when closely related species or specific strains need to be identified. Specific SCAR primers were used to monitor biocontrol fungal strains released in the field (Paavanen-Huhtala et al., 2000; Schena et al., 2000, 2002a), the ectomycorrhizal fungus *Tuber borchii* (Bertini et al., 1998), and phytopathogenic fungi (Taylor et al., 2001b; Vandemark and Barker, 2003).

Qualitative detection of fungi

Compared to conventional PCR, real-time PCR has significant advantages. This technique eliminates the requirement for post-amplification processing steps and significantly reduces the time and labour, greatly increasing the throughput of PCR testing as an automated diagnostic system suitable for large-scale analyses (Schena et al., 2002b; Schena and Ippolito, 2003). Furthermore, without ethidium bromide, health risks for operators and environmental contamination are reduced. To date, the main constraint to the diffusion of real-time PCR is the requirement for dedicated, sophisticated spectrofluorometric thermal cyclers, which are expensive to purchase and to maintain. However, the recent results on the diagnosis of bacterial diseases by using a portable real-time PCR apparatus suggest the problem is being overcome (Schaad and Frederich, 2002).

The potential of real-time PCR as large-scale detection method for phytopathogenic and antagonistic fungi can be further improved by the use of multiple primers to amplify and detect multiple templates within a single reaction (multiplex real-time PCR). In conventional multiplex PCR, discrimination relies on obtaining products of different sizes that can be distinguished by gel electrophoresis. However, different size products amplify with different efficiencies. In contrast, with real-time specific detection methods, differentiation can be achieved using different fluorescent dyes, and therefore amplicons of the same length can be used. A limit to the use of multiplex real-time PCR is the limited number of fluorophores available, however the discovery and application of non-fluorescent quencher (dark quencher) has made available wavelength emission that were previously occupied by the emission of the quenchers themselves (Mackay et al., 2002). This advance has permitted the inclusion of a greater number of spectrally discernible oligoprobes per reaction, and highlighted the need for a single dark quencher, which can quench a broad range of emission wavelengths. From a practical point of view, although several fluorophores have been used simultaneously in human medicine (Ugozzoli et al., 2002), only two fluorophores have been reported in plant pathology for the detection of fungi with specific real-time detection methods (TaqMan, Molecular beacons and Scorpion-PCR). This is partially due to reduced sensitivity of multiplex PCR compared to separate amplifications. Furthermore, multiplex PCR is less reliable for quantitative analyses. A very high level of sensitivity is essential to detect soilborne and wood colonising fungi that can be present at very low levels. TaqMan probes labelled with different fluorophores enabled the simultaneous detection of host (Douglas-fir) and pathogen (*Phaseocryptopus gaeumanii*) DNA whereas two different Scorpion primers enabled the identification of *Phytophthora nicotianae* and *P. citrophthora* in a single tube (Winton et al., 2002; Ippolito et al., 2004).

Real-time PCR seems to be more sensitive than conventional PCR. Amplification of *Rhizoctonia solani* target DNA extracted from soil was

achieved with real-time PCR, but not using conventional PCR (Lees et al., 2002). Similarly, the same level of sensitivity for specific detection of *Helminthosporium solani* in soil and in tubers was obtained with a TaqMan-based PCR (Cullen et al., 2001). Combining two sequential amplifications with conventional (first amplification) and labelled primers (second amplification) a further increase of sensitivity can be achieved without loosing the advantages of real-time PCR. Such approach (nested Scorpion-PCR) enabled the detection of *Rosellinia necatrix* (Schena et al., 2002b; Schena and Ippolito, 2003), *V. dahliae* (Nigro et al., 2002), *P. nicotianae,* and *P. citrophthora* (Ippolito et al., 2000) in naturally infected substrates (soils, roots, bark, and/or woody tissues). Nested Scorpion-PCR provided higher levels of sensitivity than conventional detection methods and required much less time.

One of the main disadvantages of fungal diagnosis based on conventional PCR is the risk of false positive due to cross contamination of reaction mixtures. Although false positives can result from sample-to-sample contamination, a more serious source of false positive is the carry-over of DNA from a previous amplification of the same target (Kwok, 1990). In real-time PCR, the reduced level of sample manipulation reduces the potential of cross contamination. When a double amplification is required (nested PCR) cross contamination can be a problem due to increased handling. However, considering that the quantity of amplified product after the first amplification is usually very low and that tubes remain closed after the second amplification, the risks of cross contaminations in real-time nested PCR remain much lower compared to conventional PCR.

A variety of naturally occurring compounds, such as humic acids, tannins, and lignin associated compounds, can interfere with PCR reactions and inhibit the amplification (Cullen and Hirsch 1998; Bridge and Spooner, 2001; Ippolito et al., 2002), although such problems have been partially overcome by development and optimization of the initial DNA extraction methods. Real-time PCR seems to be less affected by inhibitors than conventional PCR, because they mainly affect the late cycles of PCR, which are critical for product accumulation. In contrast, product accumulation is not required to give positive results in real-time assays, because detection is achieved through the generation of a fluorescent signal in the early stages of PCR (Mumford et al., 2000).

A major limitation to the use of conventional as well as real-time PCR to detect phytopathogenic and antagonistic fungi by purely molecular methods is the lack of discrimination between living and dead material. As a result, molecular profiling of natural samples can be expected to give different results compared with those obtained by traditional isolation techniques (Bridge and Spooner, 2001). Nucleases are widely diffused in the environment and can degrade DNA after the death of microrganisms. However, the degradation rate strongly depends on environmental conditions. Schena and Ippolito (2003) found that DNA of *R. necatrix* is degraded rapidly in soil and minimize the risks of false positives due to the presence of dead cells. However, further research is necessary to assess the persistence of the DNA under different environmental conditions and in relation to the structures produced by the pathogens. Indeed, although several reports indicate that nucleic acids are quickly digested by DNases in the soil (England et al., 1998), other studies have shown that DNA can persist in soil for long period of time by forming complexes with soil components (England et al., 1997). A possible approach to avoid false positive due to the detection of DNA in dead cells is the combination of real-time PCR with baiting (BIO-PCR). The biological amplification of the target microrganisms increase sensitivity of the detection and reduce problems with DNA extraction from complex environmental samples, such as soil, since nucleic acids do not need to be concentrated before PCR (Schaad and Frederich, 2002). BIO-PCR seems to be particular suitable for phytopathogenic bacteria since they are easily and quickly cultured in liquid and/or on solid nutritive media (Weller et al., 2000). This technique has also been utilised to detect phytopathogenic fungi, such as *R. solani* (Lees et al., 2002) and *P. nicotianae* (Ippolito et al., 2000) in naturally infested soils. Compared to real-time PCR alone, BIO-PCR requires more time for analysis (microrganisms needs to be grown on nutritive media for 1–4 days) and is more expensive (especially if selective media are utilised). An alternative strategy could rely on the use of RNA rather DNA as target molecule for diagnosis. RNA is degraded quickly in dead cells and does not interfere with the analysis. In order to be used as target molecule by PCR, RNA must first

be reverse transcribed into DNA (RT-PCR) (see below).

Quantitative detection of fungi

An accurate, reliable, and high throughput quantification of target DNA requires a real-time PCR approach (Lie and Petropoulos, 1998; Schmittgen, 2001). Several amplification methods have been developed with conventional PCR for quantitative analysis of phytopathogenic fungi (competitive PCR) (Hadidi et al., 1995; Mahuku and Platt, 2002). These methods are laborious and not enough accurate because amplification efficiency decreases during later PCR cycles (Ginzinger, 2002). As previously described, in real-time PCR the amplicons are measured at an early stage of the reaction when the efficiency is still constant. The number of PCR cycles necessary to generate a fluorescent signal significantly above the noise level (Cycle threshold, Ct) is inversely related to the log of the initial amount of target molecules. Quantification is automatically determined by associate software interpolating Ct values of unknown samples with standard curves prepared from known quantities of target DNA.

Quantification of soil-inhabiting fungi

A research area of particular interest is the quantification of individual fungal species in the soil. A high and significant correlation was found between inoculum density of *P. nicotianae* assessed with a selective medium (propagules per gram of soil), and the corresponding Ct values (Ippolito et al., 2004). Similarly, using a *R. necatrix* infested soil serially mixed with different amounts of uninfested soil a high correlation was found between the dilution factor (% of infested soil) and the cycle threshold (Schena and Ippolito, 2003). The biomass of two ectomycorrhizal fungal species (*Suillus bovinus* and *Paxillus involutus*) was detected in samples containing a complex microbial community and showed increasing amounts of *S. bovinus* DNA and decreasing amounts of *P. involutus* DNA over the time (Landeweert et al., 2003). Other methods used to quantify pathogen DNA in soil have been developed for *H. solani* (Cullen et al., 2001), *Colletotrichum coccodes* (Cullen et al., 2002), *Spongospora subterranea* (van de Graaf et al., 2003), *Fusarium solani* f. sp. *phaseoli* and the arbuscolar mycorrhizal fungus *Glomus intraradices* (Filion et al., 2003a). Unlike baiting and cultural methods, real-time PCR is not affected by external factors such as other fungal species that could conceal the presence of the pathogen on agar-based media. Therefore real-time PCR has the potential for determining the soil inoculum threshold levels necessary for the disease development in a number of host–pathogen combinations. Similar research should allow the development of predictive diagnostic test to identify high-risk fields where pathogens inoculum is above threshold values (Cullen et al., 2001, 2002). Quantitative analyses are of basic importance if there is a threshold level of pathogen propagules that trigger application of chemical or other control means. For example in citrus, fungicide application is thought to be economically justified if the *Phytophthora* population is higher than 15–20 propagules per gram of soil (ppg) for susceptible rootstocks (Lutz and Menge, 1986; Magnano di San Lio et al., 1988) and 30 ppg for the resistant ones (Ippolito et al., 1991).

Quantification of biocontrol agents

Biocontrol agents need to be monitored to evaluate their ability to colonise the environment, to tolerate climate changes and to assess environmental risks (Gullino et al., 1995). A strain of *Aureobasidium pullulans* (L47), effective against postharvest rot of fruits and vegetables has been monitored and quantified on the carpoplane of table grapes and sweet cherries by Scorpion-PCR (Schena et al., 2002a). The results from molecular analysis were correlated significantly with the colony forming units, assessed by culturing the antagonist on a semiselective medium. Similarly, compared to dilutions on selective media, real-time PCR provided a more sensitive method to detect and quantify in soil a potential biocontrol agent (*Plectosphaerella cucumerina*) of potato cyst nematodes (Atkins et al., 2003).

Fungal DNA quantification in host tissues

The quantification and the assessment of the colonization rate of plant pathogens in host tissues is one of the most attractive applications of real-time PCR. Quantitative detection methods are available

to quantify *H. solani* and *C. coccodes* in potato tubers (Cullen et al., 2001, 2002), *G. mosseae*, *Phytophthora infestans* and *P. citrophthora* in different hosts (Böhm et al., 1999), *Diaporte phaseolorum* and *Phomopsis longicolla* in soybean seeds (Zhang et al., 1999), *R. necatrix* in roots and bark of different hosts (Schena and Ippolito, 2003), *P. nicotianae* and *P. citrophthora* in citrus roots (Ippolito et al., 2004), *Pyrenophora teres* on infected barley seed (Bates et al., 2001; Bates and Taylor, 2001), and *Aspergillus flavus* on maize, pepper, and paprika (Mayer et al., 2003). Using two probes labelled with different fluorophores it was possible to quantify simultaneously host (Douglas-fir) and pathogen (*Phaeocryptopus gaeumannii*) DNA (Winton et al., 2002). In this case, detection of host DNA provided an endogenous reference that served as an internal positive control to adjust variations introduced by sample-to-sample differences in DNA extraction and PCR efficiency. Compared to three different alternative methods (fruiting body abundance, ergosterol content and dot blot analysis), TaqMan real-time PCR provided a more accurate calculation of the relative growth of the fungus within Douglas-fir needles and was the only method able to quantify the pathogen early in the disease cycle (Winton et al., 2003).

Due to its high sensitivity and reproducibility, real-time PCR is ideal to detect minor changes of host resistance and susceptibility. Blast resistance levels of rice cultivars were more accurately evaluated with real-time PCR, since by the time in which lesions on leaves just became visible, the growth of *Magnaporthe grisea* was 80 times higher in susceptible than in resistant cultivar (Qi and Yang, 2002). In Norway Spruce (*Picea abies*) colonised with *Heterobasidion annosum*, pathogen DNA was more restricted and localised in the lesion of a clone with high resistance, whereas in a clone with low resistance, the fungus was detected until the visible end of the lesion (Hietala et al., 2003). Similarly, in alfalfa plants infected with *Aphanomyces euteiches* (Vandemark et al., 2002) and with *Phytophthora medicaginis* (Vandemark and Barker, 2003), significant correlations were found between the amount of pathogens DNA and disease severity, suggesting that real-time PCR can be utilised for the selection of resistant plants also when samples are indistinguishable based on the visual assessment of disease severity. The same authors demonstrated that quantitative PCR could be used to study mechanisms of resistance to the pathogen. Low levels of pathogen DNA in resistant plants would characterise a mechanism that result in the inhibition of pathogen multiplication, whereas the presence of relatively high amounts of pathogen DNA should indicate a mechanism based on tolerance rather than on true resistance.

Quantification of mRNA transcription

Among the different real-time PCR applications, the quantification of messenger RNA (mRNA) to evaluate gene expression is one of the most promising. If mRNA is being measured the experiment must contain a reverse transcription step in which RNA is converted to cDNA (RT-PCR). Once the cDNA has been synthesised a standard quantitative real-time PCR reaction can be run and quantification carried out. Primers should bind to separate exons to avoid false positive results, arising from amplification of contaminating genomic DNA. If the intron/exon boundaries are unknown, or when targeting an intron-less gene, it is necessary to treat the RNA sample with RNAse-free DNAse. Generally, two quantification strategies can be performed: absolute and relative quantification (Livak and Schmittgen 2001; Pfaffl and Hageleit, 2001; Pfaffl et al., 2002). In the absolute quantification the mRNA copy number is determined by comparison with appropriate external calibration curves. The relative quantification is based on the expression ratio of a target gene versus a reference gene. Several housekeeping non-regulated reference gene have been proposed for mammalian systems (Bustin, 2002), whereas much less information is available for filamentous fungi and plant hosts. The β-tubulin and the actin genes seem to be appropriate to normalise results from gene expression in both filamentous fungi (Semighini et al., 2002) and plant hosts (Bézier et al., 2002).

The quantification of mRNA to assess the effect of different treatments on its transcription level is widely and increasingly used in medicine (Bustin, 2000, 2002; Snider et al., 2001; Ginzinger, 2002) and it is likely that it would be adapted to plant pathology. In fact, although microarrays are the most powerful methods for determining gene

expression in response to microbes, false positive results are relative common and require confirmation with independent methods such as Northern blot analysis (Kagnoff and Eckmann, 2001). However, real-time quantitative PCR is sensitive enough to detect and quantify gene expression and to reveal small changes in mRNA level (Bustin, 2002).

Plants defence themselves against fungal pathogens as well as other biotic and abiotic stress factors activating specific genes that can be revealed by quantifying mRNA (McMaugh and Lyon, 2003). Recently, real-time quantitative RT-PCR was used to evaluate the effect of elevated growth temperatures and heat stress on the expression of ascorbate peroxidase genes in *Arabidopsis* (Panchuk et al., 2002). The same approach enabled to study the role of defense-related genes (phenylalanine ammonia-lyase, stilbene synthase, polygalacturonase inhibitor protein, an acidic chitinase) in grapevine leaves and berries infected by *Botrytis cinerea* (Bézier et al., 2002). The analysis of *Polymyxa betae* glutathione-S-transferase (GST) expression in infected sugar beet showed continuing upward trend of transcript levels in susceptible plants and extremely low levels in resistant ones (Kingsnorth et al., 2003).

Another possible application of real-time quantitative RT-PCR is the quantification of the expression of specific genes from fungal pathogens and antagonists. The analysis of the relative transcript levels of ABC transporters genes by real-time RT-PCR might provide useful information about multidrug resistance in filamentous fungi as reported for *Aspergillus nidulans* (Semighini et al., 2002). In *P. infestans* the analysis of the expression of 18 genes showed that all transcripts were up regulated in germinating cysts and 12 were found to be up regulated in potato leaf during the early stages of infection (Avrova et al., 2003). Recent studies have demonstrated that the expression of an endochitinase gene of the mycoparasite *Stachybotrys elegans* increase after 2 days of contact with the pathogen *Rhizoctonia solani* (Morissette et al., 2003).

Concluding remarks

Real-time PCR has an enormous potential to address central questions in plant pathology at a level of precision that was impossible a few years ago. The sensitivity, speed, and versatility of this technique, together with the possibility of achieving quantitative analyses, are primary factors which will encourage its rapid and wide acceptance in plant pathology. It is likely that real-time PCR will become a standard method suitable for large-scale diagnosis of phytopathogenic and antagonistic fungi in extension services.

Quantitative results provide new important information not achievable with conventional PCR. In particular, although still in its embryonic phase, this technology has great potential for the quantification of mRNA to study unknown mechanisms involved in the host–pathogen interaction as well as in the pathogen's response to environmental changes.

Real-time PCR technologies are also ideal for the detection of single nucleotide polymorphisms (SNPs). These markers are increasingly used in clinical diagnosis and often are associated with specific diseases. Similar applications are also possible in plant pathology (Bates and Taylor, 2001) and will prove a useful instrument for the study of gene flow and population genetics of phytopathogenic and antagonistic fungi.

To date research has just begun to develop specific applications. Although the number of primers and fluorescent probes available for real-time PCR is still limited compared to conventional PCR, many more will be developed in the near future. As knowledge of the fungal genome expands, the opportunities to use real-time quantitative PCR in a wide variety of settings will expand. Classical PCR protocols already exist for many phytopathogenic and antagonistic fungi. Adapting these to a real-time PCR assays should be a relatively easy task.

Acknowledgements

This work was partially supported by CEGBA (Centro di Eccellenza di Genomica in campo Biomedico e Agrario) research line n.7 and by Ministero dell'Istruzione, dell'Università e della Ricerca of Italy. PRIN 2003 – Grant No. 2003074533: 'Diseases caused by soil-borne species of *Phytophthora*: aetiology, molecular diagnosis, epidemiology, and new control means'.

References

Ahmed FE (2002) Detection of genetically modified organisms in foods. TRENDS in Biotechnology 20: 215–223

Atkins SD, Clark IM, Sosnowska D, Hirsch PR and Kerry BR (2003) Detection and quantification of *Plectosphaerella cucumerina*, a potential biological control agent of potato cyst nematodes, by using conventional PCR, real-time PCR, selective media, and baiting. Applied and Environmental Microbiology 69: 4788–4793

Avrova A, Venter E, Birch PRJ and Whissona SC (2003) Profiling and quantifying differential gene transcription in *Phytophthora infestans* prior to and during the early stages of potato infection. Fungal Genetics and Biology 40: 4–14

Bates JA and Taylor EJA (2001) Scorpion ARMS primers for SNP real-time PCR detection and quantification of *Pyrenophora teres*. Molecular Plant Pathology 2: 275–280

Bates JA, Taylor EJA, Gans PT and Thomas JE (2002) Determination of relative proportions of *Globodera* species in mixed populations of potato cyst nematodes using PCR product melting peak analysis. Molecular Plant Pathology 3: 153–161

Bates JA, Taylor EJA, Kenyon DM and Thomas JE (2001) The application of real-time PCR to the identification, detection and quantification of *Pyrenophora* species in barley seed. Molecular Plant Pathology 2: 49–57

Bertini L, Agostini D, Potenza L, Rossi I, Zeppa S, Zambonelli A and Stocchi V (1998) Molecular markers for the identification of the ectomycorrhizal fungus *Tuber borchii*. New Phytologist 139: 565–570

Bézier A, Lambert B and Baillieul F (2002) Study of defense-related gene expression in grapevine leaves and berries infected with *Botrytis cinerea*. European Journal of Plant Pathology 108: 111–120

Böhm J, Hahn A, Schubert R, Bahnweg G, Adler N, Nechwatal J, Oehlmann R and Oßwald W (1999) Real-time quantitative PCR: DNA determination in isolated spores of the mycorrhizal fungus *Glomus mosseae* and monitoring of *Phytophthora infestans* and *Phytophthora citricola* in their respective host plants. Journal of Phytopathology 147: 409–416

Boonham N, Smith P, Walsh K, Tame J, Morris J, Spence N, Bennison J and Barker I (2002) The detection of *tomato spotted wilt virus* (TSWV) in individual thrips using real time fluorescent RT-PCR (TaqMan). Journal of Virological Methods 101: 37–48

Bridge P and Spooner B (2001) Soil fungi: diversity and detection. Plant and Soil 232: 147–154

Broude NE (2002) Stem-loop oligonucleotides: a robust tool for molecular biology and biotechnology. Trends in Biotechnology 20: 249–256

Brouwer M, Lievens B, Van Hemelrijck W, Van den Ackerveken G, Cammue BPA and Thomma BPHJ (2003) Quantification of disease progression of several microbial pathogens on *Arabidopsis thaliana* using real-time fluorescence PCR. FEMS Microbiology Letters 228: 241–248

Bruns TD, White TJ and Taylor JW (1991) Fungal molecular systematics. Annual Review of Ecology and Systematics 22: 525–564

Bustin SA (2000) Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. Journal of Molecular Endocrinology 25: 169–193

Bustin SA (2002) Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. Journal of Molecular Endocrinology 29: 23–39

Ciancio A, Leonetti P and Finetti Sialer MM (2000) Detection of nematode antagonistic bacteria by fluorogenic molecular probes. EPPO Bulletin 30: 563–570

Cullen DW and Hirsch PR (1998) Simple and rapid method for direct extraction of microbial DNA from soil for PCR. Soil Biology and Biochemistry 30: 983–993

Cullen DW, Lees AK, Toth IK and Duncan JM (2001) Conventional PCR and real-time quantitative PCR detection of *Helminthosporium solani* in soil and potato tubers. European Journal of Plant Pathology 107: 387–398

Cullen DW, Lees AK, Toth IK and Duncan JM (2002) Detection of *Colletotrichum coccodes* from soil and potato tubers by conventional and quantitative real-time PCR. Plant Pathology 51: 281–292

Didenko VV (2001) DNA probes using fluorescence resonance energy transfer (FRET): designs and applications. BioTechniques 31: 1106–1121

Dyer PS, Furneaux PA, Douhan G and Murray TD (2001) A multiplex PCR test for determination of mating type applied to the plant pathogens *Tapesia yallundae* and *Tapesia acuformis*. Fungal Genetics and Biology 33: 173–180

England LS, Holmes SB and Trevors JT (1998) Persistence of viruses and DNA in soil. World Journal of Microbiology and Biotechnology 14: 163–169

England LS, Lee H and Trevors JT (1997) Persistence of *Pseudomonas aureofaciens* strains and DNA in soil. Soil Biology and Biochemistry 29: 1521–1527

Eun AJC, Seoh ML and Wong SM (2000) Simultaneous quantitation of two orchid viruses by the TaqMan® real-time RT-PCR. Journal of Virological Methods 87: 151–160

Filion M, St-Arnaud M and Jabaji-Hare SH (2003a) Quantification of *Fusarium solani* f. sp. *phaseoli* in mycorrhizal bean plants and surrounding mycorrhizosphere soil using real-time polymerase chain reaction and direct isolations on selective media. Phytopathology 93: 229–235

Filion M, St-Arnaud M and Jabaji-Hare SH (2003b) Direct quantification of fungal DNA from soil substrate using real-time PCR. Journal of Microbiological Methods 53: 67–76

Finetti Sialer M, Ciancio A and Gallitelli D (2000b) Use of fluorogenic scorpions for fast and sensitive detection of plant viruses. EPPO Bulletin 30: 437–440

Finetti Sialer M, Schena L and Gallitelli D (2000a) Real-Time diagnosis in plant pathology with self-probing amplicons (Scorpions). Proceeding 5th Congress of the European Foundation for Plant Pathology (pp 94–96) Taormina-Giardini Naxos, Catania, Italy

Foster SJ, Ashby AM and Fitt BDL (2002) Improved PCR-based assays for pre-symptomatic diagnosis of light leaf spot and determination of mating type of *Pyrenopeziza brassicae* on winter oilseed rape. European Journal of Plant Pathology 108: 379–383

Fraaije BA, Butters JA, Coelho JM, Jones DR and Hollomon DW (2002) Following the dynamics of strobilurin resistance in *Blumeria graminis* f. sp. *tritici* using quantitative

allele-specific real-time PCR measurements with the fluorescent dye SYBR Green I. Plant Pathology 51: 45–54

Fraaije BA, Lovell DJ, Coelho JM, Baldwin S and Hollomon DW (2001) PCR-based assays to assess wheat varietal resistance to blotch (*Septoria tritici* and *Stagonospora nodorum*) and rust (*Puccinia striiformis* and *Puccinia recondita*) diseases. European Journal of Plant Pathology 107: 905–917

Frederick RD, Snyder CL, Peterson GL and Bonde MR (2002) Polymerase chain reaction assays for the detection and discrimination of the soybean rust pathogens *Phakopsora pachyrhizi* and *P. meibomiae*. Phytopathology 92: 217–227

Frederick RD, Snyder KE, Tooley PW, Berthier-Schaad Y, Peterson GL, Bonde MR, Schaad NW and Knorr DA (2000) Identification and differentiation of *Tilletia indica* and *T. walkeri* using the polymerase chain reaction. Phytopathology 90: 951–960

Ginzinger DG (2002) Gene quantification using real-time quantitative PCR: an emerging technology hits the mainstream. Experimental Hematology 30: 503–512

Glass NL and Donalson GC (1995) Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Applied and Environmental Microbiology 31: 1323–1330

Gonçalves MC, Klerks MM, Verbeek M, Vega J and van den Heuvel JFJM (2002) The use of Molecular beacons combined with NASBA for the sensitive detection of Sugarcane yellow leaf virus. European Journal of Plant Pathology 108: 401–407

Gullino ML, Migheli Q and Mezzalama M (1995) Risk analysis in the release of biological control agents. Plant Disease 79: 1193–1201

Hadidi A, Levy L and Podleckis EV (1995) Polymerase chain reaction technology in plant pathology. In: Singh RP and Singh US (eds) Molecular Methods in Plant Pathology (pp 167–187). CRC Press Inc. London, UK

Haugland RA, Brinkman N and Vesper SJ (2002) Evaluation of rapid DNA extraction methods for the quantitative detection of fungi using real-time PCR analysis. Journal of Microbiological Methods 50: 319–323

Henson JM and French R (1993) The polymerase chain reaction and plant disease diagnosis. Annual Review of Phytopathology 31: 81–109

Hermansson A and Lindgren PE (2001) Quantification of ammonia-oxidizing bacteria in arable soil by real-time PCR. Applied and Environmental Microbiology 67: 972–976

Heuser T and Zimmer W (2002) Quantitative analysis of phytopathogenic ascomycota on leaves of pedunculate oaks (*Quercus robur* L.) by real-time PCR. FEMS Microbiology Letters 209: 295–299

Hietala AM, Eikenes M, Kvaalen H, Solheim H and Fossdal CG (2003) Multiplex real-time PCR for monitoring *Heterobasidion annosum* colonization in norway spruce clones that differ in disease resistance. Applied and Environmental Microbiology 69: 4413–4420

Higuchi R, Dollinger G, Walsh PS and Griffith R (1992) Simultaneous amplification and detection of specific DNA sequences. Biotechnology 10: 413–417

Holland PM, Abramson RD, Watson R and Gelfand D (1991) Detection of specific polymerase chain reaction products by utilizing the 5′-3′ exonuclease activity of *Thermus aquaticus* DNA polymerase. Proceeding of the National Academy of Science the United States of America 88: 7276–7280

Howlett BJ, Brownlee AG, Guest DI, Adcock GJ and McFadden GI (1992) The 5S ribosomal RNA gene is linked to the large and small subunit ribosomal RNA genes in the oomycetes, *Phytophthora vignae*, *P. cinnamomi*, *P. megasperma* f. sp. *glycinea* and *Saprolegnia ferax*. Current Genetics 22: 455–461

Ippolito A, De Cicco V and Salerno M (1991) Aetiological and epidemiological aspects of Phytophthora citrus root rot in Apulia and Basilicata. Phytopathologia Mediterranea 30: 47–51

Ippolito A, Schena L and Nigro F (2002) Detection of *Phytophthora nicotianae* and *P. citrophthora* in citrus roots and soils by nested PCR. European Journal of Plant Pathology 108: 855–868

Ippolito A, Schena L, Nigro F and Salerno M (2000) PCR based detection of *Phytophthora* spp. and *P. nicotianae* from roots and soil of citrus plants. Proceeding 5th Congress of the European Foundation for Plant Pathology (pp. 158–160) Taormina-Giardini Naxos, Catania, Italy

Ippolito A, Schena L, Nigro F, Soleti Ligorio V and Yaseen T (2004) Real-time detection of *Phytophthora nicotianae* and *P. citrophthora* in citrus roots and soil. European Journal of Plant Pathology 110: 833–843

Ishiguro T, Saitoh J, Yawata H, Yamagishi H, Iwasaki S and Mitoma Y (1995) Homogeneous qualitative assay of hepatitis C virus RNA by polymerase chain reaction in the presence of a fluorescent intercalater. Analytical Biochemistry 229: 207–213

Kagnoff MF and Eckmann L (2001) Analysis of host responses to microbial infection using gene expression profiling. Current Opinion in Microbiology 4: 246–250

Kingsnorth CS, Kingsnorth AJ, Lyons PA, Chwarszczynska DM and Asher MJC (2003) Real-time analysis of Polymyxa betae GST expression in infected sugar beet. Molecular Plant Pathology 4: 171–176

Korimbocus J, Coates D, Barker I and Boonham N (2002) Improved detection of Sugarcane yellow leaf virus using a real-time fluorescent (TaqMan) RT-PCR assay. Journal of Virological Methods 103: 109–120

Kwok S (1990) Procedures to minimize PCR-product carryover. In: Innis MA, Gelfand DH, Sninsky J and White TJ (eds) PCR Protocols: A Guide to Methods and Applications (pp 142–145) Academic Press Inc. Harcourt Brace Jovanovich, London, UK

Lacourt I and Duncan JM (1997) Specific detection of *Phytophthora nicotianae* using the polymerase chain reaction and primers based on the DNA sequence of its elictin gene ParA1. European Journal of Plant Pathology 103: 73–83

Landeweert R, Veenman C, Kuyper TW, Fritze H, Wernars K and Smit E (2003) Quantification of ectomycorrhizal mycelium in soil by real-time PCR compared to conventional quantification techniques. FEMS Microbiology Ecology 45: 283–292

Lees AK, Cullen DW, Sullivan L and Nicolson MJ (2002) Development of conventional and quantitative real-time PCR assays for the detection and identification of *Rhizoctonia solani* AG-3 in potato and soil. Plant Pathology 51: 293–302

Lie YS and Petropoulos CJ (1998) Advances in quantitative PCR technology: 5′ nuclease assay. Current Opinion in Biotechnology 9: 43–49

Liew ECY, Maclean DJ and Irwin JAG (1998) PCR based detection of *Phytophthora medicaginis* using the intergenic

spacer region of the ribosomal DNA. Mycological Research 102: 73–80

Livak KJ and Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2_T^{-\Delta\Delta C}$ method. Methods 25: 402–408

Livak KJ, Flood SJA, Marmaro J, Giusti W and Deetz K (1995) Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridisation. PCR Methods and Applications 4: 357–362

Lutz A and Menge JA (1986) Phytophthora root rot. Citrograph 72: 33–39

Lyon E (2001) Mutation detection using fluorescent hybridisation probes and melting curves analyses. Expert Review of Molecular Diagnostics 1: 92–101

Mackay IM, Arden KE and Nitsche A (2002) Real-time PCR in virology. Nucleic Acids Research 30: 1292–1305

Magnano di San Lio G, Perrotta G, Cacciola SO and Tuttobene R (1988) Factors affecting soil populations of *Phytophthora* in citrus orchards. In: Proceedings of Sixth International Citrus Congress 2: 767–774

Mahuku GS and Platt HW (2002) Quantifying *Verticillium dahliae* in soils collected from potato fields using a competitive PCR assay. American Journal of Potato Research 79: 107–117

Martin RR, James D and Lévesque CA (2000) Impact of molecular diagnostic technologies on plant disease management. Annual Review of Phytopathology 38: 207–239

Mayer Z, Bagnara A, Färber P and Geisen R (2003) Quantification of the copy number of nor-1, a gene of the aflatoxin biosynthetic pathway by real-time PCR, and its correlation to the cfu of *Aspergillus flavus* in foods. International Journal of Food Microbiology 82: 143–151

McCartney HA, Foster SJ, Fraaije BA and Ward E (2003) Molecular diagnostics for fungal plant pathogens. Pest Management Science 59: 129–142

McKay GJ, Brown AE, Bjourson AJ and Mercer PC (1999) Molecular characterisation of *Alternaria linicola* and its detection in linseed. European Journal of Plant Pathology 105: 157–166

McMaugh SJ and Lyon BR (2003) Real-time quantitative RT-PCR assay of gene expression in plant roots during fungal pathogenesis. BioTechniques 34: 982–986

Morissette DC, Driscoll BT and Jabaji-Harea S (2003) Molecular cloning, characterisation, and expression of a cDNA encoding an endochitinase gene from the mycoparasite *Stachybotrys elegans*. Fungal Genetics and Biology 39: 276–285

Morrison TM, Weis JJ and Wittwer CT (1998) Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification. BioTechniques 24: 954–962

Morton A, Tabrett AM, Carder JH and Barbara DJ (1995) Sub-repeat sequences in the ribosomal RNA intergenic regions of *Verticillium albo-atrum* and *V. dahliae*. Mycological Research 99: 257–266

Mumford RA, Walsh K, Barker I and Boonham N (2000) Detection of *potato mop top virus* and *tobacco rattle virus* using a multiplex real-time fluorescent reverse-transcription polymerase chain reaction assay. Phytopathology 90: 448–453

Nazar RN, Hu X, Schmidt D, Culham D and Robb J (1991) Potential use of PCR-amplified ribosomal intergenic sequences in the detection and differentiation of verticillium wilt pathogens. Physiological and Molecular Plant Pathology 39: 1–11

Nigro F, Schena L and Gallone P (2002) Diagnosi in tempo reale della verticilliosi dell'olivo mediante Scorpion-PCR. Proceeding 'Convegno Internazionale di Olivicoltura' (pp. 454–461) Spoleto, Italy

Paavanen-Huhtala S, Avikainen H and Yli-Mattila T (2000) Development of strain-specific primers for a strain of *Gliocladium catenulatum* used in biological control. European Journal of Plant Pathology 106: 187–198

Panchuk II, Volkov R.A and Schöffl F (2002) Heat stress- and heat shock transcription factor- dependent expression and activity of ascorbate peroxidase in Arabidopsis. Plant Physiology 129: 838–853

Pfaffl MW and Hageleit M (2001) Validities of mRNA quantification using recombinant RNA and recombinant DNA external calibration curves in real-time RT-PCR. Biotechnology Letters 23: 275–282

Pfaffl MW, Horgan GW and Dempfle L (2002) Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Research 30: 36–46

Pramateftaki PV, Antoniou PP and Typas MA (2000) The complete DNA sequence of the nuclear ribosomal RNA gene complex of *Verticillium dahliae*: intraspecific heterogeneity within the intergenic spacer region. Fungal Genetics and Biology 29: 19–27

Qi M and Yang Y (2002) Quantification of *Magnaporthe grisea* during infection of rice plants using real-time polymerase chain reaction and northern blot/phosphoimaging analyses. Phytopathology 92: 870–876

Roberts CA, Dietzgen RG, Heelan LA and Maclean DJ (2000) Real-time RT-PCR fluorescent detection of tomato spotted wilt virus. Journal of Virological Methods 88: 1–8

Schaad NW and Frederick RD (2002) Real-time PCR and its application for rapid plant disease diagnostics. Canadian Journal of Plant Pathology 24: 250–258

Schaad NW, Frederick RD, Shaw J, Schneider WL, Hickson R, Petrillo MD and Luster DG (2003) Advances in molecular-based diagnostics in meeting crop biosecurity and phytosanitary issues. Annual Review of Phytopathology 41: 305–324

Schena L and Ippolito A (2003) Rapid and sensitive detection of *Rosellinia necatrix* in roots and soils by real time Scorpion-PCR. Journal of Plant Pathology 85: 15–25

Schena L, Finetti Sialer M and Gallitelli D (2000) Specific identification of *Aureobasidium pullulans* strain L47 using Scorpion-PCR. EPPO Bulletin 30: 559–562

Schena L, Finetti Sialer M and Gallitelli D (2002a) Molecular detection of the strain L47 of *Aureobasidium pullulans*, a biocontrol agent of postharvest diseases. Plant Disease 86: 54–60

Schena L, Nigro F and Ippolito A (2002b) Identification and detection of *Rosellinia necatrix* by conventional and real-time Scorpion-PCR. European Journal of Plant Pathology 108: 355–366

Schmittgen TD (2001) Real-time quantitative PCR. Methods 25: 383–385

Schnerr H, Niessen L and Vogel RF (2001) Real time detection of the *tri5* gene in *Fusarium* species by LightCycler™-PCR using SYBR®Green I for continuous fluorescence monitoring. International Journal of Food Microbiology 71: 53–61

Schoen CD, Knorr D and Leone G (1996) Detection of potato leafroll virus in dormant potato tubers by immunocapture and a fluorogenic 5′ nuclease RT-PCR assay. Phytopathology 86: 993–999

Semighini CP, Marins M, Goldman MHS and Goldman GH (2002) Quantitative analyses of the relative transcript levels of ABC transporter *Art* genes in *Aspergillus nidulans* by real-time reverse transcription-PCR assay. Applied and Environmental Microbiology 68: 1351–1357

Snider JV, Wechser MA and Lossos IS (2001) Human disease characterization: real-time quantitative PCR analysis of gene expression. Research Focus 6: 1062–1067

Solinas A, Brown LJ, McKeen C, Mellor JM, Nicol JTG, Thelwell N and Brown T (2001) Duplex Scorpion primers in SNP analysis and FRET applications. Nucleic Acids Research 29: 96–104

Taylor EJA, Bates J, Kenyon D, Maccaferri M and Thomas J (2001a) Modern molecular methods for characterization and diagnosis of seed-borne fungal pathogens. Journal of Plant Pathology 83: 75–81

Taylor EJA, Stevens EA, Bates JA, Morreale G, Lee D, Kenyon DM and Thomas JE (2001b) Rapid-cycle PCR detection of *Pyrenophora graminea* from barley seed. Plant Pathology 50: 347–355

Thelwell N, Millington S, Solinas A, Booth J and Brown T (2000) Mode of action and application of Scorpion primers to mutation detection. Nucleic Acids Research 28: 3752–3761

Tyagi S and Kramer FR (1996) Molecular beacons: probes that fluoresce upon hybridisation. Nature Biotechnology 14: 303–308

Ugozzoli LA, Chinn D and Hamby K (2002) Fluorescent multicolor multiplex homogeneous assay for the simultaneous analysis of the two most common hemochromatosis mutations. Analytical Biochemistry 307: 47–53

Van de Graaf P, Lees AK., Cullen DW and Duncan JM (2003) Detection and quantification of *Spongospora subterranea* in soil, water and plant tissue samples using real-time PCR. European Journal of Plant Pathology 109: 589–597

Vandemark GJ and Barker BM (2003) Quantifying *Phytophthora medicaginis* in susceptible and resistant alfalfa with a real-time fluorescent PCR assay. Journal of Phytopathology 151: 577–583

Vandemark GJ, Barker BM and Gritsenko MA (2002) Quantifying *Aphanomyces euteiches* in alfalfa with a fluorescent polymerase chain reaction assay. Phytopathology 92: 265–272

Weller SA, Elphinstone JG, Smith NC, Boonham N and Stead DE (2000) Detection of *Ralstonia solanacearum* strains with a quantitative, multiplex, real-time, fluorogenic PCR (Taq-Man) assay. Applied and Environmental Microbiology 66: 2853–2858

Welsh J and McClelland M (1990) Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Research 18: 7213–7218

Whitcombe D, Theaker J, Guy SP, Brown T and Little S (1999) Detection of PCR products using self-probing amplicons and fluorescence. Nature Biotechnology 17: 804–807

White TJ, Bruns T, Lee S and Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ and White TJ (eds) PCR Protocols: A Guide to Methods and Applications (pp 315–322). Academic Press, Inc., New York, USA

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA and Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Research 18: 6531–6535

Winton LM, Manter DK, Stone JK and Hansen EM (2003) Comparison of biochemical, molecular, and visual methods to quantify *Phaeocryptopus gaeumannii* in Douglas-Fir foliage. Phytopathology 93: 121–126

Winton LM, Stone JK, Watrud LS and Hansen EM (2002) Simultaneous one-tube quantification of host and pathogen DNA with real-time polymerase chain reaction. Phytopathology 92: 112–116

Zhang AW, Hartman GL, Curio-Penny B, Pedersen WL and Becker KB (1999) Molecular detection of *Diaporthe phaseolorum* and *Phomopsis longicolla* from soybean seeds. Phytopathology 89: 796–804