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Real-time PCR detection and quantification of soilborne fungal pathogens: the case of *Rosellinia necatrix, Phytophthora nicotianae, P. citrophthora,* and *Verticillium dahliae*

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Summary. Conventional and Scorpion primers were designed from the ITS regions to identify *Rosellinia necatrix*, *Phytophthora nicotianae,* and *P. citrophthora* and from the IGS regions to identify *Verticillium dahliae* and *V. albo-atrum*. Specificity of primers and probes was assessed using genomic DNA from a large number of fungi from several hosts and by means of BLAST analyses, to exclude the presence of similar sequences in other micro-organisms among available DNA databases (GenBank). Simple and rapid procedures for DNA extraction from naturally infected matrices (soils, roots, bark, and/or woody tissues) were utilised to yield DNA of a purity and quality suitable for PCR assays. Combining these protocols with a double amplification (nested Scorpion-PCR), the real-time detection of these pathogens was possible from naturally infested soils and from infected citrus roots (*P. nicotianae* and *P. citrophthora*), from the roots and bark of stone fruits and olive (*R. necatrix*) and from olive branches (*V. dahliae*). For target pathogens, the limit of detection was 1 pg μl^{-1} in Scorpion-PCR and 1 fg μl^{-1} in nested Scorpion-PCR. High and significant correlations between pathogen propagule concentrations and real-time PCR cycle thresholds (Ct) were obtained. Moreover, specific tests with *R. necatrix* seem to indicate that its DNA is quite rapidly degraded in the soil, excluding the risk of false positives due to the presence of dead cells.

Key words: Scorpion PCR, quantitative PCR, white root-rot, *Phytophthora* root rot, *Verticillium* wilt.

Introduction

Rosellinia necatrix (Hartig) Berlese on stone fruits and olive, *Phytophthora nicotianae* van Breda de Haan and *P. citrophthora* (Sm. et Sm.) Leon. on citrus, and *Verticillium dahliae* Kleb. on olive, are widespread and dangerous pathogens in both bearing orchards and nurseries. Heavy losses may result from the disease they cause; moreover, soil contamination by these pathogens, which are characterized by high poliphagy, may preclude replanting and/or new plantations. The available control methods (chemical control, soil fumigation, cultural practices, biological control, etc.) are expensive and not always effective. Therefore, disease-free propagating material planted into pathogen-free soils is a basic measure for preventing disease spread. In this regard, according to the European and Italian specifications on the quality of nursery productions (EU Dir. 93/48 of 23.06.93, "*Conformitas Agraria Com-*

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munitatis", CAC), a protocol for the certification of pathogen-free planting material of citrus, olive, and stone fruits, has recently been developed and several critical control points have been identified (Bazzoni *et al*., 2001; Djelouah *et al*., 2001; Saponari *et al*., 2001).

The early, rapid, and accurate identification and detection of pathogens in plants, soil, or water is essential to reduce their spread and to improve prevention, especially when pathogens have a wide host range. Moreover, quantitative analyses are of basic importance if there is a threshold level of pathogen propagules at which chemical or other control means must be applied (Ippolito *et al*., 1991).

Recently, detection methods based on real-time polymerase chain reaction (PCR) have been developed to identify and diagnose a number of phytopathogenic and antagonistic fungi (Schena *et al*., 2004). These techniques, which enable a micro-organism to be identified by a specific increase of fluorescence during PCR amplification, are more sensitive than conventional PCR (Lees *et al*., 2002), reduce the risk of false positives and promote multiplex and quantitative analyses (Bustin, 2002).

The present review focuses on real-time PCR detection methods developed for *R. necatrix*, *P. nicotianae*, *P. citrophthora,* and *V. dahliae*.

DNA extraction from host tissue and soil

The success and reliability of any PCR-based direct detection system for soil-borne pathogens largely depend upon obtaining high yields of target DNA from samples. Environmental samples pose problems for PCR, since a variety of naturally occurring compounds (such as humic acids, tannins, and lignin associated compounds) can interfere with the reaction and inhibit amplification (Cullen and Hirsch, 1998; Bridge and Spooner, 2001). Protocols to extract total DNA from soil, feeder roots, woody tissues and bark pieces were developed and adapted to *R. necatrix*, *P. nicotianae*, *P. citrophthora* and *V. dahliae* (Nigro *et al*., 2002; Schena and Ippolito, 2003; Ippolito *et al*., 2004). Methods are based on the physical disruption of cells by liquid nitrogen grinding and/or blending with a FastPrep FP120 Instrument (Qbiogene, Illkirch, Cedex, France) in the presence of glass beads. The key steps for the removal of co-extracted inhibitory compounds from the soil are the use of CTAB buffer and PVPP-Sepharose spin column chromatography. Similarly, excess polyphenols in the extraction from plant tissues are removed by adding PVP to the mixtures and using PVPP-Sepharose columns. These protocols have a small number of lyses and purification steps but maximize the yield and quality of recovered DNA (2–3 $\mu g\ g^{-1}$ of plant material and 0.5–1.5 $\mu g\ g^{-1}$ of soil), thus allowing rapid processing of many samples.

Primer selection, specificity and sensitivity

Four main real-time PCR chemistries are utilised to detect and study phytopathogenic fungi (SYBR Green, TaqMan, Molecular beacons, and Scorpion-PCR), however Scorpion-PCR seems to be the most powerful as it provides lower backgrounds and high signal strength (Thelwell *et al*., 2000; Schena *et al*., 2004). Detailed information on Scorpion-PCR chemistry are reported by Whitcombe *et al*. (1999) and Thelwell *et al*. (2000). Briefly, a conventional primer is covalently linked on its 5' end to a "stem-loop" shaped hybridisation probes with a fluorescent dye covalently attached on one end and a quencher covalently attached on the opposite end. The stem-loop tail is separated from the PCR primer sequence by a "PCR blocker", a chemical modification that prevent the Taq polymerase from copying the stem loop sequence of the Scorpion primer. The probe element (loop) is complementary to a sequence newly synthesized 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.

The nuclear-encoded ribosomal RNA genes (rDNA) provide attractive targets to design specific primers since they are highly stable, can be amplified and sequenced with universal primers, occur in multiple copies, and possess conserved as well as variable sequences (White *et al*., 1990) (Fig. 1A). Among the variable regions, ITS are the most widely sequenced in fungi (Henson and French,

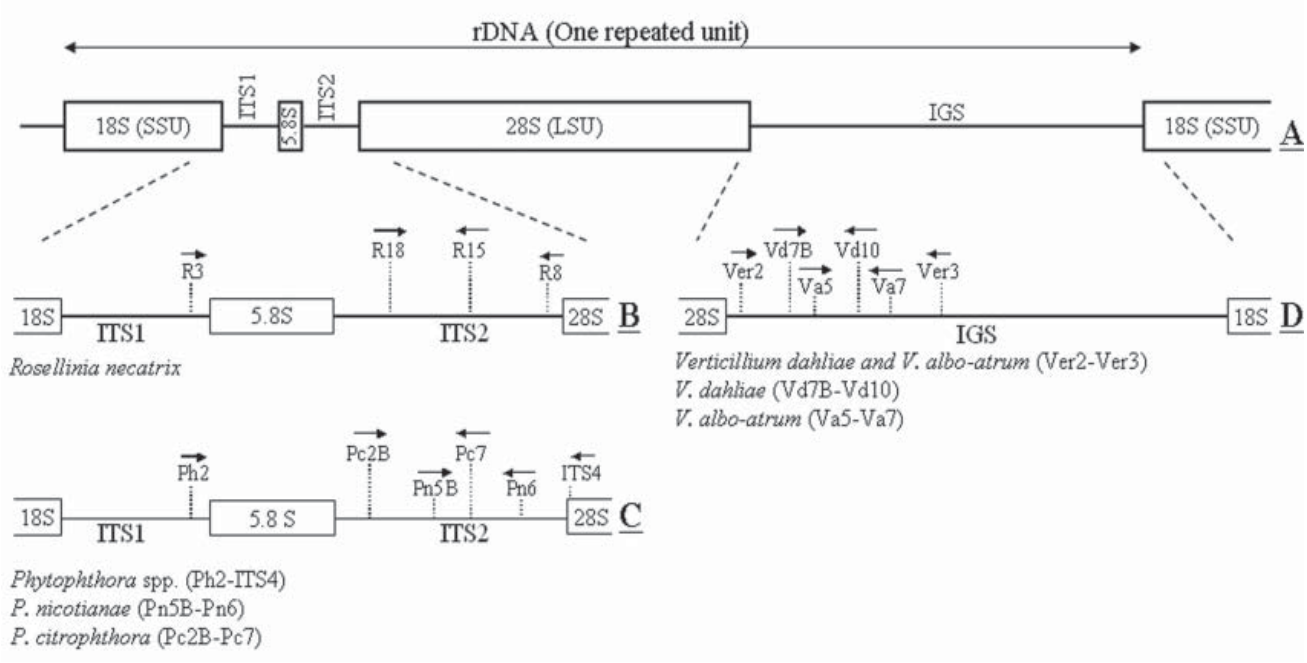

Fig. 1. Diagrammatic representation of the organization of ribosomal DNA repeat unit (A), and localization of primers used to identify and detect *Rosellinia necatrix* (B), *P. nicotianae* and *P. citrophthora* (C), and *Verticillium dahliae* and *V. albo-atrum* (D). For each pathogen specific primer pair, obtained amplifying short DNA fragments (bold character in the figure) were modified to develop a Scorpion-PCR approach. These primers were utilised alone (Scorpion-PCR) or in a nested PCR approach providing a first amplification with conventional external primers (nested Scorpion-PCR).

1993) and were utilised to design specific Scorpion primers (Whitcombe *et al*., 1999) for *R. necatrix*, *P. nicotianae* and *P. citrophthora* (Ippolito *et al*., 2002; Schena *et al*., 2002) (Fig. 1B, C). The greatest amount of sequence variation in rDNA exists within the IGS regions (Pramateftaki *et al*., 2000). Compared to ITS, IGS regions pose more difficulties for amplification and sequencing because much longer; however, they can be useful when there are not enough differences available across the ITS. This is the case with *V. dahliae* and *V. albo-atrum*, where the ITS regions are very similar making it 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), making it possible to develop conventional and Scorpion primers to identify and detect *V. dahliae* and *V. albo-atrum* (Nigro *et al*., 2002) (Fig. 1D).

The first step when evaluating primer specificity is to analyse available DNA databases so as to exclude similar sequences to occur in other microorganisms. Basic Local Alignment Search Tool (BLAST) analyses of selected primers for *R. necatrix*, *P. nicotianae, P. citrophthora* and *V. dahliae* excluded similar sequences occurring in other micro-organisms. Primer specificity was further confirmed by the amplification of DNA fragments of the expected size and by the increase of fluorescence signals that specifically target the DNA of the pathogen, but not of any other fungi (Nigro *et al*., 2002; Schena and Ippolito, 2003; Ippolito *et al*., 2004).

For all pathogens the limit of detection using 10-fold dilutions series of total DNA extracted from pure cultures was 1 pg μl^{-1} with Scorpion-PCR, and 1 fg μl^{-1} with nested Scorpion-PCR, *i.e.* combining 2 sequential amplifications first with convention-

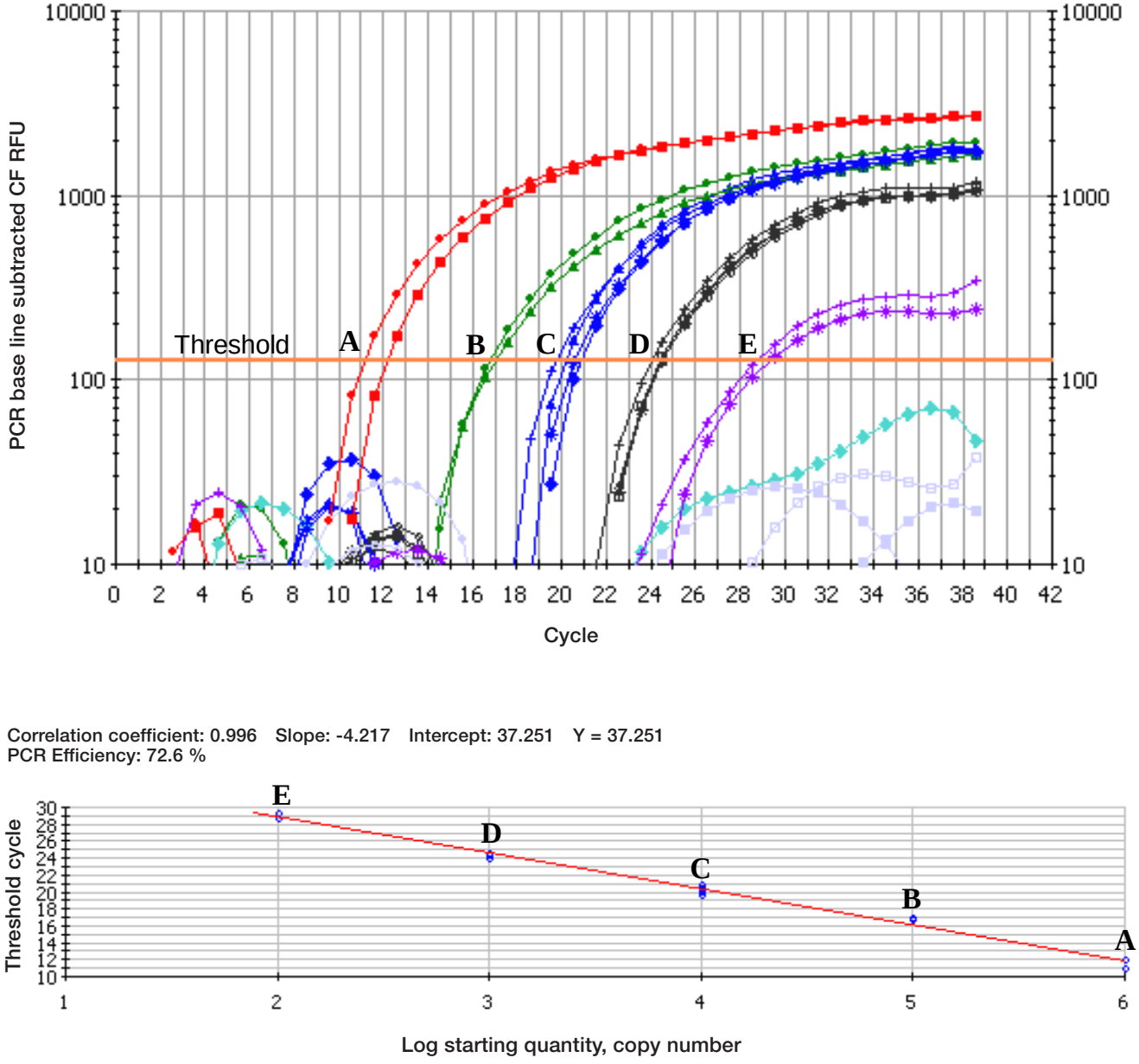

Fig. 2. Top: real-time fluorescence curves of PCR products amplified from *Phytophthora nicotianae* DNA at the standard concentrations of 10 ng (A), 1 ng (B), 100 pg (C), 10 pg (D) and 1 pg (E). Bottom**:** standard curve of the DNA concentration standards against the cycle threshold.

al (first amplification) and than with Scorpion primers (second amplification). High and significant linear correlations (r ranging from 0.95 to 0.99) were achieved between input DNA and cycle thresholds with both Scorpion-PCR and nested Scorpion-PCR (Fig. 2).

Qualitative analyses

Compared to conventional PCR, real-time PCR eliminates the requirement for post-amplification processing steps, reduces the time and labour of the analyses and increases the throughput of PCR testing as an automated diagnostic system suitable for large-scale applications. Furthermore, since it does not require ethidium bromide, the health risks for operators and environmental contamination are reduced. Moreover, independently from the method used for the DNA extraction, real-time PCR seems to be less affected by inhibitors than conventional PCR, because they mainly affect late cycles of PCR, which are critical for product accumulation. By contrast, in real-time PCR, detection is achieved by generating a fluorescent signal in the early stages of PCR assay and, therefore, a less product accumulation is required to achieve positive results.

Real-time PCR as large-scale detection method can be further improved by using multiple primers to detect more than one phytopathogenic organism within a single reaction (multiplex real-time PCR). Two Scorpion primers labelled with FAM and ROX fluorophores (multiplex real-time PCR) enabled the simultaneous identification of *P. nicotianae* and *P. citrophthora* (Ippolito *et al*., 2004).

Real-time PCR seems to be more sensitive than conventional PCR, since even minute DNA fragments can cause a significant increase in fluorescence. However, a nested approach cannot be avoided when the target DNA needs to be detected in a naturally infected matrix such as soils with a very low level of pathogen contamination (Schena and Ippolito, 2003). In that case, the combination of 2 sequential amplifications, the first with conventional and the second with labelled primers is necessary to increase sensitivity without loosing the advantages of real-time PCR (Fig. 2). Nested Scorpion-PCR enabled the detection of *R. necatrix* (Schena and Ippolito, 2003), *V. dahliae* (Nigro *et al*., 2002), *P. nicotianae,* and *P. citrophthora* (Ippolito *et al*., 2004) in naturally infected matrices (soils, roots, bark, and/or woody tissues) with higher sensitivity and in a much shorter time than with conventional detection methods, which are based on isolations on nutritive media and/or baiting. With this technique, it was possible to detect *P. nicotianae* in citrus with less than 1% of infected feeder roots and single propagules of *P. nicotianae*, *P. citrophthora* and *V. dahliae* in naturally infested soils.

A major limitation to the use of conventional as well as real-time PCR in detecting phytopathogenic and antagonistic fungi is this technique's lack of discrimination between living and dead material. As a result, the molecular diagnosis of natural samples can give different results from those obtained with traditional isolation techniques (Bridge and Spooner, 2001). Nucleases are widely diffused in environmental samples and can degrade DNA after the death of micro-organisms. However, the degradation rate strongly depends on environmental conditions. Schena and Ippolito (2003) found that DNA of *R. necatrix* is degraded rapidly in soil, thus minimizing the risks of false positives due to the presence of dead cells. However, further research is necessary to assess the persistence of DNA under various environmental conditions, and in relation to the structures produced by the pathogens. Although several reports indicate that nucleic acids are quickly digested by DNases (England *et al*., 1998), other studies report that DNA persists in the soil for long 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). However, compared to real-time PCR alone, BIO-PCR requires more time for analysis (micro-organisms must be grown on a nutrient medium for 1–4 days) and is more expensive (especially if selective media are used). An alternative strategy could be to use RNA rather than DNA as the target molecule for diagnosis. RNA is degraded quickly in dead cells and does not interfere with the analysis. In order to be used as a target molecule by PCR, RNA must first be reverse transcribed into DNA (RT-PCR) (Keer and Birch, 2003).

Quantitative analyses

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 the quantitative analysis of phytopathogenic fungi (competitive PCR) (Hadidi *et al*., 1995; Mahuku and Platt, 2002). These methods are laborious and insufficiently accurate because the amplification efficiency decreases in later PCR cycles (Ginzinger, 2002). By contrast, in real-time PCR data are collected during the exponential accumulation of PCR products, when the efficiency of the reaction is still constant. Quantification is automatically determined by associate software interpolating the Cycle thresholds (Ct) of specific samples with standard curves prepared from known quantities of target DNA. The Ct is the number of PCR cycles necessary to generate a fluorescent signal significantly above the average background fluorescence emission in the initial PCR cycles.

In a *R. necatrix*-infested soil serially diluted with different amounts of uninfested soil, a high and significant correlation (r=-0.962; $P \leq 0.001$) was found between the dilution factor (% of infested soil) and Ct values (Schena and Ippolito, 2003). Similarly, using *P. nicotianae*-infested soils serially diluted with non-infested soils, a high correlation (r=-0.98) was found between inoculum density (propagules g^{-1} of soil, assessed with the selective medium) and Ct values (Ippolito *et al*., 2004). In tests on naturally infected roots and soils, a significant correlation was also found, although with lower correlation coefficients. The lower level of correlation with naturally infected samples was expected since they obviously had different characteristics (composition, tissue texture, etc.) and pathogen propagule ratios (mycelia, zoospores, oospores, etc.) that can affect both culture and molecular detection methods (Bridge and Spooner, 2001). However, unlike baiting and cultural methods, real-time PCR is not affected by external factors such as other fungal species that conceal the pathogen in agar-based media and hence this technique allows a more rapid and accurate quantification of fungal DNA.

The high sensitivity of real-time PCR seems to be more than enough to detect target pathogens at a population level below those causing yield loss and/or severe damage. For example, to cause severe losses on citrus, the *Phytophthora* population in the soil must be higher than 15–20 propagules per gram of dry soil (ppg) for susceptible rootstocks (Magnano di San Lio *et al*., 1988; Menge and Nemec, 1997), and around 30 ppg for resistant rootstocks (Ippolito *et al*., 1991). Real-time PCR therefore has the potential for determining the soil inoculum threshold levels for disease development in a number of host-pathogen combinations. Similar research should make it possible to develop predictive diagnostic tests to identify high-risk fields where the inoculum of *R. necatrix*, *P. nicotianae* or *P. citrophthora* is above threshold values (Cullen *et al*., 2002).

Concluding remarks

Real-time PCR has an enormous potential to address central questions in plant pathology to a level of precision that was unimaginable just a few years ago. The sensitivity, speed, and versatility, together with the possibility it offers of performing quantitative analyses, make real-time PCR a method suitable for studying pathogen biology, ecology, host-pathogen interactions, and many other, more general aspects of *R. necatrix*, *P. nicotianae, P. citrophthora* and *V. dahliae*. It is likely that real-time PCR will become a standard method suitable for the large-scale diagnosis of these pathogens in extension services.

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