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Specific detection of *Phytophthora nicotianae* using the polymerase chain reaction and primers based on the DNA sequence of its elicitin gene ParA1

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Abstract

Six primers based on the sequence of the flanking and coding regions of the elicitin gene ParA1 of *Phytophthora nicotianae* were tested for specific detection of the fungus by the polymerase chain reaction (PCR). One combination, IL7/IL8, with IL7 in a flanking region and IL8 in a coding region of the gene, gave an intense 378 bp signal with a diverse collection of isolates of *P. nicotianae*, that included some from black shank disease of tobacco and others from a variety of hosts. The sequence of the amplification product obtained with an isolate that produces elicitin and one that does not, was homologous with the known sequence of the ParA1 gene. The same primer combination gave no signal with sixteen other *Phytophthora* species tested except for two isolates *P. palmivora* with which it gave a weak 800 bp signal. It gave no signal with DNA from healthy tobacco and tomato plants but *P. nicotianae* was detected in inoculated tobacco and tomato plants. Small numbers of zoospores (>100) trapped onto a nitrocellulose membrane after filtration from suspension were also detected after two successive rounds of PCR.

Introduction

Soil-borne diseases caused by *Phytophthora* spp. are often intractable due to the release into soil of resistant perennating structures, oospores and/or chlamydospores, formed by the pathogen. Healthy nursery material, fungicides and crop rotations are important elements in disease control but they require early diagnosis and detection of the pathogen either in the plant, soil or water. Rapid and accurate identification and detection of *Phytophthora* species would improve diagnosis and prophylaxis, especially where several pathogens have the same host range but are not equally severe in the disease that they cause. Moreover, measurements of inoculum in soil or irrigation water could be used to predict the scale of possible losses and for timing fungicide applications.

In the case of *Phytophthora*, detection traditionally requires the isolation and culture of the fungus from soil or diseased plant material with identification based on morphological or physiological characters. This is time-consuming and requires considerable knowledge of the fungus (Tsao, 1983). Recently, new techniques have been used to lessen or eliminate some of these difficulties. Serology has been used to detect *Phytophthora* in a number of instances (Jones and Shew, 1988; Miller et al., 1989; McDonald et al., 1990). Specific DNA probes have also been used to detect sensitively *P. nicotianae* (Goodwin et al., 1990). However the use of radioactivity prohibits a routine use of this method. Alternatively, the polymerase chain reaction (PCR), can exponentially amplify specific DNA sequences of the fungus by *in-vitro* DNA synthesis. Both serological and PCR-based techniques are sensitive and can be used to develop quick and simple diagnostic tools. According to needs, narrow and broad selectivities in detection are feasible. However, PCR primers to detect a single pathogen or many members of a group of related pathogens can be made and tested more quickly and at a lower cost than specific DNA probes or antibodies and have therefore considerable potential in a multi-purpose detection method.

A single species-specific sequence can be used to produce a PCR-based detection system, for example, highly specific oligonucleotide primers have been obtained from a 1300 base pair (bp) DNA sequence

from *P. nicotianae* and from an 800 bp sequence from *P. citrophthora* (Ersek et al., 1994). Other attempts to develop PCR-based detection tools have used the tandemly repeated ribosomal RNA (rRNA) gene as a source of DNA sequences (Picard et al., 1992; Bruce et al., 1992). Ribosomes exist in every genome and ribosomal primers are available to amplify different parts of the rRNA gene from any fungus providing a basis for further work (White et al., 1990). The spacer regions ITS1 and ITS2 of the ribosomal units are more variable in sequence than the rRNA genes and have potential for distinguishing among species (Lee and Taylor, 1992). However, this potential will remain somewhat limited until sequences from a wide range of isolates are available from such complex species as *P. megasperma*, *P. cryptogea* and *P. drechsleri* and the resultant molecular taxonomy has been clarified.

Alternatively, genes other than the rRNA genes could be the basis of PCR-based tests. *Phytophthora* spp. and some closely related oomycetes such as *Pythium* spp. are unique in that they produce a family of proteins named elicitins (Ricci et al., 1989; Huet et al., 1995). Each species of *Phytophthora* tested so far secretes different elicitins which are named after the species from which they were purified e.g. cryptogein for *P. cryptogea*, capsicein for *P. capsici* . . . Advanced studies showed that several isoforms of elicitins can also be produced by the same *Phytophthora* isolate (Le Berre et al., 1994). Using serological techniques, it has been possible to raise antibodies which cross react with elicitins from several species or specific of one species (Devergne et al., 1994). Several genes encoding elicitins have been found in *P. cryptogea* and *P. nicotianae* isolates (Ricci et al., 1993; Kamoun et al., 1993; Panabières et al., 1995). Their sequences show homology while variability occurs in intergenic spacers (Panabières, pers. comm.). Therefore primers with different levels of specificity could be designed as for ribosomal sequences.

Phytophthora nicotianae Breda de Haan, is a pathogen on more than 72 genera of plants (Hickman, 1958). In the past, this species has been subdivided into two varieties according to morphological criterions, var. *nicotianae* and var. *parasitica*; but recent studies have suggested that there is only one species (Ho and Jong, 1989). This species has also been called *P. parasitica* Dastur in which the tobacco-specialized isolates have been grouped in the variety *nicotianae* (Tucker, 1931). For reasons of anteriority, in this paper it is referred to as *P. nicotianae*. The fungus produces several types and isoforms of elicitin (Yu, 1995) and the amino acids of the principal one, parasiticein, and its encoding gene ParA1 have been sequenced (Nespoulos et al., 1992; Kamoun et al., 1993). Some specialized isolates on tobacco, causing the important black shank disease, do not appear to produce elicitins (Ricci et al., 1989), or produce them in very much smaller amounts than isolates from other hosts (Lacourt, 1994). Nevertheless these isolates also appear to possess elicitin genes as judged by Southern blots using an elicitin probe (Kamoun et al., 1993; Ricci et al., 1993).

In this study, we have assessed primers designed around the sequence of the ParA1 gene for their specificity towards *P. nicotianae* and their ability to detect that fungus in infected tomato and tobacco plants and as zoospores in water. Zoospores are an important target for detection because they spread the disease in water and infect the host after encystment. They have been detected immunologically in water or in soil after incubation and washing (Cahill and Hardham, 1994a) and are probably the most common propagule found in water; numbers can reach as high as 400 per litre in recirculated irrigation water (McDonald et al., 1994). Their accumulation onto a dipstick has been used to detect *P. cinnamomi* serologically (Cahill and Hardham, 1994b) and zoospores of *P. nicotianae* have also been detected using ELISA by filtration and trapping on a membrane (Ali-Shtayeh et al., 1991). A similar approach using filter membranes has been used to detect bacteria, except that PCR was used instead of serology to detect the bacteria (Toranzos et al., 1992). In this study we have used this latter approach to detect zoospores of *P. nicotianae* using the elicitin-based primers.

Materials and methods

Fungal isolates and cultural conditions

Nearly all the isolates (Table 1) of *Phytophthora* in the study came from the collections at SCRI and INRA Antibes. There were thirteen *P. nicotianae* isolates from various hosts and continents, chosen to give geographical and pathological diversity. Among them were producers and non-producers of parasiticein and different groups as determined by analysis of mitochondrial DNA (Lacourt et al., 1994). There were also 33 isolates of another sixteen *Phytophthora* species.

The fungal isolates were maintained on oatmeal agar sloped cultures at 4 °C, except for *P. palmivora*

Table 1. List of *Phytophthora* isolates tested

Species	Number	Country of origin	year of isolation	host	Institution where held
P. pseudotsugae	PSE 1	USA	1989	*Pseudotsuga menziezii*	IMI 331662
P. cactorum	CAC 19	England	1979	*Fragaria vesca*	SCRI
"	CAC 21	Scotland	1988	*Fragaria vesca*	SCRI
"	CAC 23	England	-	*apple*	SCRI
P. ideae	IDA 7	England	1993	*Rubus idaeus*	SCRI
P. nicotianae	26	France	1971	*Dianthus caryophyllus*	INRA Antibes
"	149	Spain	1975	*Lycopersicon esculentum*	INRA Antibes
"	329	Greece	-	*Nicotiana tabacum*	INRA Antibes
"	385	Bulgary	1992	*Nicotiana tabacum*	INRA Antibes
"	409	Argentina	1993	*Nicotiana tabacum*	INRA Antibes
"	399	USA (Georgie)	1991	*Nicotiana tabacum*	INRA Antibes
"	432	USA (North Car.)	1993	*Nicotiana tabacum*	INRA Antibes
"	183	USA (Kentucky)	-	*Nicotiana tabacum*	INRA Antibes
"	301	USA (Kentucky)	-	*Nicotiana tabacum*	INRA Antibes
"	378	Zimbabwe	1991	*Nicotiana tabacum*	INRA Antibes
"	405	Australia	-	*Nicotiana tabacum*	INRA Antibes
"	308	Cuba	1985	*Nicotiana tabacum*	INRA Antibes
"	NIC 1	-	-	-	IMI 208688
P. palmivora	PAL 1	-	-	-	IMI 250457
"	178	Venezuela	-	*Ficus carica*	INRA Antibes
"	288	Costa Rica	-	*Theobroma cacao*	INRA Antibes
"	292	Togo	-	*Theobroma cacao*	INRA Antibes
P. capscici	147	France	-	*Capsicum sp*	INRA Antibes
"	238	France	1982	*Cucurbita pepo*	INRA Antibes
P. citrophthora	CTP 1	Chile	1989	*Rubus idaeus*	IMI 334962
P. syringae	SYR 1	Scotland	1985	*Rubus idaeus*	SCRI
"	SYR 2	Scotland	1989	*Rubus idaeus*	SCRI
P. citricola	CIT 1	Scotland	1986	*Rubus idaeus*	SCRI
"	CIT 2	Ireland	1986	*Rubus idaeus*	SCRI
P. infestans	I 117	Egypt	-	*Rubus idaeus*	SCRI
"	36609	-	-	*Solanum tuberosum*	SCRI
P. fragariae	83361	-	-	-	SCRI
"	FVR 11	Scotland	1985	-	SCRI
"	FVR 70	Netherland	1993	*Rubus idaeus*	SCRI
"	FVR 72	Scotland	1993	*Rubus idaeus*	SCRI
P. megasperma	FVF 168	England	1969	*Rubus idaeus*	SCRI
"	MEG 6	Scotland	1986	*Fragaria vesca*	SCRI
P. cinnamomi	MEG 7	Scotland	1987	*Rubus idaeus*	SCRI
P. drechleri	CIN 8	USA	1993	*Rubus idaeus*	SCRI
"	DRE 1	Ireland	1988	*Persea sp*	SCRI
P. cryptogea	DRE 2	England	1987	*Rubus idaeus*	SCRI
P. cambivora	CRY 3	New Zealand	1951	*Rubus idaeus*	SCRI
"	CAM 1	Scotland	1985	*Lycopersicon esculentum*	SCRI
"	CAM 2	England	1985	*Rubus idaeus*	SCRI
P. erythroseptica	CAM 3	Scotland	1985	*Rubus idaeus*	SCRI
	ERY 1	USA		*Rubus idaeus* *Solanum tuberosum*	ATCC36302

and *P. nicotianae* which were maintained at room temperature (c. 20 °C). All were cultured on 1% V8 juice agar medium in the dark at room temperature for seven days after which the mycelium was peeled off and used to inoculate 10 ml of glucose asparagine liquid medium (GAM: modified medium of Hall et al. (1969), without yeast extract and peptone), in a Petri dish. After one week, the mycelium was harvested by filtration through filter paper (Whatman), washed with distilled water and either used immediately for DNA extraction or stored frozen (−20 °C).

Inoculation of plants

Two week-old tobacco (*Nicotiana tabacum* cv Samsun) and tomato (*Lycopersicon esculentum* cv Moneymaker) plants were removed from soil and their roots were washed. Their root tips were cut off and the plantlets were placed in a tube containing tap water and a plug of mycelium. Tomato plants were inoculated with isolate 149 and tobacco plants with isolate 329 of *P. nicotianae*. The test tubes were kept in a glasshouse at 24 °C under natural light and water was added regularly into the tubes. Symptoms developed after c. one week. The plants were stored frozen (−20 °C) until further analysis.

Six week-old tomato plants, kept under the same conditions of temperature and light, were inoculated by placing agar plugs of mycelium of isolate 149 on the cut ends of stems decapitated at various points on the plants. The progress of the infection was observed over a week and diseased tissues were collected and stored frozen (−20 °C) before analysis.

DNA extraction

Fungal DNA

Fungal DNA was extracted using a Nucleon II extraction kit (Scotlab Ltd, Glasgow) according to the manufacturer's recommendations. Between 150 and 200 mg of compressed wet-weight mycelium were extracted in a protocol scaled down for use in 1.5 ml Eppendorf tubes; this yielded 20 to 30 mg of DNA suitable for PCR amplification.

Plant DNA

Plant DNA was extracted according to a modified protocol of Edwards et al. (1991): 250 mg of plant material was ground using a pestle in an Eppendorf containing 500 ml of lysis buffer (200 mM Tris HCl, pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) plus 25 mg of Polyvinylpyrrolidone and sterile sand. After centrifuging for 3 min at × 13000 g, the supernatant was extracted with 1 volume of chloroform-isoamylalcohol (24/1, v/v) and then collected after centrifugation for 3 min at × 13000 g. DNA was precipitated with one volume of isopropanol and centrifuged at × 13000 g for 5 min. The resultant pellet was washed three times with 70% ethanol, vacuum dried and resuspended in sterile distilled water to yield 10 to 20 mg of DNA suitable for DNA amplification.

Preparation of the zoospores and fixation on a membrane

Zoospores of *P. nicotianae* were produced according to a protocol of P. Bonnet (INRA Antibes, pers. comm.). Isolates 149 and 329 were cultured on 1% V8 agar medium for one week as previously described and four agar plugs of mycelium were transferred to pea broth for one week at 24 °C under light. The resultant mycelium was cut with a scalpel into small pieces which were transferred onto 2% water agar in Petri dishes for four days under the same conditions. The dishes were placed for 30 min at 4 °C, 15 ml of sterile distilled water at 30 °C were added and after incubation for 10 min at 30 °C, the water, now containing the zoospores, was removed, the zoospores were counted using an haemocytometer and their numbers were adjusted to the desired concentration by adding distilled water.

The zoospore suspension (20 ml) was filtered through a 0.45 mm pore size nitrocellulose filter, 4.5 cm diam, which was then treated according to a modified method of Toranzos and Alvarez (1992). It was placed 'zoospore' side upwards on filter papers soaked with the following solutions and for the following times: 20 min. on 10% SDS; 5 min. on 0.5 M NaOH + 1.5 M NaCl, then on 1.5 M NaCl + 0.5 mM Tris HCl pH 7.5 and finally on 2X SSC. The nucleic acids were fixed onto the nitrocellulose membrane by heating in an oven at 80 °C for two hours and the membrane was washed in sterile distilled water and allowed to dry.

Design of PCR primers, DNA amplification by PCR and analysis of PCR products

Primer sequences

Six primers based on the sequence of the ParA1 gene were synthesised (Figure 1). Some primers were

designed to anneal to the coding regions of the ParA1 gene in an attempt to ensure that the PCR products were derived from that gene: IL3, IL8 and IL5 were of this type with the first two reading in the reverse sense and the last reading in the forward sense. Other primers were located in the flanking region upstream from the 5′ end of the gene (IL7, reading in the forward sense), the signal peptide region of the gene (IL2, reverse sense) or in the flanking region downstream from the 3′ end of the gene (IL6, forward sense). As these others were not located in the generally conserved coding region, it was expected that they might confer specificity when used in combination with primers in the coding regions. The primer sequences result from a compromise between several requirements. We chose 19 to 20 nucleotides long primer having a GC content which allows annealing temperatures c. 60 °C. As much as possible we also avoided base repetitions or hairpin and primer dimers structure, but this was not entirely possible.

Five different primer combinations were used for PCR on fungal DNA: IL2/IL5, IL3/IL5, IL2/IL6, IL3/IL6 and IL7/IL8. Only IL7/IL8 combination was used for PCR on DNA extracted from plant samples. All primers were used at the concentration of 1 mM with 200 mM of each dATP, dCTP, dGTP and dTTP (Pharmacia, Biotech), 2.5 units of *Taq* polymerase (Gibco BRL) and the PCR buffer supplied with the enzyme (20 mM Tris HCl pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$), in a final volume of 50 ml with 100 to 500 ng of DNA as template. With plant DNA, 10 mg ml^{-1} of Bovine Serum Albumin (BSA) were added. Each reaction was sealed with a few drops of mineral oil before the tubes were incubated at 94 °C for 3 min, then subjected to 35 cycles of denaturation 94 °C for 30 s; annealing at 59 °C (primer combinations IL2/IL5, IL3/IL6, IL3/IL5) or 65 °C (primer combination IL7/IL8) for 30 s.; and synthesis at 72 °C for 1.5 min. Finally, they were incubated for an additional 10 min at 72 °C and stored if necessary at −20 °C before analysis.

Amplification of the DNA fixed on the nitrocellulose membrane

A piece of the membrane 35 mm^2 and representing 2.2% of the total area of the original filter was cut out with a cork borer. The numbers of zoospores filtered through the membrane were calculated so as to give a known number of zoospores on the removed piece of membrane, assuming that the distribution of zoospores was similar across the whole membrane. The piece of membrane was placed in an Eppendorf tube with the following reagents: 1 mM of each primer IL7 and IL8, 200 mM of each dATP , dCTP, dGTP and dTTP (Pharmacia, Biotech), 10 mg ml^{-1} of BSA, 10 units of *Taq* polymerase (Gibco BRL) and the PCR buffer supplied with the enzyme (20 mM Tris HCl pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$), in a final volume of 100 ml. A two min. ramp time was allowed between denaturation, annealing and synthesis of DNA. After an initial denaturation of 3 min at 94 °C, there were 40 cycles as follows: 94 °C 30 s., 37 °C 1 min and 72 °C 2 min. The low annealing temperature in the first round PCR was used in accordance with the methodology of Toranzos and Alvarez (1992). The reaction was extended with a final step of 72 °C for 10 min. For a second round of PCR amplification, 5 ml of the first reaction mixture was added to an identical reaction mixture and amplified under standard conditions used for DNA in solution.

PCR controls

For each PCR experiment, one tube with the PCR mix but without any DNA added was run. The results were only considered when no DNA amplification was visible on gel electrophoresis for such negative controls. As a further check, PCR reactions were made separately with each single primer.

Gel electrophoresis

PCR products were separated by electrophoresis in 1% agarose gels, in TAE buffer (0.04 M Tris-acetate, 1 mM EDTA), stained in ethidium bromide and analysed under UV light (Sambrook et al., 1989).

Sequencing of PCR products

The amplified products were obtained under the conditions described above with primers IL7/IL8 and DNA of isolates 183 and 26, non-producing (tobacco black shank) and parasiticein-producing isolates respectively. They were purified using the Wizard™ DNA purification kit (Promega) according to the manufacturer's instructions. They were quantified by gel electrophoresis, by comparing the intensity of the bands with bands corresponding to known amounts of a DNA fragment digested from lambda phage DNA (Pharmacia, Biotech), of a size comparable to the amplified fragment. At least 125 ng of double strand PCR amplified DNA were used for each sequence reaction with the PRISM™ Ready Reaction Taq

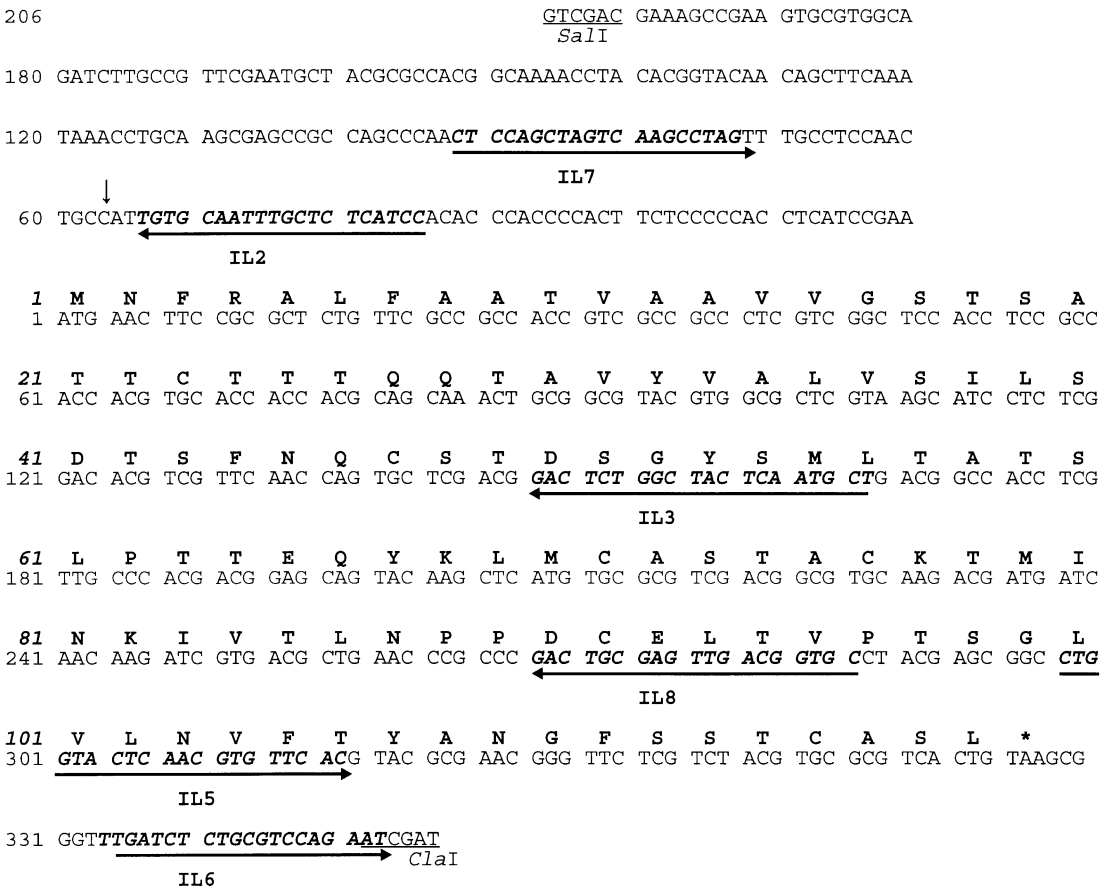

Figure 1. Sequence of the *Sal I*/*Cla I* DNA fragment containing the ParA1 gene of *Phytophthora nicotianae* that encodes the elicitin parasiticein (taken from Kamoun et al. (1993)) with the amino acid sequence of parasiticein in bold above the DNA sequence. Also shown are the origin of transcription of the gene (↓), the location, sequence (in bold italics) and the orientation (arrowed) of primers IL2, IL3, IL5, IL6, IL7 and IL8.

Dyedeoxy™ Terminator Cycle Sequence Kit (Perkin Elmer) according to the manufacturer's instructions. The standard annealing temperature was 60 °C and 3.2 pg of primer were used for each sequencing reaction. Sequences were produced using both IL7 and IL8 primers and the reactions were processed with an Applied Biosystems 373 Stretch Automated Sequencer.

Results

Primer combinations

The primer combination IL2/IL6 (see Figure 1 for details of primers) did not amplify DNA of *P. nicotianae*, even when lower annealing temperatures were tested. IL3/IL5 produced highly polymorphic patterns which were influenced strongly by concentrations of dNTPs, primers and $MgCl_2$. Combinations IL2/IL5 and IL3/IL6, gave consistent patterns with all twelve isolates of *P. nicotianae* with which they were tested but in control experiments, primers IL2 and IL3 gave some bands when used singly (data not shown). All the primer combinations involving IL2, IL3, IL5 and IL6 were therefore excluded from further investigation.

The IL7/IL8 combination amplified an intense 378 bp DNA fragment from all *P. nicotianae* isolates. In further tests with sixteen other species representing all six groups of *Phytophthora* spp. (Stamps et al., 1990), this combination only amplified DNA from *P. nicotianae* and from two of four isolates of *P. palmivora* and there was no amplification with DNA from healthy plants. The DNA product from the two isolates of *P. palmivora* was rather faint and longer (800 bp) than that produced from *P. nicotianae* and could be easily

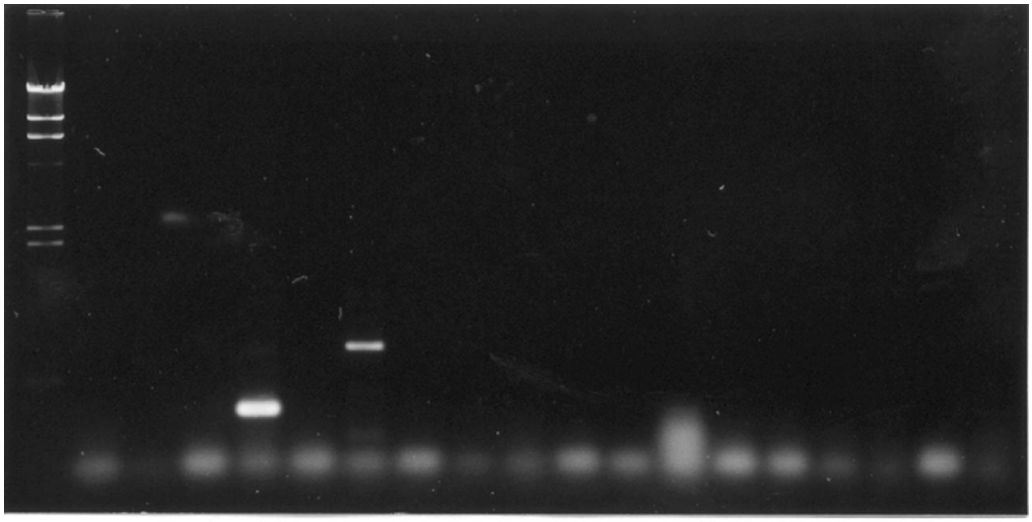

Figure 2. Agarose gel electrophoresis of amplification products obtained with the primer combination IL7/IL8 and DNA of seventeen *Phytophthora* species: lane 1: DNA marker size l/*HindIII*; lane 2: *P. pseudotsugae* (Pse 1), lane 3: *P. cactorum* (Cac 23), lane 4: *P. idaei* (Ida 7), lane 5: *P. nicotianae* (26), lanes 6 and 7: *P. palmivora* (178, Pal 1), lane 8: *P. capsici* (238), lane 9: *P. citrophthora* (Ctp 1), lane 10: *P. syringae* (Syr 2), lane 11: *P. citricola* (Cit 1), lane 12: *P. infestans* (I 117), lane 13: *P. fragariae* (FrR 70), lane 14: *P. megasperma* (Meg 6), lane 15: *P. cinnamomi* (Cin 8), lane 16: *P. dreschleri* (Dre 1), lane 17: *P. cryptogea* (Cry 3), lane 18: *P. cambivora* (Cam 1), lane 19: *P erythroseptica* (Ery 1).

distinguished from the latter (Figure 2). To confirm that IL7 and IL8 amplified part of the gene ParA1 and its 5′ flanking region, amplification products from isolates 26 and 183 (a non-tobacco and tobacco isolate respectively) of *P. nicotianae* were sequenced directly and were identical to one another and to part of the original ParA1 DNA sequence. This included part of the gene and also part of the flanking region upstream from the 5′ end of the gene (Figure 1).

Because of the small size and strong intensity of their amplified product and its absence from amplifications of DNA from other *Phytophthora* spp., the IL7/IL8 combination was chosen for further studies *in planta* and with zoospores.

Detection of the fungus in the plants

The fungus attacked the collar region of two week-old plants and subsequently the stems, giving characteristic rotting symptoms. Inoculation directly on the stem of six week-old tomato plants, resulted in clearly visible and expanding lesions.

Using the IL7/IL8 combination, no PCR amplification was observed with healthy uninoculated plants, whereas a band identical to that obtained with pure DNA from *P. nicotianae* was found in infected tomato and tobacco plants (Figure 3). PCR worked better with DNA extracted from two week-old rather than six week-old tomato tissues. However, signals were generally stronger when instead of grinding the sample in liquid nitrogen, it was crushed directly in the lysis buffer using a pestle. Improvement of the signal was also achieved when BSA was added to the PCR mix (Hummel and Herrmann, 1994). Indeed, the signal was more intense without loss of specificity and the addition of BSA to samples was adopted as standard.

Detection of the zoospores on a membrane by PCR amplification

Using PCR amplification alone to detect zoospores, two successive cycles were required to obtain a signal, even when one million zoospores were fixed to the membrane. After the second round of PCR, a signal was observed with one hundred of zoospores with the intensity of the signal increasing with increasing number of zoospores (Figure 4). When one million of zoospores were present, the signal was identical with that obtained with a single PCR was made on the pure fungal DNA. The high annealing temperature during the second round of PCR increased the specificity of DNA amplification. A negative control with water but

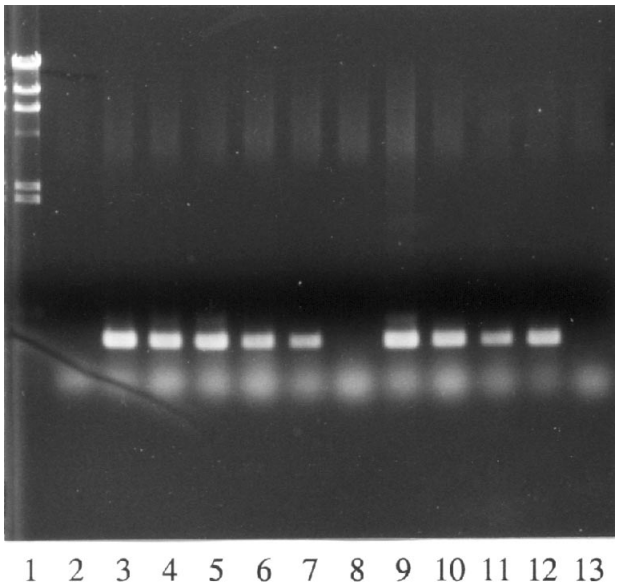

Figure 3. Detection of *Phytophthora nicotianae in planta*. Agarose gel electrophoresis of amplification products obtained with the primer combination IL7/IL8; lane 1 – DNA marker size l/*HindIII*; lane 2 – negative control with no DNA, lanes 3 and 9 positive control with pure fungal DNA of *P. nicotianae* isolates 149 and 329; all other lanes with DNA extracted from plants: lanes 4 to 7–2 week-old (lanes 4, 5) and 6 week-old (lanes 6, 7) infected tomato stems; lane 8 – uninoculated 2 week-old tomato stem; lanes 10 to 12 – stem bases of infected 2 week-old tobacco plants; lane 13 – stem base of uninoculated 2 week-old tobacco plant.

no zoospores gave no signal after the two cycles of PCR.

Discussion

To be useful for the identification and detection of a single species of *Phytophthora*, elicitin-based primers must be specific for a gene, or part thereof, which is characteristic of that species. On the other hand, primers must also be located in regions of the gene that are conserved enough to avoid problems with intraspecific variation, so that all the isolates of the same species will give the same amplification signal.

Most elicitins sequenced to date are the same size, 98 amino acid residues in *Phytophthora* elicitins and 100 in the elicitin-like proteins of *Pythium* with a high degree of similarity among them. It could be expected therefore that elicitin genes from different species would be similar in size and sequence and that primers which amplified elicitin sequences from one species would amplify similar sequences in other species. Yu (1995) reported that some isolates of *P. nicotianae* possess a multigene family of between two and ten elicitin genes with two other elicitin genes in tandem with ParA1. Tandemly repeated elicitin genes have also been reported in *P. cryptogea* (Panabières et al., 1995). Therefore, four combinations of primers IL2/IL5, IL3/IL5, IL2/IL6 and IL3/IL6 were designed to amplify non-coding regions between elicitin genes, the hypothesis being that primers amplifying from such conserved regions into variable intergenic regions would be much more likely to yield species-specific bands than the coding regions of highly conserved elicitin genes. However, these primer combinations gave no amplification products, highly polymorphic banding patterns or where patterns were consistent, one of the primers also produced bands when used alone in control reactions. Thus the results obtained with these combinations were unreliable and they were excluded from further consideration.

In contrast, primers IL7/IL8 both of which were oriented towards the ParA1 gene gave a single band with all *P. nicotianae* isolates. This strong signal was clearly different and easily distinguishable from that obtained with two of four isolates of *P. palmivora*. Furthermore, no signal was obtained with any of sixteen other *Phytophthora* species, representing all six groups within the genus (Stamps et al., 1990). Primer IL7 is most likely the one that induces specificity of the signal since the sequence of IL8 is conserved among different elicitins (Panabières et al., 1995). However it was not possible to confirm directly this fact since only the flanking sequence of the gene ParA1 was available in the data base.

The direct sequencing of the PCR products obtained with IL7/IL8 primer combination of a parasiticein non-producing, black shank isolate (Ricci et al., 1992) and a parasiticein producing isolate gave unambiguous results, indicating that the products were pure in each case and homologous with the matching region of ParA1 (Figure 1). It appears therefore that if there were different elicitin genes present, the primers were specific enough only to amplify one of them. Furthermore, the results provide strong support for the view of Kamoun et al. (1993) that both elicitin-producing and non-producing isolates have similar elicitin genes, even though they might not be equally expressed. They cannot however be taken as definitive proof as only part of the gene was amplified and sequenced.

Thus, primers directed towards the coding region of elicitin genes with one of the primers designed in

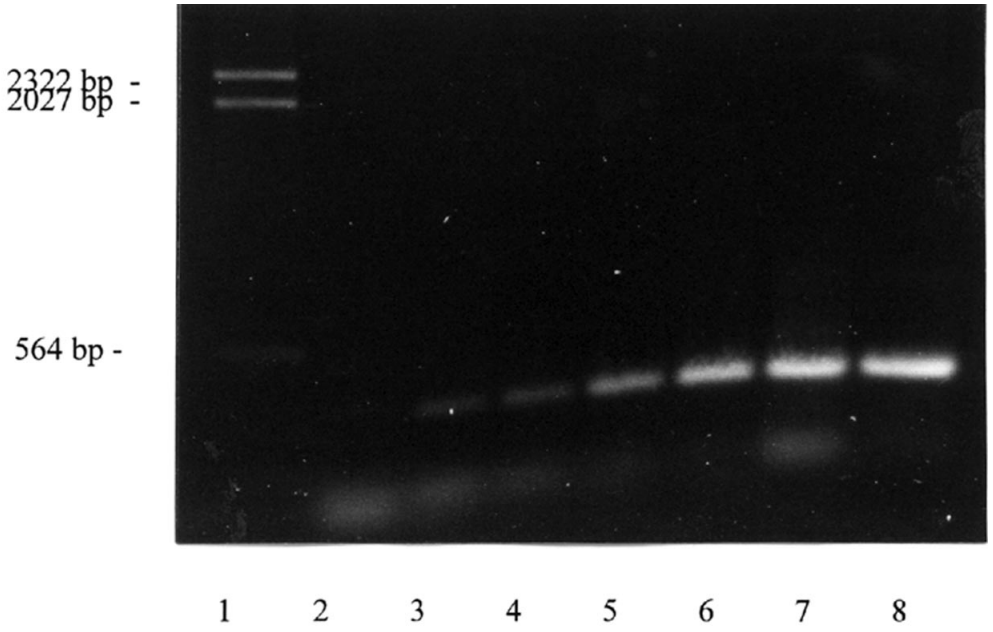

Figure 4. Agarose gel electrophoresis of a second PCR amplification of products obtained with the primer combination IL7/IL8 from various numbers of zoospores of isolate 149 of *Phytophthora nicotianae* fixed on a nitro-cellulose membrane; lane 1 – DNA marker size l/*HindIII*. Lanes 2 to 7 respectively – zoospore numbers 0, 10^2, 10^3, 10^4, 10^5, 10^6; lane 8 – amplification product of a single PCR on pure fungal DNA (isolate 149).

the coding sequence of the parasiticein and the other in non-coding sequence upstream to the 5′ end of the gene ParA1 have some potential as *P. nicotianae*-specific primers. Given that all species of *Phytophthora* probably have such elicitin genes with flanking sequences, PCR-based detection/ identification/diagnostic techniques based on elicitin genes, could be developed for all species in the genus. Only the sequence of one elicitin gene was amplified using IL7 and IL8 primers. The choice of repeated sequences such as ribosomal genes of satellite sequences offers the advantage of increasing the level of DNA amplification from the start of the PCR. However, new refinements of the PCR technique such as the nested PCR (MacManus and Jones, 1995), raises the sensitivity of this technique to a much higher scale than the use of repeated sequences. Discrete sequences, therefore, could be chosen in preference to repeated sequences if they presented the appropriate specificity.

The signal obtained with IL7/IL8 was intense and specific enough to detect the pathogen *in planta*. It was generally stronger in younger than in older tissues. Perhaps the latter were more lignified and contained phenolic compounds that inhibited the *Taq* polymerase, alternatively, lesions on young tissue may have contained more of the fungus than older ones. By adding BSA to the reaction mixture, this problem was largely overcome. *P. nicotianae* is a relatively unimportant pathogen in Scotland and only artificially inoculated host material was available for test. Nevertheless, a very similar protocol, but using rRNA gene-based, specific primers instead of elicitin genes, has been used successfully to detect *P. fragariae* and *P. idaei* in naturally infected field-grown raspberry plants (*Rubus idaeus*) (unpublished results). Again the incorporation of BSA in the reaction increased the sensitivity of the reaction. This result, taken together with the work on inoculated material, indicates that the same technique should be able to detect *P. nicotianae* in naturally infected plants and that it should be possible to identify *P. nicotianae* directly in lesions on the host using PCR with the primers IL7/IL8 without resort to isolation of the pathogen from the host. The IL7/IL8 primers have also been used with scrapings of mycelium from a pure culture of *Phytophthora* isolated from *Dieffenbachia* (dumb cane) to confirm within a few hours its identity as *Phytophthora nicotianae* (Dr T. O'Neill, ADAS, Cambridge pers. comm). Routine diagnostics and identifications should therefore be possible within a day or less.

Also feasible is the detection of zoospores on a nitro-cellulose membrane filter by PCR using a protocol based on Toranzos et al. (1992) for bacteria. Extended treatment with 10% SDS appeared to be sufficient to lyse the zoospores even if they had already encysted. It was not possible to include the whole of a filter membrane in the PCR reaction, only small pieces of it fitted into 0.5 ml Eppendorf tubes used for the PCR reaction. Thus only a small portion of the total number of zoospores applied to the membrane would have been added to the reaction mixture assuming that the zoospores, which were applied to the membrane in large volumes of water, were all trapped on the membrane and that they were evenly distributed. Nevertheless, there was a steady increase in signal obtained with PCR with increasing amounts of zoospores filtered through the membrane. After two cycles of PCR, a *P. nicotianae* signal was visible with an estimated 100 zoospores trapped on a piece of the membrane. The annealing temperature used for DNA amplification of template bound to membrane was very low in comparison with the temperature used when the DNA is in solution. This was necessary to amplify enough DNA from template whose structure is modified. This temperature was recommended by Toranzos et al. (1992) and by other authors using degraded DNA as template for PCR (Hauswirth et al., 1994). The specificity of the DNA amplification was obtained in the second round PCR. Indeed, there were no cross-reactions with zoospores of other species trapped on membranes in our work (results not shown).

The technique is still open to improvement. For example, Toranzos et al. (1992) detected very small numbers of bacteria by performing a third PCR on their samples. Using nested primers, or combining PCR amplification and dot-blot or reverse-blot hybridization also gave good results (MacManus and Jones, 1995). Thus, the results offer a realistic alternative to the use of ELISA-based tests for the detection of *P. nicotianae* in plants and as zoospores.

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