European Journal of Plant Pathology **108:** 197–207, 2002.

Specific and sensitive detection of *Phytophthora nicotianae* by simple and nested-PCR

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Accepted 29 November 2001

Key words: DNA amplification, fungal taxonomy, hydroponics, identification, oomycetes

Abstract

Phytophthora nicotianae Breda de Haan is one of the most important soil-borne plant pathogens. The identification of this pathogen based on morphological or physiological characters is time-consuming and labour-intensive and requires comprehensive knowledge of fungi. Molecular analysis of the internal transcribed spacer (ITS) regions of rDNA is a novel and very effective method of species determination. Based on this concept, conventional and single closed tube nested-PCRs were developed for the specific and sensitive detection of *P. nicotianae*. Two new specific primers, designed from the spacer regions ITS1 and ITS2, internal to the nucleotide sequence flanked by universal primers ITS4 and ITS6, were used. To evaluate the specificity of the method, 36 morphologically characterized isolates were tested. A positive reaction, characterized by an amplification product of 737 bp, was shown by all *P. nicotianae* isolates and two *P. nicotianae/cactorum* hybrids. No amplification product was observed when other *Phytophthora* species and genera were assayed. The sensitivity of this method was analysed by serial dilutions of a defined amount of fungal DNA in a healthy root extract. Nested-PCR was at least 1000 times more sensitive than conventional PCR. In addition, samples from different infection sites, origins and crops, samples from nutrient solution, water and the rockwool used in hydroponic cultures, were analysed to validate this method.

Introduction

Phytophthora nicotianae Breda de Haan, a major genus of plant pathogen within the oomycetes, has a host range of more than 72 genera of plants (Hickmann, 1958). Oomycetous organisms differ from the true fungi because of their diploid nature, the morphology of mitochondrial cristae, the flagellar heterokont apparatus of the zoospore, the biochemistry of the cell wall, their lack of epoxidation of squalene to sterols, their different metabolic pathways, and their unique molecular biological system. Because of their phylogenetic relationship to the heterokont algae, the oomycetes were transferred from the kingdom Mycetae to the kingdom Chromista (Cavalier-Smith, 1989; Barr, 1992; Erwin and Ribeiro, 1996). However, because of its many physiological and morphological similarities to the true fungi, the genus *Phytophthora* is still at times referred to as a fungus, a practice that is followed in the present work.

Hydroponic systems may encourage the development of a diverse range of plant pathogens on crop plants because of optimal reproductive, nutritive and distributive conditions (Davies, 1980; Grote and Bucsi, 1992; 1998). These pathogens may cause very similar disease symptoms, such as wilting, necrosis and root rot. Early detection and diagnosis of pathogens either in plants, soil, water, or especially in the re-circulating nutrient solutions of hydroponic cultures are crucial for the implementation of efficient control strategies. More target-specific measurements may improve the efficiency of control strategies in the future. Therefore,

rapid, specific, and sensitive methods for the detection of all important pathogens are required. Classical identification based on morphological or physiological characters is time-consuming, labour-intensive, and requires considerable knowledge of the genera involved. Consequently, false determinations are frequently made.

In order to facilitate the detection of *Phytophthora*, serological techniques have been developed that are in most cases genus specific (Jones and Shew, 1988; McDonald et al., 1990; Grote and Gabler, 1999). Specific DNA probes have also been used for the sensitive detection of *P. nicotianae* (Goodwin et al., 1990). However, the use of radioactivity prohibits routine use of this assay. Because of the lack of suitable morphological and other criteria for identification, detection methods utilizing DNA have been targeted for development. Molecular data represent a novel and highly effective means of species determination based on the comparison of restriction enzyme digest products of the internal transcribed spacer (ITS) regions of rDNA (Cooke et al., 2000).

PCR-based techniques allow the amplification of a species-specific sequence at a high level of sensitivity (White et al., 1990; Bruce et al., 1992; Picard et al., 1992; Ersek et al., 1994). A PCR-based test for the detection of *P. nicotianae*, based on primers from elicitin genes, was described by Lacourt and Duncan (1997). In this paper, the effective characterization of this pathogen in pure cultures as well as its detection in artificially and naturally infected plant material or nutrient solution was reported. Conventional and single closed tube nested-PCR using new specific primers designed from the ITS regions ITS1 and ITS2 have also been also developed (Jaeger et al., 2000; Olmos et al., 1999). These molecular approaches to the sensitive identification and detection of *P. nicotianae* open up new pathways for monitoring the epidemiology of this economically important plant pathogen.

Materials and methods

Isolates and cultural conditions

Sixteen isolates *P. nicotianae*, two *P. nicotianae/cactorum* hybrids, 21 other *Phytophthora* species or isolates, and eight different fungal isolates from other genera and species were obtained from herbaceous and woody plants. Species, origin, and host plant are listed in Table 1. For the characterization of isolates obtained, symptoms were described and a microscopical investigation was conducted. Hydroponic culture works like a water culture and favours the development of the fungus in general and in particular of sporangia. Where no specific structures like sporangia were found, some roots of the samples were incubated with sterile water at 27 °C in the dark for 2 days (water culture), or a re-isolation was made on common selective media (Tsao, 1970).

Isolates were grown at 23 °C on potato dextrose agar (Merck, Darmstadt, Germany) medium except for *P. infestans* and *P. syringae*, which were cultivated on V8 medium at 18 °C, and maintained at 10 °C on the respective culture media. Material for DNA extraction was obtained by peeling mycelium from agar, using a

Table 1. Species-specific amplification of DNA sequences from isolates of *Phytophthora* and other fungal isolates from various origins

Species	Source host/Country of origin	Amplification using PNIC primers[a]
P. nicotianae		
305.29	*Nicotiana tabacum,* Taiwana[b]	+
310.62	*N. tabacum,* India[b]	+
10664	*Spatiphyllum* spp., The Netherlands[c]	+
Pn1	*Lycopersicon esculentum,* Germany[d]	+
Pn2	*L. esculentum,* Germany[d]	+
Pn5	*L. esculentum,* Germany[d]	+
Pnp2	*N. tabacum,* Germany[d]	+
1432	*Euphorbia pulcherrima,* Germany[g]	+
1705	*Spatiphyllum* spp., Germany[g]	+
IVIA-P1	Unknown, Spain[e]	+
249	*L. esculentum,* Belgium[d]	+
268	*L. esculentum,* Belgium[d]	+

Table 1. Continued

Species	Source host/Country of origin	Amplification using PNIC primers[a]
309	*L. esculentum,* Belgium[d]	+
374	*Capsicum* spp., Belgium[d]	+
AN 96/4	*Spathiphyllum* spp., the Netherlands[f]	+
AN 97/28	*L. esculentum*, the Netherlands[f]	+
P. nicotianae/cactorum AN 99/3	*Cyclamen* spp., the Netherlands[f]	+
P. nicotianae/cactorum (IVIA-P2)[i]	*Pyrus communis*, Spain[e]	+
P. citricola (1817)	*Medicago sativa*, the Netherlands[c]	−
P. megasperma var. *megasperma* (118)	*Rubus idaeus*, the Netherlands[c]	−
P. citrophthora (IVIA-P3)	*Citrus* spp., Spain[e]	−
P. syringae (4292)	*Malus* spp., the Netherlands[c]	−
P. cryptogea (307.62)	*Godetia* spp., South Africa[b]	−
P. cryptogea (63779)	Unknown[g]	−
P. cambivora (21/95-K II)	*Chamaecyparis lawsoniana*, unknown[g]	−
P. palmivora (64972)	Unknown[g]	−
P. cinnamomi (IVIA-P4)	*Quercus rotundifolia*, Spain[e]	−
P. cactorum (IVIA-P5)	*Fragaria* spp., Spain[e]	−
P. capsici (IVIA-P6)	*Capsicum* spp., Spain[e]	−
P. infestans (Naumann)	*L. esculentum*, Germany[h]	−
P. sp. (P3)	*L. esculentum*, Belgium[d]	−
P. sp. (335)	*L. esculentum*, Belgium[d]	−
P. sp. (336a)	*L. esculentum*, Belgium[d]	−
P. sp. (336b)	*L. esculentum*, Belgium[d]	−
P. sp. (405)	*L. esculentum*, Belgium[d]	−
P. sp. (AN 97/1)	*Spathiphyllum* spp., the Netherlands[f]	−
P. sp. (AN 97/2)	*Spathiphyllum* spp., the Netherlands[f]	−
P. sp. (AN 97/22)	*Spathiphyllum* spp., the Netherland[f]	−
P. sp. (AN 98/12)	*Cyclamen* spp., the Netherlands[f]	−
Pythium polymastum (348a)	*Apium graveolens*, Belgium[d]	−
Pythium aphanidermatum (Pythium-2)	*L. esculentum*, Germany[d]	−
Fusarium oxysporum (IVIA-F2)	*Phoenix dactylifera*, Spain[e]	−
F. oxysporum (IVIA-F3)	*Citrus* spp., Spain[e]	−
Fusarium sp. (IVIA-F1)	*P. dactylifera*, Spain[e]	−
Fusarium sp. (IVIA-F5)	*P. dactylifera*, Spain[e]	−
Alternaria alternata f. sp. *citrici* (IVIA-A1)	*Citrus* spp., Spain[e]	−
Botrytis cinerea (IVIA-B1)	*Allium cepa*, Spain[e]	−

[a] +, present; −, absent.

[b]Obtained from CBS = Centraalbureau voor Schimmelcultures, Baarn, the Netherlands.

[c]Obtained from PD = Plantenkundige Dienst (Plant Protection Service), Wageningen, the Netherlands.

[d]collection at IGZ = Institut für Gemüse- und Zierpflanzenbau (Institute for Vegetable and Ornamental Crops, Großbeeren/Erfurt, Germany.

[e]Collection at IVIA = Instituto Valenciano de Investigaciones Agrarias (Valencian Institute of Agricultural Research), Moncada, Spain.

[f]Obtained from PBG = Proefstation voor Bloemisterij en Glasgroente (research Station for Floriculture and Glasshouse Vegetables), Aalsmeer, the Netherlands.

[g]Obtained from BBA = Biologische Bundesanstalt (The Federal Biological Research Centre for Agriculture and Forestry), Berlin and Braunschweig, Germany.

[h]Obtained from BAZ = Bundesanstalt für Züchtungsforschung (The Federal Breeding Research Centre for Crops), Aschersleben, Germany.

[i]Confirmed by Dr. W.A. Nan in't Veld from c).

scalpel, in plates incubated in the dark for 7 days. The mycelium was used immediately for DNA extraction or stored at −20 °C until use.

DNA extraction

DNA was isolated from pure cultures according to a conventional phenol extraction method (Lee and Taylor, 1990), and concentrations were calculated by spectrophotometry. Mycelium and plant material were homogenized using a metal bead apparatus that oscillates at high frequency (Retsch, Haan, Germany). Four methods for DNA extraction from plant samples and water were tested: the phenol-free method (Hering and Nirenberg, 1995) and three commercially available Kits: the Fungal Kit (PeQlab, Erlangen, Germany), the DNeasy Plant Kit and the DNeasy Tissue Kit (Qiagen, Hilden, Germany). The amount of DNA obtained by the latter methods was estimated semi-quantitatively, and the absence/presence of DNA banding within positive samples was checked.

Primer design

Primer design was performed according to Olmos et al. (2002) with slight modifications. Briefly, sequenced ITS regions from *P. nicotianae* were recovered using the Nucleotide Sequence Search Program provided by the National Center for Biotechnology Information (NCBI, http://www3.ncbi.nlm.nih.gov/Entrez) (Bethesda, MD, USA). Conserved regions, internal to the DNA sequence flanked by universal primers ITS4 and ITS6, were analysed using the similarity search tool Advanced BLAST 2.0 (http://www3.ncbi.nlm.nih.gov/blast/blast.cgi?Jform = 1) (Altschul et al., 1997) to find significant nucleotide homologies in the molecular data retrieved from NCBI's integrated databases GenBank, EMBL and DDBJ. Primers internal to the universal primers ITS4 and ITS6 (Cooke and Duncan, 1997) were subsequently designed using oligo program 4.0: PNIC1 5′CAATAGTTGGGGGTCTTATT 3′ and PNIC2 5′ GTATACCGAAGTACACATTAAG 3′.

Specific identification and detection of P. nicotianae *by amplification methods using* PNIC *primers*

PCR protocol. The PCR protocol was performed as follows: the reaction mix (25 µl) consisted of 10 mM Tris–HCl pH 8.8, 50 mM KCl, 2.0 mM $MgCl_2$, 1 µM of each PNIC1 and PNIC2 primers, 200 µM dNTPs, 2% formamide, 0.5 units of *Taq* DNA polymerase (Promega, Mannheim, Germany) and 2 µl of DNA template. The amplification was carried out in a Perkin Elmer 9600 cycler (PE Applied Biosystems, Weiterstadt, Germany). After a denaturation step (95 °C for 3 min), 40 cycles of amplification (94 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min) and 10 min at 72 °C were performed. Ten microlitres of PCR products were analysed by electrophoresis in 1.5% agarose gels, stained by ethidium bromide and visualized under UV light.

Conventional nested-PCR protocol. The reaction mix for external amplification (25 µl) consisted of 10 mM Tris–HCl pH 8.8, 50 mM KCl, 2.0 mM $MgCl_2$, 1 µM of each ITS4 and ITS6 primers, 200 µM dNTPs, 5% glycerol, 0.5 units of *Taq* DNA polymerase (Promega) and 2 µl of DNA template. The amplification was carried out in a Perkin Elmer 9600 cycler (PE Applied Biosystems). After a denaturation step (95 °C for 3 min), 35 cycles of amplification (94 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min) and 10 min at 72 °C were performed. One microlitre of the first round was used as template in the second round of amplification, performed according to the PCR protocol described above.

One closed tube nested-PCR protocol. This method was carried out according to Olmos et al. (1999, 2002) using a compartmentalized 0.5 ml Eppendorf tube with the end of a standard 200 µl plastic pipette tip. A small cone was inserted into the Eppendorf tube allowing the physical separation of the two PCR cocktails in the same tube.

The cocktail for the first amplification was a mixture of 30 µl consisting of 50 mM KCl, 10 mM Tris–HCl (pH 9.0 at 25 °C), 3 mM $MgCl_2$, 250 µM dNTPs, 0.1 µM of external primers (ITS4, ITS6), glycerol 5%, 1.5 units of *Taq* DNA and 2 µl of DNA template. The cocktail for the second amplification was a mixture of 10 µl consisting of 50 mM KCl, 10 mM Tris–HCl (pH 9.0 at 25 °C), 4 µM of internal primers (PNIC1 and PNIC2) and 8% formamide. Nested-PCR was carried out in a Techne PHC3 cycler (Techne, Cambridge, Great Britain) starting with a denaturation phase of 2 min at 94 °C, followed by 20 cycles of external amplification (94 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min). After the first PCR, tubes were vortexed and

centrifuged (6000*g* for 2 s). Nested-PCR consisted of 40 cycles of amplification following this profile: 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min. Amplification products were analysed and visualized under UV light.

Specific and sensitive detection of P. nicotianae *in artificially inoculated and natural infected plant samples*

Microscopy and PCR. In a first analysis of plant material, comparisons between symptom occurrence, microscopy and simple-PCR were carried out. Samples of naturally infected roots from hydroponically cultivated tomato plants (*Lycopersicum esculentum*) of 12 separated systems were checked referring to the presence/absence of symptoms, the presence of *Phytophthora* as determined by microscopy, and the presence/absence of a specific amplicon within the simple-PCR test. The samples were generally taken from the part between rotted and viable (white) roots.

In further investigations, microscopy, simple and nested-PCR techniques were compared. In three assays, tomato plants of cv. Counter were inoculated at the fourth leaf stage with a nutrient solution containing 5×10^4 propagules of the pathogen per ml (moderate inoculation) from isolate Pn2 in six repetitions (Grote and Bucsi, 1998; Grote and Claussen, 2001), to study the infection of roots or the translocation of *P. nicotianae* within the hydroponically grown plants. Root and shoot samples were examined 5 and 14 days (roots from first assay), 14 days (shoot from first assay) and 4, 8, 12 and 15 days, after inoculation (second and third assay), respectively. Sections of shoot at 0.5, 1, 2, 4 or 6 cm from the base were collected and stored as all roots samples at −20 °C until analysis.

The same comparison of methods was performed with *Spatiphyllum* cv. Ceres naturally infected with *P. nicotianae*, kindly supplied by Dr. Numansen (Proefstation voor Bloemisterij en Glasgroente at Aalsmeer, the Netherlands) and some samples of rockwool, water, roots or shoots of tomato plants cultivated in Belgium, kindly supplied by A. Vanachter from Scientia Terrae at St. Katelijne-Waver (Belgium).

Comparison of sensitivity of simple and nested-PCR. Some of the extracted DNA from pure *P. nicotianae* cultures was used to prepare a tenfold serial dilution of 600 pg DNA in a healthy root extract, to compare the sensitivity of simple and nested-PCR. A positive control was diluted in sterile water.

Results

Evaluation of DNA extraction methods

Successful DNA extraction from pure pathogen culture was performed according to Lee and Taylor (1990), yielding good quality target DNA. When DNA was extracted from plants, high amounts were obtained, but a reliable PCR pattern for the target organism was not obtained in all cases (Table 2). Only when using the Fungal Kit from PeQlab were reliable and satisfactory results in subsequent PCR reactions achieved.

Specificity of the designed primers

An amplification product of the expected size of 737 bp was obtained with DNA from all morphologically well-characterized *P. nicotianae* isolates and from the two *P. nicotianae/cactorum* hybrids tested, when the designed primers PNIC1 and PNIC2 were used. No amplification products were obtained in cocktail controls, healthy controls, with other *Phytophthora* species, or with species from other genera (Table 1 and Figure 1).

Specific and sensitive detection of P. nicotianae *in artificially inoculated and natural infected plant samples*

Microscopy and PCR analysis. The plants were analysed for the occurrence of brown rotted roots, which was mostly accompanied by shoot wilting. Such symptoms were found in 10 out of 12 samples from the tomato plants investigated, while in three cases, rotting was extensive (samples number 1, 6 and 12, Table 3). Samples number 9 and 11 did not show any symptoms and all other roots showed slight browning. For 75% of the 12 samples, microscopic

Table 2. Qualitative comparison of extraction methods and commercial kits for DNA extraction from infected plant material

DNA extraction method/kit	High DNA amount	Reliable PCR pattern
PeQlab Fungal Enzyme Kit	++	+++
Qiagen Plant Kit	+	−
Qiagen Tissue Kit	+	−
Extraction after Hering and Nirenberg (1995)	++	+(+)

−, not reliable; +, frequent; ++, more frequent; +++, very frequent.

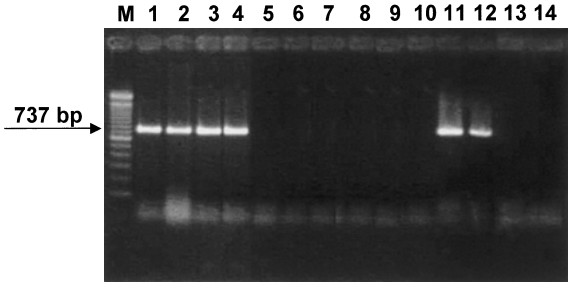

Figure 1. Amplification products of *P. nicotianae* using specific PNIC primers. M, 100 bp Marker (Gibco BRL); lanes 1–4 *P. nicotianae* species: lane 1: *P. n.* CBS 310.62; lane 2: *P. n.* PD 10664; lane 3: *P. n.* Pnp2 IGZ/BBA; lane 4: *P. n.* 5 IGZ: lanes 5–9: other *Phytophthora* species: lane 5: *P. citricola* PD 1817; lane 6: *P. megasperma* var. *megasperma* PD 118; lane 7: *P. citrophthora* IVIA-P3; lane 8: *P. cryptogea* CBS 307.62; lane 9: *P. infestans* BAZ; other fungus: lane 10: *Fusarium oxysporum* IVIA-F2; lanes 11–13: isolates, identified by PCR; lane 11: *P. nicotianae P. n.* 2 IGZ; lane 12: *P. nicotianae P. n.* PBG AN 96/4; lane 13: *Phytophthora* sp. *P* 3 IGZ; lane 14: no DNA control.

Table 3. Detection of *P. nicotianae* in naturally infected roots of hydroponically grown tomato plants (Germany) with microscopical analysis and simple PCR

Number of plant for sampling	Symptoms	Microscopy	Simple PCR
1	++	−[a]	−
2	+	−[a]	−
3	+	−[a]	−
4	+	+	−
5	+	−	−
6	++	+	+
7	+	+	+
8	+	−[a]	−
9	−	−	−
10	+	+	−
11	−	+	+
12	++	+	−

−, absent; +, present or light to moderate affected; ++, moderate to heavy affected;
[a]No *Phytophthora*.

analysis of naturally infected roots was in agreement with the results from simple-PCR analysis. For three plants, *P. nicotianae* infection was detected by both simple-PCR and direct microscopic observation. One of these plants was asymptomatic and the others showed typical symptoms. When *Phytophthora* was not observed microscopically, the PCR supplied no reaction (six plants) irrespective of the symptoms observed on the plants. However, no reaction was obtained by simple-PCR for three plants on which *Phytophthora* was observed under the microscope.

Results of three assays from different infection sites on artificially inoculated tomato plants examined under the microscope and analysed by simple and nested-PCR, are shown in Table 4. From a total of 35 samples, 97% and 94% were positively determined by microscopy and nested-PCR, respectively, but only 31% by simple-PCR. In one shoot sample collected 14 days after inoculation at 0.5 cm (assay number 1 in Table 4), the pathogen was detected only by nested-PCR.

The conventional examination of samples from different infection sites, crops and origins of naturally infected plant samples by optical microscopy enabled identification of *P. nicotianae* and *P. cryptogea* in 9 out of the 15 samples tested (Table 5). Two samples, one of which was obtained from a water reservoir used for preparing nutrient solutions for hydroponic culture (sample number 6), were negative samples without any infection. *P. nicotianae* was detected in only one out of four positive samples by simple-PCR, while with nested-PCR, all positive samples were confirmed.

Comparison of the sensitivities of simple and nested-PCRs. The results for detection of the target pathogen within plant material showed that the sensitivity was not always satisfactory. This could be performed by nested-PCR using specific and highly sensitive primers. Nested-PCR was found to be at least 1000 times more sensitive than conventional PCR (Figure 2, Table 6). The nested-PCR sensitivity was at least 60 fg of *P. nicotianae* DNA when diluted in healthy root extract. With both methods, a concentration of target DNA more than ten times lower could be detected within the positive control (Table 6).

Discussion

Symptoms of fungal diseases, such as water deficiencies in above-ground plant parts, are very similar when caused by many *Phytophthora* spp. but also as a consequence of infection by fungi such as *Fusarium* or *Pythium* spp. (Duniway, 1977). For example, roots of solanaceous plants such as tomato can be simultaneously infected by *P. nicotianae* and other *Phytophthora* species like *P. cryptogea, P. capsici* or *P. erythroseptica*, as well as other genera like *Pythium* and *Fusarium*. *P. cactorum* and *P. infestans* are the most commonly found *Phytophthora* species on tomato

Table 4. Detection of *P. nicotianae* in artificial inoculated infected tomato plant material (Germany) by optical microscopy, PCR and nested-PCR

Assay	Samples	Days after inoculation	Distance from crown (cm)	Determination by microscopy	PCR	Nested-PCR
1	Roots	5		+	+	+
		14		+	+	+
		14		+	+	+
		14		+	−	+
		14		+	+	+
	Shoot	14	0.5	−	−	+
2	Roots	4		+	+	+
		8		+	+	+
		12		+	+	+
		15		+	−	−
	Stem	4	0.5	+	+	+
			2	+	+	+
			4	+	−	+
		8	0.5	+	−	+
			2	+	−	+
			4	+	−	+
		12	0.5	+	−	+
			2	+	−	+
			4	+	−	+
		15	0.5	+	−	+
			2	+	−	+
			4	+	−	+
3	Shoot	4	0.5	+	+	+
			2	+	+	+
			4	+	−	+
		8	0.5	+	−	+
			2	+	−	+
			4	+	−	+
		12	0.5	+	−	+
			2	+	−	+
			4	+	−	+
		15	0.5	+	−	+
			2	+	−	+
			4	+	−	+
			6	+	−	−

fruits and shoots, respectively (Atherton and Rudich, 1986; Erwin and Ribeiro, 1996). For this reason, several pathogens from different genera like *Phytophthora, Pythium, Fusarium* and others, which threaten agriculturally important plants throughout the world, were included in our studies.

Most of the isolates from herbaceous and woody plants, plus those obtained from colleagues and collections, were identified confidently by the authors or the supplier with the help of morphological structures. For microscopical identification, morphological structures like sporangia, which are related to the water-borne character of the pathogen, were used (Waterhouse, 1963; Erwin and Ribeiro, 1996). Thus, sporangia were directly detected by microscopy on roots sampled from hydroponically cultivated crops, or previously washed roots were covered by sterile, ionized water and maintained for 2 days at 27 °C in darkness. Only one isolate was confirmed according to isozyme analysis by Dr. Man in't Veld (Table 1).

The DNeasy Plant and Tissue Kit from Qiagen and the extraction method of Hering and Nirenbenberg (1995) produced a relatively high amount of DNA, but only the Fungal Kit achieved a high amount of fungal DNA within the total DNA extracted, and consequently

Table 5. Detection of *P. nicotianae* in naturally infected plant material by optical microscopy, PCR and nested-PCR

Sample no.	Host/country/origin	Samples	Determination by microscopy	PCR	Nested-PCR
1	*L. esculentum*/Belgium/ Costermans Marc[a]	Roots (within rockwool slab)	+	+	+
2	*L. esculentum*/Belgium/ Somers Guy[a]	Roots (within rockwool slab)	+	−	+
3	*Spatiphyllum 'ceres'*/ the Netherlands/PBG[b]	Roots	+	−	+
		Shoot	+	−	+
4	*L. esculentum*/Belgium/ *Aernouts*[a]	Rockwool slab	−[c]	−	−
		Nutrient solution	−[c]	−	−
		Roots	−[c]	−	−
		Stem base	−[c]	−	−
		Shoot	−[c]	−	−
5	*L. esculentum*/ Belgium/ *Costermans*[a]	Rockwool slab	−[d]	−	−
		Nutrient solution	−[d]	−	−
		Roots	−[d]	−	−
		Stem base	−[d]	−	−
		Shoot	−[d]	−	−
6	Water Reservoir/ Belgium/Costermans[a]	Water reservoir	−	−	−

[a]Samples originated from Scientia Terrae, St.-Katelijne-Waver, Belgium by A. Vanachter.
[b]Sample originated from PBG = Proefstation voor Bloemisterij en Glasgroente, Aalsmeer, the Netherlands by A. Numansen.
[c]No *Phytophthora*.
[d]*Phytophthora cryptogea*.

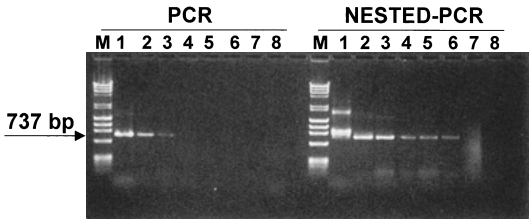

Figure 2. Sensitivity of simple and nested-PCR for detection of *P. nicotianae*. M, Markermix (biometra, Göttingen, Germany); lane 1: 600 pg of *P. nicotianae* in water; lane 2: 600 pg of *P. nicotianae* in root extract (r.e.); lane 3: 60 pg in r.e.; lane 4: 6000 fg in r.e.; lane 5: 600 fg in r.e.; lane 6: 60 fg in r.e; lane 7: healthy r.e.; lane 8: no DNA control.

Table 6. Sensitivity of detection of *P. nicotianae* by simple and nested-PCR

Dilution within	Simple-PCR	Nested-PCR
Water (positive control)		
2500 pg	+	+
250 pg	+	+
25 pg	+	+
2500 fg	+	+
250 fg	−	+
25 fg	−	+
2500 ag	−	+
Healthy root extract		
6000 pg	+	+
600 pg	+	+
60 pg	+	+
6000 fg	−	+
600 fg	−	+
60 fg	−	+

gave the best PCR pattern. Bonants et al. (1997) noted that improved PCR techniques should include simpler protocols for DNA extraction, e.g., by PCR-ELISA having polyclonal antisera or better monoclonal antibodies. However, the development of such sera and antibodies is expensive, time-consuming and labour-intensive. Another approach is to improve the sensitivity and specificity of the PCR, which allows direct detection of the target DNA within plant samples without previous DNA extraction. This approach was tried successfully for nearly all positive samples

presented here, by the use of the described nested-PCR protocol.

The ITS1 and ITS2 spacer regions of the ribosomal units are more variable in sequences than rRNA genes and have potential for distinguishing species (Lee and Taylor, 1992). Therefore, primers designed from these regions allowed the species-specific identification of all *P. nicotianae* isolates and the two *P. nicotianae/cactorum* hybrids used. However, a similar amplification product was detected in the first experiment with a *P. citricola* isolate, probably because of the close genetic relationship of the studied sequences to *P. nicotianae* sequences. The addition of 2% formamide to the cocktail mix increased the specificity and avoided the appearance of this amplicon. Morphologically and genetically similar *Phytophthora* species like *P. cactorum* could be excluded with the simple-PCR even without the inclusion of 2% formamide. The potential for species discrimination will remain somewhat limited until sequences from a wide range of isolates are available, especially for complex species like *P. megasperma*, *P. cryptogea* and *P. nicotianae* with its hybrids, and until the molecular taxonomy has been clarified (Lacourt and Duncan, 1997).

Detection by microscopy and the results from PCR (Table 3) suggest that only moderate to heavy infections can be detected by the simple-PCR method, as was previously shown for *P. fragariae* on strawberry plants (Bonants et al., 1997). To avoid false negative detection, a method with higher sensitivity was required and so nested-PCR methods were developed using PNIC specific primers as internal to the amplified fragment, recently described by Cooke and Duncan (1997) as being specific for the genus *Phytophthora* (Tables 4 and 5). Lower concentrations of pathogens may also be essential for agronomic impact on crops, because disease development is not directly related to the initial inoculum density. A stronger influence on disease development of predisposition and stress conditions during plant growth was shown by Grote and Claussen (2001) and Van der Plank (1999).

In general, nested-PCR gave the same reliability as the microscopic investigations, and could even detect latent pathogen presence as described for one artificially infected sample and low concentrations of the target organism. Therefore, reliable DNA detection methods can be used instead of time-consuming and labour-intensive microscopy, which requires considerable knowledge of plant pathogens.

The authors suppose that the false negative results obtained by the simple-PCR were due to the less sensitive method for *P. nicotianae* detection, the relatively higher amount of plant DNA, and the presence of plant inhibitors (Wilson, 1997). With the nested-PCR, the pathogen could be found in nearly all cases, confirming the higher sensitivity of the nested-PCR in practice. These results are in agreement with results of other authors who were able to detect less than 25 pg or 1 fg in media such as soil or plant material, respectively, with a nested-PCR method (Tsushima et al., 1995; Bonants et al., 1997; Coelho et al., 1997; Cooke et al., 2000).

Both PCR methods were able to detect a more than ten times lower detectable concentration of *P. nicotianae* within the positive control-DNA diluted in water, when compared with the dilution in healthy root extract, consistent with the previously described inhibitory effects of plants inhibitors (Wilson, 1997).

The results for nested-PCR applied in separate or in one closed tube were identical. This fact assumed exclusion of contamination of the samples. In general, the use of a single closed tube reduces the risk of contamination drastically (Olmos et al., 1999).

The results show that the nested-PCR enables detection of low levels of the pathogen and can therefore be used as an early warning detection and diagnostic tool to confirm the pathogen in obviously diseased plant parts. The approach described was applied because of the high potential for simultaneous DNA-based detection of several agronomically important pathogens in one test as a multiplex PCR system for monitoring and for epidemiological studies (Dyer et al., 2001).

Acknowledgements

The financial support from the Instituto Valenciano de Investigaciones Agrarias (IVIA) and from the Agricultural Ministries of the German Federal Government as well as from the Agricultural Ministries of Brandenburg and Thuringia, is gratefully acknowledged. The authors would like to thank all co-workers at the laboratories of Mycology, Bacteriology and Virology and Immunology of the Department of Plant Protection and Biotechnology of IVIA, where Dr. D. Grote stayed as part of a research project from March to December of 1999 and at the Plant Health Department of the IGZ for their support and to A. Ballester and A. Fandrey for technical assistance. We also thank Dr. R.B. Baayen and A. Numansen, as well Dr. W. Man in't Veld,

A. Vanachter, Dr. J. Gabler, Dr. H. Nierenberg and Dr. S. Werres for kindly supplying isolates.

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