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Identification and detection of *Rosellinia necatrix* by conventional and real-time Scorpion-PCR

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Abstract

Several polymerase chain reaction (PCR) primers were designed from the internal transcribed spacer (ITS) regions of the rDNA genes of *Rosellinia necatrix* to develop a PCR-based identification method. Screening the primers against two isolates of *R. necatrix* and six other *Rosellinia* species resulted in the amplification of a single specific product from *R. necatrix* for most of the primer pairs. Two primer pairs (R2-R8 and R10-R7) confirmed their specificity when tested against 72 isolates of *R. necatrix* and 93 other fungi from different hosts and geographic areas. The R10 primer was modified to obtain a Scorpion primer for detecting a specific 112 bp amplicon by fluorescence emitted from a fluorophore in a self-probing PCR assay. This assay specifically recognised the target sequence of *R. necatrix* over a large number of other fungal species. In conventional PCR, with primer pairs R2-R8 and R10-R7, 10-fold dilutions of *R. necatrix* DNA indicated a detection limit of 10 pg μl^{-1} using a single set of primers and 10 fg μl^{-1} in nested-PCR. For Scorpion-PCR, the detection limit was 1 pg μl^{-1} and 1 fg μl^{-1} in nested Scorpion-PCR, i.e. 10 times more sensitive than conventional PCR. A simple and rapid procedure for DNA extraction directly from soil was modified and developed to yield DNA of purity and quality suitable for PCR assays. Combining this protocol with the nested Scorpion-PCR procedure it has been possible to specifically detect *R. necatrix* from artificially inoculated soils in approximately 6 h.

Abbreviations: CTAB – cetyltrimethylammonium bromide; dNTPs – deoxynucleoside triphosphates; ITS – internal transcribed spacer; PCR – polymerase chain reaction.

Introduction

The white root rot caused by the fungus *Rosellinia necatrix* Prill. (anamorph: *Demathophora necatrix* Hartig), is destructive to many fruit tree species including almond, peach, plum, apple, pear, olive, cherry and avocado. Usually, symptoms of the disease consist of collar and root rot which result in different degrees of canopy decline, followed by leaf drop, wilting and death of the tree. Recently, an extensive field survey indicated an increasing occurrence of the disease in Southern Italy where susceptible species such as sweet cherry, almond and olive are cultivated in the same soil.

The effective control of white root rot is primarily based on planting disease-free plants in a pathogen-free soil. To produce disease-free propagative material, methods for rapid identification and detection of the pathogen are necessary. However, to our knowledge no specific methods for *R. necatrix* detection are available. Isolation of the pathogen from infected roots is difficult due to the lack of selective media and the occurrence of a large number of saprophytic micro-organisms. The avocado leaf disk colonisation method (Sztejnberg et al., 1987) has been utilised to isolate and assess inoculum level of *R. necatrix* in naturally and artificially infected soils, but it is a laborious procedure and

avocado leaves are not available everywhere. In addition, expertise is needed to identify the fungus (Petrini, 1992).

The polymerase chain reaction (PCR) provides a reliable alternative for the identification and detection of fungal pathogens. Internal transcribed spacer (ITS1 and ITS2) regions within ribosomal gene clusters are widely utilised to design species-specific PCR primers (White et al., 1990). However, the large-scale application of conventional PCR for the detection of micro-organisms are limited, due to post amplification procedures necessary to detect amplified fragments (Schaad et al., 1999). Real-time PCR combines the sensitivity of conventional PCR with the generation of a specific fluorescence signal only when the probe forms a stable hybrid with the complementary internal sequence of the amplicon. Real-time PCR is widely used in medicine for the diagnosis of viral and bacterial infections (Machida et al., 2000; Reischl et al., 2000) and there are increasing reports about its application in plant pathology (Weller et al., 2000; Schena et al., 2002; Ippolito et al., 2000; Bates et al., 2001; Cullen et al., 2001). Several attempts have been made to generate amplification systems in which the amplicon detection is based on fluorescence resonance energy transfer (FRET) such as Taq-Man (Lee et al., 1993) and Molecular Beacons (Tyagi and Kramer, 1996). Scorpion-PCR (Whitecombe et al., 1999) uses a unimolecular approach and the probe target binding is kinetically favoured over duplex reannealing and thermodynamically favoured over intrastrand secondary structures. These characteristics make Scorpion-PCR rapid and sensitive (Thelwell et al., 2000; Finetti et al., 2000a). Scorpion-PCR has been used to detect antagonistic (Schena et al., 2002) or pathogenic fungi (Ippolito et al., 2000) and viruses (Finetti et al., 2000).

The aim of this work was to develop specific and sensitive PCR-based methods to detect and identify *R. necatrix* in soil.

Materials and methods

Fungal isolates and DNA extraction

The isolates of *R. necatrix* were collected in Southern Italy from different hosts (27 isolates were from sweet cherries, 14 from olive, 9 from peach, 6 from almond, 6 from walnut-tree, 1 isolate was from pear, 1 from grapevine and 1 from pistachio). Additional isolates were obtained from the Centraalbureau voor Schimmelcultures (CBS, Utrecht, the Netherlands) (isolates CBS 267.30 and CBS 349.36), the Coleccion Española de Cultivos Tipo (CECT, University of Valencia, Valencia, Spain) (isolates CECT 2817 and CECT 2818), Prof. A. Sztejnberg of the The Hebrew University of Jerusalem (Rehovot, Israel) (one isolate from apple and one from avocado), and Dr. A.M. Vettraino of the Tuscia University (Viterbo, Italy) (one isolate from poplar). Other fungal isolates (Table 1) were available from the collection of the Department of Plant Protection and Applied Microbiology (University of Bari, Italy). All fungal isolates were stored on potato dextrose agar (PDA) slants at 5 °C.

To extract total DNA, fungi were grown in Petri dishes containing malt extract agar (MEA) covered with sterile cellophane sheets to facilitate collection of the mycelium. Inoculation of each fungal isolate was carried out using a 7 mm mycelial plug taken from the edge of actively growing colonies on PDA. Between 5 and 10 mg of mycelia were collected with a spatula after 5–15 days of incubation at 24 °C, suspended in 400 µl of breaking buffer (Hoffman and Winston, 1987) and extracted with 400 µl of phenol/chloroform/isoamyl alcohol (25 : 24 : 1) in the presence of 25 mg of acid-washed glass beads (425–600 µm diameter) and two sterile 5 mm stainless steel ball bearings. This mixture was vortexed at 3000 rpm for 10 min, and centrifuged for 15 min at 13,000 × g. The aqueous phase was mixed with an equal volume of chloroform/isoamyl alcohol (24 : 1), vortexed at 3000 rpm for 2 min, centrifuged for 5 min at 13,000 × g and precipitated with two volumes of 100% cold (−20 °C) ethanol. The precipitated DNA was washed with 70% cold (−20 °C) ethanol, dissolved in 50 µl of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.6), quantified using a spectrophotometer and diluted to 50 ng μl^{-1}.

Primers design and amplification

To ensure the quality of template DNA, all extracts were amplified by PCR with universal primers ITS5 and ITS4 (White et al., 1990). PCR reactions were performed in a total volume of 25 µl containing 100 ng of genomic DNA, 10 mM Tris-HCl (pH 9), 50 mM KCl, 0.1% Triton X-100, 100 µM dNTPs, 1 mM $MgCl_2$, 1 unit of *Taq* polymerase (Taq DNA polymerase, Promega Corporation, WI, USA) and 2 µM of ITS4 and ITS5 primers. The PCR reaction was incubated in a programmable thermal cycler (PCR express, Hybaid, UK) starting with 5 min denaturation at 95 °C,

Table 1. Fungal isolates

Fungus	Isolate code	Host	Year of isolation
1. *Alternaria brassicicola*	Fv359	Cauliflower	2000
2. *Alternaria citri*	Fv33	Lemon	1988
3. *Alternaria* sp.	Fv167	Pistachio	1995
4. *Alternaria* sp.	Fv169	Parsley	1995
5. *Alternaria* sp.	Fv232	Cycas	1996
6. *Alternaria* sp.	OL67	Olive	1997
7. *Alternaria* sp.*	F5	Chestnut-tree	2000
8. *Alternaria* sp.*	F20	Sweet cherry	2000
9. *Aspergillus niger*	Asp	Grapevine	2001
10. *Aspergillus* sp.*	F15	Sweet cherry	2000
11. *Botryosphaeria ribis*	Fv360	Orange	2000
12. *Botryosphaeria ribis*	Fv20	Currant	1988
13. *Botryosphaeria* sp.	A4	Grapevine	1999
14. *Botrytis cinerea*	Fv69	Grapevine	1990
15. *Botrytis cinerea*	Fv132	Kiwifruit	1993
16. *Botrytis cinerea*	Fv349	Grapevine	2000
17. *Camarosporium* sp.	Fv253	Orange	1996
18. *Cephalosporium* sp.	Fv61	Cotton	1990
19. *Cladosporium* sp.	Fv267	Not known	1997
20. *Colletotrichum* sp.	Fv68	Clementine	1990
21. *Cylindrocarpon* sp.	Fv128	Kiwifruit	1992
22. *Cytospora* sp.	Fv196	Sweet cherry	1996
23. *Endothia parasitica*	Fv46	Chestnut-tree	1989
24. *Euypa lata*	A5	Grapevine	1999
25. *Fomitiporia puntata*	A1	Lemon	1999
26. *Fusarium roseum**	F4	Almond	2000
27. *Fusarium* sp.	Fv27	Kiwifruit	1988
28. *Fusarium* sp.	Fv183	Not known	1996
29. *Fusarium* sp.	Fv231	Cycas	1996
30. *Fusarium* sp.*	F11	Sweet cherry	2000
31. *Fusarium* sp.*	F14	Sweet cherry	2000
32. *Fusarium* sp.*	F17	Sweet cherry	2000
33. *Fusarium* sp.*	F18	Sweet cherry	2000
34. *Fusicoccum amygdali*	Fv342	Sweet cherry	2000
35. *Gliocladium roseum*	Fv39	Not known	1989
36. *Gliocladium* sp.	Fv114	Kiwifruit	1992
37. *Gliocladium* sp.	Fv303	Potato	1998
38. *Gloeosporium* sp.	Fv94	Apple	1991
39. *Macrophomina* sp.	Fv313	Potato	1998
40. *Mycocentrospora cladosporioides*	OL177	Olive	2000
41. *Myrothecium roridum*	Fv50	ATCC 16297	
42. *Penicillium digitatum*	Fv51	Orange	1990
43. *Penicillium digitatum**	F7	Almond	2000
44. *Penicillium funiculosum*	Fv273	CBS 884.71	
45. *Penicillium italicum*	Fv99	Mandarin	1992
46. *Penicillium* sp.*	F16	Sweet cherry	2000
47. *Phaemoniella chlamydospora*	A2	Grapevine	1999
48. *Phialophora* sp.	Fv266	Sweet cherry	1997
49. *Phialophora parasitica*	OL25	Olive	1999
50. *Phoma* sp.	Fv156	Clementine	1995
51. *Phomopsis diospyri*	Fv205	Japanese persimmon	1996
52. *Phomopsis viticola*	A3	Grape vine	1999
53. *Phyllosticta arbuti*	Fv265	Strawberry tree	1997
54. *Phytophthora boehmeriae*	Ph131	CBS 291.29	

Table 1. Continued

Fungus	Isolate code	Host	Year of isolation
55. *Phytophthora cactorum*	Ph206	Nuts	1990
56. *Phytophthora capsici*	Ph26	Pepper	1991
57. *Phytophthora capsici*	Ph201	Pepper	2000
58. *Phytophthora cinnamomi*	Ph207	CBS 288.47	
59. *Phytophthora cinnamomi*	Ph124	CBS 271.55	
60. *Phytophthora citricola*	Ph38	CBS 295.29	
61. *Phytophthora citrophthora*	Ph199	Citrus soil	2000
62. *Phytophthora cryptogea*	Ph202	Tomato	1999
63. *Phytophthora erythroseptica*	Ph203	CBS 357.59	
64. *Phytophthora heveae*	Ph129	CBS 296.29	
65. *Phytophthora nicotianae*	Ph106	Clementine	1993
66. *Phytophthora* sp.	Ph204	Orange	1996
67. *Pleurotus ostreatus*	Fv32	Not known	1989
68. *Pythium* sp.*	F1	Almond	2000
69. *Pythium* sp.*	F2	Almond	2000
70. *Pythium* sp.*	F3	Almond	2000
71. *Rhizoctonia solani**	F10	Sweet cherry	2000
72. *Rosellinia aquila*	R31	CBS 399.61	
73. *Rosellinia limoniispora*	R33	CBS 381.86	
74. *Rosellinia mammaeformis*	R34	CBS 445. 89	
75. *Rosellinia millegrana*	R35	CBS 590.70	
76. *Rosellinia reticulispora*	R40	CBS 868.68	
77. *Rosellinia sanguinolenta*	R41	CBS 395.85	
78. *Sclerotinia* sp.	Fv75	Lemon	1991
79. *Septoria tritici*	Fv95	Wheat	1992
80. *Stemphylium* sp.	Fv123	Pistachio	1992
81. *Trichoderma coningii*	Fv283	Not known	1997
82. *Trichoderma pseudoconingii*	Fv154	Clementine	1995
83. *Trichoderma* sp.*	F9	Sweet cherry	2000
84. *Trichoderma harzianum**	F12	Sweet cherry	1999
85. *Trichoderma* sp.*	F13	Sweet cherry	2000
86. *Trichoderma* sp.*	F19	Sweet cherry	2000
87. *Trichoderma* sp.*	F8	Sweet cherry	2000
88. *Trichoderma viridae*	Fv181	Not known	1996
89. *Trichothecium* sp.	Fv236	Strawberry tree	1996
90. *Ulocladium* sp.	Fv263	Sonchus oleraceus	1996
91. *Verticillium albo-atrum*	Fv336	CBS 321.91	
92. *Verticillium dahliae*	Fv330	Olive	2000
93. *Verticillium dahliae*	OL61	Olive	1997

*Isolates obtained from *Rosellinia* infected tissue.

followed by 35 cycles at 95 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 1 min. A negative control (no template DNA present in PCR reaction) was included in each experiment. Amplicons were analysed by electrophoresis in 2% agarose gels and TAE buffer (Sambrook et al., 1989) and visualised by staining with ethidium bromide (2 $\mu g\,ml^{-1}$).

Specific primers for *R. necatrix* were designed from the ITS regions (ITS1 and ITS2). ITS sequences were available on Internet (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) for *R. necatrix* (accession numbers AB017657 and AB017658), *R. pepo* (AB017659) and *R. quercina* (AB017661). ITS sequences were aligned using the Clustalw software (EMBL, European Bioinformatics Institute) and screened for differences between three species. Eight different primer sets (Table 2) were designed using the 'Steve Rozen, Helen J. Skaletsky (1998) Primer3 software' and arranged in several possible combinations for DNA amplification (Figure 1). Specificity was assessed using genomic DNA from *R. aquila, R. limonispora, R. mammiformis, R. miligrana,*

Table 2. R. necatrix primers

Primers forward (5′–3′)		Primers reverse (5′–3′)	
R1	ATA ACT CCC AAA ACC CAT GTG A	R7	AAC CAT AGG CGA GAT GAG AAA T
R2	CAA AAC CCA TGT GAA CAT ACC A	R8	CCG AGG TCA ACC TTT GGT ATA G
R3	CGA AGT GCC CTA CCC TGT TA	R11	CAC AAC CAT AGG CGA GAT GA
R5	CAC GAA ACT CTG TTT AGC ATT GA		
R10	CCC CTG TTG CTT AGT GTT GG		

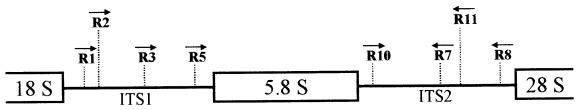

Figure 1. Diagrammatic representation of the internal transcribed spacer regions (ITS1 and ITS2) among the ribosomal DNA repeats, and localisation of primers utilised to specifically amplify DNA from *R. necatrix*. The arrows on each primer indicate the amplification direction.

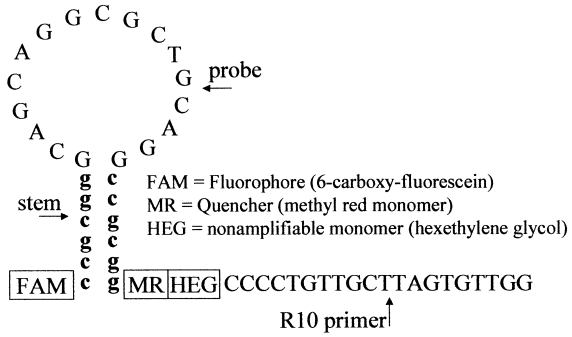

Figure 2. Schematic representation of R10 Scorpion probe. R10 Scorpion is composed of a primer element (R10), a non-amplifiable monomer (HEG), a quencher (MR), a closed stem, a probe element and a fluorophore (FAM) (Whitecombe et al., 1999).

R. reticulispora, R. sanguinolenta (Table 1) and two isolates of *R. necatrix* (positive control). Furthermore, specificity of two primer pairs (R2-R8 and R10-R7) was tested against 72 isolates of *R. necatrix* and 93 isolates of other fungi (Table 1). These two primer pairs were selected because (i) they were specific for identifying the target pathogen, (ii) enabled the development of a nested-PCR and (iii) the primers R10-R7 amplified a DNA fragment of 112 bp, suitable for Scorpion-PCR. PCR amplifications were conducted as described for the universal primers ITS5-ITS4, except for the annealing temperature, which was increased to 60 °C.

Scorpion-PCR

The R10 primer was modified according to Whitcombe et al. (1999) to obtain a Scorpion primer (R10 Scorpion) for amplicon detection by fluorescence emitted from a fluorophore through a self-probing reaction (Oswel Research Products Ltd., Southampton, UK). Primer R10 Scorpion (Figure 2) was combined with primer R7 and tested for specificity against all *R. necatrix* and other fungal isolates (Table 1). Reactions were performed in a 25 µl mixture containing 100 ng of genomic DNA, 10 mM Tris-HCl (pH 9), 50 mM KCl, 0.1% Triton X-100, 100 µM each dNTPs, 1 mM $MgCl_2$, 1 unit of *Taq* polymerase, 0.3 µM R10 Scorpion and 1 µM of reverse primer (R7). Amplification was conducted in sealed tubes in a 96-well microtiter plate (Bio-Rad, Hercules, CA, USA) and consisted of an initial denaturing step at 94 °C for 5 min followed by 40 cycles, each consisting of 45 s at 94 °C and 45 s at 60 °C. Fluorescence was monitored using a spectrofluorometric thermal cycler (iCycler Thermal Cycler, Bio-Rad, Hercules, CA, USA) for real-time data collection during annealing-extension. Relative normalised fluorescence (ΔRn) was plotted and utilised to calculate the average background fluorescence emission in the initial PCR cycles before fluorescence increased. Threshold fluorescence intensity was established at 10-fold above the standard deviation in the initial PCR cycles and any sample that reached a fluorescence value exceeding the fluorescence threshold value was considered positive. The PCR cycle at which fluorescence exceeded the threshold was defined as the cycle threshold (Ct).

To confirm the amplification of the specific fragment of the expected size, 10 µl of each PCR sample was analysed by agarose gel electrophoresis and ethidium bromide staining.

Sensitivity of the reactions

To assess sensitivity of the PCR reactions and to compare conventional PCR with Scorpion-PCR, total

DNA extracted from a pure culture of *R. necatrix* was serially diluted in the PCR reaction mixture to the final concentrations of 1 ng μl^{-1}, 100 pg μl^{-1}, 10 pg μl^{-1}, 1 pg μl^{-1}, 100 fg μl^{-1}, 10 fg μl^{-1}, 1 fg μl^{-1} and 100 ag μl^{-1}. Water was used as negative control to replace template DNA in PCR reaction. DNA dilutions were amplified both using a single set of primers and by nested-PCR. In PCR using a single set of primers amplifications were carried out with primers R2-R8, R10-R7 and R10 Scorpion-R7 (Scorpion-PCR). In the nested-PCR, 1 µl of amplified products obtained with primers R2-R8 was utilised as template for both R10-R7 and R10 Scorpion-R7 (nested Scorpion-PCR) primer pairs. Reactions and data analysis were carried out as described above. To generate standard curves each DNA concentration and no template controls were amplified in triplicate and cycle threshold values plotted against the logarithm of the initial DNA concentrations. The correlation coefficient (r^2) of the standard curve was calculated by using the least square fit method.

Detection of R. necatrix *in artificially infected soils*

The inoculum of *R. necatrix* was prepared by inoculating wheat grains with isolate R24 (Sztejnberg and Madar, 1980). An apparently healthy soil collected from a wheat-cultivated field received 3 g of infected wheat grains per 1000 g of soil. Inoculated and non-inoculated (negative control) soils were used to grow 2-year-old *Prunus mahaleb* plantlets in 6 l plastic pots. *P. mahaleb* is widely used as a sweet cherry rootstock in Southern Italy and is susceptible to root rotting (Perry et al., 1996). Plants were maintained in a greenhouse at 25 ± 2 °C (day), 15 ± 2 °C (night) and irrigated once per week. Four replicate plots were used for both inoculated and non-inoculated plants. Soil samples (≅1 kg) were collected 4 weeks after inoculation, when plantlet showed evidence of canopy decline. The soil samples were dried at room temperature for 10 days, crumbled, mixed and sieved with a 2 mm mesh. For each sample, DNA extraction was performed in triplicate by

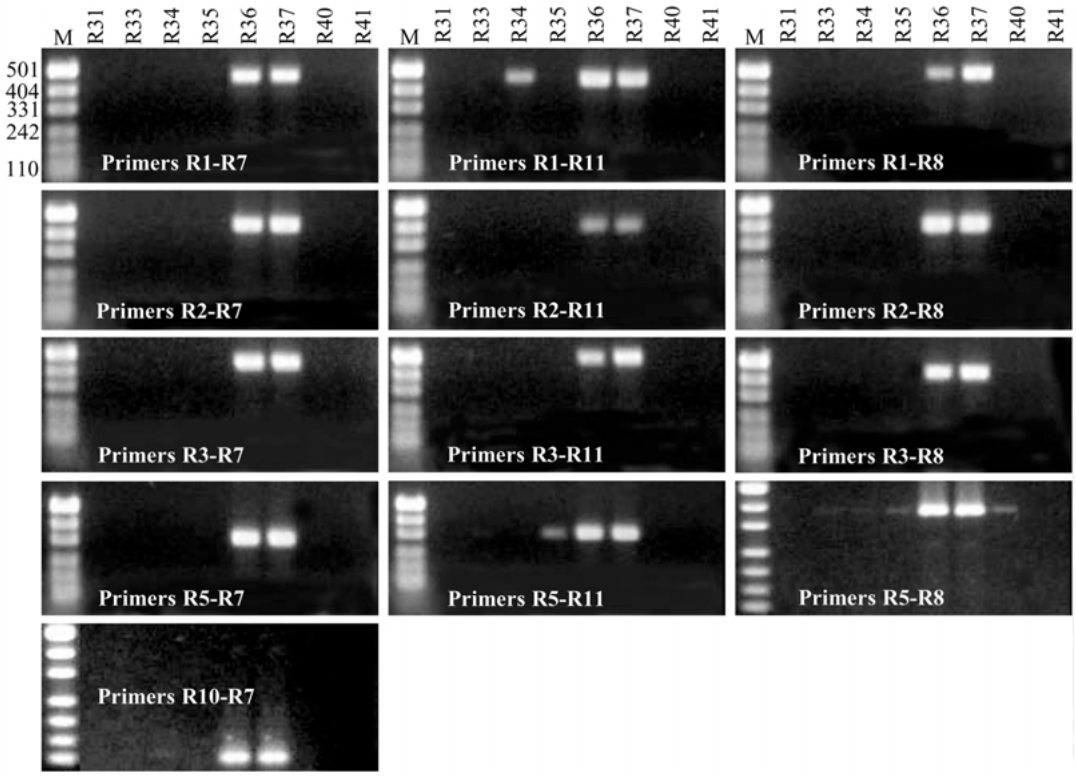

Figure 3. PCR amplification of DNA from *R. aquila* (isolate R31), *R. limoniispora* (isolate R33), *R. mammiformis* (isolate R34), *R. miligrana* (isolate R35), *R. necatrix* (isolates R36 and R37), *R. reticulispora* (isolate R40) and *R. sanguinolenta* (isolate R41) using various primer pairs. Lane M, DNA marker with fragment size in bp (pUC19 DNA/*MSP*I, MBI Fermentas, Germany).

using the protocol described by Cullen et al. (2001) with minor modifications. Soil (0.5 g) was suspended in 700 µl of extraction buffer (0.12 M Na_2HPO_4, 1.5 M NaCl, 2% CTAB) in the presence of two 5 mm stainless steel ball bearings and 0.5 g acid-washed glass beads (425–600 µm diameter). The extraction mixture was vortexed at 3000 rpm for 10 min and centrifuged at 13,000 × g for 5 min at room temperature. The upper phase was extracted with 750 µl of chloroform, precipitated with two volumes of isopropanol, washed with 70% cold ethanol, dried and resuspended in 50 µl of sterile distilled water. Before amplification, DNA was purified with Sepharose CL-6B (Pharmacia, Peapack, NJ, USA) by construction of a spin column (Bramwell et al., 1995).

One microlitre of purified genomic DNA was amplified using both a single set of primers (R2-R8 or R10 Scorpion-R7) and by nested Scorpion-PCR (first amplification with primers R2-R8, second amplification with primers R10 Scorpion-R7). Reactions and real-time data analysis were carried out as described above.

Results

DNA extraction and amplification

The protocol utilised to extract DNA from pure culture enabled the extraction of 50–150 µg of total DNA. Template DNA was suitable for PCR amplification as demonstrated by the amplification with the universal primers ITS5 and ITS4 of single fragments (including both ITS regions and the 5.8S rDNA) from all *R. necatrix* and other fungus isolates (data not shown).

Primers specificity was assessed against six different species of *Rosellinia* and two isolates of *R. necatrix*. All primer combinations amplified a unique DNA fragment of the expected size. In particular, the primer combinations R1-R7, R1-R8, R2-R7, R2-R11, R2-R8, R3-R7, R3-R11, R3-8, R5-R7 and R10-R7 resulted in highly specific DNA fragments amplified only from the two *R. necatrix* isolates (Figure 3). Primer pair R1-R11 amplified a DNA fragment from *R. necatrix* and *R. mammiformis*; primer pair R5-R11 amplified a DNA fragment from *R. necatrix* and *R. miligrana* and primer pair R5-R8 amplified a DNA fragment from *R. necatrix* and one from *R. limoniispora*, *R. mammiformis*, *R. miligrana* and *R. reticulispora* (Figure 3).

Specificity of primer pairs R2-R8 and R10-R7 was further assessed against 72 isolates of *R. necatrix* and 93 isolates of other fungal species. Both primer sets showed amplified fragments of the expected size (493 and 112 bp, respectively) only from *R. necatrix* isolates (data not shown).

Scorpion-PCR and sensitivity

Total DNA extracted from 72 *R. necatrix* isolates primed with R10 Scorpion-R7 in a real-time assay yielded a significant fluorescence increase (Figure 4A), whereas no increase was observed with DNA from other fungal species (Figure 4B). Agarose gel electrophoresis analysis and ethidium bromide staining of the Scorpion-PCR products showed a positive correlation between the fluorescence and the amplification of the expected DNA fragment (Figure 5).

In the 10-fold dilutions series of *R. necatrix* DNA, the sensitivity of Scorpion primers was 1 pg μl^{-1} (Figure 6A) and for nested Scorpion-PCR was 1 fg μl^{-1} (Figure 6B). Standard curve showed a linear correlation between input DNA and cycle threshold after both, Scorpion-PCR (Figure 7A) and nested Scorpion-PCR (Figure 7B). The correlation coefficients (r^2) of the standard curve was 0.993 and 0.991 for Scorpion-PCR and nested Scorpion-PCR, respectively.

In conventional PCR the detection limits of primers R2-R8 and R10-R7 were 10 pg μl^{-1} after the first amplification (Figure 8A and B) and 1 fg μl^{-1} after nested-PCR (Figure 8C).

Detection of R. necatrix *in artificially inoculated soils*

The protocol utilized to extract DNA from soil enabled the extraction of total nucleic acid suitable for PCR amplification in approximately 2 h. The presence of *R. necatrix* in soil was not detectable using a single set of primers with both conventional PCR (primers R2-R8) and Scorpion-PCR (primer R10 Scorpion-R7) (data not shown). In nested-PCR, the R10 Scorpion generated distinct fluorescent signals from all inoculated soil samples, whereas no fluorescence increase was observed in non-inoculated ones (Figure 9). Cycle threshold value ranged from 12 to 16 for all infected soils.

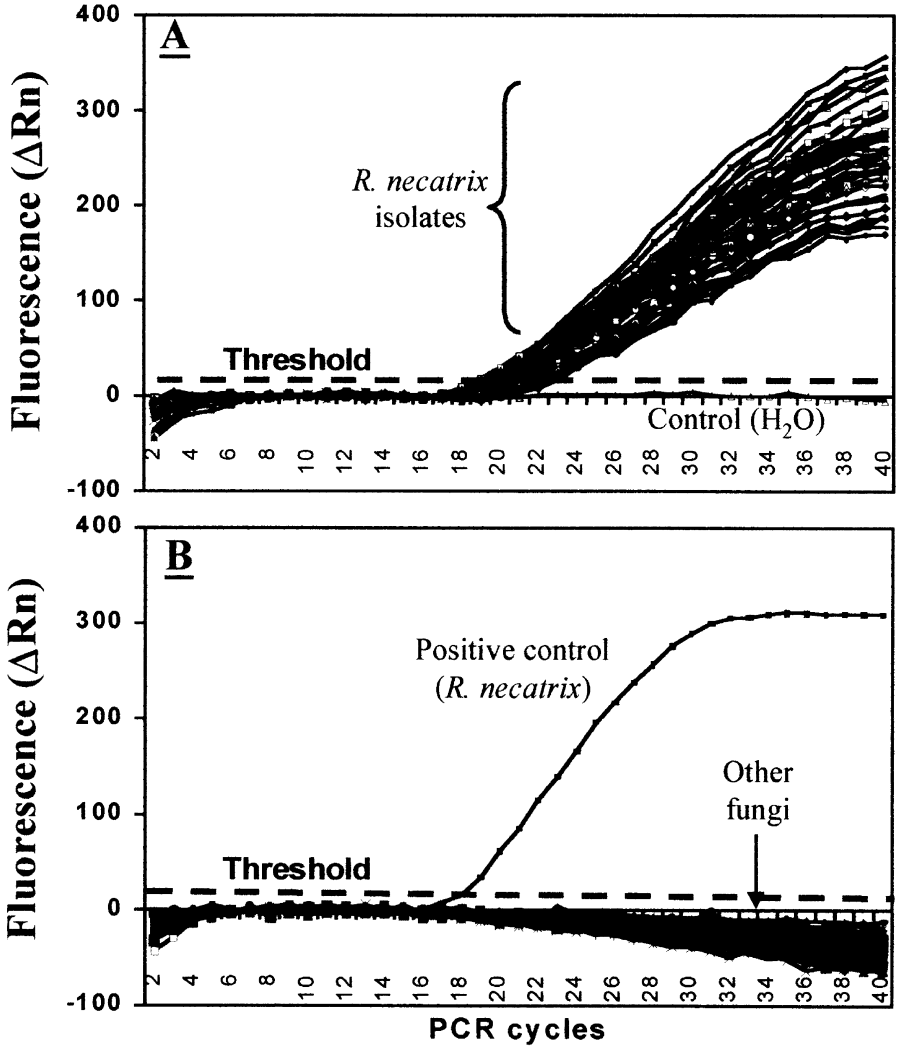

Figure 4. Specific real-time identification of *R. necatrix* isolates by Scorpion-PCR. The relative normalised fluorescence (ΔRn) obtained for *R. necatrix* isolates as compared with the water control is shown in panel A, whereas the ΔRn obtained for other fungi in comparison with one isolate of *R. necatrix* is shown in panel B. The average background fluorescence emission was calculated from cycles 5–17 in both panels.

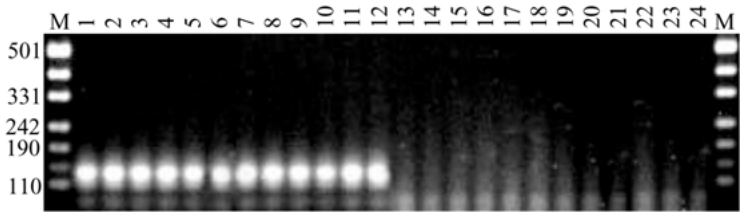

Figure 5. Specific DNA fragment of 112 bp in samples with *R. necatrix* DNA amplified with primer R10 Scorpion-R7 (lanes 1–12). Lanes 13–24 show the absence of any amplified fragment in samples with DNA extracted from other fungi. Lane M, DNA marker (pUC19 DNA/*MSP*I, MBI Fermentas, Germany).

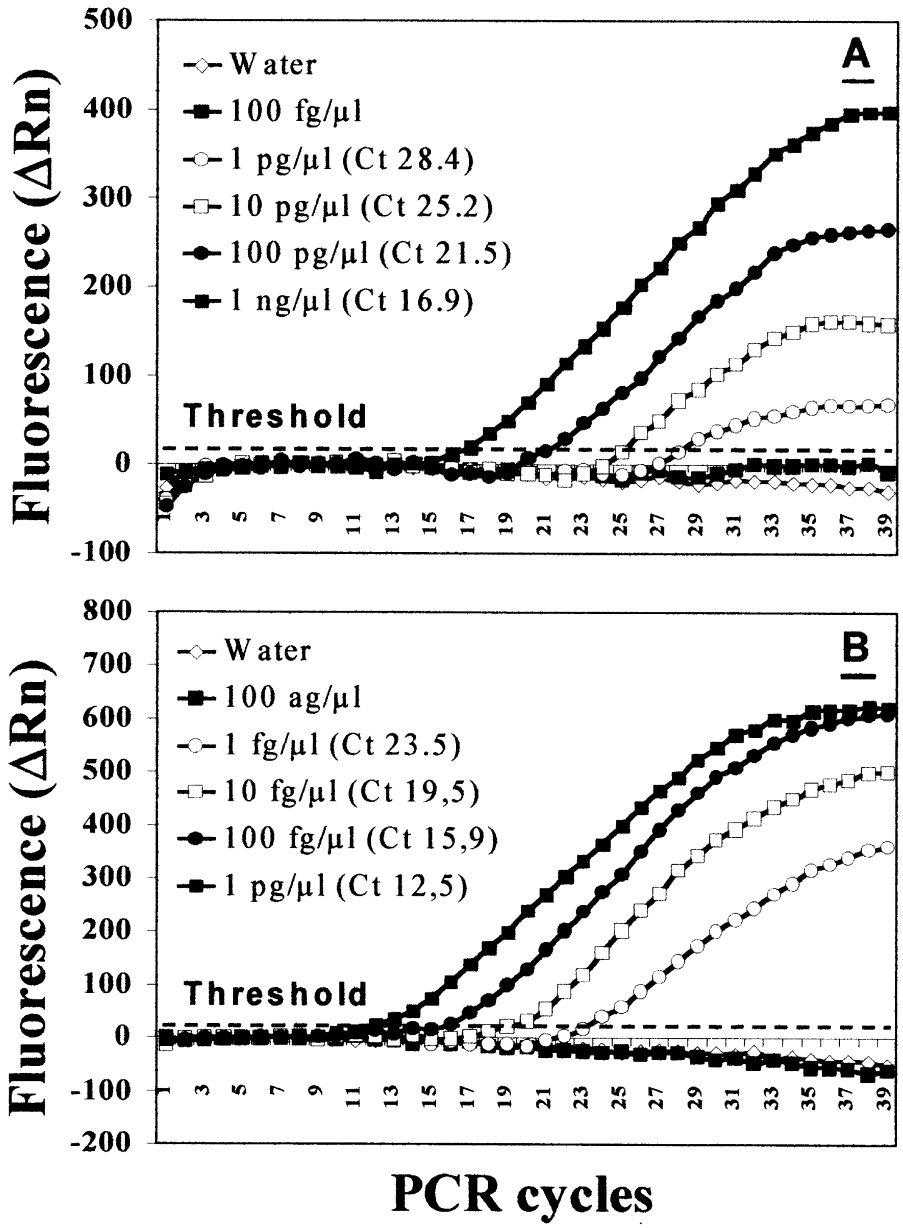

Figure 6. Sensitivity of Scorpion-PCR assessed by using 10-fold serial dilutions of *R. necatrix* DNA. The sensitivity of a single set of Scorpion primers was 1 pg μl^{-1} (A) and for the nested Scorpion-PCR was 1 fg μl^{-1} (B). The average background fluorescence emission was calculated from cycles 3 to 15 in Scorpion-PCR (A) and from cycles 2 to 8 in nested Scorpion-PCR (B).

Discussion

The main objective of this research was to develop a specific PCR test for *R. necatrix* detection. Two primer pairs (R10-R7 and R2-R8) were specific for *R. necatrix*, with amplification from a large number of isolates from different hosts and geographic areas but not from other fungal species, mainly isolated from soil or *Rosellinia* infected tissues. A single set of primers gave a detectable amplification product up to 10 pg μl^{-1}

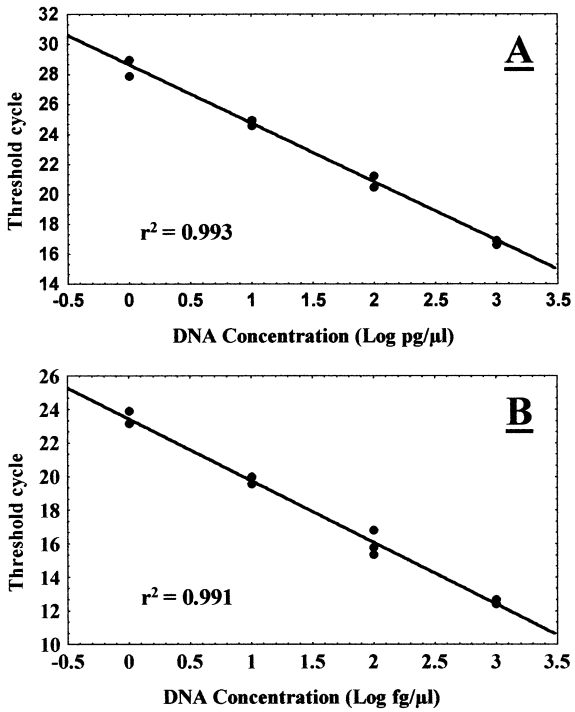

Figure 7. Standard curve and correlation coefficient (r^2) obtained by plotting the cycle threshold (see text) against the input DNA target quantity (logarithm scale) after Scorpion-PCR (A) and nested Scorpion-PCR (B).

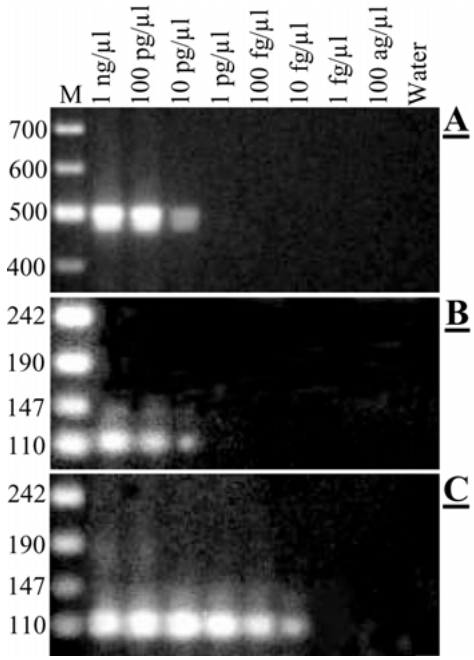

Figure 8. Sensitivity of conventional PCR assessed by using 10-fold serial dilutions of *R. necatrix* DNA. Primer pairs R2-R8 (A) and R10-R7 (B) generated distinct amplified fragments up to 10 pg μl^{-1}. In nested-PCR, using R2-R8 (first amplification) and R10-R7 (second amplification), the detection limits was 10 fg μl^{-1} (C). M = 100 bp DNA Ladder plus (A), pUC19 DNA/*Msp*I marker (B,C) (MBI Fermentas, Germany).

of template DNA in conventional PCR and 1 pg μl^{-1} in the Scorpion-PCR. The sensitivity was improved to 10 fg μl^{-1} (conventional PCR) and 1 fg μl^{-1} (Scorpion-PCR) in the nested-PCR, thus enabling the detection of *R. necatrix* in artificially inoculated soils. On the basis of these results, it appears that these primers could be used to detect *R. necatrix* in naturally infected soils and/or plant tissues.

Scorpion-PCR has been utilised to monitor *Aureobasidium pullulans* strain L47 in the carposphere (Schena et al., 2002) and *Phytophthora nicotianae* and *P. citrophthora* in citrus roots and soil (Ippolito et al., 2000). In the present work, combining a rapid protocol to extract DNA, a preliminary amplification step with conventional primers (R2-R8) and then a second amplification step with Scorpion primers (R10 Scorpion-R7), it was possible to detect *R. necatrix* in artificially inoculated soils in approximately 6 h (2 h DNA extraction, 2 h preliminary amplification and 2 h for second amplification). The use of a labelled primer does not require any post amplification step such as gel electrophoresis and ethidium bromide staining. It significantly reduces the time and labour of the analysis. The fluorescent detection of PCR products in conjunction with a built-in, 96-well format thermal cycler, can greatly increase the throughput of PCR testing and is an important step towards a PCR-based automated diagnostic system (Lockey et al., 1998).

A linear response with a high correlation coefficient was achieved between input DNA and cycle threshold both in Scorpion and nested Scorpion-PCR. These data demonstrate the accuracy of the PCR-based quantification, which may possibly allow to develop a quantitative detection system for this pathogen in soil, as reported for other fluorogenic systems (Bates et al., 2001; Cullen et al., 2001). Furthermore, the use of different fluorophores linked to primers specific for different plant pathogens may facilitate multiplex detection of micro-organisms in a single tube.

In conclusion, the high sensitivity of Scorpion-PCR combined with a fast protocol to extract DNA from soil led to development of a specific, sensitive and

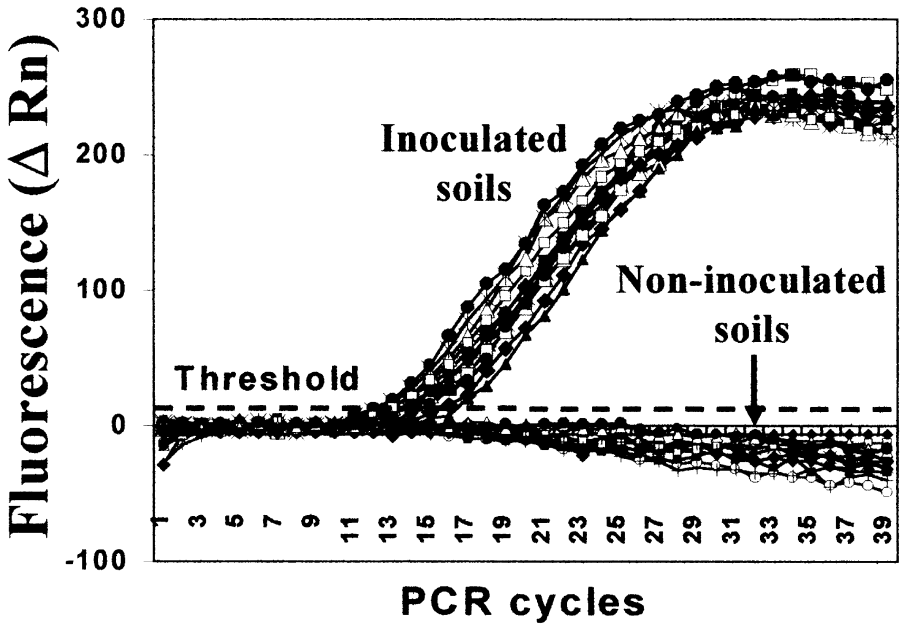

Figure 9. Real-time detection of *R. necatrix* in artificially infected soils. In nested-PCR the R10 Scorpion generated distinct ΔRn increases for all infected soils, whereas no fluorescence increase was achieved for natural soils. The average background fluorescence emission was calculated from cycles 3 to 13.

rapid detection method for *R. necatrix*. The availability of such a method has great practical importance, as checking the soil for the presence of the pathogen before planting is essential to prevent disease developing. Moreover this method could be used for large-scale testing of propagative materials for the production of disease-free plantlets.

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