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ORIGINAL ARTICLE

Development of amplified fragment length polymorphism (AFLP)-derived specific primer for the detection of *Fusarium solani* aetiological agent of peanut brown root rot

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AFLP markers, *Fusarium solani* species complex, PCR-based diagnosis, peanut, root rot.

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Abstract

Aims: The objective of this work was to design an amplified fragment length polymorphism (AFLP)-derived specific primer for the detection of *Fusarium solani* aetiological agent of peanut brown root rot (PBRR) in plant material and soil.

Methods and Results: Specific primers for the detection of the pathogen were designed based on an amplified region using AFLPs. The banding patterns by AFLPs showed that isolates from diseased roots were clearly distinguishable from others members of the *F. solani* species complex. Many bands were specific to *F. solani* PBRR, one of these fragments was selected and sequenced. Sequence obtained was used to develop specific PCR primers for the identification of pathogen in pure culture and in plant material and soil. Primer pair FS1/FS2 amplified a single DNA product of 175 bp. Other fungal isolates occurring in soil, included *F. solani* non-PBRR, were not detected by these specific primers. The assay was effective for the detection of pathogen from diseased root and infected soils.

Conclusions: The designed primers for *F. solani* causing PBRR can be used in a PCR diagnostic protocol to rapidly and reliably detect and identify this pathogen.

Significance and Impact of the Study: These diagnostic PCR primers will aid the detection of *F. solani* causing PBRR in diseased root and natural infected soils. The method developed could be a helpful tool for epidemiological studies and to avoid the spread of this serious disease in new areas.

Introduction

Argentina is a major peanut-producing country. During the 2011/12 season, peanut production exceeded 680 000 tons, with approximately 92% of the crop produced in Córdoba Province (SIIA 2011). Peanuts are used for direct human consumption and as a raw material for the production of animal feed and oil. Most of the peanuts are exported to the European Union and the USA, but some are consumed within Argentina. In Argentina, the two main peanut market types are Runners and Virginias with a smaller area planted to cultivars of Spanish market type (Busso *et al.* 2004).

Diseases caused by soil pathogenic fungi limit peanut production and can result in fields being taken completely out of peanut production (Busso *et al.* 2004). Peanut brown root rot (PBRR) was first observed in Cordoba province in 1992 (March and Marinelli 1998) and is now widespread in Argentine peanut-growing regions. Since 1992, PBRR has been of epidemic proportions in Cordoba Province. The pathogen kills adult plants resulting in large economic losses. In seasons with

long drought stress periods, this disease is the most important of peanut and may reach a 95% disease incidence in some fields (March and Marinelli 2005). The aetiological agent responsible for the disease was first reported as *Fusarium solani* (Mart.) Appel. and Wollenw. Snyd. and Hans., but the pathogen has not been well characterized. The disease has been also reported in Indonesia, Pakistan, Egypt and Australia (Semangun 1993; Saleh 1997; Elsayed Abdalla and Abdel-Fattah 2000; Fuhlbohm *et al.* 2007; Widodo and Budiarti 2009; Ahmed *et al.* 2012; Zaman and Ahmed 2012).

The *F. solani* species complex (FSSC) contains approximately 50 phylogenetic species, many of which could be distinct species (Zhang *et al.* 2006; O'Donnell *et al.* 2008; Nalim *et al.* 2012). A number of biological species also have been described as mating populations within *F. solani* and evaluated in sexual crosses (Matuo and Snyder 1972; Matuo and Snyder 1973; van Etten and Kistler 1988; Hawthorne *et al.* 1994). As for other diseases caused by soilborne pathogens, PBRR may be influenced by tillage practices and crop rotation (Bockus and Shroyer 1998). In a 2-year rotation with soybean and maize, a Paratill subsoiler that was used before peanut seeding in a no-till system improves root growth, reduces water deficits, increases native populations of biocontrol agents and reduces PBRR (Oddino *et al.* 2008). Biological control offers another tool for managing PBRR. *Trichoderma harzianum* ITEM 3636 controlled PBRR in fields naturally or artificially infested with *F. solani* PBRR, decreasing disease severity, increasing the frequency of healthy plants and boosting plant yield (Rojo *et al.* 2007). Vargas Gil *et al.* (2008) found that improvement in soil quality through appropriate cultural practice increases the soil micro-organisms that may take part in biological control of soilborne diseases, resulting in enhanced plant root health. Soilborne contamination is the most important source of inoculum for causing disease. A soil test of *F. solani* inoculum before sowing can help determine the risk of PBRR developing. In the main peanut-growing region in Argentina, soil tests are particularly valuable on rented land, with unknown field history being a requirement to rent a field for peanut cultivation.

Currently, the soil inoculum is detected by fungal isolation and enumeration on selective media, but morphological identification of these fungi on culture media is time-consuming and requires extensive knowledge of classical taxonomy. Molecular biology techniques, especially the polymerase chain reaction (PCR), have provided an alternative approach for the detection and identification of plant pathogens and many soilborne pathogenic fungi (Kageyama *et al.* 2003; Vincelli and Tisserat 2008). Such simple and specific diagnostic assays are important for studying the ecology and epidemiology of plant pathogens. The goal of this work was to design specific PCR primers based on genomic markers [amplified fragment length polymorphism (AFLP)] to develop a diagnostic PCR assay for the detection of *F. solani* PBRR in artificially and naturally contaminated samples of peanut plants and soil.

Materials and methods

Fungal isolates

Fungal isolates used in this study were isolated from peanut plants exhibiting symptoms of peanut brown root rot or supplied from culture collection and are listed in Table 1. Infected peanut plants were collected at fields in five different productions areas of Córdoba Province, Argentina. Roots were cut from the diseased peanut plants, washed under running tap water for 10 min to remove the soil and cut into 1–5-cm long pieces. The pieces were surface-disinfected with NaOCl (1%) for 1 min and then rinsed with distilled water (three times). One hundred root pieces were plated (10 pieces per Petri dish) on a medium containing pentachloronitrobenzene (PCNB) (Leslie and Summerell 2006). The PCNB plates were incubated at 24°C for 7 days with a 12-/12-h photoperiod under cool white and black light fluorescent lamps. Single spores from putative Fusarium colonies were transferred to carnation leaf agar (CLA) and potato dextrose agar (PDA) and identified by using the morphological criteria of Leslie and Summerell (2006). All strains were stored as lyophilized cultures or in 15% glycerol at −80°C in the culture collection at the Department of Microbiology and Immunology, Universidad Nacional de Río Cuarto, Cordoba, Argentina.

Soybean or beans are commonly used as a rotation crops in the field where peanut is planted in Argentina depending on the area. So that we decided to compare AFLP profiles of *F. solani* PBRR isolates with representative isolates previously characterized as *F. solani* f. sp. *phaseoli* (strain S-1008), *F. solani* f. sp. *pisi* (strain S-580), *F. tucumaniae* (NRRL 34549), *F. virguliforme* (NRRL 34552), *Haematonectria haematococca* (FRC S- 1274), which has been described as pathogens of soybean and bean (Table 1). We also compared *F. solani* PBRR isolates with *F. solani* B (greyish-blue phenotype) and J (yellowish-white phenotype) isolates, previously confirmed as peanut pathogenic through fulfilment of Koch's postulates (Oddino *et al.* 2008).

DNA isolation and AFLP

A suspension of spores of each strain (1 ml, approximately 10^6 spores ml^{-1}) from CLA was used to inoculate

Table 1 Strains of *Fusarium* species included in the amplified fragment length polymorphism analysis

Species	Number	Geographical origin	Host/substrate
F. solani PBRR*	18	Argentina, Córdoba, General Deheza	*Arachis hypogaea* L.
F. solani PBRR*	8	Argentina, Córdoba, General Cabrera	*Arachis hypogaea* L.
F. solani PBRR*	21	Argentina, Córdoba, Hernando	*Arachis hypogaea* L.
F. solani PBRR*	19	Argentina, Córdoba, Carnerillo	*Arachis hypogaea* L.
*F. solani PBRR**	11	Argentina, Córdoba, Etruria	*Arachis hypogaea* L.
F. solani (strain B and J)†	2	Argentina, Córdoba	*Arachis hypogaea* L.
F. oxysporum	1	Argentina, Córdoba, Carnerillo	*Arachis hypogaea* L.
F. oxysporum	3	Argentina, Córdoba, Etruria	*Arachis hypogaea* L.
Reference strains			
F. tucumaniae (NRRL 34549)‡	1	Argentina, Buenos Aires, Pérez Millán	*Glycine max* (SDS)
F. virguliforme (NRRL 34552)‡	1	Argentina, Santa Fe, Serodino	*Glycine max* (SDS)
F. solani f. sp. *phaseoli* (S-1008)§	1	USA, PA	*Phaseolus vulgaris* L. (root rot)
F. solani f. sp. *pisi* (S-580)§	1	USA, WA	*Pisum sativum* L.
Haematonectria haematococca (S-1274)§	1	USA, PA	*Exacum* (*Arabian violet*)
Total	88		

*PBRR: *Fusarium solani* causing peanut brown root rot;

†Representative isolates of *F. solani* that showed pathogenicity to peanut plants.

‡NRRL: The Agriculture Research Service Culture Collection, National Center for Agricultural Utilization Research, USDA/ARS, Peoria, IL, USA;

§S: Fusarium Research Center, Pennsylvania State University, University Park, Pennsylvania, USA.

complete medium contained in 250-ml Erlenmeyer flask (CM; Correll *et al.* 1987). The Erlenmeyer flasks were incubated on an orbital shaker (150 rpm) for 3 days at 25 ± 1°C. Mycelia were harvested by filtration and frozen at −20°C. Fungal DNA was extracted using a hexadecyltrimethylammonium bromide (CTAB) procedure (Leslie and Summerell 2006).

The AFLP was carried out as described by Vos *et al.* (1995) as described by Zeller *et al.* (2000) in a PTC-2000 Thermal Cycler (MJ Research Inc. Watertown, MA, USA). All buffers and DNA-modifying enzymes were used following either the manufacturer's instructions or standard protocols (Sambrook *et al.* 1989). Genomic DNA digested to completion with *Eco*RI and *Mse*I was ligated to AFLP adapters in a single overnight reaction at room temperature (21–24°C). After preamplification, AFLP fingerprints were generated with two base-pair extension primer combinations: *Eco*RI+TT/*Mse*I+CA, *Eco*RI+TT/*Mse*I+AC, *Eco*RI+GG/*Mse*I+CA and *Eco*RI+GG/*Mse*I+AC. The *Eco*RI primers used in the final specific PCR amplifications were end-labelled with [γ-^{33}P] ATP (Life Technologies, Rockville, MD, USA).

The presence or absence of polymorphic AFLP bands ranging in size from 200 to 400 bp in each gel was scored manually, and the data recorded in a binary format. All bands in this size range were scored, even those that were unique to a single individual. Bands migrating at the same position were assumed to be homologous and to represent the same allele and locus. Bands of differing mobility were treated as independent loci with two alleles (present or absent). Irresolvable bands and missing data were scored as ambiguous for use in the population genetic analyses. The Dice coefficient (Nei and Li 1979) was used calculate pairwise UPGMA genetic distances among isolates with the CLUSTER procedure on SAS (version 6.11; SAS Institute, Cary, NC, USA). The AFLP data were subjected to bootstrap analysis with 1000 replications with PAUP* version 4.0b10 (Swofford 1999) to determine whether there was significant genetic substructure or clustering among isolates as they were resolved by the AFLPs.

DNA recovery and sequencing

The autoradiograms and blotting paper were aligned and appropriate bands excised. The DNA was eluted from the bands cut out of the 3-MM gel-blotting paper (Midwest Scientific, Valley Park, MO, USA) with 50 μl double-distilled water and incubated for 1 h at 37°C. Two microlitres of the eluate was taken as template for band re-amplification. The composition of the PCR was similar to that used for amplification, but the total volume was 20 μl. The temperature profile was as follows: one cycle of 94°C for 30 s, 65°C for 30 s and 72°C for 30 s, and then, this cycle was followed by a 12-cycle step-down protocol in which the annealing temperature was lowered each cycle by 0·7°C from 65 to 56°C. After that, 23 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 60 s were performed, followed by a final extension step of 72°C for 5 min and then held at 4°C. PCR products (2 μl) were detected in 2% agarose gels in TAE 1x buffer (Tris–acetate 40 mmol l^{-1}, EDTA 1·0 mmol l^{-1}), separated by

electrophoresis and stained with ethidium bromide (5 μg ml^{-1}). The DNA ladder, 100 pb (New England Biolabs, Inc., Ipswich, MA, USA), was used as molecular size marker.

For DNA sequencing, template DNA (18 μl) was directly prepared from PCR products by purifying it with a commercial kit (DNA Wizard DNA Clean-Up kit, Promega, Madison, WI, USA) according to the manufacturer's instructions and sequenced with by Applied Biosystems Sanger Sequencing Chemistry and the ABI 3730 Sequencers.

Primer design, PCR optimization and testing for specificity

Oligonucleotide primers were designed based on the obtained sequences using Primer 3 software (S. Rozen and H. J. Skaletsky, Whitehead Institute for Biomedical Research, Cambridge, MA, USA) and tested for optimal PCR conditions. The PCR containing species-specific primers were set up with 1·25 U of Taq Gold DNA polymerase (Promega) in 20 μl reaction mixtures, containing 0·2 μmol l^{-1} of each outside primer, 0·2 mmol l^{-1} of each deoxynucleoside triphosphate (Promega), 1·75 mmol l^{-1} of $MgCl_2$ and 2 μl (approximately 10 ng) of fungal template DNA. Reactions were performed using the following PCR conditions: denaturation at 94°C for 2 min; 35 cycles of denaturation at 94°C for 30 s; annealing at 55°C for 30 s; extension at 72°C for 30 s; final extension at 72°C for 5 min, followed by cooling at 4°C until recovery of the samples. Amplification products were checked on 1·5% agarose gel stained with ethidium bromide (5 μg ml^{-1}). A negative control, containing all reagents but no DNA, was used in every set of reactions of PCR.

The specificity of the PCR primer pairs was determined individually for (i) genomic DNA extracted from pure cultures of *Fusarium* spp. (including *F. solani* isolated from peanut soil that not caused PBRR in greenhouse assays (*F. solani* non-PBRR), *F. oxysporum*, *F. equiseti*, *F. verticillioides* and *F. semitectum*), *Aspergillus*, *Penicillium* and other species isolated from peanut soil and (ii) genomic DNA extracted from pure cultures of *F. solani* strains belonging to FSSC (Table 1). PCR was tested using different concentrations of *F. solani* DNA (RC386 and J strains) at 100 ng, 10 ng, 1 ng, 100 pg, 10 pg, 1 pg per microlitre in a 20-μl PCR.

Detection of the AFLP-derived specific primer product from plant and soil samples

Nine isolates obtained from peanut plants exhibiting symptoms of peanut brown root rot were purified and inoculated to peanut plants to test whether they caused PBRR in greenhouse. For experiments in greenhouse conditions, plants were grown as previously described (Rojo *et al.* 2007). The inoculum was prepared by culturing each isolate on CM (Correll *et al.* 1987) in 9-cm plastic Petri dishes for 2 week at 25°C in the dark. From these cultures, a disc of 10 mm in diameter was used to inoculate 200 g of sorghum grains that had been autoclaved for 30 min at 121°C in a 1-l Erlenmeyer flask on two consecutive days. The inoculated sorghum was incubated at 25°C in the dark for 2 weeks and shaken daily to disperse the fungus and to ensure more uniform colonization of the grain (Mueller *et al.* 2003). Peanut seeds of a cultivar 'Alto Oleico' (PEPE ASEM INTA) were sown in plastic pots (20 cm of diameter) containing sterile soil (50% soil, 50% sand, v/v) and inoculated with fungus-infested sorghum grains. Three grains of infested sorghum seeds were placed 2 cm below a peanut seed in each plastic post. Noninfested sorghum grains were used as controls. The pots were placed in a greenhouse bench under a photoperiod day/night 13/11 h at 25 $\pm$ 3°C for 70 days. Soil was watered to saturation after planting and maintained at near field capacity throughout the study. In the test, each replicate consisted of 20 pots with two plants each. Un-inoculated plants served as a negative control. Some plants were sampled 15 days after planting. Disease incidence (DI) was scored 70 days after planting.

In addition, five naturally infected peanut plants with visible root rot symptoms were randomly collected from five peanut fields (Córdoba, Argentina). Plant materials without brown root rot symptoms were also collected. Roots of each plant (15 and 70 days after planting, field grown) were removed and washed under running water to remove any adhering soil. After washing, roots were kept in a plastic bag and stored at 4°C until use for plating or kept at −20°C for DNA isolation.

DNA was extracted from peanut root samples using DNeasy Plant Mini kit (Qiagen Inc., Valencia, CA, USA), according to the manufacturer's protocol. DNA concentration was measured using a Quant-It™ Assay Kit™ (Invitrogen Co., Carlsbad, CA, USA).

Peanut root samples obtained from the greenhouse experiments and from fields were cut into 10–15-mm pieces, surface-disinfested with 1% NaOCl solution for 5 min, rinsed three times with sterile distilled water for 3 min and plated on PCNB medium (Leslie and Summerell 2006). Representative isolates from the plate were screened for the specific marker.

To develop the PCR detection assay from soil, the concentration of conidia suspension prepared from a *F. solani* PBRR isolate RC 386 was determined using a haemocytometer. The conidial suspensions was then diluted to 10^6, 10^5, 10^4, 10^3, 10^2 and 10, before adding 1 ml of each suspension to 100 g three autoclaved soil

and incubated at 25°C for 15 and 30 days on the dark. Negative control inoculated with distilled water was included.

In addition, three natural soil samples (sandy loam) were collected from peanut fields with and without history of PBRR. Five grams of soil was sampled each time; 1 g of the sample was used for colony counting and 200 mg was used for DNA extraction. DNA was extracted from soil samples using Mo Bio PowerSoil DNA isolation kit (Mo Bio Laboratories, Carlsbad, CA, USA), used according to the manufacturer's protocol. DNA concentration was measured using a Quant-It™Assay Kit ™ (Invitrogen Detection Technologies).

All DNA samples were kept at −20°C for long-term storage or at 4°C for immediate use.

The optimal cycle parameters were an initial denaturation step at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 57°C (root) and 55°C (soil) for 30 s, extension at 72°C for 30 s and a final primer extension step at 72°C for 5 min. The master mix was prepared as described in 2·4 modified by adding bovine serum albumin (BSA) (Sigma-Aldrich Co., St Louis, MO, USA) at final concentration of 1 mg ml^{-1}. DNA template obtained from soil samples was 10–20 ng, and for peanut roots, 16-fold diluted DNA samples were used as templates. Negative controls that excluded the DNA template or 'DNA' isolated from autoclaved soil were included in every experiment to test reagent contamination.

As a positive control, DNA samples extracted from soil or plant material were amplified with the universal primers ITS1/ITS4 that recognize the internal transcribed spacers (ITS1–ITS2) of fungal nuclear ribosomal DNA (White *et al.* 1990). This control was performed to ensure the quality and integrity of the extracted DNA, to test for the presence of fungal DNA in the soil and to minimize the risk of obtaining a false-negative result. All experiments were repeated for confirmation of results.

Results

AFLP fingerprints

Amplified fragment length polymorphism fingerprinting with all four primer pairs resulted in 213 bands that could be unambiguously scored, of which 171 (80%) were polymorphic if only loci at which both alleles were present at frequencies between 5 and 95% in the entire population were included in the analysis. Dendrograms constructed from each AFLP primer pair were concordant so the data were combined to generate a single dendrogram (Fig. 1). AFLP fingerprinting similarity among the 77 representative *F. solani* PBRR isolates was high (>80% UPGMA similarity). There were 74 unique AFLP fingerprint haplotypes, and the few isolates with the same AFLP fingerprint haplotype were recovered from the same location.

The AFLP similarity between the *F. solani* PBRR isolates and the other strains included in the analysis ranged from 27 to 55%. UPGMA similarity between the 77 *F. solani* PBRR isolates and the *F. solani* f. sp. *pisi*, *F. solani* f. sp. *phaseoli* and *Haematonectria haematococca*, *F. tucumaniae* and *F. virguliforme* isolates averaged 45%, and with the *F. oxysporum* isolates from the diseased plants, it averaged 27%. Pairwise similarity between the *F. solani* PBRR isolates and proven PBRR pathogenic strains B and J was approximately 80%. Bootstrap support for the unity of the *F. solani* PBRR group is 100%, as it was for the *F. oxysporum* group. The group that included *F. solani* f. sp. *pisi*, *F. solani* f. sp. *phaseoli, Haematonectria haematococca, F. tucumaniae* and *F. virguliforme* isolates had 99% bootstrap support. Thus, *F. solani* isolates causing PBRR are clearly different from the other members of the *F. solani* species complex evaluated.

Marker identification

Comparison of AFLP fragment patterns derived from different FSSC revealed several fragments characteristic for this species. Fragments considered to be specific for the pathogen were cut out from the gel and re-amplified with the AFLP primers after elution of DNA, and one fragment was chosen and sequenced. When the DNA sequence of the PCR product unique to *F. solani* PBRR was compared with nonredundant database in GenBank, using the BLAST tool under http://www.ncbi.nlm.nih.gov/BLAST, there was no significant match with any other DNA or protein sequences.

Primer design and PCR optimization

Based on the sequences obtained, one set of specific primers for *F. solani* PBRR named FS1/FS2 had the following sequences: 5′-GCAGGTATGGCTTTTTGGAA-3′ and 5′-AGTAAACTCCGACAGGTGCAA-3′, respectively. This set of *F. solani* PBRR-specific primers produced PCR product of 175 bp with DNA from all *F. solani* PBRR isolates tested. No amplification occurred when the specific primers were tested with others *F. solani* species complex (included *F. solani* non-PBRR isolates) and other fungal isolates occurring on soil, including *Aspergillus*, *Penicillium* and *Fusarium* species (Fig. 2). Sensitivity of the primer set FS1/FS2 was 1 pg from pure template of *F. solani* PBRR total genomic DNA (Fig. 3) and down to 10^3 conidia g^{-1} soil (Fig. 4).

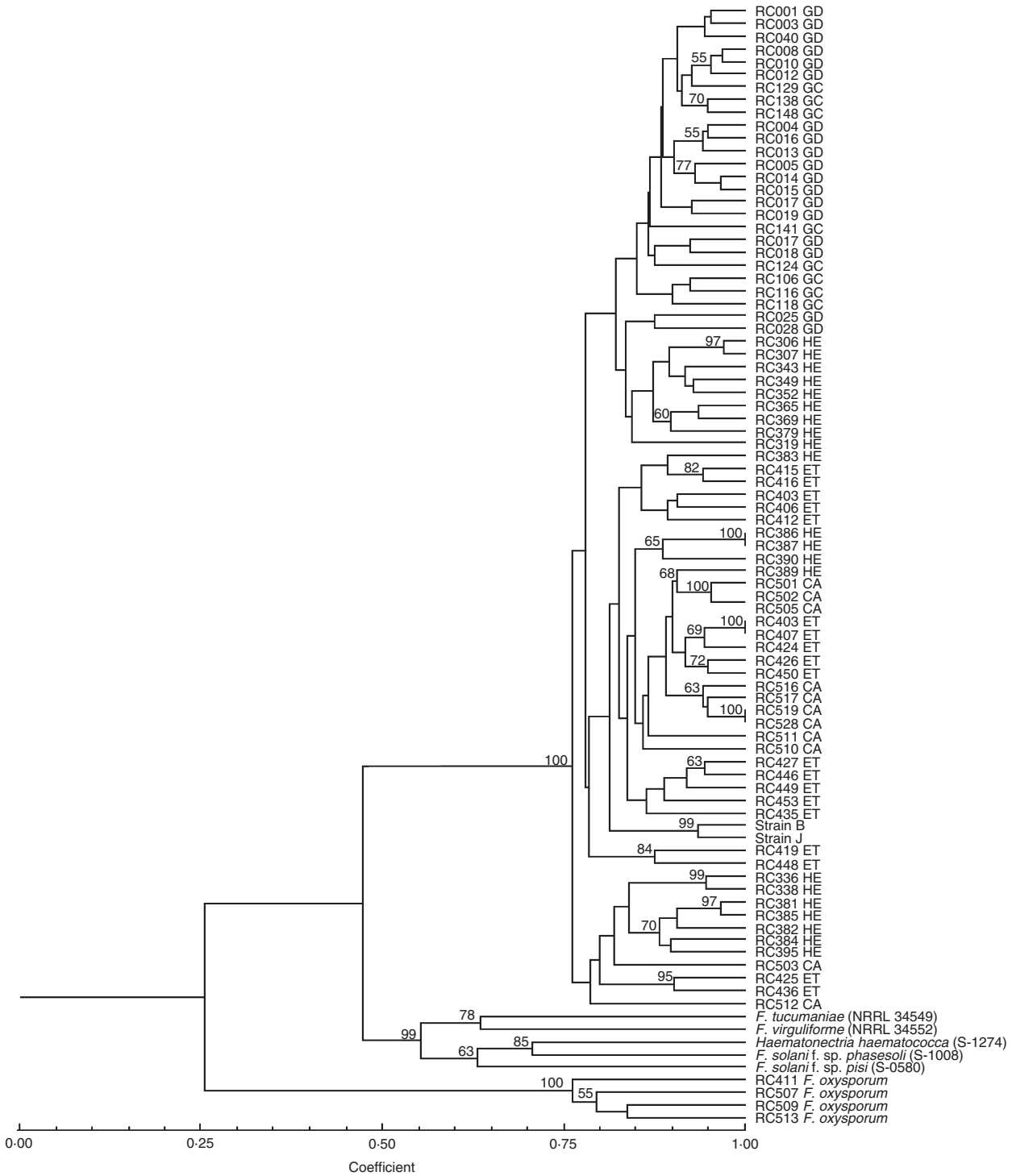

Figure 1 Network of UPGMA similarity among *Fusarium* species based on comparisons of amplified fragment length polymorphism fingerprints generated by amplification with ETT/MAC, ETT/MCA, EGG/MAC and EGG/MCA primer pairs. Support from 1000 bootstrap interactions is indicated for those clusters receiving greater than 50%. (GD: General Deheza; GC: General Cabrera; HE: Hernando; CA: Carnerillo; ET: Etruria).

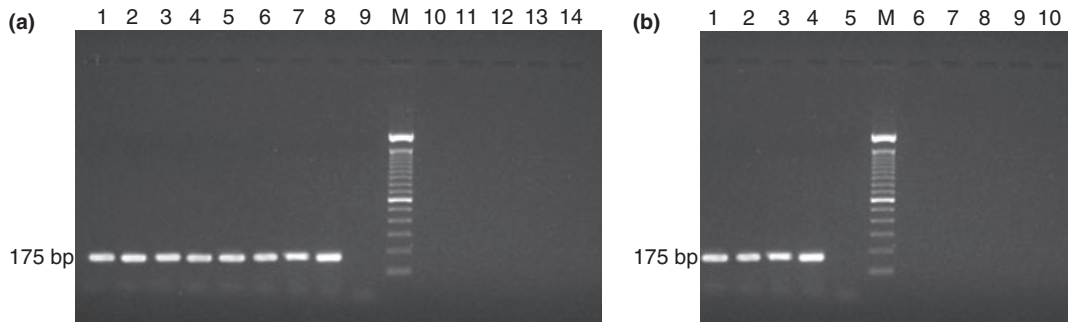

Figure 2 Agarose gel electrophoresis of PCR-amplified products using *Fusarium solani* peanut brown root rot (PBRR) *Fusarium solani* peanut brown root rot (FSPBRR)-specific primers FS1/FS2. (a) Lanes 1–8, FSPBRR isolates; lane 9, negative control; lane M, 100-bp DNA ladder; lanes 10–14, *F. solani* non-PBRR causing isolates and (b) lanes 1–4, FSPBRR isolates; lane 5, negative control; M, 100-bp DNA ladder; lanes 6–10, other fungal isolates from soil.

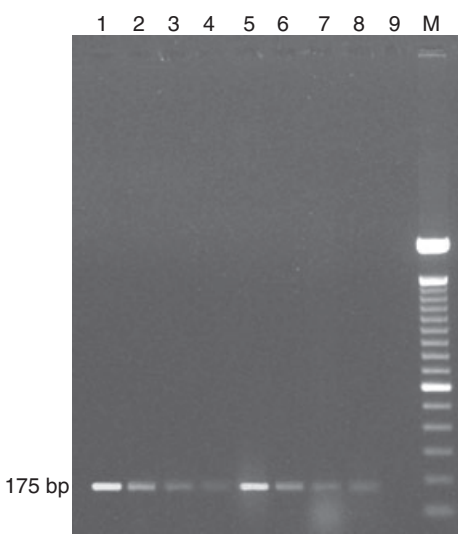

Figure 3 Sensitivity of PCR with primers FS1/FS2 using different concentrations of *Fusarium solani* peanut brown root rot (FSPBRR) DNA. Lanes 1–4, RC 386 strain DNA at concentrations of 1 ng, 100 pg, 10 pg, 1 pg ml^{-1} in a 20-μl PCR reaction; lanes 5–8, J strain DNA at concentrations of 1 ng, 100 pg, 10 pg, 1 pg ml^{-1} in a 20-μl PCR reaction; lane 9, negative control; lane M: 100-bp DNA ladder.

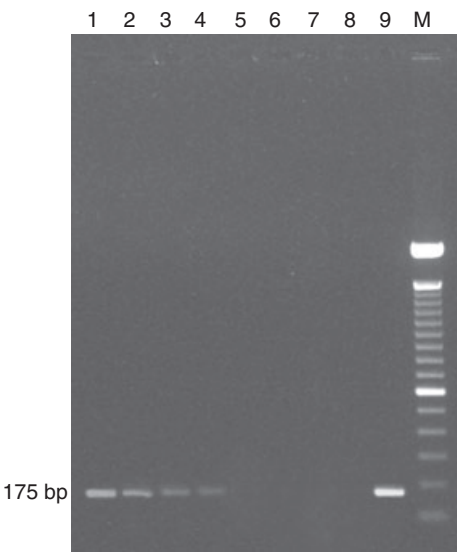

Figure 4 Sensitivity of PCR with *Fusarium solani* peanut brown root rot (FSPBRR) primers FS1/FS2 using soils containing different concentrations of FSPBRR conidia. Lanes 1–6, number of FSPBRR conidia g^{-1} soil was 10^6, 10^5, 10^4, 10^3, 10^2 and 10, respectively; lane 7, negative control (without DNA template); lane 8, negative control (uninoculated soil); lane 9, positive control (J strain DNA); lane M: 100-bp DNA ladder.

Detection of *Fusarium solani* PBRR in peanut roots

To detect DNA of *F. solani* PBRR in peanut roots, we grew peanut plant in pathogen-infested soil in the greenhouse. We observed the root rot symptoms of some peanut plants at 70 days. From diseased roots, the pathogen was isolated in PCNB medium and confirmed using FS1/FS2 primers (data not shown).

PCR assays using the FS1/FS2 primers for the direct detection of *F. solani* PBRR failed in almost all root samples using the protocol describes in 2·4. In plant tissues, various compounds (including polysaccharides and phenolic compounds) can inhibit PCR amplifications (Wilson 1997). So that we used for roots analysis the DNeasy Plant Mini Kit, and BSA was added to the PCR mixture. Under these conditions, good amplification products were not obtained. For overcoming the inhibition, DNA template was diluted. DNA extracted by the commercial kit had to be diluted 16-fold before used as a template. Using bovine serum albumin and a 16-fold-diluted DNA as templates, the pathogen was detected in field- and greenhouse-infected roots. A 175-bp PCR amplification product was obtained using primers FS1

and FS2 on all infected roots. No amplification occurred when the specific primers were tested with peanuts roots from asymptomatic adult plants (Fig. 5). Roots of inoculated soil were positive for PCR amplification 15 days after inoculation; these plants showed characteristic PBRR symptoms when the adult plants were evaluated.

Also, *F. solani* PBRR was detected in all field-grown peanut roots from diseased plants using primers FS1/FS2. No amplification occurred when the specific primers were tested with peanuts roots from asymptomatic adult plants (Fig. 6).

Detection of *Fusarium solani* PBRR in soil

A PCR using the FS1/FS2 primer pair failed to amplify the target DNA region from all template DNAs extracted from artificially contaminated soils using the protocol describes in 2·4. For DNA extraction from soil samples, we used Mo Bio PowerSoil DNA isolation kit, which has a humic substance/brown colour removal procedure that eliminates PCR inhibitors. When the kit was used to isolate fungal DNA from soil, only two of 10 DNA samples were PCR positive using fungal universal primers ITS1/ITS4. However, when bovine serum albumin (final concentration of 1 mg ml^{-1}) was added to the PCR mix, an increase in positive PCR amplification occurred in all soil samples tested. The addition of BSA (1 mg ml^{-1}) in the reaction mixture allowed the specific fragment amplification. *Fusarium solani* PBRR was detected in soil samples from all artificially and naturally infected soil (Figs 7 and 8).

In additions, 20 colonies from planting of field peanut root samples that appeared similar in cultural morphology to purified isolates of cultured of *F. solani* were selected from a PCNB medium (Leslie and Summerell 2006). Results of PCR assay using specific primers FS1/Fs2 were positive for these isolates.

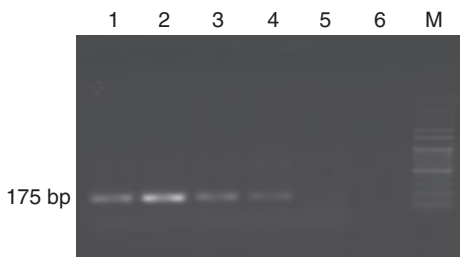

Figure 5 Specific PCR for the detection of *Fusarium solani* peanut brown root rot (FSPBRR) using specific primers FS1/FS2 in peanut roots grown in artificially contaminated soil. The plants were grown in peanut field soil inoculated with RC 319 and 386 strains. Lane 1, DNA from peanut root inoculated with RC 386 strain (diluted 1 : 50); lane 2, DNA from peanut root inoculated with RC 319 strain (diluted 1 : 32); lane 3, DNA from peanut root inoculated with RC 338 strain (diluted 1 : 50); lane 4, DNA from peanut root inoculated with RC 436 strain (diluted 1 : 50); lane 5, DNA from asymptomatic peanut root; lane 6, negative control; lane M, 100-bp DNA ladder.

Discussion

Morphologically, *F. solani* is identified by the shape of its macroconidia, chlamydospores and the presence of long phialides. The most important limitations of morphological identification of *F. solani*, however, lie in the inability to distinguish between the different biological species and the presence of genetically diverse mitotic strains sharing common morphological features. Based on host specificity, *F. solani* has been divided into 10 *formae speciales* (Matuo and Snyder 1973). *Formae speciales* are determined based on pathogenicity on specific hosts. However, pathogenicity test are cumbersome, time-consuming and often inconclusive because they are affected by factors such as the environment and genetic makeup of the host species (Correll 1991). Most of *F. solani* are heterothallic having *H. haematococca* Berk, et Br, as the teleomorph. The teleomorph states are reproductively isolated and each represents a distinct biological species (designated as mating population). Isolates are grouped into these MPs

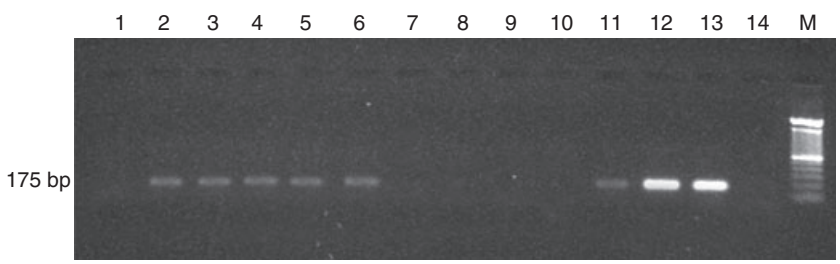

Figure 6 Specific PCR for the detection of *Fusarium solani* peanut brown root rot (FSPBRR) using specific primers FS1/FS2 in field-grown peanut roots. Lane 1, DNA from asymptomatic peanut root; Lanes 2–6, DNA from symptomatic peanut root (diluted 1 : 16); lanes 7–11, DNA from symptomatic peanut root (diluted 1 : 32); lanes 12–13, positive controls (DNA from pure culture of RC 386 and J strains, respectively); lane 14, negative control; lane M, 100-bp DNA ladder.

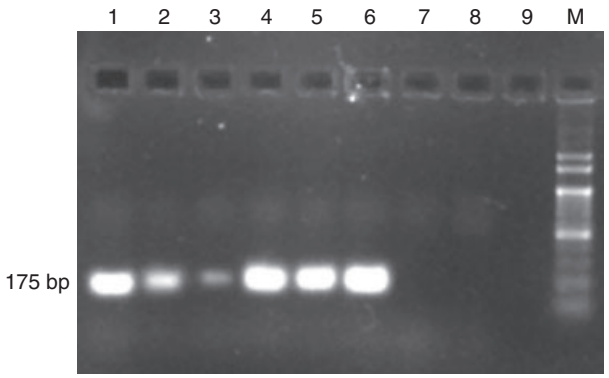

Figure 7 Specific PCR of artificially contaminated soils using *Fusarium solani* peanut brown root rot (FSPBRR)-specific primers FS1/FS2. Lanes 1–3, DNA from soil inoculated with RC 366 strain (undiluted and diluted 1 : 16 and 1 : 50, respectively); lanes 4–6, DNA from soil inoculated with RC 319, RC 338 and RC 313 strains, respectively; lane 7, DNA from soil inoculated with distilled water (negative control); lane 8, negative control; lane M, 100-bp DNA ladder.

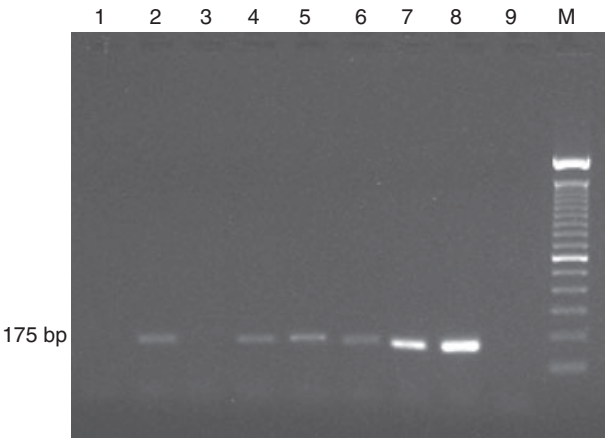

Figure 8 Specific PCR of naturally contaminated soils using *Fusarium solani* peanut brown root rot (FSPBRR)-specific primers FS1/FS2. Lane 1, DNA from soil without history of PBRR (undiluted); lane 2, DNA from soil with history of PBRR (undiluted); lane 3, DNA from soil without history of PBRR (diluted 1 : 2); lane 4, DNA from soil with history of PBRR (diluted 1 : 2); lanes 5–6, positive controls (artificially contaminated soils); lanes 7–8, positive controls (DNA from pure culture of RC 386 and J strains, respectively); lane 9, negative control; lane M, 100-bp DNA ladder.

using mating tests. However, like pathogenicity tests, mating tests are also unwieldy and time-consuming, and only positive mating tests provide guaranteed identification. To avoid the shortcomings associated with classifications based on morphology, pathogenicity and mating tests, various molecular tools have been employed to characterize *F. solani* isolates. Among these methods, analysis of DNA sequences appears to be the most informative (O'Donnell 2000).

Amplified fragment length polymorphism analyses have been used in the taxonomy and phylogeny of FSSC (Bogale *et al.* 2009). AFLPs offer one major advantage over the other DNA-based tools: it samples widely in the genome rather than considering specific regions (Majer *et al.* 1996; Baayen *et al.* 2000).

In this study, we have designed specific primers based on AFLP-PCR products for differentiating the *F. solani* PBRR from others members of the FSSC. The UPGMA analysis based on AFLP data clearly distinguished between *F. solani* PBRR evaluated. We detected many bands specific only to *F. solani* PBRR, some of them could be used to develop the specific PCR marker, but we selected one fragment because it have a suitable length for easy sequence determination.

The data indicate AFLP as a powerful tool for comparing closely related genomes and for exploiting this information to develop a specific PCR with extensive typing potential. This approach was used to distinguish species in the *Gaeumannomyces–Harpophora* species complex (Saleh and Leslie 2004), to develop PCR assays for the detection and identification of *Aspergillus carbonarius* in coffee and grape-derived products (Schmidt *et al.* 2004), for a PCR specific for *A. ochraceus* (Schmidt *et al.* 2003). More recently, the AFLP technique has been used to design specific DNA markers for environmental persistence studies of fungal strains and for Domestic Substances List (DSL) bacterial strains (Hynes *et al.* 2006; Xiang *et al.* 2010).

Although many extraction protocols and commercial kits are now available for extracting PCR amplifiable DNA, some time the type of sample, plant material and soil make difficult to develop convenient and reliable procedures (Steffan *et al.* 1988; Tsai and Olson 1991; Zhou *et al.* 1996). Extraction of DNA often results in co-extraction of humic substances that interfere with DNA detection and might preclude PCR amplification (Zhou *et al.* 1996; Wilson 1997; Braida *et al.* 2003; Schneegurt *et al.* 2003). The addition of BSA to the PCR mixture was used for the first time by Comey *et al.* (1994) as DNA extraction strategies for AFLP analysis and was used by Volossiouk *et al.* (1995) for the direct DNA extraction for PCR-mediated assays of soil organisms. BSA is cheap and effective, which binds a broad range of inhibitory substances, enhancing amplification from complex biological samples such as soil. However, this enhanced amplification efficiency results in reduced stringency of amplification leading to nontarget DNA fragments; in our work, this problem was overcome by adjusting the PCR conditions for root and soil samples. The developed method was evaluated using a range of soil samples, included soil samples of a single soil, which were artificially inoculated at different inoculums levels, as well as naturally infested soil samples from different fields. In all assays, we found that PCR inhibitors were higher in

DNA from naturally contaminated that in inoculated soil. This finding agree with Ma and Michailides's (2007) finding that reported that commercial DNA extraction kits can remove most PCR inhibitors efficaciously, but not all inhibitors in some specific cases.

Using the design primers allowed obtaining a PCR product (175 bp) only from pathogenic *F. solani* isolates and roots exhibiting brown root rot symptom but not for other isolates of *F. solani*, other pathogens or healthy root. Furthermore, some fungal species that were frequently obtained from peanut soil samples did not yield any PCR product.

The availability of such a sensitive and rapid detection method for the pathogen causing PBRR in peanut has great practical importance, due to the need to evaluate the inoculum potential of the pathogen before planting to prevent occurrence of the peanut brown root rot.

The development of this detection tool for *F. solani* causing PBRR in Argentina will aid to determine whether the disease described in different parts of the world, named peanut brown root rot or root rot, is caused by the same pathogen.

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