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A simple PCR-based method for the detection of the chickpea-wilt pathogen *Fusarium oxysporum* f.sp. *ciceris* in artificial and natural soils

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Abstract

A fast and simple polymerase chain reaction method has been developed for the detection of *Fusarium oxysporum* f.sp. *ciceris*, the causal agent of Fusarium wilt of chickpea, in natural and artificial soils. The method involves the disruption of fungal biomass by grinding dry soil, using its abrasive properties, in the presence of skimmed milk powder. The latter prevents loss of DNA by adsorption to soil particles or by degradation and reduces the co-extraction of PCR inhibitors with the DNA. After phenol/chloroform extraction, the DNA is suitable for direct PCR amplification without a precipitation step. For the efficient detection of small amounts of DNA extracted from soil, a two step amplification with nested primers was used. The dilution step reduced the effect of Taq-polymerase inhibitors. The specificity of the amplification, and consequently the yield of specific product, was increased by the use of a modified 'touch down' process during the annealing step. The method has been applied to the specific detection of wilt-inducing isolates of the pathogen in a variety of natural and artificial soils. The amplification was improved by the use of increased concentrations of skimmed milk powder in soils with high organic or clay contents.

Abbreviations: Foc – Fusarium oxysporum f.sp. *ciceris*.

Introduction

Fusarium wilt, caused by *Fusarium oxysporum* Schlechtend.: Fr. f.sp. *ciceris* (Padwick) Matuo and K. Sato (*Foc*), is the most important soilborne disease of chickpea throughout the world and particularly in the Indian Subcontinent, the Mediterranean Basin, and California (Haware, 1990; Jalali and Chand, 1992). The most practical and cost-efficient method for management of Fusarium wilt of chickpea is the use of resistant cultivars (Jalali and Chand, 1992; Nene and Reddy, 1987), the effectiveness of which is limited by the occurrence of different pathotypes and races in *Foc* (Haware and Nene, 1982; Jiménez-Díaz et al., 1989b; Kelly et al., 1994). Isolates of the pathogen fall into two pathotypes according to whether they induce the yellowing or wilt syndromes (Jiménez-Díaz et al., 1989b; Kelly et al., 1994); both syndromes are the result of vascular infections (Jiménez-Díaz et al., 1989a). In addition, the wilting pathotype is much more virulent toward certain chickpea lines (Jiménez-Díaz et al., 1989b; Trapero-Casas and Jiménez-Díaz, 1985), and it causes more severe epidemics of the disease than the yellowing pathotype (Navas-Cortés et al., 1998). Eight races of *Foc* have been identified by their differential interactions with ten chickpea lines, namely races 0, 1B/C, 1A, 2–6. Isolates of races 0 and 1B/C cause the yellowing syndrome (Jiménez-Díaz et al., 1993; Kelly et al., 1994), whereas isolates of races 1A, and 2 through 6, cause the wilting syndrome (Jiménez-Díaz et al., 1989b; Jiménez-Díaz et al., 1993; Kelly et al., 1994). Although characterization of races is important,

the most crucial initial distinction is between wilting and yellowing-associated pathotypes.

The choice of resistant chickpea cultivars for planting could be improved if rapid methods, such as PCR, were available for the characterization of wilt-inducing populations of *Foc* in soils in different areas of chickpea cultivation. The main limitations in the application of PCR for the direct detection of microorganisms in soil, have resulted from losses of DNA by degradation or by adsorption to soil particles during the extraction, as well as due to the presence of PCR inhibitors in the DNA extracts. Volossiouk et al. (1995) demonstrated the ability of skimmed milk powder to prevent DNA degradation and its adsorption to soil particles during the extraction. Indeed, they showed that skimmed milk powder efficiently replaced the more expensive ingredients of Denhardt's solution: bovine serum albumin, Ficoll and polyvinylpyrrolidone. Compounds such as polyphenols, fulvic and humic acids are commonly present in soil (Picard et al., 1992) and have been shown to co-precipitate with DNA (Tsai and Olson, 1991). These compounds are powerful inhibitors of the Taq-polymerase (Tsai and Olson, 1992). Different methods have been used to remove inhibitors; these methods include the use of polyvinylpolypyrrolidone columns (Berthelet et al., 1996) and Sephadex™ G-50 and G-200 columns (Tsai and Olson, 1992).

A method is already available for the detection of wilt-inducing fungal DNA from pure *Foc* isolates and infected plant material (Kelly et al., 1994, 1998). This is a PCR method based on the sequencing of a 1.6 kb DNA fragment generated by RAPDs (random amplified polymorphic DNA) which was used to design specific wilt-associated primers. The method was shown to be reliable after testing 28 *Foc* wilt-inducing isolates, 34 *Foc* yellowing-inducing isolates and 13 other fungal isolates including other forma speciales of *F. oxysporum* and unrelated fungal genera.

The aim of this study was to extend the use of this method to the detection of wilt-inducing isolates in different artificial and natural soils and to develop a simpler and more rapid method than those currently available.

Materials and methods

Fungal strains

Fusarium oxysporum f.sp. *ciceris* race 5 (wilt-inducing) isolate 8012, was used to artificially infest soil samples. Three wilt-inducing isolates: 8012 (race 5), 1987-W6-1 (race 5), 1992R4N (race 4); and two race 0 yellowing-inducing isolates: 7802 and 7982, were used as representative isolates to confirm the specificity of the PCR primers (Kelly et al., 1998). Isolates 7802, 7982 and 8012 were obtained from Fusarium wilt-infected chickpeas in southern Spain, and isolate 1987-W6-1 from California (Kelly et al., 1994). Isolate 1992R4N was kindly provided by M.P. Haware, International Crops Research Institute for the Semi-arid Tropics, Hyderabad, India (Kelly et al., 1994). These isolates have been used in previous studies (Jiménez-Díaz et al., 1989a; Jiménez-Díaz et al., 1989b; Jiménez-Díaz et al., 1993; Kelly et al., 1994, 1998). The fungus was grown and maintained in the conditions previously described (Jiménez-Díaz et al., 1989b; Trapero-Casas and Jiménez-Díaz, 1985). For genomic DNA, mycelia were grown in PDB (potato dextrose broth) lyophilised and ground; DNA was extracted by a method described by Raeder and Broda (1985) as modified by Bainbridge et al. (1990).

Soil samples

Several types of soil with different textures and composition were used. These were: an artificial soil made with silt and peat moss in proportion 2 : 1, and three natural soils (A, B, C) taken from three field plots respectively in which chickpeas were grown in Córdoba province of southern Spain. The natural soils were representative of soils used to grow chickpeas in southern Spain, showing the ranges of clay, silt, sand, organic matter and pH found. Soil A, from microplots at the Alameda del Obispo Research Station near Córdoba, was artificially infested, as described previously, with isolate 8012 in December 1986 to carry out experiments on the epidemiology and control of Fusarium wilt of chickpea (Navas-Cortés et al., 1998). This was an Entisol, Xerofluvents soil (27.0% clay, 9.3% silt, and 63.8% sand), pH 7.4, 1.9% organic matter, and a cation exchange capacity of 12.9 m.e.q./100 g soil. Soil B, from a naturally infested wilt-sick plot near Santaella town, had been used for screening chickpea germplasm for resistance to Fusarium wilt since 1982 (Jiménez-Díaz et al., 1991). This soil was a Vertisol, Chromoxererts (46.1% clay, 19.6% silt and 34.3% sand), pH 7.9, 1.0% organic matter and 21.2 m.e.q./100 g cation exchange capacity. Soil C, from the Agricultural Research Farm of Córdoba University, was artificially infested, as described below, with isolate 8012

in October 1992 to carry out Fusarium wilt resistance screening of chickpea lines. This soil was a Vertisol, Haploxererts soil (50.0% clay, 33.0% silt, 17.0% sand), pH 8.6, 1.4% organic matter and 36.8 m.e.q./100 g cation exchange capacity.

Ten 200 g soil samples were collected from each plot with a cylindrical auger (2.5 × 20 cm) to a depth of 20 cm after removing 3–5 cm of surface soil. Soil samples from each plot were bulked, sieved through a 2 mm-pore-size screen, and thoroughly mixed. They were stored with the residual moisture in open plastic bags at 4–5 °C for up to 1 day and then autoclaved. All soil samples were sterilised by autoclaving for 1 h at 121 °C on three consecutive days, leaving the samples at room temperature between sterilisations, and then artificially infested with race 5 isolate 8012 (wilt-inducing) of *Foc*. To infest the soil samples, the inoculum was first increased by growth for 2 weeks in an autoclaved cornmeal-sand (CMS) mixture (sand, cornmeal, and water; 9 : 1 : 2, v/v) under the same conditions used for the growth of fungal isolates. Infested CMS was thoroughly mixed with a soil sample in proportion 1 : 12, w/w. Aliquots of 550 g per soil sample were prepared. Sterilised samples without the addition of fungus were used as negative controls. The infested soil samples and the negative controls were stored in sterile closed plastic bags at 4–5 °C until used. These levels of infestation were typical of those used to test for resistance or sensitivity of cultivars in laboratory and field trials (Navas-Cortés et al., 1998). Typically 100% of suceptible cultivars showed wilting and plant death when grown in these infested soils. These levels of infestation are routinely used for resistance screening of breeding chickpea germ plasm for national and international breeding programmes.

Extraction of DNA from soil samples

For the extraction of DNA from the fungus present in soil samples, a method based on that described by Volossiouk et al. (1995) was followed with PCR amplification at different stages. Five g of each soil were dried overnight in a desiccator and then ground to a fine powder using a pestle and mortar. Samples of 75 mg were added to 1.5 ml microcentrifuge tubes and thoroughly mixed by vortexing with 200 µl of skimmed milk solution: 0.4–4.8% (w/v) skimmed milk powder (Marvel, Cadbury's, 99% fat free) suspended in sterilised distilled water. Tubes were centrifuged at 12,000*g* for 10 min to remove soil. After this stage, DNA soil extracts were sampled and used for PCR amplification. In a second set of samples the extraction method continued with a deproteinisation step. For that, 6 µl of 10% SDS were added (final concentration of 0.3% w/v) and then an equal volume of phenol : chloroform : isoamylalcohol (25 : 24 : 1); both phases were mixed for 2 min by intermittent vortexing. After centrifugation, the upper phase was used directly for amplification (Figure 1). In a third set of samples, DNA was extracted using the full method. In this case, after treatment with skimmed milk solution and centrifugation, the supernatants were transferred to a fresh tube containing 400 µl of extraction buffer (0.3% SDS, 0.14 M NaCl, 50 mM NaAc pH 5.1). After brief vortexing, samples were treated with phenol : chloroform : isoamylalcohol as described above. DNA present in the aqueous phase was then precipitated with 2.5 volumes of ethanol, and left overnight at −20 °C. The DNA was collected by centrifugation for 30 min at 12,000*g*, washed with 70% ethanol, dried at room temperature and dissolved in 50 µl of distilled water.

After DNA extraction, 5 µl of each sample were analysed by electrophoresis on a 1% agarose gel (Promega) in Tris-acetate EDTA buffer (40 mM Tris-acetate and 1 mM EDTA) as described by Sambrook et al. (1989).

Primer development and synthesis

Specific primers for wilt-inducing isolates of *Foc* were used to amplify DNA extracted from the fungus present in soil samples. To design these primers the sequence of a 1,618 bp fragment, specific for the wilting pathotype, was used. This fragment had been identified by random amplified polymorphic DNA (RAPD) (Kelly et al., 1994, 1998), cloned and sequenced (GENEMBL accession no. AF005633). Two pairs of specific PCR primers were designed from this sequence using the PRIMER routine of GCG (Devereux et al., 1984). Those primers were: WiltNF-1 (5′-ATAGCCAAGCCGACCCTCAC-3′, corresponding to nucleotides 410–429); WiltNR-1 (5′-ACGAGGTTCGTCGTTGTTC-3′, complementary sequence to nucleotides 1239–1258); WiltNF-2 (5′-TTGTATGGCGTTGGAGAGGG-3′, corresponding to nucleotides 448–467) and WiltNR-2 (5-TTGTTCAGATCGGAATCGGG-3′, complementary sequence to nucleotides 1033–1052). WiltNF-2 and WiltNR-2 were internal to the other primer pair. The specific PCR oligonucleotide primers

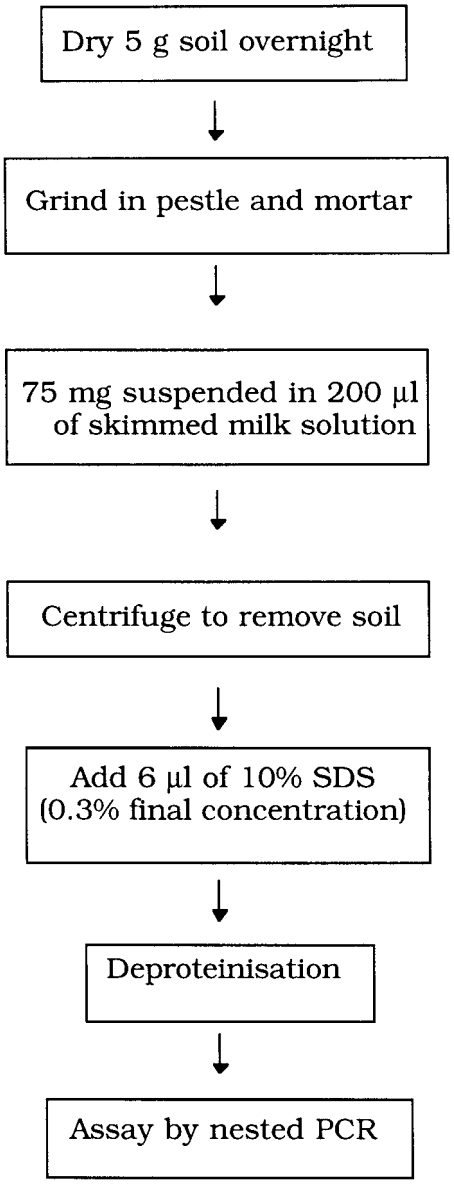

Figure 1. Simplified procedure for extracting fungal DNA from infested soil for detection by PCR.

WiltNF-1/WiltNR-1 and WiltNF-2/WiltNR-2 were synthesised by the Molecular Biology Unit at King's College, London.

Polymerase chain reaction

For the nested PCR amplification (Henson et al., 1993), the first reaction was performed in 50 µl of reaction mixtures containing: several different volumes of soil extract, 200 mM of each deoxynucleotide-triphosphate, 0.1 µm of each primer (WiltNF-1 and WiltNR-1), 1.5 mM $MgCl_2$, reaction buffer (50 mM KCl, 10 mM Tris-HCl pH 9.0 [25 °C], 1% v/v Triton X-100, Promega) and 1.5 Units of Taq DNA polymerase (Promega). Samples were overlaid with 25 µl of filter-sterilised light mineral oil (Sigma). The PCR products were diluted 10-fold and 5 µl were used for a second round of amplification using the same conditions with the internal pair of primers: WiltNF-2 and WiltNR-2. Both rounds of PCR were performed for either 20 or 25 cycles each. Rounds of greater than 30 cycles were found to produce non-specific smears of DNA. For each round of PCR, either a fixed annealing temperature of 65 °C or a modification of the 'touch down' method (Don et al., 1991) were used. In the first case the samples were heated to 94 °C for 5 min to fully denature the DNA and then subjected to 20 or 25 cycles of: 1 min of primer annealing at 65 °C, 2 min extension at 72 °C, 1 min denaturation at 94 °C, followed by a final annealing step of 1 min at 65 °C and a final extension step of 6 min at 72 °C. When the modified 'touch down' approach was used the same conditions were kept, except that the initial annealing temperature of 65 °C was decreased 1 °C every two cycles, until a temperature of 58 °C was reached and used for 11 cycles. Genomic DNA, extracted from mycelium of wilt and yellowing-inducing isolates (from *in vitro* cultures), was used to check the specificity of primers using 30 cycles with annealing temperature of 58 °C, 62 °C or 65 °C. For all amplifications, 20 µl aliquots were analysed by agarose gel electrophoresis as described above, and DNA size was determined by comparison against known standards (1 kb DNA ladder, Promega).

Results and discussion

In all the cases, DNA extracts were subjected to PCR amplification using nested primers as described above. The amplified bands were of the expected size, 605 bp, being the distance between the two internal primers, and this coincided in length with the positive control using DNA extracted from mycelium of race 5 isolate 8012. Soil extracts from sterilised soils without the addition of fungus, prepared in the same way as the samples from infested soils, were used as negative controls in the PCR. In addition no DNA controls were also used. These controls never produced visible bands (data not shown).

In order to find the simplest method that produced positive results, DNA extracts were sampled at several steps during the extraction and used for nested PCR. Figure 2 shows the results of this study. Race 5 isolate 8012 DNA was extracted from the artificial soil. Three concentrations of skimmed milk, 0.4%, 0.8% and 1.6%, were used to check if an increase in the concentration of skimmed milk would improve the results. PCR was performed at a fixed annealing temperature of 65 °C and 20 cycles in each round. More cycles of amplification were found to be unnecessary, extending the time required for detection and producing non-specific smearing of DNA. Poor amplification occurred in the absence of deproteinisation (lanes 3–8) which confirmed results showing that the addition of skimmed milk powder directly to PCR reactions caused inhibition of PCR reactions (M. García-Pedrajas, unpublished results). This is in contrast to reports showing that proteins such as BLOTTO improved PCR (De Boer et al., 1995). Uniformly better results were obtained when samples were used for amplification after deproteinisation prior to a precipitation step (lanes 9–14), or with DNA extracted using the full method (lanes 15–20). This showed that a precipitation step was not required to successfully extract and amplify DNA from soil samples. The omission of this step makes the method considerably shorter and less susceptible to contamination, as fewer steps were required to produce DNA of high enough quality for PCR.

An increase in the concentration of skimmed milk clearly improved the brightness of the amplified bands, the best results being obtained at the highest concentration, 1.6%. With artificial soil, the optimum amplification was obtained with 1.6% skimmed milk as a concentration of 1.2% gave the same level of amplification (García-Pedrajas, results not shown). In the case of DNA sampled after treatment with skimmed milk and centrifugation, but without deproteinisation (lanes 3–8), no clear visible bands were obtained below this concentration (1.6%). In order to check if the better results with higher concentrations of skimmed milk were due, at least partially, to a higher yield of DNA,

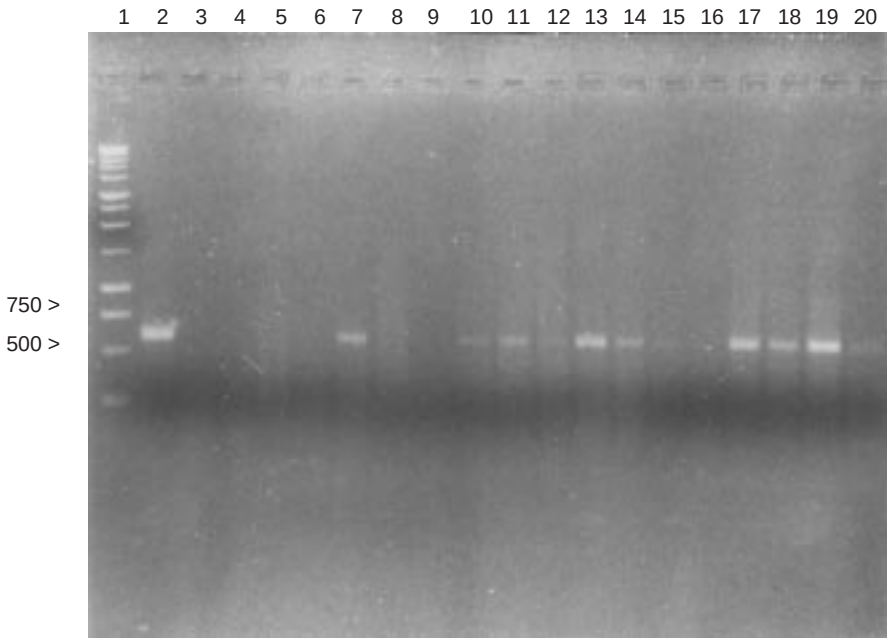

Figure 2. Detection of DNA from *Fusarium oxysporum* f.sp. *ciceris* in artificial soil by PCR using specific wilt-associated nested primers. DNA extracts were used for amplification at different stages during the extraction protocol: lanes 3–8, after treatment with skimmed milk solution and centrifugation; lanes 9–14, after deproteinisation; lanes 15–20, after DNA ethanol precipitation. Three different concentrations of skimmed milk solution were used with 1 µl of DNA extract for the amplification, either without dilution or after 10-fold dilution. Dilution occurred before amplification. Lanes: (3,9,15) 0.4% skimmed milk, no dilution; (4,10,16) 0.4% skimmed milk, 10-fold dilution; (5,11,17) 0.8% skimmed milk, no dilution; (6,12,18) 0.8% skimmed milk, 10- fold dilution; (7,13,19) 1.6% skimmed milk, no dilution; (8,14,20) 1.6% skimmed milk, 10-fold dilution. Lane (1) 1 kb ladder (Promega); Lane (2) positive control.

5 µl of each sample were loaded on a 0.7% agarose gel, but the amounts of DNA were in all cases too small to be visible on a gel (data not shown). As shown in Figure 2, the amplification was always better using 1 µl of soil extract without dilution before amplification, than when the soil DNA extract was diluted 10-fold before addition to the PCR mix, in order to dilute any possible PCR inhibitor present in the samples. Thus, any advantage achieved by such dilution was presumably lost by reducing the target DNA below a threshold level needed for amplification. This is in contrast to observations by Volossiouk et al. (1995) who found that 50-fold dilution improved amplification presumably due partly to higher levels of fungal infestation.

One of the main barriers to successful amplification of DNA extracted from soil samples is the presence of humic acids and related compounds produced by the degradation of organic matter. These compounds are known to be powerful inhibitors of the Taq polymerase (Tsai and Olson, 1992). The results presented here strongly suggest that skimmed milk prevents the co-purification of inhibitors with DNA. When DNA was extracted with the same procedure, but without the addition of skimmed milk powder, soil DNA extracts were a dark brown colour, presumably due to the presence of humic compounds. But when skimmed milk was added, samples were colourless or a faint brown colour. Figure 3 shows the results of amplification of DNA extracted from artificial soil using different concentrations of skimmed milk: 2.4% and 3.2%. At 2.4% the best results were obtained using 1 µl of soil extract (lane 1), with 2 µl producing a faint band and 4 µl showing no amplification. This result showed a clear inhibition effect; thus, when the volume of soil extract was increased the presence of inhibitors was also increased, which resulted in less amplification even though a higher amount of DNA template was used. On the other hand, when a 3.2% skimmed milk suspension was used, the inhibition effect was appreciably less; 2 µl (lane 5) produced a robust band, clearly brighter than when using 1 µl, but some inhibition effect still remained, as shown by the fact that 4 µl of soil extract produced a fainter band than that using 2 µl. This result suggests that skimmed milk powder effectively reduces inhibitors in soil extracts. Better amplification was obtained with samples of soil A, a light coloured soil with low content of organic matter from artificially-infested microplots, than with the artificial soil, a dark soil with a much higher content of organic matter (Figure 4). Dark soils with a high concentration of organic matter might therefore need a higher concentration of skimmed milk during extraction, to reduce the concentration of humic compounds in the DNA extracts.

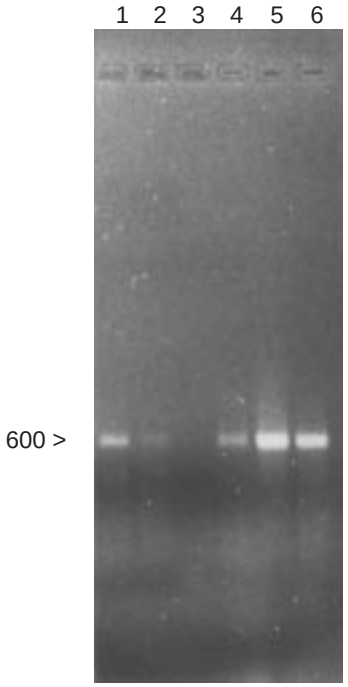

Figure 3. Detection of DNA from *Fusarium oxysporum* f.sp. *ciceris* in artificial soil using specific wilt-associated, nested primers. DNA extracts were used for amplification after deproteinisation. Two concentrations of skimmed milk and three volumes of DNA extract were used. Lanes: (1–3) 2.4% skimmed milk; (4–6) 3.2% skimmed milk. (1,4) 1 µl DNA extract; (2,5) 2 µl soil extract; (3,6) 4 µl soil extract.

Another factor which affected detection was the efficiency of DNA extraction from the soil, which could also explain the difference in results for the different types of soil shown in Figure 4. The fungal walls were disrupted by grinding dry soil, which would be much more effective in sandy soils, as sand is naturally abrasive, leading to a better yield of DNA and consequently to more PCR product. That could explain why the poorest results were obtained with soil C, which contained a high proportion of clay and the lowest proportion of sand of the three soil samples used in the study. This problem could be overcome by the addition of acid-washed sand to clay soils before grinding. Secondly, skimmed milk has been described as an agent for blocking charged sites on soil particles and

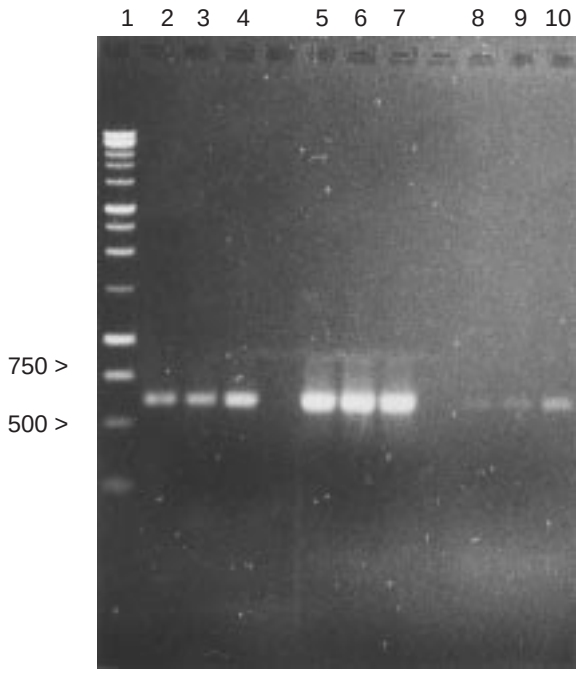

Figure 4. Detection of DNA from *Fusarium oxysporum* f.sp. *ciceris* in one artificial and two natural soils using specific wilt-associated, nested primers. DNA extracts were used for PCR after deproteinisation. Three concentrations of skimmed milk were used. Lanes: (1) 1 kb Ladder (Promega); (2–4) artificial soil; (5–7) natural soil A; (8–10) natural soil C; (2,5,8) 3.2% skimmed milk; (3,6,9) 4.0% skimmed milk; (4,7,10) 4.8% skimmed milk.

preventing the adsorption of DNA. Milk proteins are also used as blocking agents in Southern hybridisation (Sambrook et al., 1989). The adsorption of DNA to soil particles will occur to a higher extent in clay, with small charged particles (O'Donnell and Hopkins, 1993); thus a higher concentration of skimmed milk might be needed to 'block' a clay soil than a sandy soil. This effect could explain why an increase in the concentration of skimmed milk from 3.2% to 4.8% in soil C (high clay, low sand) produced better results in the yield of PCR product, while with soil A (low clay, high sand), at the concentration of 3.2%, a plateau had already been reached.

For a given extraction method, PCR conditions can greatly affect the results. Nested PCR was essential to produce clear visible bands and no DNA was detectable after the first amplification cycle (data not shown). The first reaction was followed by a dilution step and a second amplification reaction using an internal pair of primers. The use of a different, more internal, pair of primers in the second round of PCR increased the efficiency, as fragments with damaged ends, synthesised in the first amplification, can also work as templates (Henson et al., 1993). The use of nested primers allowed the amplification of visible PCR products when the amounts of DNA template were small. This was especially important when DNA samples contained inhibitors. An increase in the volume of soil extract, in order to increase the amount of DNA template, frequently produced the reverse result, as the presence of inhibitors was increased as well. With nested PCR, small volumes of DNA extract can be employed as small amounts of template produce visible products. The first reaction need only result in a few rounds of DNA amplification because this is followed by dilution of the inhibitors and more efficient amplification in the second reaction. When 25 cycles per round, instead of 20 and a modified 'touch down' approach were used (Figure 5), brighter bands were obtained with all type of soils and the amplification was already robust in all the cases using skimmed milk at 3.2%. This improvement was not obtained with an increase in the number of cycles without the use of 'touch down' (data not shown). 'Touch down' has been described as a method to increase the amount of specific product (Don et al., 1991) but in this case the gradual reduction of annealing temperature, after the initial stringent conditions, presumably increases the efficiency of amplification even in the presence of some inhibition. High annealing temperatures in the first cycles favour the synthesis of specific products, and a decrease during the successive cycles favours the annealing of primers to this specific product which increases the yield of DNA. This would explain the better result obtained with this approach in comparison with that using a fixed annealing temperature of 65 °C. Amplification could not be improved by using a lower fixed annealing temperatures as smears of non-specific, high molecular weight products were obtained (data not shown).

All the steps during the extraction have been simplified in order to produce a rapid and simple method. Thus liquid nitrogen, ion exchange columns and expensive chemicals were all found to be unnecessary. This simplified method (Figure 1) worked successfully in soils of very different composition in which the presence of the fungus was well established and severe Fusarium wilt of chickpea had occurred. Thus, this method would be of use to determine the occurrence of the wilting pathotype of *Foc* in a natural soil. This

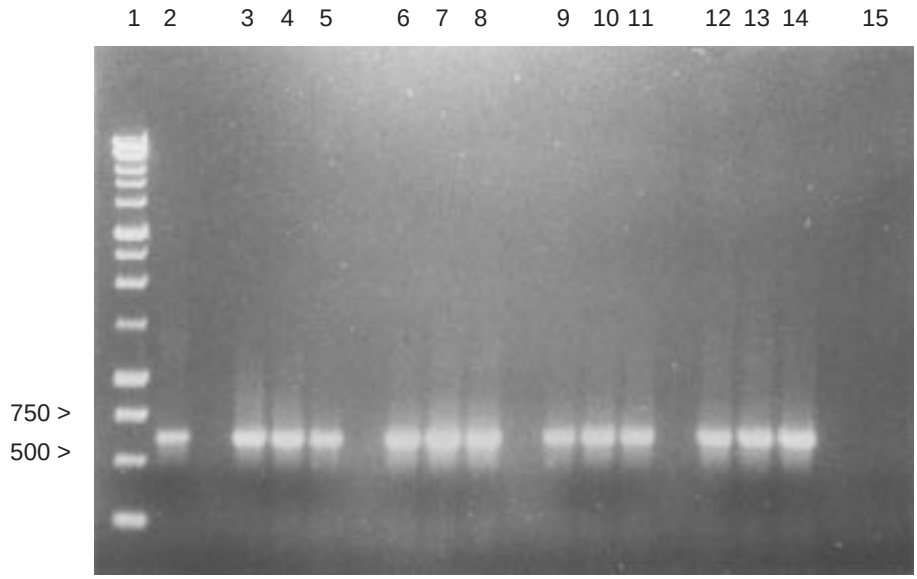

Figure 5. Detection of DNA from *Fusarium oxysporum* f.sp. *ciceris* DNA by PCR in one artificial and three natural soils using specific, wilt-associated, nested primers. The PCR was made using a modified 'touch down' for the annealing step. Three concentrations of skimmed milk were used. Lanes: (1) 1 kb ladder (Promega); (2) positive control; (3–5) artificial soil; (6–8) natural soil A; (9–11) natural soil C; (12–14) natural soil B; (3,6,9,12) 3.2% skimmed milk; (4,7,10,13) 4.0% skimmed milk; (5,8,11,14) 4.8% skimmed milk.

would help to decide whether to avoid sowing chickpea in such infested soil, when only highly susceptible cultivars are available; or making a choice of the appropriate cultivar if moderate resistance is available (Navas-Cortés et al., 1998).

The basic strategy for using this method is therefore to assess the type of soil to be tested. Soils which contain a low level of sand will result in inefficient disruption of the hyphae, and therefore up to 30% wt/wt of acid-washed sand could be added. Soils with high clay content will require up to 4.8% skimmed milk and those with high organic content will also need higher levels. The simplified method shown in Figure 1 should then be used. This is a model system and it should be possible to adapt this for use with any set of nested primers for a range of different DNA targets such as ribosomal RNA genes or specific genes. For the analysis of races of *Foc*, race-specific primers will be required and these are currently being developed using race-associated bands obtained for RAPD analyses (M. García-Pedrajas and R. Jiménez-Díaz, unpublished data). More work is needed in order to fully exploit the potential of this method for the management of Fusarium wilt of chickpea, including the quantitation of fungal DNA and the establishment of inoculum density thresholds for positive detection of the pathogen in soil. Research is currently in progress aimed at these objectives.

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