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QUICK DETECTION OF *ASCOCHYTA LENTIS* FROM LENTIL SEEDS USING POLYMERASE CHAIN REACTION (PCR) BASED TECHNIQUES

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Abstract

Two Polymerase Chain Reaction (PCR) specific primers, ASCO1 and ASCO2 were developed for the detection of *Ascochyta lentis* from infected lentil seeds. PCR amplification with primers ASCO1 and ASCO2 resulted in amplification of an approximately 400bp product with *A. lentis* and *A. rabiei* isolates but not with *A. fabae* and other lentil pathogens viz., *Sclerotinia sclerotiorum, Stemphylium botryosum* and *Colletotrichum lindemuthianum*. The specific primers were amplified with the whole DNA of the *A. lentis* infected seeds but not with healthy seeds suggesting that the specific primers will provide a valuable tool for accurate and quick detection of *A. lentis* in lentil seeds.

Introduction

Ascochyta lentis is an important seedborne pathogen of lentil (Morrall & Sheppard, 1981; Khan *et al.*, 1983; Kaiser & Hannan, 1986; Ahmed & Beniwal, 1988; Kaiser, 1992; Nasir & Bretag, 1997). Infected seeds not only provide the fungus with an important survival mechanism but are also the main source for the spread of the fungus into *Ascochyta* free areas (Morrall & Sheppard, 1981). *A. lentis* survived for one and half year in infected pod and seeds on the soil surface but lost viability in 29 weeks when the debris was buried in soil (Kaiser & Hannan, 1986). Seeds infected with *A. lentis* lose viability quicker than healthy seeds. *A. lentis* survived in infected lentil seeds stored for 4 years at temperature 20, 5,-18 and -160 to -196°C (Kaiser *et al.*, 1989).

Seed health testing through traditional plating methods and in glasshouse by growing seeds in pots is laborious and time consuming. Recently, the PCR has proved to be a powerful tool in the diagnosis of plant pathogens. The sensitivity, speed and versatility of PCR are primary factors in its wide acceptance in plant pathology as well as many field of biology (Annamalai *et al.*, 1995). Similarly a new approach has been introduced in PCR for fungal diseases diagnosis based on the amplification of fungal rDNA using ITS primers (Lee & Taylor, 1992; Brown *et al.*, 1993). There are reports where ribosomal genes and the spacers between them provide attractive targets for molecular detection and phylogenetic studies because they occur in high copy numbers, posses conserved as well as variable sequences and can be amplified and sequenced with universal primers based on their conserved sequences (Barry *et al.*, 1991; Bruns *et al.*, 1991; Lee *et al.*, 1992a; Stackebrandt *et al.*, 1992; Ward *et al.*, 1993).

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The objective of this research was to develop a PCR primer for specific amplification of *A. lentis* and to develop a quick assay for the detection of *A. lentis* from infected seeds of lentil.

Materials and Methods

Sources of isolates: *A. lentis* and *A. rabiei* were isolated from naturally infected lentil and chickpea seeds respectively, collected from the experimental plot at National Agriculture Research Center, Islamabad, Pakistan during 1996-97 crop seasons. Lentil and chickpea extract media were used for the growth of the fungus in culture. Single spore culture of each isolate was obtained on lentil and chickpea extract media (Nasir & Bretag, 1997; Alam *et al.*, 1987). Some other fungal pathogens were obtained from the culture collection of the Ministry of Agriculture Forestry and Fishery (MAFF), Japan (Table 1).

Extraction of DNA: Each isolate was grown on V-8 Juice medium (200ml V-8 juice, Campbel Soup Co., 3g $CaCO_3$ per liter of distilled water) at 22°C for one week. Mycelial plugs (3mm) were prepared from mycelial colonies. Liquid complete medium containing 100ml Ca $(No_3)_2.4H_2O$ (10g/100ml), KH_2PO_4 (2g), $MgSO_4.7H_2O$ (2.5g), NaCl (1.5g/100ml), yeast extract (1g), casein hydrolysate acid (0.5g), casein hydrolysate enzymatic (0.5g) in 1 liter were autoclaved in 500ml Erlenmeyer flask. The media was inoculated with 2-3 fungal plugs and incubated on a rotary shaker (150-200rpm) for 6 days at 25°C. Mycelium was harvested by filtration on Whatman #4 filter paper and frozen at -85°C for 4 hrs. The lyophilized mycelia were grounded to a fine powder in liquid nitrogen using pestle and mortar. About 400mg of mycelial powder were shaken smoothly to completely dissolve in 800μl of lysis buffer (50mM Tris/HCl, pH 7.5, 50mM EDTA, pH 8.0, 3% SDS, 1% mercaptoethanol) then incubated at 65°C for 30 minutes and centrifuged @ 12000 rpm for 10 min., at 4°C. The supernatant was extracted twice with phenol: chloroform: isoamylalcohol (25:24:1) and one time with chloroform: isoamylalcohol (24:1) and then centrifuged @ 14000 rpm for 15 min., at 4°C. T E buffer (10mM Tris Hcl pH 8.0, 1mM EDTA pH 8.0) was added to the supernatant up to 500μl volume, then mixed with 50μl of 3M NaOAc and 900μl of absolute ethanol (99.5%) followed by invert mixing several times for precipitation of DNA. It was left on ice for 20-30 min., and then centrifuged @ 12000 rpm for 10 min., at 4°C. The pellets were washed with 70% ethanol. The eppendorf tubes were drained on tissue paper and allowed to air dry for 10-15 min.

Purification of DNA: The DNA was cleaned up by PEG precipitation. One microlitre of RNase was added to remove RNA and incubated at 37°C for 1hr. DNA was extracted using phenol, chloroform and ethanol precipitation as described above. TE was added up to 500μl volume then mixed with 50μl of NaOAc and 900μl of absolute ethanol, invert mixing several times and kept on ice for 20-30 min. Pellets were washed with 70% ethanol and after drying 160μl of sterile distilled water was added to dissolve the pellets again. 110μl of PEG 8000/2.5M NaCl was added to the solution, mixed well and incubated on ice for 20-30 min., for precipitation of DNA then centrifuged @ 12000 rpm for 10 min., drained and washed with 70% ethanol. The dried pellets were resuspended in TE buffer.

Table 1. Isolates of *Ascochyta lentis* and other fungi used to screen the polymerase chain reaction primers (ASCO1,2) for amplification specific to *A. lentis*.

Isolates	Species	Host	Product with specific primer
I-A	*Ascochyta lentis*	Lentil	+
I-2	*A. lentis*	Lentil	+
K	*A. lentis*	Lentil	+
B-2	*A. lentis*	Lentil	+
PK-10	*A. lentis*	Lentil	+
Dhudial	*A. lentis*	Lentil	+
C	*A. lentis*	Lentil	+
H (NIAB)	*A. lentis*	Lentil	+
AR-1	*Ascochyta rabiei*	Chickpea	+
AR-2	*A. rabiei*	Chickpea	+
305020	*Ascochyta fabae*	Fababean	–
305943	*Sclerotinia sclerotiorum*	**	–
305980	*Phoma destructiva*	Tomato	+
MMG6	*Fusarium solani*	Many crops	–
305241	*Rhizoctonia solani*	Many crops	–
305097	*Curvularia trifolili*	Trifolium	–
305541	*Alternaria brassicae*	Brassica	–
305562	*Stemphylium botryosum*	**	–
305426	*Glomerella tucumanensis*	Sugar cane	–
305441	*Cladosporium phlei*	Timothy	–
BZ1013	*Cochliobolus carbonum*	Maize	–
305161	*Mycosphaerella melonis*	Melon	+
306123	*Phomopsis longicolla*	Soybean	–
306289	*P. obscurans*	Strawbery	–
306283	*Colletotrichum acutatum*	Strawbery	–
CdG-1	*C. dematium*	Gazania	–
Glo3	*C. gloeosporioides*	Strawbery	–
306157	*C. lindemuthianum*	**	–
305383	*C. trifolii*	Alfalfa	–

**Infect many plant species including lentil.

Polymerase chain reaction: PCR was conducted in 20μl reaction volumes. Each reaction consisted of 1μl of template DNA, 2μl 10xPCR buffer (TaKaRa), 1.6μl of 2.5mM dNTPS (TaKaRa), 0.8μl each of 10nM internal transcribed spacer (ITS)1 and ITS 4 primers, 14.5μl of sterile distilled water and 0.1μl Taq Polymerase (5U/μl, TaKaRa). All reactions were performed in small eppendorf tube in thermal Cycler (TaKaRa). Thermal cycling parameters were initial denaturation at 95°C for 2 min., followed by 5

cycles consisting of denaturation at 95°C for 1 min., annealing at 50°C for 1 min., and extension at 72°C for 1 min., followed by 30 cycles consisting of denaturation at 95°C for 1 min., annealing time at 55°C 1 min., and extension at 72°C for 1 min. A final extension at 72°C for 10 min., followed. PCR products were analyzed by gel electrophoresis on 1% agarose in 1xTAE (40mM Tris-acetate pH 8.0, 1mM EDTA). Gels were stained with ethidium bromide (10μg/ml) and photographed under UV illumination with Polaroid type 667 film.

PCR-RFLP by restriction enzymes digestion: Aliquots 3μl of PCR products of *A. lentis* were digested with 5U of restriction endonuclease (TaKaRa) in 15μl reaction volume with manufacturer's recommended buffer and temperature. Enzymes viz., *AluI, HaeIII, HhaI, MboI* and *TaqI* were used. Reaction mixture was incubated at 37°C for 4 to 5 hrs. Digested DNA was analyzed by gel electrophoresis on 2% refine Agarose (Nu-Sieve 3:1 agarose, FMC Bio Products) in 1xTAE buffer and gel was stained and photographed as described earlier.

RAPD, ISSR and CPS1 & 5 analysis: Six isolates of *A. lentis* were selected as weak and highly virulent isolates (3 from each) on the basis of pathogenic reaction on 7 different genotypes of lentil (Hussain *et al.*, 1999). Three RAPD (Randomly Amplified Polymorphic DNA) primers of 10-nucleotide, Internal simple sequence repeat (ISSR) ISSRI & ISSRII, the fragments of cyclic peptide synthetase (CPS) CPS1 and CPS5 were initially screened. Only ISSR2 and CPS5 yielded reproducible fragment patterns of multiple distinct bands and were therefore used to examine the remaining isolates. Polymerase chain reaction (PCR) was conducted in 20μl reaction volumes. Each reaction consisted of approximately 1μl of template DNA, 2μ 10xPCR buffer (TaKaRa), 1.6μl of 2.5mM dNTPS (TaKaRa), 0.8μl each of 10nM ISSRII and CPS5 primers, 14.5μl of sterile distilled water and 0.1μl Taq Polymerase (5U/μl, TaKaRa). All reactions were performed in small eppendorf tube in Thermal Cycler. Thermal cycling parameters were initial denaturation at 94°C for 1 min., followed by annealing at 36°C for 1 min., and extension at 72°C for 2 min., and a final extension at 72°C for 10 min. PCR products were analyzed by gel electrophoresis on 1% agarose in 1xTAE. Gel was stained and photographed as described earlier.

Cloning and sequencing of PCR products: PCR product obtained with ITS primers was cloned in the pCR 4-TOPO Vector (Promega Co.) with the recommended procedures by manufacturer. The pCR-4-TOPO plasmid inserted with the PCR product was introduced to the competent cells of *Eschericia coli* supplied by the manufacturer. After growing them on LBA medium, five colonies were picked up for PCR and checked inserted fragments in plasmid through colony-PCR. Colony-PCR was done by putting a small amount of bacterial body to PCR solution and then amplified as described above. PCR, gel electrophoresis and staining condition were also same as mentioned above. Minipreparation kit (Quiagen, Co.) was used for extraction of plasmid DNA that was inserted for the expected fragments. PCR products were sent for automated DNA sequencing on an ABI Prism System automated sequencer (Model 377, version

TCCGTAGGTGAACCTGCGGAAGGATCATTACCTAGAGTTTGTGGGCTTTGCCTGCTATCT

CTTACCCATGTCTTTTGAGTACTTACGTTTCCTCGGTGGGCTCGCCCGCCGATTGGACAA

ATTTAAACCCTTTGCAGTTGCAATCAGCGTCTGAAAAACATAATAGTTACAACTTTCAAC

AACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGA

ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGTATTCCA

TGGGGCATGCCTGTTCGAGCGTCATTTGTACCTTCAAGCTTTGCTTGGTGTTGGGTGTTT

GTCTCGCCTCTGCGTGTAGACTCGCCTCAAAACAATTGGCAGCCGGCGTATTGATTTCGG

AGCGCAGTACATCTCGCGCCTTGCACTCATAACGACGACGTCCAAAAGTACATTTTTTAA

CACTCTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGA

GGA

Fig. 1. Sequences of rDNA-ITS region of *A. lentis*. The region with under line indicate the specific sequences of *A. lentis*, complementary to the primers.

3.0.1b2, TaKaRa). Sequence alignment was done with the CLUSTAL V program (Higgins *et al.*, 1992). Search was performed for the most similar sequence in the sequence data banks with the help of BLAST search algorithm (Altschul *et al.*, 1990).

Design of specific primers for *A. lentis*: Two PCR primers, ASCO1, a 23 base sequence (5'-GGCCCATGTCTTTTGAGTACTTA) and ASCO2, a 21 base sequence (5'-GGTCGTCGTTATGAGTGCAAG), were designed specifically for *A. lentis* based on the ITS sequences (Fig. 1). The design of the primers was aided by the computer program amplify to check for potential nonspecific primering sites and primer dimer formation. The primers were designed so they had sufficient GC contents and enough length to withstand highly stringent PCR conditions. The conditions of PCR amplification with the specific primers were same as those described above. PCR was done with DNA preparations from all the isolates of *A. lentis* and other fungi which are pathogenic to lentil crop as listed in Table 1.

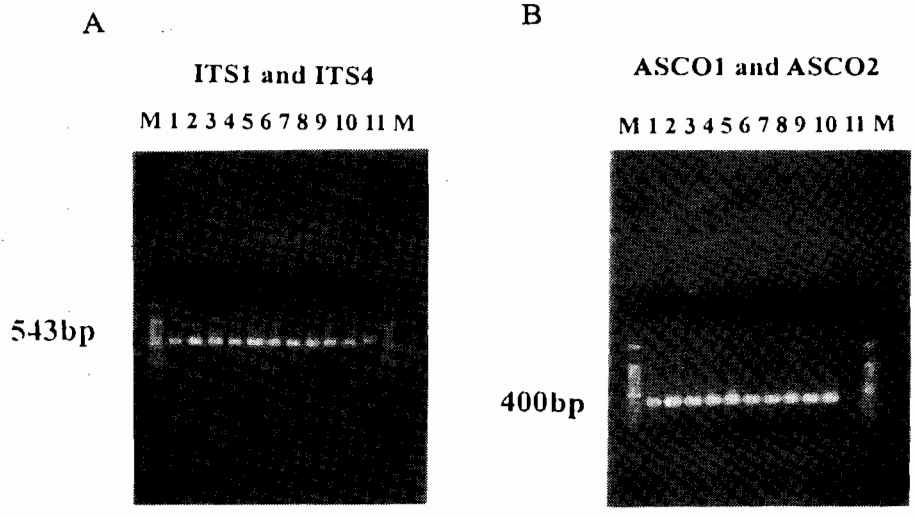

Fig. 2. Agrose gel showing PCR amplification with ITS1/ITS4 (A) and specific primers ASCO1/ASCO2 (B). Ladder DNA 100 bp(laneM); *Ascochyta lentis* (lane 1-8); *Ascochyta rabiei* (lane 9-10) and single isolate of *Ascochyta fabae* (lane 11).

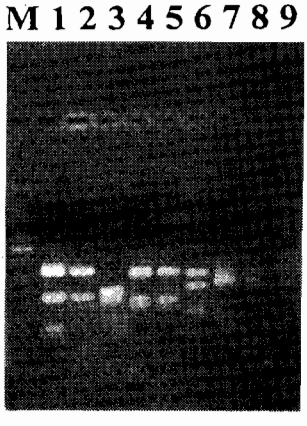

Fig. 3. Agrose gel showing the Polymorphism of PCR-RFLP with various restriction enzymes. Restriction enzyme digestion were electrophoresed on 2% agrose gel M: 100pb ladder Marker; lane1: *A. lentis* (MboI); lane2: *A. rabiei* (MboI); lane3: *A. fabae* (MboI); lane4: *A. lentis* (HhaI); lane5: *A. rabie* (HhaI); lane6: *A. fabae* (HhaI); lane7: *A. lentis* (TaqI); lane8: *A. rabiei* (TaqI); land9: *A. fabae* (TaqI).

Detection of *A. lentis* from seeds: Both healthy and naturally infected seeds were used in the experiments. The extraction and purification of DNA from seeds was similar as mentioned earlier. Minipreparation Kit (Promega, Co.) was used for extraction of DNA from both healthy and diseased seeds to avoid the presence of inhibitory substances in the DNA extracts.

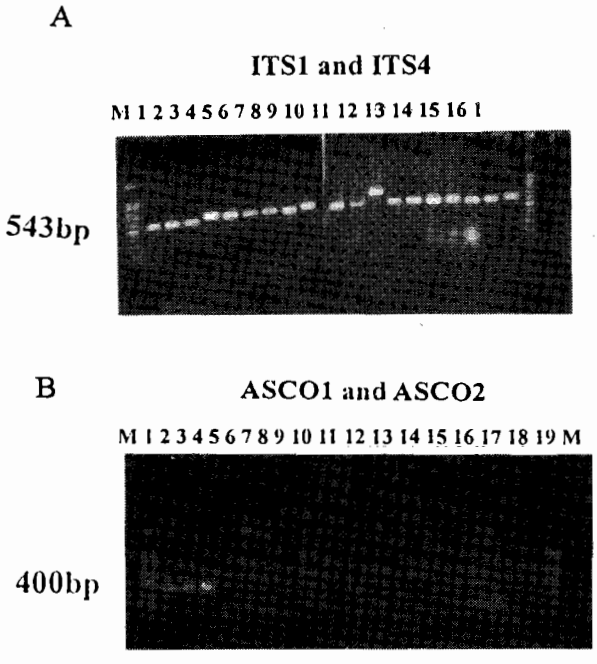

Fig. 4. Agrose gel showing PCR amplification with ITS1/ITS4 (A) and specific primers ASCO1/ASCO2 (B). Ladder 100 bp(lane M); *Ascochyta lentis* (lane1); *Phoma destructiva* (lane2); *Mycospherella melonis* (lane3); *Curvularia trifolli* (lane4); *Alternaria brassicae* (lane5); *Stemphylium botryosum* (lane6); *Glomerella tucumanensis* (lane7); *Cladosporium phlei* (lane8); *Cochliobolus carbonum* (lane9); *Sclerotinia sclerotiorum* (lane10); *Fusarium solani* (lane11); *Rhizoctonia solani* (lane12); *Colletotrichum dematium* (lane13); *C. acutatum* (lane14); *C. gloesporioides* (lane15); *C. lindemuthianum* (lane16); *C. trifoliorum* (lane17); *Phomopsis longicola* (lane18); *P. obscurans* (lane19).

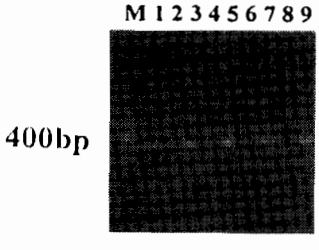

Fig. 5. PCR amplification from g DNA of healthy and infected seed with specific primers, ASCO1 and ASCO2. Ladder DNA 100bp (laneM); *A. lentis* (lane1); DNA of healthy seeds (lane2); DNA of diseased seeds (lane3); lane4 to 9 are replications of lane2 and lane3.

Results

PCR and restriction enzyme digestion: PCR with primers ITS1 and ITS4 were successful in amplifying a single specific site from each isolate. The isolates of *A. lentis* showed one size, about 543bp. Two isolates *A. rabiei* also produced same base pair whereas single isolate of *A. fabae* produced different ITS region which was higher in base pair than *A. lentis* and *A. rabiei* (Fig. 2A). After restriction enzymes digestion, all the isolates from *A. lentis* and *A. rabiei* showed the same banding pattern with all the enzymes, whereas restriction enzyme digestion patterns of *A. fabae* were different from *A. lentis* and *A. rabiei* (Fig. 3). Some other plant pathogens showed variant ITS region of different base pair (Fig. 4A).

RAPD, ISSR and CPS1 & 5 analysis: RAPD, ISSR and CPS primers were used to differentiate the highly and weak virulent pathotypes of *A. lentis*. RAPD, ISSRI and CPSI could not be distinguished amongst the isolates whereas ISSRII and CPS5 produced several amplification products. There was no correlation between ISSRII and CPS5 patterns and the pathogenic groups were established on the basis of virulence (data is not presented).

Sequence analysis: Sequences from the ITS primers of *A. lentis* isolates were aligned with previously published sequences from a similar region of *Phoma glomerata*. A BLAST database search showed that the most similar sequence in the database was *Phoma glomerata* sequence that showed 97% similarity.

Specificity of PCR primers for *A. lentis*: After comparing the *A. lentis* sequences with sequences of *P. glomerata*, two PCR primers, ASCO1, a 23 base sequence (5'-GGCCCATGTCTTTTGAGTACTTA) and ASCO2, a 21 base sequence (5'-GGTCGTCGTTATGAGTGCAAG), were designed specifically for *A. lentis* from lentil. In PCR with primers ASCO1 and ASCO2 at an annealing temperature of 50 and 55°C, the expected PCR product (about 400bp) was observed in all isolates of *A. lentis* and *A. rabiei* whereas isolates representing *A. fabae* yielded no amplification product with the ASCO1 and ASCO2 primer combination (Fig. 2B). Similarly no amplification was observed with other isolates of plant pathogens. Only *Phoma destructiva* and *Mycosphaerella melonis* showed amplification with these specific primers but these pathogens cause disease in tomato and melon, respectively (Fig. 4B).

Detection of *A. lentis* in lentil seeds: Of the variety of methods used for isolating DNA from healthy and naturally infected seeds the minipreparation kit method showed better results by which DNA suitable for PCR was obtained. A single PCR product approximately 400bp in size was detected in *Ascochyta* blight infected seeds from NARC in 1996-97. No product was detected in healthy seeds (Fig. 5).

Discussion

In studies to develop a primer for the detection of seed borne inoculum of *Ascochyta lentis*, amplification with the ASCO1 and ASCO2 primers produced approximately 400bp product with isolates of *A. lentis* and *A. rabiei*. Both species were host specific. *A. rabiei* causes ascochyta blight only in chickpea. Similarly, *A. fabae* which causes ascochyta blight in fababean did not amplify with specific primers, despite its belonging to the same genus. Restriction digests of the resulting PCR products with *MboI, HhaI, HaeIII, AluI* and *TaqI* did not differentiate between *A. lentis* and *A. rabiei*. The results of the present studies showed that *A. lentis* and *A. rabiei* produce similar rDNA ITS sequences, however we did not sequence *A. rabiei* to examine sequence homology between the two species.

DNA of certain plant pathogen viz., *Sclerotinia sclerotiorum, Stemphylium botryosum* and *Colletotrichum lindemuthianum* which cause infection in lentil crop (Qureshi *et al.*, 1985; Simay, 1990) were also tested with ASCO1 and ASCO2 but did not amplify with the primers. Similar results were obtained when DNA of other isolates of different genera were used in the experiments. Only *Phoma destructiva* and *Mycosphaerella melonis* were amplified with specific primers but these pathogens cause diseases in tomato and melon, respectively.

Whereas seed health testing through traditional plating methods and in glass house by growing seeds in pots are laborious and time consuming, the seed borne bacterial and viral pathogens have been detected using PCR, which has been found to be faster and more sensitive than other detection techniques (Audy *et al.*, 1996; Leite *et al.*, 1995; Maes *et al.*, 1996; Schaad *et al.*, 1995; Schoen *et al.*, 1996). Detection of seed borne fungal pathogens by PCR has not been extensively reported. Most of the work has been done with *Phytophthora* species through molecular tools including isozymes analysis, restriction fragment length polymorphism in nucleic and mitochondria DNA, PCR, serological assays, DNA probes and PCR of ITS regions and nuclear small and large subunit rRNA have been developed for evaluation of intraspecific and interspecific variation (Ersek *et al.*, 1994; Forster *et al.*, 1990; Goodwin *et al.*, 1989; Lee & Taylor, 1992; Lee *et al.*, 1993; Oudemans & Coffey 1991; Panabieres *et al.*, 1989).

The extraction of DNA from seeds is a critical step for the use of PCR testing. Isolation of DNA from infected seeds and infected plant tissues are very difficult due to the presence of inhibitors (Chen *et al.*, 1996; Demeke & Adams, 1992; Schilling *et al.*, 1996). The immunocaptured cultures for bacteria coupled with PCR have been effective in overcoming inhibitors (Schaad *et al.*, 1995; Schoen *et al.*, 1996). We have also faced similar hurdle for amplification of PCR product from extracted DNA. A variety of methods for isolating DNA from infected seed were studied. The greatest problem we experienced was the presence of a substance in the DNA which interfered with PCR. The DNA minipreparation proved to be an effective alternative. Such minipreparation could increase the efficiency of PCR detection by reducing PCR inhibitors while reducing the time for DNA extraction. A single PCR product approximately 400bp in size was detected in infected seeds of lentil. ITS region of healthy and diseased seeds was different from *A. lentis* isolates, probably due to the presence of saprophytic fungi in seeds.

The dissemination of ascochyta blight pathogens of lentil, chickpea and fababean in disease free countries is through germplasm exchange. Some time it produced serious disease out break which was disastrous (Kaiser, 1972; Morrall & Mckenzei, 1974; Morrall & Shepperd, 1981). Priority should therefore be given to prevent the introduction of these pathogens because they survived for many years when infected seed was stored at ultra low temperature (Kaiser, 1997). More sensitive techniques are needed to detect the blight pathogens in seed. The ASCO1 and ASCO2 primers will provide an efficient alternative to identify *A. lentis* inoculum in lentil seeds.

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