1632 Development of sensitive molecular diagnostic tools for detection of economically important fungal pathogens of cotton

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PCR protocols for detection and differentiation of strains of *Rhizoctonia solani*, *R. bataticola*, *Ramularia areola* and *Alternaria macrospora*, four economically important fungal pathogens of cotton were developed. Based on nucleotide sequence of the internal transcribed spacer regions of ribosomal RNA genes of these pathogens four sets of primers were developed. Primers pRSol and pRBat were specific to strains of *R. solani* and *R. bataticola* and supported amplifications of rDNA fragments of 255 and 400 bp, respectively. Primer pRare indiscriminately detected four strains of *R. areola* isolated from each of the only four cultivated species of cotton by supporting amplification of an universal amplicon of 372 bp. Strains of *A. macrospora* could be identified by amplification of a DNA fragment of 542 bp using primer pAmac and differentiated from other species of *Alternaria* by PCR-RFLP of the rDNA product with *Ban*II, *Hae*III and *Mse*I restriction endonucleases.

Cultivated cotton (*Gossypium arboreum*, *G. herbaceum*, *G. hirsutum* and *G. barbadense*) in India suffers from large number of diseases that affect both above and underground parts of the plant causing considerable losses in quality and yield (Hillock, 1992; Srinivasan, 1994; Chakrabarty and Mayee, 2004). Besides bacterial and viral diseases, fungal diseases provide a real challenge to successful cultivation of cotton. Fungal foliar diseases such as Grey mildew, which is caused by *Ramularia areola*, was predominantly a pathogen of diploid cotton (*G. arboreum* and *G. herbaceum*) which now infects tetraploid cotton (*G. hirsutum* and *G. barbadense*) as well (Mukewar et al. 1994). The disease causes extensive defoliation and has become a menace in central and South India. Leaf spot and blight caused by *Alternaria macrospora* is another destructive foliar disease of cotton that affects production of cotton in different regions (Srinivasan, 1994). Root rot and wilt, caused by the soil-borne fungal plant pathogens, *Rhizoctonia* spp. and *Fusarium oxysporum* f.sp. *vasinfectum*, are two major diseases that exist in different cotton growing regions of the country (Srinivasan 1994 ; Monga and Raj, 2003).

Identification of the cause and prevalence of a disease is very essential for adequate and timely plant disease management, which in turns depends on accurate diagnosis and early detection of the pathogen. Often it may be desirable to examine the soil for prevalence of any potential pathogen even before the crop is sown. Early detection enables one to make decisions regarding cultivar choice and chemical control that can be used most effectively to prevent development of a potential plant disease epidemic (Ward et al. 2004). Diagnosis of the causal agent is also important for studies on epidemiology (NOTE: Some diseases and/or declines have been studied without knowing the biological cause such as Para wilt of cotton (Raj et al. 1991), yield loss relationships and designing new strategies for disease management. Traditional or classical methods of disease diagnosis and pathogen identification could be relatively slow, often requiring skilled taxonomists to reliably identify the pathogens at the genus or species level. Delays are damaging when quick diagnosis is

needed so that appropriate disease control measures may be taken to prevent plant injury especially when high value cash crops like cotton and other important plant species are at stake.

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Advances in Biotechnology have intensified efforts in recent years to develop novel methods for detection and identification of plant pathogens. Nucleic acid has increasingly been used in recent years to develop diagnostic assay for plant pathogens (Ward et al. 2004). Molecular approaches mainly the polymerase chain reaction have been used widely as the tool for detection of fungal pathogens (Martin et al. 2000, Schaad and Frederick, 2002). Rapid PCR assay based on amplification of sequence of internal transcribed spacer (ITS) region of rDNA or pathogenicity genes have been developed and used for detection of several plant pathogens (Henson and French 1993). Molecular techniques, if not alone, can be used in conjunction with classical methods where the latter approaches can at least narrow pathogen diagnosis to genus level. Once genus is narrowed by morphology, symptomatology, host-specificity, etc., then PCR can be used to differentiate species.

We developed PCR based diagnostic methods to detect strains of *R. solani*, *R. bataticola*, *Ramularia areola* and *A. macrospora*

MATERIALS AND METHODS

Fungal strains and maintenance. The sources of fungal species for which diagnostic tools were developed are given in Table 1. Fungal strains, except that of *R. areola*, were grown and maintained on potato dextrose agar (PDA). For long term storage, they were stored in mineral oil at 4^{o}C in 15 ml screw-capped Corning glass tubes. For DNA isolation the fungal strains except that of *R. areola* were grown in potato dextrose broth (PDB). PDB (100 ml) was inoculated with a 5 mm diameter plug of culture agar cut from the edge of 5 days old culture of each isolate grown on a Petri dish. The inoculated broth was incubated at 28 ± 2^{o}C for 7 days.

Isolation of genomic DNA. The mycelial mat was filtered through Whatman No 1 filter paper and dried at room temperature. The genomic DNA was extracted from fresh mycelium by a modified DNA extraction protocol (Chakrabarty 2004). Approximately 0.5 g of dry mycelial mat was transferred to a clean sterile mortar. Added 1.5 g of White quartz sand (HiMedia, India), 2.5 ml extraction buffer (100 mM Tris, pH 8.0, 20 mM EDTA, 0.5 M NaCl, 1% SDS, 0.5M glucose) and 1.25 ml buffer saturated phenol/chloroform/isoamyl alcohol (25:24:1) at pH 8. The mixture was ground thoroughly with a pestle and the homogeneous slurry was transferred into several microfuge tubes using a wide-bore tip and centrifuged at 13,000 rpm for 5 min at room temperature. The aqueous phase from each tube was transformed to 1.5 ml microfuge tubes to a volume of 750µl and re-extracted with equal volume of chloroform/isoamyl alcohol (24:1). The contents of the tube were mixed by inverting several times followed by centrifugation at 13000 rpm for 5 min. The aqueous phase was again transferred to a new tube and the DNA was precipitated with 0.1 volume 3M sodium acetate (pH 5.2) and 1 volume isopropanol at room temperature for 10 min. The DNA was pelleted by centrifugation at 13,000 rpm for 10 min at 40^{o}C, rinsed with 70% ethanol, and resuspended in 200 µl of TE (10 mM Tris, 1 mM EDTA, pH 8.0) buffer containing 20 µg/ml RNAse. Using this method, genomic DNA was extracted from strains of *A. macrospora*, *R. solani*, and *R. bataticola*. Spores from the surface of the lesions of mildew infected leaves were scraped with a sterile tooth-pick moistened with sterile distilled water. The spore mass were boiled for 5 min and used as the template in PCR reaction.

ITS-PCR and cloning of rDNA sequences. PCR amplification of rDNA sequences for all fungal species was conducted in 50 µl reaction volumes using conserved ITS1 and ITS4 primers (White et al. 1990). Each reaction consisted of 2 µl of 50 ng/µl DNA template, 5 µl of 10X PCR buffer, 0.5 µl of 25mM dNTPs, 1.5 µl of 15 mM $MgCl_2$, 0.3 µl of 1.25U Taq DNA polymerase, 1 µl each of 10 µM primers ITS1(5' TCC GTA GGT GAA CCT GCG G 3 ') and ITS 4 (5' TCC TCC GCT TAT TGA TAT GC 3 ') and 38.7µl sterile distilled water. The PCR protocol was standardised to amplify rDNA sequences from a strain each of *R. solani, R. bataticola, A. macrospora* and four strains of *R. areola* infecting four cultivated species of cotton:, *G. arboreum*, *G. herbaceum*, *G. hirsutum* and *G. barbadense*. The standardised protocol had cycling parameters of initial denaturation at 94°C for 4 min followed by 33 cycles of denaturation at 94° C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1.5 min. A final extension at 72 °C for 5 min was done at the end of amplification. Negative controls were used to test for false priming and amplification.

A 10-µl PCR amplification product for each of the fungal species was visualized in a 1%

agarose gel and viewed under UV light following staining with ethidium bromide.

Cloning of rDNA fragments. Gel purified fragments of ~ 650 bp comprising partial sequences of 18S and 28S rRNA genes, and complete sequences of ITS1, 5.8S and ITS2 of each fungal strain were cloned in pGEMT (Promega, Madison, WI, USA), following manufacturer's protocol, unless stated otherwise. The ligation reaction was incubated overnight at 40°C. The ligation mix was transformed in Escherichia coli (XL-1 Blue) by heat shock method. The tube containing the competent cells (200 µl) was removed from –70°C and allowed to thaw on ice. Ligation reaction mixture (2 µl) was added to the tube of competent cells following incubation on ice for 5 min. The cells were subjected to heat shock at 42°C for 30 second and transferred on ice for 2 min. Heat-shocked cells were dispensed in 250 µl LB in micro centrifuge tube. The transformation mix was incubated at 37°C in an orbital shaker at 220 rpm for 45 min to allow expression of the plasmid. The entire transformation mixture was then plated on LB agar containing Ampicillin (70ug/ml), Xgal (80 µg/ml) and IPTG (50 µM). The plate was incubated overnight at 37° C. The recombinant clones were identified by blue white colony selection. The white putative recombinant colonies were streaked on LB agar supplemented with Ampicillin (70 µg/ml). The plasmid isolation from the putative transformants was done by the rapid miniprep protocol (Chakrabarty, unpublished). The recombinant clones were confirmed by digesting plasmid DNA with *Aat*II and *Pst*I.

Sequencing of ITS amplicons and multiple alignment of sequence data. The cloned ribosomal RNA genes and the ITS regions of each fungal strains were sequenced using T7 and SP6 vector based primers at M/S Bangalore Genie Pvt. Ltd. Bangalore (India). The rRNA sequences of each fungal pathogens, comprising of partial sequences of 18S rRNA and 28S rRNA; and complete sequences of ITS 1, 5.8S rRNA and ITS 2 were submitted in GenBank. The DNA sequences of each accession were aligned among themselves as well as with other published sequences available in GenBank using BlastN and http://www.justbio.com.

Development of species-specific primer and PCR detection protocol. Following multiple alignments of the rDNA sequences, regions of dissimilarity in ITS 1 and ITS 2 sequences were determined and used to design primers specific to three fungal species: *R. solani, R. bataticola* and *R. areola* and an *Alternaria* genus-specific primer for *A. macrospora*. To test specificity of primers in detecting strains of respective cotton pathogen only, the genomic DNA of each pathogen was subjected to PCR amplification with each set of primer. For *A. macrospora*, sequence variability with respect to other *Alternaria* species

infecting other economically important plants was not good enough for designing species-specific primers. Therefore, restriction fragment length polymorphism analyses of amplified rDNA fragments with different restriction enzymes were used to differentiate *A. macrospora* from other *Alternaria* species. PCR amplified ITS regions of *A. macrospora* and seven other *Alternaria* species were digested with restriction enzymes viz., *Bal*I, *Bal*II, *Ban*II, *Cla*I, *Hae*III, *Hin*dIII, *Hph*I, *Mbo*I, *Mse*I, *Nla*IV, *Sac*I, *Tfi*I, *Sal*I, *Sau*3aI, *Sma*I, *Xho*I and *Xma*I. Restriction digestion reaction was carried out in 15 µl volumes and consisted of 0.5 µl restriction endonuclease (5U/µl), 1.5µl restriction buffer (10X), 11µl sterile distilled water and 3µl of PCR product. The digestion was carried out at 37^{o}C for 2 h. The digested PCR product was resolved on 2 percent agarose gel, stained with ethidium bromide (0.5 µg/ml) and visualized under UV to analyze nucleotide polymorphism in amplified fragment.

RESULTS

DNA based PCR diagnostic protocols were developed to identify four fungal pathogens of cotton, including *R. solani, R. bataticola, A. macrospora* and *R. areola* (Table 1). PCR Amplification of cotton fungal species with conserved primers ITS1 and ITS4 yielded an ~600 base pair rDNA product which were cloned in plasmid pGEMT (Fig. 1 a & b). Analysis of rDNA fragments from fungal strains revealed presence of partial sequences of 18S and 28S rRNA genes and complete sequences of ITS 1 and ITS 2 along with 5.8S rRNA gene. The sequences of the entire ITS 1/5.8S/ITS 2 regions together with short termini from large and small subunit genes, were obtained for each of the four pathogens. The sequences were deposited in GenBank and accession numbers obtained for each of them (Table 1). There was significant variation in the sequences of the ITS regions, especially within ITS1 and ITS 2, although several highly conserved regions were present in both regions. Regions of significant sequence variability in *R. solani, R. bataticola, R. areola* and *A. macrospora*, were good enough to design species-specific oligonucleotide primers for strains of first three species. Four different sets of primers capable of differentially detecting these four pathogens were designed. The pathogens, the primers and the sizes of the diagnostic amplicons are given in Table 2.

Primers pRsol, pRbat, pAmac and pRare could specifically detect strains of *R. solani, R. bataticola, A. macrospora* and *R. areola* by amplification of rDNA fragments of 255, 400, 542 and 372 bp, respectively (Fig. 2). Primers pRsol and pRbat can specifically amplify strains of *R. solani* and *R. bataticola*, respectively. pRsol successfully detected strains of *R. solani* tested but did not detect *R. bataticola* strains infecting cotton (Fig. 3a). On the other hand pRbat could amplify a DNA fragment of 400 bp from strains of *R. bataticola* but not from the strains of *R. solani* collected from different cotton growing zones of the country (Fig. 3b).

Primer pRare indiscriminately detected four strains of *R. areola*, each isolated from *G. hirsutum, G. barbadense, G. arboreum* and *G. herbaceum* by universal amplification of a DNA fragment of 372 bp. pAmac amplified a rDNA fragment of 542 bp from strains of *A. macrospora.* Each set of primer supported amplification of the strains of respective target pathogen but failed to detect members of other three pathogens tested (Fig. 4a-d).

The primer pAmac however, was not specific to *A. macrospora* of cotton but supported amplification of the rDNA fragment from several species of *Alternaria*, such as *A. alternata* strains from sorghum and sunflower, *A. longipes, A. porri, A. dianthicola, A. citri* and *A. brassicae* (Kadam 2005). Lack of adequate variability in nucleotide sequences in the ITS region of different species of *Alternaria* did not allow designing species-specific primers for *A. macrospora*. Strains of *A. macrospora* could however, be identified and differentiated by

possession of two unique restriction endonuclease sites such as *Ban*II and *Mse*I in the rDNA repeat unit. These two enzymes sites are not present in any other *Alternaria* species studied. There was a single *Ban*II site in the ITS1 region of *A. macrospora* which cleaved the linear PCR amplified rDNA repeat into two fragments of 448 and 127bp size (Fig. 5). The rDNA region also possessed two *Mse*I sites one each in ITS2 region and 28S rRNA gene that generated three fragments of 418, 136 and 21bp size. Also unlike in all *Alternaria* spp. which possessed single *Hae*III site, *A. macrospora* had two *Hae*III sites one each in ITS1 and ITS2 regions and generated three DNA fragments of 368, 140 and 67 bp. Besides, comparison of the rDNA sequences amplified using conserved ITS1 and ITS4 primers in *A. macrospora* against other Alternaria species, revealed that the former has the highest number of nucleotides (575 bp) in the ITS region.

DISCUSSION

Four sets of pathogen-specific primers developed as a part of this study enabled successful diagnosis of cotton-specific strains of *R. solani, R. bataticola* and *R. areola*, while PCR-RFLP method could differentiate strains of *A. macrospora* from several other species of this pathogen. Detection of polymorphism using PCR-RFLP analysis of the ribosomal DNA- ITS region has been successfully used for identification of several species of fungi (Martin et al. 2000). This simple technique requires only minute amounts of DNA and two specific conserved primers flanking the ITS region of rDNA genes. This is one of the groups of genes most frequently targeted for phylogenetic studies and codes for rRNA. The main reasons for the popularity of rDNA are that it is a multicopy, non-protein-coding gene, whose repeated copies in tandems are homogenized by concerted evolution and is therefore treated as a single locus gene. Furthermore, the ribosomes are present in all organisms and ribosomal RNA genes are the most commonly used target for fungal and bacterial diagnostics (Ward et. al 2004). The amplified products of ITS region of 11 fungal species from different crops (Kadam 2005), including strains of *R. solani, R. bataticola, A. macrospora* and *R. areola* reported in the present study, ranged between 569-575 bp, coinciding with the sizes obtained from similar fungal pathogens from other strains of the same species. The multiple alignments of the rDNA sequences using sequences available in GenBank and sequences from this study revealed significant variability in ITS1 and ITS2 regions directly allowing us to design species-specific primers. Considerably greater sequence variations is found in the internal transcribed spacer (ITS) regions between the rRNA genes within a rRNA repeat unit (Henson and French 1993). Nazar et. al. (1991) found adequate sequence differences in the ITS regions of the cotton wilt fungi, *Verticillium dahliae* and *V. alboatrum*, to design primers that specifically amplify the DNA of each species. Primers based on differences in ITS 1 sequences of *Leptosphaeria maculans* allowed specific amplification of weak or virulent isolates of this fungal pathogen (Xue et. al. 1992). Specific primers were also designed and developed based on the ribosomal genes to detect and differentiate several species of the genus *Phytophthora,* an economically important fungal pathogen of crop plants Ristaino et. al. 1998; Appiah et. al. 2004). For fungal species such as *Alternaria alternata, A. longipes, A. dianthicola, A. citri, A. brassicae, A. macrospora and A. porri*, where significant variability in the nucleotide sequence of rDNA did not exist, inter and intra-specific variation was evaluated by analysis of the ITS region of rDNA using restriction fragment length polymorphism. Cleavage of amplified fragments with specific restriction enzymes revealed extensive polymorphism that allowed further differentiation of these *Alternaria* species. *A. macrospora* possessed certain unique restriction sites like *Ban*II and *MSe*I. The presence of these unique restriction sites are consistent with observed nucleotide sequence variability in the rDNA sequences of these *Alternaria* species that included addition or deletion of several conserved nucleotides. Such substitutions are responsible for obliteration of some conserved restriction sites or creation of some unique sites. The inter-specific variation among several species of *Phytophthora* infecting cocoa could be clearly distinguished by restriction analysis

of the PCR amplified rDNA regions with unique restriction enzymes (Appiah et al 2004). The PCR-RFLP analysis of rDNA-ITS region has also been successfully used for identification and differentiation of several species of ectomycorrhizal fungi (Amicucci et al.1996, Eliane et al. 2002). Previously, Chakrabarty et al (2005) developed PCR based diagnostic protocols for detection of *Xanthomonas axonopodis* pv. *malvacearum* and cotton leaf curl virus, two major pathogens of cotton, by developing primers based on their pathogenicity genes. A PCR protocol for detection of *Alternaria radicina* on carrot seed was developed by Pryor and Gilbertson, (2001). The primers were designed based upon the sequence of a cloned RAPD fragment of the pathogen.

The results obtained during the present investigation showed that the internal transcribed spacer regions of the ribosomal RNA gene sequences can be used to design species-specific diagnostic tools. Furthermore, the ITS-restriction fragment length polymorphism analysis has potential to serve as markers for differentiation of closely related species or the strains belonging to same species.

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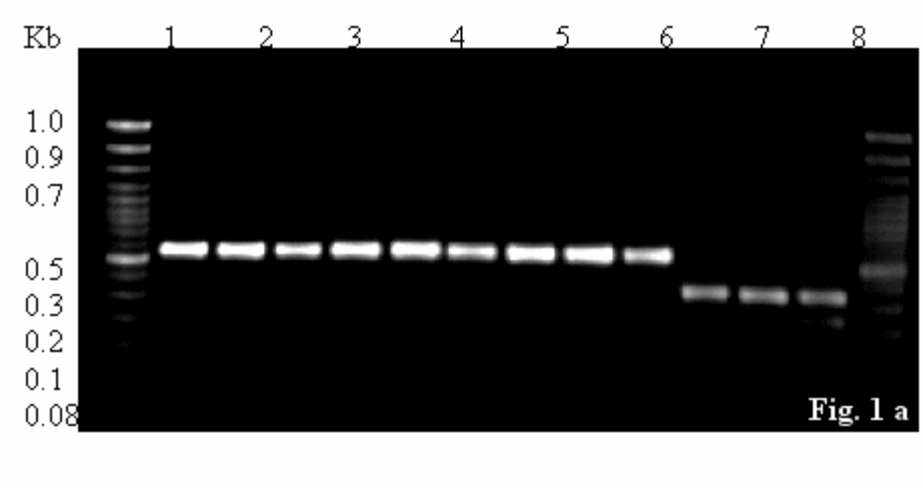
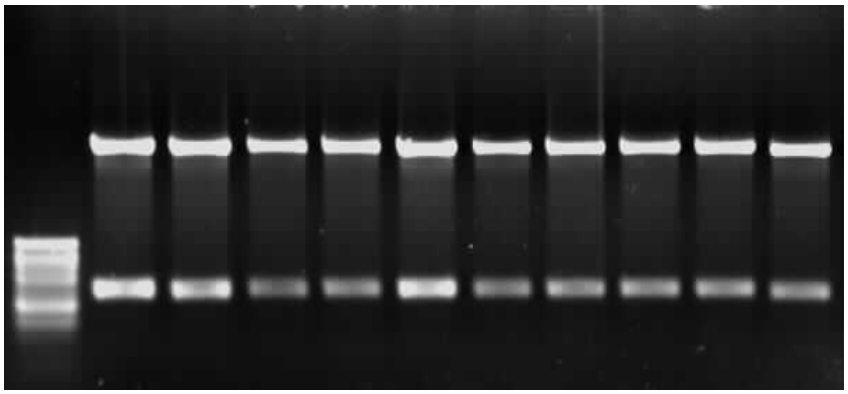
FIGURES





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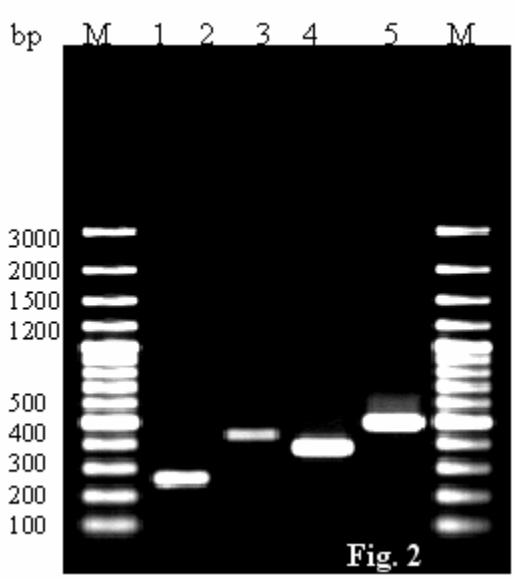

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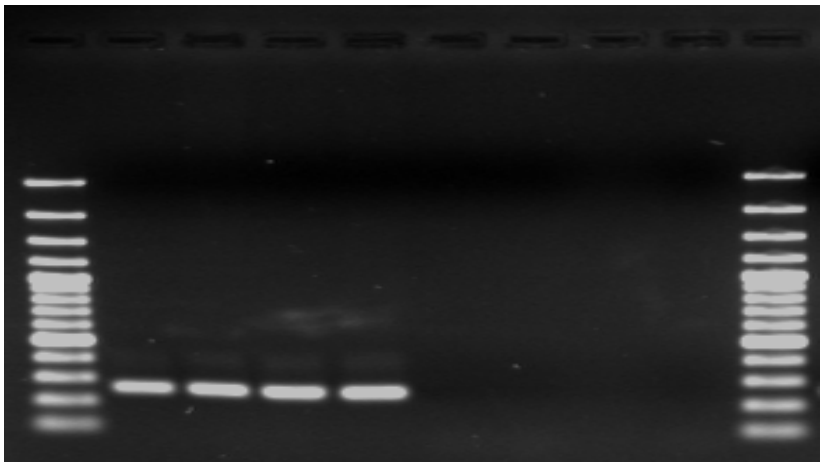
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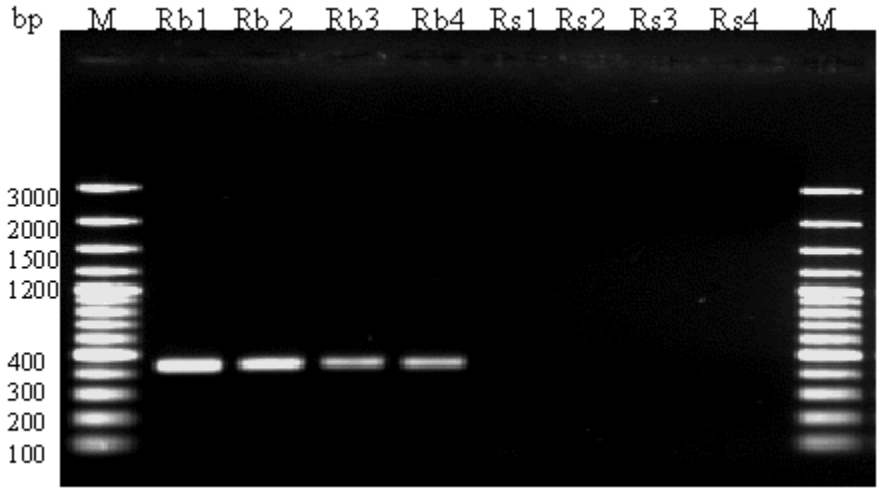
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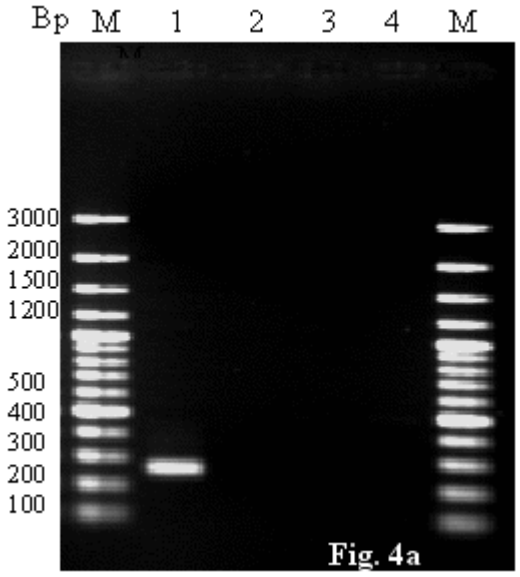

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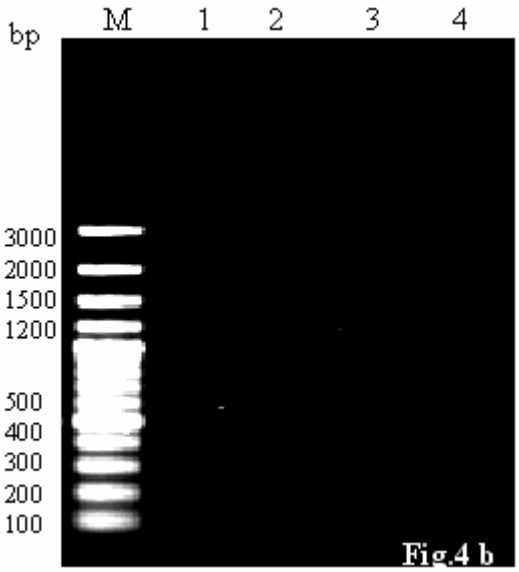

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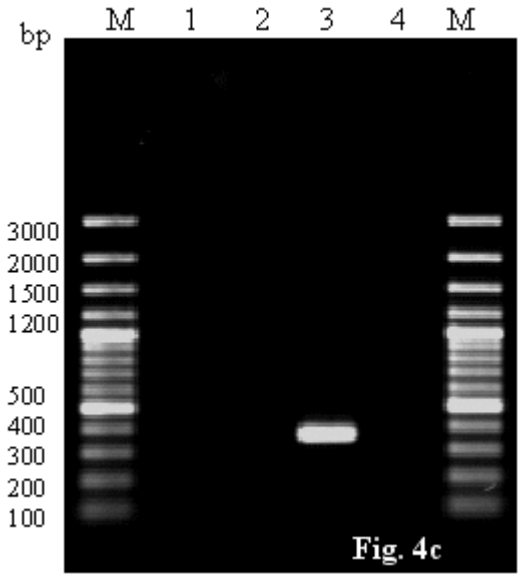

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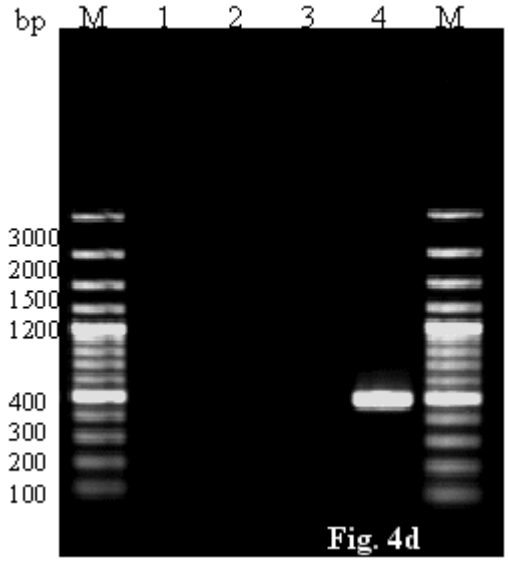
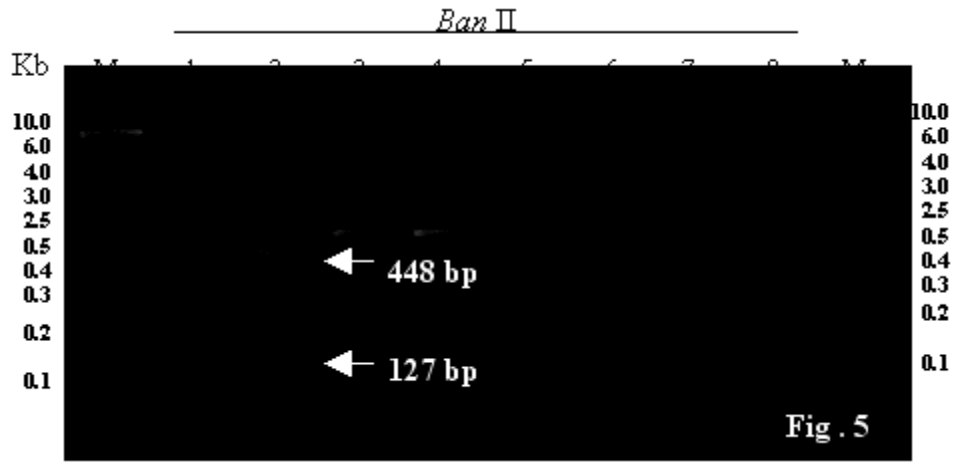


Fig. 4d



LEGENDS

Fig. 1 a-b: PCR amplification of ITS regions of rDNA of several fungal pathogens using primers ITS 1 and ITS 4 and their cloning. Ribosomal DNA amplified using conserved primers ITS1 and ITS4 (a). Recombinant Plasmids digested with *Aat*II and *Pst*I to release the cloned rDNA fragments (b). Lane 1, DNA ladder; 2-8: *Rhizoctonia solani, R. bataticola, Ramularea areola* strains of *G. aboreum, G. herbaceum, G. hirsutum and G. barbadense*, *Alternaria macrospora*.

Fig. 2: Detection of cotton pathogens using species-specific primers. Lanes 1 and 6, 100 bp DNA ladder; 2-5, *Rhizoctonia solani, R. bataticola, Ramularia areola, Alternaria macrospora*.

Fig. 3 a-b: Detection of strains of two species of *Rhizoctonia* with species-specific primers. Amplification of strains of *Rhizoctonia solani* with primer pRsol (a). Lanes 1 and 10, 100bp DNA ladder; 2-5, *R. solani* strains Rs1, Rs2, Rs3, Rs4; lanes 6-9, *R. bataticola* strains Rb1, Rb2, Rb3, Rb4. Amplification of strains of *Rhizoctonia bataticola* with primer pRbat (b). Lanes 1 and 10, 100bp DNA ladder; 2-5, *R. bataticola* strains Rb1, Rb2, Rb3, Rb4; lanes 6-9, *R. solani* strains Rs1, Rs2, Rs3, Rs4.

Fig. 4a-d: Specific detection of strains of four fungal pathogens of cotton with species specific primers: pRsol (Panel a), pRbat (Panel b), pRare (Panel c) and pAmac (Panel d). Lanes 1 and 5, DNA Marker; 2-4, strains of *Rhizoctonia solani, R. bataticola, Ramularia areola* and *Alternaria macrospora.*

Fig. 5: Digestion of PCR amplified rDNA product of *Alternaria* species with *Ban*II restriction endonuclease. Lane 1 and 9, 1kb DNA ladder; 2,8, *Alternaria alternata* (shorghum), A*. macrospora, A. brassicae, A. porri, A. citri, A. longipes* (strain RLC 16.3), *A. longipes* (strain 21.5)*, A. alternata* (sunflower).

Table 1: The fungal strains, sources and GenBank accessions of their ITS sequence.

Sr. No	Pathogen species/ strain	Sexual/ asexual form	Source	GenBank Accession
1	*Rhizoctonia solani* Kuhn.	*Thanatephorus cucumeris*(Frank) Donk	Infected roots of cotton	DQ339103
2	*Rhizoctonia bataticola* (Taub.), Butler	*Macrophomina phaseolina* (Tassi) Gold.	Infected roots of cotton	DQ339102
3	*Alternaria macrospora* Zimm.	-	Infected leaves of cotton	DQ156342
4	*Ramularia areola* Atk. (hirsutum)	*Mycosphaerella areola* (Ehrlich and Wolf.)	Mildewed leaves of *G. hirsutum*	DQ459076
5	*R. areola* Atk. (barbadense)	*Mycosphaerella areola* (Ehrlich and Wolf.)	Mildewed leaves of *G. barbadense*	DQ631897
6	*R. areola* Atk. (arboreum)	*Mycosphaerella areola* (Ehrlich and Wolf.)	Mildewed leaves of *G. arboreum*	DQ459081
7	*R. areola* Atk. (herbaceum)	*Mycosphaerella areola* (Ehrlich and Wolf.)	Mildewed leaves of *G. herbaceum*	DQ459082

Table 2. The fungal pathogens, diagnostic primers and sizes of the amplified products.

Sr. No	Pathogen species	Primers	amplicon (bp)
1	*Rhizoctonia solani*	pRsol	255
2	*Rhizctonia bataticola*	pRbat	400
3	*Ramularia areola*	pRare	372
4	*Alternaria macrospora*	pAmac	542