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# DIRECT PCR BASED DETECTION OF *MACROPHOMINA PHASEOLINA* FROM SOIL OF JUTE FIELD

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## Abstract

*Macrophomina phaseolina* (Tassi) Goid. is a devastating fungal pathogen which infects a large number of economically important crops. In jute, it mainly causes seedling blight, leaf spot, and stem rot. The pathogen is seed borne as well as soil borne. *Macrophomina phaseolina* pathogen was detected in soil samples collected from jute field by a direct PCR method (dPCR) without DNA isolation using ITS2 and ITS3 region specific primers designed during the present study. The soil samples were prepared in a lysis buffer and were incubated at $65^0$C for 15 min. The optimized buffer system was composed of 200 mM tris (hydroxymethyl aminomethane (Tris)-Cl (pH 8.0), 3.5 mM ethylene diamine tetra acetic (EDTA) (pH 8.0), 4 M NaCl and 200 µg/ml Proteinase K. Further, β-marcaptoethanol (1% v/v) was added into the buffer.

*Macrophomina phaseolina* (Tassi) Goid. is a dreaded pathogen that generally causes damping off, seedling blight, collar rot, stem rot, charcoal rot, and root rot in many economically important crops such as maize, sorghum, common bean, green gram, jute, cotton, soybean, sunflower, sesame, etc (Muchero *et al.* 2011). It causes yield losses of up to 35-40% in cases of severe infection (Roy *et al.* 2008). The pathogen is mainly detected in soil samples by cultivation on media followed by microscopy. But these methods are laborious, time consuming and not reliable. Presently, fungal pathogens are being detected by PCR which is a rapid and reliable technique. Although PCR is a very sensitive method for the detection of soil borne pathogens, the isolation of pure DNA is a prerequisite. Isolation of pure DNA from soil is cumbersome, and the impurities present in the soil may hinder PCR amplification. Therefore, in the present investigation, *M. phaseolina* was detected by a direct PCR method, avoiding DNA extraction from soil samples using ITS primers.

The pathogen *M. phaseolina* was isolated from an infected jute (*C. olitorius*) plant (cultivar JRO 524) collected from the CRIJAF research farm, Barrackpore, India. For isolation of DNA from *M. phaseolina*, a 5 mm disc of 2-day-old culture was grown for seven days at 25 ± 1°C in 250 ml conical flask containing 50 ml of potato dextrose broth (pH 5.5).

Soil sample was prepared in lysis buffer for direct PCR without DNA extraction. Different components and their concentration in the lysis buffer system were optimized following cell lysis method (Li *et al.* 2011) with major modifications. Approximately 100 mg soil samples were taken. Samples were washed with 100% ethanol for 7 min. Samples were placed in 1.5 ml eppendrof tube and 500µl of lysis buffer was added and incubated at 65°C for 15 min. The optimized buffer system composed of 200 mM tris (hydroxymethyl) aminomethane hydrochloride (Tris)-HCl (pH 8.0), 3.5 mM ethylene diamine tetra acetic (EDTA) (pH 8.0), 4 M NaCl and 200 µg/ml Proteinase K. Further, β-marcaptoethanol (1% v/v) was added into the buffer. Incubated sample was centrifuged at 12000 x g for 15 min. and the lysate was carefully taken out. The lysate was used for PCR amplification. For isolation of DNA from *M. phaseolina* pure culture the mycelia were filtered through Whatman No.1 filter paper and CTAB based lysis buffer system (Biswas *et al.* 2014) was used.

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The ITS primers ITS-1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS-4 (5'-TCCTC-CGCTTATTGATATGC-3') (White *et al.* 1990) were used for direct PCR amplification of soil lysate. PCR was performed in a total volume of 50 ml containing 5 ml of 10X PCR buffer (100 mM, Tris-HCl, pH 8.3, 15 mM $MgCl_2$, 250 mM KCl), 1U Taq DNA polymerase (Bangalore Genei, India), 25mM dNTP mixture, 50 pmol of each ITS-1 and ITS-4 primers, and 5 µl lysate. PCR was carried out for 35 cycles of denaturation at 94°C for 1 min. annealing at 50°C for 30 s and extension at 72°C for 1 min. 20 s with a final extension step at 72°C for 10 min, using T100 BioRad Thermal Cycler.

The amplified PCR products were selected for sequencing. For direct sequencing, five amplified products were electrophoresed on a 1% agarose gel and the fragment was extracted and purified using the Prepagene kit (BioRad). Sequencing was carried out on an ABI automated DNA sequencer, using cycle sequencing with the ABI prism Dye termination cycle sequencing ready reaction kit following the protocol recommended by the manufacturer. The resulting ITS sequences were analyzed for homologies and for further analysis. BioEdit software version 7.0 analyzed that sequences obtained from five samples were identical to each other and one of them was deposited at NCBI, Accession no. KC427133.

The sequence data was converted to SeqWeb (version 2; Accelrys, Cambridge, UK) format and aligned. Oligonucleotide primers specific for *Macrophomina* species were identified by ITS1 sequence comparison. The primer analysis Primer BLAST software (NCBI, USA) was used to select PCR primers. Two pairs of highly specific primers (ITS2 and ITS3) were synthesized (Table 1). Ten different fungal/bacterial species were tested with the ITS2 and ITS3 primers for their validation. PCR amplification for specificity was performed following previously described methods.

**Table1. Sequences of developed ITS 2 and ITS 3 region specific primers.**

| Primer name | Sequence | Bases | Tm |
|---|---|---|---|
| ITS2 | 5'GCCTGTTCGAGCGTCATTCG3' | 20 | 57°C |
| | 5'TGTCCGAAGCGAGGTGTATT3' | 20 | |
| ITS3 | 5'GTCCGAAGCGAGGTGTATTCT3' | 21 | 62°C |
| | 5' TCGAGCGTCATTTCAACCT3' | 19 | |

About 350 bp of ITS1/ITS 4 amplicon from *M. phaseolina* species was sequenced and used for designing primers (Fig.1). Two pairs of primers were designed from ITS1 and ITS 4 variable regions of *M. phaseolina* species and amplified with the rDNA primers ITS2 or ITS3. The PCR conditions were optimized for each primer pair to amplify the purified genomic DNA from which they were designed. Raising the annealing temperature and shortening annealing time provided better specificity. For example, the theoretical annealing temperatures of forward and reverse primers ITS2 and ITS3 were found to be 57 and 62°C respectively. The ITS 2 and ITS 3 region gave 250 bp and 270 bp amplicon with *M. phaseolina*, respectively. Ten different fungal/bacterial species viz. *Fusarium oxysporum* f sp *lini, Alternaria alternata, Trichoderma viride, Aspergillus niger, Rhizoctonia solani, Bacillus subtilis, Pseudomonas fluorescence, Colletotrichum capsici, Beauveria bassiana, Pythium aphanidermatum* other than *Macrophomina phaseolina* were tested against the currently designed primers but no amplification was obtained (Fig. 2).

PCR based detection of *M. phaseolina* was initiated with ITS primers using ITS1 as the forward primer located at the 3' end and ITS4 as the reverse primer at the 5' end. These primers

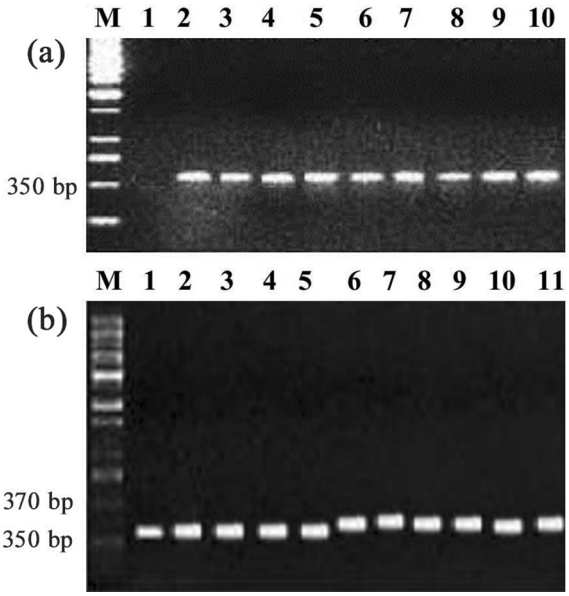


Fig. 1. Amplification of *M. phaseolina* from soil samples by direct PCR with ITS 1 and ITS 4 primer (a) and with ITS 2 and ITS 3 primers (b), a) lane M : 50- bp ladder, lane 1:control, lane 2-10: positive samples with ITS 1 and ITS 4 primer. b) lane M : 50- bp ladder, lane1-5: PCR positive samples with ITS2 primer and lane 6-11: PCR positive samples with ITS3 primer.

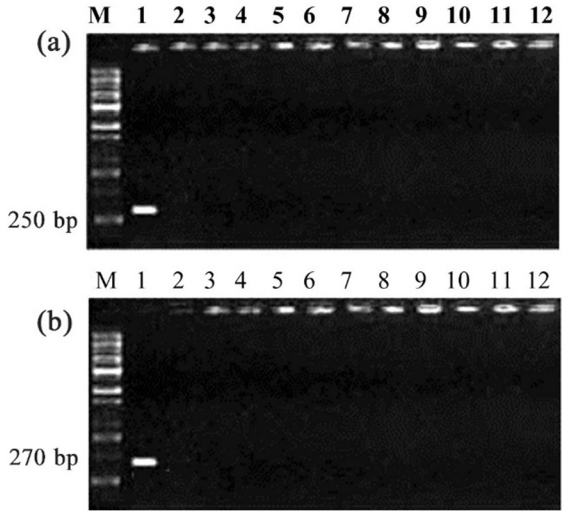


Fig. 2. Validation of developed ITS 2 primer pair (a) and ITS 3 primer pair (b) by testing for different microorganisms. lane M**:** 50- bp ladder, lanes 2-11: different fungal and bacterial samples lane12: control.

amplify the entire ITS region. The reverse primer ITS4 was not intended to amplify targets, however, based on sequence comparisons, it appeared that it could be a poor match to many other plants. Reverse primers in the 5' section of LSU (large subunit) of ITS 1 region developed by Egger (1995) were also tried. A reliable PCR amplification was achieved broadly targeting *M. phaseolina* ITS 2 and ITS 3 specific region by developing a pair of primer (ITS2 and ITS3) that

worked well with ITS1 and close to the annealing site for ITS4. The ITS2 and ITS3 primer pair effectively excluded plant sequences, amplified only specific targets. A robust pair of *M. phaseolina* specific primers (ITS2 and ITS3) was also developed that could serve as fast detection primers in PCR reactions with target sites. Additionally, synthesized primer pairs allowed separate amplification of ITS1 and ITS2 region i.e 5.8S sequence completes the overlapping region. Direct PCR of soil sample lysates with newly developed ITS 2 and ITS 3 region specific primers generated 250 and 270 bp amplicons respectively from all the soil samples (Fig. 1b).

Although dPCR methods which obviate DNA isolation steps were developed (Sharma *et al.* 2012) mainly to amplify plant genomic DNA, are recently being used for detecting plant pathogens. A fungal plant pathogen, *Botrytis cinerea* was detected in grape berries by dPCR without DNA extraction (Gindro *et al.* 2010). We also detected the stem rot pathogen of jute *Macrophomina phaseolina* by a direct PCR method from the infected leaf as well as the stem of jute plants (Biswas *et al.* 2014). In the present investigation *M. phaseolina* was detected from soil by a direct PCR method which is low cost, and rapid. This is the first report of detecting this pathogen from soil by direct PCR. Soil is a natural store house of diverse microorganisms and it necessitates the use of more specific primers. Therefore, ITS 2 and ITS 3 region specific primers were developed that are more specific and thus the method would enable more efficient and reliable detection of the pathogen from soil.

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**References**

Biswas C, Dey P, Mandal K, Satpathy S and Karmakar PG 2014. In planta detection of *Macrophomina phaseolina* from jute (*Corchorus olitorius* L.) by a sodium acetate-based direct PCR method. Phytoparasitica **42**(5):673-676.

Egger KN 1995. Molecular analysis of ectomycorrhizal fungal communities. Can. J. Bot. **73**: S1415-S1422

Gindro KG, Pezet R, Viret O and Richter H 2005. Development of a rapid and highly sensitive direct PCR assay to detect a single conidium of *Botrytis cinerea* Pers.:Fr. *in vitro* and quiescent forms in planta. Vitis **44**: 139-142.

Li H, Xu H, Zhao C, Sulaiman Y and Wu C 2011. A PCR amplification method without DNA extraction. Electrophoresis **32**: 394-397.

Muchero W, Ehlers JD, Close TJ and Roberts PA 2011. Genic SNP markers and legume synteny reveal candidate genes underlying QTL for *Macrophomina phaseolina* resistance and maturity in cowpea (*Vigna unguiculata* (L) Walp.) BMC Genomics **12**: 8.

Roy A, De RK and Ghosh SK 2008. Diseases of bast fibre crops and their management. *In*: Jute and allied fibre updates, (Eds) Karmakar PG, Hazra SK, Ramasubramanian T, Mandal RK, Sinha MK and Sen HS, pp. 217-241, Central Research Institute for Jute and allied Fibres, Kolkata

Sharma R, Kumar V, Mohapatra T, Khandelwal V and Vyas GK 2012. A simple and non-destructive method of direct PCR for plant systems. Journal of Plant Biology **55**: 114-122.

White TJ, Bruns T, Lee S and Taylor JW 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In*: PCR Protocols: A Guide to Methods and Applications. (Eds) Innis MA, Gelfand DH, Sninsky JJ, White TJ, pp.315-322, New Academic Press Inc, New York.

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