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In planta detection of *Macrophomina phaseolina* from jute (*Corchorus olitorius*) by a sodium acetate-based direct PCR method

Chinmay Biswas · Piyali Dey · Kunal Mandal · Subrata Satpathy · P. G. Karmakar

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Abstract *Macrophomina phaseolina* (Tassi) Goid. is the most important pathogen of jute and primarily causes seedling blight, leaf spot and stem rot. The pathogen was detected from field samples by a simple method of direct PCR (dPCR) which obviates the steps of DNA extraction. The leaf bits were treated with a lysis buffer at 65°C for 25 min, whereas the stem pieces were initially incubated at 65°C for 5 min followed by incubation at 60°C for 25 min and the lysate was used as PCR template. Based on the type of tissue, the composition and concentration of lysis buffer systems were optimized. For leaf samples the optimized buffer system composed of 20 mmol l^{-1} tris (hydroxymethyl aminomethane (Tris)-Cl (pH 8.0), 1.5 mmol l^{-1} ethylene diamine tetra acetate (EDTA) (pH 8.0), 1.4 mol l^{-1} sodium acetate and 200 μg/ml proteinase K. Further, 3% PVP (w/v) and β-mercaptoethanol (1% w/v) were added into the buffer. In case of stem samples, PVP was not applied and higher concentrations were used for other components. *M. phaseolina* could be detected from both leaf and stem samples generating amplicon of 350 bp. This is the first report of detecting *M. phaseolina* by a direct PCR method without DNA extraction.

Keywords ITS primer · dPCR · Sequencing

C. Biswas (✉) · P. Dey · K. Mandal · S. Satpathy · P. G. Karmakar
Central Research Institute for Jute and Allied Fibres (CRIJAF), Barrackpore, Kolkata 700120, India
e-mail: chinmaybiswas@rediffmail.com

Jute is the second most important fibre crop after cotton in the world and, unlike cotton, the fibre is extracted from the stem. It is grown mainly in the Southeast Asian countries like India, Bangladesh, Nepal, China, Indonesia, Thailand, Myanmar, and a few South American countries. It is used in making sacks, ropes, bags, carpets, shoes, geo-textiles, jewellery and home decorations (Kundu 1956). Jute is attacked by a number of pathogens, of which *Macrophomina phaseolina* (Tassi) Goid. is the most important one (Roy *et al.* 2008). It is a devastating necrotrophic fungal pathogen with worldwide distribution which infects more than 500 crop and non-crop plant species (Mihail & Taylor 1995). Maize, sorghum, common bean, green gram, jute, cotton, soybean, sunflower and sesame are the major crops which are affected by this pathogen (Muchero *et al.* 2011). *M. phaseolina* can affect the jute crop at any stage of growth, causing seedling blight, leaf spot and stem rot (Biswas *et al.* 2011). Average yield loss due to this disease is about 10%, but it can go up to 35–40% in cases of severe infection (Roy *et al.* 2008). Jute is also attacked by many other fungal diseases such as anthracnose (*Colletotrichum* sp.), black band (*Botryodiplodia theobromae*), soft rot (*Sclerotium rolfsii*), etc. All these pathogens are conventionally detected by colony morphology through microscopy. However, this is not a sensitive and specific technique. Recently PCR has been used for rapid and reliable detection of many plant pathogens. However, in jute high contents of mucilage and secondary metabolites hinder DNA isolation and PCR amplification. To address these problems we developed a DNA isolation technique

for detecting *M. phaseolina* from jute seeds by PCR, which is a rapid and reliable technique (Biswas *et al.* 2013). However, it is time consuming to isolate pure DNA from jute plants. Therefore, in the present investigation we detected *M. phaseolina* from jute plants by a direct PCR method without DNA isolation.

The pathogen *M. phaseolina* was isolated from an infected jute (*Corchorus olitorius*) plant (cultivar JRO 524) at the research farm of Central Research Institute for Jute and Allied Fibres, Barrackpore, Kolkata, India. The fungus culture was maintained at 25°C on potato dextrose agar (PDA). For isolation of DNA from *M. phaseolina*, a 5-mm disc of 2-day-old culture was grown for 7 days at 25 ± 1°C in a 250 ml conical flask containing 50 ml potato dextrose broth (pH 5.5). The mycelia were filtered through Whatman No.1 filter paper and DNA was isolated by the CTAB method following Biswas *et al*. (2013).

Naturally infected *tossa* jute plants were used in the present study. The samples were collected in July 2013 from the experimental field of the Central Research Institute for Jute and Allied Fibres (CRIJAF), Kolkata, India (22.45ºN, 88.26ºE; 3.14 m above msl). Samples were collected from infected plants showing symptoms as well as from asymptomatic healthy plants. The samples were stored at –80°C.

Different components and their concentration in lysis buffer systems were optimized using cell lysis method (Li *et al*. 2011) with major modifications. First, leaf samples (both infected as well as healthy) were cut into small pieces (<3 mm). Leaf sections were washed with 95% ethanol for 5 min. Two or three leaf sections were placed in a 1.5 ml eppendorf tube and 200 µl of lysis buffer was added and incubated at 65°C for 25 min. The optimized buffer system was composed of 20 mmol l^{-1} tris (hydroxymethyl aminomethane (Tris)-Cl (pH 8.0), 1.5 mmol l^{-1} ethylene diamine tetra acetic (EDTA) (pH 8.0), 1.4 mol l^{-1} sodium acetate and 200 µg ml^{-1} proteinase K. Then, 3% PVP (w/v) and β-mercaptoethanol (1% w/v) were added to the buffer. The incubated sample was centrifuged at 12,000 × *g* for 15 min and the lysate was carefully removed. Lysate was used for PCR amplification.

For stems, a very small amount of tissue was taken and the stem samples were cut into sections < 1.5 mm and washed with 100% ethanol for 10 min. The composition of the lysis buffer was 50 mmol l^{-1} tris (hydroxymethyl aminomethane (Tris)-Cl (pH 8.0), 10.5 mmol l^{-1} ethylene diamine tetra acetic (EDTA) (pH 8.0), 3.0 mol l^{-1} sodium acetate and 300 µg ml^{-1} proteinase K. β-mercaptoethanol (2% v/v) was added in buffer which helped to remove secondary metabolites. Three hundred µl of preheated (65°C) lysis buffer was added to the sample and initially incubated at 65°C for 5 min followed by incubation at 60°C for 25 min. The incubated sample was centrifuged at 11,000 × *g* for 15 min.

For isolation of DNA from *M. phaseolina*, monoconidial culture was grown in PDA broth (pH 5.5) for 7 days at 25 ± 1°C. The mycelia were filtered through Whatman No.1 filter paper and a small amount of mycelia was used for study. Composition of lysis buffer was 20 mmol l^{-1} tris (hydroxymethyl aminomethane (Tris)-Cl (pH 8.0), 2.5 mmol l^{-1} ethylene diamine tetraacetic (EDTA) (pH 8.0), 1.4 mol l^{-1} NaCl and 2 µl of 5U/ µl lysozyme. 200 µl of preheated (65°C) lysis buffer was added to the sample and initially incubated at 65°C for 5 min followed by incubation at 60°C for 15 min. The incubated sample was centrifuged at 10,000 × *g* for 10 min and the lysate was carefully removed. Lysate was used for PCR amplification.

Lysate was extracted from naturally infected as well as healthy stems and leaves and was assessed by PCR using *M. phaseolina* specific primer pair MpKFI (5′-CCG CCAGAGGACTATCAAAC-3′) and MpKRI (5′CGTC CGAAGCGAGGTGTATT-3′) (Babu *et al.* 2007). Pure *M. phaseolina* lysate was used as positive control. The volume of PCR was 25 µl, which contained 10 × expanded high fidelity buffer (10 mmol l^{-1} Tris–HCl, 50 mmol l^{-1} KCl, pH 8.3) (New England Biolabs, Beverly, MA, USA), 25 mmol l^{-1} MgCl2 (Biolabs), 200 mmol l^{-1} of each dNTP (Biolabs), 0.5 µl mol l^{-1} of each primer (forward and reverse primer), 10 ng µl^{-1} genomic DNA and 5 U µl^{-1} Taq DNA polymerase. The amplification was carried out using a gradient thermal cycler (Bio-Rad, Hercules, CA, USA). The amplification profile was 5 min initial denaturation at 94°C, denaturation at 94°C for 1 min, annealing at 56°C for 60 s and elongation at 72°C for 45 s followed by 30 cycles and a final elongation at 72°C for 7 min. PCR products were visualized in 1.6% agarose gel stained with ethidium bromide.

In the present study jute stem rot pathogen *M. phaseolina* could be detected from a leaf as well as a stem by direct PCR without prior DNA isolation. All the infected leaf and stem samples were amplified generating 350 bp amplicons (Fig. 1). The 350 bp amplicons from five stem-rot-infected jute samples and

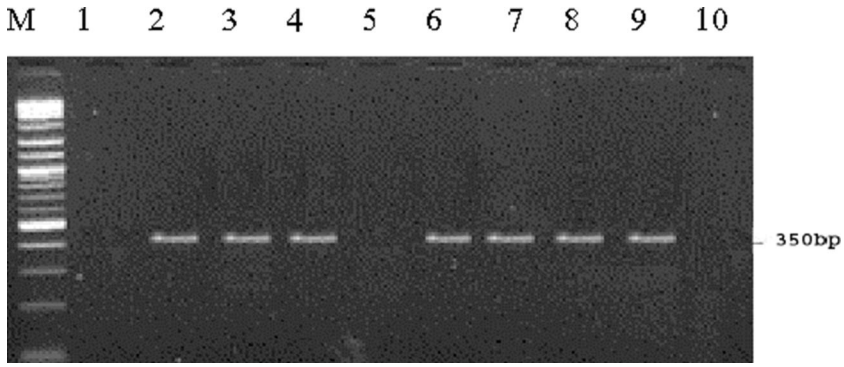

Fig. 1 Direct PCR amplification of *Macrophomina phaseolina* infected stem and leaf samples of jute. Lane M: 100bp ladder; Lane 1: healthy stem sample; Lanes 2–4: infected stem samples; Lane 5: healthy leaf sample; Lanes 6–8: infected leaf samples; Lane 9: *M. phaseolina* pure culture; Lane 10: water control

M. phaseolina pure culture PCR samples were eluted through PCR clean-up system (Promega, Madison, WI, USA) and cloned. Then the clones were sequenced. BLASTn analysis revealed that the CRIJAF isolate had 100% identity with Chile isolate 4 consisting of internal transcribed spacer 1 and partial sequence of 5.8S ribosomal RNA gene and complete sequence of internal transcribed spacer 2 (Accession No: JX203633).

To detect the sensitivity of the dPCR method, the lysate from 1 mg of infected leaf and stem sections was diluted in different concentrations, *viz*., 1:500, 1:1000, 1:1500, 1:2000, 1:2500, 1:3000, 1:3500 and 1:4000. The differentially diluted lysates were used as templates in PCR under the same PCR conditions. DNA could be amplified from the lysate prepared from 1 mg tissue up to 1:3000 dilutions in the case of a stem sample (Fig. 2). In the case of a leaf sample, the detection limit was 1:3500 (Fig. 2).

PCR has become a very popular technique in medical as well as agricultural research (Gindro *et al.* 2005). It has applications in research concerning disease diagnostics, plant diversity analysis, marker-assisted selection, genetic purity testing, etc. PCR generally requires prior isolation of good quality pure DNA and involves many steps which make the whole process time consuming and expensive. Some direct PCR methods which obviate these preparatory steps from PCR-based amplification have been developed (Bellstedt *et al.* 2010; Sharma *et al.* 2012), mainly to amplify plant genomic DNA. Direct PCR has been very useful in detecting different bacteria, including *E. coli,* from untreated environmental samples (Fode-Vaughan *et al.* 2001, 2003; Olive 1989). However, very few plant pathogens have been reported to be detected by direct PCR from infected plants. In the present investigation the stem rot pathogen of jute – *Macrophomina phaseolina* – could be detected by a direct PCR method from an infected leaf as well as stem. The method did not require tissue grinding; instead, leaf bits were placed directly in a lysis buffer and then the lysate was used as the PCR template. This is the first report of direct PCR detection of *M. phaseolina* without DNA isolation. This

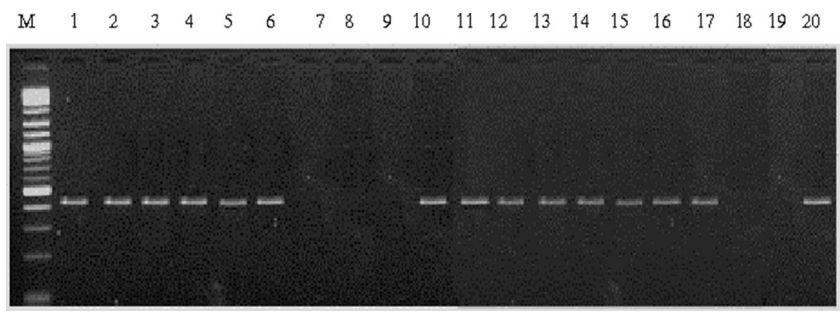

Fig. 2 Detection limit of Direct PCR in *Macrophomina phaseolina*-infected stem and leaf samples of jute. Lane M: 100bp ladder; Lanes 1–8: Different dilutions of the stem lysate used as PCR template, *viz.,*1:500, 1:1000, 1:1500, 1:2000, 1:2500, 1:3000, 1:3500 and 1:4000, respectively; Lane 9: negative control; Lane 10: positive control; Lanes 11–18: Different dilutions of the leaf lysate used as PCR template, *viz*., 1:500, 1:1000,1:1500, 1:2000, 1:2500, 1:3000, 1:3500 and 1:4000, respectively; Lane 19: negative control; Lane 20: positive control

rapid and low cost method would enable easy and early detection of the dreaded pathogen so that the control measures can be adopted in time to be effective.

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