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PCR based early detection of *Ganoderma* sp. causing basal stem rot of oil palm in India

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Basal stem rot (BSR) is one of the most serious diseases of oil palm and the incidence could be as high as 25 per cent (Singh, 1991; Ariffin *et al*., 2000). In India, the disease is caused by either *G. lucidum* or *G. applanatum* (Anonymous, 2008). Cross infectivity of this pathogen is already reported (Turner, 1981; Mandal *et al.,* 2003) and the BSR occurrence in oil palm in India is noticed where under planting is done in the infected coconut gardens. The disease is rapidly spreading in the oil palm growing states of India. One of the major problems associated with this disease management is the lack of early diagnosis. By the time symptom appears, the palm is beyond recovery. If early identification of infected palms was impossible, they can be treated and the disease can be managed effectively. Hence an early detection technique is felt essential for effective management of basal stem rot in oil palm.

Ganoderma is a soil borne fungi under the family of Ganodermataceae, which mostly cause infection through the root. Hence the inoculum can be detected first in the root than from any other part of the palm. In the present study, a PCR based confirmatory diagnostic technology for detection of *Ganoderma* has been attempted using the root samples of oil palm.

To standardize the PCR protocol for identification of *Ganoderma*, BSR affected palms were located and infected tissues were collected from the base of the stem. *Ganoderma* isolates were purified in 2 per cent potato dextrose agar (PDA) medium in Petri plates. Mycelia (200 mg) from two weeks old *Ganoderma* isolates were used for DNA extraction using the miniprep method using 1 mL pre-heated DNA extraction buffer containing 50 mM Tris-HCl, 10 mM EDTA, 100 mM NaCl and SDS 1 per cent. Crude DNA preparation was treated with RNase and phenol:chloroform (1:1) for purification.

Amplification of the DNA samples was carried out using two sets of virulent *Ganoderma* specific primers - *Gan*1 (5'-TTG ACT GGG TTG TAG CTG-3') and *Gan*2 (5'-GCG TTA CAT CGC AAT ACA-3') (Utomo and Neipold, 2000); and *Gan* ET (5'-GAG TTG TCC CAA TAA C-3') and ITS3 (5'-GCA TCG ATG AAG AAC GCA GC-3'), which was derived from the ITS region of *Ganoderma* (Bridge *et al*., 2000). DNA (5 ng), dNTPs (50 mM each), primers (5 ng each) and *Taq* (0.5 U) polymerase were mixed to a reaction volume of 12.5 µL for amplification. PCR condition for both the sets of primers was set as 95 ^{O}C - 5 min.; 40 cycles of 94 ^{O}C- 35 sec., 52 ^{O}C- 35 sec., 72 ^{O}C- 40 sec.; finally 72 ^{O}C - 10 min for amplification. To the amplified product, 1.5 µL bromophenol blue dye was added and 10 µL sample was electrophoresed in 2 per cent agarose gel. The gel was stained with ethidium bromide and viewed in a gel documentation system.

All the *Ganoderma* isolates showed amplifications with both the primer pairs. *Gan*1 - *Gan*2 produced a 167 bp DNA fragment and GanET-ITS3 produced a 320 bp DNA fragment. However,

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the 320 bp fragment was more prominent. To detect the presence of infection, healthy looking root samples from infected palms were collected and DNA was extracted using the method mentioned above as well as by CTAB method. DNA preparation was purified using RNase and phenol:chloroform (1:1) and subjected to amplification by PCR using the procedure mentioned above. But no amplification could be noticed. The possible reason for non-amplification might be due to insignificant amount *Ganoderma* DNA present in the DNA preparation in comparison to the plant DNA.

Thereafter, the healthy looking root samples from infected palms were surface sterilized for 1 min with 0.1 per cent mercuric chloride and cut in 1 cm pieces. The root pieces were cultured on 2 per cent PDA media in Petri plates. They were incubated at 28 ^{O}C temperature for 5 days and mycelial growth was visible on and around the root pieces (Fig. 1). Visible mycelial growth was not prominent in all the cases; however, the purpose was to proliferate the organism present in the root for a period of 5 days. Surface of the root pieces, the surrounding surface area of the PDA media were scraped out gently along with mycelia (wherever present) with a sterile blade and around 50 mg of material was used for DNA extraction using miniprep method described earlier. Both the primers were used for DNA amplification and PCR products revealed the desired fragment of 167 bp and 320 bp by *Gan*1-*Gan*2 and *Gan*ET-ITS3 primers respectively (Fig. 2). It is important to note that, when root samples were incubated for 3 days and followed the procedure, amplifications were noticed

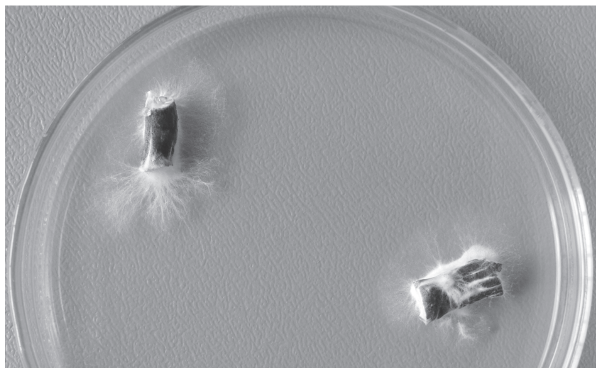

Fig. 1. Root samples showing mycelia growth, five days after inoculation

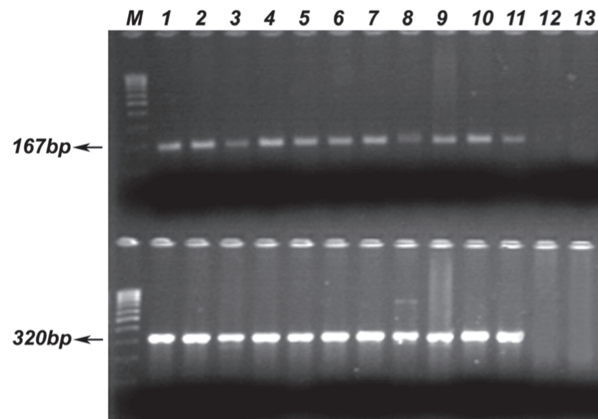

Fig. 2. Amplification of *Ganoderma* samples using *Gan*1-*Gan*2 and *Gan*ET-ITS3 primers. Legend: Lane M: 100 bp ladder; Lanes 1 to 10: 167 bp (top) and 320 bp (bottom) fragments produced by amplification of DNA extracted from the material surrounding root samples (healty looking root from BSR infected palms) 5 days after incubation in 2% PDA using *Gan*1 - *Gan*2 and *Gan*ET – ITS3 primers respectively. Lane 11: positive control (DNA from confirmed Ganoderma isolates); Lane 12: Negative control.

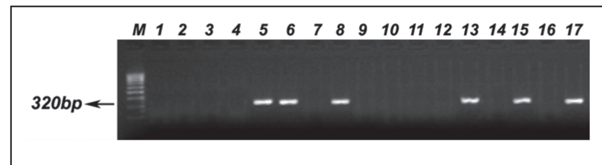

Fig. 3. Amplification of random root samples using *Gan*ET-ITS3 primers. Legend: Lane M: 100 bp ladder; Lanes 1 to 15: amplification of DNA extracted from randomly collected root samples, 5 days after incubation in 2% PDA. Lane 16: negative control; Lane 17: positive control.

in most of the cases and in all the cases with prominence when incubated for 4 days. However, 5 days incubation is recommended with an allowance of an additional day.

Subsequently, 15 root samples were collected randomly from the healthy looking oil palms from different basal stem rot affected gardens located in West and East Godavari districts of Andhra Pradesh and they were incubated for 5 days in 2 per cent PDA medium as mentioned above. DNA from those samples was subjected to PCR amplification using *Gan*ET-ITS3 primers (Fig. 3). Five samples out of fifteen showed the presence 320 kb fragment. The same samples were reconfirmed using *Gan*1- *Gan*2

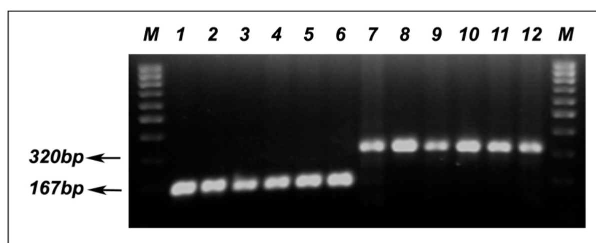

Fig. 4. Confirmation of *Ganoderma* infection in the healthy looking root samples using *Gan*ET-ITS3 and *Gan*1-*Gan*2 primers. Legend: Lane M: 100 bp ladder; Lanes 1 to 5: amplification of random root samples having *Ganoderma* infection using *Gan*ET – ITS3 primers; Lanes 6: positive control using *Gan*ET – ITS3 primers; Lanes 7 to 11: amplification of same samples (lane 1-5) using *Gan*1 – *Gan*2 rimers; Lanes 12: positive control using amplification using *Gan*1 – *Gan*2 primers.

primers also (Fig. 4). When root bits were incubated for 5 days in PDA media, the negative samples also showed some mycelial growth. This might be due to the presence of some other soil borne fungi, but definitely not *Ganoderma* as confirmed by PCR.

Some progress on development of precise technique for early detection of *Ganoderma* were reported through enzyme-linked immunosorbent assay (ELISA) using polyclonal antibody and PCR techniques elsewhere (Utomo and Neipold, 2000; Darmono and Suharyanto, 1995). In India, BSR in oil palm was reported only from Tripura state (Sankaran *et al*., 2005). But the disease may be prevalent, which goes unrecorded, as sporocarps are not produced during early stage of infection. However, incidence of the disease in oil palm was noticed during 2002 (Kochu Babu and Mandal, 2003) from Andhra Pradesh and Kerala. Thereafter, it is increasing in alarming way, especially in the East and West Godavari districts of Andhra Pradesh, which necessitates the need for an authentic early diagnostic tool. This report validates and confirms the PCR based early detection of *Ganoderma* causing basal stem rot disease in oil palm, which is accurate and robust. The significance of the study lies on routine survey and random root samples collection from different oil palm gardens. Early detection would be possible three to five days after collection of samples by using this methodology. Subsequently, appropriate control measures can be taken up to save the affected palms as well as spread of the disease from one palm to other. Thus, this is an important step in managing BSR disease of oil palm in the country.

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