PCR TECHNIQUE FOR DETECTION OF *GANODERMA*

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The basal stem rot (BSR) caused by species of *Ganoderma*, a basidiomycete fungus, remains the major disease of oil palm in Southeast Asia. In Malaysia, serious incidence of BSR is no longer restricted to Peninsular Malaysia, but also in Sabah and Sarawak (Ariffin and Idris, 2002). Diagnosis of *Ganoderma* infection in oil palm is based on the presence of basidiomata of the pathogen on the stem base, or frond bases or roots. To facilitate various studies on *Ganoderma* in oil palm, Ariffin and Idris (1991) have developed the *Ganoderma*-selective medium (GSM) that could selectively isolate the pathogen from any parts of infected tissues, directly from the field, with or without surface sterilization. With GSM, it was possible to detect more oil palm infected with *Ganoderma* not showing any obvious symptom of infection externally using drilling technique (Ariffin *et al.*, 1993).

An alternative approach, using molecular techniques, could offer several advantages in providing a more specific and sensitive detection of the target organism in the host tissues. Repetitive DNA polymorphism analysis, oligonucleotide hybridization to amplified ribosomal DNA (rDNA) spacers, and polymerase chain reaction (PCR) amplification of ribosomal DNA and internal transcribed spacer (ITS) regions have been used to detect and identify the pathogen. PCR-based identification has many advantages over other molecular and traditional isolation methods because the procedure is rapid and less time consuming. Several primers for PCR detection of *Ganoderma* in oil palm for diagnosis of BSR have been developed (Bridge *et al.*, 2000; Utomo and Niepold, 2000; Yamoaka *et al.*, 2000). In our laboratory, a PCR primer, the PER44-123, was constructed from ITS region rDNA and used with LR1 primer to yield an approximately 580 bp product specific to *Ganoderma boninense* (Yamoaka *et al.*, 2000). We show here that the amplification of *Ganoderma* DNA by the PCR method is a reliable and convenient way to detect and identify different isolates of this pathogen to oil palm.

FUNGAL ISOLATES

The isolates used in this study are shown in *Figures 3* and *4*. The fungal cultures were grown on potato dextrose agar (PDA) or malt extract agar (MEA) in standard 9 cm sterile petri dishes and incubated in an incubator at 23ºC-28ºC. After five to seven days of incubation, plugs of mycelium (7 mm diameter) were cut from the actively growing mycelium and transferred to petri dishes or conical flask each containing 25 or 50 ml of czapek dox medium or glucose yeast extract medium. Each petri dish or conical flask was seeded with three mycelial plugs and incubated at 23ºC-28ºC for five to seven days. The mycelial cultures were harvested by vacuum filtration through a Buchner funnel, rinsed with cool sterile distilled water (4ºC), and the mycelium collected on sterile Whatman No. 4 filter paper and then blotted dry between sterile Whatman No. 1 filter paper for 2-3 hr at room temperature. The fresh mycelia were then frozen at -20ºC for 1-2 hr and then freeze dried (-40ºC) for one to two days. The samples were stored in freezer at -20ºC until required.

DNA EXTRACTION AND PCR AMPLIFICATION

Total genomic DNA was extracted using a modification of the method of Idris (1999) and a polyvinyl pyrolidine/cetrimide of Cubero *et al.* (1999). Amplifications were performed in a DNA thermal cycler (Perkin Elmer, Model 9600 series) programmed as described by Yamaoka *et al.* (2000). PCR products were resolved in a 1% agarose gel and visualized with ultraviolet light after ethidium bromide staining.

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SENSITIVITY OF DETECTION

DNA of *G. boninense* was extracted from mycelium and was quantified using a spectrophotometer. The DNA was diluted to 1, 5, 10, 50, 100, 500 and 1000 pg/ul. One microlitre of each dilution was used for PCR reactions as described. The targeted DNA of *G. boninense* was amplified by PCR using PER44-123 and LR2 primers at concentrations of template DNA as low as 10 pg/ul *(Figure 1)*. DNA was not amplified when the template DNA was reduced to concentrations of 5 or 1 pg/ul.

Figure 1. Sensitivity of the rDNA primers PER44-123 and LR1 to detect G. boninense *DNA. Lanes 1-7,* G. boninense *template DNA at 1000, 500, 100, 50, 10, 5 and 1 pg/ul, respectively; and Lane 8, negative control of sterile distilled water. M is 50 bp DNA ladder.*

A product of identical size (580 bp) to that amplified from fungal DNA was produced when primers PER44-123 and LR1 were used in PCR with total nucleic acid extracted from *G. boninense* basidiopores, and diseased oil palm stem and frond base tissues *(Figure 2)*. A dilution series of total genomic DNA from diseased stem tissues was performed. The results indicated that as little as 0.2 ng of template DNA (per g fresh weight) was adequate for detection of *G. boninense*. No amplification product was observed with DNA extracted from healthy tissues. This suggested that the product amplified contained the target sequence of the fungal DNA from diseased tissue. Besides that, a dilution series of basidiopores of *G. boninense* was also created and numbers were confirmed by microscopic counts. Results indicated that basidiospores DNA could be amplified by PCR to give products that could be detected on ethidium bromide-stained agarose gels. PCR products were consistently visible when 1000 or more basidiospores were supplied, although on occasions they were detectable from 100 to 1000 basidiospores.

SPECIFICITY OF PRIMERS

Primer PER44-123 and primer LR1 were used in PCR with genomic DNA extracted from *G. boninense* and 11 others species of *Ganoderma* including *G. philippii, G. lucidum, G. weberianum, G. applanatum, G. oregonense, G. chalceum, G. pfeifferi, G. tornatum, G. miniatocinctum, G. zonatum* and *G. resinaceum*

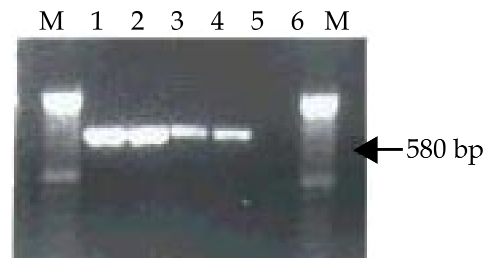

Figure 2. PCR amplification products with primers PER44-123 and LR1. Lane 1, positive control of G. boninense *DNA; Lane 2,* G. boninense *basidiospores; Lane 3, DNA extracted from diseased stem; Lane 4, DNA extracted from diseased frond base; and Lane 5, negative control of sterile distilled water. M is 50 bp DNA ladder.*

(Figure 3). A fragment of approximately 580 bp was amplified with *G. boninense, G. miniatocinctum* and *G. zonatum*, and failed to amplify from other species of *Ganoderma*. DNA from all samples gave product (approximately 167 bp) with the primers Gan1 and Gan2 indicating the presence of amplifiable DNA. As reported by Idris (1999), *G. boninense, G. miniatocinctum* and *G. zonatum* were found pathogenic to oil palm and other species were not pathogenic.

Several other fungal species which are occasionally found saprophytically on rotten palm tissues such as roots, stem and fruit bunches including *Aspergillus, Botryodiplodia, Gymnopilus, Fusarium, Marasmius, Phytium, Penicillium, Pycnoporus, Rhizopus, Rhizoctonia, Schizophyllum, Thielaviopsis, Trichoderma* and *Volvariella*, have been tested using primers PER44-123 and LR1 to check for any *cross-reaction*. A PCR fragment of 580 bp was amplified from isolate of *G. boninense, G. zonatum* and *G. miniatocinctum*, but no amplification product of other fungi used in this study *(Figure 4)*.

CONCLUSION

The PCR detection method described here could be used as a practical screen for detection and identification of *Ganoderma* pathogenic to oil palm. Primer PER44-123 was used with LR1primer to produce a product of approximately 580 bp. Sensitivity of the primers for PCR was high, and DNA was detectable at concentrations as low as 10 pg/ul. The primer was also useful for detection of basidiospores and the *Ganoderma* in naturally diseased tissues.

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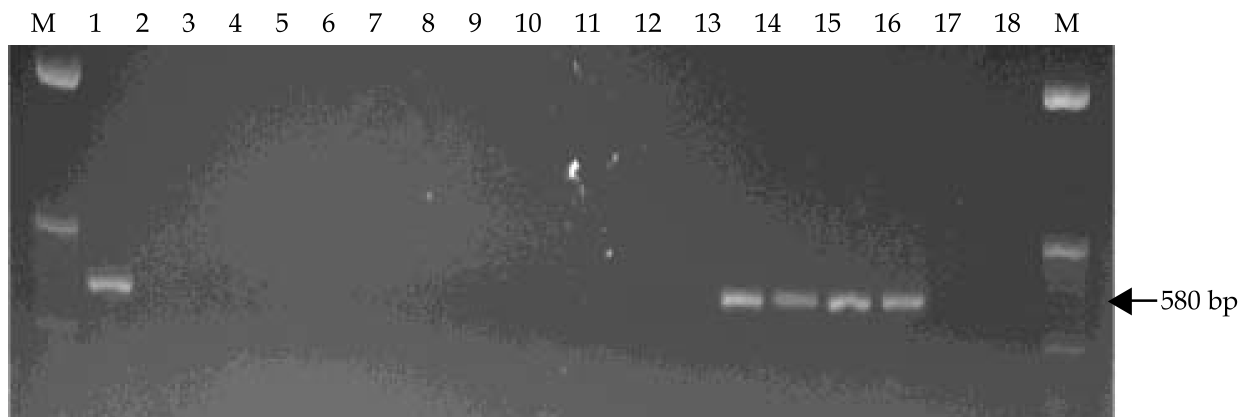

Figure 3. Specificity tests of the rDNA primers PER44-123 and LR1 with DNA extracted from G. boninense *and 11 other species of* Ganoderma. *Lane 1,* G. boninense*; Lane 2,* G. philippii*; Lanes 3-4,* G. lucidum*; Lane 5,* G. weberianum*; Lanes 6-7,* G. applanatum*; Lane 8,* G. oregonense*; Lane 9,* G. chalceum*; Lane 10,* G. pfeifferi*; Lanes 11-12,* G. tornatum*; Lanes 13-14,* G. miniatocinctum*; Lanes 15-16,* G. zonatum*; Lane 17,* G. resinaceum *and Lane 18, negative control of sterile distilled water. M is 50 bp DNA ladder.*

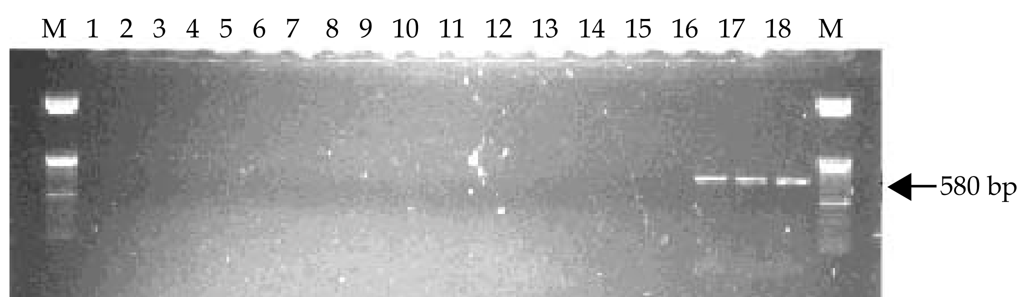

Figure 4. Specificity tests of the rDNA primers PER44-123 and LR1 with DNA extracted from three species of Ganoderma *and 14 saprophytic fungi. Lane 1, negative control of sterile distilled water; Lanes 2-18,* Aspergillus *sp.,* Botryodiplodia theobromae, Gymnopilus *sp.,* Fusarium *sp.,* Marasmius palmivorus, Phytium *sp.,* Penicillium *sp.,* Pycnoporus *sp.,* Rhizopus *sp.,* Rhizoctonia *sp.,* Schizophyllum commune, Thielaviopsis paradoxa, Trichoderma *sp.,* Volvariella *sp.,* G. miniatocinctum, G. zonatum *and* G. boninense. *M is 50 bp DNA ladder.*

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