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Following basal stem rot in young oil palm plantings

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Abstract

The PCR primer GanET has previously been shown to be suitable for the specific amplification of DNA from *Ganoderma boninense*. A DNA extraction and PCR method has been developed that allows for the amplification of the *G. boninense* DNA from environmental samples of oil palm tissue. The GanET primer reaction was used in conjunction with a palm-sampling programme to investigate the possible infection of young palms through cut frond base surfaces. *Ganoderma* DNA was detected in frond base material at a greater frequency than would be expected by comparison with current infection levels. Comparisons are made between the height of the frond base infected, the number of frond bases infected, and subsequent development of basal stem rot. The preliminary results suggest that the development of basal stem rot may be more likely to occur when young lower frond bases are infected.

Key words: *Ganoderma boninense*, molecular diagnostics, ITS, Papua New Guinea

Introduction

The ribosomal RNA (rRNA) gene cluster is found in all eukaryotic organisms. The gene cluster consists of a number of different components that are the subunit genes, the internally transcribed spacers (ITS) and the intergenic spacer (IGS). The genus *Ganoderma* consists of a number of ITS defined groups [1], and the ITS regions for the basal stem rot pathogen (BSR) *Ganoderma boninense* are distinct among these [2].

The taxonomy of *G. boninense* has been extensively studied, and the species has recently been placed together with other palm associated species in a clade based on ITS sequence similarity [1, 3–5]. ITS sequences have been used extensively to determine relationships in numerous fungi and in many cases distinct short sequences have been found that can be used to develop molecular diagnostics [6–9].

The use of ITS sequences is particularly appropriate for the detection of *G. boninense* due to the clear distinctions between ITS clades in the genus, and the ready availability of the comparative sequences. A further important consideration is that *G. boninense* occurs on oil palm as dikaryotic mycelium and basidiocarps, that subsequently give rise to monokaryotic basidiospores. The rRNA gene cluster is generally considered to be resistant to crossover and segregation events, and it can therefore be expected to be conserved through both mitotic and meiotic processes [10, 11].

The ITS2 region is particularly useful in the systematics of *Ganoderma*, and the differences in the sequence of this region can be used to differentiate between individual species. In the oil palm basal stem rot pathogen *G. boninense* there are three different sections at the 3′ terminus of the ITS2 region that are unique to the species. These

short sequences vary in length and GC content, and one is particularly suited to the generation of a polymerase chain reaction (PCR) primer. Based on this information Bridge et al. developed the *G. boninense* specific primer, GanET, from sequence analysis of a range of isolates from oil palm in Papua New Guinea [2, 12].

The GanET primer has been shown to be specific for *G. boninense* when used in a PCR reaction in conjunction with a universal ITS primer. In addition to specificity for *G. boninense* when tested *in vitro* with cultures and herbarium material, the GanET PCR method has also been shown to be capable of detecting *G. boninense* DNA from environmental samples in the presence of DNA from other organisms including oil palm [5, 12].

The GanET PCR method is being used as part of a larger study on the spread and control of *G. boninense* in oil palm in Papua New Guinea. The GanET primer has been used to identify *G. boninense* in samples of palm tissue, and these results have been used to investigate potential infection sites on the oil palm [12]. This study is ongoing and the initial results suggest that recently cut frond bases may provide a site of infection. One of the limitations of a single survey is that the results reflect the situation at the time of the survey, and single time point studies can only provide limited information on the establishment and spread of a pathogen. In order to address this and provide some time series based information, two blocks of oil palms were repeatedly sampled over a 3-year period. The results of the sampling were compared to the age of palm, the location of the fungus on the palm, previous and subsequent sampling, and subsequent development of disease symptoms. This paper reports the results of such a 3-year survey.

Materials and methods

Sampling

Two blocks of palms at the Numundo plantation on the New Britain Palm Oil estates at Kimbe, West New Britain, Papua New Guinea, were selected for this study. One block was made up of 2-year-old palms, and one block comprised of 5-year-old palms. The 5-year-old palms were sited in an area where windrowed debris containing sporulating *Ganoderma* brackets had been recently removed. At the time of sampling the 2-year-old palm site still contained windrowed debris and *Ganoderma* brackets. None of the palms showed any signs of *Ganoderma* infection when sampled. A sampling strategy of removing immediately subsurface internal tissue from the frond bases was used. Sampling was started with one of the most recently pruned frond bases, and extended in a spiral to the base of the palm (see Figure 1). This gave approximately five separate samples from each of the 5-year-old palms, and 2–3 samples from the 2-year-old palms that had shorter stems. In total 191 frond bases were sampled across the two blocks, these were screened with the GanET primer method.

Target frond bases were trimmed back to provide a fresh internal surface between 0.25 and 1 cm inside the frond base, and a further sample of approximately 0.5 cm^3 was taken from the internal tissue. Samples were stored under ethanol or propanol in the field prior to DNA extraction.

Figure 1. Sampling strategy for frond bases showing spiral of frond bases examined after sampling.

DNA extraction and PCR conditions

Samples were removed from alcohol, freeze-dried and ground in a mortar and pestle. Total genomic DNA was extracted from the ground samples using the polyvinyl-polypyrolidone/cetrimide method described by Cubero et al. [13].

PCR amplification of samples was undertaken using the primer GanET [2] together with the universal primer, ITS3 [6]. The reactions were undertaken in 50 μl volumes in a reaction mixture consisting of 4 μl dNTPs (each at 5 mM), 2 μl (10 ng) DNA, 3 μl of 25 mM $MgCl_2$, 5 μl buffer, 2.5 μl GanET (25 pmol), 2.5 μl ITS3 (25 pmol), 30.75 μl H_2O and 0.25 μl *Tth* enzyme (0.5 U).

PCR was carried out in a programmable thermocycler (MJ Research) with a programme comprising of 40 cycles of an initial denaturation at 95 °C for 1 min, followed by an annealing step at 50 °C for 1 min, and an extension step at 72 °C for 1 min. This was followed by 10 min at 72 °C for the final extension. The PCR products were separated by electrophoresis in a 1.2% agarose gel in Tris–acetate–EDTA buffer (TAE), and stained with ethidium bromide.

Results

The GanET/ITS3 primer pair amplified a particular 321 bp region of the ITS2 spacer from *Ganoderma boninense* (see Figure 2). Samples that produced a single band of the same size as that obtained from the DNA of pure *Ganoderma* cultures were considered to be positive.

Year 2000

Twenty-two 2-year palms were assessed and these gave rise to 40 samples. Of these 15% of samples were found to contain *G. boninense* DNA, which equated to 27% of palms being infected. Thirty-one 5-year palms were assessed and 151 samples were screened. 7.3% of the samples were positive for *G. boninense* DNA, and this equated to 25.8% of the palms being infected.

Year 2002

Ten 7-year-old palms that had previously tested positive were re-assessed and 19 samples were obtained. Of these, eight samples were positive, corresponding to six infected palms. Of these six infected palms, one had clearly died and was extensively rotted, and three others showed typical characteristics of *Ganoderma* infection including a basal dry rot. Nine 4-year-old palms were assessed, and this resulted in 18 samples. Two of the samples, each from different palms gave positive results, corresponding to 22% infected palms.

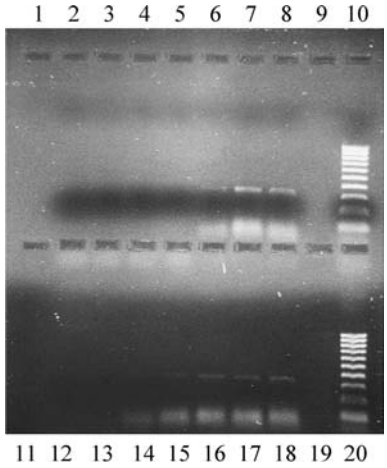

Figure 2. PCR products derived from GanET/ITS3 amplification of total DNA from frond bases of young palm. Lanes 1–5 and 11–14 −ve; lanes 6–8 and 15–18 +ve. Lanes 9 and 19, negative controls; lanes 10 and 20 100 bp markers.

Discussion

The results of the molecular screening clearly indicate that *G. boninense* DNA is present in the palm tissue immediately beneath the cut frond bases, and that this can be detected in asymptomatic palms. Previous studies have shown that *G. boninense* is rarely present in deep palm tissue in asymptomatic palms (unpublished result), and therefore positive results are more likely to indicate that the *Ganoderma* is entering the palm, as opposed to the spread of a deep infection. The number of samples taken each year was reduced so that for 2002, only palms that had previously tested positive were screened. These results showed that the positive results did not always persist, and this is further supported by the number of palms (4) that subsequently showed disease symptoms. This reduction is also illustrated in the results from the block of younger palms, where positive results only persisted in two of nine palms previously infected.

An analysis was made of the numbers of positive samples obtained, and the height of the frond base above ground level. In the 2000 samples, 73% of the positive samples from the 5-year-old palms were obtained from frond bases numbered 3, 4 or 5 above ground level. These correspond to the most recently pruned, and would not necessarily be indicative of an established infection, as the tissue had only recently been exposed. Whereas the lower frond bases had been exposed for much longer and so the persistence of *Ganoderma* on lower frond bases could indicate an established infection. One of the problems in considering the spread and development of any plant disease, and basal stem rot in particular, is the time scale of the total disease process, and the time scale of any movement of the inoculum and the growth of the fungus.

In the 2002 samplings only two out of seven of the positive samples (29%) were from the upper frond bases, and the remainder were from the frond bases near ground level. At this stage palms were beginning to show diseases symptoms and those that had clear infections also gave positive results from the lower frond bases. Similarly, both positive samples obtained from the block of younger palms were from lower frond bases.

Fruiting bodies of *G. boninense* produce considerable numbers of airborne spores in the oil palm plantation environment [14]. These spores are therefore present in the atmosphere in proximity to *G. boninense* fruiting bodies. In the palm blocks sampled here, windrowed material containing *G. boninense* fruiting bodies was present at planting, but was subsequently removed. It is therefore likely that spores were present in the immediate vicinity of the palms when the initial pruning occurred. Although fruiting bodies were removed with the windrowed material, the presence of *G. boninense* in upper frond bases, exposed after the removal, suggests that either *G. boninense* spores persist in the environment, or that there is a continuing influx of spores from other areas. As a result, spores can come into contact with freshly exposed palm tissue, and therefore, there is a possible mechanism for subsequent infection. The reduction in the number of positive samples with time, may reflect a reduction in spore numbers after fruiting body removal, or may suggest that the presence of *G. boninense* in palm tissue does not always persist and lead to subsequent infection and disease. This latter explanation would seem most likely, as some palms that contained *G. boninense* in 2000, gave negative results in 2002, and did not develop disease symptoms. Where palms showed consistently positive results or disease symptoms, the positive molecular diagnosis was predominantly made from lower or ground level frond bases. This suggests that the site of infection may be significant in determining whether the disease develops in a palm. One interpretation of the results obtained, is that cut frond bases are continually exposed to *Ganoderma* spores, even after the removal of obvious infectious material. Failures of *Ganoderma* in the higher frond bases suggest that the main infection route is through the lowest one or two frond bases, and that when the fungus is present in a lower frond base there is a greater likelihood of the palm subsequently developing basal stem rot.

The early 2000 results show relatively high levels of infection of 25–27%, in an area where disease incidence in mature palms from previous plantings was below 3%. This figure may reflect the high levels of spores present in the atmosphere from fruiting bodies in the original windrowed material. Certainly of the 31 5-year-old palms that were initially sampled, only four showed disease symptoms after 2 years (13%), although three others continued to give positive results from lower frond bases. This may indicate that the area sampled was an untypical 'hotspot' for infection, or that the disease incidence rises with subsequent plantings. In the younger palms, none showed disease symptoms, although the 22% of positive palms is very similar to the figures obtained for the older block.

It must be stressed that the results obtained here are from two relatively small data sets, and that they may not be indicative of the situation over a wider scale. However, these results, when linked to the recent findings on spore dispersal [14, 15, 16] indicated that spore inoculation of freshly cut palm surfaces may provide an important disease mechanism. Although the results obtained here must be regarded as preliminary, it may be concluded that it may be beneficial to ensure removal of any infected or potentially infected debris, together with any *G. boninense* fruiting bodies from an area prior to planting out young oil palms and their subsequent early pruning. If possible, early pruning should be delayed as long as possible, and any

early pruning, especially at planting out, could place the palms at risk.

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