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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 lower frond bases are infected.

Introduction

Basal Stem Rot of Oil Palm is caused by the single species pathogen, *Ganoderma boninense* (Pat).

The taxonomy of *G. boninense* has been extensively studied. The species has recently been placed in an ITS based clad together with other palm associated species Moncalvo *et al*., 1995a; b; c; [1,2,3] Moncalvo, 2000 [4]. As the genus consists of a number of ITS defined groups, and the ITS regions for the basal stem rot pathogen (BSR) *Ganoderma boninense* is distinct among these. This genus is a particularly good candidate for ITS based detection Bridge *et al*., 2000. [5] The use of these, as primers have been used to develop species-specific diagnostics as many species-specific DNA sequences have been identified within the ITS regions of various fungi. Bridge and Arora 1994; Edel, 1998 [6]; Levesque et al., 1994 [7]; White *et al*., 1990; [8]; Mills *et al*; 1992 [9]; The ITS regions were looked at, because of the ready availability of the comparative sequences and also because of the success obtained with this approach in other plant pathogenic fungi. The other consideration was, *Ganoderma* on oil palm occurs as dikaryotic mycelium and basidiocarps that give rise to monokaryotic basidiospores. Because the rRNA gene cluster is generally considered to be resistant to crossover and segregation events, it is therefore expected to be conserved through both mitosis and meiosis. Hillis and Dixon; 1991 [10]; Hibbet, 1992 [11];

The ribosomal RNA gene cluster is found in all eukaryotic organisms. The different components of the cluster; the subunit genes, the internally transcribed spacers and the intergenic spacer, have been widely used for generation of a range of molecular diagnostics for fungi.

ITS has 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.

In the genus *Ganoderma*, the ITS2 region is particularly useful, 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 3 different sections at the 3' terminus of the region that are unique to the species. One of these sections is particularly suited to the generation of a PCR primer. Bridge *et al*.2000 [5], Pilotti *et al* 2002 [12]

This primer is specific to *G. boninense* when used in conjunction with a universal ITS primer. DNA from environmental samples was tested and compared to the other *Ganoderma* species. Due to the uniqueness of the primer, DNA and PCR methods were compared to arrive at a protocol that was suitable for detecting the pathogen in the environment.

As part of a larger study on the spread and control of *G. boninense* in oil palm in Papua New Guinea, GanET was used to investigate potential infection sites and rates Pilotti *et al* 2002 [12]. This study is ongoing and the results suggest that recently cut frond bases may provide a site of infection. Time series based sampling is necessary to follow the establishment and spread of the fungus in the plant.

This paper reports the results of such a 3-year survey.

Materials and Methods

Sampling

Having shown that the methodology could detect *Ganoderma* in the frond bases where there was an obvious infection, the work was broadened to examine multiple samples from blocks of young palms over a period of time. Two blocks of palms at Numundo plantation of the New Britain Palm Oil estates 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 6-year-old palms. The 6-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 still contained windrowed debris and *Ganoderma* brackets. None of the palms showed any signs of *Ganoderma* infection when sampled. The sampling strategy of removing immediately sub-surface internal tissue from the frond bases was used. In this case sampling was started with one of the most recently pruned frond bases, and extended in a spiral to the base of the palm. This gave approximately 5 separate samples from each of the 6-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 2 blocks, these were screened with the GanET primer method.

Figure 1. Sampling Strategy for frond Bases.

Before developing the screening procedure, the potential site of infection was needed to be considered, and based on some of the results obtained in the OPRA study, it was decided to test oil palm tissue from frond bases. Initially frond bases adjacent to areas of basal stem rot were sampled to provide positive controls. Target frond bases were trimmed back to provide a fresh Internal surface between 0.25 and 1cm^3 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, once taken to the laboratory; the samples were removed from the alcohol, freeze-dried and ground up using a pestle and mortar.

Total genomic DNA was extracted from the ground up samples using a polyvinyl pyrolidine/cetrimide method Cubero *et al*., 1999 [13]

PCR amplification of samples was undertaken using the primer GanET with a universal primer, ITS3 White *et al*., 1990 [6]. The reactions were undertaken in 50μl volumes in a reaction mixture consisting of; 4μl dNTPs, 2μl DNA, 3μl $Mgcl_2$, 5μl buffer, 2.5μl GanET, 2.5μl ITS3, 30.75μl H_2O and 0.25μl tth enzyme.

PCR was carried out on a programmable thermocycler (MJ Research)

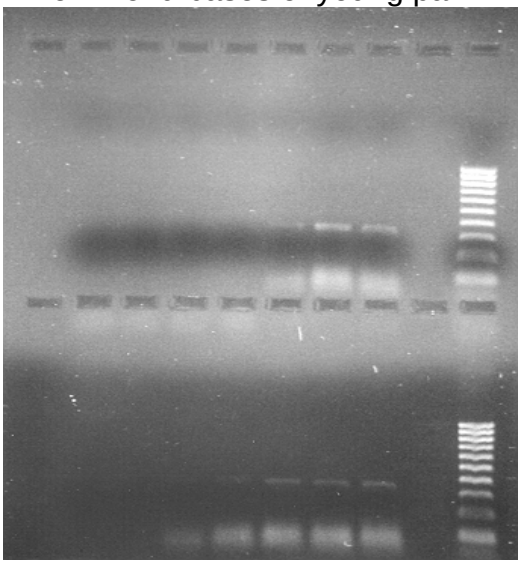
Programme was as follows, initial denaturation 95°C for 1 min, followed by annealing at 50°C for 1min, and extension at 72°C for 1 min, for 40 cycles. This was followed by 10mins at 72°C for the final extension.

The PCR products were separated by electrophoresis in a 1.2% agarose gel in Tris-acetate-EDTA buffer (TAE), and stained with ethidium bromide (1.2μl of ethidium bromide in a 30ml gel).

Results

The GanET/ITS3 primer pair amplifies a particular 321bp region of the ITS2 spacer from *Ganoderma boninense*. 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.

Figure 2. PCR products derived from GanET/ITS3 amplification of total DNA From frond bases of young palm.



Screening

Year	Place	Age of Palm
2000	Numundo	2 year (windrow and brackets present) + 5 year (had windrow + brackets until approx 2 months before sampling)
2002	Numundo	7 year (2 year palms no windrow but No later samples)

Year 2000

Twenty-two 2-year palms were assessed and these gave rise to 40 samples. Of these 15% were found to contain *G. boninense* DNA, which equated to

27% 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, 8 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.

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 a symptomatic palms. Previous studies have shown that *G. boninense* is rarely present in deep palm tissue in a symptomatic 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 2 of 9 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 has only recently been exposed. Whereas the lower frond bases had been exposed for much longer and the persistence of *Ganoderma* 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 2 out of 7 of the positive samples (29%) were from the upper frond bases, and the remainder were from the frond base at 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 Sanderson, 2004 [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 provide the main infection route 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 3 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 Sanderson & Pilotti 1997 [15]; Sanderson et al., 2000 [16]; Sanderson 2004 [14] indicate 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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