Some Latest R&D on *Ganoderma* Diseases in Oil Palm

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Abstract

Diseases caused by *Ganoderma boninense* are considered the greatest threat to sustainable palm oil production in SE Asia but the host-pathogen interaction has not been fully investigated at the cellular level. The pathogen growing from colonised blocks as small as $3cm^3$ could successfully infect oil palm seedlings provided the inoculum was attached to the roots. Reproducible infection of roots with an aggressive isolate GMR3 showed penetration and infection of intact roots, followed by rapid progression throughout the root and then colonization of the lower stem (bole). Transmission electron microscopy showed invasion of the root cortex, with no evidence of selective progression through the vascular system or lacunae. In newly colonised tissue, the fungus appeared as a hemibiotroph, with numerous large hyphae occupying entire cells. In the bole, this led to a complete depletion of previously abundant starch grains. Subsequently, in the roots and colonized bole widespread, necrotrophic, enzymatic attack of all layers of the host cell walls occurred. A third developmental stage was the formation of an extensive, melanized, tough mycelium or pseudo-sclerotium which surrounded roots and comprised many very thick-walled cells. Macroscopic observation of the bole of randomly felled, commercial palms provided confirmatory evidence that multiple infections originated in the roots before spreading into the palm base. The pathogen did not grow well in soil (except when sterilised) and is therefore unlikely to spread from palm to palm by mycelial extension. It is likely to remain in debris to avoid competition from natural soil microflora. The field implications of these studies is discussed.

Introduction

The greatest threat to sustainable oil palm production in South East Asia is from *Ganoderma* diseases, caused by the white rot fungus *Ganoderma boninense* (Flood *et al.,* 2000). Basal stem rot (BSR) is the most common manifestation of *Ganoderma* disease in the region. BSR is characterised by a decay of the bole, production of aerial symptoms such as multiple spears and production of brackets or fruit bodies on the base of the trunk. In severe cases, the palm falls over.

Most severe losses from BSR occur in Indonesia and Malaysia with lower incidences being recorded in Africa, Papua New Guinea and Thailand (Idris *et al*., 2004). In Malaysian coastal areas an average of 50% yield losses from 80% of 13-year-old plantings was recorded by Lim *et al.* (1992). The disease has long been known in these coastal areas, but a survey has also reported typical levels of disease incidence of 30% on 13-year-old palms in both inland and peat soils (Rao *et al.*, 2003). In Indonesia, estates encounter similar problems. In North Sumatra, by the time of replanting (25 years) 40-50% of palms are lost in some fields (Sumatra Bioscience data base) with the majority of standing palms showing disease symptoms but where oil palm stumps were left in the ground at replanting then more serious palm losses due to *Ganoderma* have been observed in some fields (up to 25% occurred within 7 years**) (**Subagio & Foster**,** 2003). Losses begin to have a financial effect once the disease affects more than 10% of the stand (Hasan & Turner, 1998). On average there is a decline of the yield of the fresh fruit bunch (FFB) of 0.16t/ha for every palm lost, and when the stand had declined by 50% the average FFB yield reduction was 35% **(**Subagio & Foster**,** 2003).

There has been a general consensus within the oil palm industry that the primary route of infection for BSR is through roots, as is the case with other basidiomycete root-invading pathogens, notably *Heterobasidion annosum* (Woodward *et al*., 1998), cause of root and butt rot of conifers in temperate regions and *Armillaria* spp., which are pathogenic to numerous tree species (Onsando, 1997). Thus, any woody material colonised by *G. boninense* is a potential source of inoculum for the current stand and any colonised debris left from one stand might provide inoculum for the next planting (Hasan & Turner, 1998). Consequently, much emphasis

has been placed on land preparation at replanting time with different practices favoured by different estates (Flood & Hasan, 2004), although in North Sumatra, boles and stems from the previous stand are generally uprooted and windrowed. Resistant material holds great promise for future management of BSR in oil palm in S E Asia (Idris *et al*., 2004; Durand-Gasselin et al., 2005; Breton *et al*., 2006; Breton *et al*., 2009a;) and there is ongoing work in Sumatra Bioscience and Socfindo (see Breton *et a*l 2010; Setiawati, *et al*., 2010- this volume). Biological control of the pathogen should also be considered as part of any management strategy (Susanto *et al.,* 2005)

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In this paper, we will present some information on the mode of infection of the pathogen into oil palm roots under controlled glasshouse conditions and by observing naturally infected palms in the field in Indonesia. We also report some experimental work on the growth of the pathogen through soil and frond debris and the discuss implications of our results to the field situation.

Materials and methods

Fungal isolates and growth

For full details of the *Ganoderma boninense* isolates used see Rees (2006). Isolates were collected from various Lonsum Estates in North Sumatra. Isolate (GMR3) was selected for studies on infection of palm seedlings as it was a very aggressive isolate. It was isolated from an infected oil palm in Gunung Malayu Estate in North Sumatra using *Ganoderma* Selective Medium (GSM) (Ariffin & Seman 1991) and maintained on Potato Dextrose Agar (PDA) as described previously by Rees *et al*. (2007). BLRS1 and GMB3 were obtained from brackets from Bah Lias estate and Gunung Malayu Estate respectively.

Oil palm seedlings

18th month old oil palm seedlings were used for inoculation/ infection studies and were commercial Deli dura x AVROS pisifera crosses supplied by Sumatra Bioscience, North Sumatra. Palms were maintained under glasshouse conditions as described by Rees *et al*. (2007).

Preparation of wood blocks and inoculation of oil palm seedlings

Oil palm and rubber wood blocks were obtained from BLRS, North Sumatra and treated by a method adapted from Adaskaveg *et al*. (1990) as described by Rees *et al*. (2007). Wood blocks were cut to 3 cm^3 and sterilised before inoculation with *G. boninense* and incubated for 4 weeks. Colonised blocks were then physically attached to oil palm primary roots using Parafilm to facilitate infection. Progress of colonisation of the blocks was monitored as described by Rees *et al*. (2007). After 5 months, infection of the bulb and basal stem followed a typical pattern and images of macro-morphological symptoms were taken and sectioning from disease tissue for Transmission Electron Microscopy (TEM) was undertaken.

Random Felling of Mature Palms

Observations were made of from natural infections in the field. Palms were third replantings (oil palm on oil palm). Symptomless trees of 5, 7, 9, 13, and 15 years were identified. The boles of the trees were dug out and the root mass was observed for signs of the pathogen. The root mass was then removed with axes until the bole tissue could be seen and then this was examined for BSR decay. Also a transverse section was taken across the bole. The trunk was also cut longitudinally to observe any lesion and the boles cut into longitudinal slices to examine the extent of decay.

Transmission electron microscopy

Samples were collected and prepared chemically as detailed in Rees *et al*., 2009. Ultra thin sections were cut with a microtome diamond knife. Cut sections were stained using uranyl acetate and Reynolds lead citrate before viewing by transmission electron microscopy (JEOL TEM1200EX transmission electron microscope).

Growth of *G. boninense* in field soil and organic debris

In vitro growth of *G. boninense* was determined in soil and frond debris (FD) collected from Bah Lias Estate, North Sumatra. Half of the material was autoclaved for 1 h at 121ºC then dispensed into sterile 100 ml polypropylene containers, with the remainder left un-sterilised. *Ganoderma boninense* previously grown on sterile wheat grains (boiled for 5 min to hydrate) for 7d at 28C was then used as inoculum. To ensure that lack of aeration did not limit growth, tubes were

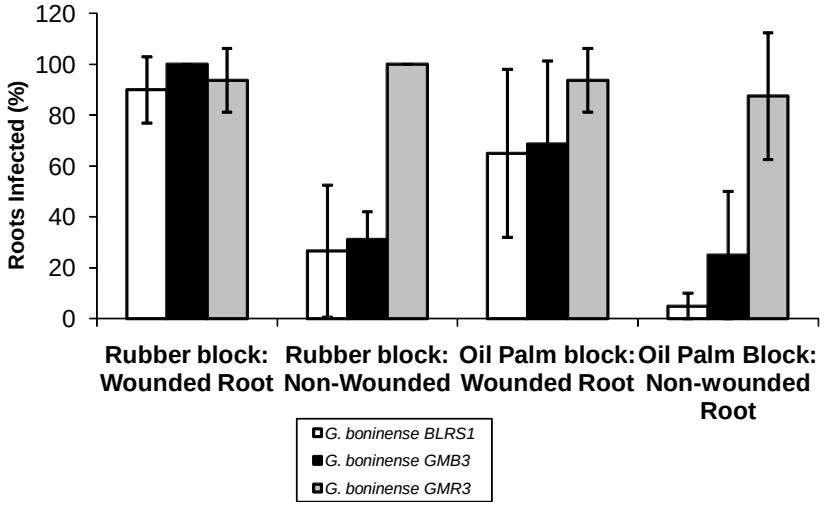
inoculated either at the top of the tube near the cap or at the bottom. In addition, cut oil palm root segments (3 x 2 cm primary roots) were placed in the centre of half of the containers (randomly selected) of each treatment (autoclaved material/non-autoclaved) to determine if root exudates had any effect on fungal growth. Five replicate tubes were prepared for each treatment and *G. boninense* mean mycelial growth determined after 2, 4, 6 and 8 days.

Results

Studies on the mode of infection of seedlings and field palms.

Various trials were conducted in the UK and Indonesia to examine the effect of size of inoculum on infection of oil palm seedlings. For full details see Rees *et al*., 2007. The larger the inoculum size then the more rapid was symptom development but colonised blocks (rubber wood and oil palm wood) as small as 3cm^3 could successfully infect oil palm seedlings (without wounding) provided these blocks were attached to the roots (Figure 1).

Figure 1: Infection of oil palm seedlings using attached, small, rubber wood and oil palm wood blocks in the presence and absence of root wounding.



Key: *Error bars represent standard deviation of means from roots of five inoculated seedlings.*

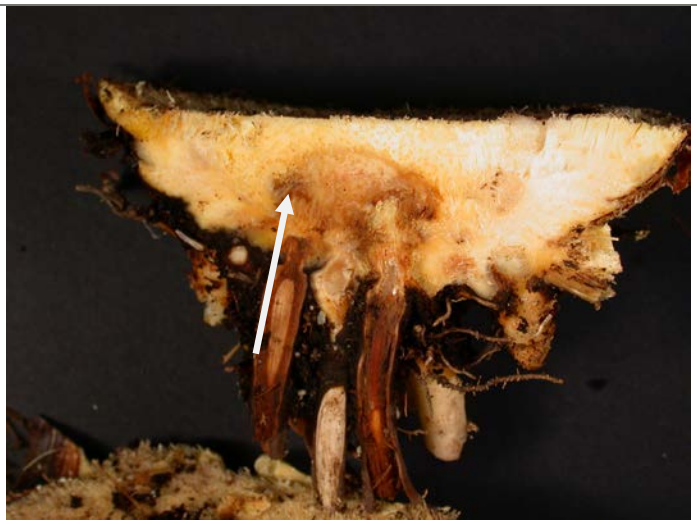
The experiment also highlighted variability in aggressiveness between the isolates; GMR3 was the most aggressive isolate ($p<0.001$) and caused infection in over 80% of seedlings for all treatments including non wounded roots (Figure 1). This isolate was the subsequently used for infection studies. Infection of wounded roots was significantly higher than non-wounded roots ($p<0.001$).

Detailed examination of the mode of infection of *G. boninense* was reported by Rees *et al.,* 2009 using light microscopy and transmission electron microscopy. Root infection occurred subsequent to firm attachment of *Ganoderma* hyphae to the root surface. The attachment was either localised to the initial point of contact or sometimes the fungus completely enveloped the root at the point of contact. The fungal mass often became encrusted and pigmented with melanin, forming pseudo-sclerotia. This extremely tough tissue was often connected to brackets that formed from infected stem bases. Transmission electron micrographs of this material showed that some of the cells had developed massive, lamellated walls and appeared to have lost their cytoplasm, whilst others had normal cell walls and contained typical cytoplasmic organisation.

Following very close contact with the root surface, penetration of the root epidermis and exodermis occurred. Heavily infected tissue within the root was evident on sectioning by brown discoloration primarily in the cortex, but in some instances *G. boninense* mycelium re-emerged through ruptures in the epidermis of infected roots. The advancing edge of infection was determined by use of *Ganoderma*-selective medium (GSM) to isolate the pathogen from the infected tissue, as described by Rees *et al*. (2007). Using this method it was possible to follow the rate of root colonisation by the fungus and examine the progress of infection through the roots and into the lower stem or bole. GMR3 showed rapid colonization through root (necrosis progressed at an average of 4.4 cm/month) (Figure 2a).

The infected region in the stem base showed brown discoloration with the perimeter of the infected region delimited by a darker brown band (Figure 2b). Immediately in advance of infection was often a small area of yellow tissue which has been observed by other authors including Darmono (1998)

Figure 2: Infection of oil palm seedlings roots (a)and invasion into the bole(b)

a)	
(b)	

Key: *Arrow indicate direction of decay from root into the bole. Photographs from Rees (2006)*

Natural infection of mature palms within plantations

Progress of infection from the roots into the bole was also observed by felling of asymptomatic commercial palms in Gunung Malayu Estate, North Sumatra. Palms were felled and their roots removed to observe the bole and observe sites of infection. Random felling of palms from different aged plantings indicated that most palms remain healthy up to 12 years after planting. The boles of all the sampled palms below 12 years appeared unblemished, and pale-cream in colour (Figure 3a), but after this time BSR infection increased in incidence. Figure 3b shows multiple brown disease lesions on the bole tissue after the roots had been excised. Multiple roots appeared to have been infected giving rise to these discrete lesions. Closer observation revealed that lesions clearly progressed from infected roots (Figure 3c and d) to establish in the bole tissue. As infections progress, these separate lesions coalesce forming larger zones of decay over much of the bole tissue. Such extensive internal symptoms were often associated with the onset of foliar symptoms and bracket formation.

Figure 3: Observations on infection of mature palms in the field

c)			d)

Key: *3a) View of bole from healthy palm; 3b) View of bole from diseased palm; 3c) Longitudinal section of a lesion in an infected bole showing decay from infected root(arrowed) spreading into the bole; 3d) Cross- section of a lesion in an infected bole sowing decay spreading from infected root into the bole. Photographs: Flood 2004*

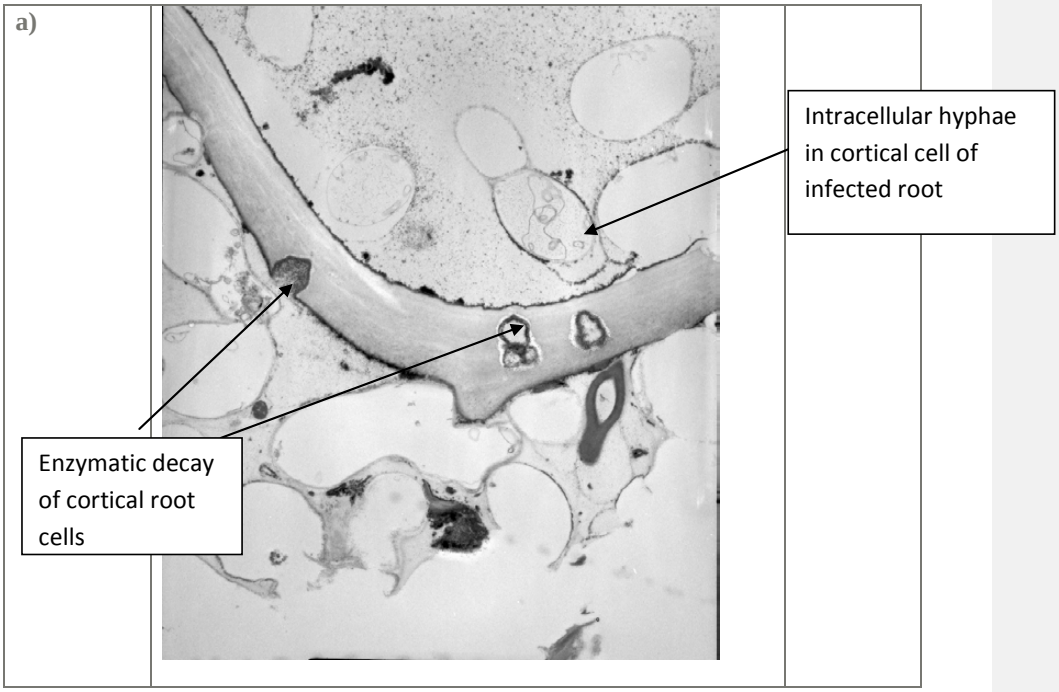
Microscopy of root infection

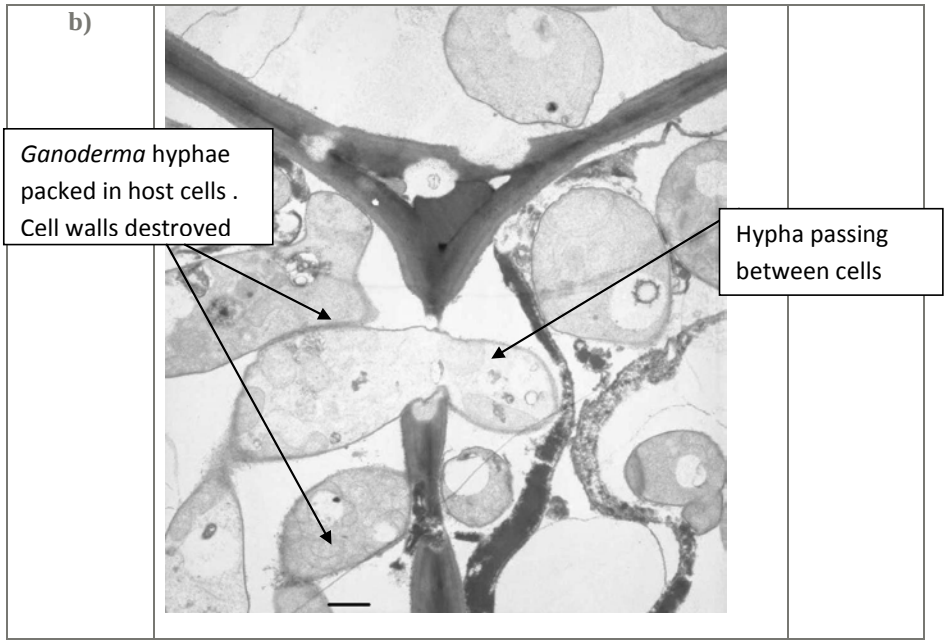
A number of substantial barriers comprising secondary cell walls must be breached for *G. boninense* to gain access to more vulnerable root tissues. For further details see Seubert (1997) and Rees *et al.,* 2009. Penetration of the outer layers of the root by *G. boninense* was not easily viewed microscopically due to the non-synchronous nature of infection. Entry was followed by entry of the hyphae into the more easily degraded inner cortex and longitudinal progression along roots. During early colonisation of host tissue, *G. boninense* appears to act as a hemibiotroph, with abundant, enlarged hyphae within newly colonised cells mainly in the inner cortex. This was followed by considerable breakdown of cortical cell walls (Figure 4a). The cell wall is attacked in multiple localised areas by the pathogen. All cell wall layers were attacked which resulted in the complete breakdown of the cell wall including the middle lamella. This facilitated inter- and intra-cellular and intra-mural colonisation of the oil palm root.

Microscopy of infection of the basal stem

As with observations from colonized root tissue, infected cells from the reaction zone of basal palm tissue were first colonised by numerous, enlarged fungal hyphae before subsequent widespread destruction of the host cell walls (Fig. 4b).

Figure 4: Transmission electron microscopy of cell wall degradation by *G. boninense* during infection





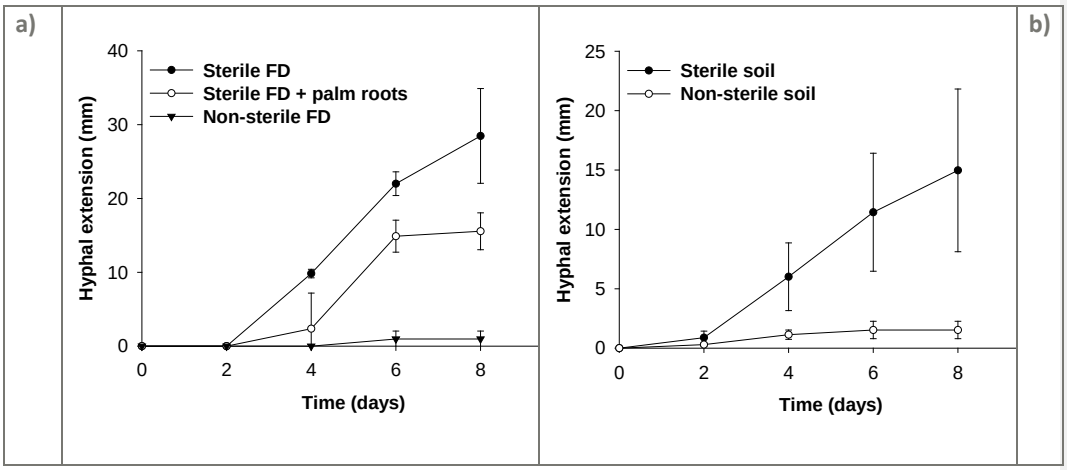
Studies on the colonization of soil and frond debris by *G.boninense*

Investigations were made on the ability of *G.boninense* to actively grow through soil. Flood *et al*., (2002) had hypothesised that the pathogen could actively search for food bases through the soil as with other root-infecting basidiomycetes. Flood *et al* (2002) had also suggested that one way basidiospores could be involved in infection of oil palm was through colonization of debris trapped in frond axils. In order to test these hypothesises, soil and frond debris were collected at Bah Lias Estate and growth of the pathogen in both substrates (sterilised and un sterilised) was investigated.

Unless sterilised, soil and FD were unable to support significant growth of *G. boninense* (Figure 5). Growth in sterilised FD was rapid and evident as mycelial cords emanating from the inoculum source. Hyphae progressed *ca.* 30 mm within 8 days (Figure 5). Growth in sterilised soil was slower and achieved *ca*. 15 mm after 8 days (Figure 5). Oxygenation did not appear to be limiting, as growth was equivalent regardless of inoculum placement at the bottom or top within

growth chambers. *Ganoderma* did not grow in sterilised soil supplemented with roots, whereas sterilised FD supplemented with roots supported good growth in initial stages and had reached 15 mm after 6 days, although as mycelium approached the roots, growth was inhibited.

Figure 5: Hyphal extension of *G. boninense* within sterile and non-sterile frond debris and plantation soil from North Sumatra and the influence of added palm roots.



Key: a) *FD = frond debris, ● = Sterile FD, ○ = Sterile FD + roots, ▼ = Non-sterile FD. Three hyphal extension measurements were made from each growth chamber per time point and error bars represent standard deviation of means from 5 replicates (growth chamber).* ***b)*** *● = Sterile soil, ○ = Non-sterile soil. More soil was available than FD and therefore 20 replicates were used for sampling in this case.*

Discussion

Research presented here provides further evidence for the initiation of BSR infection from a point of primary contact in the roots ultimately penetrating into the basal stem of infected palms. These observations were made under controlled inoculation conditions in glasshouse conditions in the UK and from felling of commercial palms in North Sumatra. The larger the

inoculum the more rapid will be the development of symptoms (Rees *et al*., 2007) but even inocula as small as 3cm^3 will induce disease in unwounded oil palm roots provided that inoculum is in intimate contact with the root. Ariffin *et al* (1995) similarly reported that artificial inoculum only slightly bigger than an average oil palm root could cause infection of seedling oil palms while Singh (1991) traced and demonstrated infection of some palms had been initiated from small bundles of diseased roots from a former stand of oil palm. This has important implications for management of the disease as it is impossible to remove all inoculum sources from the field (Hasan & Turner, 1998). Nevertheless, replanting time offers a good opportunity to remove as much of the inoculum as possible from the field before planting the new generation of palms. If inadequate sanitation is conducted eg where boles were not removed properly in the field, seedling palms can be exposed to large amounts of infected debris close to their planting points (Flood *et al.,* 2000) and will rapidly develop characteristic symptoms.

G. boninense is not a good competitor as demonstrated from the results presented here and by Rees et al (2007) and is thus, very unlikely to actively seek new food sources through mycelial spread as suggested by Flood *et al* (2002). Mycelial spread occurs with other pathogenic root infecting fungi such as *Armillaria spp*. which produce rhizomorphs that are directly involved in infection of roots of susceptible trees species. *G. boninense* appeared to produce monopodially branched rhizomorph-like structures in sterilised soil and palm frond debris (Rees, 2006) but these were not formed in non-sterilised soil or frond debris and there are no reports of rhizomorphs under field conditions.

These experimental studies indicating that *Ganoderma* does not actively grow in non sterilised soil, could also explain previous molecular analysis of *G. boninense* isolates (Miller *et al.* 2000; Miller *et al*., 1999) and somatic compatibility studies (Ariffin e*t al.*, 1996) which indicate high genetic variability of *Ganoderma* isolates within BSR infected palms. If mycelial spread was occurring, then a single genet (individual) could be expected to be seen rather then this high variability. It is highly probable that the pathogen remains present in infected debris (avoiding competition from other soil microflora) and that infection mainly occurs when healthy palm roots come into contact with infected debris (roots or bole debris). Darmono (1998, 2000) has described the presence of sclerotium-like structures which he called *Ganoderma* resting bodies

(1-5 cm across) in oil palm tissue. The resting bodies were capable of forming fruiting bodies and of infecting oil palm seedlings and can provide a survival mechanism for the pathogen in plant tissue from one generation of oil palm to another.

As with other molecular studies, Rees (2006) found isolates from BSR lesions taken from oil palm boles had different genetic profiles ie were different individuals. Multiple infections were seen in the boles of field palms and these originated from multiple root infections. Such multiple infections could account for variability observed in pathogen isolates from palms infected with BSR as hypothesised by Flood *et al.,* 2002. Eventually, these discrete decay lesions coalesce resulting in large areas of decay in the bole and destruction of the elements conducting water/ nutrients which is then manifested as the characteristic aerial symptoms.

Therefore, the need to remove as much of the infected debris as possible from the field remains a key part of management of the disease. Removal of infected palms within the estate as they are identified is one option as is good sanitation at replanting time eg placing material carefully on top of the ground (windrows) as opposed to allowing some of the infected material to remain buried and planting seedlings as far as possible from windrows (Flood *et a*l., 2005). Fallowing for 1year has also been shown recently to significantly reduce infection in the next generation (Virdiana & Flood, 2010, in press). As the pathogen is not a good competitor, allowing the natural microbial population to reduce the effective inoculum at replanting such as by having a fallow period should be considered as a management option for this disease. Further trials on fallowing in combination with other practices are ongoing at Sumatra Bioscience.

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Using TEM microscopy, it was observed that following penetration of the outer barrier layers, *G. boninense* colonisation occurs mainly through the inner, thin-walled cortex. Heavily infected roots often have intact outer cell layers disconnected from an un-degraded stele. Nevertheless, ultimately the thickened cell walls of the outer cortex and the vascular cylinder were also partially destroyed as colonisation progressed and remaining root fragments disintegrated in the soil. *Sariah et al.,* (1994) considered that infection of vascular tissue predominated in the colonization of oil palm by *Ganoderma* although colonisation of all other tissues was observed

but Rees *et al*., (2009) found no evidence of *Ganoderma* preferentially using the vascular tissue, or lacunae, to facilitate progress through the host.

For successful penetration and degradation of intact roots, production of an array of cell wall degrading enzymes (CWDE) is likely to be required to penetrate the outermost tissues, comprising of cellulose, lignin and suberin One month after inoculation, when the pathogen had adhered to the roots, removal of the mycelium revealed bleaching underneath (probably indicative of the oxidative breakdown of lignin and the white rot status ascribed to this genus) (Adaskaveg *et al.,* 1990) and macroscopic evidence of enzymic degradation of the tough root outer cell layers, which was confirmed by transmission electron microscopy (TEM)

Rees (2006) found enzyme activities from *G. boninense* covering all the major structural polymers of oil palm cell walls. TEM of infected roots and bole tissue (Rees *et al.,* 2009) and presented here suggests that CWDEs are important in the extensive degradation of oil palm cell walls during pathogenesis by *G. boninense* and are likely pathogenicity factors for this interaction. Cell wall degradation occurred at discrete locations with attack of all wall layers, including the middle lamella region, which in secondary walls is high in lignin content. Areas of cell wall attack were sometimes not adjacent to fungal hyphae. Attack of plant cell walls, resulting in development of holes through all cell wall layers is indicative of simultaneous wood decay with the pathogen producing enzymes that can attack all cell wall layers. Similar patterns of cell wall attack have been observed from simultaneous degradation of *Laurelia philipiana* wood by a *Ganoderma* sp. (Agosin, 1990) and of date palm wood by both *Ganoderma colossum* and *P. chrysosporium* (Adaskaveg *et al*., 1991; Blanchette, 1984).

Rees *et al* (2009) have further suggested that progress *via* the root cortex to stem base initially appears to involve some sort of hemibiotrophy where the host is colonized without apparent reaction but this phase breaks down into necrotrophy with associated extensive host cell wall breakdown. Infection appears to involve a series of developmental switches. This initial hemi-biotrophic phase in infection of both root cortex and stem base, involves largely intracellular colonization by swollen hyphae. This phase is then followed by an aggressive necrotrophic phase associated with extensive host wall degradation. Hemibiotrophy is a very common strategy for

plant pathogens but to our knowledge is not well described for basidiomycetes. Also the formation of melanized mycelium occurred both within host tissues (possibly in response to host defences) and on a much larger scale external to roots in the form of very tough pseudo-sclerotia. The latter may have survival functions as the empty cells with massive, melanised cell walls enclose thin-walled hyphae with active cytoplasm and from which *G. boninense* could be routinely reisolated.

Observation of *Ganoderma* development and survival in the basal tissue of oil palm suggested the presence of the zones described in oil palm by Darmono (1998) as reaction zones and transition zones. Reaction zones were first described by Shain (1967) as a dynamic interface between living sapwood and decayed wood colonised by a pathogen, but Pearce (1991) and Boddy (1992) suggested that these are static boundaries. They further suggested that when reaction zone boundaries fail, a volume of wood is colonised with little or no expression of characteristic reaction zone responses, until ultimately a new reaction zone boundary is established. *G. boninense* decay of oil palm might support this theory as areas of decayed tissue are delimited by thick-walled melanised hyphae (Darmono, 1998; Hasan *et al*., 2005) possibly in response to formation of static reaction zones. The reaction zone appears to restrict or coincide with fungal advance and may have elevated levels of antimicrobial compounds, as found in the sapwood of many tree species (Pearce, 1996). These putative barrier zones are ultimately breached by the pathogen and rapid progress occurs until formation of the next reaction zone, which results in a concentric-like decay pattern (see Figure 3c). The zones are sometimes delimited by melanized mycelium or pseudo-sclerotium, which presumably perform a protective function.

Immediately ahead of the reaction zone in oil palm is an area that was termed the 'yellow zone' by Darmono (1998). Darmono suggested this zone was a consequence of fungal activity such as enzyme production, but no evidence of fungal activity or cell wall degradation was observed in this study. Instead, ultrastructure revealed that transition zone cells had increased cellular activity with large amounts of vesicular budding at the plasma membrane. Host utilisation of energy reserves such as starch polymers in the transition zone, (starch grains were undergoing substantial modification according to TEM) may contribute to the energy needs of oil palm

defence responses. Water potential is also postulated to affect progress of infection by decay fungi (Rayner & Boddy, 1988). Pearce *et al*. (1997) found by nuclear magnetic resonance (NMR) that water levels were elevated in the reaction zone of *Ganoderma adspersum* decay of *Acer pseudoplatanus* and suggested that build-up of elevated water levels in the reaction zone was probably osmotically driven. Starch hydrolysis around the lesion could be used to generate this condition in the reaction zone. Adaskaveg *et al*. (1991) recorded high levels of starch in date palm wood and we similarly detected chemically very high starch content of oil palm seedlings that was rapidly depleted during early infection (Rees, 2006). Host or pathogen utilization of starch is not clear at this stage, but a very significant energy resource is apparently contributing to this host-pathogen interaction.

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