COMPARATIVE STUDY OF LIGNIN IN ROOTS OF DIFFERENT OIL PALM PROGENIES IN RELATION TO *Ganoderma* BASAL STEM ROT DISEASE

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ABSTRACT

Basal stem rot (BSR) disease caused by Ganoderma boninense *Pat., a serious constraint to oil palm (*Elaeis guineensis *Jacq.) in South-east Asia, namely Malaysia and Indonesia, is also becoming a threat in Africa and Latin America. Currently, no complete management is available although* Ganoderma *tolerant oil palm is likely to have a crucial role in the management of the disease in the future. In this study, oil palm progenies exhibiting different reactions to* Ganoderma *BSR were investigated. Lignin in their roots was detected by phloroglucinol-HCl. The intensity of burgundy red colour developed after staining varied among progenies, indicating differences in lignin content but this was a subjective qualitative approach. A quantitative approach following the modified Klason method was then used. Uninoculated progenies TK 714 and TK 716 were significantly different in lignin content at six to seven months but 10 months later, lignin contents of all uninoculated progenies were similar, while that of inoculated plants exhibited some small but significant differences. Nevertheless, the accumulation of lignin did not correlate well with susceptibility or tolerance to BSR and consequently, lignin content may not be a reliable trait to characterise oil palm progenies for* Ganoderma *tolerance or susceptibility in screening tests.*

INTRODUCTION

Oil palm is the highest oil yielding crop with an average of 5 t of oil per hectare per year, which corresponds to 10 times the yield of soyabean, about

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seven times the yield of sunflower and 6.3 times that of rapeseed. To produce 1 t of oil, oil palm requires less land, only 0.26 ha, while soyabean, sunflower and rapeseed need 2.2, 2.0, and 1.5 ha, respectively (Anon, 2010). In terms of energy balance, oil palm leads many other crops. With a total input of 19.2 GJ ha^{-1} yr^{-1}, oil palm produces 182.1 GJ ha^{-1} yr^{-1}. This corresponds to a ratio output to input of about 9.5, an output achieved by few agricultural systems (Chong, 2010). For example, the output to input energy ratios of soyabean and rapeseed are only 2.5 and 3.0, that is nearly four and three times, respectively, less than that of oil palm. This reflects the crop's efficiency and indicates that oil palm is an

important crop in meeting the increasing demand for oils and fats.

However, the oil palm industry in Asia, especially in Malaysia and Indonesia, which are the leading producers of palm oil, is threatened by basal stem rot (BSR) caused by *Ganoderma boninense* (Idris, 1999; Flood and Hasan, 2004; Pilotti, 2005). The losses due to *Ganoderma* vary from yield reduction to palm death. Yield reduction of up to 46% and losses of 85% of palms have been recorded in Malaysia (Singh, 1991). In Indonesia, 1% disease incidence has been assumed to cause USD 38 million loss annually to the economy (Flood and Hasan, 2004). In Cameroon, 53% losses have been recorded due to both *Ganoderma* BSR and *Fusarium* wilt in 25-year old oil palm stands (Tengoua and Bakoume, 2005). Many interventions have been attempted to manage *G. boninense* but most have proven inefficient or cost-ineffective. These include the use of a balanced fertiliser, N, P, K (Mohd Tayeb *et al.*, 2003), manual application of calcium nitrate (Flood and Hasan, 2004), mounding around the stem base (Wan, 2007), digging of trenches around infected palms (Flood and Hasan, 2004), use of systemic fungicides (Ramasamy, 1972; Jollands, 1983; Khairudin, 1990; Lim *et al.*, 1990), sanitation and clean clearing (Idris *et al.*, 2004; Flood *et al.*, 2005). Breeding for tolerance or resistance to BSR is also advocated as a strategy for managing the disease (Anjara *et al.,* 2013). The underlying basis for resistance/tolerance of oil palm material to BSR remains unclear but progenies with potential tolerance/resistance are suspected to have high lignin content (Paterson *et al.*, 2008) yet no attempt has been made to investigate this.

The role of lignin in plant defence has been extensively and thoroughly investigated in other crops (Hijwegen, 1963; Vance *et al.*, 1980; Nicholson and Hammerschmidt, 1992; Raiskila, 2008; Bhuiyan *et al.*, 2009), but not yet in oil palm. This study was designed to assess the lignin content in roots of six oil palm progenies reported to have different tolerance/susceptibility towards BSR disease caused by *G. boninense.*

MATERIALS AND METHODS

Plant and Fungal Materials

Six oil palm progenies were obtained from the Federal Land Development Authority (FELDA) Agricultural Services Sdn Bhd, Sungai Tekam, Jerantut, Pahang, Malaysia. They were classified by FELDA with regard to tolerance/susceptibility to *Ganoderma* BSR (*Table 1*).

Inoculum Preparation and Inoculation

The rubber wood block (RWB) inoculum was prepared following the method routinely used by MPOB (Idris *et al.*, 2000). Briefly, freshly cut RWB (6 cm × 6 cm × 6 cm) were oven-dried at 80°C for 48 hr and sterilised at 121°C for 30 min. The RWB were then placed individually in a heat-resistant polyethylene bag, with 60 ml of malt extract agar (MEA), autoclaved at 121°C for 30 min and left to cool overnight, with a regular manual rotation to allow the medium to evenly cover the RWB before solidification. After cooling, the RWB were inoculated with a 7- to 10-day old pure culture of *G. boninense* PER 71 (supplied by MPOB). One agar plate was divided into eight pieces (of about 7.95 cm^2) and each RWB received four inoculum pieces (one on each lateral surface). Inoculated RWB were kept in the dark at 28°C for three months to allow external and internal colonisation by *G. boninense.*

The experimentation was conducted at Field 2, Universiti Putra Malaysia campus, Serdang, Selangor, in a shaded nursery with 50% light penetration. Inoculation of 6- to 7-month old oil palm seedlings with *Ganoderma* RWB inoculum consisted of placing the bulb and washed roots of each seedling firmly in contact with an inoculated RWB which was placed in soil (3:2:1 ratio of top soil/sand/organic matter) in a polybag (25 cm × 30 cm). The RWB and roots were covered with soil. The inoculated and un-inoculated seedlings received normal care (daily watering and monthly fertilisation) for 10 months.

TABLE 1. GENETIC BACKGROUND AND RANKING OF THE PROGENIES TESTED

Progeny code	Genetic background	Tolerance/susceptibility to *Ganoderma*
TK 715	GHP 8 × AB 1/10 (Deli *Dura* × *Pisifera* Yangambi)	Most tolerant
KA 4	DR 10/8 × EB 3/20 (Deli *Dura* × *Pisifera* Yangambi)	Tolerant
TK 716	FFP 10 × AB 1/10 (Deli *Dura* × *Pisifera* Yangambi)	Moderately tolerant
SC 5	CDN 95 × CEN 118 (Deli *Dura* × *Pisifera* Yangambi)	Susceptible
SC 10	HQE 36 × ML 161 (Deli *Dura* × *Pisifera* Yangambi)	Susceptible
TK 714	EOA 6 × AB 1/10 (Deli *Dura* × *Pisifera* Yangambi)	Most susceptible

Lignin Histochemical Analysis by Wiesner Reaction

Lignin staining was initially conducted on un-inoculated seedlings. Transverse thin sections of primary roots from each progeny were obtained by carefully cutting with a razor blade between the elongation zone and differentiation zone. Two to three drops of a freshly prepared 2% phlorolucinol-HCl (0.2 g phloroglucinol dissolved in 3.3 ml of 95% ethanol and 6.7 ml of 36.5% HCl) (Morrison *et al.*, 1994) were added. The intensity of burgundy-red colour developed was observed using the microscope Meiji Technology Japan, 5000 and photographed with digital camera Olympus E – 420 associated to the software Quick Photo Camera 2.5.

Lignin Quantification

Determination of lignin content in oil palm seedling roots was performed on un-inoculated seedlings and on inoculated seedlings at 10 months after inoculation using the modified Klason method (Yoshihara *et al.*, 1984). One gramme dry, secondary root samples were ground up to pass through a 1 mm mesh size sieve and then weighed in a 50 ml beaker. The 10 ml of 72% H_2SO_4 were added and stirred with a small glass rod. The mixture was left at room temperature for 2 hr with occasional stirrings. After this first hydrolysis, the mixture was transferred to a 500 ml flask, added with 375 ml of distilled water to make final concentration of H_2SO_4 of 3% and autoclaved at 121°C for 30 min. The acid insoluble lignin was allowed to settle overnight and the solution was vacuum filtered and washed with 250 ml of hot distilled water. Filtration was done using crucibles No. 1, as used in Animal Nutrition Laboratory, Department of Animal Science, Faculty of Agriculture, Universiti Putra Malaysia, for routine analysis of lignin in forage. The weight of the crucibles (W1) was recorded after oven-drying at 105°C for 1 hr 30 min and cooling in a desiccator for 30 min. Other steps were modified to comply with the laboratory conditions. Thus, instead of oven-drying the filtered samples in the crucibles at 110°C for 1 hr, the drying was performed at 105°C for 12 hr to achieve constant weight. Samples were then cooled in a desiccator, weighed (W2) to quantify the ash-mixed Klason lignin (W3 = W2-W1), and incinerated in a furnace at 550°C for 4 hr, cooled and weighed to determine the acid insoluble ash (W3-W1). The ash correction was performed to Klason lignin to get accurate and reliable quantitative summative values. The percentage of ash-corrected acid insoluble lignin (AIL) was calculated by the following formula (Sluiter *et al.*, 2007):

AIL (%) = [W2-W1-(W3-W1)] × 100 / ODW = (W2-W3) × 100/ODW, where

W1 = weight of oven-dry crucible (g); W2 = weight of oven-dry crucible with acid insoluble lignin and acid insoluble ash (g); W3 = weight of oven-dry crucible with acid insoluble ash (g); ODW = weight of oven-dry root sample (g).

A fraction of the filtrate was collected before the washing step, kept in a refrigerator at 4°C for the determination of acid soluble lignin. Five millilitres of the filtrate were thoroughly mixed with 5 ml of 3% H_2SO_4 and the absorbance was recorded at 205 nm with UV-VIS spectrophotometer, Model UV-700 Pharmaspec, Shimadzu Corporation, Japan. Three percent of H_2SO_4 was used as a blank. The percentage of acid soluble lignin (ASL) was calculated by the following formula (Sluiter *et al.*, 2007):

ASL (%) = A × volume filtrate × dilution factor × 100/ε × 1000 × ODW = (A/110) × volume filtrate × dilution factor × 100/1000 × ODW where, A = absorbance reading at 205 nm; ε = 110 is the absorptivity or extinction coefficient. In this formula, the term A × dilution factor / ε × 1000 = A × dilution factor /110 × 1000 = C is the concentration of lignin in g litre^{-1} in the filtrate. The figure 1000 is for the conversion of litre in millilitre. The total lignin (TL) content was therefore the sum of acid insoluble lignin and acid soluble lignin: TL (%) = AIL + ASL.

Experimental Design

The six treatments (inoculated progenies) and their controls (un-inoculated progenies) were arranged in a randomised complete block design (RCBD) with three replications of 12 seedlings per replicate. Data were compared by the analysis of variance (ANOVA) using SAS 9.2. When significant, means comparison was performed by Duncan's Multi Range Test (DMRT) at 0.05 significant level.

RESULTS AND DISCUSSION

Lignin Staining

Progenies SC 5 and SC 10, both known as susceptible, and progeny KA 4 classified as tolerant gave very high intensity of burgundy colour (*Figure 1*), indicative of cinnamyl aldehyde markers of lignin. This constitutes a proof of high lignin content in the roots of those progenies. The intensity of the colouration was low in TK 714, the most susceptible progeny, while TK 715, the top tolerant progeny, and TK 716, the moderately tolerant progeny exhibited a moderate intensity.

The histochemical determination of lignin is a good qualitative method to detect the presence of this compound in plant tissues. However, as indicated by others (Nur Sabrina, 2011), it seems not to be a useful discriminatory tool to confidently compare different treatments or different planting materials
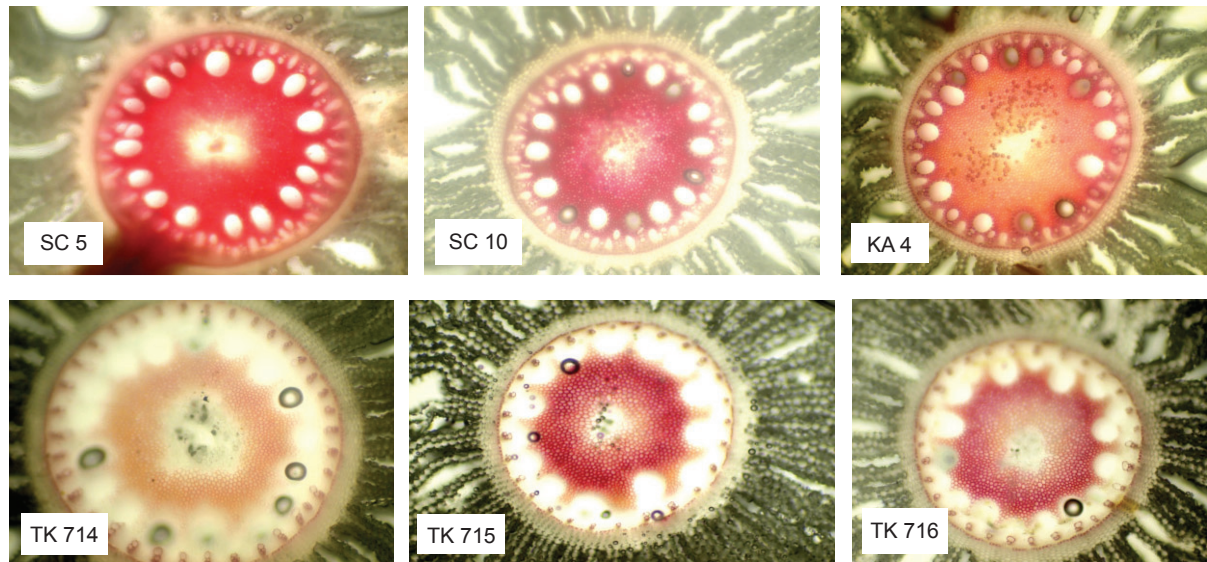
Figure 1. Histochemical staining using Phloroglucinol-HCl for detection of lignin in the roots of six oil palm progenies. Note the variation in the intensity of the colouration.

since it is based on subjective visual assessment; a quantitative appreciation method was needed.

Lignin Quantification

Lignin content in seedlings at six to seven months (prior to inoculation). The analysis of lignin content in the roots of un-inoculated oil palm progenies at six to seven months (*Table 2*) did not reveal any significant difference (P > 0.05) between standard control (SC 5), tolerant and most tolerant progenies. Also, the most susceptible progeny, TK 714, and the moderately tolerant one, TK 716 differed significantly in their root lignin content but did not differ from other progenies. Furthermore, the contrast analysis of susceptible progenies versus tolerant progenies was not significant (P > 0.05), meaning that the lignin content of susceptible progenies did not differ significantly from the lignin content of tolerant progenies. This seems to suggest that in the absence of *G. boninense* attack, lignin quantification in roots might not differentiate susceptible and tolerant oil palm progenies.

Lignin content in inoculated and uninoculated seedlings (10-month post-inoculation). Ten months

post-inoculation (when seedlings were 17 months old), all progenies tested had similar root lignin content to the levels seen in uninfected roots (*Figure 2*). This reinforces our previous hypothesis that lignin quantification in roots of uninoculated seedlings does not adequately discriminate susceptible and tolerant oil palm progenies.

Following inoculation, some differences (some significant and others not) occurred in terms of root lignin content amongst the different progenies. For example, progenies SC 5 (standard control) showed the highest lignin content but was not statistically different from that of progenies SC 10 (standard control), KT 714 (the most susceptible), KT 716 (moderately tolerant). The lignin content of KA 4 (tolerant), the lowest recorded, was significantly different from that of other progenies but similar to that of KT 715 (most tolerant) progeny according to DMRT, but the non significance of paired t-test shows that no significant difference (P > 0.05) exists between inoculated KA 4 progeny and un-inoculated progenies. A general observation from the experimentation indicates that root lignin content of inoculated seedlings was higher than that of uninoculated seedlings. This observation may indicate that when attacked by *Ganoderma*, oil

TABLE 2. LIGNIN CONTENT IN THE ROOTS OF UNINOCULATED OIL PALM PROGENIES AT SIX TO SEVEN MONTHS

	Progenies					
	SC 5	**SC 10**	**KA 4***	**TK 714**	**TK 715***	**TK 716***
Lignin content (%)	31.4 ±0.3 [ab]	31.4 ±1.2 [ab]	31.3 ±0.1 [ab]	30.5 ±1.0 [b]	31.9 ±0.1 [ab]	32.4 ±0.6 [a]

Note: Values with the same letter are not significantly different at α = 0.05 (DMRT); * Indicates tolerant progenies; values are the means of three replicates ±standard deviation.
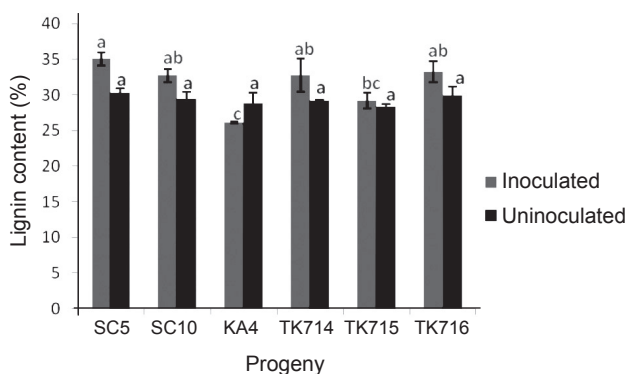
Figure 2. Lignin content in inoculated (10 months after inoculation) and uninoculated oil palm progenies. Values in the same colour with the same letter are not significantly different at α = 0.05 (DMRT); values are the means of three replicates; bars represent standard error.

palm generally produces more lignin as a defence mechanism. However, following inoculation, more tolerant progenies seem to have a lower lignin content compared to susceptible ones. One explanation would be that under *Ganoderma* attack, susceptible progenies may react by synthesising more lignin than the tolerant progenies even though that lignin will be soon or later degraded by lignolytic enzymes secreted by the pathogen. Paired t-test (inoculated versus uninoculated) conducted to verify the change in lignin as a result of inoculation showed a significant difference (P<0.05) only between inoculated SC 5 progeny and un-inoculated ones, with inoculated SC 5 having higher lignin in roots. The same paired t-test performed in other inoculated versus uninoculated progenies, either susceptible or tolerant, did not reveal any significant difference. Alternatively, the results may indicate that *Ganoderma* tolerant oil palm progenies have different defence strategies rather than solely lignification. Lignin is considered important as a first line defence against successful penetration of invasive pathogens (Bhuiyan *et al.*, 2009) and monolignol biosynthesis is also critically important in effective cell wall apposition formation (Bhuiyan *et al.*, 2009). Many intermediates in the lignin biosynthetic pathway *e.g.* caffeic acid, p-coumaryl acid and ferulic acid were found to be produced twice in amount in resistant compared to susceptible olive varieties affected by leaf-spot disease (Rahioui *et al.*, 2013). Also, it has been demonstrated that syringic acid, caffeic acid, and at a lesser extent, 4-hydroxybenzoic acid exert an anti-fungal effect on *G. boninense* (Chong *et al.*, 2009a, b; Chong, 2010). Interestingly, impregnation of wood with coniferyl alcohol followed by standardised decay test with the white-rot fungus, *Coriolus versicolor* showed that coniferyl alcohol significantly hindered fungal decay (Raiskila, 2008). This underlines the involvement of phenylpropanoid and monolignol biosynthetic pathway in plant resistance to white rot pathogens.

Lignin is considered important in disease resistance because it is not normally degraded by most of the pathogens, except by lignolytic fungi (Blanchette, 1994). However, *Ganoderma boninense* is lignolytic fungus producing laccases, lignin-peroxidases and manganese-peroxidases to efficiently degrade the recalcitrant lignin. Given that there seems to be no correlation between lignin content and tolerance (as demonstrated here), it would suggest that increased lignin accumulation is not important against this pathogen.

CONCLUSION

It can be concluded that lignin production is not correlated with tolerance in this host-pathogen interaction and lignin accumulation and detection may not be used to screen oil palm for tolerance or susceptibility to *G. boninense*. The scientific basis of *Ganoderma* tolerance still needs to be investigated. Phenylpropanoid and monolignol biosynthetic pathway products and derivatives seem to be a better and promising way to explore and identify oil palm progenies producing efficient anti-fungal metabolites against *G. boninense*.

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