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CERATOCYSTIS WILT OF *ACACIA MANGIUM* IN SABAH: UNDERSTANDING THE DISEASE AND REDUCING ITS IMPACT

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A canker and wilt disease caused by the fungal pathogen *Ceratocystis manginecans* has devastated *Acacia mangium* plantations in Southeast Asia. The disease develops when the pathogen enters wounds of the stems of the trees. The wounds are caused by wind damage, branch pruning, animal feeding and borer infestation. Various insects, including nitidulid beetles and scolytine wood borers, have been shown to be closely associated with the development of this disease, although a vector relationship has not been established. The disease has never been found on the roots of trees and isolations from soil in heavily infested plantations have failed to yield cultures of *C. manginecans*. Research was initiated in 2012 has focused on developing an inoculation protocol to select *A. mangium* with tolerance to infection by *C. manginecans*. In order to achieve this, an isolate of the pathogen identified using DNA sequencing technology and having a high level of aggressiveness was selected. Preliminary trials showed that inoculations need to be conducted on established trees with well-developed vascular tissues, ideally one-year-old, and that tests on small plants are meaningless. The ideal inoculation technique involved inserting a single plug of *C. manginecans* mycelia into wounds made on the stems of one-year-old trees and monitoring the results during the subsequent 12 months, at which time most trees would have died. Over a period of approximately eight years, inoculations were performed on 6 000 such trees representing 140 *A. mangium* families. The small number of surviving trees was retained by grafting and subsequent vegetative propagation. Re-inoculation of these putatively tolerant trees has led to the identification of approximately 50 clones having high levels of disease tolerance. These trees can now be used to establish seed orchards and for hybridisation with *Acacia auriculiformis*, which is known to be substantially less susceptible to *C. manginecans* than *A. mangium*. The results suggest that it may be possible to pursue plantation forestry utilising *A. mangium*, most likely as a hybrid partner with *A. auriculiformis*.

Keywords: *Ceratocystis manginecans*, *Acacia mangium*, fungal pathogens, inoculation, hybridisation, *Acacia auriculiformis*

INTRODUCTION

Acacia mangium was first planted at large scale in the humid tropics of South Asia in early 1990s. This is comparatively recent, at least when one considers the long history of plantation forestry involving non-native species, such as *Pinus* and *Eucalyptus*, which for some countries dates back to late 1800s. The impressive growth of *A. mangium* in countries, such as Indonesia, Malaysia and Vietnam, led to the rapid expansion of plantations of this species, supplying large pulp mills and other timber-based industries.

In 2014, it was estimated that 2.5 million hectares had been planted with *Acacia* spp. across Indonesia, Malaysia, Thailand, Vietnam and parts of China (Harwood & Nambiar 2014). This was predominantly of *A. mangium* variety, but it also included *Acacia crassicarpa* particularly in low-lying sites, and *Acacia auriculiformis* and its hybrids with *A. mangium*. The species had become the mainstay of important forest industries. In terms of growth and productivity, there was little competition for this remarkable tree. Losses due to disease and insect pests were relatively minor in the early years of *A. mangium* plantation development, which is typical of plantation forestry based on non-native species (Payn et

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al. 2015, Burgess & Wingfield 2016). One of the first diseases to raise concern in these plantations was Ganoderma root rot caused by the native pathogen *Ganoderma phillipii* (Glen et al. 2009, Coetzee et al. 2011, Page et al. 2020). While relatively large patches (root disease centres) could be found, these were typically localised and the overall impact over larger areas has been manageable.

In 2003, a serious disease was detected in *A. mangium* plantations in Teso East (Riau area, Sumatra). The trees were found to have died very rapidly after routine pruning to reduce branching. A species of *Ceratocystis* was isolated from these trees (Wingfield unpublished). Subsequent surveys in 2004 in Riau showed that large numbers of young trees were dying very rapidly after pruning. A serious disease problem was clearly emerging. An intensive research programme was thus established by the April Group (https://www.rgei.com/our-business/april/april) in Indonesia to investigate the cause of this disease as well as to develop strategies to reduce its impact.

Beginning in 2005, isolations, as a result of periodic surveys, of diseased *A. mangium* trees in Riau were routinely undertaken. These consistently yielded cultures of a *Ceratocystis* sp., a genus of fungi well-known to cause canker wilt diseases of trees (Kile 1993, Seifert et al. 2013). The cultures were identified based on DNA sequence representing two phylogenetic lineages in *Ceratocystis*. One of these was *Ceratocystis manginecans* which had been found to kill large numbers of mango (*Mangifera indica*) trees in Oman (van Wyk et al. 2007, Al Adwai et al. 2013) and an undescribed species named *Ceratocystis acaciivora* (Tarigan et al. 2011a). Later research applying more robust phylogenetic tools led to the conclusion that *C. acaciivora* was actually the same species as *C. manginecans,* and it was later reduced to a synonymy with *C. manginecans* (Fourie et al. 2015). *C. manginecans* is therefore, accepted as the appropriate name for the fungus killing *A. mangium* in South East Asia (SEA).

Tarigan et al. (2011a) conducted the first pathogenicity trials with *C. manginecans* on *A. mangium*. Importantly, these studies provided the first unequivocal evidence that the fungus was the cause of the rapidly declining *A. mangium* plantations in Riau. There were also regular reports of a similar problem in other parts of Indonesia as well as in Malaysia and Vietnam (Wingfield unpublished). It was evident that the *A. crassicarpa,* plantations particularly in Indonesia, were only minimally affected and pathogenicity trials confirmed that this species was substantially less susceptible to infection by *C. manginecans* than *A. mangium* (Tarigan et al. 2011b).

As Ceratocystis Canker and Wilt Disease (CCWD) developed and became a threat to *A. mangium* plantations in SEA, it became clear that it would no longer be viable to propagate this species profitably (Harwood et al. 2015, Nambiar et al. 2018). The new plants grew relatively well for the first two years, but by three years of age, they were typically collapsing and this was true across most of the region (Figure 1). This serious problem required a rapid response that generally included felling trees in the worst-affected areas and replacing them with *Eucalyptus*. This was mostly of *Eucalyptus pellita*, which had emerged as an excellent option for deployment in the region (Brawner et al. 2010, Nambiar et al. 2018, Nasution et al. 2019).

Research considering options to deal with the collapse of *A. mangium* in SEA has been conducted at various levels by companies that have planted this species. Although replacing them with *Eucalyptus* was an obvious approach, the excellent performance, excellent pulping properties and ease for establishing *A. mangium* prior to the appearance of CCWD were important considerations. Furthermore, there is a good argument for these companies not to rely solely on a single genus of tree for plantation forestry (Nambiar et al. 2018).

In the case of Sabah (Malaysia), an intensive programme aimed at identifying putative disease tolerance in *A. mangium* was launched by members of Borneo Forestry Co-operative (BFC, https://www.borneoforestrycoop.com). The current review summarises the findings of studies on CCWD that has decimated *A. mangium* plantations in Sabah. These studies have focused on understanding the pathogens, its means of spread and the development of a disease-screening protocol to identify tolerance to infection by *C. manginecans*. Most of the trials were conducted in plantations belonging to Sabah Softwoods Berhad (located in eastern Sabah), which was one of the pioneers of plantation forestry in the wet tropics. When the studies were first initiated, very little was known regarding the interaction of *C. manginecans* and *A. mangium*. Over the course of eight years, more

than 20 field trials were established including approximately 1500 families of *A. mangium* as part of a staged research programme. Each of these stages incorporated results of the earlier ones in order to build a complete understanding of the pathogen that exists today. Specific details and results of the extensive screening trials that have been conducted during this period are discussed by Lapammu et al. (2023).

DISEASE DEVELOPMENT AND SYMPTOMS

Symptoms of CCWD on *A. mangium* in Sabah was initially found among young trees that had been pruned - a routine operation to promote the development of single stems or on trees that had been wounded by animals, such as squirrel, monkey and elephant. Infections could easily be traced from the wounds and these rapidly developed both upwards and downwards within the vascular tissue of the tree stems. These infections also commonly extend to the branches and entire trees wilt rapidly post infection (Figure 1). The inner bark of newly infected trees typically has lens-shaped necrotic lesions (cankers) scattered randomly throughout most of the trees and illustrating the rapid movement of the pathogen upwards in the stems via the vascular tissue (Figure 2). Cross sections through these stems show a streaking pattern of infection typical of *Ceratocystis* spp. When the infected bark or cambium was incubated for 24 hours, structures of the sexual state (ascomata) of the pathogen were commonly formed on the areas having lesions (Figure 2).

Some *Ceratocystis* spp. and related fungi are well-known to be soil-borne and are associated with infections of the roots and root collars of their hosts (Kile 1993, Wingfield et al. 2013b). It is for this reason, trees showing symptoms of CCWD are routinely inspected for infections of the roots or symptoms of disease moving upwards from the soil level. This mode of infection

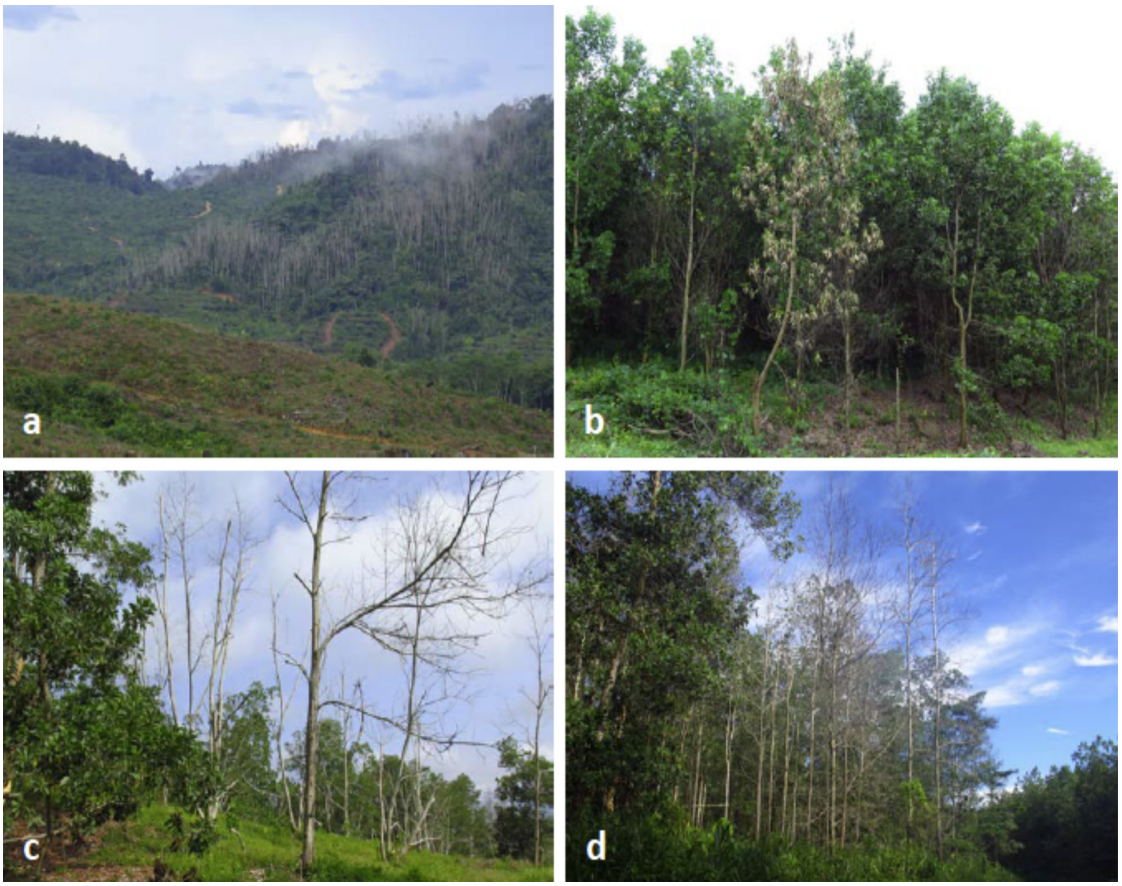

Figure 1 Plantation scale symptoms of Ceratocystis canker and wilt disease on *Acacia mangium* in Sabah: a) hillside showing large scale death of trees, b) early symptoms usually include rapid wilting, c, d) patches of dead trees in established plantations

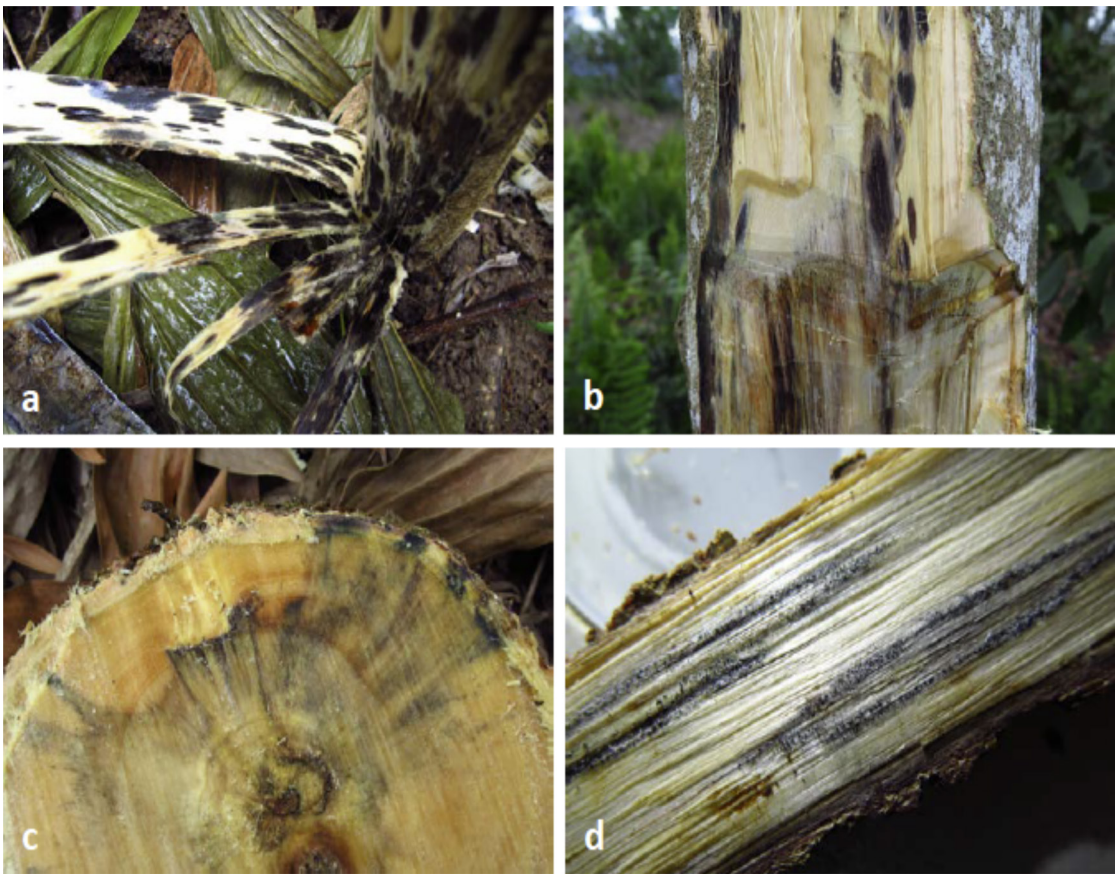

Figure 2 Internal symptoms on *Acacia mangium* trees infected by *Ceratocystis manginecans*: a) bark peeled back from a dying tree showing distinct patches of necrotic tissue associated with the pathogen moving through the vascular tissue and subsequently rising to the cambium, b, c) streaked pattern of discolouration typical of movement of the pathogen through the vascular tissue, d) *C. manginecans* sporulating on the surface of cambial cankers soon after the tree bark was removed

was never found. In contrast, infections have consistently occurred on the main stems of trees and arose from wounds on those parts of trees (Figure 2).

An important aspect of CCWD on *A. mangium* is the association with damage to trees caused by animals, especially monkeys and squirrels. Physical damage to tree stems provided a multiplicity of wounds that were rapidly infected by *C. manginecans*, which could be easily re-isolated from the infected tissue. All evidence points to the fact that animal damage, at least at the start of the disease epidemic, have led to a very substantial increase in the incidence of CCWD, apparently associated with a substantial increase in inoculum of the pathogen. This in turn led to massive increases in populations of two scolytine (Coleoptera: Scolytinae) bark beetles *Euwallacea perbrevis* (Lynn et al. 2020, Lynn et al. 2021) and *Xylosandrus crassiusculus*.

Acacia mangium trees showing early symptoms of CCWD were consistently and heavily infested by *E. perbrevis* and *X. crassiusculus* (Figure 3). However, it was also common to find trees infested by these insects in the absence of obvious signs of infection by *C. manginecans*. In these cases, the insects appeared to be attracted to dead branch stubs, often with dead wood extending to the centre of the trees. Cambial tissue associated with insect penetration sites was discoloured and isolations from these lesions commonly yielded cultures of *C. manginecans*.

Symptoms of CCWD on *A. mangium* in Sabah were first noticed in 2011 and a positive identification of the pathogen based on DNA sequence data was achieved in 2012 (Wingfield unpublished). The disease spread rapidly from the first observation of the disease in the area, and by the end of 2012, it was clear that it was no longer possible to grow *A. mangium* profitably.

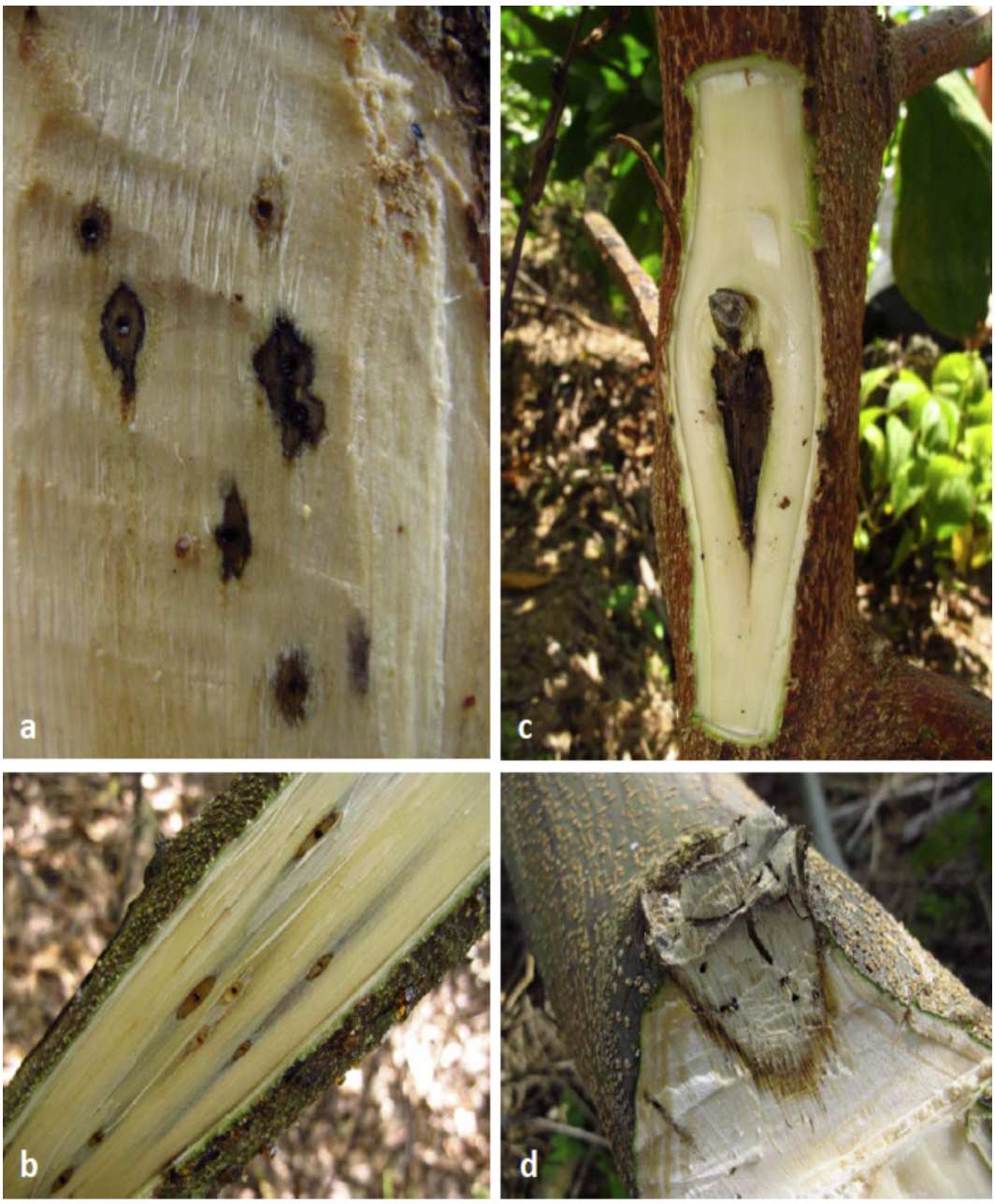

Figure 3 Symptoms illustrating the association of wood borers and *Ceratocystis manginecans* on recently infected *Acacia mangium*: a) discrete cankers in the fresh bark associated with recent borer infestation, b) cankers in the cambium associated with sites where borers penetrated the wood and vascular streaking associated with *C. manginecans* infection, c, d) borer infestation on the bases of dead branches and associated *C. manginecans* infection

A decision was then made to fell all trees over three years old (the time when plantation collapse became obvious) and to cease all new planting of this species. All felled areas where then planted with *Eucalyptus*, mainly *E. pellita*. The same approach was widely adopted in Indonesia where *A. mangium* plantations were also failing (Nambiar et al. 2018).

IDENTIFICATION OF THE PATHOGEN

The genus *Ceratocystis* dates back to the description, in 1898, of the causal agent of black rot of sweet potato in the USA as *Ceratocystis fimbriata* (Marincowitz et al. 2020). This genus has had a difficult taxonomic history, having been confused with the morphologically similar

but phylogenetically distinct genus *Ophiostoma* (Upadhyay 1981, Seifert et al. 2013). The distinction of these two genera was eventually resolved when phylogenetic inference based on DNA sequence data became available to resolve this important question. It thus, emerged that these very relevant fungal genera resided in different orders of fungi (Spatafora & Blackwell 1994, de Beer et al. 2014).

Ceratocystis, even after its distinction from *Ophiostoma,* remained taxonomically confusing for many years. This was despite the recognition that the genus included numerous groups of morphologically, phylogenetically and ecologically different fungi (Wingfield et al. 2013, de Beer et al. 2014). This eventually led to a major revision of *Ceratocystis* in which those species related to the type species, *Ceratocystis fimbriata*, causal agent of black rot of sweet potato, was segregated in *Ceratocystis sensu stricto*, including at the time approximately 32 species (de Beer et al. 2014). Consistent with this taxonomy, species of *Ceratocystis s.s.* are mostly pathogens, and exclusively of angiosperm plants. Common examples include *Ceratocystis platani*, the cause of a serious canker and wilt disease of *Platanus* in Europe (Engelbrecht & Harrington 2005, Ocasio-Morales et al. 2007, Tsopelas et al. 2017), *Ceratocystis albifundus*, an important pathogen of non-native *Acacia mearnsii* and *Protea* species in Southern Africa (Roux & Wingfield 2009, Lee et al. 2016), and two newly described species *Ceratocystis lukuohia* and *Ceratocystis huliohia* resulting in a devastating disease of *Meterosideros polymorpha* in Hawaii (Barnes et al. 2018).

The appropriate name for the causal agent of CCWD remains a matter of debate. The fungus is a close relative of the causal agent of sweet potato black rot, *Ceratocystis fimbriata sensu stricto* (Marincowitz et al. 2020). Based on phylogenetic inference, isolates from *A. mangium*, mango and two genera of legume trees in Oman and Pakistan, can clearly be separated from *C. fimbriata* and a number of related species (Al Adawi et al. 2013). Populations of these isolates from SEA also show relatively high levels of gene and genotypic diversity (Fourie et al. 2016). A complicating factor is that these isolates are sexually compatible in culture with those of *C. fimbriata s.s.*, the sweet potato pathogen (Baker et al. 2003, Engelbrecht & Harrington, 2005), which has recently been proposed as a *forma specialis* (host pathogenic variety) of *C. fimbriata* (Valdetaro et al. 2019). However, there is also clear evidence that these crosses result in segregation for host (Fourie et al. 2018). This suggests that the fungus infecting *A. mangium* and that we choose to treat as *C. manginecans,* has speciated and might appropriately be treated as a species different from that infecting sweet potato.

A question that has arisen in studying CCWD on *A. mangium* is whether isolates from this tree are able to infect sweet potato; likewise, whether isolates from sweet potato, commonly found in Sabah markets, can infect *A. mangium.* Inoculation trials to test this question have been conducted (Barnes et al. 2023) and these have shown clear evidence of host specialisation where *A. mangium* isolates killed these trees but sweet potato isolates had no effect on them.

We believe that there is sufficient evidence to treat the causal agent of CCWD on *A. mangium* as *C. manginecans*. Early studies including whole genome sequence data support this view (Kanzi et al. 2020), but these must clearly be expanded. In addition, further studies including comparisons of large populations of isolates will help to resolve this intriguing question (Oliveira et al. 2015), and these are currently underway. At the present time, to conflate the name of the cause of CCWD on *A. mangium* with the fungus causing black rot of sweet potato would cause considerable confusion. This would be at the research level, but also in terms of disease management, where such confusion has regularly arisen. Furthermore, for the application of quarantine procedures, applying a confused pathogen identity would bear risks relating to the movement of this pathogen globally.

MEANS OF PATHOGEN SPREAD

Very little is known regarding the means of spread of *C. manginecans* in *A. mangium* plantations of SEA. Hypotheses must thus, be based on knowledge of how other species of *Ceratocystis* are dispersed. This topic was treated in some detail by Wingfield et al. (2013b), and it has been studied for various species of *Ceratocystis* and their relatives. *Ceratocystis* spp. are perhaps best known as insect associates, a feature that is facilitated by their long-necked sexual structures (ascomata) that produce sticky spores at their apices. These fungi also typically produce strong fruity aromas, which are highly attractive to insects that visit tree wounds, such as picnic beetles (Nitidulidae) and

flies (Diptera). Consequently, there is substantial evidence for the involvement of nitidulids as vectors of some *Ceratocystis* spp. (Appel et al. 1990, Heath et al. 2009a).

Bark and ambrosia beetles (Scolytinae) are well-known associates of fungi including species previously accommodated in *Ceratocystis* (Ploetz et al. 2013, Mayers et al. 2015, Wingfield et al. 2017). However, there is no experimental evidence showing a vector relationship between these beetles and *Ceratocystis s.s.* This would negate the role of *X. crassiusculus* and *E. perbrevis* associated with the early symptoms of CCWD on *A. mangium* as vectors of the causal pathogen. However, it is well-known for some *Ceratocystis* spp. such as *C. platani* and *C. lukuohia*, that wood boring insects infesting infected trees produce frass (wood dust) from which the pathogen can easily be isolated (Soulioti et al. 2015, Tsopelas et al. 2017, Roy et al. 2019).

A number of preliminary trials have been conducted in Sabah to consider the means of spread of *C. manginecans* in *A. mangium* plantations (Figure 4). These included the role of nitidulid beetles as vectors, the possible role of ambrosia beetles and vectors and whether the pathogen could have a soil-borne phase. These are briefly discussed below.

Nitidulid beetle vectors

Nitidulid beetles are commonly found on recently infected *A. mangium* trees. These trees usually display a foamy fermentation exudate from their stems and nitidulid beetles are commonly found breeding in this substance (Figure 4). Despite considerable efforts, trapping meaningful numbers of nitidulid beetles for isolation purposes has not been successful.

Figure 4 Nitidulid beetle (Coleoptera: Nitidulidae) and wood borer association with *Ceratocystis manginecans* infections: a, b) trees infected by *C. manginecans* commonly exude a foamy yeast/bacterial substance from their stems, which is rapidly colonised by nitidulid beetles (arrow) from which *C. manginecans* can be isolated, c) borer infestation of infected trees results in the production of frass 'tubes" contaminated with *C. manginecans*, d) freshly exposed cambium due to elephant activity contaminated by borer frass and associated colonies of *C. manginecans*

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However, small numbers of these insects collected from infected stems or bark flaps associated with artificially induced wounds have been found to consistently yield cultures of *C. manginecans*. It must thus, be assumed that these insects play a role in transferring propagules of the pathogen to freshly made wounds on trees. However, this view needs to be supported by much stronger experimental evidence than is currently available.

Wood borers as vectors

Approximately 100 individuals representing a mixed collection of *X. crassiusculus* and *E. perbrevis* were collected as they emerged from one-year-old trees that had been artificially inoculated with *C. manginecans* and were in various stages of death. These insects were all placed on carrot discs in an effort to isolate the pathogen. None of the isolations yielded a positive result. In contrast, samples of frass (Figure 4) collected from 20 of the same trees and treated in the same way all yielded cultures of *C. manginecans*. These preliminary results strongly suggest a role for wind-borne frass, acting as an important source of inoculum as it lands on freshly made wounds on trees. This hypothesis was tested by visiting a site where the bark had recently been stripped from tree stems by elephants. These wounds were carefully inspected and found to be contaminated by beetle frass and they were also infected by *C. manginecans* (Figure 4).

Soil-borne propagules

All evidence based on symptoms negates the likelihood that *C. manginecans* is a soil-borne pathogen in plantations of *A. mangium*. An experiment was conducted to test this view. A total of 20 aggregated soil samples (from five distinct collection points) was collected under the canopies of a large number of two-year-old *A. mangium* trees dying subsequent to having been inoculated with *C. manginecans*. These soil samples were subjected to a carrot-baiting technique (Moller & deVay 1968) routinely used to isolate *Ceratocystis* spp. None of these samples yielded cultures of the pathogen; this despite the obviously very heavy inoculum load on the trees below which the soil samples had been collected. The consistent absence of any basal or root infections on trees dying due to *C. manginecans* infection is also in line with this observation.

Observations relating to the dissemination of *C. manginecans* in plantations of *A. mangium* in Sabah are all based on preliminary and minimally replicated studies. However, there is reasonably well supported evidence that nitidulid beetles play a role in long-distance dissemination of the pathogen between wounds on infected trees. Likewise, frass produced by wood-boring insects infesting trees infected by *C. manginecans* appears to be another source of wide-spread dissemination of the pathogen. Clearly, as the number of trees infected by *C. manginecans* has grown (initially from small numbers and closely linked to wounding via pruning and animal damage) the pathogen has become well-established. This apparently has led to infection levels reaching a "tipping point" where entire plantation areas have collapsed due to the disease.

ESTABLISHMENT OF A RELIABLE INOCULATION PROTOCOL

In establishing a meaningful inoculation protocol to screen *A. mangium* for tolerance to infection by *C. manginecans*, a number of factors were taken into consideration. These included: the need to use cultures of the pathogen that had been reliably identified and appropriately represented its aggressiveness; the age of trees that would provide a fair reflection of disease tolerance; an appropriate means to inoculate trees, and a reliable system to evaluate results including the time needed to do so. These questions were addressed using a suite of pilot trials.

Appropriate age of trees for inoculation

In 2012 when disease screening work was being considered, it was accepted that species of *Ceratocystis* are vascular wilt pathogens. Thus, any trees used for inoculation-based screening would need to have reasonably well-developed vascular systems. Furthermore, CCWD caused by *C. manginecans* is a disease that first appears on trees of about six months of age (3–5 cm diameter) and is never seen in small plants. Thus, the temptation to utilise small plants in large nursery or laboratory trials, which would have been most simple, was avoided. This was later shown to be a well-founded decision when it was discovered that even inoculating relatively large trees in field trials led to high levels of relatively rapid mortality (Figure 5). Thus, the findings

of Brawner et al. (2020) that small plants and petiole inoculation tests require field verification, are well founded.

After five years of undertaking field inoculations, a study was carried out in 2019, to verify that small plants should not be used to test for host susceptibility. For this experiment, 10 small plants of seven clones representing four of the most tolerant and three of the least tolerant trees, which had been selected from field trials, were used in an artificial inoculation trial. These plants were an average of 150 mm in height and approximately 5mm in diameter at soil level. Within four weeks of inoculation, 36 of the 40 plants representing the higher end of tolerance and 25 of the 30 plants representing the lower end of the tolerance scale had died (Figure 5). In contrast, the 21 plants (two clones from the higher tolerance group) inoculated as controls were all healthy. In order to complete Koch's postulates, *C. manginecans* was re-isolated from all the dead plants and not from any of the control plants. The

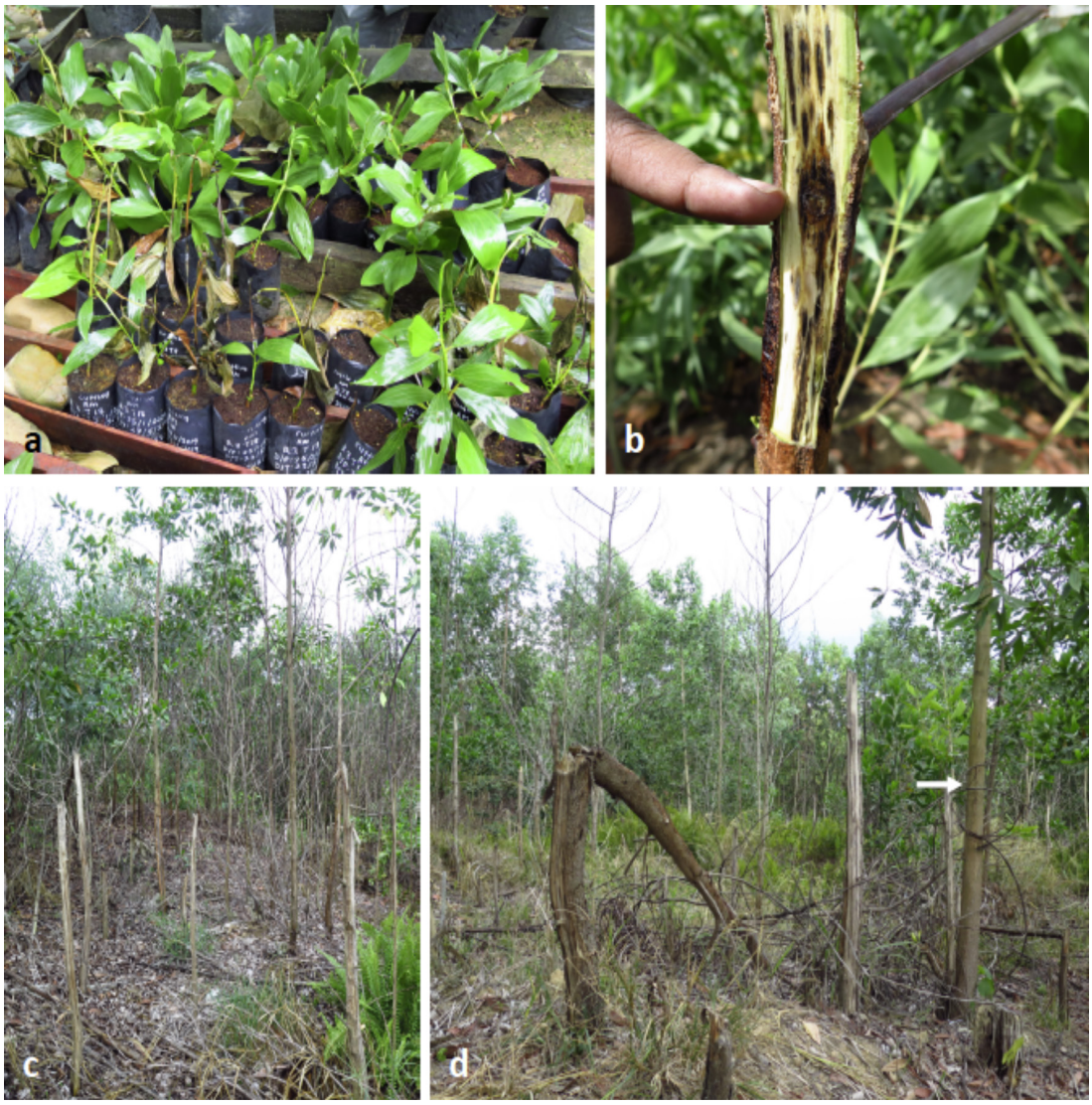

Figure 5 Young plants and established trees inoculated with *Ceratocystis manginecans*: a) dead and dying *Acacia mangium* plants vegetatively propagated from trees shown to be disease tolerant in field inoculations and illustrating that small-plant inoculations fail to reflect field tolerance to *C. manginecans*, b) inoculations on six month-old trees after six weeks showing rapid development of disease, c) one-year old trees inoculated with *C. manginecans*, the majority of which die within six months of inoculation, d) a single living *A. mangium* tree (arrow) in a plot of trees inoculated at one-year of age and photographed one year later, illustrating the aggressiveness of the pathogen and low number of trees surviving single-point inoculations

results suggested that inoculating small plants fails to provide a proxy for disease tolerance under field conditions. More importantly, it is likely to result in an inaccurate view of disease tolerance when starting plantations (Raffa et al. 2023).

Inoculum and inoculation procedure

A culture of *C. manginecans* to be used for screening was selected in a preliminary trial. In this case, 20 six-month-old *A. mangium* trees each grown from seed were inoculated with three different isolates that had been identified based on their DNA sequence analyses (Figure 7). These trees had all became severely diseased and there was no obvious difference in the results for the different isolates used. One of these isolates was then used in a first trial conducted between April and October 2014, the results of which are presented by Brawner et al. (2015). This isolate was used in all subsequent inoculations, after first being passed through an inoculated tree and re-isolated to retain pathogen aggressiveness (Lapammu et al. 2023). The cultures for these re-isolated strains covering nine years have been retained in the culture collection (CMW) of Forestry and Agricultural Biotechnology Institute for future studies.

Figure 6 Lesions resulting from inoculations with *Ceratocystis manginecans* on one-year-old *Acacia mangium* and recorded after six months: a, b) extensive lesion development on more susceptible trees, most of which died within a year, c) small lesion on a tree with higher levels of tolerance to infection, d) trees most tolerant to infection typically have well developed callus tissue surrounding lesions

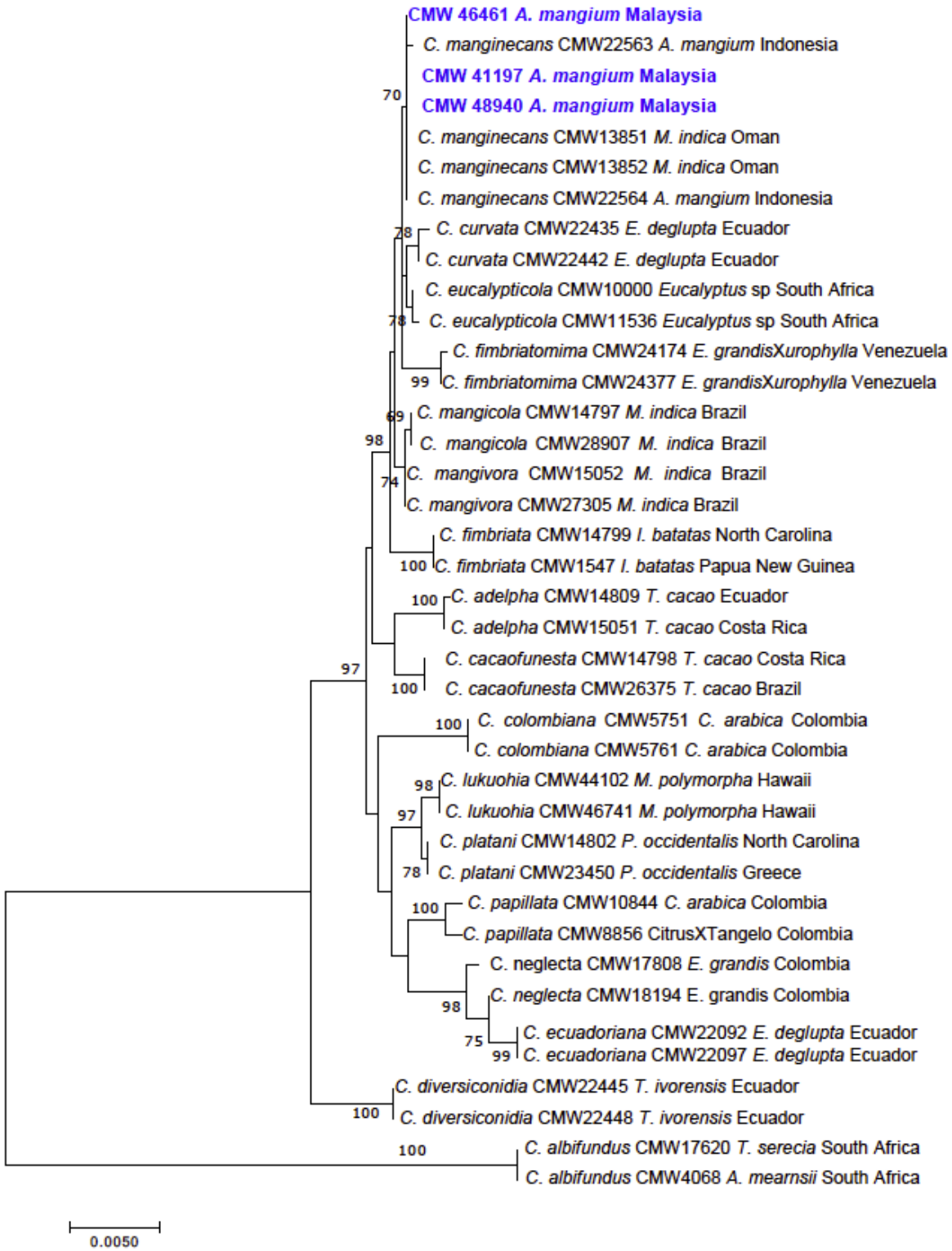

Figure 7 Molecular phylogenetic analyses of the combined bt1, ef1, ms204 and rpb2 gene regions showed that the fungal isolates obtained from *A. mangium* in Malaysia are those of *Ceratocystis manginecans* (in bold and blue). The four gene regions for the Malaysian isolates were PCR amplified and sequenced following the methods in Fourie et al. (2015) and Liu et al. (2018). These were added to the aligned sequence data base generated by Barnes et al. (2018, TreeBASE Accession No. S22005). The tree was 'constructed' using Maximum Likelihood (ML) based on the GTM Model in MEGA V. 7 (Kumar et al. 2016). ML bootstrap values (from 500 replicates) showing support for branches >60% are indicated in the branches. *Ceratocystis albifundus* was used as the outgroup taxon. The tree with the best log likelihood value of -6332.04 was chosen for this figure

All inoculations on trees were made using a single inoculation point at breast height and with a wound made using a punch of 10mm diameter (Figure 5). Discs of agar taken from fresh actively growing cultures were used for inoculations. Pilot trials showed that it is essential to produce a wound that penetrates the bark sufficiently deep to expose the wood just below the cambial layer. Shallow wounds only exposing the cambial layer were shown in these pilot trials to give inconsistent results.

The first set of inoculations used by Brawner et al. (2015) utilised measurements of lesion length taken after approximately six weeks. In order to make these measurements (also used in a subsequent inoculation in September 2015), it was necessary to strip the bark from the tree stem (Figure 5). The result was that infections spread rapidly after measurements were taken, and they died quickly. This result led to two trials where 2000 trees were inoculated and where the lesions were exposed for measurement but where an equal number of trees was left without stripping the bark. After approximately six months, both sets of trees were dying in large numbers (Figure 5, 6). A decision was then made to inoculate trees and to evaluate the results based only on survival. It was thus found that an ideal tree age for inoculation was approximately one-year-old and that six-month-old trees were unduly susceptible to provide reliable results. Furthermore, it was evident that trees could be inoculated on the stems with a relatively small (10 mm diameter plug of inoculum) single insertion of the pathogen. Under these challenging conditions of relatively large trees and single relatively small inoculation sites, most trees were found to die within a year after inoculation (Figure 5). Consequently, all inoculation trials subsequent to the first three trials (2014, 2015 and 2017 respectively) utilised one-year-old trees and results were read as trees surviving after 12 months.

SELECTION FOR DISEASE TOLERANCE IN *A. MANGIUM*

At the time of the discovery of CCWD on *A. mangium* in Indonesia, it was clear that *A. crassicarpa* had a low level of susceptibility to *C. manginecans*. This was confirmed in artificial inoculation studies (Tarigan et al. 2011a & 2011b). During the course of studying CCWD in Sabah, an inoculation trial was conducted to compare the relative susceptibility of *A. mangium*, *A. auriculiformis* and *A. crassicarpa* (Barnes et al. 2023). The results of that trial showed high levels of tolerance in the latter two species. This points to an opportunity to ultimately produce hybrids between *A. auriculiformis* and *A. mangium* that are well-suited for vegetative propagation. Ultimately, these hybrids will need to be made from individuals of the parent species having high levels of tolerance to *C. manginecans*. This will be particularly relevant to *A. mangium* that is clearly highly susceptible to the pathogen.

One of the most important observations made regarding screening *A. mangium* for tolerance to infection by *C. manginecans* was that inoculation of greenhouse size or even small trees in the field do not provide reliable results (Figure 5). Similarly, using detached branches or tissue culture for screening has shown that they are not useful. Planting large blocks representing families of *A. mangium* and allowing natural infection to proceed may be a possibility. However, this approach would require waiting for at least one rotation for results and it would suffer from the problem of disease escape. Artificial inoculation of one-year-old trees and assessing tree survival after one year, when tree death had reached a plateau, has been found to provide reliable results.

Statistical details and heritability data for an extensive suite of screening trials using the above-mentioned approach have been discussed in other studies (Lapammu et al. 2023). These trials were based on three large trials in 2014, 2016 and 2017 where trees were inoculated. The first two of these trials involved 100 families of *A. mangium* with 20 trees per family (2000 trees in each of the trials). The third inoculation carried out in 2017 included four families with 500 trees per family. In addition, all trees in a clonal seed orchard (900 in total) planted in 2014 were inoculated in 2016. Trials were assessed for survival after one year and annually thereafter. After one year, the number of surviving trees in all trials was relatively small, and tree death continued, although relatively slowly.

Ninety four candidate trees were selected that had survived field inoculation and were subjected to clonal verification trials. Eighty-six of these were from the 2014 field inoculation trial (Figure 5) and a further eight were from a clonal seed orchard where all the trees had been inoculated (Lapammu et al. 2023). It is notoriously difficult to produce clones from established (mature) *A. mangium* and the only option was to do this via

grafting to seedlings of *A. mangium*. The objective here was to produce 20 replicate clones of the trees that had survived inoculation. This is an ongoing process and will continue for trees surviving in all of the three major inoculation trials.

By 2019, over 200 clones had been propagated from trees that had survived the 2014 inoculation trial or the clonal seed orchard inoculation trial. These trees were planted in clonal verification trials and these were inoculated at one year of age. These trials ultimately yielded 50 clones (42 from the 2014 inoculation) and eight from the clonal seed orchard and where most trees had survived the second inoculation (Lapammu et al. 2023). These 50 clones have thus, been subjected to an extremely heavy inoculation pressure and are considered to represent a high level of tolerance to *C. manginecans*.

THE WAY FORWARD

An extensive suite of studies conducted over a period of eight years have revealed substantial findings and crucial information regarding the biology of *C. manginecans* infecting *A. mangium* in Sabah. This knowledge can be applied in other parts of SEA where *C. manginecans* has also devastated plantations of *A. mangium*. Overall, the results have shown that there is some level of tolerance to infection in *A. mangium,* although this appears to be relatively low.

All evidence from field observations suggest that *A. auriculiformis* is relatively tolerant to infection by *C. manginecans*. Pilot trial inoculations have also confirmed this fact. Thus, it is reasonable to expect that hybridisation between *A. auriculiformis* and the highly disease tolerant *A. mangium* clones (produced over a period of more than six years in Sabah) will result in progeny that will sustain infection by the pathogen. Trees in progeny trials could then be experimentally tested for disease-tolerance using the techniques established in this study. Those trees with the highest levels of tolerance and having superior growth characteristics could then be vegetatively propagated to produce high-quality and CCWD- tolerant planting stock. Ideally, *A. auriculiformis* trees selected as hybrid parents should also be tested for tolerance to infection by *C. manginecans*.

Eucalyptus pellita and hybrids between this species and *E. grandis* or *E. urophylla* have been widely planted in the humid tropics of SEA. This trend has grown strongly in recent years and particularly in response to the collapse of *A. mangium* due to CCWD. Recent field observations have shown that *E. pellita* harbours a level of susceptibility to infection by *C. manginecans*. This has raised concern that *Eucalyptus* plants could face a future similar to that of *A. mangium*. This is unlikely to occur for a number of reasons. First, susceptibility of *Eucalyptus* to *C. manginecans* infection appears to be strongly clone-related. Hence, selection for CCWD tolerance and thus applying disease-avoidance should be relatively simple. This is similar to experiences with other *Eucalyptus* diseases where losses are relatively easily avoided by selecting tolerant planting stock (Wingfield et al. 2008 & 2013a). Second, it is also relevant to recognise the vast genetic diversity in *Eucalyptus* and the many species that can serve as hybrid partners in developing disease-tolerant clones.

Acacia mangium is very sensitive to wounding and appears to have a relatively poor capacity to heal. This was clearly seen in the pruning trials conducted by Tarigan et al. (2011b). Where this species or hybrids of *A. mangium* with *A. auriculiformis* are planted in the future, every effort must be made to avoid wounds to the stems. This also applies to *A. crassicarpa* that has a higher level of tolerance to *C. manginecans,* but after wounding, susceptible trees commonly die. Consequently, serious reconsideration must be made to operations that result in stem wounds, such as pruning. This applies equally to *Eucalyptus* grown for solid timber products.

New *Ceratocystis* diseases of trees are emerging in many parts of the world. This applies to trees in natural forest ecosystems, such as in the case of *C. platani* on *Platanus* in Europe (Ploetz et al. 2013, Tsopelas et al. 2017) and *C. lukuohia* on *Metrosideros polymorpha* in Hawaii (Barnes et al. 2018), and to plantation forestry, such as in the case of this study and elsewhere (Heath et al. 2009b, Ferreira et al. 2011, Roux et al. 2020). This provides a strong argument to avoid the accidental introduction of *Ceratocystis* spp. into new areas and thus, to clearly understand the pathways of movement that would allow this to occur. Strict quarantine regulations and adaptation of these rules capturing the most recent technologies must also take these factors into consideration (McTaggart et al. 2016).

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CONCLUSION

The results of these studies, undertaken over a relatively long period of time have shown that it is possible to select *A. mangium* planting stock that has a high level of tolerance to infection by *C. manginecans*. However, the levels of disease tolerance are relatively low, and it seems unlikely that this tree will easily be propagated as a pure species in areas where *C. manginecans* occurs. This is especially also due to the fact that *A. mangium* cannot be serially propagated vegetatively and thus, pure clones of the species are unlikely to be an option at an operational level. However, there is good evidence to suggest that hybrids between *A. mangium* and *A. auriculiformis*, which suffer less from maturation, can be vegetatively propagated commercially as clones, and can be a future option. The success of these hybrid clones will however, require that they originate from parent trees with high levels of tolerance to infection by *C. manginecans*. The disease tolerant *A. mangium* clones produced in this study will promote such an opportunity and it is worth pursuing, as suggested by the current findings.

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