



Screening clonally replicated *Acacia mangium* breeding populations for tolerance to *Ceratocystis* canker and wilt disease

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Abstract

Three screening trials of clonally replicated *Acacia mangium* seedlings were evaluated for survival and lesion length following inoculation with locally collected strains of *Ceratocystis* in Indonesia. Tolerance in the population was low with 6.7% of the 1033 clones represented by more than 4 ramets surviving repeated inoculations. Differences in tolerance among populations were slight; however, populations with consistently higher survival and shorter lesion lengths were from Papua New Guinea rather than Queensland. Estimates of the proportion of the experimental variation attributable to differences among parents (heritability) were low to moderate for both survival and lesion length. Estimates of the proportion of the experimental variation that was attributable to differences among clones (repeatability) were greater but typically similar to the heritability estimates, indicating that initial improvements from selection will primarily be derived from identifying tolerant parents. While genetic correlations among experiments were positive, estimates could not exclude the existence of host–pathogen interactions. Two validation trials of the tolerant clones were assessed 9 months after establishment; these trials verified that one-third of the clones identified in the nursery screening were also tolerant to *Ceratocystis* in field trials. The experiments confirmed that nursery screening may be used to quickly focus efforts on parents that produce more tolerant progeny, screening additional seedlings to increase selection intensity rather than using clonal replication to increase accuracy would lead to greater improvements in tolerance and field trials are required to verify disease tolerance at later ages.

Keywords *Acacia mangium* · *Ceratocystis manginecans* · *Ceratocystis fimbriata* · Tolerance · Heritability · Repeatability

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Introduction

Ceratocystis species are virulent pathogens of a wide range of plants and have been associated with serious diseases in many tree species (Barnes et al. 2018; Tarigan et al. 2011; Ploetz et al. 2013; Al Adawi et al. 2013; Bakshi 1950; Valdetaro et al. 2015). These pathogens have altered the species composition of natural forests and caused substantial economic losses in horticultural and forest tree crops (Wingfield et al. 1993; Harrington et al. 2011). While the taxonomy of the pathogen is still being clarified (Oliveira et al. 2015; Fourie et al. 2016; Wingfield et al. 1993), there is a strong consensus that the canker and wilt disease of *Acacia mangium* Willd. that has led to extensive forest loss is caused by *Ceratocystis*.

An outbreak of a canker and wilt disease caused by *Ceratocystis manginecans* (Tarigan et al. 2010) has expanded across extensive areas of *A. mangium* Willd.

plantation forests in the wet tropics of Southeast Asia (Brawner et al. 2015; Harwood and Nambiar 2014). The causal agent of the disease was initially described as *C. acaciivora* (Tarigan et al. 2011) but was later determined to be conspecific with the well-known pathogen of mango trees, known as *C. manginecans* by some (Fourie et al. 2014, 2016) and *C. fimbriata* by others (Guimaraes et al. 2021). The disease in acacia plantations is commonly associated with damage caused by monkeys and evidence indicates that the rapid spread of the disease has also been facilitated by ambrosia (*Coleoptera: Scolytinae*) and nitidulid beetles (*Coleoptera: Nitidulidae*) transferring the pathogen among trees. Wind-blown frass appears to be contributing to the rapid spread of disease, as it has in other disease outbreaks caused by *Ceratocystis* species (Barnes et al. 2018).

The *Acacia* forests that have been most severely impacted were established for fibre production in the humid equatorial tropics of Indonesia and Malaysia. *A. mangium* plantations expanded rapidly in the tropics over the past three decades following trials that identified this species as highly productive and suitable for the development of planted forests (Turnbull et al. 1983; Harwood and Williams 1991; Griffin et al. 2011). Since the 1970s, more than 2.4 million hectares of *Acacia* plantations had been developed in Southeast Asia and *A. mangium* was particularly favoured for its ease of management and high productivity across a diverse range of mineral soils in the humid tropics (Harwood and Nambiar 2014). Significant reductions in productivity have, however, been reported with each successive rotation and reductions have frequently been associated with mortality caused by fungal pathogens. In Sumatra, productivity fell from mean annual increments of 25 - 35 m³/ha/year in initial plantings to 10–20 m³/ha/year in subsequent rotations. Productivity losses were primarily attributed to a progressive build-up of root rot caused by *Ganoderma philippii* (Glen et al. 2009). More recently, *Ceratocystis* canker and wilt disease has led to such severe productivity losses that *A. mangium* is now infrequently planted for fibre production in the wet tropics of Southeast Asia (Brawner et al. 2015; Thu et al. 2014; Tarigan et al. 2011).

Over the past few years, losses caused by *Ceratocystis* canker and wilt disease have led most companies in Southeast Asia to move to alternative species in areas where *A. mangium* was once highly productive. The land-use transition caused by *Ceratocystis* canker and wilt has been dramatic with a large part of the more than 1 million hectares of the *A. mangium* plantation estate in Indonesia and Malaysia converted to *Eucalyptus pellita* Muell. and related hybrids over the past 5 to 10 years (Brawner et al. 2010; Guimaraes et al. 2010). Unfortunately, *Ceratocystis* has recently been isolated from *E. pellita* in both Indonesia and Malaysia. Mortality associated with *Ceratocystis* infection has also been reported in a number of other species across parts of

Southeast Asia (Fourie et al. 2016; Pornsuriya and Sunpapao 2015; Chi et al. 2019).

Following widespread mortality in industrial and small-holder *A. mangium* plantations in Sumatra, the Australian Centre for International Agricultural Research (ACIAR) supported the development of three experiments to evaluate the genetic architecture of tolerance to the *Ceratocystis* canker and wilt disease in breeding populations managed by the Indonesian government's Centre for Forest Biotechnology and Tree Improvement Research and Development (CFBTI). The experiments were designed to evaluate the potential of developing a rapid and cost-effective screening system for identifying individuals that tolerated inoculation with the pathogen. Rapid screening is necessary if *A. mangium* seedlings that survive inoculation are to be deployed directly as physiological ageing dramatically reduces productivity if cuttings are produced from selections greater than 2 years old. Cloning seedlings that survived controlled inoculations would provide an immediate source of *A. mangium* planting stock if an accurate and repeatable system could be developed for screening seedlings with a vascular wilt pathogen.

Evaluating resistance using natural infection in the field would be the preferred option given its low cost and simplicity; however, this approach suffers from problems associated with uneven infection in field trials both spatially and temporally. This study presents results from screening for disease tolerance in three clonally replicated progeny trials of open-pollinated families from four *A. mangium* breeding populations managed by CFBTI. Clonally replicated seedlings were used to provide a better understanding of the genetic architecture of *Ceratocystis* tolerance in order to contrast the effectiveness of selecting among families or selecting among clones within families to develop a *Ceratocystis* canker and wilt-tolerant breed of *A. mangium*. Field trials used to validate the nursery screening trials were also established, with one trial maintained to allow for natural infection and the other inoculated 9 months after planting. Results from these trials are discussed with respect to the development of *Ceratocystis* screening systems to identify disease-tolerant *A. mangium*.

Methods

CFBTI managed a collaboration among three Indonesian industry partners to screen *A. mangium* seedlings for tolerance to inoculation with locally collected isolates of *Ceratocystis manginecans*. The trials were established by industry partners, with two trials located near Pekanbaru and one trial located near Palembang in Sumatra, Indonesia. The intention of this public/private cooperation was to develop a canker and wilt-tolerant breed of *A. mangium* that could be utilised by local stakeholders and the forest industry. Seed from the

A. mangium breeding programme managed by CFBTI on behalf of the Indonesian government was shared among partners and 3 to 7 cuttings of each seedling were propagated for disease screening. The most productive families from the CFBTI breeding programme were shared with all partners and a similar set of families was provided to each partner. Partners screened clonally propagated seedlings from these populations in three nursery trials. Two field trials were subsequently established with clones that survived the nursery screening to verify their tolerance. A description of the populations is provided in Fig. 1.

Acacia germplasm

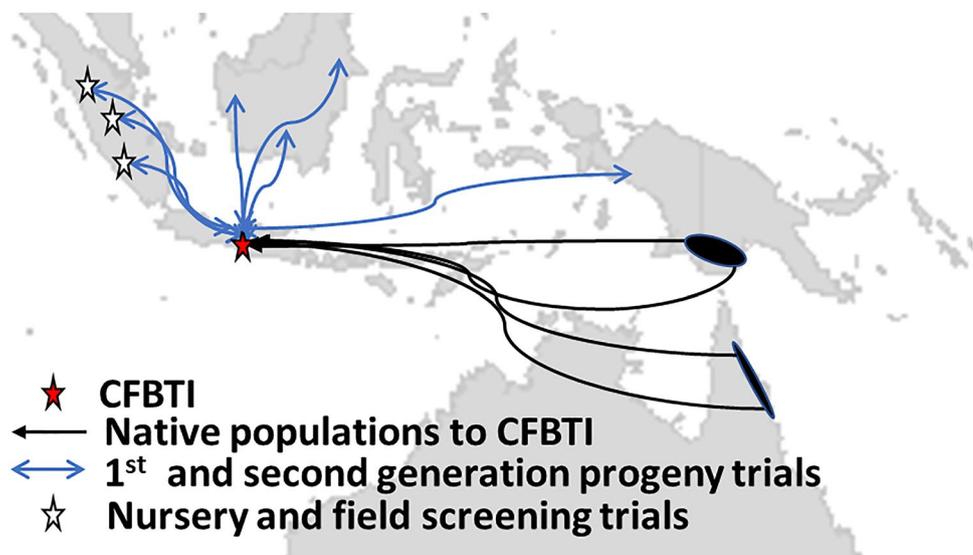
Germplasm evaluated in the screening trials was derived from progeny trials established as part of a project between the Indonesian Ministry of Forestry and the Japan International Cooperation Agency in the 1990s (Kurinobu 1996; Final report on seed source evaluation, unpublished). Seed was collected from 70 trees selected for growth and form in CFBTI's second-generation seedling seed orchards and open-pollinated families were distributed to each partner (Table 1). These seedling seed orchards were thinned progeny trials established with seed from selections identified in the first-generation progeny within provenance trials that evaluated native range *A. mangium* populations. These second-generation trials are comprised of the four most productive provenances identified in the first-generation trials: (A) East of Southeast Papua New Guinea; (B) West of Southeast Papua New Guinea; (C) Southern Cape York, Queensland, Australia; (D) Northern Cape York, Queensland, Australia.

Table 1 Number of open-pollinated families from four populations of *Acacia mangium* inoculated with *Ceratocystis* in three disease screening trials

Population/seed source	Trial 1	Trial 2	Trial 3	Unique families
A – West PNG	15	10	15	20
B – East PNG	15	10	10	15
C – North Cape	15	10	15	20
D – South Cape	15 <td 10	10	15	
Total	60	40	50	70

Ceratocystis isolates were collected within the estates of each partner with 20, 5 and 20 isolates evaluated for trials 1, 2 and 3, respectively. Isolates were grown in culture to produce sufficient inoculum to evaluate pathogenicity in seedlings derived from seedlots that have been used in each partner's operational nurseries. Partners evaluated the aggressiveness of isolates by inoculating 25 seedlings with each isolate and assessing lesion length 3 weeks after inoculation. Each partner selected the most isolate that produced the largest lesion in this isolate evaluation trial and these isolates were used for the screening in the nursery and field trials. A different isolate was used by each partner to screen the common CFBTI families. The three isolates that were used are maintained in the CFBTI collection with accession numbers AA-C013, UGM-8 and CTA138, corresponding to trials 1, 2 and 3, respectively. The identity of cultures was verified by sequencing β -tubulin genes and the ITS region of the ribosomal DNA and comparing the sequences to those of known cultures of this fungus (Tarigan et al. 2010).

Fig. 1 Description of germplasm and trials used to identify the 70 families supplied for screening in nursery and subsequent field trials. Germplasm sourced from East and West Papua, North and South Cape York was evaluated in first-generation progeny trials across Indonesia with seed from selections returned to CFBTI. Seed was distributed to industry partners in Sumatra for clonal replication and disease resistance screening in the three nursery trials and subsequent evaluation of survivors in nearby field trials



Experimental design

Nursery trials

Open-pollinated seeds from selections made in CFBTI's *A. mangium* breeding programme were distributed to partners to produce seedlings that were replicated by vegetative propagation. Each seedling was propagated following topping to produce shoots that were rooted as stem cuttings and a minimum of 4 ramets per clone was required for inclusion in the screening. Across all three trials, an average of 5.1 ramets of each clone were used to evaluate 1033 clones so that a total of 5177 ramets were inoculated. An average of 14.8 clones was evaluated for each of the 70 open-pollinated families. Cuttings from each clone were set weekly with a target to produce 5 ramets of each clone for the nursery trials. The mother plants were managed as hedges so that cuttings were available for validation in field trials once the nursery trials were completed.

Inoculation and assessments in nursery trials

Inoculum was cultured to obtain sufficient material so that all plants within a replication of a trial could be inoculated in 1 day. Each ramet was wounded by inserting a 0.5-mm needle into the mid-point of the stem 15 cm above the root collar. Inoculation was carried out by extracting approximately 1 mm³ of inoculum from the edges of freshly grown cultures and pressing the plug of inoculum into the stem of the rooted cuttings with the needle. The entire site of inoculation was sealed tightly by wrapping the stem with 1.5-cm-wide parafilm, which was used to press the inoculum into the stem and reduce the chance of contamination or desiccation. Tolerance to fungal development was evaluated as the ability of the rooted cuttings to restrain fungal growth with small or nonexistent lesions indicating tolerance.

Survival was assessed weekly and internal lesion length was measured when mortality was recorded; lesion length measurements were taken from all seedlings. Lesion length in all surviving plants was assessed in millimetre using a ruler when the overall survival of the trial reached 50%. Plants in trial 2 were much larger as rooted cuttings were transferred from 150-cc containers into 1-l polybags 5 months after setting and cuttings were set 9 months prior to the date of inoculation by which time the average height was 115 cm. Plants in trials 1 and 3 were transferred from 150-cc containers into 1-l polybags 4 months after setting and were inoculated 5 and 5.5 months after setting when plants averaged 49 and 61 cm, respectively.

Further screening in the nursery and validation in field trials

Two different types of validation trials were established to verify tolerance in field trials using plants produced for nursery trial 1 and nursery trial 3. No field trials were established with selections from nursery trial 2. The goal was to identify a set of clones with differential tolerance to the pathogen that could be used to validate the nursery screening in replicated field trials.

Clones evaluated for validation of nursery trial 1 were derived from the clonally replicated screening trial as well as from excess seedlings from the trial that were inoculated prior to clonal replication and screening. For the field trial derived from nursery trial 1, two of the 4 clones with 5 ramets surviving repeated inoculations in the main nursery trial were included as part of a set of 20 tolerant clones with the other 18 tolerant clones were derived from 1754 excess seedlings. Excess seedlings were not initially converted into hedges for the main nursery screening experiment and seedlings in 150-cc planting tubes were inoculated at the same time as the nursery trial. Survivors were then converted into hedges for further propagation and an additional 4 ramets were inoculated. Therefore, all 20 tolerant clones established in the field validation trial survived 5 inoculations in the nursery. Further validation in the nursery was achieved by inoculating cuttings from 23 of the clones selected from the nursery trial 1 and excess seedlings. For further validation of the 20 tolerant clones included in the field trial, additional cuttings were produced and each clone was inoculated 10 more times as additional cuttings became available.

In total, a set of 40 clones were selected from nursery trial 1 and excess seedlings for field trial validation. There were 3 sets of clones selected for validation: 20 clones with 100% survival classified as tolerant from the nursery trial (2 clones) and excess seedlings (18 clones), 10 clones with survival between 41 and 80% in the clonally replicated nursery trial were classified as intermediate and 10 clones with survival less than 40% in the nursery trial were classified as susceptible. Four ramets of each of these 40 clones were established in field trials and no inoculation was undertaken. Natural infection and other factors led to mortality and survival was assessed 2 years after establishment.

From nursery trial 3, 19 clones with 100% survival were selected for validation in two field trials. An additional 5 clones from the partner's internal breeding programme were also included. Each trial contained 5 ramets of each clone established as single-tree-plots in five replications on two sites. When the trees were 9 months old, four of the five replications in each trial were inoculated with the same culture used in the nursery screening. Survival was assessed 3, 7, 11 and 21 weeks after inoculation. Trees

that were missing clearly died from other causes or had no *Ceratocystis* symptoms were included as missing values.

Statistical analysis

Data from the assessment of the nursery trials were analysed with ASReml-R (Gilmour et al. 2009) to produce breeding value predictions, estimates of population effects and genetic parameter estimates: heritability, repeatability and genetic correlation estimates (Brawner et al. 2011, 2010; Baltunis et al. 2009). Summary statistics for the nursery and field trials were estimated using the base R software (R Core Team 2017). Clonal replication was used to allow for the estimation of repeatability and the open-pollinated family structure provided the pedigree that allowed for heritability estimates and genetic correlation estimates between trials. Repeatability reflects the level of variation among clones and heritability reflects the level of variation among parents relative to the total observed variation in the trial (Baltunis et al. 2009).

A complete linear model and various reduced models were used to evaluate the importance of the genetic strata represented in the trial (Lee et al. 2014; Pegg et al. 2013), where reduced models were used to test the significance of random effects. The complete linear model used to analyse all three trials included fixed effects for the model intercept, each trial and replication within each trial, with random effects included for maternal effects associated with the additive relationship matrix as well as clonal or residual genetic effects associated with an identity matrix. The maternal genetic effects were associated with an unstructured variance–covariance matrix that allowed for the partitioning of genetic variance ($\hat{\sigma}_{\text{Additive}}^2$) by trial and covariance among pairs of trials. Variance components were estimated with restricted maximum likelihood (Gilmour et al. 2019). As clones within families were unique to each trial, there was no clonal covariance among trials and a diagonal variance–covariance matrix was used to partition the residual genetic variance ($\hat{\sigma}_{\text{Clone}}^2$) by trial. Taylor series approximations were used to estimate standard errors of genetic parameters and Wald F -tests were used to determine the significance of fixed effects included in the mixed model (Kenward and Roger 1997). Likelihood ratio tests were used to estimate the significance of random effects by comparing the likelihood of complete and reduced models (Brawner et al. 2011). The variance component estimates for these random effects were used to provide genetic parameter estimates.

Heritability estimates (\hat{h}^2) from the generalised linear model used for the binomial survival data were calculated as follows:

$$\hat{h}^2 = \frac{\hat{V}_A}{\hat{V}_P} \approx \frac{\hat{\sigma}_{\text{Additive}}^2}{\hat{\sigma}_{\text{Additive}}^2 + \hat{\sigma}_{\text{Clone}}^2 + \hat{\sigma}_{\text{Link}}^2}$$

where the error variance was fixed and equated to the variance of the link function ($\hat{\sigma}_{\text{Link}}^2$) as $\pi^2/3$. For the normally distributed lesion length assessments, the error variance was estimated directly from the model. The additive variance $\hat{\sigma}_{\text{Additive}}^2$ was estimated directly from an individual model and given the lack of relationships among families was equal to 4 times the variance among open-pollinated parents. Repeatability estimates (\hat{H}^2) included the residual genetic or clone within family variance, ($\hat{\sigma}_{\text{Clone}}^2$), in the numerator of the heritability estimate. Between experiment and site–site genetic correlations (r_g) were estimated as the ratio of the between-trial parental covariances to the square root of the product of trial-specific parental variances,

$$r_{g(i,j)} = \frac{\hat{\sigma}_{\text{Additive } i,j}}{\sqrt{\hat{\sigma}_{\text{Additive } i}^2 \times \hat{\sigma}_{\text{Additive } j}^2}}$$

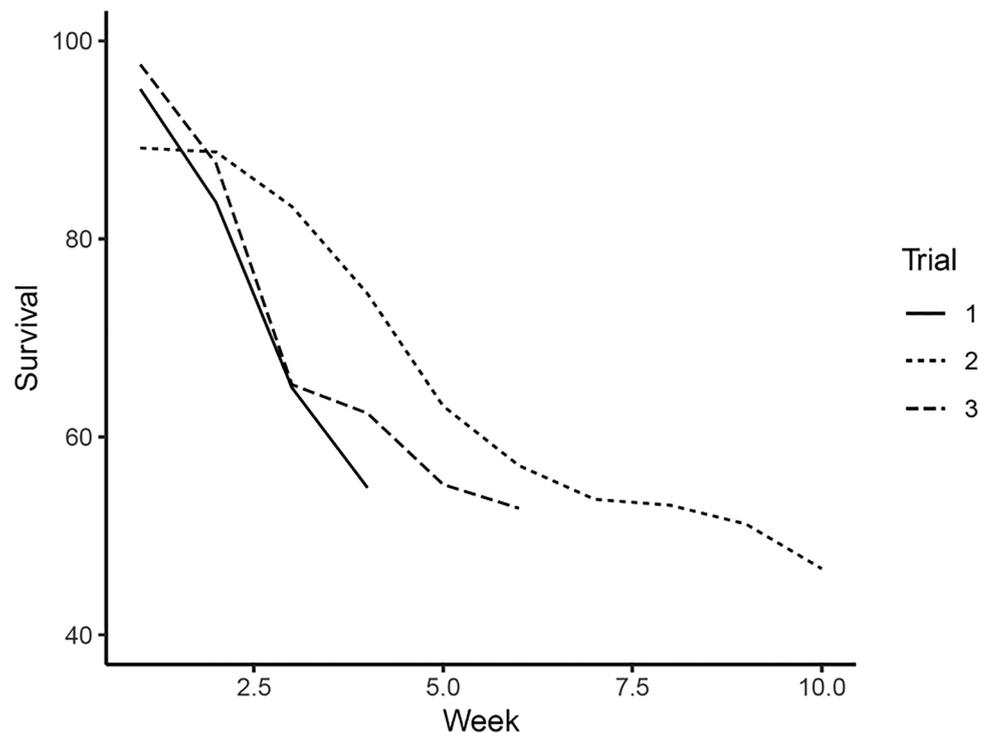
Solutions of the mixed models provided breeding value predictions for the parents evaluated in each of the three nursery trials were used to illustrate between-trial genetic correlation estimates. Fixed effect solutions were used to centre the parental predictions to the trial average by adding the model intercept, trial and the average of the replication fixed effect solutions associated with each trial to the breeding value predictions for parents evaluated in each trial. Survival predictions were returned from the logit scale to percentile predictions using the inverse logit; where: $\text{Survival} = \exp(F_i + R_i) / (1 + \exp(F_i + R_i))$, where F_i is the sum of the fixed effect solutions associated with each trial and R_i is the solution for the random maternal effect associated with each parent in each trial (Schall 1991). Bivariate analyses to directly estimate genetic correlations between the binomially distributed survival trait and the normally distributed lesion length assessments were not possible using ASReml 4.1 (Brawner et al. 2011). Descriptive statistics and analysis of variance tests for tests of significance were produced with R.

Results

Nursery trials of clonally replicated seedlings

Of the 1033 *A. mangium* clones from the CFBTI breeding programme that were inoculated in nursery trials of clonally replicated seedlings and represented by at least 4 ramets, there were 61 clones (5.9%) with 0 mortality. Mortality progressed more rapidly in the two trials where plants were smaller. Figure 2 demonstrates the marked difference among

Fig. 2 Reduction in percent survival in clonally replicated progeny trials of *Acacia mangium* following inoculation with *Ceratocystis* in three nursery screening trials in Indonesia



trials in the time taken to reach 50% mortality with trial 1 requiring just 4 weeks and trial 3 requiring 11 weeks.

Differences among breeding populations

Significance tests for differences among fixed population effects were undertaken using the reduced model that included populations classified as the four seed sources managed by CFBTI. The differences among sources for survival were slight and probably significant ($p < 0.09$) with contrasts among populations showing the survival of the Southern Cape York population was lower than the survival of other three populations (Table 2). There were no differences among the populations for external lesion length assessments. Interestingly, the Southern Cape York Peninsula population produced the lowest survival and the longest external lesion lengths, while both sources from Papua New Guinea presented numerically higher survival and smaller lesion lengths.

Table 2 Survival and external lesion length of the four seed sources included in the three screening trials; treatments followed by different letters indicate significant differences

Population/seed source	Survival (%)				Lesion length (cm)			
	All	Trial 1	Trial 2	Trial 3	All	Trial 1	Trial 2	Trial 3
A – West PNG	47.2 ^a	37.3 ^a	49.0 ^a	52.8 ^a	14.9 ^a	14.0 ^a	20.7 ^a	10.0 ^a
B – East PNG	46.4 ^a	38.1 ^a	49.8 ^a	53.6 ^a	14.2 ^a	13.3 ^a	20.0 ^a	9.3 ^a
C – Cape North	44.7 ^a	35.8 ^a	47.3 ^a	51.1 ^a	15.2 ^a	14.3 ^a	21.0 ^a	10.3 ^a
D – Cape South	37.6 ^b	29.3 ^b	40.0 ^a	43.7 ^b	16.1 ^a	15.2 ^a	21.9 ^a	11.2 ^a

Means having the same letter within column are not significantly different at $\alpha = 0.05$

Genetic parameter estimates

Genetic parameter estimates from a pooled analysis of all data indicate there is a significant level of genetic control for both survival and external lesion length ($p < 0.01$) across all trials (Table 3). When considering survival across all three trials, 26% of the phenotypic variation could be attributed to differences among parents and 31% of the phenotypic variation could be attributed to the combination of differences among parents and differences among the clones derived from these parents. The strength of the genetic signal varied across trials with trial 2 demonstrating the highest heritability and repeatability estimates for both survival and lesion length. The level of genetic control estimated for survival was consistently greater than the level of genetic control estimated for lesion length.

The significance of h^2 (heritability) and H^2 (repeatability) estimates was tested using likelihood ratio tests comparing a complete mixed model to reduced models that excluded the

Table 3 Heritability (h^2) and repeatability (H^2) estimates from all trials and each trial individually, with standard errors of parameter estimates provided in parentheses

Heritability	All trials	Trial 1	Trial 2	Trial 3
Survival (%)				
h^2	0.26 ± (0.05) ***	0.20 ± (0.04) ***	0.38 ± (0.10) ***	0.15 ± (0.06) **
H^2	0.31 ± (0.05) ***	0.22 ± (0.04) *	0.45 ± (0.08) **	0.19 ± (0.06) *
Lesion length (cm)				
h^2	0.14 ± (0.04)***	0.06 ± (0.02)**	0.28 ± (0.07)***	0.02 ± (0.01) ^{ns}
H^2	0.20 ± (0.04)***	0.08 ± (0.02) ^{ns}	0.38 ± (0.06)*	0.02 ± (0.01) ^{ns}

*** $p < 0.01$, ** $p < 0.05$, * $p < 0.1$, *ns* not significant

maternal or clonal effects, respectively. While h^2 provides an estimate of the percent of experimental variance in tolerance explained by differences among parents, H^2 provides an estimate of the percent of variance attributable to differences among parents and clones. The significance tests for H^2 reflect the significance of additional genetic variance that is produced by clonal replication rather than the significance of the total genetic variance. For example, while the 6% of experimental variance in lesion length explained by parental effects (h^2) in trial 1 was considered to be significant, the additional 2% increase in genetic variance produced by accounting for clonal replication was insignificant.

The square root of h^2 and H^2 are estimates of the accuracy of genetic predictions for tolerance in parents or clones, respectively, where the accuracy is the correlation between true and predicted breeding values. As the pedigree assumes there is no relatedness among the open-pollinated families, significance tests for H^2 provide an indication of the importance of increases in prediction accuracy provided by clonally replicating seedlings. In two of the trials, there was no significant increase in the accuracy of lesion length predictions resulting from clonal replication. Conversely, clonal replication consistently produced statistically significant increases in the accuracy of survival predictions in all three trials.

Associating the variance component estimates with their underlying genetic effects (Cockerham 1963) indicates that non-additive genetic effects were consistently smaller than additive genetic effects for both survival and lesion length. Given that the assumption of half-sib relatedness among all members of open-pollinated families is unlikely to hold, it is highly likely that these estimates of

additive variance are inflated. Nevertheless, these results clearly indicate that selecting among parents will provide much of the improvement in tolerance that may be attained using these disease screening methods.

The genetic correlation estimates produced from a combined analysis of all three trials indicated similarities in both survival and lesion length were moderate and parents identified with high survival or short lesion lengths in one trial produced similar results in the other trials. However, the standard errors of genetic correlation estimates were high for all trial pairs and likelihood ratio tests indicated genetic correlations were all significantly different from 0 (survival: $r_{g(1,2)} = 0.32 (\pm 0.25)$, $r_{g(1,3)} = 0.42 (\pm 0.29)$, $r_{g(2,3)} = 0.49 (\pm 0.30)$; lesion length: $r_{g(1,2)} = 0.41 (\pm 0.38)$, $r_{g(1,3)} = 0.35 (\pm 0.25)$, $r_{g(2,3)} = 0.78 (\pm 0.35)$). These experiments were unable to rule out the significance of host–pathogen interactions. More specifically, the family by trial interaction was not significant as the trial is associated with the different pathogens used to evaluate the same families across the three trials. There is some evidence of similarity in host response with Figs. 3 and 4 demonstrating the positive relationships among parental predictions of lesion length and survival between pairs of experiments. Of particular interest was CFBTI family 34 from the West Papua population, identified with a filled circle in Figs. 3 and 4, which had the highest average survival across all three trials as well as the shortest lesion length in trials 2 and 3. The impact of low heritability estimates on the spread of parental breeding value prediction is obvious for lesion length in trials 1 and 3. While it is not possible to imply causation, the height of seedlings evaluated in trials

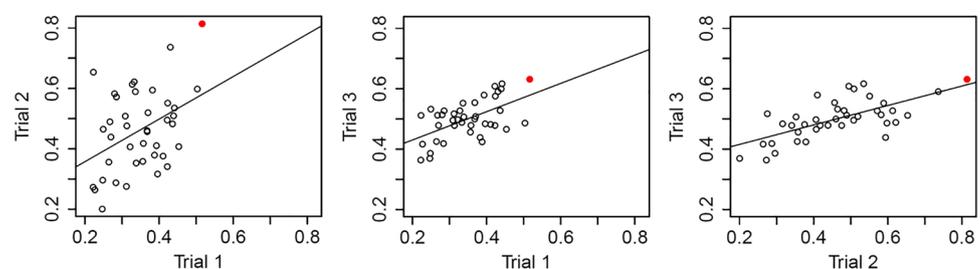
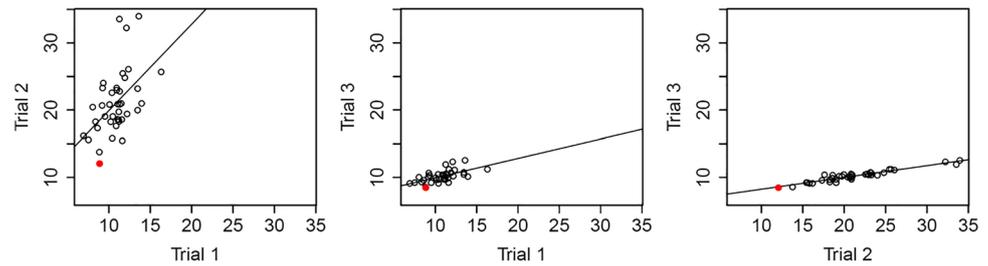
Fig. 3 Relationships between parental breeding values in pairs of trials depicting the observed genetic correlations for survival. A filled circle identifies the family with the highest survival and smallest lesions in all three trials

Fig. 4 Relationships among parental breeding values in pairs of trials describing the observed genetic correlations for lesion length. A filled circle identifies the family with the highest survival and smallest lesions in all three trials



1 and 3 was much smaller than the height of seedlings in trial 2 and this likely impacted the accuracy of estimates.

Further validation in the nursery and in-field validation trials

For trial 1, the excess 1754 seedlings that were not included in the main nursery experiment were inoculated and survivors were clonally replicated for further inoculation. Just 1.1% of these seedlings were identified as resistant with 20 clones surviving all 5 inoculations. Further validation in the nursery that was undertaken by inoculating 23 tolerant clones an additional 10 times identified 4 clones with survival greater than 50%. Just one clone survived 8 of the 10 inoculations demonstrating the extreme challenge posed when inoculating small plants. This reflects the observation in the main nursery trials, where the larger seedlings in trial 2 produced more accurate estimates of tolerance. The field trial was assessed for survival 24 months after establishment in the field, where the average disease incidence in the tolerant, intermediate and susceptible treatments was 27.5%, 35.9% and 46.2%, respectively. While the treatments ranked as expected, differences were not significant. Survival assessments were confounded by mortality from root rot (*Ganoderma* sp.), stem breakage caused by monkeys and other unidentified causes.

Two field trials containing rooted cuttings of tolerant selections from trial 3 and susceptible controls were established and assessed 3, 7, 11 and 21 weeks after inoculation. There was 0 mortality associated with *Ceratocystis* wilt in either trial 3 weeks after inoculation of the 9-month-old trees; however, 24 and 47% of the trees exhibited wilt symptoms and there were no significant differences in external lesion lengths in field trials 1 and 2 at 2.5 and 3.7 cm respectively. At 7 weeks after planting, one tree with wilt symptoms was dead in both trials and 93 and 89% of trees exhibited wilt symptoms with significant differences in external lesions observed between field trial 1 and field trial 2 ($p < 0.01$, 33.3 and 18.1 cm respectively) and among clones ($p < 0.01$). At 11 weeks after planting, mortality associated with wilt symptoms increased to 33 and 27% in field trials 1 and 2 respectively but there was no increase in the number of trees with wilt symptoms. The external

lesion length observed among clones differed significantly ($p < 0.03$) and the lesion length of 55.9 and 38.7 cm differed significantly between field trial 1 and field trial 2 ($p < 0.01$), respectively. Just four of the clones had 0 mortality in both trials and the average lesion length of these four clones was significantly smaller, 33.1 cm compared to 56.4 cm for the remainder of the clones ($p < 0.03$).

Discussion

Selection for tolerance to *Ceratocystis* species has been used to successfully develop improved varieties of various crops: mangoes (*Mangifera*) (Ribeiro et al. 1995), cacao (*Theobroma*) (Simmonds 1994), sweet potato (*Ipomoea*) (Martin 1954) and coffee (*Coffea*) (Castilla 1982). While developing a *Ceratocystis*-tolerant breed of *A. mangium* may be possible, it will be a difficult process given the low levels of tolerance observed in the species and the low to moderate levels of genetic variation (Brawner et al. 2015). The low heritability estimates are directly translated to low estimates of the accuracy associated with assessing seedlings for a vascular wilt disease. Improved accuracy was evident in screening trial 2, where larger saplings with developed vascular tissue were used. While comparisons across trials are anecdotal and seedling size was not directly assessed in discrete experiments, comparing results across trials indicate saplings rather than seedlings should be used for screening (Wingfield et al. 1993). Given the impact of physiological ageing associated with waiting for vascular tissue to develop prior to screening, a lack of resources available to screen sufficient individuals and propagation efficiencies from mature hedges obviates the ability to deploy seedlings following screening. In addition, the low level of tolerance and low heritability estimates indicate seedlings derived from tolerant clones are unlikely to have sufficient tolerance for direct deployment of *A. mangium* seedlings. Nevertheless, *A. mangium* individuals that demonstrate tolerance to *Ceratocystis* canker and wilt may be used to hybridise with other species to produce seedlings for evaluation in screening trials.

Of the clones represented by four or more ramets that were evaluated in these screening trials, only 6.5% had no mortality. In addition to establishing these clones in

validation trials in the field, the most tolerant material has also been propagated and transferred to CFBTI. Disease-tolerant individuals identified in the three screenings have been consolidated into the CFBTI breeding arboretum and are being managed for seed production and hybridisation. This breeding arboretum will be used for the production of open-pollinated seed for further testing and the selections will also be incorporated into the *Acacia auriculiformis* × *A. mangium* hybridisation programme managed by CFBTI. Higher levels of tolerance observed in nursery screenings of *A. auriculiformis* in other nursery screening trials in Indonesia and Vietnam (Trang et al. 2018, Brawner et al. 2020) suggest that hybrids will have greater tolerance compared to *A. mangium*.

Combining a rapid screening system with a suitable propagation strategy will be required to deploy disease-tolerant *A. mangium* at scale, given the reductions in productivity caused by physiological ageing limits the time that individual trees may be deployed as clones. By the time in-field screening trials would be evaluated, propagation rates and productivity are greatly reduced. Alternative screening systems are being evaluated to increase the accuracy and reduce the time required for screening (van Wyk et al. 2010; Newhouse et al. 2013). Although time may be reduced considerably with nursery screening techniques, verification of stability between seedling, sapling and mature tree assessments of lesion length is needed to provide confidence in indirect screening methods using seedlings, branches or detached phyllodes. As well, a greater understanding of the variability in host response to inoculation with different strains of the pathogen is required.

While the positive genetic correlations among trials using different isolates provide evidence that tolerance is stable when families are screened with different strains used by the different partners, the evidence is weak and many races of the pathogen have been described (Ferreira et al. 2010; Witthuhn et al. 1999; Oliveira et al. 2015; Fourie et al. 2016). Further screening of a differential set of tolerant and susceptible clones using various strains of the pathogen is underway so that the importance of differences in strains and their interaction with hosts may be better understood.

The methodology used in the nursery screening must be improved to identify clones that can repeatedly tolerate infection with the disease if reforestation with pure *A. mangium* is required. If the repeatability of the nursery screening could be significantly improved, vegetative propagation following screening may be used for reforestation at scale. However, the low levels of survival, low repeatability estimates and problems maintaining the vigorous growth associated with juvenility in *A. mangium* make the deployment of highly tolerant clones following validation in nursery trials an unlikely proposition.

This study provides findings that differ from South African disease screening experiments using *A. mearnsii*, where resistant individuals were identified but no differences among families were found (Roux et al. 2000). In all three experiments of this study, the major source of genetic variation was from differences among families rather than differences among clones within families. This study indicates that parents which produce seedlings with a greater tolerance to inoculation may be identified using nursery screening; however, the accuracy of the screening system requires large numbers of seedlings to be evaluated repeatedly. Further work with inoculation in the field rather than reliance on natural infection will be required to provide direct comparisons of the efficacy of the nursery and field screening methods. Once the correlation between nursery and field inoculations is available, direct comparisons may be made to evaluate the trade-offs of either method for the development of a *Ceratocystis*-tolerant breed of *A. mangium*.

The study provides several results to consider when developing methodologies to identify individuals with *Ceratocystis* tolerance. In some situations, such as areas where monkeys have become accustomed to feeding on the cambium of *A. mangium* and produce large wounds where the pathogen may readily enter the vascular tissue, it is unlikely that increasing tolerance will be sufficient to reduce damage to a level that is acceptable to forest managers. In other areas where mortality from the disease is less prevalent, a disease-tolerant breed of *A. mangium* may make reforestation with the species a viable option again. However, given the very low levels of tolerance present in *A. mangium*, hybridisation with species such as *A. auriculiformis* Benth. will likely provide greater levels of tolerance in a shorter period than what would result from breeding pure *A. mangium*. Project partners have taken findings from this trial to guide the screening of many seedlings from a diverse set of *A. auriculiformis* populations so that the most tolerant individuals may be consolidated with the *A. mangium* selections for hybridisation and further breeding. Exchanges of acacia genetic resources have also provided genetic diversity that will be evaluated for tolerance.

Conclusions

Screening of clonally replicated *A. mangium* seedlings for tolerance to *Ceratocystis* canker and wilt disease was completed in a relatively short time frame and provided evidence that tolerance following inoculation is heritable. However, very low levels of tolerance and low to moderate heritability estimates for tolerance traits indicate that developing a *Ceratocystis*-resistant breed of *A. mangium* will be difficult. The heritable variation for survival and lesion length found in this screening has provided evidence that parents with greater tolerance may

be identified. The slight increase in genetic control produced by clonally replicating seedlings provides little evidence that clonal replication will deliver significant increases in the accuracy of screening for tolerance.

It is recommended that further protocol development work to improve the disease screening methodology is undertaken and that improved methods are used to evaluate a diverse range of *A. mangium* families for tolerance to *Ceratocystis* infection. Given a fixed level of resources available for screening, evaluating more families rather than clonally replicating individuals will deliver greater improvements in tolerance. However, given the very low levels of tolerance in *A. mangium* in Indonesia, the focus of resistance breeding in acacia has shifted to screening *A. auriculiformis* populations and the production of hybrids for subsequent screening. It is hoped that hybrids between selections from the more tolerant *A. mangium* families and selections from more tolerant *A. auriculiformis* families will provide material so that acacias can once again be deployed at scale in the wet tropics of Southeast Asia.

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Author contribution All the authors planned the study and revised the manuscript. JTB designed the experiments, analysed the data and drafted the manuscript. SS, AN and AR supplied germplasm and coordinated the trial network. HI, MT, MY, SR and EBH developed the protocols used to undertake the experiments and provided data from the screening trials.

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Declarations

Ethics approval This study does not involve any human or animal testing.

Conflict of interest The authors declare no competing interests.

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