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Tolerance of *Pinus patula* full-sib families to *Fusarium circinatum* in a greenhouse study

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The pitch canker fungus, *Fusarium circinatum*, has caused large-scale mortality of young *Pinus patula* Schiede and Deppe ex Schltdl. and Cham. seedlings in nurseries in South Africa since 1990. Diseased seedlings have been inadvertently carried to the field, which in turn have died and has reduced stocking below an acceptable level. Tree breeders have suggested that the only long-term solution to limit infection by this pathogen is to identify and deploy tolerant *P. patula* families. A commonly used technique to identify tolerant clones is to artificially inoculate open-pollinated progeny from orchard clones with *F. circinatum* under greenhouse conditions. In these trials, large variation in tolerance to the pathogen among seedlings within open-pollinated families was observed and this could be influenced by the pollen parent. Therefore, identifying individual full-sib families, where both parents are known, should improve the identification of tolerant families, which can then be repeated. In this study, cuttings from control-pollinated *P. patula* seedling hedges were inoculated with *F. circinatum* in a greenhouse. The results showed large family variation where some of the full-sib families were similar in tolerance to *P. elliottii* Engelm. var. *elliottii* seedlings. Therefore, it is recommended that breeders focus on identifying specific family combinations that are more tolerant to *F. circinatum*.

Keywords: cuttings, *Fusarium circinatum*, greenhouse inoculation, *Pinus elliottii*, *Pinus patula*

Introduction

Pinus patula Schiede and Deppe ex Schltdl. and Cham. is the most important pine species used to establish plantations in South Africa (DAFF 2008) because of its superior growth and wood properties (Morris and Pallett 2000, Vermaak 2007). However, during the last 20 years its deployment has been severely hampered by the pitch canker fungus, *Fusarium circinatum*, which causes mortality of young seedlings in nurseries and after planting (Wingfield et al. 2002, Mitchell et al. 2011). In order to reduce the negative impact of this pathogen on *P. patula*, forest owners can either replace this species with alternatives, that are more tolerant of *F. circinatum*, such as *P. elliottii* Engelm., *P. taeda* L. or *P. tecunumanii* (Schw.) Eguiluz and Perry (Mitchell et al. 2012b) or hybridise it with tolerant species (Mitchell et al. 2012a). In most cases, however, these alternative species or hybrids are best suited to subtropical and warm temperate sites. This complicates the replacement of *P. patula* as it currently remains the best species for planting in colder climates in South Africa. In order to continue to deploy *P. patula* in these regions, without the risk of it becoming infected with *F. circinatum*, it is necessary to identify tolerant individuals that can be grafted in new seed orchards, or used in a controlled-pollination program.

In previous greenhouse studies, where open-pollinated *P. patula* families have been screened as seedlings, large within-family variation in tolerance has been observed among the seedlings. These differences are probably because of the fact that male parents have widely different levels of tolerance to the pathogen. Assuming that this variation is because of the influence of the pollen parent, then identifying those full-sib families that produce progeny with tolerance to infection will be more beneficial than identifying mother trees with good general combining ability. In these cases, tolerant full-sib families can repeatedly be made. Given the limitation of control-pollinated seed, such families would likely be deployed as cuttings from seedling hedges.

In this study, cuttings from a number of control-pollinated full-sib *P. patula* families were inoculated with *F. circinatum* in a greenhouse. A number of common parents were used to determine the mean tolerance of a parent. The overall aim was to assess the variation in tolerance to *F. circinatum* between full-sib families sharing a common parent and to identify full-sib families that could be used in a pine plantation breeding programme.

Methods and materials

Plant material

Sixty control-pollinated full-sib (identity of both parents known) *P. patula* families, from 29 parents, were established as seedling hedges in 2008 for the production of rooted cuttings. Of the 29 parents, 14 were used as the female and 23 were used as the male in the controlled crosses. Eight of the parent clones were used as both the female and male in

the controlled crosses. The shoots from these hedges were routinely harvested and rooted. Those cuttings that had rooted during the first half of 2009 were used in this trial. The 60 families were arranged as treatments in a randomised complete block design with four replications. The average number of cuttings per treatment was 51. Open-pollinated seedlings of *P. patula* and *P. elliottii* from commercial seed orchards were included as controls. In September 2009, the plants were transported to a greenhouse at the University of Pretoria, South Africa, specifically erected for the purpose of screening pine families for tolerance to *F. circinatum*.

Inoculations

The plants were inoculated with a combination of three *F. circinatum* isolates (CMW 3577, 3578 and 3579) maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI) that were shown to be highly virulent in previous studies (Porter 2010). The isolates were grown on half-strength potato dextrose agar (2 g potato extract, 10 g dextrose and 7.5 g agar l^{-1} distilled water) prior to inoculation. The cultures were flooded with sterile water and the spore concentrate diluted to 50 000 spores ml^{-1}. The diluted spore suspension of each isolate was then combined equally. The plants were wounded by removing the apical bud and inoculated by applying 10 µl (500 spores) of the mixed inoculum to the wounded surface. The inoculated plants were watered daily and assessed for lesion development eight weeks after inoculation. At the time of assessment the seedling height, from the root collar to the wounded tip, was also recorded.

Statistical analysis

The statistical software package GenStat version 14 (Payne et al. 2011) was used to analyse the data. The following factors were analysed: (1) Group (where full-sib families, as cuttings, were compared with the *P. patula* and *P. elliottii* seedling controls), (2) *P. patula* female parents, (3) *P. patula* male parents and (4) individual full-sib families. Summary statistics were carried out on the variables (Height, Lesion length and Dieback) to calculate means and the standard error of the mean (SEM) for each treatment. A correlation matrix was generated between Height, Lesion length and Dieback to determine the relationship that each had to the other.

The factors Group, Female, Male and Full-sib family (assigned as fixed effects) were analysed individually by analysis of variance (ANOVA) using lesion length as the variable (see model 1). The control treatments were included in all analyses. Given the small sample size of some of the individual full-sib families after mortality ($n < 30$) SEMs were generally large and the individual full-sib families with a standard error greater than 3 mm were excluded from the full-sib family comparison. A separate ANOVA was carried out where families were nested within parents (parent/family) to assess family variation within a parent (see model 2).

A Duncan's multiple range test was used to differentiate treatment differences at the 5% significance level in each case. Heritability (h^2_{fs}), using lesion length as the variable, was calculated for the complete dataset of 60 full-sib families using the Model Least-Squares and Maximum Likelihood program (LSMLMW and MIXMDL PC-2 Version) developed by Harvey (1990), where a coefficient of relationship of 0.5 was used. The seedling controls (*P. patula* and *P. elliottii*) were excluded from heritability analyses.

Model 1

$$Y_{ij} = \mu + \beta_i + t_j + \varepsilon_{ij}$$

where Y_{ij} = the trait's value in the jth treatment in the ith block, μ = the population mean, β_i = the random effect because of the ith block [$\beta_i \sim N(0, \sigma^2_j)$], t_j = the fixed effect for the jth treatment ($\sum \tau_i = 0$), and ε_{ij} = the random error effect of the jth treatment in the ith block [$\varepsilon_{ij} \sim N(0, \sigma^2_\varepsilon)$].

Model 2

$$Y_{ijk} = \mu + \beta_i + p_j + pf_{jk} + \varepsilon_{ijk}$$

where Y_{ijk} = the trait's value in the kth family in the jth treatment in the ith block, μ = the population mean, β_i = the random effect because of the ith block [$\beta_i \sim N(0, \sigma^2\beta)$], p_j = the fixed effect for the jth parent ($\sum p_j = 0$), pf_{jk} = the fixed interaction effect of the kth family within the jth parent ($\sum pf_{jk} = 0$), and ε_{ijk} = the random error effect of the kth family within the jth parent in the ith block [$\varepsilon_{ijk} \sim N(0, \sigma^2_\varepsilon)$].

Results

Visible lesions could be seen one week after inoculation. After eight weeks approximately one-third of the inoculated *P. patula* plants had died. This was most likely because of the very small size of the cuttings (82 mm) and seedlings (86 mm) (Table 1). Although many of the *P. patula* cuttings had died, sufficient numbers (mean of 39 plants per family) remained to distinguish treatment differences and obtain meaningful results. None of the *P. elliottii* seedlings died.

Lesion length and percentage dieback were positively correlated and significant ($r = 0.911$, $p < 0.001$). Height

Table 1: Mean values and standard errors for height, lesion length and percentage dieback for all full-sib *P. patula* crosses (as cuttings), and *P. elliottii* and *P. patula* controls (as seedlings). Treatments with a different letter are significantly different (Duncan's multiple range test). *n* = Number of plants

Group	Height (mm)				Lesion length (mm)				Dieback (%)[1]			
	Mean	SE	Duncan	*n*	Mean	SE	Duncan	*n*	Mean	SE	Duncan	*n*
Full-sib families	81.8	0.53	B	3 020	22.7	0.41	A	1 989	27.7	0.59	A	1 989
P. elliottii	137.1	2.92	A	56	8.6	1.19	B	56	15.7	1.17	B	56
P. patula	86.0	2.79	B	56	30.5	2.78	C	42	35.9	3.50	C	42

[1] Dieback (%) was adjusted for height

correlated negatively with dieback ($r = -0.217$, $p < 0.001$) and had a small, but significant, influence on lesion length ($r = 0.081$, $p < 0.05$). A comparison of unadjusted versus adjusted values for height was made and, depending whether the covariate (height) was included in the model, the adjusted lesion length for the *P. elliottii* seedling control (in particular) was not constant. Given the small effect that height had on lesion length ($r = 0.081$), unadjusted lesion length was used to compare treatment means.

The *P. elliottii* seedling control was significantly more tolerant (8.6 mm) than the combined mean of all *P. patula* cutting treatments (22.7 mm), which were more tolerant than the *P. patula* seedling control (30.5 mm) (Table 1). There were large differences among the parents tested. In the case of the female parents, which were represented by at least three full-sib progenies, lesion length ranged from 15.8 to 34 mm (Figure 1). Although there were 23 male parents used in the study, only 10 were represented by at least three full-sib families. These produced lesions ranging from 18.8 to 26.8 mm (Figure 1).

The greatest variation in tolerance was between the full-sib families assessed. The lesion lengths of 38 families (that had a SEM of less than 3 mm) ranged from 5.8 to 30.8 mm (Figure 2). Family AP12 × AP29 (13.4 mm), and those below it, was as tolerant as *P. elliottii* (8.6 mm) (Duncan's multiple range test) (Figure 2). There was significant variation among full-sib families that shared a common parent where some full-sib families were more tolerant than either parent (Figure 3). Heritability (h^2_{fs}) for the full-sib families was estimated at 0.199 ± 0.055.

Discussion

As expected, the results of this study demonstrate that the variation in tolerance within open-pollinated families to *F. circinatum* is partially controlled by the pollen parent. Although none of the parents (the mean of a minimum of three full-sib families with a common parent) were as tolerant as the *P. elliottii* seedlings, there were some full-sib *P. patula* families tested as cuttings that were as tolerant as *P. elliottii*. In South Africa, *P. elliottii* is considered 'tolerant' by comparison with *P. patula* as demonstrated by these results. However, it is well known that other species, such as *P. oocarpa* Schiede and *P. jaliscana* Pérez de la Rosa, demonstrate higher levels of tolerance to *F. circinatum* (Hodge and Dvorak 2000), and recent studies show significant variation between *P. elliottii* families that have been bred in South Africa (Mitchell et al. 2012b).

The combined mean for all of the families as cuttings reflected a higher level of tolerance than the mean of the *P. patula* seedling control. This is most likely because of the increased maturation state of cuttings as has been observed with the increased tolerance of *P. taeda* cuttings

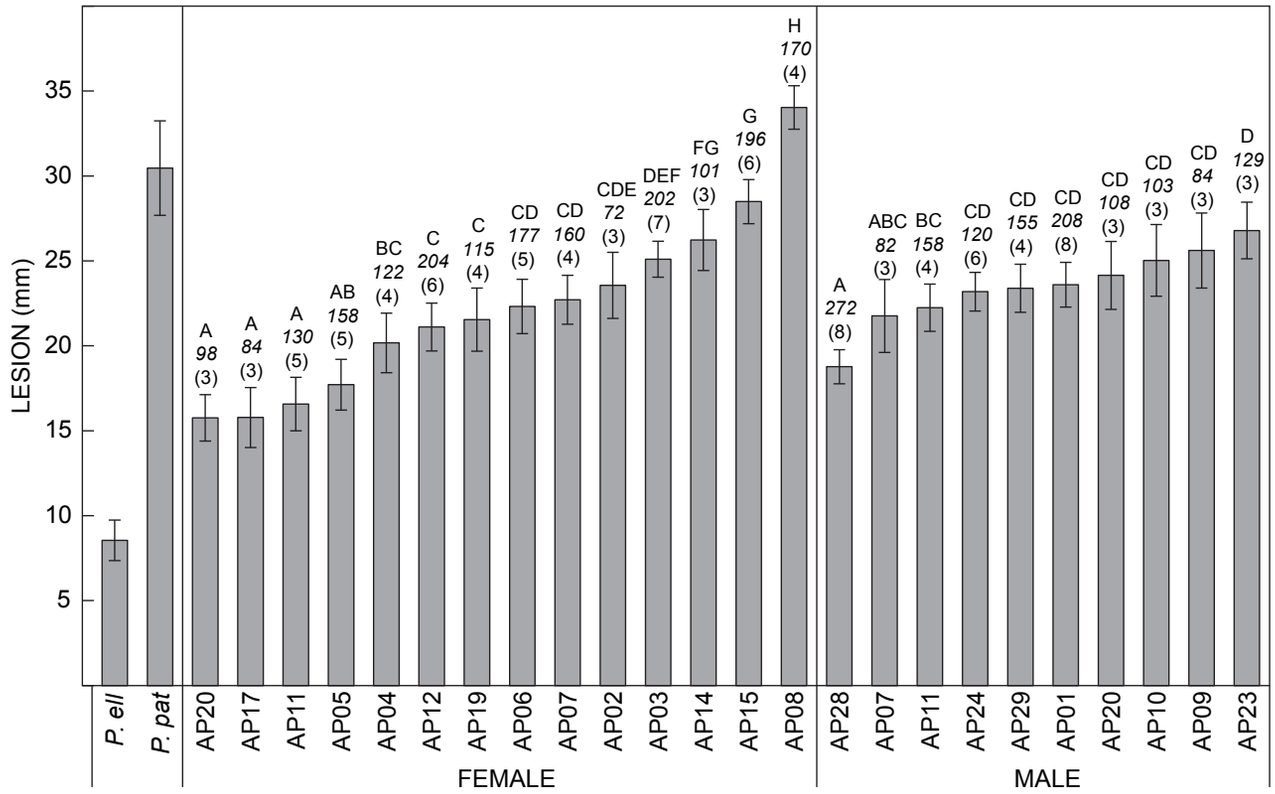

Figure 1: Ranking of the *P. patula* female and male parents, as cuttings, relative to *P. patula* and *P. elliottii* seedling controls. Parent clones with a different letter (within Female or Male groups) are significantly different (Duncan's multiple range test). The numbers in italics represent the number of plants per parent clone, and the numbers in parentheses represent the number of full-sib families that share a common parent. Error bars represent the SE

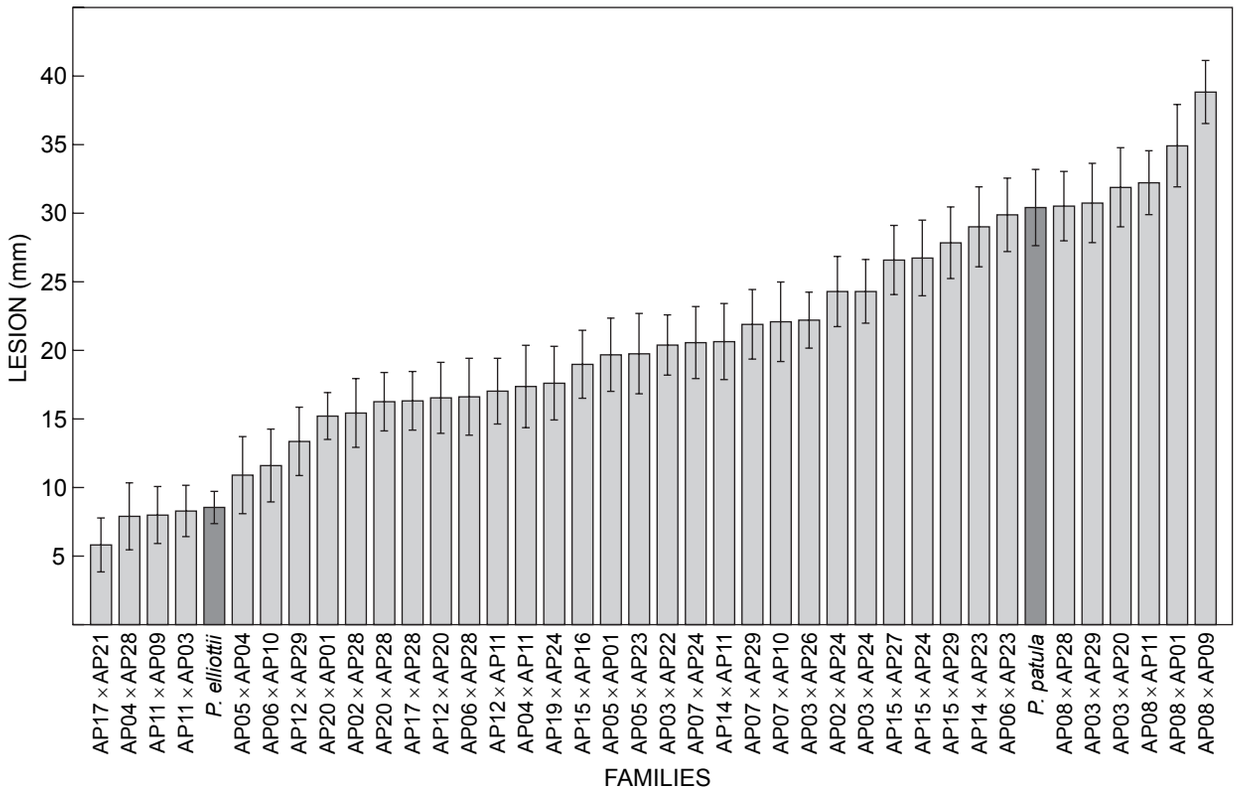

Figure 2: Full-sib *P. patula* family ranking from most to least tolerant. All families with a standard error of the mean greater than 3 mm are not shown. Narrow-sense heritability for the full-sib families was estimated at 0.199 ± 0.055. Error bars represent the SE

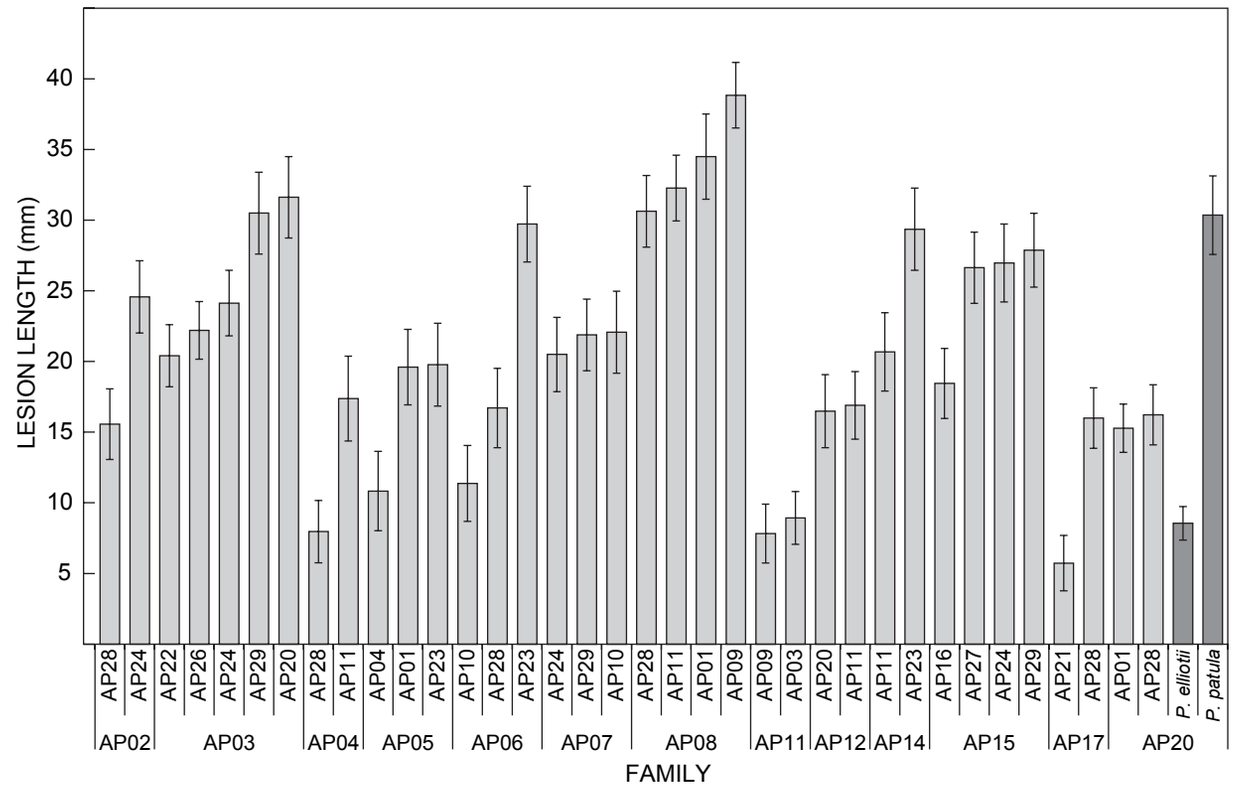

Figure 3: Variation in tolerance among *P. patula* full-sib families, which share a common female parent, relative to the *P. elliottii* and *P. patula* seedling control. Error bars represent the SE

to fusiform rust caused by *Cronartium quercuum* (Frampton et al. 2000). This suggests that, if the full-sib families were tested as seedlings, fewer full-sib families would have been as tolerant as the *P. elliottii* control.

Because the progeny of several of the full-sib crosses were more tolerant than either parent, it is probable that the specific combination of two different clones (referred to as specific combining ability) contributes more to the large phenotypic variation observed as has been reported for the *P. patula* × *P. tecunumanii* hybrid (Mitchell et al. 2012a). If this is true for *P. patula*, then it will be necessary to screen specific crosses and not just the parents for tolerance to *F. circinatum* in the future. None of the *P. patula* parents tested could be described as tolerant (i.e. as tolerant as *P. elliottii*). Ideally, a greater number of parents of open-pollinated families, which would be representative of a larger number of crosses, would enable better identification of tolerant parents with good general combining ability.

There has been concern among breeders that the general susceptibility of *P. patula* to *F. circinatum* cannot be sufficiently improved to overcome the substantial damage because of nursery infection and subsequent post-planting mortality. For this reason, some organisations are considering terminating *P. patula* breeding programs. However, the meaningful heritability ($h^2_{fs} = 0.2 \pm 0.05$) and wide range in tolerance with relatively few (38) full-sib families tested, indicates that improvement can be made through selection.

Despite the fact that hybrids such as *P. patula* × *P. tecunumanii* are performing very well (Nel et al. 2006), and are more tolerant to *F. circinatum* than the pure species (Roux et al. 2007), *P. patula* currently remains the most important softwood species on sites that experience frequent frost in South Africa. It is, therefore, likely that because of the advanced breeding of *P. patula*, where fourth-generation (F_4) selections have been identified for orchard establishment in South Africa, further improvement in the species will emphasise disease tolerance and the performance of parents as hybrid partners.

Conclusion

This study has focused on opportunities to produce *P. patula* that is tolerant of *F. circinatum* in nurseries and in the field. Nursery and establishment infection currently accounts for the most significant losses because of the pathogen in South Africa, but recent outbreaks of pitch canker on mature trees in plantations (Coutinho et al. 2007) represents a real threat. *Pinus radiata* D.Don is severely damaged by *F. circinatum* in plantations in the western and southern Cape but there have not been similar outbreaks in *P. patula* plantations. However, this manifestation of the disease remains a substantial threat to *P. patula* and providing resistance to infection through genetic selection will be important (Wingfield et al. 1999).

Ultimately, new seed orchards of *P. patula*, composed of clones tolerant to infection by *F. circinatum*, are likely to be planted in South Africa. Obtaining seed from such new orchards will not be possible for at least 10 years. In the interim, identifying specific parental combinations should provide a means of deploying *P. patula* that has some level of tolerance to infection by *F. circinatum*. Although it would be useful to repeat the trial on which this study was based, preferably by including additional families, other research has shown a relatively high genetic correlation ($r_A = 0.9$) between repeated greenhouse experiments testing seedling tolerance to *F. circinatum* (RGM unpublished). This suggests that the specific full-sib families that had similar levels of tolerance to *P. elliottii* in this study should be propagated as cuttings and tested commercially.

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