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Comparison of the tolerance of *Pinus patula* seedlings and established trees to infection by *Fusarium circinatum*

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Since the first appearance of *Fusarium circinatum* in South Africa in 1990, foresters have been challenged with poor field survival of *Pinus patula* seedlings at establishment. One of the best long-term solutions is to improve the genetic tolerance of *P. patula* to infection by the pathogen. Currently, large numbers of families are routinely screened for their tolerance to *F. circinatum* by infecting open-pollinated seedlings from orchard clones in a greenhouse and assessing lesion development. In this study, nine-year-old *P. patula* trees from 96 families were inoculated with *F. circinatum* in the field. Their levels of tolerance were assessed and compared to those observed in seedlings originating from seed harvested from the same trees. The field results were also compared with those from previous greenhouse screening trials where seedlings from a number of the same families had been inoculated with *F. circinatum*. The results showed that there was a strong phenotypic (r = 0.71) and genetic (r_g = 0.94) correlation in the performance of the families common in both the greenhouse studies. A comparison of the tolerance of the families, screened as both seedlings and as trees, was also meaningful (r = 0.40). Furthermore, the seedlings raised from seeds collected from the infected *P. patula* trees, that ranked more tolerant than the mean of the *P. elliottii* trees, were similar in tolerance to *P. elliottii* seedlings in the greenhouse trial. Our results indicate that utilising seedlings from clones known to be tolerant should improve the tolerance of mature trees to infection by *F. circinatum*.

Keywords: disease screening, *Fusarium circinatum*, heritability, *Pinus patula*, pitch canker, seedlings, trees

Introduction

Pinus patula is South Africa's most important pine species with an annual planting of approximately 15 000 ha (DAFF 2010) or 25 million seedlings. While it is an outstanding species for planting in many parts of South Africa, it is more susceptible to fungal pathogens such as *Diplodia pinea* (Swart et al. 1985; Smith et al. 2002) and abiotic factors such as fire (de Ronde and du Plessis 2002) and high temperatures during the summer months (Allan and Higgs 2000) than the alternative *P. elliottii*. However, the single most important limiting factor restricting the planting of *P. patula* is its susceptibility to the pitch canker fungus, *Fusarium circinatum* (Mitchell et al. 2011).

Poor seedling survival (Crous 2005) and the cost of replanting young *P. patula* stands (Mitchell et al. 2011) has caused foresters to question the value of continued planting of this species in high disease areas. It is accepted by many that the only long-term solution will be to improve the genetic resistance of *P. patula* to *F. circinatum*. Success in creating tolerant germplasm has been achieved by hybridising *P. patula* with tropical species such as *P. tecunumanii* and *P. oocarpa* (Roux et al. 2007) and these hybrids have gained popularity in recent years. *Pinus patula* × *P. tecunumanii* and *P. patula* × *P. oocarpa* are, however, limited to the warmer sites of South Africa, leaving *P. patula* as the preferred species for regions that experience frequent frost events (Mitchell et al. 2012b).

Breeders have attempted, for a number of years, to identify *P. patula* seed orchard clones that produce tolerant open-pollinated progeny that can be commercially deployed. These studies are conducted in a greenhouse where seedlings are wounded and inoculated with spores of *F. circinatum*. In these studies, none of the *P. patula* families, when tested as seedlings, have been found to be as tolerant as *P. elliottii*. In other studies, inoculating seedlings of various species in the greenhouse (Hodge and Dvorak 2000; Mitchell et al. 2012a) compare well with the tolerance of the same species in the field (Roux et al. 2007). Therefore, it can be expected that if tolerant *P. patula* clones do exist, identifying them as seedlings in greenhouse inoculation studies will be a relatively easy and a cost-efficient means of improving field survival as well as reducing the risk of pitch canker on mature trees.

The objectives of this study were to determine (1) whether screening families as seedlings in a greenhouse will provide information about the tolerance of the same families as mature trees, and (2) whether there is sufficient variation in the tolerance of *P. patula* to *F. circinatum* to identify tolerant families. To achieve these objectives, two successive greenhouse trials were initiated to test a large number of families, several of which were common to both greenhouse screening events. These results were then compared to the tolerance of the same families tested as nine-year-old

trees. Furthermore, we compared the tolerance of seedlings raised from seeds collected from the mature trees to infection by *F. circinatum* in the greenhouse.

Materials and methods

Greenhouse studies

In this research, we conducted three different greenhouse studies. The first two studies utilised open-pollinated seed from 78 and 63 *P. patula* seed orchard clones, respectively. The first group was sown in early 2006 in preparation for inoculation in November of that same year and the second group in the winter of 2006 in preparation for inoculation in March 2007 (Table 1). Seventeen of the *P. patula* families were common to both studies. Although the first study did not include a control species, the second trial included seedlings from open-pollinated *P. elliottii* and *P. radiata* seed orchards, and cuttings of *P. patula* × *P. tecunumanii* (low elevation source; LE), *P. patula* × *P. oocarpa*, *P. patula* × *P. greggii* var. *greggii*, *P. patula* × *P. caribaea*, *P. elliottii* × *P. caribaea*, *P. tecunumanii* (LE) × *P. caribaea* and *P. tecunumanii* (LE) × *P. oocarpa*.

Seedlings for the first two studies were grown in plastic Unigro 98® trays with square-shaped removable inserts in composted pine bark at the Komatiland Forests (KLF) research nursery near Sabie. Each insert had a cavity size of 90 cc. The plants were fertilised with granular 2:3:2 (22) N:P:K fertiliser approximately three times during the nursery period. No record was kept of the quantities of fertiliser applied on each occasion. No fungicides were applied to the seedlings during their establishment. Once the seedlings/cuttings were ready for planting, the treatments were arranged in a randomised complete block (RCB) design with four replications (Table 1).

The third greenhouse study utilised seed collected in August 2009 from 18 open-pollinated *P. patula* trees that were scored as either tolerant or susceptible to *F. circinatum* (see below). All the available seed from six tolerant trees and 12 susceptible trees was sown in May 2010 at the York Timbers Klipkraal nursery near Sabie. The seedlings were raised in composted pine bark in Unigro® trays described previously, and under similar conditions as the KLF nursery, for a period of 10 months. Open-pollinated *P. elliottii* seedlings from a second-generation seed orchard were included as a control. At the end of the nursery phase, the seedlings from each tree were arranged in plots in a RCB design and replicated four times. Depending on the number of seedlings available, each plot consisted of 14 to 28 plants with an average plot size of 21 plants (84 seedlings per treatment).

When the seedlings were of a suitable size for out planting, they were transported to a greenhouse screening facility that is specifically used for inoculation studies at the Forestry and Agricultural Biotechnology Institute of the University of Pretoria. The plants in the first two studies were inoculated with a combination of three virulent isolates (CMW 3577, 3578 and 3579) of *F. circinatum*, while only one isolate (CMW 3579) was used in the third study. This was to accommodate the fact that, although CMW 3579 is highly virulent, it can be outcompeted by isolates CMW 3577 and CMW 3578 when applied to the same wound (Porter 2010). In all cases, inoculum was prepared by growing the fungi on half-strength potato dextrose agar medium (PDA; 2 g potato extract, 10 g dextrose, and 7.5 g agar per litre distilled water) for 7 d at 25 °C. The spores of each isolate were then harvested by flooding the cultures with sterile distilled water and their concentration quantified using a haemocytometer. For the first two studies, the spore suspensions for each of the three isolates were mixed together equally to a final concentration of 50 000 spores ml^{-1}, while the third study utilised 50 000 spores ml^{-1} prepared from the single isolate. In the greenhouse, 10 µl (500 spores) of the freshly prepared inoculum was applied to the wounded surface of a seedling after removing the apical bud (a few millimetres below the seedling tip) using secateurs. Once inoculated, the plants were watered daily. After eight weeks, lesion development and height of each seedling was recorded (in mm).

Field study

During 2001, open-pollinated seedlings from 96 seed orchard clones of *P. patula* were planted in unreplicated

Table 1: Trial design and dates for inoculation of *Pinus patula* seedlings with *F. circinatum*

Details	Greenhouse 1	Greenhouse 2	Field	Greenhouse 3
Date inoculated	Nov 2006	Mar 2007	Dec 2009	Dec 2010
Date assessed	Jan 2007	May 2007	Mar 2010	Feb 2011
Families tested	78	63	96	17
Mean plot size	22 (seedlings)	14 (seedlings)	10 (trees)	21 (seedlings)
Replications	4	4	1	4
Mean no. observations per family	88	56	30	84
Controls	None	*P. elliottii* *P. patula* *P. radiata* *P. patula* × *P. tecunumanii* LE *P. patula* × *P. oocarpa* *P. patula* × *P. caribaea* H *P. patula* × *P. greggii* var. *greggii* N *P. tecunumanii* LE × *P. oocarpa* *P. tecunumanii* LE × *P. caribaea* H *P. elliottii* × *P. caribaea* H	*P. elliottii*	*P. elliottii* *P. patula*

square plots of 25 trees per family by KLF near Sabie. The family plots were arranged in a rectangular shape (16 plots long and six plots wide) and the seedlings were planted at a spacing of 3 m × 3 m. When the trial was nine years old, four to 12 trees were chosen (based on the availability of cones) in each family plot and marked for use in this study. Most families were represented by 10 trees and a total of 923 trees were selected. Ten *P. elliottii* trees, of the same age from a compartment adjacent to the trial site, were selected as a control.

In December 2009 (mid-summer) the trees were inoculated with the same three isolates (CMW 3577, 3578 and 3579) used for the first two greenhouse studies. Inoculum was prepared by growing the isolates on half-strength PDA for 7 d at 25 °C. The trees were wounded at breast height (1.3 m above ground level) by removing part of the thick outer bark and extracting a 5 mm plug of phloem to the depth of the cambium from three equally spaced sides of the tree. Each wound was inoculated with a separate isolate of the pathogen. A 5 mm agar plug with mycelium (removed with a cork borer from the actively growing culture) was placed so that the hyphae made contact with the cambium. The identity of each isolate was recorded by painting the stem of the tree above the wound with a colour unique to each isolate. The agar plugs were sealed with masking tape to protect the inoculum from desiccation. Twelve weeks after the trees were inoculated the wounds were exposed by removing the bark above and below the point of inoculation and recording the length of each lesion (in mm). The circumference of each tree was also assessed at the height of the inoculation point.

Twenty-three months after inoculation, the trees were felled to prevent the possible spread of *F. circinatum.* Bark and wood samples were collected from the inoculation sites on several of the fresh logs that had been stacked at the roadside. These were submitted to the Tree Pathology Cooperative Program (TPCP; http://www.fabinet.up.ac.za), University of Pretoria, where they were placed on *Fusarium* selective medium (Peptone PCNB agar; Nash and Snyder 1962, modified by Nelson et al. 1983) for a period of 5 d. Fungal cultures, typical of the genus *Fusarium*, were transferred to Spezierller Nährsstoffarmer agar (known as SNA) (Nirenberg 1976), where they were cultured for a further 10 d. These were then viewed under a light microscope to detect the presence of morphological structures unique to *F. circinatum* (Viljoen et al. 1997).

Statistical analysis

The statistical software package SAS® Enterprise Guide 4.3 (SAS Institute, Inc., Cary, NC, USA, 2010) was used to analyse the data in all the trials. In keeping with previous studies (Mitchell et al. 2012a), lesion length was used as the variable to describe treatment differences.

Each of the greenhouse trials was analysed separately. After standardising and correcting the data for the effect of replication, a Pearson correlation matrix was generated as a measure of the strength of the relationship of height, lesion length and percentage dieback. The data (lesion length) was subjected to an analysis of variance (ANOVA) to determine the level of significance between family means. A Duncan multiple range test was used to identify treatment differences at the 5% significance level. Narrow-sense individual-seedling heritability (h_i^2) was calculated using the Model Least-Squares and Maximum Likelihood program (LSMLMW & MIXMDL PC-2 Version) (Harvey 1990) where a coefficient of relationship of 0.25 was used. The *P. elliottii* and *P. radiata* seedlings, as well as the cuttings of the various hybrids, were excluded from heritability analysis in the second trial.

After analysing the results from the first two greenhouse studies, the tolerance of the 17 families common to both trials was compared. A Pearson correlation matrix was used as an indication of the strength of the relationship based on phenotypic observations. The Proc Varcomp procedure (SAS® Enterprise Guide 4.3) was carried out to estimate the family variance components in each data set. Data sets containing the family means for each trial were created. The data sets were then merged and the correlation procedure (Proc Corr) was conducted to estimate the covariance of the family means. The genetic correlation (Falconer 1989) and standard error of the genetic correlation (Becker 1992) were calculated using the formulae shown below where 'site A' and 'site B' refer to the first and second greenhouse screening data sets, respectively.

$$r_{g_{\text{siteA\_siteB}}} = \frac{\text{cov}_{f_{\text{siteA\_siteB}}}}{\sqrt{\sigma^2_{f_{\text{siteA}}} \times \sigma^2_{f_{\text{siteB}}}}}$$

where $r_{g_{\text{siteA\_siteB}}}$ is the genetic correlation for lesion length between sites A and B, $\text{cov}_{f_{\text{siteA\_siteB}}}$ is the covariance of family means for lesion length between sites A and B, $\sigma^2_{f_{\text{siteA}}}$ is the family variance of lesion length at site A, and $\sigma^2_{f_{\text{siteB}}}$ is the family variance of lesion length at site B.

$$\text{SE}_{r_g} = \frac{1 - r_g^2}{\sqrt{2}} \times \sqrt{\frac{\text{SE}_{h_x^2} \times \text{SE}_{h_y^2}}{h_x^2 \times h_y^2}}$$

where SE_{r_g} is the standard error of the estimated genetic correlation between site A and B; r_g is the estimated genetic correlation between site A and B; h_x^2 and h_y^2 are the heritability estimate for lesion length of sites A and B, respectively; and $\text{SE}_{h_x^2}$ and $\text{SE}_{h_y^2}$ are the standard errors of the heritability estimate for lesion length of sites A and B, respectively.

For the field study, a Pearson correlation matrix was calculated between tree stem circumference and lesion length for each isolate, and the means of the combined isolates. As circumference had a weak, but positive, correlation with the combined lesion length (r = 0.14, $p < 0.001$) the ANOVA tests were conducted on the corrected (for circumference) lesion length values testing family (n = 97 (including the *P. elliottii* control), isolate (n = 3) and their interaction in a single model (see below). A Duncan multiple range test was used to distinguish family and isolate differences.

$$w_{ijk} = \mu + f_i + s_j + fs_{ij} + e_{ijk}$$

where w_{ijk} is wound or lesion length (either corrected or not) of isolate j of tree k of family i, μ is the population mean, f_i

is the random effect of family i, s_j is the random effect of isolate k, fs_{ij} is the interaction effect between family i and isolate j, and e_{ijk} is the random error effect.

There were 12 families common to the first (2006) greenhouse trial and the field trial, and 16 families common to the second (2007) greenhouse trial and the field trial. The phenotypic correlation between the families common to the greenhouse and field trials was determined using the same procedures described for the two greenhouse studies. This was done separately for each greenhouse study and the field. The data for both greenhouse studies were then merged to allow for a comparison of 23 common families between the greenhouse trials and the field trial. These procedures were carried out on unadjusted greenhouse values compared with both adjusted and unadjusted field values in separate analyses.

Results

Greenhouse studies

In the first two greenhouse studies, seedling height correlated negatively ($p < 0.021$, $r = -0.14$; and $p < 0.001$, $r = -0.37$, respectively) with dieback but did not correlate significantly with lesion length in the first ($r = 0.04$) or the second greenhouse study ($r = 0.02$). Therefore, unadjusted lesion length was used to compare treatments. The lesion length of the 78 families screened in the first (2006) greenhouse study ranged from 5 mm (AP044) to 14.7 mm. The narrow-sense heritability (h_f^2) estimate was 0.25 ± 0.05 (Table 2). In the second (2007) greenhouse study, *P. elliottii* and those hybrids between *P. elliottii*, *P. patula*, *P. caribaea* var. *hondurensis*, *P. oocarpa* and *P. tecunumanii*, were significantly more tolerant than the mean of all *P. patula* families (Table 3). *Pinus radiata*, and the hybrid between *P. patula* and *P. greggii* var. *greggii*, were significantly more susceptible than *P. patula* (Table 3). The lesion length of the 63 *P. patula* families in the second greenhouse trial ranged from 10.2 to 34.2 mm. The most tolerant family (AP004) was as tolerant as the *P. elliottii* control (9.1 mm) and 20 families were as susceptible as the *P. radiata* control (32.2 mm). The narrow-sense heritability (h_f^2) estimate was 0.52 ± 0.09 (Table 2).

The phenotypic correlation generated by comparing the 17 common families of *P. patula* in the two greenhouse studies was meaningful ($r = 0.71 \pm 0.002$), as was the genetic correlation ($r_g = 0.94 \pm 0.03$) (Table 4, Figure 1). Family AP004, which was as tolerant as the *P. elliottii* control in the second greenhouse study, was the second-most-tolerant family in the first greenhouse study (Table 5). Families AP057 and AP064 were considered susceptible in both the first and second greenhouse studies (Table 5) and families AP067, AP036, AP039, AP065 and AP055, which were intermediate, ranked similar in both studies (Table 5).

Table 2: Narrow-sense heritability (h_f^2) estimates in the greenhouse studies

Screening event	Families	h^2 ± SE
2006 greenhouse	78	0.25 ± 0.05
2007 greenhouse	63	0.52 ± 0.09
2006 + 2007 greenhouse	35	0.40 ± 0.09

Table 3: Tolerance of *P. patula*, compared with *P. elliottii*, *P. radiata* and several hybrids in the 2007 greenhouse trial. Values are the mean ± SE

Treatment group	*n*	Height (mm)	Lesion (mm)	Dieback (%)
P. tecunumanii LE × *P. oocarpa*	22	94.0 ± 5.1	3.2 ± 0.3[a]	4.4 ± 0.4[a]
P. tecunumanii LE × *P. caribaea*	52	128.4 ± 2.1	5.1 ± 0.7[a]	4.2 ± 0.6[a]
P. patula × *P. caribaea*	23	110.3 ± 3.1	5.2 ± 0.4[a]	4.78 ± 0.4[a]
P. elliottii	53	180.6 ± 2.1	5.6 ± 0.6[a]	3.1 ± 0.3[a]
P. elliottii × *P. caribaea*	44	193.4 ± 3.0	6.1 ± 0.7[a]	3.2 ± 0.4[a]
P. patula × *P. oocarpa*	45	124.0 ± 5.4	7.5 ± 1.1[a]	6.2 ± 0.9[a]
P. patula × *P. tecunumanii* LE	67	116.6 ± 3.3	8.2 ± 0.8[a]	7.6 ± 0.8[a]
P. patula	4 371	142.5 ± 0.4	24.6 ± 0.2[b]	18.5 ± 0.2[b]
P. radiata	66	99.5 ± 2.6	34.7 ± 1.8[c]	35.2 ± 1.9[c]
P. patula × *P. greggii* var. *greggii*	35	63.8 ± 2.2	36.9 ± 2.2[c]	50.2 ± 3.3[d]

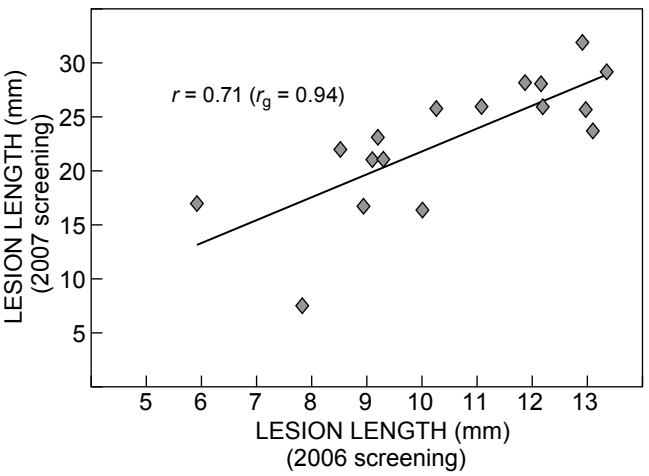

Figure 1: Phenotypic correlation (based on lesion length) of the 17 families common in the two greenhouse studies. The value in parentheses represents the genetic correlation

Table 4: Summary of the phenotypic (r) and genetic (r_g) correlations of the families common in the 2006 and 2007 greenhouse trials, and the phenotypic (r) correlation between those the families common to the greenhouse and field trial. Values in parentheses are based on lesion length after adjusting for circumference. For a given variable, means that do not share a common letter are statistically different ($p < 0.05$; Duncan's multiple range test)

Site A	Site B	Families	r (phenotypic)	r_g (genetic)
G/H 1	G/H 2	17	0.71 ± 0.00	0.94 ± 0.03
G/H 1	Field	12	0.56 ± 0.06 (0.44 ± 0.15)	
G/H 2	Field	16	0.47 ± 0.07 (0.47 ± 0.07)	
G/H 1 and 2	Field	23	0.43 ± 0.04 (0.40 ± 0.06)	

In the third greenhouse study that included progeny of the most tolerant and susceptible trees identified in the field study (see below), seedling height had a negative effect on percentage dieback ($r = -0.28$, $p < 0.001$) and correlated positively with lesion length ($r = 0.12$, $p < 0.001$). Lesion length and percentage dieback were significantly correlated ($r = 0.89$, $p < 0.001$). The lesion length and dieback values were therefore adjusted for height. The seedlings of the three *P. patula* trees that ranked more tolerant than the mean of the *P. elliottii* trees in the field (AP11-6, AP29-2 and AP12-3) were equally tolerant as the *P. elliottii* seedlings in the greenhouse (Table 6). All of the *P. patula* trees that had a lesion length longer than the mean length of the 10 *P. elliottii* trees (55 mm produced progeny that were more susceptible than the *P. elliottii* seedlings (Table 6). The lesions on the seedlings from the most susceptible trees continued to develop and, by month four, most of these seedlings were dead while those from the most tolerant trees were producing new shoots beneath the lesion (Figure 2). None of the seedlings in the *P. elliottii* control died (Figure 2).

Field inoculation study

Overall, the mean lesion length of all the *P. patula* trees was 95.9 ± 0.7 mm and the mean of the *P. elliottii* trees was 54.5 ± 2.3mm. There was significant ($p < 0.001$) variation between the families with the lesion length of the most tolerant family (AP168), which measured 70.1 ± 5.9 mm, and most susceptible family (AP163) which measured 121.0 ± 4.7 mm (Figure 3). The lesion length for the trees of the most tolerant family (AP168) ranged from 38.0 to 111.0 mm and within the most susceptible family (AP163) the range was from 99.7 to 150.5 mm. The most tolerant *P. elliottii* tree had a lesion length of 36.0 mm and the least tolerant *P. elliottii* tree had a lesion length of 67.2 mm. The most tolerant *P. patula* tree in the trial had a mean lesion length of 30.3 mm and the most susceptible *P. patula* tree had a mean lesion length of 162.7 mm. Of the 923 *P. patula* trees, 30 had lesion lengths that were smaller than the 10 *P. elliottii* trees (54.5 mm) and 67 trees had lesion lengths smaller than the least tolerant *P. elliottii* tree (67.2 mm). Approximately 5% of the trees had lesion lengths less than 60 mm and were considered tolerant based on the mean lesion length of the 10 *P. elliottii* trees. The 30 most tolerant trees (with lesion lengths less than 55 mm) were from 25 of the 96 families.

There were significant ($p < 0.001$) isolate effects. Isolate CMW3579 produced a mean lesion length of 98.3 mm, which was more aggressive than CMW 3578 (96.2 mm) and CMW 3577 (92.1 mm). There was no family × isolate ($p = 0.45$) interaction. Although the trees produced resin around the inoculation points, the infection sites never developed into large cankers. In addition, trees in the field did not develop any typical symptoms of pitch canker such as shoot and branch dieback seen elsewhere (Coutinho et al. 2007). Furthermore, *F. circinatum* could not be reisolated from the wood samples 23 months after the inoculation date by which stage new tissue and bark had grown over the lesion.

A comparison of the 12 families in the first (2006) greenhouse trial, that were common in the field trial, produced a phenotypic correlation (r) of 0.44 ± 0.15 (adjusted) (Table 4). The phenotypic correlation (r) between the 16 common families in the second (2007) greenhouse trial and the field trial was 0.47 ± 0.07 (Table 4). The phenotypic correlation (r) between the 23 families in the combined greenhouse studies (2006 and 2007) and field was 0.40 ± 0.06 (Table 4).

Table 5: The ranking of families common in different screening events. For a given variable, means that do not share a common letter are statistically different ($p < 0.05$; Duncan's multiple range test)

Tree	Field		Greenhouse		
	Rank	Lesion ± SE (mm)	*N*	Lesion ± SE (mm)	Dieback ± SE (%)
AP11-6	7	41.0 ± 6.0	79	20.0 ± 1.8^{fg}	21.8 ± 2.0^{f}
AP29-2	23	51.0 ± 1.8	88	15.7 ± 2.1^{g}	18.1 ± 2.3^{f}
AP12-3	26	52.0 ± 1.4	78	16.6 ± 1.9^{g}	17.3 ± 2.3^{f}
P. elliottii[1]		54.5 ± 2.3	75	17.5 ± 1.9^{g}	20.8 ± 1.9^{f}
AP84-2	48	63.7 ± 3.2	93	27.4 ± 2.1^{de}	31.0 ± 2.5^{de}
AP69-5	51	64.0 ± 9.7	28	33.9 ± 2.7^{bcd}	37.7 ± 3.3^{bcd}
AP21-3	54	64.7 ± 7.4	68	31.2 ± 2.5^{cde}	36.1 ± 2.6^{cde}
P. patula[1]		95.9 ± 0.7	75	32.4 ± 2.2^{cd}	35.5 ± 1.6^{cde}
AP22-1	812	126.3 ± 14.0	87	31.1 ± 2.2^{cde}	34.8 ± 2.3^{cde}
AP51-2	865	127.3 ± 1.6	51	36.0 ± 2.3^{abc}	40.3 ± 2.6^{abc}
AP73-2	870	127.7 ± 15.3	73	24.4 ± 2.1^{ef}	29.5 ± 2.0^{e}
AP15-10	873	128.3 ± 3.5	74	43.2 ± 2.3^{a}	46.8 ± 2.3^{a}
AP22-2	885	130.7 ± 12.2	44	35.1 ± 2.5^{bc}	40.1 ± 3.1^{abc}
AP81-1	895	132.7 ± 5.2	31	36.6 ± 2.2^{abc}	41.1 ± 2.7^{abc}
AP15-2	896	133.3 ± 8.2	73	40.3 ± 1.8^{ab}	44.7 ± 1.9^{ab}
AP03-2	898	134.7 ± 7.6	79	32.8 ± 2.3^{bcd}	36.3 ± 2.2^{cde}
AP51-3	901	135.0 ± 5.4	52	34.5 ± 3.0^{bcd}	38.7 ± 3.1^{bcd}
AP56-2	907	138.0 ± 11.0	80	36.5 ± 1.8^{abc}	40.6 ± 1.9^{abc}
AP87-2	911	141.0 ± 16.8	81	38.6 ± 1.8^{abc}	42.4 ± 1.8^{abc}
AP61-8	920	162.7 ± 7.2	87	30.9 ± 2.2^{cde}	36.2 ± 2.5^{cde}

[1] The mean values for the *P. elliottii* and *P. patula* treatments in the field are based on the mean of the 10 *P. elliottii* trees and the mean lesion of all 920 *P. patula* trees

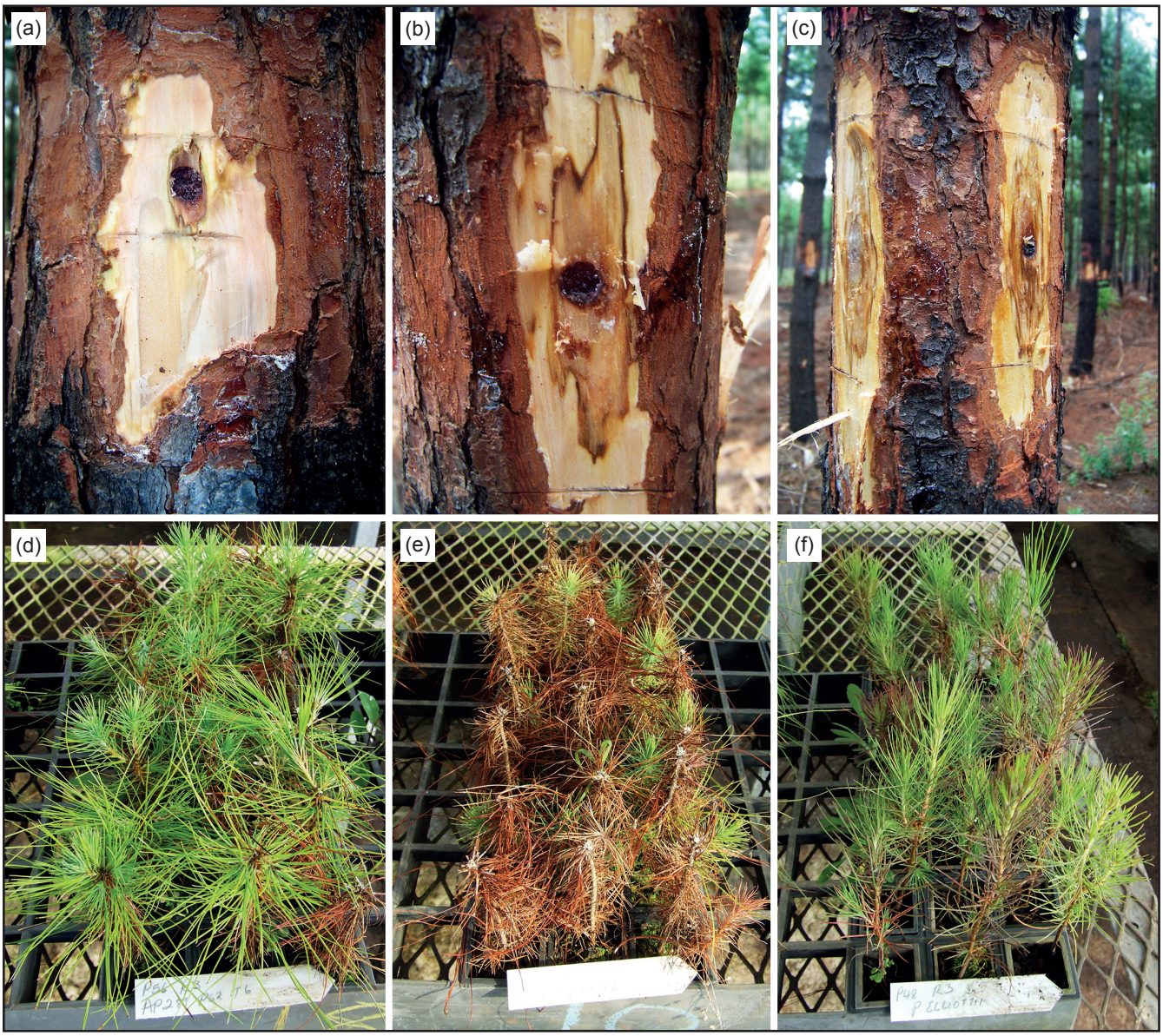

Figure 2: Individual trees in the field trial ranged from tolerant (a) to highly susceptible (b and c). Bottom: seedlings raised from the seed collected from the seventh-most-tolerant tree (d), from the most susceptible tree (e), and *P. elliottii* control (f) showed similar levels of tolerance as the parent trees

Discussion

The results of these studies show clearly that there is sufficient variation within *P. patula* to identify families that can be considered comparable to *P. elliottii* in their tolerance to *F. circinatum*. The significant genetic correlation (0.94) between the two repeated greenhouse studies, and moderate (0.25) to high (0.52) narrow-sense individual-seedling heritability seen in each, indicates that more tolerant clones can be identified as seedlings by screening large numbers of open-pollinated families in greenhouse trials. Other recent studies support this (Nel et al. 2014). Despite the fact that the correlation between the greenhouse studies was significant, the rank in tolerance of some families was dissimilar. Therefore, it would be wise to repeat a greenhouse screening event at least once before identifying tolerant families.

The meaningful comparison of the ranking of the 23 common families represented in the field and the greenhouse trials ($r = 0.40 \pm 0.06$), indicates that greenhouse screening provides an indication of the tolerance of mature trees to *F. circinatum*, as has been reported for other pines (Barrows-Broaddus and Dwinell 1984, Blakeslee and Rockwood 1999). This was especially evident by the fact that the most tolerant trees in the field produced seedlings that were as tolerant as *P. elliottii* seedlings to infection (see Figure 2a–f). This is supported by other studies where heritability values of up to 0.86 have been recorded from field trials (Rockwood et al. 1988, Blakeslee and Rockwood 1999).

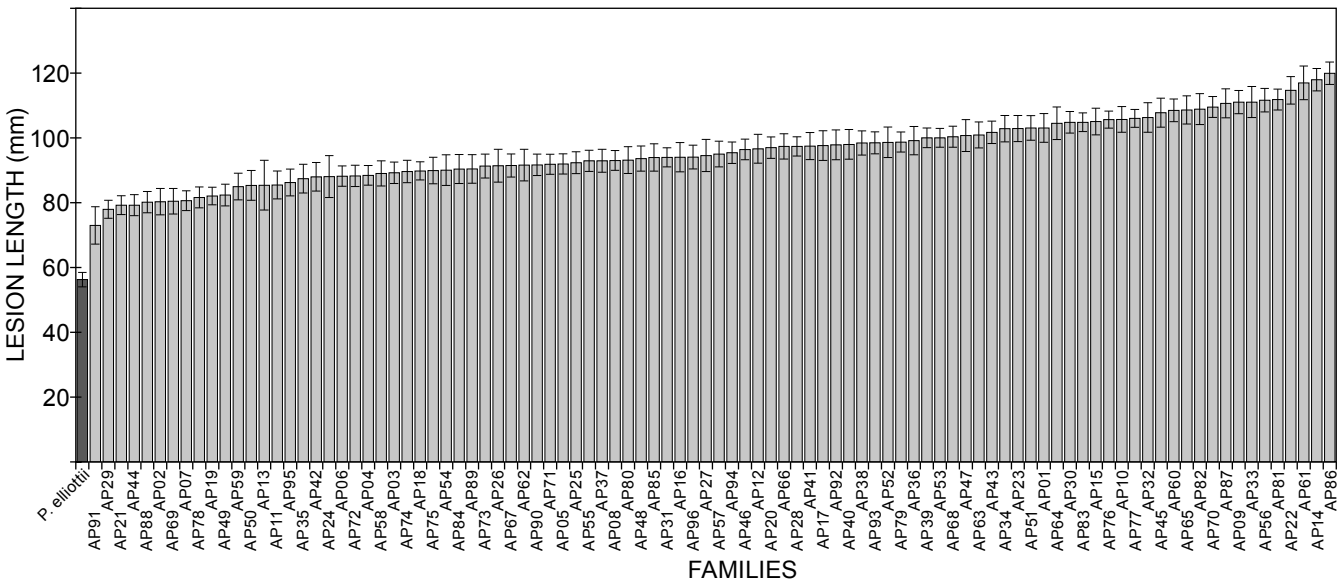

Figure 3: Mean lesion length of the 96 *P. patula* families tested in the field, from most to least tolerant. The *P. elliottii* control is far left. Error bars represent the SE of the mean

It is evident that a very large number of *P. patula* clones need to be screened in order to identify those that are similar to the general tolerance of *P. elliottii* in the field. In this study, we estimate that approximately 5% of *P. patula* trees currently deployed are likely to be as tolerant as *P. elliottii*. This figure is also quoted for planted *P. radiata* trees (Storer et al. 1999). The 30 trees that ranked more tolerant to *F. circinatum*, than the mean of *P. elliottii* in the field trial, represent just over 3% of the total number of *P. patula* trees screened.

In previous field inoculation studies, susceptible *P. radiata* trees developed signs of pitch canker after inoculating branches with *F. circinatum* (Storer et al. 1999). Perhaps it is because we inoculated stems and the fact that *P. patula* is more tolerant than *P. radiata* to *F. circinatum* (Hodge and Dvorak 2000) that typical signs of pitch canker were not observed. The recovery of young *P. patula* trees after being inoculated with *F. circinatum* has also been reported previously (Viljoen et al. 1995). This clearly demonstrates that trees are more tolerant to infection than seedlings.

The results of our field inoculation studies confirm those of Porter (2010) that *F. circinatum* isolate CMW 3579 is more aggressive than CMW 3578 or CMW 3577. Together with the fact that there was no interaction between host and isolate, we concur with Porter (2010) that inoculating *P. patula* seedlings or trees with only CMW 3579 in future screening studies should achieve rapid and reliable results. This may not be the case for different species of pine. While Gordon et al. (1999) also reported an absence of interaction between the host and isolate for *P. radiata*, Blakeslee and Rockwood (1999) reported that this phenomenon exists for susceptible *P. elliottii* and *P. taeda* families. Clearly, further research on this topic is warranted.

The findings presented here provide a basis for estimating the number of families and individuals per family that should be screened for their tolerance to *F. circinatum* to identify a sample of trees for a new orchard comprised of tolerant clones (see Rockwood et al. 1988). If we consider that 25 of the 96 families tested in the field (i.e. ±26%) produced the 30 most tolerant trees based on a sample size of 10 trees per family, then potentially 50–60 trees may have been identified from the 96 families if 20 trees per family were inoculated. In this case, it is likely that these tolerant trees would not be restricted to only 25 families. Several studies have shown that there is a good correlation between the ranking of clones in repeated inoculation events (Gordon et al. 1999; Storer et al. 1999) particularly if they were considered tolerant when they were inoculated for the first time (Sammon et al. 1999). Therefore, we can expect that 'tolerant' clones will retain their classification once grafted in a new orchard.

Historically, disease tolerance has not been considered a selection criterion in *P. patula* but it is becoming increasingly important to do so. We expect that future breeding efforts will focus on identifying a subpopulation of clones that are tolerant to *F. circinatum*, and that new selections will be identified from their progeny that grow well. Similar to the improved tolerance of the *P. patula* × *P. tecunumanii* hybrid to *F. circinatum* (Mitchell et al. 2013) (Table 3), which is due to the good tolerance of *P. tecunumanii* to *F. circinatum* (Mitchell et al. 2012a), tolerant *P. patula* clones could be used to control-pollinate *P. patula* clones with good growth but poor tolerance to *F. circinatum*. Including genetic material from more recent seed introductions will become increasingly important to continue the deployment of *P. patula* as a pure species, as will identifying hybrid partners and alternative species, which can replace some of the area currently suited to *P. patula*.

Conclusions

The results of the two greenhouse studies are strongly correlated ($r = 0.71$; $r_g = 0.94$). Although the correlation

between the family ranking in the greenhouse and field was lower ($r = 0.40$), these results demonstrate the value of greenhouse screening studies to identify families that will be more tolerant to *F. circinatum* infection as mature trees, particularly those at the extreme range of tolerance.

The percentage of *P. patula* trees that displayed a similar tolerance level to *F. circinatum* as *P. elliottii* in the field was estimated at only 5%. Thus, in order to identify sufficient numbers of *P. patula* clones for plantations, which have similar levels of tolerance to *F. circinatum* as *P. elliottii*, a large population of potential orchard clones will need to be screened as seedlings in the greenhouse.

It was encouraging that the narrow-sense individual-seedling heritability was moderate (0.25) to high (0.52) in the two greenhouse screening events. This indicates that breeding for tolerance to *F. circinatum* is possible. Thus, identifying clones that are more tolerant based on the performance of their open-pollinated progeny as seedlings can lead to healthier plants if seed is harvested from orchards of such trees. Given the heritability levels that were estimated, it is expected that subsequent selection for tolerance to *F. circinatum*, within such a population, will allow for further improvement of tolerance levels.

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