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Field survival and growth of clonal, micropropagated *Eucalyptus marginata* selected for resistance to *Phytophthora cinnamomi*

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Abstract

Clones of jarrah (*Eucalyptus marginata*), micropropagated from glasshouse-grown seedlings selected for resistance or susceptibility to *Phytophthora cinnamomi*, were planted in a former bauxite mine-site in the jarrah forest and inoculated with *P. cinnamomi*. Mortality after 13 years in resistant clones was 0–30%, while that of susceptible clones was 40–100%. Mean heights of resistant clones after 13 years were 7.8–13.6 m, while heights of surviving susceptible clones were 0.9–6.7 m. The resistance character of the seedling ortets was transmitted consistently to the clones. The field mortality of clones of some rare, apparently resistant seedlings selected from susceptible half-sib families was low after 1 year, but approached that of the susceptible clones after 2 years. The results show that *Phytophthora*-resistant jarrah ortets can be selected using stem-inoculation of glasshouse-grown seedlings; the resistance of the resulting clones has been validated in the field in an inoculation trial.

Keywords: *Eucalyptus marginata*; *Phytophthora cinnamomi*; Resistance; Dieback; Micropropagation; Jarrah forest

1. Introduction

Jarrah (*Eucalyptus marginata* Donn ex Sm.) is Western Australia's most important hardwood timber-producing species, and is endemic in the south-west region of the state. The ''jarrah dieback'' disease caused by the root-rotting water-mould *Phytophthora cinnamomi* Rands (Podger, 1972) has caused serious damage to the jarrah forest, with the most severely affected areas being located in the higher rainfall zone on the western edge of the Darling Scarp, and further south in the Donnybrook Sunklands (Shearer and Tippett, 1989).

The existence of genetically based resistance to *P. cinnamomi* in jarrah has been demonstrated in pot and field trials, using seedlings from open-pollinated mother trees selected in the forest (Stukely and Crane, 1994). There was wide variation in resistance levels between these ''families'' of half-sib seedlings, and also between individual seedlings within families. Narrow-sense heritability of the resistance was very high at both family (0.78–0.85) and individual tree (0.43 ± 0.18) levels, and this is believed to be under polygenic control (Stukely and Crane, 1994). Selected *P. cinnamomi*-resistant lines of jarrah could be useful in rehabilitation of degraded jarrah forest sites.

Jarrah can be propagated by tissue culture (McComb and Bennett, 1982), and resistance to *P. cinnamomi* has been shown to exist in some micropropagated clonal lines of jarrah (Bennett et al., 1993). The purpose of this study was to compare the field survival and growth of jarrah clones derived from seedlings that had been selected for resistance or susceptibility to *P. cinnamomi* in glasshouse screening trials, in order to validate the resistant selections for use in the restoration planting of dieback-affected jarrah forest sites. Some of the mortality results were included in preliminary reports (McComb et al., 1990; Stukely et al., 2001).

2. Materials and methods

2.1. Selection of *E. marginata* lines

Half-sib families of 14-month-old pot-grown *E. marginata* seedlings were stem-inoculated with *P. cinnamomi* type A2 (IMI 264384) as described previously (Stukely and Crane, 1994). Necrotic lesions developing in the stems were measured for 14 days, after which the stems were pruned to remove

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infected tissue and to encourage healthy axillary growth from the basal regions of the plants for use in tissue culture.

The relative levels of ''resistance'' or ''susceptibility'' to *P. cinnamomi* of 16 seedling families were determined by comparing their mean stem lesion lengths (Stukely and Crane, 1994). Families whose mean lesion lengths were significantly smaller than that of a standard susceptible family were designated ''resistant''. Within families, individual seedlings showing outstanding resistance (restricted lesions), or high susceptibility (long lesions), were selected. The seedlings selected as resistant had lesions close to or smaller than the lower 95% confidence limit for their family mean. Three categories of selected seedlings were included as ortets for micropropagation: resistant individuals from resistant families (RR), apparently resistant individuals from susceptible families (RS), and susceptible individuals from susceptible families (SS). Ortets were numbered such that the prefix indicates the open-pollinated mother tree from which the seedling family was derived; thus, Clones 1-030, 1-065, and 1-098 were all derived from the same mother tree in the forest (Tree 1), and these three ortets are half-sibs.

2.2. Micropropagation

Sterilised shoots of 16 selected seedlings were multiplied and rooted in vitro (McComb et al., 1990). Rooted shoots were potted and grown on in peat/sand. After 4–5 months the clones were transferred to an unheated shade house for a minimum of 4 weeks, and then to an open nursery area near the field plots for 6 weeks for final hardening before planting out.

2.3. Field trial

A slightly sloping site was selected in a former bauxite mine-site in the jarrah forest near Dwellingup (32°41′S; 116°05′E) in which *P. cinnamomi* had been active prior to mining 2 years earlier. The area has a Mediterranean climate with a mean annual rainfall of 1258 mm, which falls mainly in the period May–October.

The site was prepared for planting as described by Bartle and Slessar (1989). Jarrah clones were planted on the sides of the rip-lines at 4 m × 4 m spacing in June 1988. They were fertilised by placing a 200 g tablet of diammonium phosphate (DAP) (N = 17.5%, P = 20%) ∼250 mm diagonally upslope from the plant on the side of the rip-line. The experimental design consisted of randomised blocks with single-tree plots and 10 replicates of 16 clones. Six RR, seven SS, and four RS clones were used, including a duplicate set of one RS Clone (11-093). Extra individuals of some clones were planted as surround rows adjacent to the plots.

Three plants died in 3 months prior to inoculation. Roots of the dead plants were excavated, surface-sterilised (by immersion in 70% ethanol for 30 s followed by four rinses in distilled water) and plated onto *Phytophthora*-selective P_{10}VPH agar (Tsao and Guy, 1977). In September (Spring), all clones in the plots and the surround rows were inoculated: each plant was encircled with four isolates of *P. cinnamomi* type A2, which had been shown earlier to be pathogenic to jarrah seedlings (Stukely and Crane, 1994), by burying four infested *Pinus radiata* D. Don branch plugs to a depth of ∼5 cm (Butcher et al., 1984). The isolates were: 251N12 (isolated from *P. radiata*), Sc 381(IMI 264385) (from *Allocasuarina fraseriana* (Miq.) L. Johnson), DCE 210 (from *E. marginata*), and 480R1 (from *Banksia* sp.). The soil was moist at the time of inoculation.

The trial was checked periodically for mortalities for 13 years. In the first 2 years, assessments were done at approximately monthly intervals, and the roots of all dead plants were excavated and tested for *P. cinnamomi* infection. Samples of roots and root-collar tissue from all dead clones were surface-sterilised (as described above) and plated onto P_{10}VPH agar. Inoculum plugs were retrieved from the soil beneath the dead plants where possible; they were surface-sterilised, halved, and plated with the cut surface down onto P_{10}VPH agar to check the survival of the pathogen. Survival assessments were then done annually to year 7, and again at year 13. Heights of the surviving clones were measured after 2, 7, and 13 years.

2.4. Statistical analysis

Clonal mortality levels and mean heights of the surviving 13-year-old clones were compared using a *t*-test. Diagnostic tests of the residuals indicated that the normality and homoscedasticity assumptions required for the *t*-test were met (for mortality, Shapiro–Wilk $W = 0.95$, $p = 0.44$ and for height, $W = 0.92$, $p = 0.17$, indicating approximate normality; in both cases studentised residuals were between −2.0 and 1.5 with constant variance).

3. Results

Survival of the clones from field planting to the time of inoculation was 98%; *P. cinnamomi* was not isolated from the roots of the three plants (of three different clones) which died prior to inoculation. *P. cinnamomi* was isolated from the roots of 94% of clones which died in the first 2 years after inoculation. Three months after inoculation (December 1988), *P. cinnamomi* was still readily isolated from the pine inoculum plugs excavated with dead plants. Recoveries from the plugs diminished as the soil dried in the Summer months to April; however, they improved during Spring 1989, 1 year after inoculation. The pathogen was occasionally isolated from plugs until March 1990 (18 months after inoculation). By this time, many plugs had rotted and were increasingly difficult to find.

Two months after inoculation, seven plants had died: four in Clone 13-416 (SS), one in Clone 11-394 (SS), and two in Clone 11-050 (RS). Mortalities increased rapidly through the first Summer–Autumn, particularly in the SS clones (Figs. 1a and 2). Within 7 months of inoculation, mortality in SS Clone 13-416 had reached 100%. Deaths continued to occur in subsequent years, mostly in the SS and RS clones. In the RR clones, there were few losses with no mortalities recorded in RR Clones 1-030, 1-065, and 12-072 over the 13-year period. Average mortality in the RR clones after 13 years was lower than in the SS and RS clones overall ($p < 0.001$). After 1 year, mortality in the RS clones was

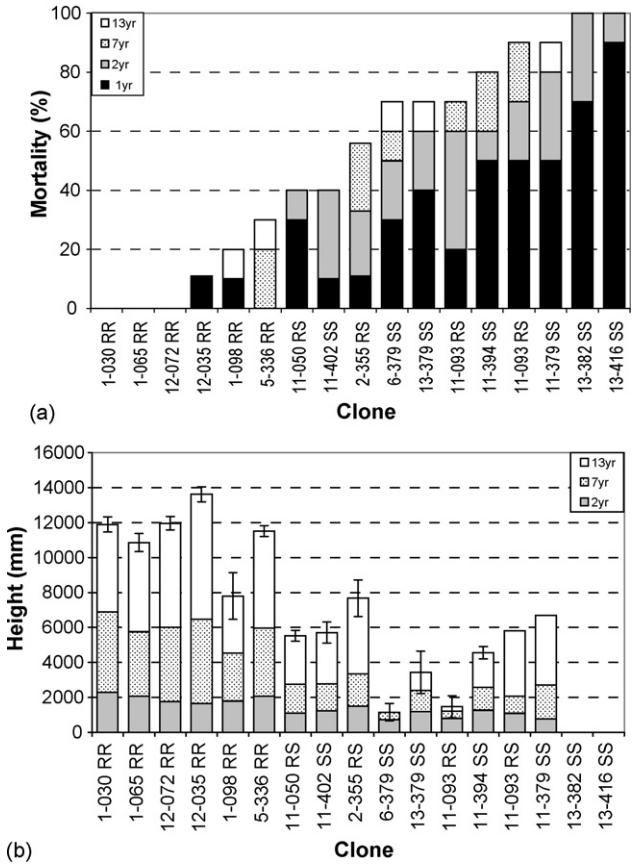

Fig. 1. (a) Mortality of clones of *Eucalyptus marginata*, in the three resistance categories RR, RS, and SS, at 1, 2, 7, and 13 years after they were transplanted to the field and inoculated with *Phytophthora cinnamomi*. The clones are ranked on their mortality after 13 years. The resistance category relates to the selected inoculated seedling from which the clone was propagated: resistant individuals from resistant families (RR), apparently resistant individuals from susceptible families (RS), and susceptible individuals from susceptible families (SS). (b) Mean heights of surviving clones of *Eucalyptus marginata*, in the three resistance categories RR, RS, and SS, at 2, 7, and 13 years after they were transplanted to the field and inoculated with *Phytophthora cinnamomi*. The clones are listed in the same order as in (a). Error bars represent the standard errors of the means.

at an intermediate level between the RR and SS clones (Figs. 1a and 2). However, after 7 years, the average mortality of RS clones had risen to a level approaching that of the SS clones (Fig. 2) with a maximum of 90% for Clone 11-093 (second set) (Fig. 1a). Mortality of the two sets of plants of RS Clone 11-093 was 70 and 90%, respectively, after 13 years, and since clones were used, this variation reflects factors related to site and *P. cinnamomi* inoculum survival. The degree of variability in 13-year mortality between all the RR clones was comparable, at 0–30%.

Extra clones planted in rows surrounding the trial generally showed very similar mortality levels to the same clones in the plots, for example, RR Clone 1-065 (24 plants in the surrounds, with no mortality recorded to 13 years in either the surrounds or the plots). However, SS Clone 11-402 (23 plants in the surrounds) showed 74% mortality at 13 years (compared with 40% in the plots).

The mean heights of surviving clones after 2, 7, and 13 years are shown in Fig. 1b. Overall, the mean heights of RR clones

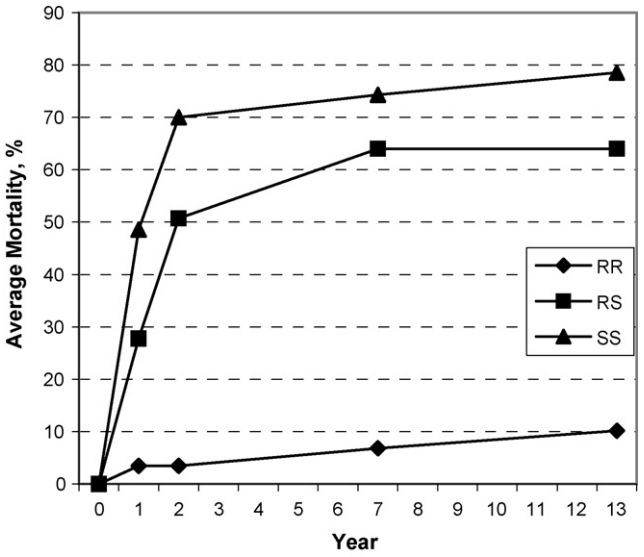

Fig. 2. Average mortality with time, after transplanting to the field and inoculation with *Phytophthora cinnamomi*, for clones of *Eucalyptus marginata* in the resistance categories RR, RS, and SS.

were greater than those of the surviving SS and RS clones combined ($p < 0.001$), and this margin increased steadily with time (Fig. 3).

Three of the RR clones reached mean heights of over 2 m after 2 years, and at 13 years the mean height of Clone 12-035 was 13.6 m (Fig. 1b). This clone produced a mean annual increment of height of 1.047 m over 13 years, and the tallest plant then measured 15.2 m. The mean height at 13 years for the smallest RR Clone 1-098 was 7.8 m, which is substantially smaller than that of its half-sib Clones 1-030 (11.9 m) and 1-065 (10.9 m). In contrast, the greatest mean annual increment of height for RS and SS clones was 0.59 m, achieved by RS Clone 2-355. The mean 13-year heights for RS Clone 11-093 (second set) and SS Clone 11-379 were 5.8

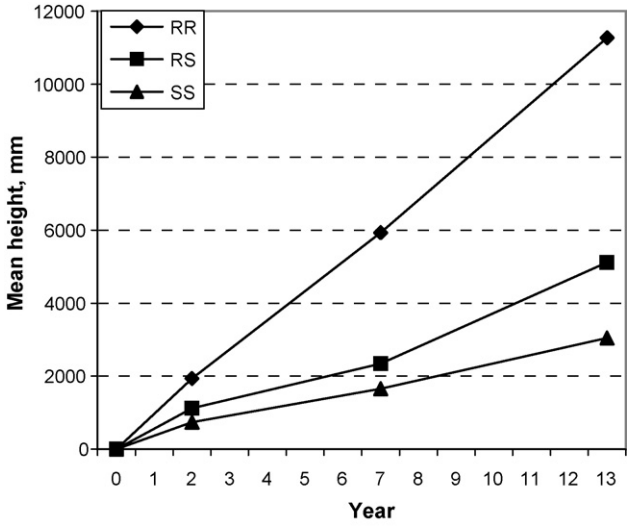

Fig. 3. Average height with time, after transplanting to the field and inoculation with *Phytophthora cinnamomi*, for surviving clones of *Eucalyptus marginata* in the resistance categories RR, RS, and SS.

and 6.7 m, respectively (Fig. 1b), but it should be noted that each of these mean heights represents only one surviving tree.

The form of the clones was generally poor, with almost all plants of all clones developing multiple stems with two to four stems at a height of less than 2 m. The reason for this is not understood, but appears to be associated with clones as this form is less common in seedlings planted on similar sites.

4. Discussion

Clonal *E. marginata*, micropropagated from seedlings selected for their outstanding levels of resistance to *P. cinnamomi* (category RR) in glasshouse screening trials, exhibited very low (0–30%) mortality 13 years after planting in a field site inoculated with *P. cinnamomi*. In contrast, the mortality of clones of *P. cinnamomi*-susceptible seedlings (category SS) was high (40–100%). Furthermore, the heights of 2–13-year-old RR clones, and their mean annual increments of height, were consistently superior to those of the surviving SS and RS clones.

The *P. cinnamomi*-resistance character of the RR and SS seedling ortets was thus transmitted consistently to their micropropagated clones. The field performance of these clones has validated their selections, which were made using a stem-inoculation test on glasshouse-grown seedlings (Stukely and Crane, 1994).

Clones of rare, apparently resistant individuals (category RS) selected from susceptible half-sib seedling families initially showed intermediate mortality levels, however, these later increased to levels approaching those of the SS clones (Fig. 2). The ''resistant'' performance of these ortets in relation to other seedlings of the same families in the original screening trials, as indicated by stem lesion length, may have been due to: a physiological difference at the time of inoculation, differences in microenvironment in the pots, or experimental error. It is equally possible that ''resistant'' seedlings selected in resistant families (category RR) could have been under these same influences, and could therefore have presented a false resistant response. Clearly, such plants should not be included as resistant selections, and this trial has shown that the RR clones selected here showed a true resistant response. The results suggest that the consistent demonstration of the resistant response by inoculated seedlings within a family is a good indicator of family resistance, and resistant individuals for propagation should be selected only from families designated as resistant.

The mechanism of resistance of jarrah to *P. cinnamomi* is not well understood, but appears to be under polygenic control (Stukely and Crane, 1994). Cahill et al. (1992) showed that the primary roots of a RR jarrah Clone (5-336) were better able to restrict and confine colonisation by *P. cinnamomi* than the roots of susceptible seedlings. They also established that the activity of phenylalanine ammonia-lyase (PAL) and levels of lignin and phenolics in roots increased after inoculation of RR Clones (5-336, 1-030), but not SS Clones (11-379, 11-402) or unselected seedlings (Cahill et al., 1993). The reaction of RR jarrah clones is similar to that observed in the field-resistant eucalypt species *Corymbia calophylla* R. Br. (Cahill and McComb, 1992).

Genetically based resistance to *P. cinnamomi* has also been demonstrated in *P. radiata* (Butcher et al., 1984), an important plantation timber species in southern Australia and New Zealand. A program of selection of resistant lines of *P. radiata* for planting in *Phytophthora*-infested areas has been completed. The use of seed from *P. cinnamomi*-resistant families to plant *Phytophthora*-infested sites gave a 24% increase in basal area after 10 years, as well as better survival and tree form, compared with trees grown from unselected seed (Butcher and Stukely, 1997). The validation of the selection of jarrah resistant to *P. cinnamomi* reported here has enabled a similar screening program to be carried out with jarrah. We have completed screening a wide range of jarrah provenances, resulting in the selection of some 60 resistant lines of jarrah, 45 of which are unrelated (Stukely et al., 2001).

Clonal jarrah can be used in two ways to restore the jarrah forest in former mine-sites and in dieback-affected areas. *P. cinnamomi*-resistant clones can be planted directly in the target sites; alternatively, the clones can be used to establish seed-orchards which will later supply seed for either the direct seeding of forest sites, or for the nursery production of seedlings for transplanting to forest sites.

A genetically diverse population of dieback-resistant jarrah is desirable for use in restoring degraded forest sites. However, the level of success in root production by jarrah shoots in tissue culture is highly variable (0 to ~80%), although the performance of individual clones is consistent (Stukely et al., 2001). Clones with poor root production in vitro also show poor survival on transfer to soil, and they are also likely to suffer high mortality after transplanting to the field, due to restricted root development. High-impact dieback ''graveyard'' sites in the forest often present very harsh conditions for plant establishment, and the survival of jarrah clones planted in these sites has been highly variable, ranging from <5 to 80% (Stukely, unpublished). Controlled pollination is not an option for large-scale production of resistant jarrah, as rates of seed set are low following artificial pollination (Stukely and Byrne, unpublished). Therefore, the strategy adopted for the deployment of *P. cinnamomi*-resistant jarrah has been to use the selected RR clones to establish seed orchards (Stukely et al., 2001). The relatively poor form displayed by the clones will thus also be avoided in the forest, through the use of seedlings. Indeed, the multi-stemmed form of the clones could be advantageous in a seed orchard where easy access to the tree crowns is essential for seed collection and other operations.

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