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Temperature and inoculation method influence disease phenotypes and mortality of *Eucalyptus marginata* clonal lines inoculated with *Phytophthora cinnamomi*

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Abstract. Survival of 1-year-old plants of three clonal lines of *Eucalyptus marginata* (jarrah), two ranked as resistant (RR1 and RR2) and one as susceptible (SS1) to *Phytophthora cinnamomi*, was assessed after pathogen inoculation with either mycelial-mats underbark or zoospores on the stem. Plants were grown at 15, 20, 25 and 30°C. Method of inoculation did not produce comparable mortalities of the clonal lines, particularly at 25 and 30°C. At these temperatures, all three clonal lines had 100% mortality when inoculated underbark, but when inoculated with zoospores, RR1 had 60% survival and lines SS1 and RR2 had 100% mortality. Generally, the level of resistance of all clonal lines declined with increasing temperature. RR2 had consistently higher mortality than SS1, and is therefore not considered resistant. Lesion development was also measured in detached stems of RR1 and a susceptible clonal line (SS2) each inoculated underbark with four different *P. cinnamomi* isolates. Stems were assessed for lesion development at 20, 25 and 30°C for 4 days. For all four isolates, detached stems of RR1 generally had smaller lesions than those of SS2, particularly at 30°C. The increase in lesion length with increasing temperature was greatest for SS2. Detached stems may have potential in screening for jarrah resistant to *P. cinnamomi* and allows identification of susceptible clonal lines at 30°C.

Additional keywords: collar rot, disease assessment, girdling lesion, hemibiotroph, dieback

INTRODUCTION

In the southwest of Western Australia (WA), *Eucalyptus marginata* Donn. ex Smith (jarrah) is an economically important hardwood-timber and dominant native tree in approximately 3.3 million ha of forest (Dell and Havel 1989). Large areas of jarrah forest have been severely affected or killed by the introduced pathogen, *Phytophthora cinnamomi* Rands (Shearer and Tippett 1989). However, individual jarrah trees have survived on infested sites and these have been used as parents for progeny, which were screened for resistance to *P. cinnamomi* (Stukely and Crane 1994). The seedlings of half-sib families from these trees were screened by underbark stem inoculation with one isolate of *P. cinnamomi* in a glasshouse. Individual seedlings of families that developed small lesions were designated resistant (RR; resistant lines from resistant families), while those with large lesions were designated susceptible (SS; susceptible lines from susceptible families). Summer inoculations of 14-month-old seedlings were found to produce the best discrimination among the families (Stukely and Crane 1994). The families' rankings of resistance to *P. cinnamomi* were confirmed by inoculations of soil in the glasshouse and field with colonised woody plugs from *Pinus radiata* stems (McComb *et al*. 1990, 1994; Stukely and Crane 1994). Individual RR and SS seedlings were then micropropagated to produce clonal lines (McComb *et al*. 1990).

Much of the *P. cinnamomi* infested jarrah forest contains bauxite ore bodies, some of which are mined by Alcoa World Alumina Australia. With the availability of resistant clonal lines of jarrah, minesites and other areas degraded by *P. cinnamomi* can be rehabilitated to re-establish jarrah in severely damaged communities. The processes of mining and rehabilitation results in a substantial change to the jarrah forest soil, which alters the *P. cinnamomi* disease interaction compared to that observed in the adjacent jarrah forest (Colquhoun and Hardy 2000). For example, after heavy rainfall events, the riplines that are produced to encourage better water drainage and reduce erosion, can become lined with fine clay particles that often result in surface water ponding due to poor drainage (Hardy *et al*. 1996; O'Gara *et al*. 1996). Ponded riplines were often associated with invasion of jarrah collars or stems by *P. cinnamomi* (Hardy *et al*. 1996). Stems may also be infected in jarrah forest areas with impeded drainage during winter (O'Gara *et al*. 1996). O'Gara *et al*. (1996, 1997) demonstrated that zoospores of *P. cinnamomi* were able to penetrate the stem periderm of jarrah seedlings both in a glasshouse and in the forest.

Many pathogenicity studies with Australian indigenous plant species comparing underbark stem inoculations with soil inoculations have reported on the similarity of disease induced in the host regardless of the inoculation method used (Dixon *et al*. 1984; McCredie *et al*. 1985; Dudzinski *et al*. 1993; Stukely and Crane 1994). They all concluded that stem inoculations were a legitimate test for screening plant species for resistance to *P. cinnamomi*. The resistance rankings of jarrah clonal lines, designated as RR or SS by stem inoculations, did not change when the white roots of 10-month-old plants were inoculated with zoospores (Cahill *et al*. 1993). However, it is not known if the relative resistance rankings of the clonal lines would change when stems or collars are infected by zoospores, such as in rehabilitated minesites. Underbark inoculations of stems, as used for the initial screening of seedlings for resistance to *P. cinnamomi*, has not been conducted on the jarrah clonal lines.

As with most plant pathogens, temperature plays an important role in the growth (Shepherd and Pratt 1974; Zentmyer *et al*. 1976; Hüberli *et al*. 2001), reproduction (Zentmyer *et al*. 1979), survival and pathogenesis (Shea 1975; Shearer and Tippett 1989) of *P. cinnamomi*. During the early stages of minesite rehabilitation, seedlings are exposed to increased temperature and moisture compared to adjacent forest sites due to the absence of canopy protection (Stoneman *et al*. 1995). Of further concern, is the prediction that Australian summers may become warmer and wetter due to the greenhouse effect (Chakraborty *et al*. 1998). These are ideal conditions for *P. cinnamomi* to cause disease. It has been shown that lesions increased with increasing temperature within the range of 10 to 30°C for inoculations of seedling roots and excised roots of jarrah trees (Grant and Byrt 1984; Shearer *et al*. 1987). To date, the effect of temperature on host-pathogen interactions in jarrah clonal lines selected for resistance to *P. cinnamomi* has not been assessed. Therefore, it is not known if the clonal lines are also resistant under conditions that are more favourable to disease.

In this paper, we compare and contrast disease progression and temperature interactions in RR and SS clonal lines of jarrah inoculated with *P. cinnamomi* using the zoospore inoculation technique developed by O'Gara *et al*. (1996) and an underbark technique. We also assessed whether detached stems could be used as a rapid bioassay to evaluate the effect of temperature on disease development in clonal lines.

METHODS

Experimental design Three inoculation experiments with *P. cinnamomi* were conducted using clonal lines of jarrah growing at different temperatures. In Experiments 1 and 2, three clonal lines (two RR and one SS) were grown at four temperatures and plants were either inoculated with mycelial-mats underbark (Experiment 1) or with zoospores (Experiment 2) of one isolate of *P. cinnamomi*. Experiments 1 and 2 were not run concurrently. Plants in Experiments 1 and 2 were randomised in a complete block design within four temperature-controlled environment cabinets (Environ Air EA7BH; SRG Cabinet Sales, Greenacre, NSW, Australia) at 15±2, 20±3, 25±1 and 30±3°C. For both experiments, each of the four cabinets contained a total of 36 plants, with ten inoculated and two control plants for each clonal line. In zoospore inoculations, there were 7 and 8 inoculated plants for RR2 at 15-25°C and 30°C, respectively, while for the remaining treatments, 10 inoculated plants were used. In Experiment 3, detached stems of two clonal lines (one RR and one SS clonal line) of jarrah were underbark inoculated with mycelial-mats of four isolates of *P. cinnamomi* and incubated at 20±1, 25±1 and 30±1°C. There were 12 inoculated replicate stems and two controls per treatment. A preliminary experiment with detached stems found that at 15°C no lesions were formed after 4 days and the extension beyond the visible lesion (EBL) was less than 0.5 cm. Consequently, this temperature was not included in the experiment.

Experiments 1 and 2: Effect of temperature and inoculation method on disease

Plant material and growth conditions Clonal lines of jarrah, previously ranked as RR or SS to *P. cinnamomi* (McComb *et al*. 1990), were supplied by the Marrinup Nursery (Alcoa World Alumina Australia, Pinjarra, WA). Three clonal lines [1J30 (RR1), 121E47 (RR2) and 11J402 (SS1)] of 1-year-old jarrah were potted and grown in a glasshouse as described in Hüberli *et al*. (2000) 4 months prior to inoculation. All lateral shoots were pruned and lateral buds were regularly removed to encourage the growth of single stemmed plants with no side branches.

Forty-eight near uniform plants per clonal line were selected for each experiment and different sized plants were distributed evenly among the four cabinets. In Experiment 1, the heights of the plants ranged from 37 to 42 cm with maximum stem diameters of 3.4 to 3.7 mm. In Experiment 2, plants had heights ranging from 54 to 71 cm and maximum stem diameters of 3.7 to 5.1 mm. The cabinets were set at 600-800 μEinsteins $s^{-1}m^{-2}$ of irradiance on a day/night cycle of 14.5/9.5 h. All plants were acclimatised for 1 week prior to inoculation. The plants at 15 and 20°C were hand watered daily to free draining and the plants at 25 and 30°C were watered twice daily due to rapid evaporation.

Inoculum production and inoculation The isolate MP94-48 of *P. cinnamomi* was passaged through a jarrah seedling 3 weeks prior to inoculation to ensure that the isolate had not lost its pathogenicity as a result of prolonged subculturing (Erwin and Ribeiro 1996). Stems of plants in Experiment 1 were underbark inoculated 5 cm from the soil surface with 1 cm diameter mycelial-mats, which were colonised Miracloth (Calbiochem Corporation, La Jolla, CA, USA) discs, using the methods of O'Gara *et al*. (1996). Controls were inoculated with sterile Miracloth discs. The plant tissue in contact with the inoculum disc is referred to as the site of inoculation (SOI) for all underbark inoculations. In Experiment 2, two weeks prior to moving the plants into the growth cabinets, a watertight inoculum receptacle was constructed, as described by O'Gara *et al*. (1996), around the suberised stems approximately 5 cm from the soil surface. Periderm formation was induced in the lower 10 cm portion of all stems by lightly sanding with fine sandpaper 2 weeks prior to receptacle construction (Burgess *et al*. 1999). Sterile deionised water was placed into the receptacles 24 h prior to inoculation to soak the stems and removed 2 h before inoculation.

In Experiment 2, zoospore inoculum was produced aseptically as described by O'Gara *et al*. (1997). A 30 mL zoospore suspension (about 5200 zoospores/mL) or deionised water (controls) was carefully poured into each receptacle. This volume ensured that at least 1 cm of the exposed stem was immersed in the zoospore suspension; this is referred to as the region of inoculation (ROI). The volume of receptacles at 25 and 30°C was refilled every second day due to high evaporation rates. After 6 days of exposure to the zoospore suspension or deionised water, the solutions and then the receptacles were carefully removed to prevent zoospores entering the soil.

Monitoring plant symptoms The number of days taken for plants to reach irreversible wilting and death was recorded in both experiments. Irreversible wilting was when the plant shoot had wilted and was unable to recover turgidity, and death as when all leaves were crisp and dry. At death, plants were

harvested and stem sections plated onto NARPH (Hüberli *et al*. 2000), an agar selective for *Phytophthora*, to confirm the presence of *P. cinnamomi*. In Experiment 1, the longitudinal acropetal lesion on the stem was measured from the mid-SOI and circumferential spread (°) of lesions at the SOI was estimated. These were measured 3, 7, 11, 17, 27, 35, 46 and 63 days after inoculation for all treatments, with additional measurements made at 5 and 10 days after inoculation for plants at 25 and 30°C. Basipetal lesions were not measured. Longitudinal lesions will henceforth be referred to as 'lesions', and the circumferential spread of lesions (also known as tangential spread) as 'circumferential spread'.

For Experiment 1, leaf stomatal conductance was measured for one fully expanded leaf per plant throughout the experiment using a Delta-T porometer Model AP4 (Delta T Devices Ltd., Burnwell, Cambridge, England). Porometer measurements of all plants were taken 1 day after inoculation and then at weekly intervals until the experiment ended. Stomatal conductance of control plants varied considerably between measurements, therefore, the data is presented as a percentage difference compared with the control plants.

Harvest of surviving plants Surviving plants in Experiments 1 and 2 were harvested 105 and 86 days after inoculation, respectively. Prior to harvest, all acropetal lesions were measured and circumferential spread estimated as for dying plants. In Experiment 1, stems were cut into ten 0.5 cm acropetal sections from the lesion margin. Stem sections were then cut longitudinally and plated, cut surface face down, sequentially onto NARPH. The remaining piece of stem was stored at 10°C for 4-7 days, and additional stem sections were plated if the original plating yielded *P. cinnamomi* from all ten sections. In Experiment 2, stems were cut into 1 cm acropetal sections as opposed to 0.5 cm sections used for Experiment 1. The reason for this was that plants in Experiment 2 did not have lesions and therefore, a larger portion of the stem was plated to determine if and how far the pathogen had progressed. Seven acropetal stem sections were cut from beyond the ROI.

Growth of the pathogen from stem pieces was assessed after 3 and 5 days incubation at 24°C. The total number of stem sections from which *P. cinnamomi* was recovered was used to determine the length of extension beyond the lesion (EBL) or beyond the ROI/SOI in the absence of lesions. Colonisation refers to the total longitudinal length of stem invaded by the pathogen, which in lesioned stems included the lesion and EBL, while in stems without lesions consisted only of the extension beyond the ROI/SOI.

Experiment 3: Effect of temperature on lesion development in detached stems

Isolates Four isolates of *P. cinnamomi* were selected based on results of a rehabilitated forest minesite inoculation which examined the capacity of ten isolates to form lesions in intact lateral branches of RR1 (Hüberli, 1995). MP94-48 produced large lesions, MP116 produced medium lesions, and MP94-09 and MP112 produced small lesions. All isolates were of A2 mating type and from the same clonal lineage. Details of isolation and maintenance of cultures have been described previously (Hüberli *et al*. 2001).

Plant material Approximately one-year-old green stems between 0.8-1.5 cm in diameter were collected in June (early winter) from four-year-old jarrah trees of clonal lines RR1 and 11J379 (SS2). The clonal lines were growing on a rehabilitated bauxite pit at the Willowdale minesite (116°2'E, 32°55'S) of Alcoa World Alumina Australia, located 110 km south of Perth, Western Australia. Stems were cut into approximately 60 cm lengths, placed into moist hessian bags in insulated iceboxes, transported to the laboratory, and inoculated within 6-12 h of the harvest.

Inoculum production and inoculation Stems were trimmed into 20 cm sections and the side shoots and leaf nodes were trimmed as close to the stem as possible. Once cut, stem ends were immediately dipped into melted wax in order to minimise desiccation. Prior to inoculation, the stems were surface-decontaminated with 70% ethanol. Stems were inoculated with mycelial-mats underbark, as in Experiment 1, at the mid-region of each stem and the wound sealed with Parafilm (American National Can, Chicago, USA) to prevent desiccation. Controls were inoculated with sterile Miracloth discs. Stems were incubated in the dark at 20, 25 and 30°C in humid chambers to prevent further desiccation. The chambers were disinfected plastic trays (39 x 27 x 6 cm) lined with moist paper towels, and sealed in plastic bags. For each clonal line, twelve replicate stems per isolate and two controls were randomly allocated to each temperature treatment.

Lesion and colonisation assessment The total length of the lesion extending above and below the SOI was measured at 2, 3 and 4 days after inoculation. After each measurement, a random sample of four stems per isolate and two controls for each temperature were cut into 0.5 cm sections, plated onto NARPH and colonisation determined as described for Experiment 1.

Statistical analysis Following Tabachnick and Fidell (1996), data for parametric tests were screened for assumptions of homoscedasity, normality and non-correlations of means and variances. The means of time to death for each treatment in Experiments 1 and 2 were subjected to analysis of variance (ANOVA). Means were taken because the individual samples within each growth cabinet are not truly independent replicates, so the data form a nonreplicated experimental design with the growth cabinets as the experimental units (Milliken and Johnson 1989). Those plants that survived the treatments to 105 (Experiment 1) and 86 (Experiment 2) days post-inoculation were given that score. To analyse the nonreplicated cabinet treatments, the error term of the three-way interaction (clonal line x temperature x inoculation method) was used for testing the significance of all lower order interactions and main effects. The highest order interaction cannot be tested in this design. Plant height and stem diameter at the SOI were not used as covariates as they did not correlate with survival data of all the treatments, with the exception of one treatment (SS1 inoculated with zoospores at 20°C). In Experiment 3, the mean lesion lengths were subjected to ANOVA with time of lesion measurement (days 2, 3 and 4) as repeated measures factors using the same approach as for Experiments 1 and 2. All significant main effects and interactions for the two ANOVAs were compared using the Least Significant Difference (LSD) test ($P = 0.05$). Given that the overall design was not replicated, an ANOVA was specified for the effect or interaction of interest and the LSD test conducted within that. For Experiment 3, post hoc tests could not be performed for interactions with the factor time because it involves a repeated measures term and a normal fixed factor. There are no agreed protocols for post hoc tests in such circumstances. Pearson's correlation coefficients for different variables were calculated.

RESULTS

Experiments 1 and 2: Effect of temperature and inoculation method on disease

Survival The clonal line and temperature main effects were highly significant and no interactions were significant (Table 1). The LSD test for the factor clonal line, failed to show which of the lines were significantly different at the conventional $P < 0.05$ (RR1 vs. RR2 $P = 0.07$, RR1 vs. SS1 $P = 0.17$, RR2 vs. SS1 $P = 0.62$). From these *P*-values and the lower overall mortalities of RR1 (Figure 1), it seems that the most likely cause of the significant clonal line effect are the differences between RR1 and RR2, closely followed by RR1 and SS1.

At 25 and 30°C, all plants of the three clonal lines died within 13 days when inoculated underbark, while all plants of RR2 and SS1 died within 11 days when inoculated with zoospores. At these temperatures, however, RR1 had 60% survival when inoculated with zoospores (Figure 1). Additionally, RR1 had 90% and 100% survival at 20°C when inoculated underbark and with zoospores, respectively. For these treatments, RR2 and SS1 had less than 40% survival, with RR2 always having two more deaths than SS1. At 15°C, mortality of underbark inoculated RR1 were initially lower compared to RR2 and SS1, although by 105 days RR1 had reached 40% mortality as had SS1. At 15°C, RR1 and SS1 inoculated with zoospores had 100% survival, while RR2 had 40% mortality.

There were no significant differences in time to death at 15 and 20°C ($P = 0.18$) and at 25 and 30°C ($P = 0.80$). However, time to death at 15 and 20°C were significantly higher than at 25 and 30°C ($P < 0.001$). This highlights the marked increase in mortality between 20 and 25°C for both inoculation methods (Figure 1).

Recovery of *P. cinnamomi* from dying and surviving plants inoculated underbark was 75% or higher (Table 2). Plants inoculated with zoospores had recoveries comparable to underbark inoculations, with the exception of dying RR2 and surviving RR1 from which *P. cinnamomi* was isolated at less than 50% (Table 2). It is unlikely that these exceptions were 'inoculation escapees' as inoculations were completely randomised and the remaining zoospore inoculated treatments had relatively high recoveries (Table 2).

Mean colonisation of stems of surviving plants at 15 and 20°C was up to 14 times longer in plants inoculated underbark than those inoculated with zoospores (Table 2). Surviving plants

inoculated with zoospores were symptomless, while those inoculated underbark had lesions. In the latter plants, lesions corresponded with colonisation of the pathogen in the stem. Control plants survived all treatments, were symptomless and *P. cinnamomi* was never isolated.

Days elapsed to irreversible wilt and death of plants were highly correlated for both underbark and zoospore inoculated experiments ($r = 0.99$, $P < 0.01$). Plants expressing irreversible wilt died on average 1-4 (Experiment 1) and 2-6 (Experiment 2) days later. Consequently, only data for mortality are presented.

Disease symptoms of underbark inoculated plants Lesions in all plants at 25 and 30°C were brown to black and continuous. At 15 and 20°C, lesions were often difficult to identify as they were frequently discontinuous, with lesions breaking out at stem nodes beyond symptomless tissue. Some plants also formed lesions that were creamy to white and the margins of these lesions were hard to recognise.

There was a strong positive correlation between lesion length and circumferential spread for dying plants at 15, 25 and 30°C ($r > 0.72$, $P < 0.001$; for all three temperatures) and surviving plants at 15°C ($r = 0.86$, $P < 0.001$). At 20°C, correlations were weak for dying ($r = 0.5$, $P = 0.01$) and surviving ($r = 0.38$, $P = 0.06$) plants. Mortality could not be correlated with lesion length or circumferential spread as they were measured on different days. However, rapid lesion development and circumferential spread was followed by a rise in mortality except at 20°C (Figures 1 and 2).

At 20°C, the lesion length of one RR1 plant progressed rapidly and was more than twice that of the other clonal lines after 7 days (Figure 2). Unlike lesion development, circumferential spread was contained at 180° for RR1 until 7 days, while for RR2 and SS1 it increased to more than 280°. At 11 days, the plant of RR1 was girdled and died 2 days later (Figures 1 and 2). While lesion extension was low and contained at 20°C for SS1, circumferential spread progressed as rapidly as RR2 (Figure 2). Lesion lengths in RR2 were more than double that of SS1 after 12 days.

At 15°C, lesion development and circumferential spread of RR1 initially progressed slowly compared to RR2 and SS1 until 27 days (Figure 2). However, after 27 days, lesion lengths increased by three-fold and circumferential spread almost doubled. This was associated with death at 38 days.

For dying plants, lesion development, circumferential spread, stomatal conductance and death progressed rapidly at 25 and 30°C compared to 15 and 20°C for all clonal lines (Figures 1 and 2). No differences in disease phenotypes were observed among clonal lines. At 25°C, girdling occurred in all plants of the three clonal lines within 11 days after inoculation and at 30°C, it occurred by 5 days.

For surviving plants, lesion lengths and circumferential spread were greater at 20°C than at 15°C (Figure 3). At 15°C, disease phenotypes were similar for all clonal lines. At 20°C, lesions were more than three times larger in RR1 than in RR2 and SS1, but were contained after 35 days (Figure 3). However, lesions of RR2 and SS1 began to extend rapidly beyond 35 days. Girdling of the stem occurred for one plant of RR2 after 63 days and two plants of RR1 after 35 days at 20°C. These plants still remained alive at 105 days after inoculation.

Stomatal conductance was not a sensitive and reliable measure of plant stress as a result of disease impact under the cabinet conditions. Controls often had relatively high readings despite plants being noninfected and symptomless. A level of above 300% stomatal conductance indicated that death of the plant was imminent and was associated with irreversible wilting of the plant (Figure 2). Levels in surviving plants never reached 300% (Figure 3) as in dying plants (Figure 2), although RR2 plants were three- to seven-fold more stressed 101 days after inoculation than SS1 and RR1 plants, respectively (Figure 3). Stomatal conductance could not be correlated with other disease phenotypes as they were measured on different days. However, rises in mortality, lesion length and circumferential spread were reflected by rises in stomatal conductance (Figures 1-3).

Experiment 3: Effect of temperature on lesion development in detached stems

Main effects of clonal line, temperature and time, as well as the interactions clonal line x temperature and temperature x time were significant (Table 3). Isolates did not vary significantly ($P = 0.08$) in their capacity to form lesions.

Overall, RR1 had consistently smaller lesions than SS2 ($P < 0.001$) (Figure 4). At 30°C, the ranges in lesion length produced by the four isolates in RR1 and SS2 did not overlap as at 20 and 25°C. Lesion lengths increased with increasing temperature (20 vs. 25°C $P = 0.18$, 25 vs. 30°C $P = 0.05$, 20 vs. 30°C $P = 0.002$) and increased with time after inoculation ($P < 0.001$, for all three times). This relationship was most obvious for SS2 (Figure 4). The largest difference among lesion

lengths produced at different temperatures within a clonal line was observed 4 days after inoculation. At 2 days, lesion lengths of all isolates were similar at different temperatures, especially for RR1.

Lesion and colonisation lengths in detached stems were highly correlated ($r > 0.88$, $P < 0.001$) at all three days of measurement. The EBL ranged from 5 to 10 mm and, therefore, only lesion length data are presented. Control stems did not form lesions and *P. cinnamomi* was never isolated.

DISCUSSION

This is the first report for jarrah clonal lines to show that mortality and other disease phenotypes are influenced by temperature and inoculation method. These findings supersede our previous statement from preliminary results that resistance ratings were equivalent regardless of whether plants were inoculated underbark or with zoospores (Colquhoun and Hardy 2000). The clonal lines used in the current study have previously been selected for resistance or susceptibility using other isolates of *P. cinnamomi* in underbark inoculations in a glasshouse and in soil inoculations in the field (McComb *et al*. 1990; Stukely and Crane 1994). We have shown that a resistant clonal line may succumb to a *P. cinnamomi* isolate if environmental conditions favour disease development.

Our inoculations were carried out under different environmental conditions and with a different isolate of *P. cinnamomi* to that originally used to screen the individual seedlings from which the clonal lines were derived (McComb *et al*. 1990; Stukely and Crane 1994). Australian isolates were found to vary in their capacity to cause disease in these clonal lines subsequent to the commencement on clonal line selections (Dudzinski *et al*. 1993; Hüberli *et al*. 2001). Variability among isolates tested against 1.5-year-old trees of a resistant clonal line ranged from killing all trees within 59 days to inducing no symptoms in trees 182 days after underbark inoculation (Hüberli *et al*. 2001). In that study, the original isolate used for the initial screen (McComb *et al*. 1990; Stukely and Crane 1994) killed only one out of six plants within 182 days, which indicates that this isolate may not be as aggressive as the isolate used in the current study.

Our underbark inoculations at 15 and 20°C and zoospore inoculations at 15 to 30°C support earlier work (McComb *et al*. 1990; Stukely and Crane 1994) that clonal line RR1 is more resistant. RR1 may have potential for use in the rehabilitation of *P. cinnamomi* infested areas. However, clonal lines RR2 and SS1 were both found to be more susceptible than RR1 under most conditions in our tests. RR2 has not passed all field validation tests to be released as a resistant clone, and in field trials SS1 had the lowest mortality of all susceptible lines tested (M. Stukely, Department of Conservation and Land Management, WA, personal communication). This may be why RR2 and SS1 were similar in susceptibility in our experiments. We would not recommend the use of RR2 as a resistant selection in rehabilitation programmes in diseased minesites and jarrah forest.

Disease severity increased with increasing temperature in our experiments confirming work with 9-week-old jarrah seedlings (Grant and Byrt 1984) and detached jarrah roots (Shearer *et al*. 1987). They also showed that disease severity measured as length of root tissue invaded was greatest at around 25 to 30°C. However, in their studies mortality was not measured. In contrast, we measured mortality and found a large increase in deaths between 20 and 25°C for 1-year-old potted plants. Our work is different with respect to age of the host.

In axenic cultures, Halsall and Williams (1984) found that 18-22°C was the optimal temperature range for zoospore production, but zoospores were still produced at 30°C. Our study showed that zoospores infected and caused rapid deaths of all SS1 and RR2 plants and some individuals of RR1 at 25 and 30°C. It indicates that ponded riplines during warmer months of the year would provide ideal conditions for disease. Stems, as well as collars and lignotubers, of jarrah are susceptible to infection by zoospores from ponded riplines (Hardy *et al*. 1996; O'Gara *et al*. 1997). Harris *et al*. (1985) have also suggested that riplines may favour *P. cinnamomi* disease development in a Victorian seed orchard of *E. regnans*.

Previously designated resistant clonal lines when inoculated underbark all died rapidly in our study at 25 and 30°C. In glasshouse trials during summer when Stukely and Crane (1994) tested for resistance of the seedlings from which the clonal lines are derived, they reported that maximum temperatures never exceeded 28°C. We found that minimum glasshouse temperatures averaged at 20°C during summer (Hüberli *et al*. 2000), which is probably similar to those during the experiment of Stukely and Crane (1994). In the current study, at continuous temperatures of 25 and 30°C deaths occurred rapidly in all clonal lines when inoculated underbark, while at 20°C RR1 showed some resistance to disease. These findings indicate that for underbark inoculated plants the resistance mechanisms are effective at average temperatures less than 25°C. Underbark inoculations in

glasshouse experiments may be selecting for resistant clonal lines that are more effective at moderate temperatures. Clearly then, further work needs to address the effect of temperature on resistance of clonal lines.

None of the disease measures, mortality, lesion length, pathogen recovery, circumferential spread or stomatal conductance, used in our study were able to unequivocally discriminate disease resistance among clonal lines in underbark inoculations of potted plants at all temperatures. However, they did reflect changes in rate of disease development. In contrast, for zoospore inoculations we were able to use mortality and pathogen recovery to discriminate among clonal lines. For example, at all temperatures RR1 had lower mortalities and lower pathogen recovery from plant tissue. We believe that for the rapid screening of large numbers of *P. cinnamomi* isolates and/or plant genotypes the underbark inoculation method is a suitable test to use. However, for a more refined differentiation of isolate variation and/or resistance of plant genotypes the zoospore inoculation method is a more appropriate screening tool.

Underbark inoculation is a very severe test. Not only does it bypass the prepenetration and penetration stages of the *P. cinnamomi* infection process, but it also exposes phloem tissue to a high inoculum load. This may be why at highly favourable conditions for the pathogen, resistance was overcome in all clonal lines. Inoculation of plants with zoospores is probably more similar to the prepenetration and penetration phases of disease development in minesites and forests than underbark inoculation. The inoculum load is lower and may allow host defence mechanisms to contain the pathogen during penetration and early colonisation of bark cortical cells and progressive cell layers towards the phloem. This may explain the low mortality at 25 and 30°C of RR1 when inoculated with zoospores compared to the 100% mortality when inoculated underbark. The pathogen was also isolated at consistently lower rates in zoospore inoculated RR1 at all temperatures suggesting that this clonal line may be less susceptible to penetration of the zoospores, via the periderm. Alternatively, RR1 may have restricted and contained pathogen colonisation once it had penetrated, such that the pathogen was nonviable or dormant and did not regrow on agar. We would suggest that in future more emphasis be placed on zoospore inoculations for further assessment of clonal lines. The stem inoculation method (this paper; O'Gara *et al*. 1996, 1997) allows individual plants in screening trials to be retained in populations as inoculation sites can be placed well above the collar and diseased portions can pruned before the pathogen colonises stem bases and/or shoots die.

Lack of lesions in surviving and symptomless plants inoculated with zoospores did not mean that there was no *P. cinnamomi* colonisation. The pathogen was recovered up to 5 cm from the ROI in symptomless plants. This phenomenon has been described for jarrah plants inoculated with zoospores under other environmental conditions (O'Gara *et al*. 1997; Hüberli *et al*. 2000). In these symptomless plants having viable pathogen, lesions may develop when conditions become conducive and death may result. It is important that further assessment of resistance takes into consideration the possibility of colonisation without lesion formation.

We found the zoospore inoculation method both cumbersome and time consuming. Recently, Lucas *et al*. (2001) demonstrated that clonal jarrah can be successfully infected with *P. cinnamomi* by placing mycelial mats onto nonwounded stems. Other studies are currently investigating whether inoculation by this method is identical to the zoospore inoculation method. If it is found to be identical, then clonal lines could be rapidly assessed in temperature factorial experiments. The nonwounding inoculation method could possibly also be used in the detached stem screening test.

We found a large temperature effect in early lesion development in detached jarrah stems. In particular, the best discrimination between RR1 and SS2 was at 30°C after 2 days. That 30°C gave the best discrimination is in agreement with hyphal growth on callus tissue derived from susceptible and resistant clonal lines of jarrah (McComb *et al*. 1987). In our study, the susceptible clonal line SS2 had larger lesion responses to increasing temperature than the RR1 line. This indicates that detached stems could possibly be used as a preliminary screen for determining which plant genotypes are highly susceptible or resistant to *P. cinnamomi* at high temperatures. It would allow efficient testing of a large number of *P. cinnamomi* isolates with the same plant genotype. However, more isolates and plant genotypes need to be assessed to confirm the validity of this test. While a callus-pathogen screening method to select for resistant trees may be a legitimate test it has the disadvantage of the long time required to establish callus cultures. Our preliminary excised stem test has advantages over the callus-pathogen screening method in that stem tissue is readily available, can be used with seedling progeny, takes only 2 days and is less labour intensive and costly.

Interpretations of disease in jarrah clonal lines are complicated by the responses of the host, the

pathogen and the host-pathogen interactions to environmental factors, and possibly, to the inoculum load or inoculation method. That underbark inoculations did not discriminate among clonal lines particularly at high temperatures and that zoospore inoculation identified RR1 as more resistant than RR2 and SS1 at all temperatures indicates that the current underbark selection process for resistant jarrah lines may be inadequate. Further tests are needed to examine the climatic conditions under which resistance in jarrah is durable with a range of isolates varying in pathogenicity. In particular, tests need to address the possible disease threats posed by zoospore infection of the stems in rehabilitated bauxite minesites and in jarrah forest sites with impeded drainage. Our findings have important implications for breeding and selection programmes for resistance to *P. cinnamomi* in warm moist regions and in environments where climatic changes may shift to more conducive conditions. They also have important implications in management of riplines for ponding and planting positions in establishment of *Eucalyptus* spp. in infested or disease prone sites.

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Table 1 ANOVA of time to death (days) of *Eucalyptus marginata* (jarrah) clonal lines RR1, RR2 and SS1 stem inoculated with either mycelial-mats underbark or with zoospores of *Phytophthora cinnamomi* isolate MP94-48 in growth cabinets at 15, 20, 25 and 30°C. The MS error term used was from the three way interaction of clonal line x temperature x inoculation method. Clonal lines RR1 and RR2 are classified as resistant to *P. cinnamomi*, and clonal line SS2 as susceptible by McComb *et al*. (1990).

Effect	DF	F	*P*-value
Clonal line	2, 6	16.04	0.004[A]
Temperature	3, 6	45.20	0.0002[A]
Inoculation method	1, 6	0.64	0.45
Clonal line x Temperature	6, 6	1.20	0.42
Clonal line x Inoculation method	2, 6	4.90	0.06
Temperature x Inoculation method	3, 6	3.13	0.11

[A]Significant interaction at $\alpha = 0.05$.

Table 2 Isolation of *Phytophthora cinnamomi* from stems of dying and surviving resistant (RR; McComb *et al*. 1990) and susceptible (SS) clonal lines of *Eucalyptus marginata* (jarrah) stem inoculated with either mycelial-mats underbark or zoospores of isolate MP94-48 in growth cabinets at different temperatures. Surviving plants were harvested 105 (mycelial-mats underbark inoculation) and 86 (zoospore inoculation) days after inoculation.

Inoculation	Temp. (°C)	Clonal line of jarrah	Dying plants	Surviving plants	
			Isolation of *P. cinnamomi* from stem (%)	Isolation of *P. cinnamomi* from stem (%)	Mean colonisation length (cm)[A]
Mycelial-mats underbark	15	RR1	100	100	4
		RR2	100	100	3
		SS1	100	100	3
	20	RR1	100	100	9
		RR2	75	100	9
		SS1	83	100	14
	25	RR1	90	–[C]	–[C]
		RR2	100	–	–
		SS1	90	–	–
	30	RR1	100	–	–
		RR2	100	–	–
		SS1	100	–	–
Zoospores	15	RR1	–[B]	20	0.5
		RR2	67	75	0.5
		SS1	–	70	2.0
	20	RR1	–	20	2.0
		RR2	83	100	1.0
		SS1	83	50	0
	25	RR1	100	50	1.0
		RR2	29	–	–
		SS1	90	–	–
	30	RR1	75	17	1.0
		RR2	50	–	–
		SS1	70	–	–

[A]Colonisation length determined by plating 0.5 cm (mycelial-mat underbark inoculation) or 1 cm (zoospore inoculation) acropetal stem sections sequentially onto agar selective for *Phytophthora* from the site or region of inoculation.
[B]– denotes no dead plants within column.
[C]– denotes no surviving plants within column.

Table 3 ANOVA of lesion lengths measured after 2, 3 and 4 days in detached stems of *Eucalyptus marginata* (jarrah) clonal lines RR1 and SS2 inoculated underbark with mycelial-mats of four isolates of *Phytophthora cinnamomi* and incubated in temperature controlled cabinets set at 20, 25 and 30°C. The MS error term used was from the three way interaction of clonal line x isolate x temperature. Clonal line RR1 is classified as resistant to *P. cinnamomi*, and clonal line SS2 as susceptible by McComb *et al*. (1990).

Effect	DF	F	*P*-value
Clonal line	1, 6	82.85	0.0001[A]
Isolate	3, 6	3.78	0.08
Temperature	2, 6	35.45	0.0005[A]
Time	2, 6	99.93	0.00002[A]
Clonal line x Isolate	3, 6	0.40	0.76
Clonal line x Temperature	2, 6	7.75	0.02[A]
Clonal line x Time	2, 6	2.51	0.16
Isolate x Temperature	6, 6	1.09	0.46
Isolate x Time	6, 6	0.46	0.82
Temperature x Time	4, 6	6.80	0.02[A]
Clonal line x Isolate x Time	6, 6	0.67	0.68
Clonal line x Temperature x Time	4, 6	0.11	0.97
Isolate x Temperature x Time	12, 6	0.32	0.96

[A]Significant interaction at $\alpha = 0.05$.

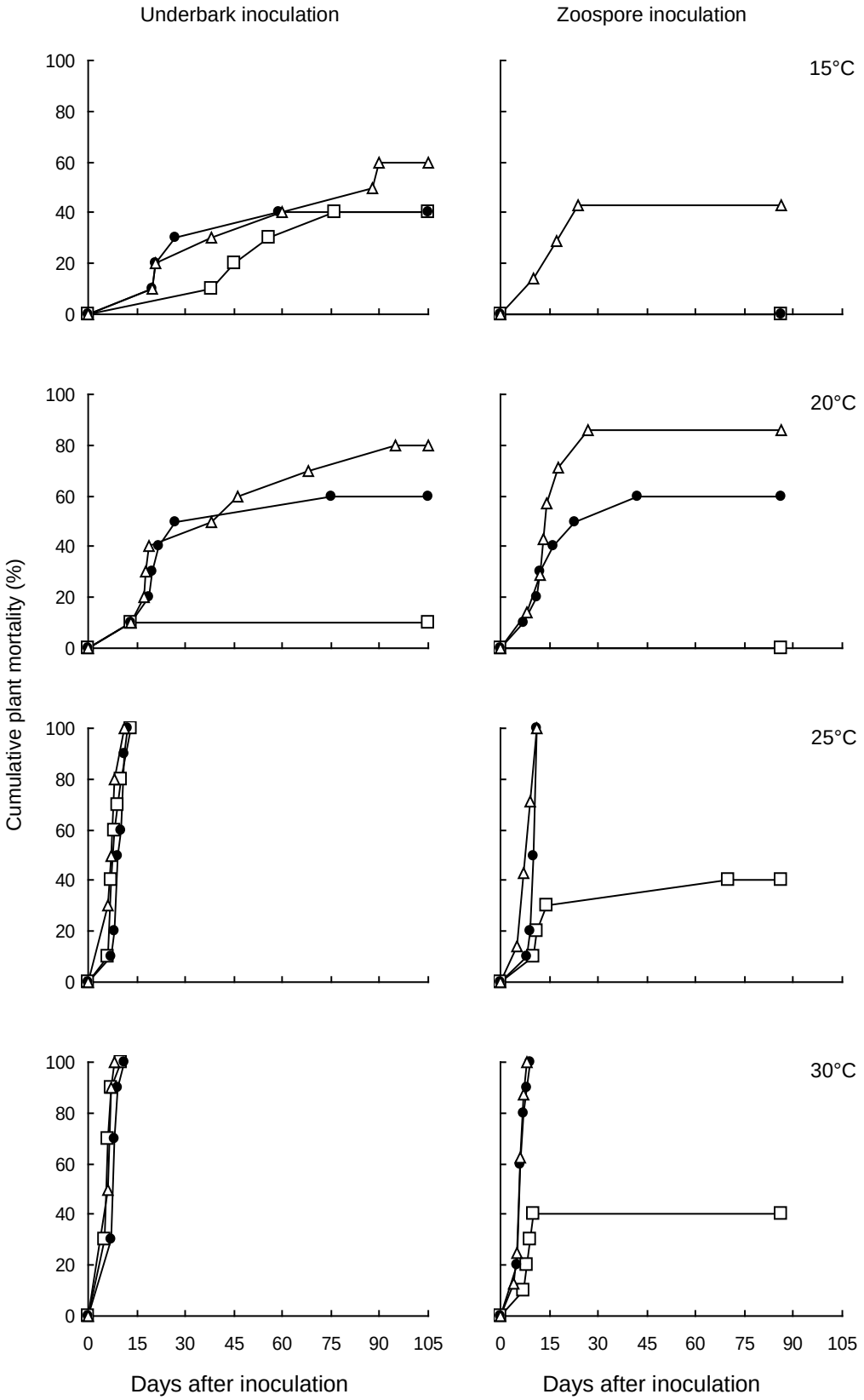

Figure 1 Effect of temperature on death of *Eucalyptus marginata* clonal lines RR1 (□), RR2 (Δ) and SS1 (●) stem inoculated with either mycelial-mats underbark or zoospores of *Phytophthora cinnamomi* isolate MP94-48. Clonal lines RR1 and RR2 are classified as resistant to *P. cinnamomi*, and clonal line SS1 as susceptible by McComb *et al*. (1990). Ten plants per clonal line at each temperature were inoculated, with the exception of RR2 inoculated with zoospores where 7 and 8 plants were used at 15-25°C and 30°C, respectively.

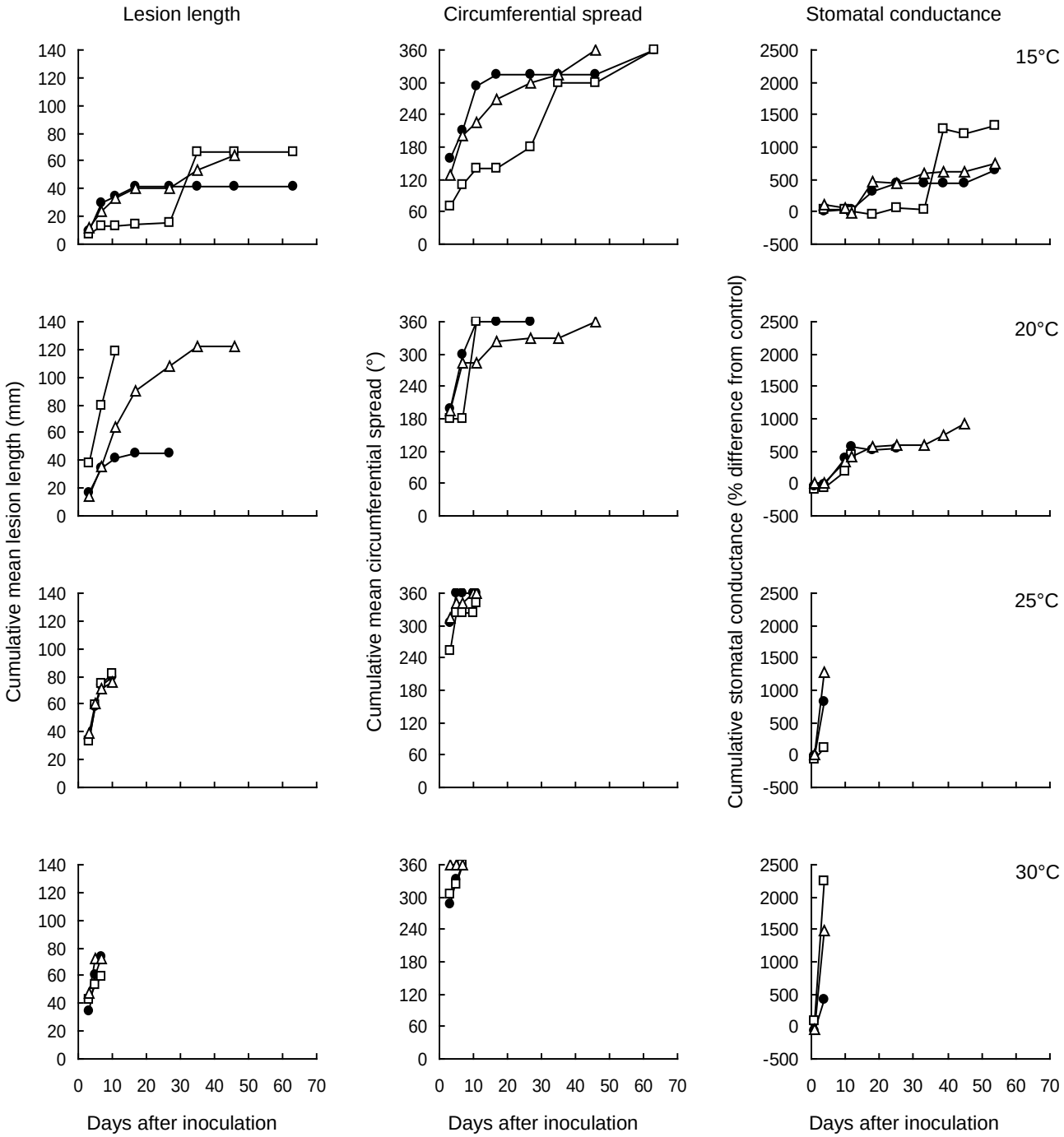

Figure 2 Effect of temperature on mean lesion length, circumferential spread and stomatal conductance of dying RR1 (□), RR2 (Δ) and SS1 (●) clonal lines of *Eucalyptus marginata* underbark stem inoculated with mycelial-mats of *Phytophthora cinnamomi* isolate MP94-48. Clonal lines RR1 and RR2 are classified as resistant to *P. cinnamomi*, and clonal line SS1 as susceptible by McComb *et al*. (1990). Plants dying after 63 days were not included. At 15°C, n = 3 for RR1, n = 4 for RR2 and SS1; at 20°C, n = 1 for RR1, n = 6 for RR2, n = 5 for SS1; and at 25 and 30°C, n = 10 for all clonal lines.

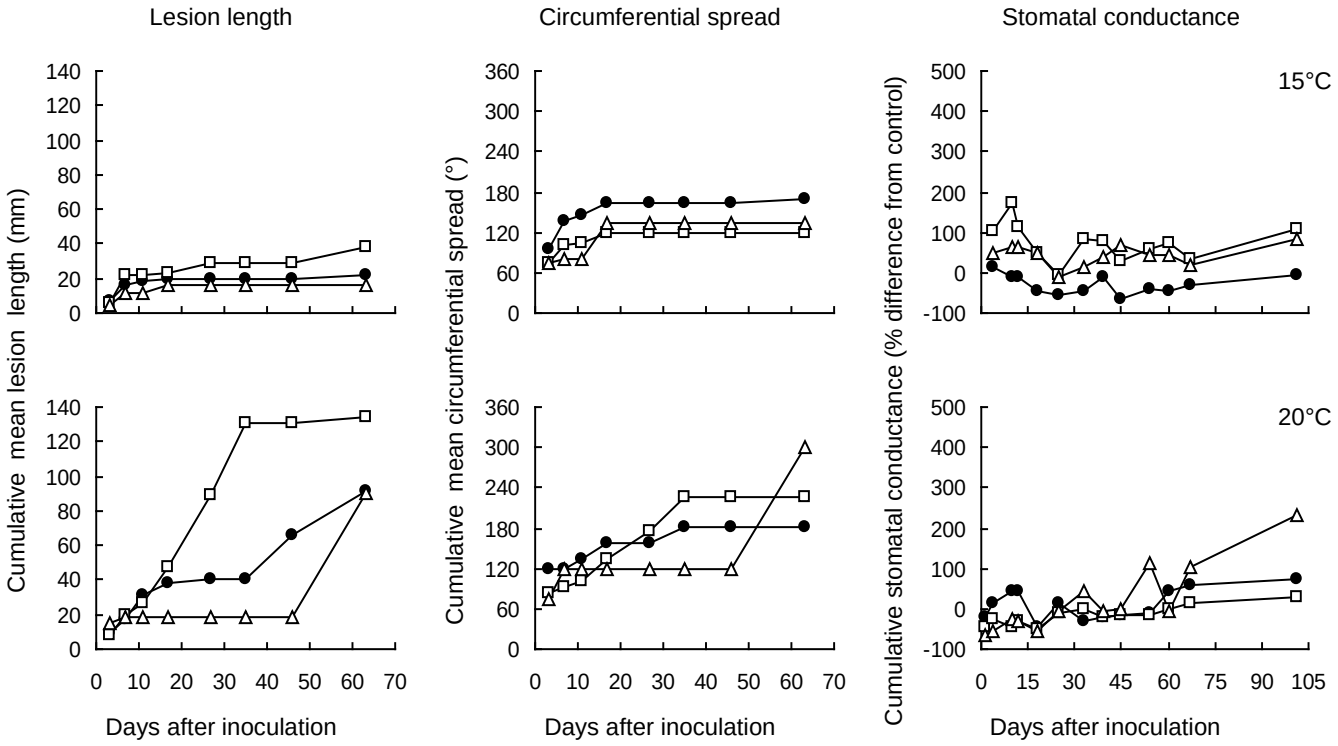

Figure 3 Effect of temperature on mean lesion length, circumferential spread and stomatal conductance of surviving RR1 (□), RR2 (Δ) and SS1 (●) clonal lines of *Eucalyptus marginata* underbark stem inoculated with mycelial-mats of *Phytophthora cinnamomi* isolate MP94-48. Clonal lines RR1 and RR2 are classified as resistant to *P. cinnamomi*, and clonal line SS1 as susceptible by McComb *et al*. (1990). Note that plants of all clonal lines were dead at 25 and 30°C. At 15°C, n = 6 for RR1 and SS1, n = 4 for RR2; and at 20°C, n = 9 for RR1, n = 2 for RR2, n = 4 for SS1.

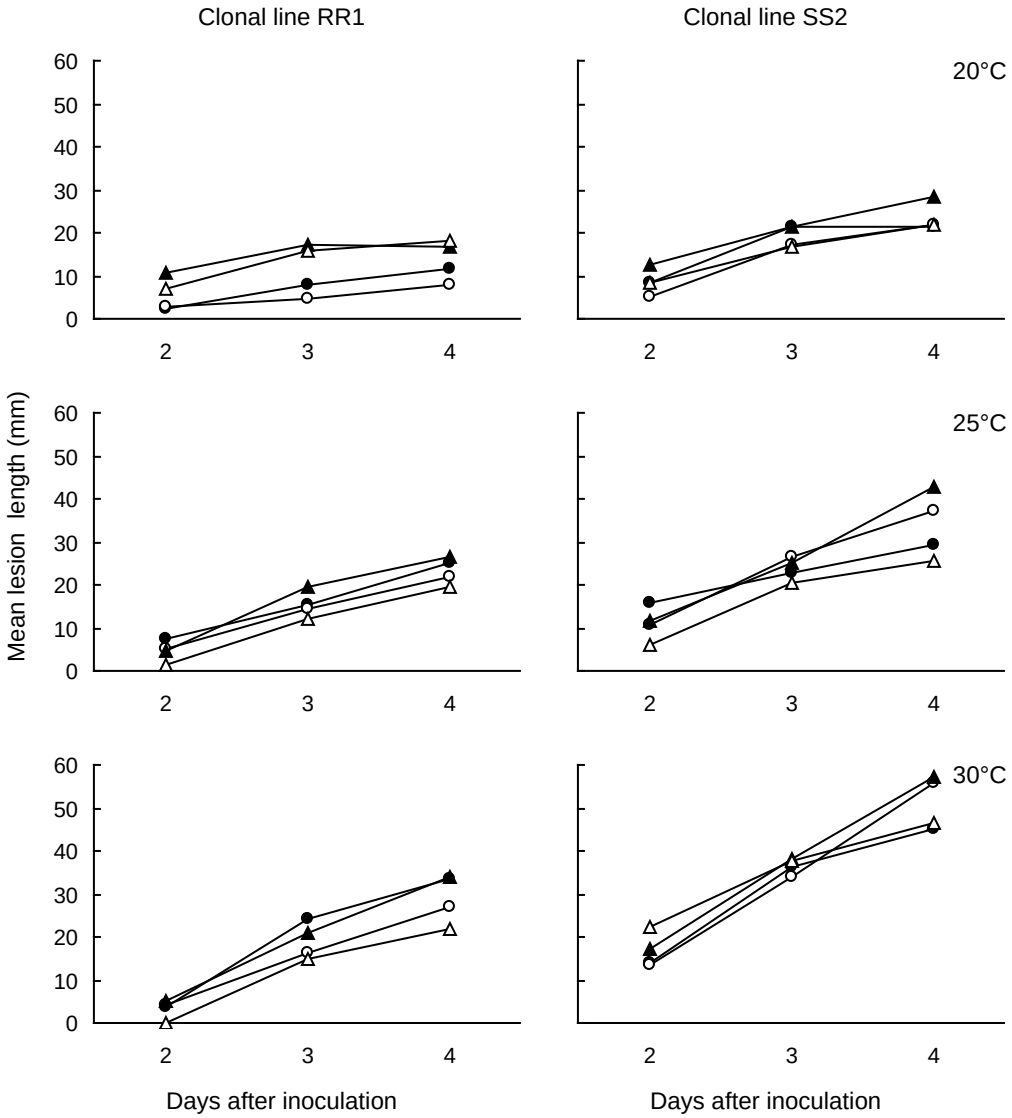

Figure 4 Effect of temperature on lesion length in detached stems of clonal lines RR1 and SS2 of *Eucalyptus marginata* inoculated with mycelial-mats underbark of isolates MP94-09 (●), MP94-48 (○), MP112 (▲) and MP116 (Δ) of *Phytophthora cinnamomi*. Clonal line RR1 is classified as resistant to *P. cinnamomi*, and clonal line SS2 as susceptible by McComb *et al*. (1990). Four inoculated stems per clonal line at each temperature were measured per day.