Resistance of Micropropagated *Eucalyptus marginata* to *Phytophthora cinnamomi*

D. M. CAHILL, I. J. BENNETT, and J. A. McCOMB, School of Biological and Environmental Sciences, Murdoch University, Perth, Western Australia 6150

ABSTRACT

Cahill, D. M., Bennett, I. J., and McComb, J. A. 1992. Resistance of micropropagated *Eucalyptus marginata* to *Phytophthora cinnamomi*. Plant Dis. 76:630-632.

Eighty-eight percent of primary roots of a "resistant" micropropagated line of *Eucalyptus marginata* were able to restrict and confine colonization by *Phytophthora cinnamomi*. In contrast, no roots of susceptible seedling *E. marginata* restricted the pathogen. Lesions in roots of the field-resistant *E. calophylla*, the clonal line of *E. marginata*, and the unselected seedlings of *E. marginata* were 35 ± 11 mm, 51 ± 23 mm, and 125 ± 13 mm, respectively, 7 days after inoculation with *P. cinnamomi*. There was a highly significant ($P < 0.01$) difference between mean lesion length in roots of the clonal *E. marginata* and lesion length in roots of the unselected seedlings and a significant difference ($P = 0.05$) between clonal *E. marginata* and *E. calophylla* root lesion lengths. Screening seedling populations of *E. marginata* for resistance to *P. cinnamomi* followed by selection and micropropagation of "resistant" individuals may be a very useful strategy for developing resistance in an otherwise susceptible species.

The *Eucalyptus marginata* Donn ex Sm. (jarrah) forest of southwestern Western Australia has been severely affected by the root pathogen *Phytophthora cinnamomi* Rands (19). In Australia, many native plant species are susceptible to this pathogen, and resistance has only been shown in certain *Eucalyptus, Acacia*, and monocotyledonous species (4,5,9,18,21,22). Resistance has recently been demonstrated in several avocado rootstocks (8,17) and in callus tissues derived from selected mature trees of *Pinus echinata* Mill. (11) and *E. marginata* (15). In addition, considerable genetic variation in resistance of *Pinus radiata* D. Don. to *P. cinnamomi* has enabled an ongoing breeding program for resistance (2).

The roots of field-resistant species are able to restrict and contain *P. cinnamomi* within a relatively small region behind the root tip (5,9), and new roots frequently form from healthy tissue just above the lesion (18). The slowing of lesion growth and eventual restriction of the pathogen is not accompanied by the hypersensitive response; however, the hypersensitive-like response of *Acacia pulchella* R. Br. to invasion by *P. cinnamomi* is an exception (23). Interpretation of the interaction of *P. cinnamomi* with its hosts is complicated, and the terms *resistance* and *susceptibility* are often difficult to define, because across the range of host species there is a continuum of degrees of resistance ranging from full susceptibility to full resistance (4).

Compared with other species of the genus *Eucalyptus, E. marginata* is highly susceptible to *P. cinnamomi*, although limited resistance in mature trees has been shown to result from the formation of periderm (20). Resistance has also been recently shown in several lines of micropropagated clonal *E. marginata* in field trials (14) and in glasshouse experiments (1).

The research presented here was undertaken to establish whether lesion containment was part of the resistance response of a clonal line of *E. marginata* that had been shown to survive in the field in soil infested with *P. cinnamomi* and to possess a high degree of resistance in preliminary laboratory experiments. For comparative purposes, unselected seedlings of *E. marginata* (susceptible) and *E. calophylla* R. Br. (marri, field-resistant) were also examined.

MATERIALS AND METHODS

Pathogen isolate and zoospore production. An isolate of *P. cinnamomi* (A2 compatibility type) was obtained from diseased roots of *E. marginata* and used in all experiments. Cultures were maintained on 20% V8 juice agar. Zoospores were produced with the method of Byrt and Grant (3) and were diluted with sterile distilled water to approximately 10^3 zoospores ml^{-1} for root inoculation.

Plants and growth conditions. *Seedlings.* Unselected seed of *E. marginata* was obtained from the Department of Conservation and Land Management, Western Australia; seeds of *E. calophylla*, from Vaughans Wildflower Seeds, Gingin, Western Australia. Seeds were surface-sterilized in a 5% sodium hypochlorite solution, with stirring (1 hr for *E. marginata* and 5 min for *E. calophylla*), and then washed three times with sterile distilled water.

Seeds were sown in flats in moist vermiculite, and then 5–6 weeks later the seedlings were transferred to plastic root trainers (38 × 50 × 184 mm, "WG7," Arthur Yates & Co., Cannington, Western Australia) containing a sterile 1:1 (v/v) mixture of vermiculite and acid-washed sand.

Clonal E. marginata. Clonal line 5-336 was selected from seedlings that had been screened for *P. cinnamomi* resistance in the glasshouse (14; M. Stukely, *personal communication*). The screening technique involved inoculation of seedlings 9- to 12-mo-old by placing mycelium in a shallow wound in the stem. Plants were assessed for lesion development after a set period (7–13 days after inoculation), and stems showing restricted lesion development (i.e., the most "resistant") were pruned to remove infected tissue and encourage axillary growth for use in tissue culture. Sterilized shoots were multiplied and rooted in vitro (13,16), and after transfer to soil they were hardened in seedling trays, then planted in sand and vermiculite in root trainers.

All clonal plants and the seedlings were grown under constant temperature in a glasshouse (25 C ± 5 C) and were watered with nutrient solution (12) three times weekly. After 6–7 mo of growth, plants were removed from the root trainers, and their tops and root systems were pruned. The pruned plants were replanted into polyvinylchloride root observation boxes (50 × 50 × 350 mm), which had a removable face and thus facilitated inoculation and observation of roots with minimal disturbance (9). The boxes were placed in a constant-environment chamber (25 C day, 22 C night, 12:12 photoperiod, 20–30 $\mu mol\ m^{-2}\ s^{-1}$) at an angle of 35% to the vertical, and plants were supplied with nutrient solution.

Inoculation and lesion measurements. Two to three weeks after transfer to the root boxes, new, white, unsuberized primary roots were produced. As many as three roots grew between the removable face and the soil surface. One root per plant was inoculated by placing 10 μl of zoospore suspension on the root tip (7). A set of plants that were treated with distilled water in place of inoculum

Present address of first author: Plant Cell Biology Group, Research School of Biological Sciences, Australian National University, GPO Box 475, Canberra ACT 2601, Australia.

Accepted for publication 24 January 1992.

served as controls in each experiment.

In experiment 1, the development of lesions was followed in roots of plants of each type by measuring lesion lengths directly with a clear plastic ruler at noon each day for 7 days after inoculation. In experiment 2, which used another group of plants, extension of the fungus within roots was assessed to determine whether lesions were contained by measuring lesion lengths 6 days after inoculation and then again 7 days after inoculation. In both types of experiments, total extension of the fungus was measured 7 days after inoculation. Roots were gently washed free of sand and vermiculite and severed. They were then surface-sterilized with 70% alcohol for 1 min, rinsed in sterile distilled water, blotted dry, divided into 5-mm segments, and sequentially plated onto cleared 20% V8 juice agar in 9-cm diameter plastic petri dishes. Plates were sealed and stored in the dark at 25 C and observed after 24 hr for the presence of *P. cinnamomi* hyphae.

All experiments were repeated at least twice, and the results were pooled.

RESULTS

Symptom development. Inoculation of roots of *E. marginata* (clonal and seedling) and *E. calophylla* with *P. cinnamomi* zoospores produced a brown lesion extending from the root tip within 12–16 hr. The roots of *E. marginata* seedlings became water-soaked in appearance within 48 hr after inoculation. Identifying the lesion margin in *E. marginata* roots required careful observation to distinguish the darker, lesioned tissue from the lighter brown, healthy root tissue. The health of secondary lateral roots, which arose every few millimeters along the main root and were readily infected as the pathogen progressed along the root, aided the identification of the lesion margin. Lesions of *E. calophylla* were darker than those of *E. marginata* and were clearly visible against the healthy white root tissue.

Pathogen growth in roots. Lesion extension in roots of seedlings of the two species differed markedly. Lesions in roots of the *E. calophylla* seedlings became restricted and were contained, whereas those in roots of *E. marginata* continued to extend. Lesions in roots of the clone grew at a much slower rate than those in the roots of the *E. marginata* seedlings and, like those in roots of *E. calophylla*, became restricted (Fig. 1).

E. calophylla seedlings. Within 3–4 days after inoculation lesion extension slowed, and by 6 days after inoculation lesions were contained. The mean lesion length was 35 ± 11 mm (n = 17) on day 7.

E. marginata seedlings. Lesion extension continued over the 7-day experimental period, and lesions were not contained. Lesions extended into the stem of several plants. The rate of lesion extension was approximately 20 mm day^{-1}, and 7 days after inoculation the mean lesion length was 125 ± 13 mm ($n = 14$).

E. marginata clone. Three days after inoculation, lesion extension had slowed from an initial rate of 20 mm day^{-1} to 1–2 mm day^{-1} before lesions became contained. At 7 days after inoculation the mean lesion length was 51 ± 23 mm.

Roots of the uninoculated control plants continued to grow and were white and healthy.

Lesion containment in *E. marginata* clone 5-336. In experiment 2 the lesion lengths were measured only on the 6th and 7th days after inoculation, and the data obtained confirmed the above observations for roots of *E. calophylla* and *E. marginata*. The lesion lengths in roots of *E. calophylla* ranged from 10 to 55 mm, with a mean lesion length on the 7th day after inoculation of 38 ± 10 mm ($n = 18$); in roots of *E. marginata*, lesions ranged from 100 to 150 mm in length, with a mean of 130 ± 13 mm ($n = 23$). Sixty-seven percent of the roots of the clones tested had lesion lengths that were less than or equal to those recorded in *E. calophylla* roots. The range of lesion lengths (5–150 mm) was greater in the clonal line than in roots of the seedling *E. marginata* or *E. calophylla*. Of the 42 individual plants of clone 5-336 tested, only five roots had lesions that were still extending 7 days after inoculation.

There was a highly significant difference ($P < 0.01$, Duncan's new multiple range test) between the mean root lesion length of the clone and those of the *E. marginata* seedlings. There was also a significant difference ($P = 0.05$) between the mean root lesion length of the clone and the *E. calophylla* seedlings. The frequency distribution of lesion length for the total number of roots inoculated was similar for seedlings of *E. calophylla* and *E. marginata* clone 5-336 but shifted to a greater lesion length for unselected *E. marginata* seedlings (Fig. 2).

Roots of the clonal plants that had restricted lesion growth had as little as 20% of their length infected, whereas those few roots in which the lesion was still expanding 7 days after inoculation had greater than 80% of their length infected. The mean length of infected root for the clone was 50% of total inoculated root length. There was no correlation ($P = 0.05$) between the percentage of root length infected and root length ($r = 0.0043$) (Fig. 3).

DISCUSSION

We have shown that plants of *E. marginata* derived from a seedling that was selected for resistance to the root pathogen *P. cinnamomi* using underbark stem inoculation and then cloned through tissue culture have the ability to restrict the spread of *P. cinnamomi* within their roots. This containment of the pathogen in the primary, unsuberized root is similar to that found in the field-resistant species *E. calophylla*. Eighty-eight percent of inoculated roots of the *E. marginata* clone showed restricted

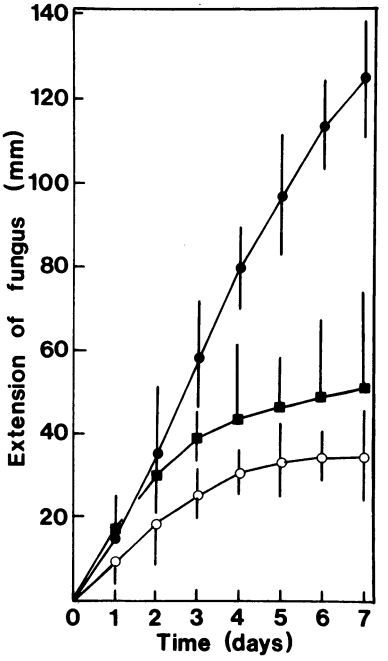

Fig. 1. Lesion extension in individual roots of *Eucalyptus calophylla* ○, *E. marginata* seedlings ●, and *E. marginata* clone 5-336 ■. Bars are the standard error of the mean of each data point.

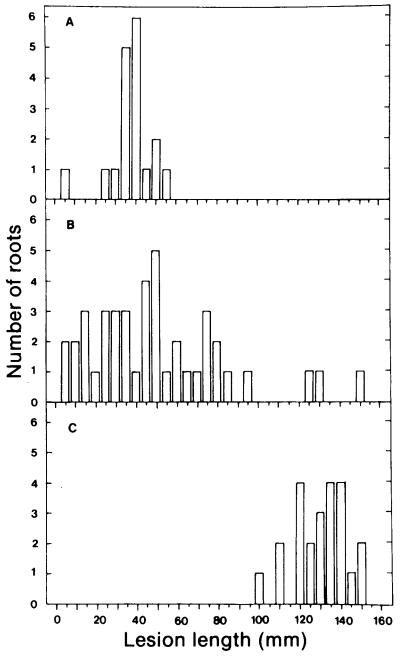

Fig. 2. Frequency distribution of the number of roots showing lesions of different lengths. **(A)** *Eucalyptus calophylla*. **(B)** *E. marginata* clone 5-336. **(C)** *E. marginata* seedlings. Lesion lengths were measured 7 days after inoculation with *Phytophthora cinnamomi* by placing root pieces onto 20% V8 juice agar.

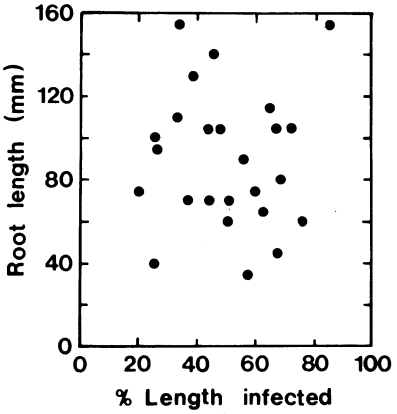

Fig. 3. Scatter diagram of root length versus percentage of root length infected for a single root from each of 25 plants of *Eucalyptus marginata* clone 5-336 7 days after inoculation. The correlation coefficient $r = 0.0043$ was not significant at $P = 0.05$.

lesion development and cessation of the spread of the pathogen within 7 days. This result is in direct contrast to the unlimited lesion development demonstrated in roots of unselected seedlings of *E. marginata*. The present work may be compared with the study of Grant and Byrt (9), in which only four of 160 (2.5%) of unselected seedlings of *E. marginata* showed the capacity to restrict invasion of root tissue by *P. cinnamomi*. Our results show the power of selection for resistance by drawing on a species' inherent genetic variability followed by micropropagation as a tool for developing clonal lines resistant to *P. cinnamomi*.

The mechanisms by which roots are able to restrict the spread of *P. cinnamomi* are not well understood. Anatomical barriers, whether preformed or induced, may play a role, as has been shown for callose (6,10), the formation of periderm (17,20), and lignin (4), especially in secondary tissues. There is increasing evidence to suggest that resistance to *P. cinnamomi* is, however, predominantly physiologically or biochemically based (4,9,11).

The results reported here, which indicate that lesion length in roots of clone 5-336 of *E. marginata* did not correlate with root length (i.e., the percentage of root length infected in those roots that had restricted the pathogen varied from 20 to 70%), also support this conclusion and strongly suggest that pathogen confinement is not related to the developmental state of the root (e.g., presence of secondarily thickened tissues). It is unlikely, therefore, that preformed anatomical barriers are important in the resistance of roots of clone 5-336 to *P. cinnamomi*, a contention supported by previous work with *Eucalyptus* spp. (4,9,21).

We have recently found that the activity of the key enzyme in the phenylpropanoid pathway, phenylalanine ammonia-lyase (EC 4.3.1.5), is increased in the resistant reaction of roots of *E. calophylla* to *P. cinnamomi*, and that increased lignification in conjunction with the synthesis of new phenolic compounds also occurs (Cahill and McComb, *unpublished*). It is possible that similar responses are invoived in the resistant reaction of roots of clonal *E. marginata* with *P. cinnamomi*.

Although lesions were restricted in roots of the majority of the clonal *E. marginata* plants used in our experiments, there were a few "lesion escapes" (12% of roots infected), which resulted in lesions comparable in length to those found in the roots of the susceptible seedlings. It is not known whether these lesions may have eventually been restricted or, indeed, whether all roots on those plants would have behaved similarly to inoculation.

The durability of the general resistance displayed by clonal *E. marginata* is now being critically evaluated in work that has been extended to examine resistance in several more clonal lines of *E. marginata*, both in the laboratory and in field trials.

ACKNOWLEDGMENTS

We thank M. Stukely and C. Crane of the Conservation and Land Management Department, Western Australia, for provision of *E. marginata* material for micropropagation, and Helen Lund for assistance with tissue culture.

LITERATURE CITED

1. Bennett, I. J. 1988. Application of tissue culture to improvement of *Eucalyptus marginata* (jarrah). Ph.D. thesis. Murdoch University, Perth, Australia.
2. Butcher, T. B., Stukely, M. J. C., and Chester, G. W. 1984. Genetic variation in resistance of *Pinus radiata* to *Phytophthora cinnamomi*. For. Ecol. Manage. 8:197-220.
3. Byrt, P. N., and Grant, B. R. 1979. Some conditions governing zoospore production in axenic cultures of *Phytophthora cinnamomi* Rands. Aust. J. Bot. 27:103-115.
4. Cahill, D. M., Legge, N., Grant, B. R., and Weste, G. M. 1989. Cellular and histological changes induced by *Phytophthora cinnamomi* in a group of plant species ranging from fully susceptible to fully resistant. Phytopathology 79:417-424.
5. Cahill, D. M., and Weste, G. M. 1983. Changes in respiration of seedling roots inoculated with *Phytophthora cinnamomi*. Phytopathol. Z. 106:51-62.
6. Cahill, D. M., and Weste, G. M. 1983. The formation of callose deposits as a response to infection with *Phytophthora cinnamomi*. Trans. Br. Mycol. Soc. 80:23-29.
7. Cahill, D. M., Weste, G. M., and Grant, B. R. 1986. Changes in cytokinin concentrations in xylem extrudate following infection of *Eucalyptus marginata* Donn ex Sm. with *Phytophthora cinnamomi* Rands. Plant Physiol. 81:1103-1109.
8. Gabor, B. K., and Coffey, M. D. 1990. Qualitative analysis of the resistance to *Phytophthora cinnamomi* in five avocado rootstocks under greenhouse conditions. Plant Dis. 74:882-885.
9. Grant, B. R., and Byrt, P. N. 1984. Root temperature effects on the growth of *Phytophthora cinnamomi* in roots of *Eucalyptus marginata* and *E. calophylla*. Phytopathology 74:179-184.
10. Hinch, J. M., and Clarke, A. E. 1982. Callose formation in *Zea mays* as a response to infection with *Phytophthora cinnamomi*. Physiol. Plant Pathol. 21:113-124.
11. Jang, J. C., and Tainter, F. H. 1990. Cellular responses of pine callus to infection by *Phytophthora cinnamomi*. Phytopathology 80:1347-1352.
12. Ladiges, P. Y. 1977. Differential susceptibility of two populations of *Eucalyptus viminalis* Labill. to iron chlorosis. Plant Soil 48:581-597.
13. McComb, J. A., and Bennett, I. J. 1982. Vegetative propagation of *Eucalyptus* using tissue culture and its application to forest improvement in Western Australia. Pages 721-722 in: Plant Tissue Cult. Proc. Int. Congr. Plant Tissue Cell Cult., 5th. A. Fujiwara, ed.
14. McComb, J. A., Bennett, I. J., Crane, C., and Stukely, M. J. C. 1991. Selection and propagation of jarrah dieback resistance. Combined Proceedings of the International Plant Propagators Society 40:86-90.
15. McComb, J. A., Hinch, J. M., and Clarke, A. E. 1987. Expression of field resistance in callus tissue inoculated with *Phytophthora cinnamomi*. Phytopathology 77:346-351.
16. Murashige, T., and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497.
17. Phillips, D., Grant, B. R., and Weste, G. M. 1987. Histological changes in the roots of an avocado cultivar, Duke 7, infected with *Phytophthora cinnamomi*. Phytopathology 77:691-698.
18. Phillips, D., and Weste, G. M. 1984. Field resistance in three native monocotyledon species that colonise indigenous sclerophyll forest after invasion by *Phytophthora cinnamomi* Aust. J. Bot. 32:339-352.
19. Shearer, B. L., and Tippett, J. T. 1989. Jarrah dieback: The dynamics and management of *Phytophthora cinnamomi* in the jarrah (*Eucalyptus marginata*) forest of south-western Australia. West. Aust. Dep. Conserv. Land Manage. Bull. 3. 76 pp.
20. Tippett, J. T., and Hill, T. C. 1984. Role of periderm in resistance of *Eucalyptus marginata* roots against *Phytophthora cinnamomi*. Eur. J. For. Pathol. 14:431-439.
21. Tippett, J. T., Hill, T. C., and Shearer, B. L. 1985. Resistance of *Eucalyptus* spp. to invasion by *Phytophthora cinnamomi*. Aust. J. Bot. 33:409-418.
22. Tippett, J. T., Holland, A. A., Marks, G. C., and O'Brien, T. P. 1976. Penetration of *Phytophthora cinnamomi* into disease tolerant and susceptible eucalyptus. Arch. Microbiol. 108:321-342.
23. Tippett, J. T., and Malajczuk, N. 1979. Interaction of *Phytophthora cinnamomi* and a resistant host, *Acacia pulchella*. Phytopathology 69:764-772.