GROWTH STIMULATION OF *WOOLLSIA PUNGENS* BY A NATURAL ERICOID MYCORRHIZAL FUNGAL ENDOPHYTE

J. H. Palmer[1], B. M. Horton, W. G. Allaway and A. E. Ashford

School of Biological, Earth and Environmental Sciences, The University of New South Wales, Sydney, NSW 2052, Australia.
[1.] E-mail: palmer1@tpg.com.au

Abstract

Woollsia pungens (Cav.) F. Muell. seedlings were germinated and grown under axenic conditions on either agar gel or Phytagel in closed transparent polystyrene culture boxes. Two months after sowing, when the seedlings were in the juvenile stage, culture boxes were inoculated with a fungal isolate MG60 which had previously been isolated from naturally-grown *W. pungens*. The inoculum and medium surfaces were then covered with a thin layer of autoclaved soil. After 5 months further growth in natural daylight the seedlings were harvested. Hair roots were fixed, stained, and scored for mycorrhizal colonisation by the presence of intracellular hyphae and fungal coils. Seedling growth was adversely affected by agar gel, with leaf yellowing and lack of mycorrhiza formation. Seedlings grown in non-inoculated Phytagel culture boxes appeared more vigorous than those reared on agar, although a reddish leaf pigment and some chlorosis were evident and measured growth was not statistically significantly different. All the sampled hair root systems of seedlings reared in inoculated Phytagel culture boxes scored positive for intracellular fungal hyphae or fungal coils characteristic of ericoid mycorrhizas. These structures were present in 55% of the hair roots examined from the inoculated culture boxes. The inoculated seedlings growing on Phytagel possessed tall shoots with normal leaf colour, and almost twice as many leaves at harvest as the non-inoculated Phytagel controls, indicating a positive effect of MG60 on seedling growth probably via nutrient uptake. This trial has demonstrated that Phytagel covered by natural soil is a suitable growing medium for *W. pungens* and that inoculation with the fungus MG60 results in the formation of ericoid mycorrhizas and has a beneficial effect on seedling growth.

Key words: Epacridaceae, Ericaceae, ericoid, hair root, *Hymenoscyphus*, mycorrhiza, *Woollsia*.

J. H. Palmer, B. Horton, W. G. Allaway and A. E. Ashford (2007). Growth stimulation of *Woollsia pungens* by a natural ericoid mycorrhizal fungal endophyte. *Australasian Mycologist* 26 (1): 1-8.

Introduction

The shrubby epacrid *Woollsia pungens* (Cav.) F. Muell. is a common plant of coastal and inland heaths in eastern Australia. Although formerly classified as Epacridaceae, epacrids are now regarded as a lineage within Ericaceae (see references in Cairney & Ashford 2002). As with the majority of other members of this family, *W. pungens* naturally forms mycorrhizas of the ericoid type (Kemp *et al.* 2003). These occur only on hair roots, which are specialised fine roots. Mycorrhizas are considered to promote establishment and growth of epacrids in natural habitats by enhancing nitrogen and phosphorus nutrition and by facilitating access to organic sources of these nutrients in the soil (Bell & Pate 1996; Read 1996). The true mycorrhizal nature of the association (i.e. mutualistic symbiosis rather than just association with a fungus) has not, however, been proven in *W. pungens*. A

definitive test would be to grow the plant under axenic conditions, inoculate with a mycorrhizal fungal isolate, and demonstrate formation of mycorrhizal structures and a plant growth response (cf. Leake & Read 1991).

Woollsia pungens is difficult to grow from seed, because of low germination rates and poor establishment (Jupp 2004). Palmer & Ashford (2004) reported that natural soil from a site where *W. pungens* was growing appeared beneficial to seedling establishment in cultivation and suggested that this might be the result of the soil containing a fungus able to form mycorrhizas. Although mycorrhizal associations have been widely reported for epacrids in the wild (Read 1996, Cairney & Ashford 2002, Davies *et al.* 2003), there has often been a lack of success in inducing mycorrhizas under axenic conditions. However McLean *et al.* (1998) were successful in inducing mycorrhizas to form in *Epacris impressa* Labill. in axenic culture. In this paper we describe how we germinated and grew *W. pungens* seedlings in axenic culture in the presence of autoclaved natural soil, and successfully inoculated them with a fungus that had been isolated from field-grown roots of *W. pungens* by Midgley *et al.* (2002). A feature of a successful ericoid mycorrhizal association in Ericaceae is the formation of fungal coils in the periplasmic space of the invaded epidermal cell in the hair root (Cairney & Ashford 2002). We used the presence of intracellular hyphae and fungal coils in epidermal cells as evidence of mycorrhiza formation to measure the success of the inoculation on *W. pungens*.

Materials and Methods

Seed and soil sources

Seed was collected from a forested shale sandstone site in the Georges River National Park in September 1999 and used in this trial in 2004. Damaged seed, dust and non-seed material were removed by manual sorting. Soil was obtained from another natural *W. pungens* population site, at La Perouse in Sydney, and was sieved to remove coarse plant debris and sterilised by autoclaving at 121°C for 15 min.

Germination and plant culture

The cleaned seed was surface sterilised in 20 mL of 2.5% calcium hypochlorite solution for 2 h, surface rinsed and shaken five times in sterile deionised water over a 1 h period. After standing for 2 h, the seed was rinsed again with five more changes of sterile deionised water. Seeds were resuspended by shaking and sub-samples were drawn into a syringe in a fixed volume of solution (1 mL) from the final rinse and pipetted into clear sterile polystyrene non-ventilated culture boxes (Phytatrays, 114 x 86 x 89 mm deep including lid, Sigma-Aldrich, Castle Hill, NSW) containing 50 mL of either 1% agar or 1% Phytagel (a clear gel composed of glucuronic acid, rhamnose and glucose, Sigma-Aldrich), made up in deionised water without any added minerals or nutrients, as the supporting gel base. On the day of sowing, seeds were counted in four randomly selected agar and four randomly selected Phytagel culture boxes, to estimate the number sown per culture box. Preliminary trials showed that darkness was necessary for optimal germination, so the culture boxes were kept in darkness for 3 weeks at 23°C. They were then moved to subdued daylight in the laboratory at the same temperature for 2 weeks while the seedlings emerged. The culture boxes of seedlings were then placed in randomised order in clear polyethylene bags with closed (but not sealed) ends, in a shaded, temperature-controlled glasshouse (temperature range 12-24°C) with natural lighting. After 1 month in the glasshouse, the percentage germination on each medium was estimated by counting the seedlings in the same culture boxes used in the seed count. On the same day the fungal isolate was added to the 'inoculated' treatments, and the boxes were re-randomised. After a further 3 months, a small volume of sterile deionised water was added to each culture box to augment the water supply, and the boxes were again re-randomised. The plants were harvested 5 months after inoculation (i.e., 7 months after seed sowing). There were four culture boxes each of: agar controls (not inoculated); Phytagel controls (not inoculated); agar inoculated; and Phytagel inoculated. To minimise contamination, all manipulations, including watering, were done in a laminar flow cabinet, and the lids of the culture boxes were always

closed before removing them from the laminar flow cabinet and returning them to the closed polyethylene bags in the glasshouse.

Fungal culture, inoculation and soil addition

The fungal isolate MG60 was used for inoculation. This had originally been isolated from the root system of a *W. pungens* plant growing in a natural ecosystem, and the isolate had subsequently been maintained on 2% malt agar with sub-culture every 4–6 weeks (Midgley *et al.* 2002). At the time of inoculation, the first leaves were beginning to unfold from the apical bud and seedlings were entering the juvenile stage of development. The juvenile stage, which lasts for 1–2 years, is characterised by lack of branching in the shoot and the production of numerous minute strap-like leaves of relatively uniform size (Palmer & Ashford 2004). Small strips of MG60 mycelium, about 4 x 2 mm, were cut from the leading edge of the colony, where the fungus was most actively growing. Ten to fifteen strips were then placed on the surface of the growing medium in each culture box that was to be inoculated. Sterile soil (30–40 g) was sieved through a sterile sieve to cover the strips and the growing medium to a depth of about 4 mm. The soil surface was stabilised with a light spray of sterile deionised water and the lids replaced. All manipulations were done in a laminar flow cabinet.

Harvesting and fixation

After 5 months in the glasshouse, the number of living seedlings in each culture box was recorded and they were then preserved *in situ* by immersion in 5% glutaraldehyde in 0.08 M piperazine-n,n'-bis(2-ethanesulfonic acid) (PIPES) buffer pH 7. After 24 h, the glutaraldehyde solution was drained off and replaced by 0.08 M PIPES buffer. The culture boxes were stored under refrigeration at 4°C. Residual fixative inhibited bacterial or fungal growth.

Hair root analysis

Four to twelve seedlings survived in the agar culture boxes. Depending on the numbers present, four or five randomly chosen seedlings were used for examination from each culture box. Seedling numbers in the Phytagel culture boxes ranged from 6 to 37, and five randomly chosen seedlings were selected from each culture box. Seedlings were lifted from each of the culture boxes and immersed in three successive rinses of deionised water to remove soil and humus from their hair roots. This operation retained each seedling's hair root system intact; there was no root growth into either of the gels and no evidence of hair root loss in the rinsing water. After removing the shoot above the hypocotyl, the complete root system attached to the base of the hypocotyl was cleared by immersion in 10 mL of 10% KOH solution and autoclaving at 112°C for 2 min. The KOH was removed by rinsing in deionised water and the hair roots stained with 0.1% Chlorazol Black E in lactic acid: glycerol: water (1:1:1) solution for 3 days, after which time they were de-stained in lactic acid: glycerol: water (1:1:1) solution for 2 days, rinsed in glycerol: water (1:1) and mounted on microscope slides in glycerol: water (1:1) (Brundrett *et al.* 1996). Slides were examined using a Zeiss Axiophot microscope with Nomarski optics. Fungal coils were identified as hyphae coiled within the epidermal cells of the hair roots (Figure 1a).

For seedlings sampled from the agar culture boxes, all hair roots that had developed beyond the initiation stage (>1 mm long) were examined for the presence of the fungus. The hair roots of the five seedlings taken from each of the Phytagel culture boxes were also scored for the presence of the fungus, using the same procedure but restricting the scoring to 10 randomly selected roots on each root system. Scoring was by scanning each root for the presence of intracellular fungal hyphae and/or coils within three or more adjacent epidermal cells. If present, the root was scored as positive.

Measurements of shoots and leaves

Results for shoots and leaves are based on observations and measurements made on all the seedlings in each culture box. The mean shoot height for each culture box, measured in mm above the hypocotyl, was calculated. Because of the difficulties involved in counting the large number of minute spirally arranged leaves produced by each shoot, an alternative procedure was used. Mean leaf number per mm of stem was calculated for each culture

box by counting the number of leaves in a 10 mm unit stem length for a random sample of four shoots, and dividing by 10. This value was then multiplied by the mean shoot height for each culture box, and the resulting estimate of mean leaf number per seedling was used as a quantitative measure of shoot productivity ("shoot productivity index"). This procedure is justified for *W. pungens*, as the seedlings produce similarly sized juvenile leaves throughout the seedling stage (Palmer & Ashford 2004). Also, the mean leaf number per seedling was multiplied by the number of seedlings surviving to harvest, to obtain an estimate of total number of leaves per culture box, which could be used to assess overall treatment response.

Statistical analysis

Statistical methods followed Zar (1974). Proportional data were arcsine-transformed; where there was heteroscedasticity, data were logarithmically transformed. Results were analysed by analysis of variance (ANOVA), followed if required by Student-Newman-Keuls multiple range testing. Where only two means were to be compared, Student's *t* tests were used.

Results

Germination and seedling survival

There were 87 $\pm$ 12 seeds per culture box (mean $\pm$ SEM, n = 8), and the percentage germination for agar was 20.0 $\pm$ 4.4, and for Phytagel 25.1 $\pm$ 3.7 (n = 4). The low germination values, which were not statistically significantly different, reflected the three-year age of the seed stock. There appeared to be an increase in seedling survival to harvest on Phytagel compared with agar (Table 1) but this was not statistically significant.

Table 1. Mycorrhizal colonisation and growth in cultured *Woollsia pungens* on two media with and without inoculation with fungal isolate MG60.

		Percentage of hair roots with fungal endophyte	**Number of seedlings per culture box at harvest**	**Mean shoot height in mm**
Agar	Control	0	5.8 $\pm$ 1.4	8.3^{b} $\pm$ 2.3
	Inoculated	0	6.0 $\pm$ 0.7	3.3^{a} $\pm$ 0.9
Phytagel	Control	8.0^{a} $\pm$ 2.4	10.8 $\pm$ 3.8	12.7^{b} $\pm$ 0.7
	Inoculated	55.0^{b} $\pm$ 10.8	19.8 $\pm$ 7.0	27.9^{c} $\pm$ 3.2
p (*t* test)		0.004		
p for treatments (ANOVA)			0.063	0.00007

Mean $\pm$ standard error of the mean are shown (n = 4). Within a column different superscripts indicate differences significant at $p < 0.05$ by Student's *t* test (Phytagel, percentage of hair roots with fungal endophyte), or by Student-Newman-Keuls test (mean shoot height in mm).

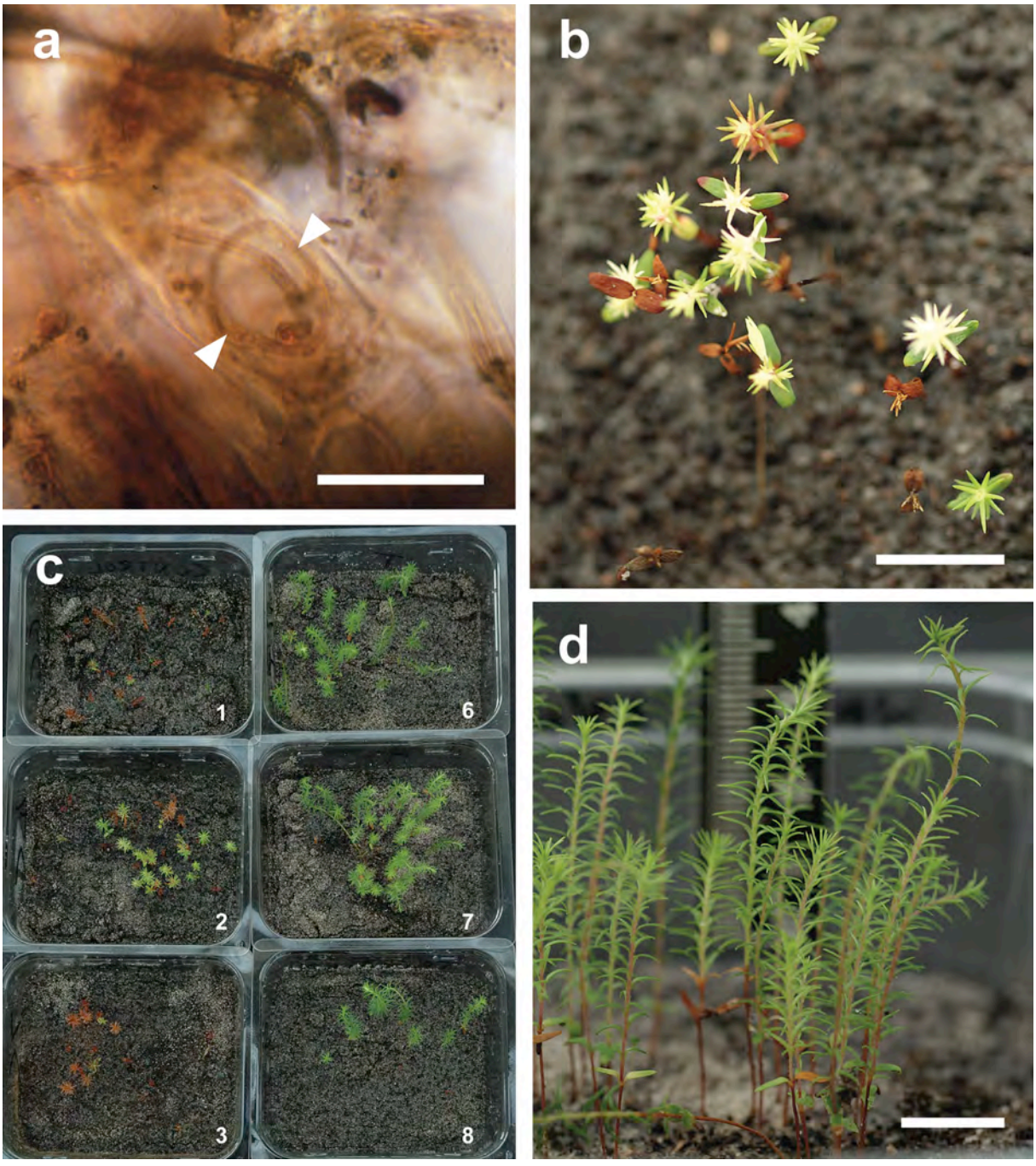

Figure 1. *Woollsia pungens* and ericoid mycorrhizal isolate MG60 in axenic culture. (a) Fungal coil (arrowed) in an epidermal cell of a *Woollsia pungens* hair root harvested from an inoculated Phytagel culture box. Bar: 10 µm approx. (b) *Woollsia pungens* seedlings in an agar control culture box photographed from above at harvest. The seven brown seedlings are dead. The 10 live seedlings all show little stem elongation and abnormally small chlorophyll deficient leaves which are confined to the apical bud. Bar: 10 mm approx. (c) *Woollsia pungens* seedlings on Phytagel at harvest in culture boxes (114 x 86 mm, lids removed for photograph). The seedlings in control culture boxes (1, 2, 3) show reduced shoot growth and reddish or pale green leaves, in contrast to the healthy appearance of those in inoculated culture boxes (6, 7, 8). (d) Side view of seedlings in inoculated Phytagel culture box 7 at harvest. All show the normal juvenile growth habit for *Woollsia pungens* with unbranched stems bearing numerous minute, green, strap-like leaves. Bar: 10 mm.

Table 2. Effect of inoculation with isolate MG60 on productivity in *Woollsia pungens* cultured on Phytagel.

	Mean number of leaves per 1 mm of shoot	**Shoot productivity index (estimated number of leaves per seedling)**	**Overall treatment response (estimated total number of leaves per culture box)**
Control	1.7 ± 0.2	21.7^{a} ± 1.1	231.8 ± 79.1
Inoculated	1.7 ± 0.1	47.1^{b} ± 7.6	966.5 ± 378.1
p (*t* test)	0.71	0.045	0.153

Mean ± standard error of the mean are shown (n = 4). Within a column different superscripts indicate differences significant at $p < 0.05$.

Hair root and mycorrhiza production

In agar culture boxes, no mycorrhizal structures were found. Sampled seedlings on agar, whether control or inoculated, possessed only a few hair roots. These were extremely short, ranging in length up to about 3 mm, and were generally unbranched. All the hair roots of the sampled seedlings from agar were scanned for fungal hyphae and coils in epidermal cells, but none were seen (Table 1).

In controls on Phytagel, mycorrhizal structures were found in only a few hair-roots: intracellular hyphae and/or fungal coils were detected in 16 of the 200 scored roots (Table 1).

In inoculated Phytagel culture boxes, the sampled seedlings possessed extensive multi-branched hair root systems, which in some cases were more than 20 mm long. All 20 root systems examined scored positive for intracellular hyphae and/or coils (Figure 1a), and on average 55% of the roots possessed intracellular hyphae and/or coils (Table 1). The difference in incidence of mycorrhizal structures between control and inoculated Phytagel culture boxes was statistically significant (Table 1).

Shoot growth

Shoot height was stunted in agar and Phytagel controls and in inoculated culture boxes on agar (Table 1). This was because of the arrest of stem elongation, which normally occurs after the hypocotyl has attained its maximum length of 5-10 mm. On agar, with or without inoculation, leaves were abnormally small and had not emerged from the apical bud. Their pale yellow colour indicated low chlorophyll levels (Figure 1b). Because the seedlings on agar were in such poor condition, further measurements on agar treatments were not pursued.

On Phytagel, in the controls, the majority of seedlings were also small because of low post-germination stem growth (Figure 1c, Table 1). Many showed reddish coloration, while others, although greener than those on agar, were nevertheless slightly chlorotic (Figure 1c). The seedlings in the four inoculated Phytagel culture boxes, however, resembled typical juvenile plants, with healthy shoot growth and numerous leaves of normal size and colour (Figure 1c). Stem elongation had resulted in tall shoots (Figure 1d) with a mean height of 27.9 ± 3.2 mm, more than twice that of the controls (Table 1). This increase in height was statistically significant, and it was not caused by a promotion of internode expansion since mean leaf number per unit stem length was not significantly affected by inoculation (Table 2). It could be accounted for if inoculation promoted the rate of shoot growth, resulting in more leaves and stem being produced during the growing period

Shoot productivity

The estimated number of leaves per seedling on Phytagel, an index of shoot productivity, was more than doubled by inoculation with MG60, and this was statistically significant (Table 2). However, the increase in overall treatment response (the estimated total number of leaves per culture box), while apparently large (Table 2), was not statistically significant because of the great variability.

Discussion

On the basis of criteria set out in Leake & Read (1991) the experiment demonstrated that isolate MG60 and *Woollsia pungens* did form ericoid mycorrhizas. The presence of fungal coils in the hair roots of all the sampled seedlings from the inoculated Phytagel culture boxes shows that the experimental procedure was successful in enabling the fungus to invade hair roots of *W. pungens* and form typical ericoid mycorrhizal structures (cf. Briggs & Ashford 2001). The low incidence of coils and intracellular hyphae in the controls demonstrates that potential contamination from the autoclaved soil was low and indicates that inoculation with MG60 was responsible for the ericoid mycorrhiza formation and plant responses in the inoculated culture boxes. The doubling of mean number of leaves in the inoculated culture boxes has shown the ability of the fungal isolate MG60 to promote growth in *W. pungens*. This is possibly by increasing nitrogen and/or phosphorus acquisition (Chen *et al.* 1999): the added autoclaved soil would be expected to contain organic and/or insoluble pools of nitrogen and phosphorus, and a successful ericoid mycorrhiza could make these available (Bell *et al.* 1994, van Leerdam *et al.* 2001; cf. also Mitchell & Gibson 2006). Hence establishment of an ericoid mycorrhizal association early in seedling growth may be beneficial for the successful establishment of *W. pungens* in natural communities.

Midgley *et al.* (2002) stated that fungal isolate MG60 belonged to their RFLP type I, which has considerable sequence identity with known ericoid mycorrhizal endophytes. This RFLP type was the most spatially widespread isolate from the *Woollsia pungens* root system they had collected from a natural ecosystem (Midgley *et al.* 2002), and it showed 99.6% ITS sequence identity with an isolate from *Epacris impressa* that formed ericoid mycorrhizas in the axenic synthesis experiments of McLean *et al.* (1998, 1999). Midgley *et al.* (2002) tentatively suggested that their RFLP type I may be in the *Hymenoscyphus* complex, which includes fungi that form ericoid mycorrhizas (see Cairney & Ashford 2002). Thus RFLP type I, which includes MG60, is likely to be an ericoid mycorrhizal fungal endophyte (Midgley *et al.* 2002). To verify mycorrhizal status, however, requires that colonisation of the root system by the isolate and formation of mycorrhizal structures is accompanied by enhanced plant growth (Leake & Read 1991, Smith & Read 1997, Cairney & Ashford 2002). In this study we have found that MG60 infects *W. pungens* seedling roots, forming typical ericoid mycorrhizal coils, and that it produces a marked growth improvement. The study therefore shows that the fungal isolate MG60 does have its suggested role as an ericoid mycorrhizal fungal endophyte.

An adverse effect of using agar as the supporting medium on post-germination seedling growth in *Woollsia pungens* has been shown in this trial. Similar results were obtained in two preliminary trials using an agar base without added soil under both axenic and non-axenic conditions. We conclude that *W. pungens* seedlings are sensitive to agar gel for an unknown reason. It is of interest that this adverse response was not alleviated (but apparently made worse, Table 1) by the presence of the fungal isolate. McLennan (1935) described inhibition of normal root system development in *Epacris impressa* by agar. Similarly Malcolm Reed (pers. comm.) claimed reduced survival of epacrids such as *Leucopogon* spp. when germinated in axenic conditions on an agar gel, raising the possibility that this may be a common phenomenon in epacrids. In contrast, the trial has shown that Phytagel covered with appropriate soil provides a favourable growing medium for *W. pungens* and for mycorrhiza formation.

Acknowledgements

We thank David Midgley and John Cairney (University of Western Sydney) for the fungal isolate used for inoculation, and Robert Miller (Cumberland Flora and Fauna Interpretive Services, 13 Park Road, Bulli, NSW 2516) for collecting the seed under licence from the National Parks and Wildlife Service of New South Wales. We are grateful for the support of this project by a research grant from the Australian Flora Foundation.

References

Bell, T.L. & Pate, J.S. (1996). Nitrogen and phosphorus nutrition in mycorrhizal

Epacridaceae of south-west Australia. *Annals of Botany* **77,** 389-397.

Bell, T.L., Pate, J.S. & Dixon, K.W. (1994). Response of mycorrhizal seedlings of SW Australian sandplain Epacridaceae to added nitrogen and phosphorus. *Journal of Experimental Botany* **45,** 779-790.

Briggs, C. & Ashford, A.E. (2001). The structure and composition of the thick wall in the epidermal cells of hair roots in *Woollsia pungens* (Cav.) Muell. (Epacridaceae). *New Phytologist* **149**: 219-232.

Brundrett, M., Bougher, N., Dell, B., Grove, T. & Malajczuk, N. (1996). *Working with mycorrhiza in forestry and agriculture.* ACIAR Monograph 32. ACIAR, Canberra. 374 pp.

Cairney, J.W.G. & Ashford, A.E. (2002). Biology of mycorrhizal associations of epacrids (Ericaceae). *New Phytologist* **154,** 305-326.

Chen, A., Chambers, S.M. & Cairney, J.W.G. (1999). Utilisation of organic nitrogen and phosphorus sources by mycorrhizal endophytes of *Woollsia pungens* (Cav.) F. Muell. (Epacridaceae). *Mycorrhiza* **8,** 181-187.

Davies, P.W., McLean C.B. & Bell, T.L. (2003). Root survey and isolation of fungi from alpine epacrids (Ericaceae). *Australasian Mycologist* **22,** 4-10.

Jupp, N. (2004). Seed: a valuable resource! Part 5. Viability trials for Epacridaceae. *Hunter Flora* **12,** 7-8.

Kemp, E., Adam, P. & Ashford, A.E. (2003). Seasonal changes in hair roots and mycorrhizal colonization in *Woollsia pungens* (Cav.) F. Muell. (Epacridaceae). *Plant and Soil* **250,** 241-248.

Leake, J.R. & Read, D.J. (1991). Experiments with ericoid mycorrhiza. *Methods in Microbiology* **23,** 435-459.

McLean, C.B., Anthony, J., Collins, R.A., Steinke, E. & Lawrie, A.C. (1998). First synthesis of ericoid mycorrhizas in the Epacridaceae under axenic conditions. *New Phytologist* **139,** 589-593.

McLean, C.B., Cunnington, J.H. & Lawrie, A.C. (1999). Molecular diversity within and between ericoid endophytes from the Ericaceae and Epacridaceae. *New Phytologist* **144,** 351-358.

McLennan, E.I. (1935) Non-symbiotic development of seedlings of *Epacris impressa* Labill. *New Phytologist* **34,** 55-63.

Midgley, D.J., Chambers, S.M. & Cairney, J.W.G. (2002). Spatial distribution of fungal endophyte genotypes in a *Woollsia pungens* (Ericaceae) root system. *Australian Journal of Botany* **50,** 559-565.

Mitchell, D.T. & Gibson, B.R. (2006). Ericoid mycorrhizal association: ability to adapt to a broad range of habitats. *Mycologist* **20,** 2-9.

Palmer, J. & Ashford, A. (2004). *Woollsia pungens*: Germination and seedling growth. *Australian Plants* **22,** 243-252.

Read, D.J. (1996). The structure and function of the ericoid mycorrhizal root. *Annals of Botany* **77,** 365-374.

Smith, S.E., & Read, D.J. (1997). *Mycorrhizal Symbiosis.* Academic Press, San Diego, CA. 605 pp.

Van Leerdam, D.M., Williams, P.A. & Cairney, J.W.G. (2001). Phosphate-solubilising abilities of ericoid mycorrhizal endophytes of *Woollsia pungens* (Epacridaceae). *Australian Journal of Botany* **49,** 75-80.

Zar, J.H. (1974). *Biostatistical Analysis.* Prentice-Hall, Englewood Cliffs, NJ. 620 pp.