

Improving the colonization capacity and effectiveness of ectomycorrhizal fungal cultures by association with a host plant and re-isolation

B. D. THOMSON, N. MALAJCZUK, T. S. GROVE AND G. E. StJ. HARDY¹*

CSIRO Division of Forestry, Private Bag, P.O. Wembley, W.A. 6014, Australia

¹ Bunnings Treefarms, P.O. Box 444, Manjimup, W.A. 6258, Australia

The ability of an ectomycorrhizal fungus to colonize plant roots (colonization capacity) and to increase plant growth at a deficient supply of P (effectiveness) declines after repeated subculture on agar media. We attempted to revitalize selected isolates of ectomycorrhizal fungi by inoculating them onto a compatible host (*Eucalyptus globulus*) and reisolating them from ectomycorrhizas and basidiomes.

The growth on agar of the reisolated fungal cultures and the original fungal cultures was measured over 14 d. Seedlings of *E. globulus* were also inoculated with either the original fungal isolates or their reisolates and were grown in pots containing a P-deficient sand, in a temperature-controlled glasshouse. Seedlings were harvested 49 and 93 d after planting and were assessed for mycorrhizal colonization, dry weights and P concentrations.

The reisolates generally grew at a faster rate on agar, and colonized plant roots more quickly, than the original isolates. Plants inoculated with the reisolates also had increased dry weights by day 93, which could be attributed to increased P uptake by the plant. We concluded that reisolating ectomycorrhizal fungi from mycorrhizas and basidiomes can increase the colonization capacity and effectiveness of isolates which have been grown on agar media for extended periods. This result, and the high cost of maintaining cultures, emphasizes the need to examine alternative methods of storage of fungal isolates.

Inoculating seedlings with ectomycorrhizal fungi can increase the growth of trees, particularly under conditions of limiting soil P (Bougher, Grove & Malajczuk, 1990; Jones, Durall & Tinker, 1990). Fungal isolates which colonize tree roots rapidly and extensively appear to have the greatest capacity to increase plant growth at a deficient supply of P (effectiveness) (Cline & Reid, 1982; Heinrich & Patrick, 1986). It follows from this that any factor which decreases an isolate's ability to colonize roots will also decrease its effectiveness.

Laiho (1970) and Marx & Daniel (1976) observed that after several years of repeated subculture on agar media, isolates of ectomycorrhizal fungi can lose their ability to colonize plant roots. Speakman (1982) obtained similar results with plant pathogens. 'Passaging' older cultures through a compatible host (i.e. inoculating them onto the host and reisolating them from colonized tissue) revitalized isolates which had lost some of their ability to colonize roots (Marx, 1981). Marx proposed that 'revitalization via host passage should be done at least every 4 years to maintain a high level of symbiotic potential for specific isolates'. He did not determine whether an increase in the colonization capacity of re-isolates resulted in an increase in the effectiveness of these fungal cultures.

In our laboratory, we have observed decreased growth rates of ectomycorrhizal fungi on agar after less than 12

months of repeated subculture. This decrease can be associated with a reduced effectiveness of the fungi in increasing plant growth (see below). In the present experiment, we attempted to regain the colonization capacity and effectiveness of some of our older ectomycorrhizal cultures by inoculating them onto a compatible host and re-isolating them from ectomycorrhizas and basidiomes.

MATERIALS AND METHODS

Fungal cultures

Six ectomycorrhizal fungi were chosen for re-isolation based on observations that their growth rates on agar had decreased after repeated subculture: two isolates of *Laccaria laccata* (Scop. ex Fr.) Berk. & Br. (A and B) and single isolates of *Hebeloma westraliense* Bough. Tomm. & Mal., *Scleroderma verrucosum* Pers., *Setchelliogaster* sp. nov. and *Pisolithus tinctorius* (Pers.) Coker & Couch. At the time of re-isolation, these isolates had been growing on modified Melin–Norkrans medium (MMN) (Marx, 1969) at 25 °C and subcultured every 2–3 months for 4, 4, 3, 4, 4 and 5 yr, respectively. The fungi were originally isolated from stipe vegetative tissue of basidiomes collected under native stands of eucalypts.

Four of the ectomycorrhizal isolates (*L. laccata* A and B, *S. verrucosum* and *Setchelliogaster* sp.) have previously been tested for their ability to increase the growth of *Eucalyptus globulus*

* Present address: School of Biological and Environmental Sciences, Murdoch University, Murdoch, W.A. 6150, Australia

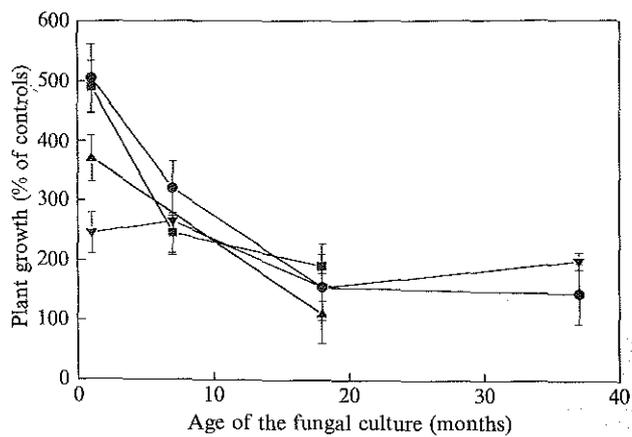


Fig. 1. Growth response of *Eucalyptus globulus* to inoculation with ectomycorrhizal fungi [*Laccaria laccata*; two isolates, A (●) and B (▲), *Scleroderma verucosum* (■) and *Setchelliogaster* sp. (▼)] in relation to age of the fungal cultures. Results have been derived from four separate experiments and responses measured relative to the growth of uninoculated plants in each study. Vertical bars represent $2 \times$ S.E.M.

Labill. in a P-deficient sand (four separate pot experiments conducted in the last 4 yr). The growing conditions used in each of these experiments were the same as those used in the present experiment (see below). Results from these experiments demonstrate that the effectiveness of the fungal isolates had decreased as the cultures aged (Fig. 1).

Re-isolation of fungal cultures

E. globulus seeds (1.4–1.6 mm diameter) were surface sterilized in a solution of 10% H_2O_2 and a trace of Tween 80 (1–2 drops l^{-1}) for 15 min. The seeds were then rinsed in deionized water five times before being transferred aseptically to agar plates containing $500 \mu M$ $CaSO_4$ and $3 \mu M$ H_3BO_3 . Plates were incubated in the dark for 5 d at 25° . Germinated seeds which were free from contamination were transferred aseptically to polycarbonate jars (65 mm diameter, 80 mm high) containing pure cultures of one of the six ectomycorrhizal isolates growing on MMN.

Jars containing the *E. globulus* seedlings and ectomycorrhizal cultures were incubated in a growth cabinet (22 – 23° , 16 h photoperiod, $350 \mu E m^{-2} s^{-1}$) for 10–14 d. At the end of this period, colonized seedlings were either aseptically transferred to other polycarbonate jars containing 100 ml vermiculite and 45 ml MMN medium (without agar) or were transplanted into pots in the glasshouse (as for main experiment below). Seedlings in vermiculite were incubated in the growth cabinet for 8 wk. Colonized roots were aseptically removed from these seedlings and were plated onto MMN medium in an attempt to re-isolate the fungi from colonized tissue. Seedlings in the glasshouse were grown for 6 months. When fruiting of isolates occurred in these pots (3–6 months after transplanting), re-isolations were also attempted from stipe vegetative tissue of the basidiomes.

The growth of reisolated and original fungal isolates on agar was compared. Plugs (5 mm diameter) of actively

growing culture of each isolate were transferred onto plates of MMN agar and the radial growth of these fungal colonies was measured at 3, 5, 7, 10, 12 and 14 d. There were three replicate plates of each fungal isolate.

Pot experiment

Experimental design. The experiment was a complete randomized block design with 16 treatments, two harvests and three replicates. The treatments were: uninoculated, inoculated with original isolates of each fungus, inoculated with reisolates from colonized roots of each fungus and inoculated with reisolates from basidiomes of *H. westraliense*, *L. laccata* B and *Setchelliogaster* sp.

Soil preparation. A yellow sand collected from the Spearwood dune system north of Perth, Western Australia (pH 6.2 in water, Bray-extractable P less than $2 mg kg^{-1}$ sand) was sieved through a 2 mm stainless steel mesh and steamed for 1.5 h at 70° . The sand was oven-dried at 70° and then placed into 2 kg plastic pots (14 cm diameter) lined with plastic bags. Basal nutrients ($mg kg^{-1}$ sand: $CaSO_4 \cdot 2H_2O$, 51.5; K_2SO_4 , 111.6; $MgSO_4 \cdot 7H_2O$, 33.7; $MnSO_4 \cdot 4H_2O$, 16.9; $CuSO_4 \cdot 5H_2O$, 8.2; $ZnSO_4 \cdot 7H_2O$, 9.2; $CoCl_2 \cdot 6H_2O$, 0.34; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.46; $Na_2B_4O_7 \cdot 10H_2O$, 1.1) were added in solution to each pot. These nutrients were allowed to dry on the sand surface and were then thoroughly mixed through the pot together with $8 mg P kg^{-1}$ sand (P sufficient for approximately 15% of maximum plant growth) applied as a powder of $Ca(H_2PO_4)_2 \cdot H_2O$. Each pot was watered to container capacity (10% w/w) with deionized water 1 wk before planting and was maintained at this water content for the duration of the experiment. The surface of each pot was covered with aluminium insulation foil to prevent algal growth and excessive evaporation and heating.

Inoculation and planting. *E. globulus* seeds were surface sterilized, germinated and grown in polycarbonate jars containing pure cultures of the fungal isolates as described above. Uninoculated control seedlings were grown in jars without fungal cultures. After 10–14 d growth, colonized and control seedlings were transplanted into pots in the glasshouse. Four seedlings were planted into each pot through holes in the aluminium covers. A small plug of inoculum (approximately 0.2 g of agar and fungus) was also placed against the seedling roots to ensure maximum success with the inoculation procedure. Following establishment, seedlings were thinned to two per pot. Nitrogen was added at 2-wk intervals to each pot as NH_4NO_3 in solution ($51.5 mg NH_4NO_3 kg^{-1}$ sand) to give a total level of applied NH_4NO_3 of $360.5 mg kg^{-1}$ sand. Pots were placed in a temperature-controlled glasshouse (December–March) with air temperatures ranging from 10° to 30° .

Harvest. Plants were harvested 49 and 93 d after transplanting the seedlings into pots. At each harvest, shoots were cut at the sand surface, oven-dried at 70° and weighed. Roots were washed free of sand and divided into coarse root and fine root (less than 0.2 mm) fractions. A sub-sample of the fine roots

was taken for assessing mycorrhizal colonization. The remaining root fractions were combined, oven-dried at 70° and weighed. The fine root sub-sample was stained with lactic-glycerol trypan blue for at least 7 d and the line intercept method of Newman (1966) was used to assess fine root length with and without mycorrhizas. Mycorrhizas appeared as short thickened roots which were stained heavily with trypan blue. Phosphorus concentrations were determined in the dried shoot and root fractions by digestion of ground material in H_2SO_4/H_2O_2 and analysis according to the method of Salt (1968).

RESULTS

Fungal growth on agar

Growth rates on agar differed among the ectomycorrhizal isolates (Fig. 2). These differences occurred between species as well as between isolates of the same species (*L. laccata* A and B).

The re-isolated fungal cultures initially grew more rapidly on agar than the original cultures, with the exception of the re-isolate of *Setchelliogaster* sp. from a basidiome (Fig. 2). For *L. laccata* B and *Setchelliogaster* sp., the re-isolates from colonized roots grew at a faster rate than the re-isolates from basidiomes.

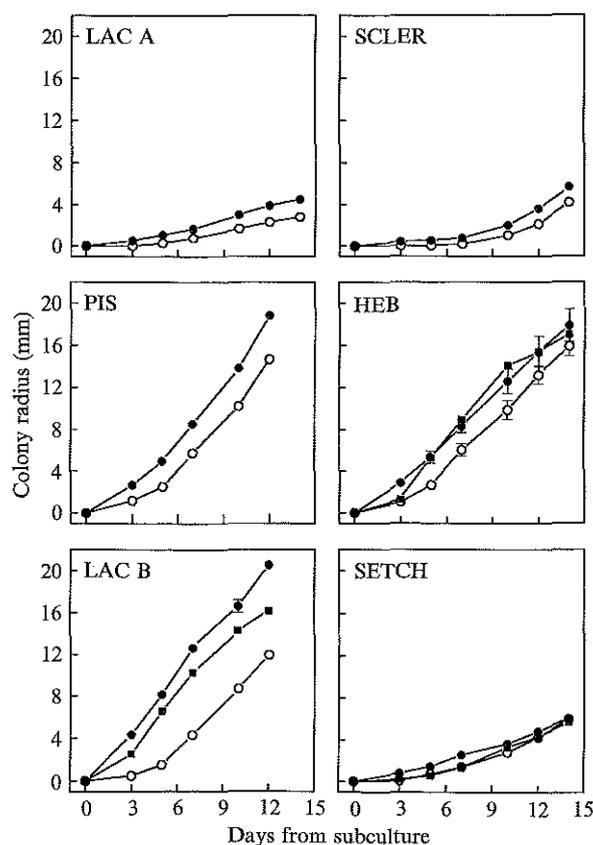


Fig. 2. Growth on agar of ectomycorrhizal fungi [*Laccaria laccata* (LAC) A and B, *Scleroderma verrucosum* (SCLER), *Pisolithus tinctorius* (PIS), *Hebeloma westraliense* (HEB) and *Setchelliogaster* sp. (SETCH)] following subculture from the original isolate (○), the re-isolates from colonized roots (●) or the re-isolates from basidiomes (■). Vertical bars represent $2 \times$ S.E.M.

Mycorrhizal colonization

The roots of uninoculated control plants were nonmycorrhizal at 49 d, but by day 93, 29% of their fine root length was colonized by an unidentified contaminant ectomycorrhizal fungus (or fungi) (Fig. 3). Inoculated plants had the same or a greater amount of mycorrhizal root at the same stage, the total amount varying with the fungal isolate. Colonization by the inoculant fungi could be distinguished from that by the contaminant fungus on the basis of morphology.

Re-isolates of the ectomycorrhizal fungi colonized a greater proportion of root length at 49 d (and to a lesser extent at 93 d) than original isolates, with the exception of both reisolates of *H. westraliense* and the re-isolate of *Setchelliogaster* sp. from colonized roots (Fig. 3). Differences between isolates in their colonization rates may have been more pronounced at an earlier harvest. Colonization by re-isolates of *L. laccata* B and *Setchelliogaster* sp. from basidiomes was greater than that by re-isolates from colonized roots. This effect, and the similar rates of colonization by the isolates of *H. westraliense*, do not correspond with growth rates on agar of these isolates.

Plant growth and P nutrition

Inoculation with the ectomycorrhizal fungi had little effect on plant growth at 49 d but by 93 d inoculated plants (particularly those inoculated with the reisolates) were larger than uninoculated ones (Fig. 3). The increased growth of inoculated plants at 93 d was associated with a higher P content in plants at both harvests and initially (day 49) with an increased P uptake per m of fine root (Table 1). Phosphorus concentrations in inoculated plants were also higher than in uninoculated plants at 49 d, but by day 93 these differences had been lost due to effects of increased plant growth in diluting plant P (Fig. 3).

Mycorrhizal isolates which colonized roots most rapidly were generally most effective at increasing P uptake and growth of plants (Fig. 3, Table 1). Thus, reisolates of the ectomycorrhizal fungi were generally more effective than original isolates. There were no differences, however, in effectiveness among the three *H. westraliense* isolates which corresponds with similar rates of colonization by each of these fungi. The re-isolate of *L. laccata* B from a basidiome was more effective than the re-isolate from colonized roots, which also corresponds with a greater rate of colonization by this isolate.

DISCUSSION

Our results are consistent with those of Marx (1981), who demonstrated that by 'passaging' older ectomycorrhizal cultures through a compatible host he was able to revitalize fungi which showed a decreased ability to colonize roots. We observed that cultures of ectomycorrhizal fungi which were re-isolated from colonized roots and basidiomes grew more rapidly on agar, and colonized roots more rapidly, than original cultures which had been repeatedly subcultured. Furthermore, these re-isolates were more effective at increasing P uptake and growth of plants than the original isolates. Other work has demonstrated that the most effective ectomycorrhizal fungi are those which colonize roots rapidly and extensively

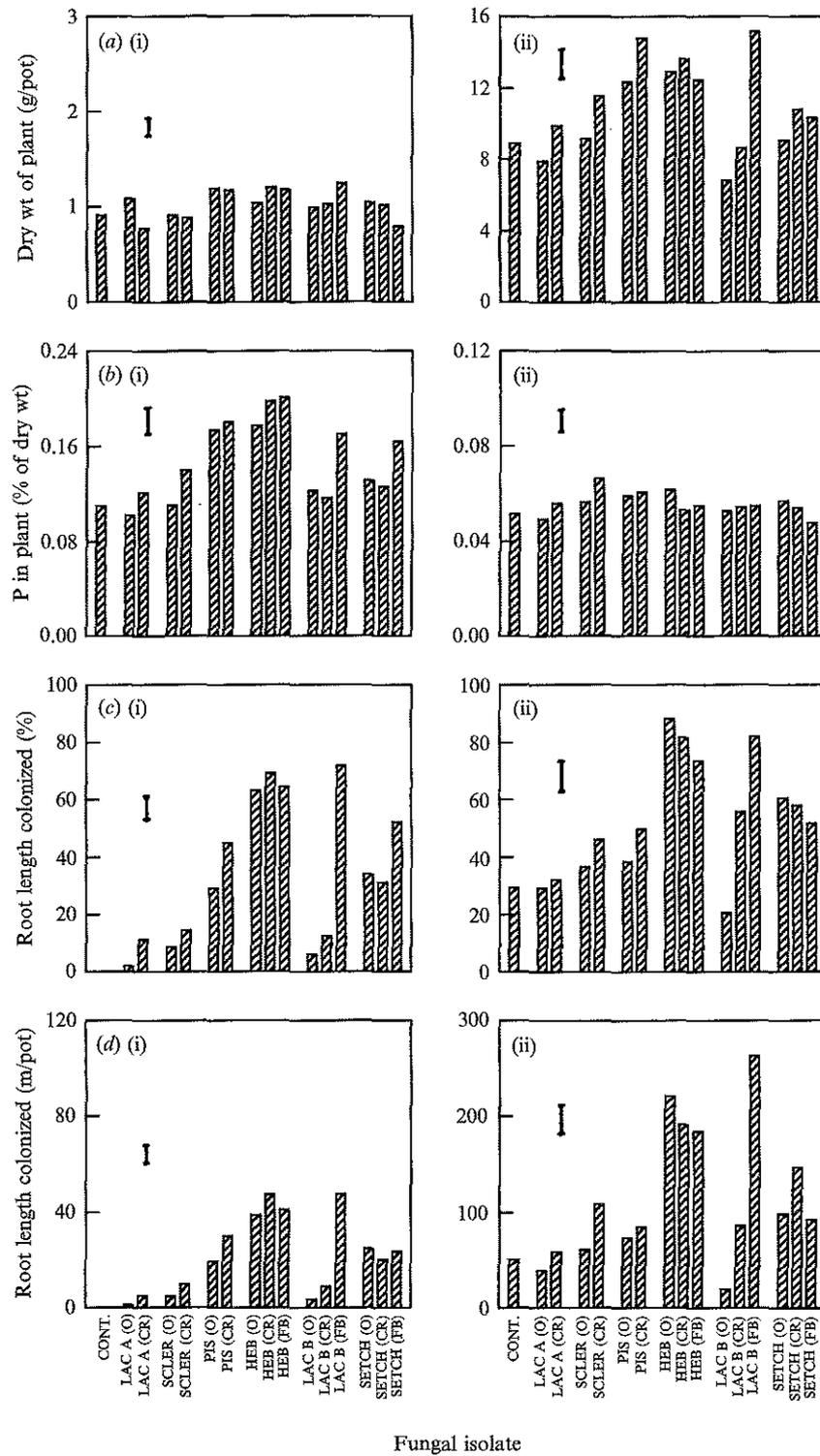


Fig. 3. Growth, P concentrations and mycorrhizal colonization of *Eucalyptus globulus* inoculated with ectomycorrhizal fungi [*Laccaria laccata* (LAC) A and B, *Scleroderma verrucosum* (SCLER), *Pisolithus tinctorius* (PIS), *Hebeloma westraliense* (HEB) and *Setchelliogaster* sp. (SETCH)]. Fungal isolates were either the original culture (O), re-isolates from colonized roots (CR) or re-isolates from basidiomes (FB). Plants were harvested (i) 49 d and (ii) 93 d after planting. Vertical bars represent $2 \times$ s.e.m. Note different scales.

(Cline & Reid, 1982; Heinrich & Patrick, 1986). However, Marx (1981) found no consistent relationship between the rate of growth of isolates in pure culture and the capacity of isolates to form ectomycorrhizas. In our study also, the colonization rates of isolates did not always correspond with their growth rates on agar.

Marx (1981) observed that revitalization of fungal cultures via host passage was successful when cultures were 4 yr old or more. He concluded from this that 'revitalization via host passage should be done at least every 4 years'. We observed a decline in the effectiveness of ectomycorrhizal fungi after less than a year in culture, implying that re-isolation should be

Table 1. P content, fine root length and P uptake per m of fine root of *Eucalyptus globulus* inoculated with selected ectomycorrhizal fungi

| Fungal isolate | P in plant (mg/pot) | | Root length (m/pot) | | P uptake (mg ⁻¹ m) of root | |
|------------------------------------|---------------------|---------------------|---------------------|----------------------|---------------------------------------|---------------------|
| | D49 | D93 | D49 | D93 | D49 | D93 |
| Control | 1.01 ^a | 4.60 ^{abc} | 53.7 ^a | 164.6 ^b | 0.019 ^a | 0.028 ^{ab} |
| <i>Laccaria laccata</i> A (O) | 1.11 ^{ab} | 3.77 ^{ab} | 67.0 ^a | 138.8 ^{ab} | 0.017 ^a | 0.027 ^a |
| <i>Laccaria laccata</i> A (CR) | 0.93 ^a | 5.49 ^{cd} | 46.3 ^a | 183.2 ^{bc} | 0.020 ^a | 0.030 ^{ab} |
| <i>Scleroderma verrucosum</i> (O) | 1.01 ^a | 5.06 ^{abc} | 62.9 ^a | 151.5 ^b | 0.016 ^a | 0.033 ^{ab} |
| <i>Scleroderma verrucosum</i> (CR) | 1.25 ^{ab} | 7.49 ^{fg} | 66.0 ^a | 232.1 ^{cd} | 0.019 ^a | 0.032 ^{ab} |
| <i>Pisolithus tinctorius</i> (O) | 2.08 ^c | 7.27 ^{ef} | 68.2 ^a | 189.9 ^{bc} | 0.030 ^{bc} | 0.038 ^b |
| <i>Pisolithus tinctorius</i> (CR) | 2.15 ^c | 8.85 ^e | 67.1 ^a | 171.5 ^b | 0.032 ^{bc} | 0.052 ^c |
| <i>Hebeloma westraliense</i> (O) | 1.85 ^{bc} | 7.91 ^{fg} | 60.7 ^a | 250.7 ^d | 0.031 ^{bc} | 0.032 ^{ab} |
| <i>Hebeloma westraliense</i> (CR) | 2.43 ^c | 7.04 ^{ef} | 68.5 ^a | 235.1 ^{cd} | 0.035 ^c | 0.030 ^{ab} |
| <i>Hebeloma westraliense</i> (FB) | 2.35 ^c | 6.93 ^{def} | 63.8 ^a | 251.1 ^d | 0.037 ^c | 0.028 ^{ab} |
| <i>Laccaria laccata</i> B (O) | 1.22 ^{ab} | 3.53 ^a | 64.8 ^a | 93.9 ^a | 0.019 ^a | 0.038 ^b |
| <i>Laccaria laccata</i> B (CR) | 1.19 ^{ab} | 4.74 ^{abc} | 74.4 ^a | 151.3 ^b | 0.016 ^a | 0.031 ^{ab} |
| <i>Laccaria laccata</i> B (FB) | 2.13 ^c | 8.32 ^{fg} | 65.7 ^a | 319.5 ^e | 0.032 ^{bc} | 0.026 ^a |
| <i>Setchelliogaster</i> sp. H (O) | 1.35 ^{ab} | 5.16 ^{bc} | 73.4 ^a | 165.5 ^b | 0.018 ^a | 0.031 ^{ab} |
| <i>Setchelliogaster</i> sp. (CR) | 1.30 ^{ab} | 5.81 ^{cde} | 66.6 ^a | 252.9 ^d | 0.020 ^a | 0.023 ^a |
| <i>Setchelliogaster</i> sp. (FB) | 1.30 ^{ab} | 4.78 ^{abc} | 48.4 ^a | 196.2 ^{bcd} | 0.027 ^b | 0.024 ^a |

Fungal isolates were either the original culture (O), re-isolates from colonized roots (CR) or re-isolates from fruiting bodies (FB). Control seedlings were uninoculated. Plants were harvested 49 d (D49) and 93 d (D93) after sowing.

Values within columns followed by the same letter are not significantly different ($P < 0.05$; Duncan's new multiple range test).

done on a more regular basis. Giltrap (1981) observed that the ability of some isolates of ectomycorrhizal fungi to form immature fruit bodies in pure culture disappeared 3–4 months after isolation of the strains. Maintenance of the full ability of ectomycorrhizal fungi to express genes involved in mycorrhiza formation and functioning may require continuous interaction with the host plant. Thus, there could be an early decline in mycorrhizal effectiveness following isolation and growth of fungi in pure culture. This possibility and the high cost of maintaining ectomycorrhizal culture collections raises the question of alternative methods of storage of fungal isolates. Various methods have been developed to preserve fungal cultures, including lyophilization (Staffeldt, 1961), covering cultures with mineral oil (Onions, 1976), silica gel (Onions, 1976) or sterile cold water (Marx & Daniel, 1976), freeze-drying (Khan & Boyd, 1968), and other cryogenic methods (Bromfield & Schmitt, 1967; Slesman, Larsen & Safford, 1974). Some of these methods have been compared by Smith & Onions (1983).

The colonization capacity and effectiveness of *L. laccata* B and the colonization capacity of *Setchelliogaster* sp. were increased more by re-isolating these fungi from basidiomes than from colonized roots. These differences may reflect differences in the length of time the ectomycorrhizal fungi were associated with the host plant before re-isolation (8 wk for colonized roots versus 12–24 wk for basidiomes). They may also reflect differences in the physiological status of the host plant prior to re-isolation (growing conditions in the glasshouse and in the growth cabinet were quite different). Alternatively, there may be a direct advantage in re-isolating ectomycorrhizal fungi from basidiomes, although we do not know what this advantage would be. Fungi were re-isolated from stipe vegetative tissue of basidiomes to eliminate the possibility of genetic variation between isolates.

It is difficult to determine the full extent to which we were able to restore the effectiveness of the ectomycorrhizal fungal

cultures following association with *E. globulus* and re-isolation. Growth responses to inoculation are likely to be underestimated in comparison with responses observed in previous experiments because uninoculated plants were colonized by a contaminant ectomycorrhizal fungus (or fungi).

The results of our work have important implications for the future screening of ectomycorrhizal fungi for use as inoculants. Fungi which are effective at increasing plant growth at the time of screening may be ineffective by the time they are used as inoculants. Without appropriate re-isolation and storage techniques, mycorrhizal fungi would have to be selected for their ability to maintain their effectiveness after extended periods of growth on agar media (e.g. *H. westraliense* did not appear to lose its effectiveness while growing in pure culture). Caution should be exercised in interpreting the results of any studies comparing ectomycorrhizal fungi which have been maintained for long periods in pure culture.

We would like to thank Inez Tommerup for helpful discussions and Janine Catchpole for excellent technical assistance. We are also grateful to Bunnings Treefarms Pty Ltd who provided financial support for this research.

REFERENCES

- Bougher, N. L., Grove, T. S. & Malajczuk, N. (1990). Growth and phosphorus acquisition of karri (*Eucalyptus diversicolor* F. Muell.) seedlings inoculated with ectomycorrhizal fungi in relation to phosphorus supply. *New Phytologist* **114**, 77–85.
- Bromfield, K. R. & Schmitt, C. G. (1967). Cryogenic storage of conidia of *Peronospora tabacina*. *Phytopathology* **57**, 1133.
- Cline, M. L. & Reid, C. P. P. (1982). Seed source and mycorrhizal fungal effects on growth of containerized *Pinus contorta* and *Pinus ponderosa* seedlings. *Forest Science* **28**, 237–250.
- Giltrap, N. J. (1981). Formation of primordia and immature fruiting bodies by ectomycorrhizal fungi in culture. *Transactions of the British Mycological Society* **77**, 204–205.

- Heinrich, P. A. & Patrick, J. W. (1986). Phosphorus acquisition in the split-root system of *Eucalyptus pilularis* Smith seedlings. II. The effect of ectomycorrhizas on seedling phosphorus and dry weight acquisition. *Australian Journal of Botany* **34**, 445–454.
- Jones, M. D., Durall, D. M. & Tinker, P. B. (1990). Phosphorus relationships and production of extramatrical hyphae by two types of willow ectomycorrhizas at different soil phosphorus levels. *New Phytologist* **115**, 259–267.
- Khan, T. N. & Boyd, W. J. R. (1968). Long term preservation of *Drechslera teres* by freeze drying. *Phytopathology* **58**, 1448–1449.
- Laiho, O. (1970). *Paxillus involutus* as a mycorrhizal symbiont of forest trees. *Acta Forestalia Fennica* **106**, 1–73.
- Onions, A. H. S. (1976). Storage of fungi by mineral oil and silica gel for use in the collection with limited resources. In *Proceedings of the Second International Conference on Culture Collections, São Paulo, Brazil* (ed. A. F. Pestana de Castro, E. J. Dasilva, V. B. D. Skerman & W. W. Leveritt), pp. 104–113. Unesco/UNEP/ICRO/WFCC/World Data Centre for Micro-organisms, Australia.
- Marx, D. H. (1969). The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. I. Antagonism of mycorrhizal fungi to root pathogenic fungi and soil bacteria. *Phytopathology* **59**, 153–163.
- Marx, D. H. (1981). Variability in ectomycorrhizal development and growth among isolates of *Pisolithus tinctorius* as affected by source, age, and reisolation. *Canadian Journal of Forest Research* **11**, 168–169.
- Marx, D. H. & Daniel, W. J. (1976). Maintaining cultures of ectomycorrhizal and plant pathogenic fungi in sterile water cold storage. *Canadian Journal of Microbiology* **22**, 338–341.
- Newman, E. I. (1966). A method for estimating the total length of a root in a sample. *Journal of Applied Ecology* **3**, 139–145.
- Salt, P. D. (1968). The automatic determination of phosphorus in extracts of soils made with 0.5 M sodium hydrogen carbonate and 0.01 M calcium chloride. *Chemistry and Industry* **4**, 584–586.
- Sleesman, J. P., Larsen, P. O. & Safford, J. (1974). Maintenance of stock cultures of *Helminthosporium maydis* (races T and O). *Plant Disease Reporter* **58**, 334–336.
- Smith, D. & Onions, A. H. S. (1983). A comparison of some preservation techniques for fungi. *Transactions of the British Mycological Society* **81**, 535–540.
- Speakman, J. B. (1982). A simple, reliable method of producing perithecia of *Gaeumannomyces graminis* var. *tritici* and its application to isolates of *Phialophora* spp. *Transactions of the British Mycological Society* **79**, 350–353.
- Staffeldt, E. E. (1961). Observations on lyophil preservation and storage of *Pythium* species. *Phytopathology* **51**, 259.

(Accepted 2 December 1992)