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The survival and development of inoculant ectomycorrhizal fungi on roots of outplanted *Eucalyptus globulus* Labill.

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Abstract

The survival and development of two inoculant ectomycorrhizal fungi (*Hebeloma westraliense* Bough. Tom. and Mal. and *Setchelliogaster* sp. nov.) on roots of outplanted *Eucalyptus globulus* Labill. was examined at two ex-pasture field sites in the south-west of Western Australia. Site 1 was a gravelly yellow duplex soil, and Site 2 was a yellow sandy earth. Plants were grown in steamed or unsteamed soil, in root bags designed as field containers for young growing trees. Three, 6 and 12 months after outplanting, plants were removed from these bags and assessed for dry weights of shoots and ectomycorrhizal colonization of roots.

The inoculant ectomycorrhizal fungi (identified on the basis of the colour and morphology of their mycorrhizas) survived on roots of *E. globulus* for at least 12 months after outplanting at both field sites. At Site 1, however, colonization of new fine roots by the inoculant fungi was low (less than 20% of fine root length). Inoculation had no effect on the growth of *E. globulus* at this site. In contrast, at Site 2 the inoculant ectomycorrhizal fungi colonized up to 30-50% of new fine root length during the first 6 months after outplanting. There was a corresponding growth response to ectomycorrhizal inoculation at this site, with a close relationship ($r^2 = 0.82^{**}$) between plant growth at 12 months and root colonization at 3 months. Plant growth at 12 months was related less closely with root colonization at 6 or 12 months. Root colonization by 'resident' ectomycorrhizal fungi increased with time at both field sites. At Site 2, this increase appeared to be at the expense of colonization by the inoculant fungi, which was reduced to less than 10% of fine root length at 12 months. Steaming the soil had little effect on colonization by the inoculant ectomycorrhizal fungi at either field site, but decreased colonization by the resident ectomycorrhizal fungi.

Introduction

In the field, plant growth responses to ectomycorrhizal inoculation will depend not only on the effectiveness of introduced ectomycorrhizal isolates (their ability to increase plant growth) relative to ectomycorrhizal fungi already present in the soil ('resident' fungi), but also on the survival and development of these introduced isolates on roots with time (Dodd and Thomson, 1994). Several workers have demonstrated that inoculant ectomycorrhizal fungi can survive on plant roots for up to two years after outplanting, but that these fungi are gradually replaced by the resident ectomycorrhizal flora (Dahlberg, 1990; Dahlberg and Stenstrom, 1991; Danielson and Visser, 1989; Stenstrom and Ek, 1990; Villeneuve et al., 1991). Other workers have demonstrated that inoculant ectomycorrhizal fungi can be completely replaced by resident ectomycorrhizal fungi soon after outplanting (Chu-Chou, 1979; Chu-Chou and Grace, 1981). Survival of introduced ectomycorrhizal fungi for long periods of time (greater than two years) does not appear to be a requisite for plant growth responses to ectomycorrhizal inoculation

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(Garbaye et al., 1988). However, the lack of survival of inoculant fungi at some sites may partly explain why inoculation with effective ectomycorrhizal isolates has not always increased the growth of trees in the field (Castellano and Trappe, 1991).

In the present study, we examined the survival and development of two inoculant ectomycorrhizal fungi on roots of outplanted *Eucalyptus globulus* Labill. at two ex-pasture field sites in the south-west of Western Australia. Both fungal isolates (*Hebeloma westraliense* Bough. Tomm. and Mal. and *Setchelliogaster* sp. nov.) have previously been shown to be effective in increasing the growth of *E. globulus* in the glasshouse (Thomson et al., 1994), and to colonize *E. globulus* seedlings extensively in the nursery (Hardy, unpubl. data).

Materials and methods

Field sites

Site 1

Situated near Tone River, Chowerup (Nelson Location No. 7342; CALM 1:50000 Plan, Perup). The site had a mean annual rainfall of 700 mm, and was located on agricultural land where clover-based pasture had grown for many years. The pasture had received annual dressings of phosphate fertilizer. The soil profile at the site was classified as a gravelly yellow duplex soil. Chemical properties of the surface 0-10 cm of this profile (dried and sieved to < 2 mm) were: pH 5.75 in water (1:5 soil:water), total P 0.070% and total N 0.437% (Technicon Industrial method No. 334-74W/B), organic C 7.58% (Walkley, 1947), and Bray-extractable P 108 μg g^{-1} of soil (Bray and Kurtz, 1945).

Site 2

Situated near Boyanup (Wellington Location No. 576; CALM 1:50000 Plan, Kirup). The site had a mean annual rainfall of 1100 mm, and was also located on agricultural land where phosphate fertilizers had been applied to clover-based pasture over many years. The soil profile at the site was classified as a yellow sandy earth. Chemical properties of the surface 0-10 cm of this profile (dried and sieved to < 2 mm) were: pH 5.05 in water, total P 0.050%, total N 0.223%, organic C 3.38%, and Bray-extractable P 51 μg g^{-1} of soil.

Seedling production and inoculation

A medium comprising sphagnum peat and perlite (1:1 v/v) and a basal application (mg L^{-1} of medium) of $FeSO_4$ (288), FeO (588), dolomite (784), gypsum (523), and micronutrient mix (105) was thoroughly mixed and added to 64-cell (45 cm^3/cell) polyurethane seedling trays (Smith and Nephew, Australia). Each tray was steamed for 1 hour at 60 °C. Five-10 Mycobeads™ (non-homogenized mycelium of ectomycorrhizal fungi encapsulated in alginate beads - a product of Biosynthetica Australasia Ltd.) of either *H. westraliense* or *Setchelliogaster* sp. were then placed at a depth of 2.5 mm in each cell of the seedling trays using a Fluid Drilling Machine (Fluid Drilling Ltd., Stratford-on-Avon, England). Uninoculated trays did not receive Mycobeads. *E. globulus* seeds (presoaked in water at 22 °C for 1 hour) were sown into the seedling trays (1-2 seeds/cell) also using the Fluid Drilling Machine. Following sowing, each seedling tray was covered with a thin layer of steamed vermiculite.

Seedlings were thinned to one per cell soon after emergence. Seedling trays were watered daily by overhead micro-irrigation and liquid-fertilized twice-weekly with (mg L^{-1}): KH_2PO_4, 16.5; urea, 274.0; KNO_3, 297.0; $CaCl_2$, 67.0; $MgSO_4$, 148.3; $CuSO_4$, 0.44; $ZnSO_4$, 0.33 and $NaMoO_4$, 0.043. Each tray received 500 mL of nutrient solution on each occasion. The rates of N and P in this liquid-fertilization were calculated to be sufficient for approximately 50% of maximum plant growth, and have been demonstrated to result in extensive ectomycorrhizal development on roots of eucalypts (Grove, unpubl. data). In all other respects, seedlings were maintained under conventional production nursery conditions at the Five Acre Nursery, Manjimup, Western Australia.

Prior to outplanting (12 weeks after sowing), seedlings were visually assessed for ectomycorrhizal colonization of roots. Only seedlings colonized extensively by the inoculant ectomycorrhizal fungi were used for the field studies.

Experimental design and set-up

Uninoculated seedlings of *E. globulus* and seedlings inoculated with either *H. westraliense* or *Setchelliogaster* sp. were planted into steamed or unsteamed soil at both field sites, and their growth and ectomycorrhizal colonization monitored 3, 6 and 12 months after outplanting. There were three replicates of each treatment,

making a total of 54 seedlings at each site. Seedlings were randomized within replicate blocks.

Fifty-four holes, 25 cm wide×30 cm deep, were dug on a 2 m×2 m spacing at both field sites. The soil from each hole was sieved through a 4 mm stainless-steel mesh and added to 14 L, (25 cm diameter) root control bags (Root Control Systems Pty Ltd, Australia) made from synthetic fabric. These bags were designed as field containers for young growing trees. Water and nutrients can move freely through them, but root growth outside the bag is restricted. The bags were considered ideal for fungal survival studies because plant roots can be easily harvested. Sieving the soil in each root bag was not expected to affect the inoculum (primarily spores) potential of resident ectomycorrhizal fungi in the ex-pasture field soils (Malajczuk, personal communication).

Once filled with soil, each root bag was either returned directly to the hole from which the soil had been taken, or was steamed at 70 °C for 1 hour and then returned to the hole. Uninoculated and inoculated *E. globulus* seedlings were planted into these bags in June (start of the winter growing season), and each seedling fertilized with a spot application of 100 g of Agras No. 1 (17.5% N, 7.6% P, 16% S). This fertilizer was placed 5 cm below the soil surface, approximately 20 cm from the base of each seedling. Twelve months after outplanting, tree growth inside the root bags was comparable with tree growth outside the root bags in a near-by field trial.

Plant harvest

Plants were harvested 3, 6 and 12 months after outplanting. At each harvest, stem diameter was measured at a height of 20 cm above the soil surface, and tree biomass determined using allometric functions (Grove et al., 1991). Each root bag was removed from the ground, and roots carefully removed by dry-sieving over a 2 mm sieve. These roots were then wet-sieved over a series of fine sieves to remove coarse root (> 0.5 mm diameter) and fine root (< 0.5 mm diameter) fractions. The original peat pot from the nursery was still intact at 3 months, and this was separated from the rest of the roots at sieving and a sample of fine roots taken for assessing ectomycorrhizal colonization (see below). A sample of fine roots was also taken from the fine root fraction collected from the rest of the bag (new fine roots formed after outplanting), and this was assessed for ectomycorrhizal colonization. The remaining fine root and coarse root fractions were oven-dried at 70 °C and weighed.

Fine root length with and without ectomycorrhizas was determined on the fine root samples using the line intercept method of Newman (1966). Ectomycorrhizas appeared as shorter, thickened roots. Different types of ectomycorrhizas were distinguished on the basis of their colour and morphology: Type I, yellow-brown mycorrhizas typical of *H. westraliense* (Bougher et al., 1991); Type II, red-brown mycorrhizas typical of *Setchelliogaster* sp. (Malajczuk, unpublished data); and Type III, white mycorrhizas typical of *Scleroderma* spp. (Malajczuk et al., 1982).

Results

Dry weights

Inoculating plants with *H. westraliense* and *Setchelliogaster* sp. had no effect on shoot dry weights in either steamed or unsteamed soil at Site 1 (Fig. 1). By contrast, at Site 2 plants grown in steamed soil and inoculated with *Setchelliogaster* sp. were larger ($p<0.05$) than uninoculated plants at 6 and 12 months, and plants grown in unsteamed soil and inoculated with *H. westraliense* were larger ($p<0.05$) than uninoculated plants at 12 months (Fig. 1).

Shoot dry weights were generally increased by the steaming treatment at both field sites (Fig. 1). This effect was evident from 3 months at Site 1, and from 6 months at Site 2.

Ectomycorrhiza formation

Three months after outplanting, fine roots of inoculated plants at both field sites were colonized extensively in the intact peat pots by the inoculant ectomycorrhizal fungi (identifed on the basis of the colour and morphology of their mycorrhizas (see above)) (Figs. 2 and 3). Inoculated and uninoculated plants were also colonized by a contaminant ectomycorrhizal fungus (possibly from the nursery), which could not be distinguished from colonization by *H. westraliense* (Type I mycorrhizas). We assumed that roots of uninoculated and inoculated plants were colonized to the same extent by this contaminant fungus. Colonization by the *H. westraliense* inoculant was then estimated as the difference between uninoculated and inoculated plants in fine root length colonized by Type I mycorrhizas. This estimation is likely to be conservative

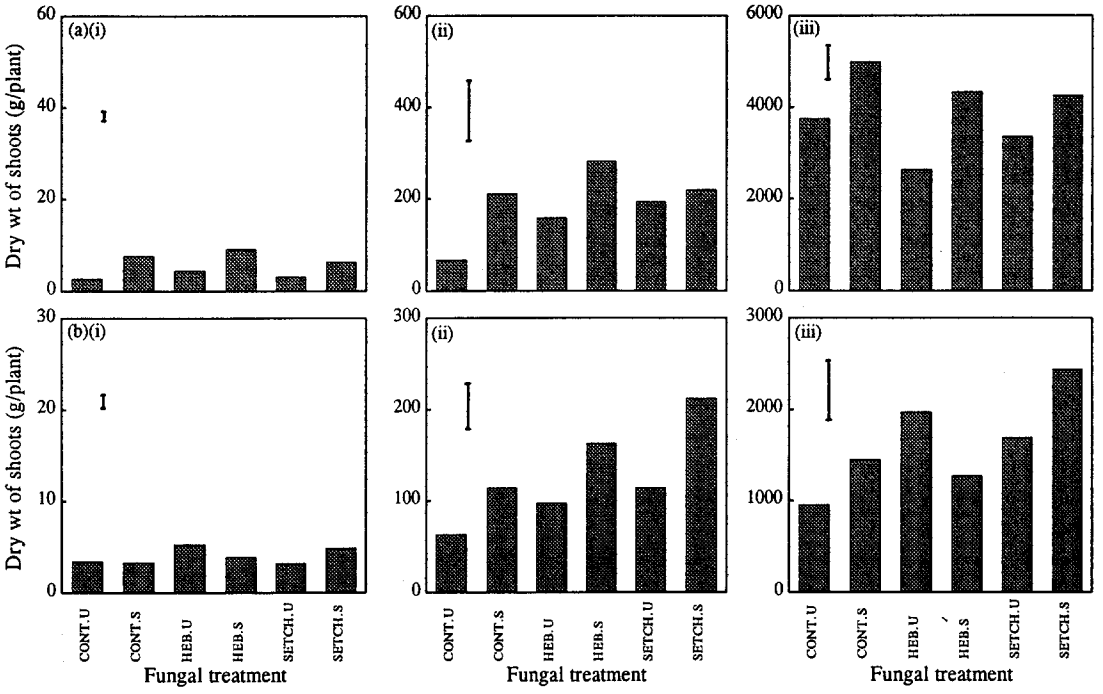

Fig. 1. Dry weights of shoots of *Eucalyptus globulus* grown at (**a**) Field Site 1 and (**b**) Field Site 2, and either uninoculated (CONT.) or inoculated with *Hebeloma westraliense* (HEB) or *Setchelliogaster* sp. (SETCH). The soil was either steamed (S) or unsteamed (U). Plants were harvested at (i) 3, (ii) 6 and (iii) 12 months after outplanting. Vertical bars represent 2 × SEM.

because for plants inoculated with *Setchelliogaster* sp. colonization by the contaminant fungus was decreased compared with uninoculated plants.

Outside the peat pots and in the rest of the root bag, colonization by the inoculant ectomycorrhizal fungi was very low at Site 1 at 3 months, but at Site 2 had colonized up to 50% of new fine root length (Figs. 2 and 3). The contaminant with Type I mycorrhizas also colonized roots outside the peat pots at 3 months. There was a close relationship ($r^2 = 0.82^{**}$) between root colonization by the inoculant ectomycorrhizal fungi at 3 months and plant growth responses to inoculation at 12 months. Root colonization at 6 or 12 months was related less closely with growth responses to inoculation at 12 months.

Six and 12 months after outplanting, the inoculant ectomycorrhizal fungi were surviving on roots at both field sites. However, colonization by these fungi was always low at Site 1 (less than 20% of fine root length colonized) and decreased with time at Site 2 (up to 30% of fine root length colonized at 6 months, but less than 10% of fine root length colonized at 12 months) (Figs. 2 and 3). At least part of the decrease in the proportion of root length colonized by inoculant ectomycorrhizal fungi with time could be due to increased root growth between 3 and 12 months (total fine root length colonized by inoculant fungi (cm 10 cm^{-3} of soil) actually increased during the same period (Figs. 2 and 3). Moreover, from 6 months after outplanting at both field sites inoculated and uninoculated plants were colonized by resident ectomycorrhizal fungi with Type III mycorrhizas. This colonization, which would have been in direct competition with colonization by the inoculant ectomycorrhizal fungi, increased with time (Figs. 2 and 3).

Steaming decreased Type III mycorrhizas at 6 and 12 months for Site 1, and at 6 months for Site 2 (Figs. 2 and 3). Steaming had little effect on colonization by the inoculant ectomycorrhizal fungi at either field site, with the exception of Type I mycorrhizas at Site 2 which were reduced in steamed soil at 3 months.

Discussion

At both field sites, the inoculant ectomycorrhizal fungi survived on roots for at least 12 months after outplanting but were being replaced to varying extents by res-

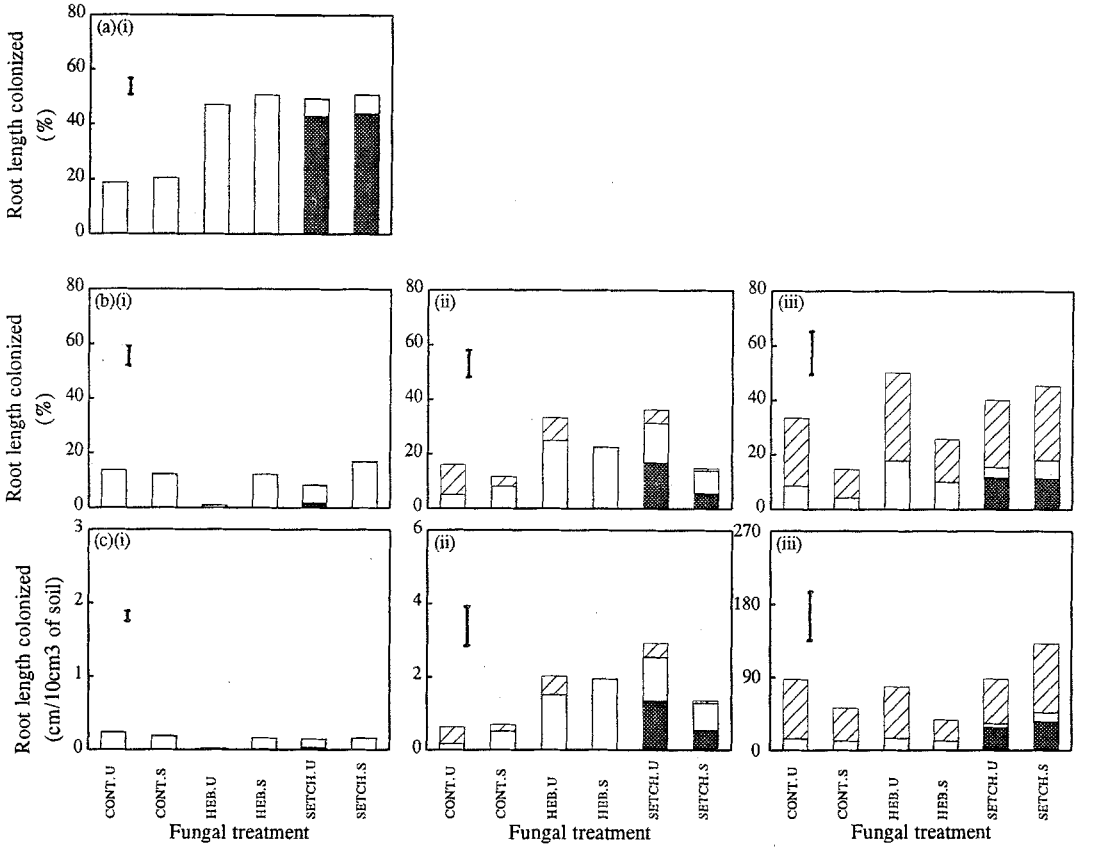

Fig. 2. Ectomycorrhizal colonization of fine roots of *Eucalyptus globulus* grown at Field Site 1, and either uninoculated (CONT.) or inoculated with *Hebeloma westraliense* (HEB) or *Setchelliogaster* sp. (SETCH). The soil was either steamed (S) or unsteamed (U). Plants were harvested at (i) 3, (ii) 6 and (iii) 12 months after outplanting, and colonization assessed in (**a**) intact peat pots (3 months only) and (**b, c**) the rest of the root bag. Mycorrhizas were classified into three types (see text): □, Type I; ■, Type II; ▨, Type III. Vertical bars represent 2 × SEM.

ident ectomycorrhizal fungi. This result is consistent with the results of others (Dahlberg, 1990; Dahlberg and Stenstrom, 1991; Danielson and Visser, 1989; Stenstrom and Ek, 1990; Villeneuve et al., 1991), who also demonstrated that inoculant ectomycorrhizal fungi are gradually replaced by resident ectomycorrhizal fungi after outplanting. At Site 2, the inoculant fungi colonized new fine roots (fine roots which developed outside the original peat pots) to a greater extent than at Site 1, and this corresponded with a positive growth response to inoculation at Site 2 and not at Site 1. However, even at Site 2 colonization by the inoculant fungi was low at 12 months (less than 10% of fine root length colonized). Garbaye et al. (1988) demonstrated that the survival of introduced ectomycorrhizal fungi for long periods of time is not a requisite for growth responses to ectomycorrhizal inoculation.

The survival and development of introduced ectomycorrhizal fungi on roots in competition with resident ectomycorrhizal fungi will depend on a range of factors including, fungal genotype (Marx et al., 1985; McAfee and Fortin, 1987), interactions with other soil microorganisms including resident ectomycorrhizal fungi (Amaranthus and Perry, 1987; Bowen and Theodorou, 1978; Garbaye and Bowen, 1987), soil type (Harvey et al., 1987; Last et al., 1984; Marx et al., 1977), soil moisture (Mamoun and Olivier, 1993) and climatic conditions such as temperature (Samson and Fortin, 1986). Further work is required to identify those factors which have the greatest influence on the survival and development of introduced ectomycorrhizal fungi on tree roots in field soils. The response of ectomycorrhizal isolates to these factors may become an important part of the screening and selection of fungi for use as inoculants (Dodd and Thomson, 1994).

There was a close relationship between plant growth responses to inoculation at 12 months and root colonization by the inoculant ectomycorrhizal fungi at

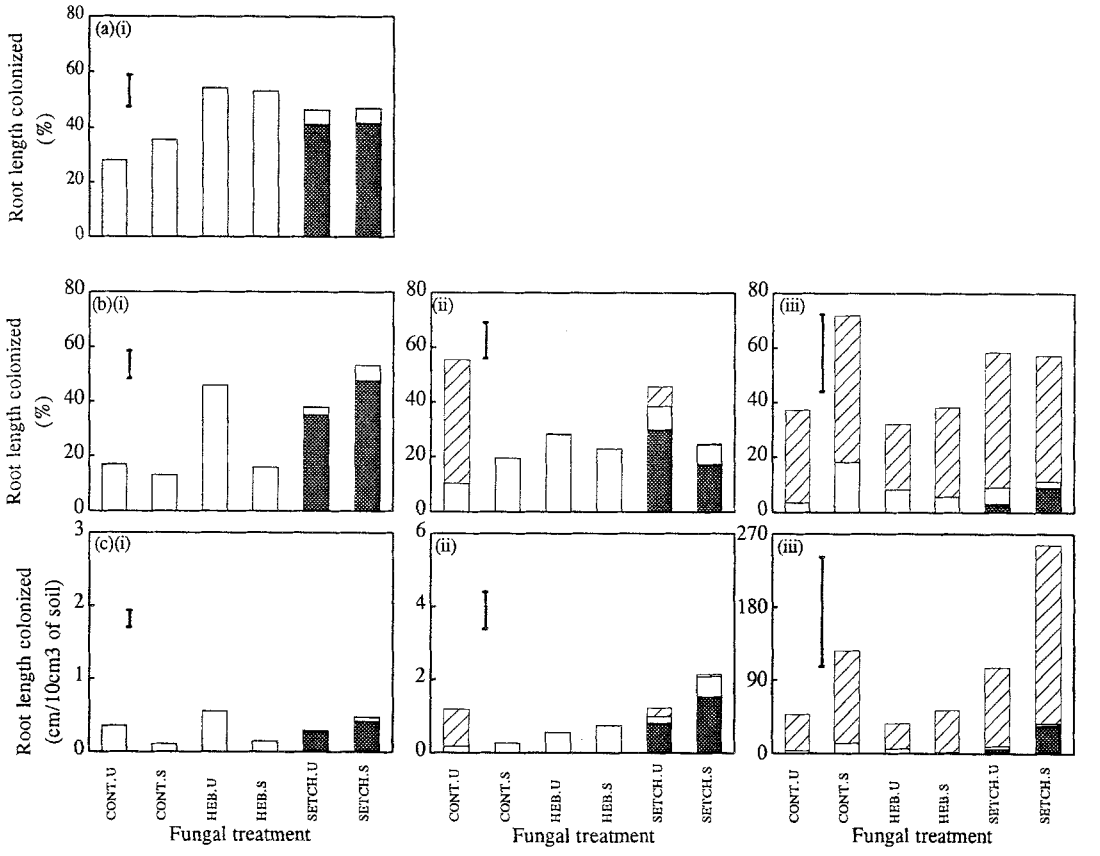

Fig. 3. Ectomycorrhizal colonization of fine roots of *Eucalyptus globulus* grown at Field Site 2, and either uninoculated (CONT.) or inoculated with *Hebeloma westraliense* (HEB) or *Setchelliogaster* sp. (SETCH). The soil was either steamed (S) or unsteamed (U). Plants were harvested at (i) 3, (ii) 6 and (iii) 12 months after outplanting, and colonization assessed in (**a**) intact peat pots (3 months only) and (**b**, **c**) the rest of the root bag. Mycorrhizas were classifed into three types (see text) : □, Type I; ■, Type II; ▨, Type III. Vertical bars represent 2 × SEM.

3 months. This result is perhaps not surprising. Much of the annual uptake of nutrients by eucalypt species growing in the south-west of Western Australia occurs in the spring and early summer (Dell and Wallace, 1983; Grove and Malajczuk, 1994), which coincides with 3 months after outplanting. Nutrients taken up by the plant during this period may be stored and used later for growth in summer and early autumn when nutrient uptake from the soil is restricted by low soil moisture (Grove and Le Tacon, 1993). In the first year after outplanting, ectomycorrhizas are therefore likely to have their greatest influence on the nutrient status of the plant in the first 6 months. Inoculant ectomycorrhizal fungi may need to be selected for their ability to rapidly colonize new roots following outplanting as well as their ability to survive in the field.

The present work was restricted by our inability to distinguish between colonization by *H. westraliense* and colonization by the contaminant fungus (possibly from the nursery), which both had Type I mycorrhizas. We assumed that roots of uninoculated and inoculated plants were colonized to the same extent by the contaminant fungus, and in this way obtained conservative estimates of colonization by the *H. westraliense* inoculant. More precise techniques (e.g. genetic markers (Martin et al., 1994)) will be required to distinguish between ectomycorrhizal fungi in ecological studies involving several fungal types.

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