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MYCORRHIZAL DEVELOPMENT IN *EUCALYPTUS CAMALDULENSIS* DEHNH

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Abstract: Effects of arbuscular mycorrhizal (AM) and ectomycorrhizal (EM) inoculation on mycorrhiza formation and development in *E. camaldulensis* seedlings were assessed in this study. Two experiments, the first with AM fungi and the second with EM fungi, were set up successively using vermiculite-peat (VP) as growth medium, and 2.5 mg l^{-1}, 5.0 mg l^{-1}, 10 mg l^{-1} and 20 mg l^{-1} phosphorus (P) Ingestad's nutrient solution in each experiment. The AM experiment using three AM inocula (including *Glomus clarum* Nicolson and Schenck. (isolate BR148-1) and *Gigaspora rosea* Nicolson and Schenck. (isolate FL105-5) and one from a trap culture of Bangladeshi soil) resulted in 30-50% colonisation; most colonisation was by *G. clarum* BR148-1 and was the highest at 10 mg l^{-1} P (>50%). In a similar experiment using five isolates of *Pisolithus tinctorius* (Pers.) Coker and Couch., only isolate K55 resulted in colonisation >15% most of which occurred at 2.5 mg l^{-1} P (>25%) while the other isolates resulted in <1% colonisation. This study showed that *E. camaldulensis* tended to be colonised more readily by arbuscular- than ecto-mycorrhizal fungi in the initial period of seedling growth. There were indications that the species could become ectomycorrhizal after 16 weeks of growth in glasshouse, but that proper care should be taken to select appropriate and compatible EM inoculants.

Key words: Arbuscular mycorrhizas; Ecto-mycorrhizas; *Eucalyptus camaldulensis*; *Glomus clarum*; *Gigaspora rosea*; *Pisolithus tinctorius*

Introduction

The term mycorrhiza is used to describe non-pathogenic associations between fungi and the roots of higher plants (Smith and Read, 1997). Mycorrhizas are mainly of two types: (a) arbuscular mycorrhizas (AM) which grow inter- and intra-cellularly, and (b) ectomycorrhizas (EM) where the fungus forms an external hyphal mantle surrounding the root (Smith and Read, 1997). Surveys of mycorrhizal literature have established that plants within a genus usually have the same type of mycorrhizas, that is, EM, AM, etc., or else they remain non-mycorrhizal (Brundrett *et al*., 1996) and these relationships are generally also consistent within a family (Harley and Harley, 1987; Newman and Reddell, 1987; Brundrett and Abbott, 1991). Many Australian trees and shrubs such as some *Eucalyptus* and *Acacia* species have been reported to have both EM and AM associations (Warcup, 1980; Malajczuk *et al*., 1981; McGee, 1986; Chilvers *et al*., 1987;

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Reddell and Warren, 1987; Brundrett and Abbott, 1991). Among the eucalyptus that form EM and AM associations, *Eucalyptus camaldulensis* is reported to be one (Vishwakarma and Singh, 1996ab; Meshram *et al*. 1997; Jamaluddin and Chandra, 1997). *Eucalyptus camaldulensis* is particularly important for the production of domestic products, such as poles, posts and timber, but above all, and increasingly, fuelwood. Because of its economic importance and because it is planted as an exotic, inoculation of this species with mycorrhizal fungi may be required depending on the availability of inocula. The effect of mycorrhizal inoculation on growth of this species is therefore of potential importance. However, before attempting any experiments that may take an account of the effect of mycorrhizal colonisation on growth, it is important first to explore the colonisation potential of both AM and EM fungi in *E. camaldulensis* seedlings. Studies on this particular species are very few. Malajczuk and Hartney (1986) compared EM (by using *Pisolithus tinctorius, Hydnangium carneum, Scleroderma verrucosum* and *Laccaria laccata*) formation on micropropagated plantlets and seedlings of *E. camaldulensis* and observed much more uniform mycorrhizal formation on micropropagated plantlets than seedlings. Dixon and Hiol-hiol (1992) found that *P. tinctorius* inoculated seedlings of *E. camaldulensis* resulted in 100% colonisation. According to Jamaluddin and Chandra (1997), *E. camaldulensis* in undisturbed plantations in India exhibited root colonisation of up to 59% by AM fungi. They have also reported that in *E. camaldulensis*, the initial colonisation takes place by AM which is later replaced by various EM fungal species. Vishwakarma and Singh (1996a) reported more than 50% colonisation in *E. camaldulensis* seedlings inoculated with seven AM fungi as compared with the non-inoculated seedlings. However, they did not quantify the nutrient regimes used in their experiments. In none of these studies was there a systematic attempt to assess the developmental aspects of AM and EM in *E. camaldulensis*. Also these studies did not properly address the host nutrition aspect which may be critical for mycorrhizal development in *E. camaldulensis*.

The aim of this study was, therefore, to look at the effects of AM and EM fungal inoculation on mycorrhiza formation and development in seedlings of *E. camaldulensis* under different nutrient regimes. The specific objectives were (1) to identify nutrient availabilities for glasshouse experimentation concerning mycorrhizal colonisation of *E. camaldulensis*, (2) to screen the species with a range of fungi/fungal strains to assess which fungi are able to form mycorrhiza with it, and (3) to investigate interactions between fungi and nutrient availability.

Materials and Methods

Growth Conditions: The experiments were carried out between May 1997 and October 1997 in a glasshouse at the Institute of Terrestrial Ecology, Edinburgh, UK with a day/night thermal regime of 20/15±2^{o}C and a light regime varying between 400-800 µmol photons m^{-2} s^{-1}. Seeds of *E. camaldulensis* Dehnh. of SILVERTON (UMBER.CK) provenance were obtained from Australian Tree Seed Center, Commonwealth Scientific and Industrial Research Organisation (CSIRO), Canberra, Australia. Seeds of *E. camaldulensis* were pre-soaked in 0.1% Thiram (a dithiocarbamate fungicide) for 24 h, dried and sown into sterile trays containing sterilised vermiculite-peat (VP). Autoclaved

VP mixture was used for growth of *E. camaldulensis* seedlings where vermiculite, peat and tap water were mixed in the proportion 45:10:175 by mass before sterilising. Plastic pots (two litter capacity) were used for raising the seedlings. After three weeks of germination, one seedling was transplanted from the tray into each pot. A nutrient solution modified [according to Mason *et al*. (1999a,b,c)] from Ingestad's solution for birch (Ingestad, 1971) was supplied to the plants. The proportions of N, P and K were 100:16:55. Four nutrient concentrations (Ingestad's 2.5, 5.0, 10 and 20 mg l^{-1} phosphorus, P) were used where all nutrient elements were proportionally adjusted. Plants were supplied twice a week with the solution with a gradually increased dosage every three weeks so that a total of 2 mg, 4 mg, 8 mg and 16 mg phosphorus had been added to various nutrient treatment pots respectively by the end of the experiment.

Inoculum Preparation and Inoculation of Seedlings: The experiments involved a range of arbuscular mycorrhizal (AM) and ectomycorrhizal (EM) fungi. The isolates, which were cultured for inoculation purposes, are listed in Tables 1 and 2.

Table 1. Origin of arbuscular mycorrhizal inocula.

Inoculum	Isolate code	Country of origin	Place of origin	Date of collection	Plant of origin
Glomus clarum Nicolson and Schenck.	BR148-1*	Brazil	Rain forests	Not known	Not known
Gigaspora rosea Nicolson and Schenck.	FL105-5*	USA	Gainesville	Not known	*Glycine max*
A mixed culture	---------	Bangladesh	Cox's Bazar	March 1996	*Eucalyptus camaldulensis*

***** The single species cultures were provided by INVAM (International Culture Collection of Arbuscular and VA Mycorrhizal Fungi, College of Agriculture and Forestry, West Virginia University, USA).

Table 2. Origin of ecomycorrhizal inocula.

Inoculant fungus	Isolate code	Country of origin	Place of Origin	Date of collection	Plant of origin
Pisolithus tinctorius (Pers.) Coker and Couch.	PTE * (IMI368132)	Philippines	Not known	1990	*Eucalyptus camaldulensis*
do	K55 * (IMI368154)	Portugal	Obidos	1993	*Eucalyptus globulus*
do	PT3	Tasmania	Murdunna	1990	*Eucalyptus globulus*
do	PT7*	Scotland	Glasshouse experiment	1993	*Eucalyptus globulus*
do	PT8*	Scotland	Glasshouse experiment	1993	*Eucalyptus globulus*

* Isolates PT7 and PT8 were cultured from young sporocarps produced by *E. globulus* –inoculated with isolate PT3 in the pot experiments at a glasshouse in the Institute of Terrestrial Ecology (ITE), Edinburgh, UK. Isolates PTE and K55 were supplied by the University of Kent, Kent, UK which were deposited under International Mycological Institute (IMI), Surrey, UK, collection.

For AM inoculum preparation, a sterilised mixture of loam, sand and grit in the proportions 2:2:1 (supplied by Scottish Agricultural Industries (SAI) Horticulture Limited, Auchterarder, Perthshire) was used as a growth medium for pot cultures. The proportions of N, P and K in the loam were 150:10:85. For all AM fungi, the source inoculum used in the pot culture was a mixture of colonised roots and soil (approximately 20 g for each inoculant). Pot cultures of mycorrhizal cowpea (*Vigna unguiculata* L.) and millet (*Pennisetum typhoides* L.) were established and were maintained for four months in a growth cabinet at temperature of 15-25^{o}C and under fluorescent light (16 h of irradiance; at 240-260 µmol m^{-2} s^{-1}). Pot cultures were harvested and roots of cowpea and millet were sub-sampled for assessment of mycorrhizal colonisation (details of assessment are given later). Pots where root samples were found to be ≥60% mycorrhizal (i.e. ≥60% of the total root length being colonised) were used for collecting roots and soil as inoculum. For EM inoculum preparation, fungal isolates were cultured initially on agar plates of modified Melin-Norkran's (MMN) solution at room temperature. These cultures were then used to inoculate 500 ml flasks containing vermiculite-peat soaked in 180 ml MMN (Mason, 1980). Flasks were maintained at room temperature for four months prior to use as inocula. AM inoculation was effected by adding 5 g portions of soil-root mixture inoculum to the planting hole and EM inoculation by adding 4 g portions of mycelial VP to the planting hole. Control seedlings received autoclaved portions of the same inoculum.

***Mycorrhizal Assessment*:** Pots were soaked overnight in water and roots from each pot were washed from soil by applying a gentle flow of water so that no fine roots were lost. Three sub-samples, each one cm long, corresponding with the top, middle and bottom parts respectively of the root system were taken. For AM assessment, sampled roots were stained using the method of Phillips and Hayman (1970) with modifications given by Koske and Gemma (1989). The percentage of root length colonised was determined under a low power microscope using the grid-line intersect method (Tennant, 1975) modified by Giovanetti and Mosse (1980). Mycorrhizal colonisation was scored by the presence of vesicles, arbuscules or hyphae at the point of intersection between root and grid. For EM assessment, the sub-sampled roots were placed in Petri dishes in water for examination of the tips of all short roots under a dissecting microscope. The tips were categorised according to whether they were mycorrhizal or not. An EM root tip was defined as a short root with a mycelial mantle, however thin. When no EM colonisation was observed in the sub-samples, the remaining roots were checked for mycorrhizal root tips.

Statistical Analysis: GENSTAT version 5.3 (Lawes Agricultural Trust, Rothamsted, Harpenden, Hertfordshire, UK) was used for statistical analysis and Microsoft Excel 97 for graphics.

Results

Effects of arbuscular mycorrhizal inoculation and nutrient regime on colonization: Casual observations of seedlings (from some extra-experimental seedlings inoculated at the same time in the same way as the experimental seedlings, and maintained alongside

the experiment) indicated that AM colonisation started to spread (>10% of total root) by 6 weeks after inoculation. At the end of 14 weeks, extent of colonisation by the three AM fungi varied between 30-50% (of the total root length). There was a significant interactive effect of inoculation and nutrient treatments (*P*= 0.028) on colonisation of *E. camaldulensis* seedlings by different inoculants. Colonisation by *Glomus clarum* isolate BR148-1 or the Bangladesh culture did not vary significantly between 2.5 mg l^{-1} P and 20 mg l^{-1} P or between 5.0 mg l^{-1} P and 10 mg l^{-1} P. The extent of colonisation was the highest at 10 mg l^{-1} P, which varied significantly (*P*= 0.028) from those at the former two. Colonisation by *Gigaspora rosea* isolate FL105-5 was not significantly different among the four nutrient treatments (Fig. 1).

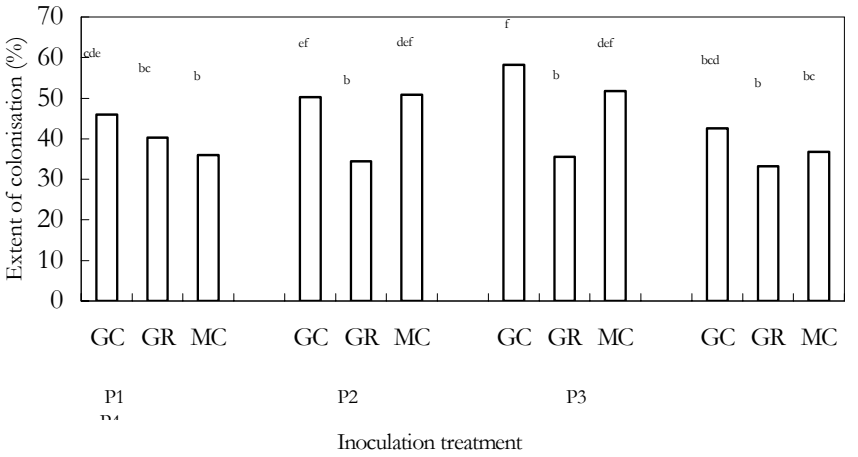

Fig. 1: Mean extent of arbuscular mycorrhizal (AM) colonisation (%) of *E. camaldulensis* seedlings under three AM inoculation treatments: GC, *Glomus clarum* BR 148-1; GR, *Gigaspora rosea* FL 105.5; MC, a mixed AM culture from Bangladesh and four nutrient treatments: phosphorus 2.5 mg l^{-1} (P1), 5.0 mg l^{-1} (P2), 10 mg l^{-1} (P3), 20 mg l^{-1} (P4). Means with different letters are significantly ($p < 0.05$) different.

On an overall basis, the three inoculant fungi had significantly (*P*= <0.001) different colonisation potential with *G. clarum* BR148-1 having the highest (up to 50% of the total root length), followed by the Bangladesh culture (up to 44%) and *G. rosea* FL105-5 (up to 36%) respectively. Considering all the fungi together, highest colonisation was found to have occurred in 10 mg l^{-1} P (36.40%), which was significantly (*P*= 0.004) higher compared to those at 2.5 mg l^{-1} P (30.57%) or 20 mg l^{-1} P (28.1%). Colonisation at 10-mg l^{-1} P (36.40%) was not significantly different from that at 5.0-mg l^{-1} P (34.48%). Colonisation at 20 mg l^{-1} P was the lowest although it did not differ significantly from that at 2.5 mg l^{-1} P. There was no significant regression between extent of colonisation and nutrient concentration (r^2 =0.0009, $F_{1,57}$=0.03, *P* =0.903).

Effects of Ectomycorrhizal Inoculation and Nutrient Regime on Colonization: Out of the five fungal inoculants, only *Pisolithus tinctorius* isolate K55 resulted in considerable

colonisation. Casual observations of seedlings (from extra-experimental seedlings inoculated at the same time in the same way as the experimental seedlings and maintained alongside the experiment) indicated that EM colonisation started to spread (>10% of total root) by 16 weeks after inoculation. At the end of 18 weeks, *P. tinctorius* isolate K55 resulted in significant colonisation (up to 27% of the root tips mycorrhizal). The other inoculants, although remaining alive (as live mycelia were found with the washed roots) at harvest, did not result in any considerable colonisation (only <1% of the root tips mycorrhizal). Most colonisation was found to have occurred at 2.5 mg l^{-1} phosphorus (P) nutrient treatment (27% of the root tips mycorrhizal) which was significantly (P =0.007) higher than that at either 10 mg l^{-1} P (11.6%) or 20 mg l^{-1} P (7.8%). There was no significant difference between colonisation at 2.5-mg l^{-1} P (27%) and that at 5.0-mg l^{-1} P (20.0%). Regression between the extent of colonisation by the isolate K55 and nutrient concentration was significant (Fig. 2) (r^2= 0.417; $F_{1,18}$= 12.85; P= 0.002); extent of colonisation decreased with an increase in nutrient concentration.

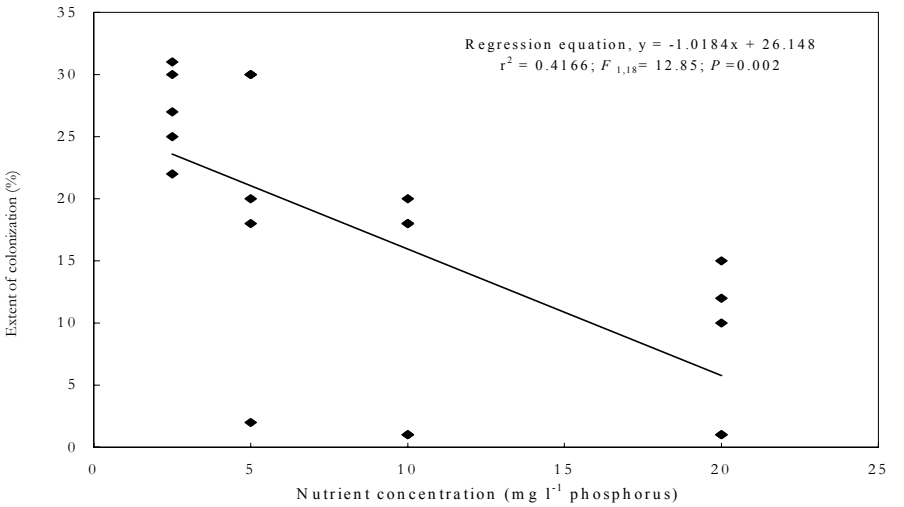

Fig. 2. Regression of the extent of colonisation (%) by *Pisolithus tinctorius* isolate K55 on nutrient concentration.

Discussion

While it was found that *E. camaldulensis* seedlings formed AM as early as 6 weeks of growth, there were indications that *E. camaldulensis* seedlings tended to be ectomycorrhizal after 16 weeks of growth in glasshouse, although even after 16 weeks EM colonisation was significantly lower compared to AM colonisation. A similar trend has been observed in a Brazilian *Eucalyptus* plantation (for example, in *E. viminalis* plantation by Bellei *et al*., 1992) where AM predominated up to 7-8 months after plantation establishment before being taken over by EM. Therefore, a later development of EM in *E. camaldulensis* seedlings in this study was similar to what other workers have found in other *Eucalyptus* species.

Although variation among different AM fungi in terms of their potential in colonising *E. camaldulensis* roots at different nutrient (P) regimes was observed, there was no significant relationship between extent of colonisation and nutrient (expressed in terms of P) concentration. Extent of colonisation by any mycorrhizal fungus decreases with an increasing availability of soluble P in the growth medium, a trend that has been observed in many mycorrhizal experiments (Smith and Read, 1997). In general, colonisation by AM fungi at P concentrations ranging from >20 to 100 ppm were reported to have resulted in mycorrhiza formation in tropical forest tree seedlings (Sieverding, 1991), and P concentrations higher than the upper limit of this range may therefore not be favourable for effective mycorrhiza formation. In this experiment, the range of nutrient regimes (from 2.5 to 20 mg l^{-1} P) was probably too narrow to reflect such a trend. Therefore, one interesting thing was observed: except for *G. rosea* FL105-5, the extent of colonisation by the other two inoculant fungi were at their maximum at 5 and 10 mg l^{-1} P nutrient regimes, but decreased significantly both at the lower nutrient (2.5 mg l^{-1} P) and at the higher nutrient regime (20 mg l^{-1} P) indicating that the range of nutrient concentrations applied spanned the optimum for the two fungi. Colonisation by *G. rosea* FL105-5 did not differ between the nutrient treatments. This indicated that different AM fungi may have different responses to different nutrient availabilities. The decrease in mycorrhizal colonisation in response to phosphate application arises from the resulting increase in tissue P concentration (Sanders, 1975; Menge *et al*., 1978). The nutrient concentration of host seedlings at 20 mg l^{-1} P may, therefore, have affected quantity or quality of root metabolites or root exudates (Twaraya *et al*., 1996) which resulted in low colonisation in those seedlings.

In the EM experiment, out of the five fungal isolates, only *P. tinctorius* K55 resulted in colonisation (up to 27% of the root tips). The other isolates of *P. tinctorius* used in the experiment were not effective colonisers of *E. camaldulensis* seedlings, as they resulted in little colonisation (<1% of the fine root tips). Most of the colonisation was found at 2.5 mg l^{-1} P. This confirms the other findings where lower P concentrations (2-4 ppm) are reported to be more favourable for EM formation in *Eucalyptus* by *P. tinctorius* (see Burgess *et al*, 1993; Mason *et al*., 1999b). In the initial two months of the experiment, the availability of light was between 700-800 µmol m^{-2} s^{-1} during daylight hours, but later due to cloudy weather throughout the rest of the experimental period the amount of light available fell down to 400-500 µmol m^{-2} s^{-1} even with the supplementary light provided from mercury vapour lamps. However, although many earlier glasshouse studies of *Eucalyptus* EM did not report the light regimes used, it was found in a very recent study done by Jones *et al*. (1998) that *E. coccifera* successfully formed both EM and AM at 400-500 µmol m^{-2} s^{-1} in a growth cabinet. A similar experiment with *E. globulus* with the isolate *P. tinctorius* PT3 has been reported to have resulted in >70% colonisation where availability of light was maintained at 1800 µmol m^{-2} s^{-1} (Mason *et al*. 1999b). The highest extent of colonisation by the isolate *P. tinctorius* K55 observed in this study was around 27% (of fine root tips). The other isolates of *P. tinctorius* used in the experiment resulted in little colonisation (<1% of the fine root tips).

As noted earlier, highest EM colonisation was observed at 2.5-mg l^{-1} P in this study. In the study of Mason *et al*. (1999b), *E. globulus* seedlings were grown at similar P

concentration but at a higher N regime (187.5-mg l^{-1}) than that used in this experiment (21-mg l^{-1}). This high N regime has resulted in a higher colonisation in *E. globulus* seedlings. However, Burgess *et al*. (1994) reported a higher level of colonisation in *P. tinctorius* inoculated *E. grandis* seedlings at similar P (4-mg kg^{-1} in the form of $Ca(H_2PO_4)_2.H_2O$) and N (150 mg per pot in the form of NH_4NO_3) concentrations. Therefore, a low N supply to mycorrhizal seedlings in this experiment as compared to that of Burgess *et al*. (1994) perhaps was not suitable for extensive mycorrhizal formation. There was a significant relationship between the extent of colonisation by *P. tinctorius* isolate K55 and nutrient concentration (Fig. 2). Extent of colonisation by any mycorrhizal fungus decreases with an increasing availability of nutrient (P) in the growth medium and it has been observed in many mycorrhizal experiments (see Bougher *et al*., 1990; Mason *et al*., 1999b).

Conclusion

The work undertaken here involved the study of mycorrhizal colonisation pattern by AM and EM fungal inoculation of *E. camaldulensis* seedlings. This study has indicated that *E. camaldulensis* can form both AM and EM but their development may depend on maturity of the host or the affinity of the host to particular mycorrhizal fungi. In this study, there was an indication of AM-EM succession in *E. camaldulensis* seedlings as they developed. This aspect need to be considered during transplanting seedlings from nursery to the field. In the field, availability of inoculum and competition with indigenous fungi and other microbial organisms may largely control the efficacy and persistence of mycorrhizal symbionts. For example, succession from AM to EM may become delayed if availability of suitable EM inoculum is sparse or absent. If EM can ensure a higher survival and growth rates of *E. camaldulensis* as plantations mature, inoculation of seedlings with effective EM fungi (which can outcompete indigenous fungi) should be carried out in the nursery stage. However, this clearly needs to be tested in the field. Therefore, complementary studies including both indigenous and effective exotic fungal isolates should be carried out before embarking on a large-scale nursery inoculation. This kind of study can be best done in the field, the result of which may readily be available for use in nursery inoculation programmes.

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