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Effect of Dual Arbuscular-ectomycorrhizal Inoculation on Mycorrhiza Formation and Growth in *E. camaldulensis* Dehnh. Seedlings Under Different Nutrient Regimes

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ABSTRACT

Seedlings of *Eucalyptus camaldulensis* Dehnh. were inoculated with arbuscular mycorrhizal (AM) fungus *Glomus clarum* Nicolson and Schenck. Isolate BR148-1 and ectomycorrhizal (EM) fungus *Pisolithus tinctorius* (Pers.) Coker and Couch isolate K55. Four inoculation treatments-AM fungal, EM fungal, dual (AM & EM fungal) and an un-inoculated control and three nutrient treatments were combined in a factorial experiment. Assessment on mycorrhizal colonisation and their effects on growth of seedlings were done at 14 and 24 weeks harvests. AM colonisation between AM fungal and dual or EM colonisation between EM fungal and dual inoculation treatments did not vary significantly. The highest AM and EM colonisation were up to 50% and 10%, respectively of the root lengths being colonised. The dual inoculation treatment contributed to a significantly higher total dry mass of the seedlings than the AM treatment; however both the AM and the dual inoculation treatments were associated with a significantly lower dry mass compared to the un-inoculated control.

Key Words: *Eucalyptus camaldulensis* dehnh.; *Glomus clarum* Nicolson and Schenck; *Pisolithus tinctorius* (Pers.) Coker and Couch; *Arbuscular mycorrhiza*; Ectomycorrhiza; Dual inoculation

INTRODUCTION

Eucalyptus species have been reported to form both ectomycorrhizal (EM) and arbuscular (AM) associations (Warcup, 1980; Malajczuk *et al*., 1981; McGee, 1986; Chilvers *et al*., 1987; Reddell & Warren, 1987; Brundrett & Abbott, 1991). In a field experiment with *E. dunnii* Maiden, Oliveira *et al*. (1997) found that mycorrhizal colonisation of *Eucalyptus* seedlings depended on the AM or EM inoculum potential of the particular site. They found three patterns: (1) pattern A was on an agricultural site, preceded by the AM-forming soy bean [*Glycine max* (L.) Merr.]- the relatively large incidence of AM five months after planting progressively decreased, while that of EM increased; (2) pattern B followed the strategy of AM/EM forming *E. viminalis* Labill.- the incidence of AM remained minimal, while that of EM reached a relatively high plateau; and (3) pattern C followed the strategy of EM-forming *Pinus taeda* L.- both AM and EM progressively increased but were never abundant. According to Reddell and Malajczuk (1984), not only inoculum availability, but also the nutrient status of litter, soil and the presence of compounds inhibitory to fungal growth are all critical in determining the abundance of any one or both of the two types of mycorrhizas. Moyersoen and Fitter (1999) found that the pattern of dual AM and EM colonisation depends on the identity of the host species particularly with reference to their habitats, where soil types and moisture regimes play a major role besides inoculum availability. However, there is some controversy about the relative importance of EM and AM associations in plants that have both types of mycorrhiza (Brundrett *et al*., 1996). There have been reports that eucalypt seedlings may initially have AM associations, which are replaced by EM associations as they mature (Gardner & Malajczuk, 1988). Various field reports suggest that both AM and EM may exist in the same root system. Lapeyrie and Chilvers (1985) suspect that predominantly EM tree species such as *Eucalyptus* may be capable of brief AM episodes in the seedling stage and the AM may be important to the early establishment of plants in low nutrient or calcareous soils. They have attributed the succession from AM to EM during the growth of dual EM/AM plants to a competitive displacement of AM by EM. Chilvers *et al*. (1987) considered AM fungi well adapted to rapid primary colonisation and perpetuation within individual roots but inferior to EM fungi for secondary colonisation because of slow hyphal spread via root branches. Cázares and Smith (1996) hypothesised that AM fungi readily colonise typically EM hosts that establish early in plant community succession in areas, where EM propagules are sparse or absent. However, hosts that establish later in plant community succession are less readily colonised by EM.

There have been very few studies on dual inoculation of *Eucalyptus* in general and *E. camaldulensis* in particular. Even those studies are contradictory. Amorim and Machovej (1990) (as cited in Lapeyrie *et al*., 1992) found a

depressive growth effect when *E. grandis* W. Hill. ex Maiden seedlings were co-inoculated with AM and EM fungi, whereas a dual inoculation of *P. tinctorius* and AM fungi of *E. camaldulensis* and *E. tereticornis* resulted in increased biomass (by 137.8 & 164.85%, respectively) compared with the un-inoculated controls (Vishwakarma & Singh, 1996). However, they did not quantify the nutrient regime (s) they used in their experiment. This study investigated the dual AM/EM inoculation of *Eucalyptus*. The main objective of the study was to assess the development of both types of mycorrhizas (AM & EM) in the same root systems of *E. camaldulensis* at various nutrient concentrations. The specific objectives were to assess effects of dual AM/EM on colonisation and the interaction between nutrient availability and dual mycorrhizal colonisation and it's effect on growth.

MATERIALS AND METHODS

Experimental design. The experiment contained a factorial combination of four inoculation treatments (AM fungal, EM fungal, AM + EM fungal & an un-inoculated control) and three nutrient treatments: 12 treatments in all. Ten randomised blocks each containing one pot per treatment was set up, with a single seedling per pot. One AM fungus, *Glomus clarum* Nicolson and Schenck. Isolate BR148-1 (Brazil, host plant not known) and one EM fungus *Pisolithus tinctorius* (Pers.) Coker and Couch. Isolate K55 (Portugal, host *E. globulus*) were used as inoculant fungi. These fungi were found to be suitable for mycorrhiza formation in seedlings of *E. camaldulensis* in a series of experiments that preceded the present study (Misbahuzzaman, 1999; Misbahuzzaman & Ingleby, 2000; Misbahuzzaman & Wilson, 2002a, b).

Growth conditions. The experiment was carried out between May 1998 and October 1998 in a glasshouse at the Institute of Terrestrial Ecology, Edinburgh, UK. In general, seedlings were grown with day/night thermal regime of 20/15 ± 2°C and a light regime ranging between 400-800 µmol photons m^{-2} s^{-1}. Seeds of *E. camaldulensis* Dehnh. of Silverton (UMBER. CK) provenance were pre-soaked in 0.1% Thiram (a dithiocarbamate fungicide) for 24 h, dried and sown in to sterile trays containing sterilised vermiculite-peat (VP). Seeds were germinated in a growth cabinet at a temperature of 15 - 25°C and under fluorescent light (16 h irradiance, at 240 - 260 µmol m^{-2} s^{-1}). Seedlings were grown in sterilised vermiculite-peat (VP). Vermiculite, peat and tap water were mixed in the proportions 45: 10: 175 by mass and sterilised. The mixture was autoclaved at 121°C and 1.06 kg cm^{-2} for one hour. Two litre plastic pots were used for raising the seedlings. One seedling was transplanted from the tray in to each pot. In case of AM, inoculation was carried out by inserting 5 g portions of soil-root (colonised cowpea & millet roots) mixture and in case of EM by mycelial vermiculite-peat in to the planting hole. Control seedlings received autoclaved portions of the same inoculum. A nutrient solution modified from Ingestad's solution for birch (Ingestad, 1971) was supplied to the plants. The proportions of N, P and K were 100: 16: 55. Three different nutrient treatments containing 0.5, 2.5 and 10 mg l^{-1} P were applied, where all other nutrient elements were proportionally adjusted. Plants were supplied twice a week with the solution with a gradually increased dosage every three weeks so that the amounts of phosphorus added to pots of each individual treatment were 0.75 mg, 3.75 mg and 15 mg, respectively at the end of the final harvest.

Harvesting and mycorrhizal analysis. Seedlings were harvested at 14 and 24 weeks, respectively (five blocks each time). Shoots were cut at soil level and separated into stem and leaves. For each seedling, stem diameter above the root-collar, shoot height (from the soil surface to the base of apical bud) and leaf number (all leaves) were measured. Total leaf area was determined for each seedling using a Delta -T area meter (Delta-T Devices, Cambridge, England). Stems and leaves of each seedling were also dried at 80°C for 3 - 4 days (Burgess *et al*., 1994) and the dry mass recorded. Shoot dry mass and total dry mass were calculated. For assessment of mycorrhizal colonisation, pots were soaked overnight in water and roots from each pot were washed free (over a 2 mm sieve with a 0.25 mm sieve underneath to collect any root fragments that became detached) from soil by applying a gentle flow of water so that no fine roots were lost. The complete root-system was laid out on top of a graduated glass plate with the root collar set at 0 cm. Three sub-samples, each one cm long, were taken from 1 - 2, 7 - 8, and 13 - 14 cm on the grid corresponded with top, middle and bottom of the pot. Each sub-sample and the remaining sample were then uniformly blotted on filter paper and their fresh mass determined. The remaining root fractions of each root system were dried at 80°C and dry mass recorded so that total root dry mass could be estimated from the proportions of fresh mass. For assessment of AM colonisation, sampled roots were stained using the method of Phillips and Hayman (1970) with modifications given by Koske and Gemma (1989). The modified syringe method of Claassen and Zasoski (1992) was used for handling the samples during staining. 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). For assessment of EM colonisation, root systems were washed and sub-sampled as aforementioned. The sub-sampled roots were then placed in Petri dishes in water for examination of the tips of all short roots under a dissecting microscope. An ectomycorrhizal root tip was defined as a short root with a mycelial mantle, however thin. When no ectomycorrhizal colonisation was observed in the sub-samples, the remaining roots were checked for mycorrhizal root tips. Dry masses for the root sub-samples were recorded following the same procedure as described for remaining fragments.

Statistical analysis. A two-way ANOVA was carried out,

where the factors were- nutrient regime and inoculation. When data were found not to have a normal distribution, necessary transformations (for example, square-root transformation for ratios, arcsine transformation for mycorrhizal percentages & log transformation for all other variables) were carried out to normalise distributions and enable statistical comparisons of means. Normality and Heterogeneity of variances for data were tested using the Kolmogorov-Smirnov test and the Bartlett's test, respectively in Minitab version 12.1 (Minitab Inc., USA, 1998). Means were compared by Fisher's least significant difference test when the results of Fisher's *F*-test from ANOVA were significant at $P \leq 0.05$. Throughout the study, GENSTAT version 5.3 (Lawes Agricultural Trust, Rothamsted, Harpenden, Hertfordshire, UK) was used for statistical analysis and Microsoft Excel for graphics.

RESULTS

Influence of inoculation and nutrient regime on mycorrhizal colonisation. There was no significant interactive effect of nutrient regime and inoculation on either AM or EM colonisation at any harvest. Also there was no significant difference in colonisation by *G. clarum* BR148-1 between the AM and the dual inoculation treatments, or by *P. tinctorius* K55 between the EM and the dual inoculation treatment at any harvest. Colonisation by *G. clarum* BR148-1 varied significantly among the nutrient treatments with a higher colonisation in the higher nutrient concentration (for example, 50% root length colonised in 10 mg l^{-1} P) at the 14-weeks harvest (Fig. 1A). Colonisation by *P. tinctorius* K55 varied significantly between the nutrient treatments with 2.5 mg l^{-1} P having the highest (5%), while 0.5 mg l^{-1} P had < 1% of the root tips being colonised at the 14-weeks harvest (Fig. 1C). At 24 weeks, there was no significant difference in AM colonisation between the three nutrient treatments (mean colonisation was up to 40% of total root length) (Fig. 1B). At 24 weeks, EM colonisation at 0.5 mg l^{-1} P still remained < 1%, while at 2.5 mg l^{-1} P and at 10 mg l^{-1} P 10% and 5% of the root tips being mycorrhizal were recorded, respectively (Fig. 1D). Overall, the two inoculant fungi used had significantly different colonisation potential with *G. clarum* BR148-1 having the highest at a maximum of 50% of the total root length and *P. tinctorius* K55 at a maximum of 10% of total number of root tips.

Influence of mycorrhizal inoculation on growth. At the 14-weeks harvest, there was a significant interactive effect of nutrient and fungus on shoot dry mass. In the 0.5 mg l^{-1} P nutrient treatment, shoot dry mass of the seedlings in the dual inoculation treatment was significantly ($P < 0.05$) higher than that for the AM inoculation treatment. Although shoot dry mass of the seedlings at 2.5 mg l^{-1} P varied significantly between AM and EM or dual and EM (with EM, higher shoot dry mass), at 10 mg l^{-1} P shoot dry mass did not differ significantly between the three fungal inoculation treatments. Either the AM or the dual inoculation treatment had significantly lower shoot dry mass as compared to the EM inoculation treatment or the un-inoculated control at each of the three nutrient treatments (Fig. 2). There was no significant difference between the EM inoculation treatment and the un-inoculated control with respect to shoot dry mass of the seedlings at any of the three nutrient treatments (Fig. 2). However, there was no significant interactive effect of nutrient regime and inoculation on any variable of seedling growth at the 24-weeks (final) harvest.

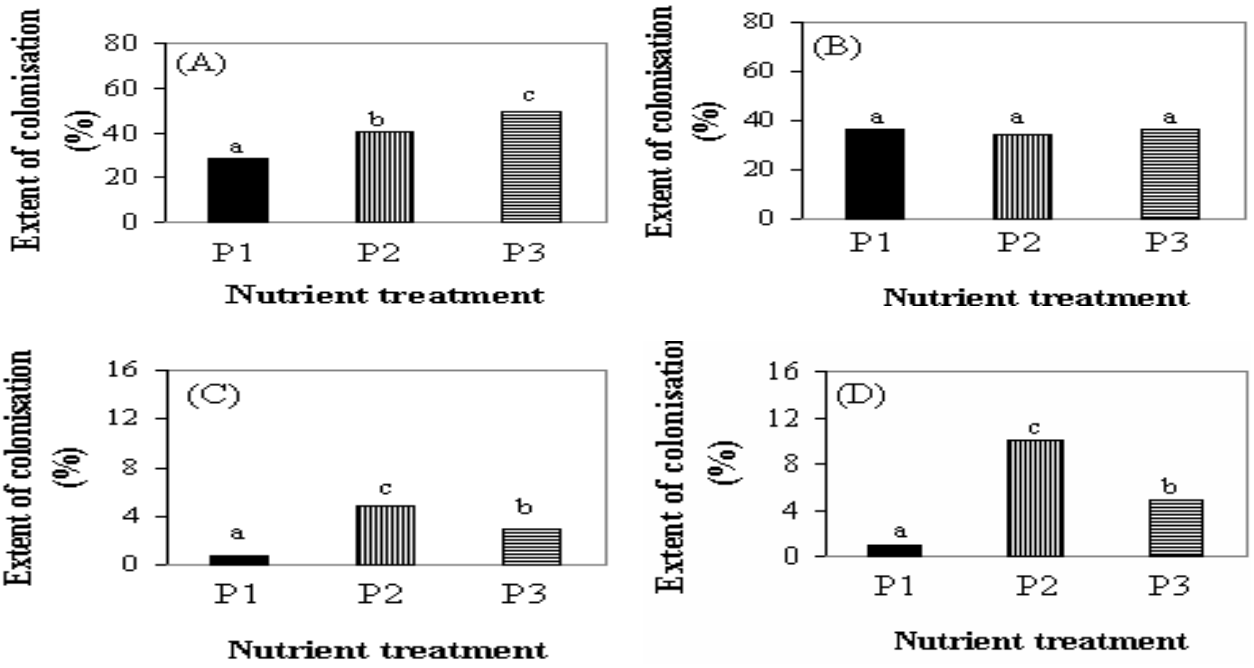
At the 14 weeks harvest, mycorrhizal colonisation resulted in negative growth responses in terms of height (Fig. 3A), stem diameter (Fig. 3B) and leaf area (Fig. 3C) and also in terms of shoot dry mass (Fig. 3D), root dry mass (Fig. 3E) and total dry mass (Fig. 3F). Although both AM and dual inoculation treatments contributed to the negative effects on growth, the dual inoculation treatment produced significantly larger leaf area (Fig. 3C) and shoot dry mass (Fig. 3D), root dry mass (Fig. 3E) and total dry mass (Fig. 3F) as compared to the AM inoculation treatment. There was no significant difference between the effects of EM inoculation treatment and the un-inoculated control on any variable of growth of the seedlings.

The 24 weeks harvest indicated that mycorrhizal colonisation has resulted in negative growth responses in terms of stem diameter (Fig. 4A), shoot dry mass (Fig. 4B), root dry mass (Fig. 4C) and total dry mass (Fig. 4D). The AM and dual inoculation treatments were again associated with all of the depressive effects on different variables of seedling growth. While these two inoculation treatments did not have a significantly different effect on stem diameter and shoot dry mass, the dual inoculation treatment was associated with significantly higher root dry mass and total dry mass as compared to the AM inoculation treatment. As at the 14-weeks harvest, there was no significant difference between the effects of EM inoculation treatment and the un-inoculated control on any of the variables.

DISCUSSION

Although there was significant variation in AM colonisation of *E. camaldulensis* seedlings at different nutrient regimes at the 14-weeks harvest (Fig. 1), they were not significantly different at the 24-weeks harvest. This indicated that nutrient regimes where P concentrations were in the range of 0.5 to 10 mg l^{-1} was still within a minimum limit for an adequate rate of AM colonisation. This does not contradict the results found in the experiments conducted preceding the height experiment, where a drop in colonisation rate was observed when nutrient concentration exceeded from 10 to 20 mg l^{-1} P (Misbahuzzaman, 1999). However, the variation between AM colonisation at different nutrient regimes levelled off at the end of 24 weeks of growth. Seedlings supplied with lower nutrient regimes (0.5 & 2.5 mg l^{-1} P) probably accumulated enough nutrients over time (24 weeks) to support a higher extent of AM

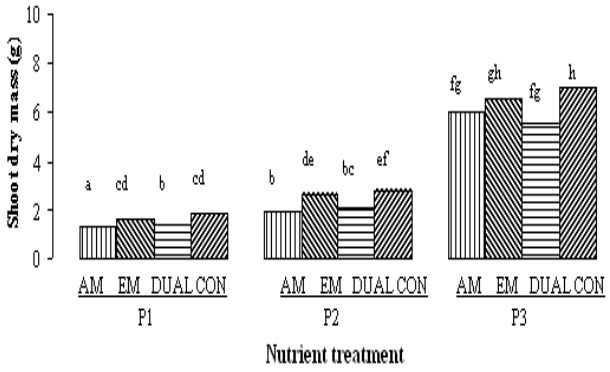
Fig. 1. Effects of nutrient treatments (P1, 0.5 mg l^{-1} phosphorus or P; P2, 2.5 mg l^{-1} P and P3, 10 mg l^{-1} P) on extent of (1A) arbuscular or AM colonisation at the 14-week harvest; (1B) AM colonisation at the 24-week harvest; (1C) ectomycorrhizal or EM colonisation at the 14-week harvest; and (1D) EM colonisation a the 24-week harvest of *E. camaldulensis* seedlings. Means with different letters in case of each part of the figure are significantly different



colonisation. However at the highest nutrient concentration (10 mg l^{-1} P), accumulation of nutrients over time perhaps tended to inhibit the extent of AM colonisation. Extent of EM colonisation at the 14-weeks harvest was very low (average 3% of the root tips) and were found mostly in 2.5 mg l^{-1} P (up to 5%) and 10 mg l^{-1} P (3%), which slightly improved over 24 weeks with 2.5 mg l^{-1} P giving 10 % and 10 mg l^{-1} P, 5%. It was found that the rate of extent of EM colonisation well exceeded that of gain in root biomass over 24 weeks. This has happened probably because of an affinity of the EM inoculant to colonise roots of relatively older seedlings that is in agreement with what Lapeyrie and Chilvers (1985) found in their AM/EM experiment with *E. dumosa*. Observation of a higher EM colonisation in *Eucalyptus* seedlings as they matured in the field has been reported in a number of studies (Gardner & Malacjzuk, 1988; Bellei *et al*., 1992).

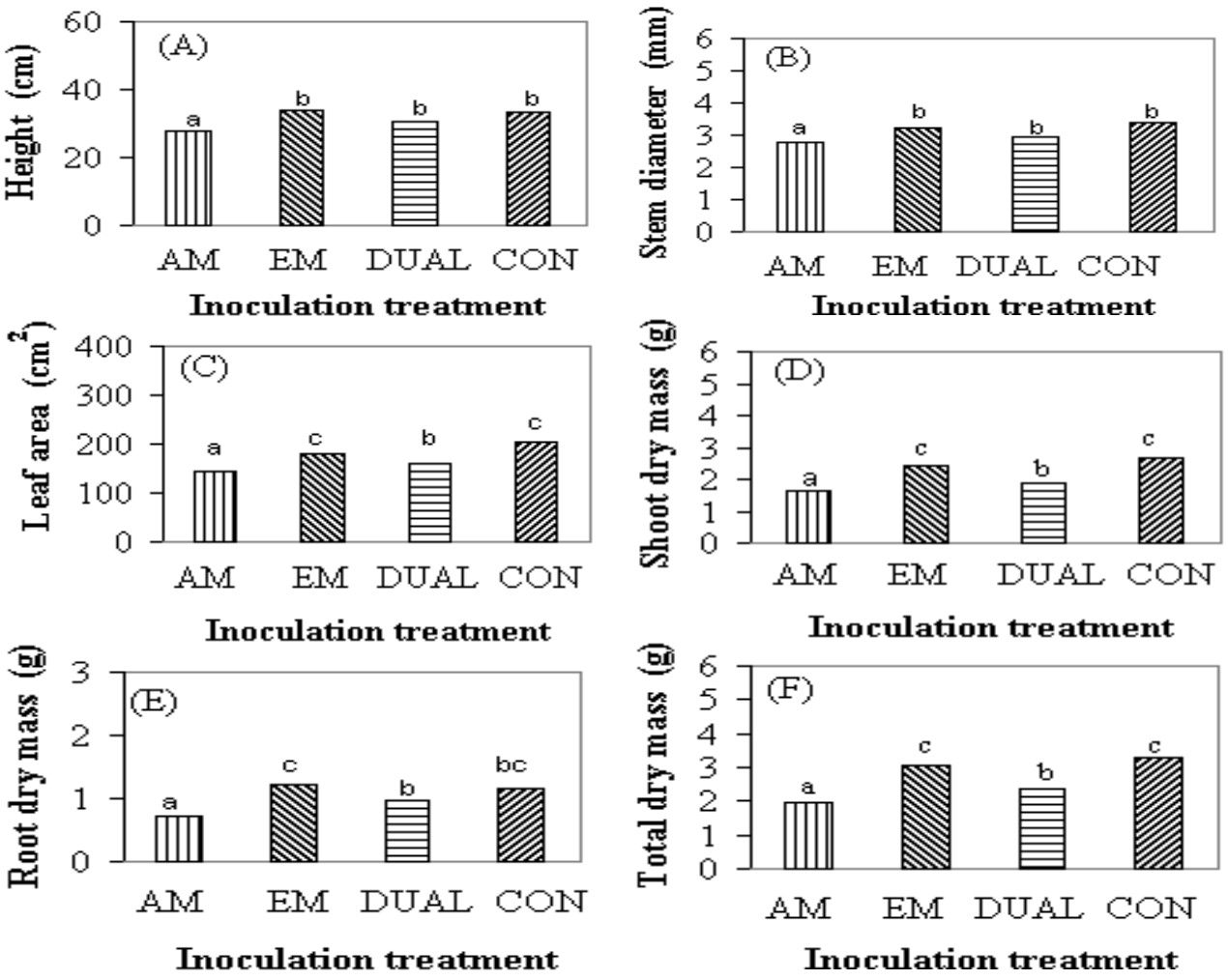
The AM and dual mycorrhizal colonisation led to a negative growth response at both harvests of the *E. camaldulensis* seedlings. At both harvests, the dual inoculation treatment contributed to a significantly higher total dry mass of the seedlings than the AM treatment; however both the AM and the dual inoculation treatments were associated with a significantly lower dry mass compared to the un-inoculated control. Both shoot and root dry mass for the dual inoculation treatment, were

Fig. 2. Effects of arbuscular (AM), ectomycorrhizal (EM), dual (AM and EM) colonisation and control (CON) on shoot dry mass of *E. camaldulensis* seedlings at different nutrient regimes (P1, 0.5 mg l^{-1} phosphorus or P; P2, 2.5 mg l^{-1} P and P3,10 mg l^{-1} P). Means with different letters are significantly different



significantly higher than those for the AM treatment in the 14-weeks harvest, so that the resulting total dry mass was also higher. Although only root dry mass for the dual inoculation treatment was higher compared to the AM treatment in the 24-weeks harvest, total dry mass was also higher. This indicated that the dual inoculation may have

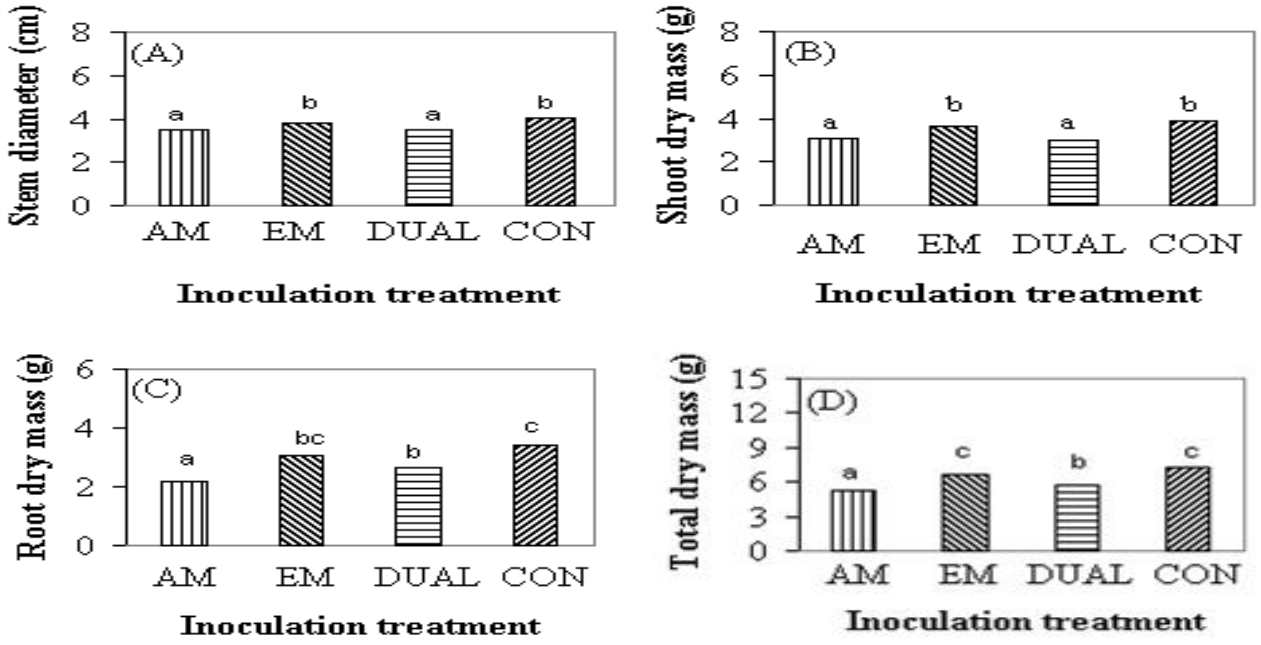
Fig. 3. Effects of inoculation treatment (arbuscular: AM, ectomycorrhizal: EM, dual: AM and EM and control: CON) on (a) height; (B) stem diameter; (C) leaf area; (D) shoot dry mass; (E) root dry mass; and (F) total dry mass of *E. camaldulensis* seedlings under different nutrient regimes (P1, 0.5 mg l^{-1} phosphorus or P; P2, 2.5 mg l^{-1} P and P3,10 mg l^{-1} P) at 14-week harvest. Means with different letters in case of each part of the figure are significantly different



resulted in a relatively high accumulation of biomass in the below-ground part of the seedlings at the end of 24 weeks of growth compared to that at 14 weeks of growth of seedlings.

Similar results were found in the experiments of Amorim and Muchovej (1990) (as cited by Lapeyrie *et al*., 1992) where seedlings of *E. grandis* were inoculated with a mixed AM-EM inoculum. After two months, EM inoculation stimulated plant growth compared with the un-inoculated control and stimulated growth of seedlings co-inoculated with AM fungal isolates compared with the treatment with AM alone. In this experiment, the AM colonisation was around 40% (of the total root length). Although the EM inoculation resulted in a low colonisation (< 10% of the root tips), it appeared to have an effect on seedlings in the dual inoculation treatment enabling them to attain more biomass as compared to those in the AM inoculation treatment. It is very difficult to explain here, whether the dual inoculation was beneficial or otherwise. EM mantle is known to prevent pathogens and other fungi from entering roots (Zak, 1964; Marx, 1971). It would be tempting to argue that, even with a very limited EM formation, there was a kind of inhibitory effect by the EM fungus on the AM fungus. However, this cannot be explained in terms of colonisation rate by the AM fungus. AM colonisation in the dual inoculation treatment did not differ significantly from that in the AM inoculation treatment. Therefore, an enhanced attainment of biomass in the dual inoculation treatment as compared to the AM treatment may be considered as a positive effect of AM/EM interaction at least relative to the results from the study of Vishwakarma and Singh (1996), where dual (AM/EM) inoculation of both *E. camaldulensis* and *E. tereticornis* seedlings resulted in significantly higher biomass compared to either the single inoculation treatments or the un-

Fig. 4. Effects of colonisation treatment (arbuscular: AM, ectomycorrhizal: EM, dual: AM and EM and control: CON) on (A) stem diameter; (B) shoot dry mass; (C) root dry mass; and (D) total dry mass of *E. camaldulensis* seedlings under different nutrient regimes (P1, 0.5 mg l^{-1} phosphorus or P; P2, 2.5 mg l^{-1} P and P3, 10 mg l^{-1} P) at 24-week harvest. Means with different letters in case of each part of figure are significantly different



inoculated control.

According to a recent study on a tropical tree species, *U. guineensis* by Taylor (1998), whether AM can be replaced by EM or *vice versa* as seedlings grow in the field, depends on the inoculum potential of either type. Therefore, depending on the availability of inoculum of both AM and EM in the growth medium, both types of mycorrhiza can remain active. Occurrence of either AM or EM may also depend on soil nutrient condition, for example, AM can be found more in mineral- or nutrient-rich soils, while EM can be found in soils rich in organic matter (Smith & Read, 1997).

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