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Research Paper

Effect of ectomycorrhizal inoculation on *Eucalyptus grandis* seedlings

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ABSTRACT

Eucalyptus grandis is the typical mycorrhiza nutritional type tree that plays an important role in man-made forest's land capability maintenance and stability of ecological system. The aim of this study was to evaluate the effect of inoculation with ectotrophic mycorrhiza fungi (ECMF) on the root colonization, growth, chlorophyll (Chl) content, photosynthesis, and nutrient uptake *of E. grandis*. Three ECMF were inoculated into *E. grandis* using a multiple factor orthogonal test. The results showed that over 50% *E. grandis* were infected with ECMF inoculation. The seedling height, stem base, and fresh weight of the treated plants were higher than those of the controls. The contents of Chl a, b, and a + b varied in different treatments and relatively high after ECMF inoculation. Photosynthetic rate (Pn), CO_2 concentration (Ci) and Stomatal conductance (Gs) were positively correlated with ECMF but not transpiration rate (E). N, P, Ca and Mg contents were higher in ECMF treatments than in sterile treatments in seedlings. Moreover, ECMF treatments increased soluble sugar content and stabilized free amino acid content to a high degree in *E. grandis* seedlings. Therefore, mycorrhizal treatment may be used in sustainable forestry programs to improve the growth of *E. grandis*.

Key words: Ectotrophic mycorrhiza, *Eucalyptus grandis*, inoculation.

INTRODUCTION

Eucalyptus spp., which originated in Australia, are important species relevant to commercial use in forms of wood, cellulose, food, and medicine in many countries (Pagano and Scotti, 2008). *E. grandis*, the main species in the genus Eucalyptus. It is fast growing and cold resistant, and it also contributes to the land capability maintenance of manmade forests and the stability of ecological systems (Pan et al., 2011). E. grandis is widely cultivated in short-rotation pulpwood forests and forestry projects in China (Pan et al., 2011).

Ectotrophic mycorrhiza fungi (ECMF) form symbiotic relationships well with plant roots (He et al., 2007; Franco et al., 2014). Previous studies confirmed that ECMF can be efficiently inoculated and beneficial to host plants. Inoculation with ECMF can significantly increase the average height and collar of Mongol Scotch pine seedlings (Song et al., 2007). It can improve the net photosynthetic rate and chlorophyll content (Zhao et al., 1997; Song et al., 2007; Zhang et al., 2013), as well as promote the nutrient uptake of plants. Inoculation with *Suillus luteus* (L, : Fr.) Gray, *Cantharellus cibarius* Fr., *Pisolithus tinctorius* (Pers.), *P. tinctorius* (Pers.) Coder et Couch, and *Cenococcum geophilum* Fr. stimulates the absorption of N, P and K in *Pinus massoniana* seedlings. In addition, inoculation with ECMF can enhance the water use efficiency and drought resistance of plants (Zhang et al., 2011).

Some Eucalyptus species can form different types of mycorrhizas, such as Arbuscular mycorrhizas and ectotrophic mycorrhiza (ECM), and predominantly correlate with ECM in both native forests and plantations (Gong et al., 1992; Brundrett et al., 2005; Pagano and Scotti, 2008; Pagano et al., 2009). Zhu et al. (2001) investigated 17 species of ECMF associated with Eucalyptus in Sichuan and nine species associated with *E. grandis*. Besides, the two genera *Pisolithus* and *Scleroderma*, which have been found under *E. grandis* forest were confirmed readily colonizing and enhancing the growth of *Eucalyptus* spp. (Chen et al., 2006; Huang et al., 2008; Zheng et al., 2003; Pan et al.,

Table 1: Infection ratio under different treatments.

No	Infection rate (%)	No.	Infection rate (%)	No	Infection rate (%)
1	0	30	0	15	0.59±0.015efDE
5	0	31	0	11	0.6±0.020fE
9	0	32	0	2	0.61±0.031fEF
13	0	18	0.5±0.045aA	7	0.61±0.015fEF
17	0	22	0.51±0.015abAB	16	0.61±0.030fEF
21	0	19	0.52±0.01abcABC	24	0.64±0.030gF
25	0	14	0.53±0.015bcABC	8	0.64±0.015gF
26	0	10	0.56±0.015cdBC	12	0.64±0.015gF
27	0	20	0.56±0.015dCD	3	0.75±0.026hG
28	0	23	0.56±0.015dCD	4	0.79±0.015iH
29	0	6	0.56±0.020deCD		

Different lowercase letters mean significant at the 0.05 level and different capital letters mean significant at the 0.01 level, LSD.

2011). However, little is known about the inoculation effect of ECMF on *E. grandis*.

Therefore, in the present study, we isolated three ECMF from the *E. grandis* forest and investigated their effects on root colonization, growth, chlorophyll content, photosynthesis, and nutrient uptake of *E. grandis*. Besides, the effect of the suitable combination of ECMF was also determined.

MATERIALS AND METHODS

Strains

ECMF *Scleroderma areolatum* (*S. citrinum* Pers.), *Vascellum pretense* (*S. areolatum* Ehrenb.), and *P. tinctorius* [*Pisolithus tinctorius* (Pers.) Coker & Couch.] were used in this study. These fungi were isolated, cultured, and purified from the fruiting body of puffballs collected under the *E. grandis* forest of Sichuan.

Cultivation

Pure cultures of the three ECMF were cultivated using potato dextrose agar (Hangzhou Microbial Reagent Co., Hangzhou, Zhejiang, China) medium. Each species was aseptically cultured in 300 mL of potato dextrose broth in a 500 mL flask with glass bead. ECMF were prepared for inoculation at 120 rpm on a rotary shaker (Forma, USA) at 25°C for 7–15 d.

Seedling and soil preparation

Clone seedlings of *E. grandis* with an average height of 10 cm were purchased from a nursery (Leshan, Sichuan). Each seedling was grown in a plastic pot (35 cm inner diameter of bottom, 40 cm inner diameter of top, and 40 cm in depth) with surface soil in the nursery of Sichuan Agricultural university (Ya'an, Sichuan). The soil was air-dried and sieved with a mesh aperture of 2 mm. Then the sieved soil was fumigated with 10% formalin for 7 d. Besides, the plastic pots was disinfected with 0.1% $KMnO_4$ and rinsed with distilled water.

Experimental design

The experiment was conducted from May to September, 2014. A total of 32 treatments were designed using a multiple factor orthogonal test, and each treatment contained eight replicates. Different treatments for seedling inoculation with ECMF are shown in Table 1. The cloned seedlings of *E. grandis* were transplanted into pots. Meanwhile, each inoculated seedling was treated with a different amount of mycorrhiza inoculums, and each non-inoculated seedling was fertilized with a different amount of compound fertilizer (10% N, 5% P, and 10% K) or sterile culture. The seedlings were watered whenever needed.

Assessment of seedlings

The seedlings were harvested after 60 d of inoculation. The roots were gently washed with double stilled water and cut into 1 cm-long root segments. Randomly selected root segments and the root mycorrhizal colonization rate of each treatment were determined using the methods of number of zhu et al. (2010). After 120 d, the seedling height, stem base, and total fresh weight were measured in each treatment.

Measurement of chlorophyll concentration

The upper leaves of *E. grandis* in each treatment were used

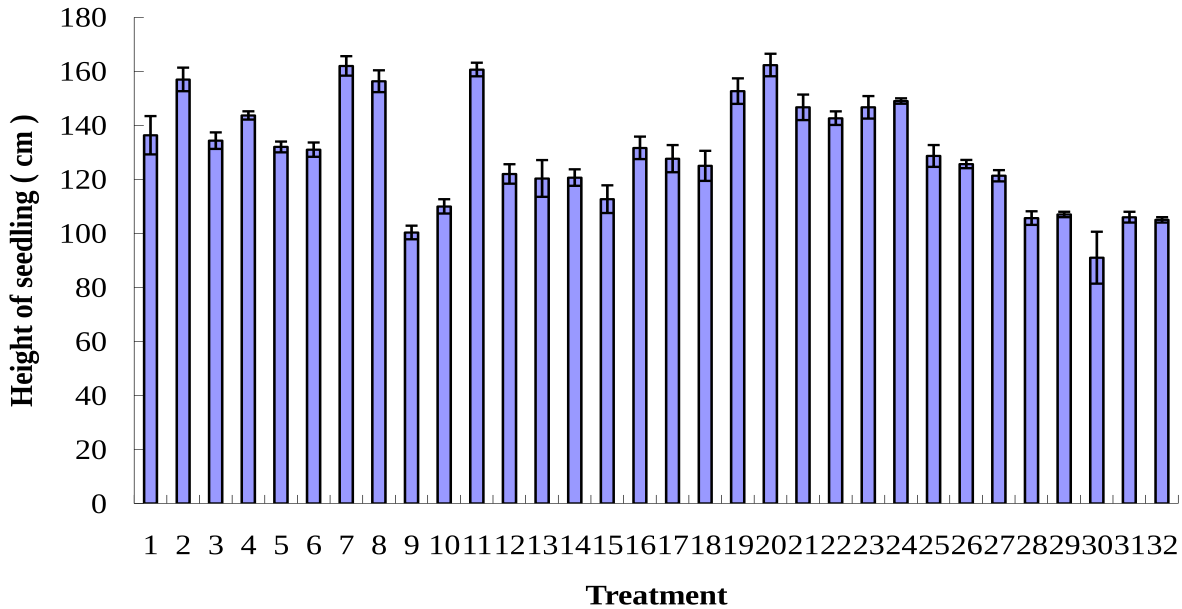

Figure 1: Seedling heights of *E. grandis* seedlings under different treatments. Each value is mean of triplicate samples ± standard deviation (SD).

to assay Chl content after 120 d. The extraction from 0.5 g of leaves without main veins was incubated in 20 ml of extraction solution (acetone: anhydrous ethanol = 1:1) at 4°C overnight. The absorbance was measured at 663 and 645 nm. Each treatment was performed in three replicates, and the contents of Chl a, b, and a + b were calculated as previously described (Zai et al., 2012).

Measurement of photosynthesis

Leaf gas-exchange parameters, including the net photosynthetic rate (Pn), stomatal conductance (Gs), transpiration rate (E), and interstitial density of CO_2 (Ci), were measured using a portable photosynthesis system LI-6400 after inoculation for 60, 120, and 180 d.

Measurement of nutrient concentration

Total N was determined using the Kjeldahl method, total P was measured using the Mo–Sb colorimetric method and total K, Ca, and Mg were analyzed through atomic absorption spectrometry in accordance with the national standard (GB7886-87). The contents of soluble sugar, reducing sugar and total protein were determined through Anthrone colorimetry, the Fehling's reagent colorimetric method and Coomassie brilliant blue staining, respectively (Zhang et al.,, 2003), and free amino acid was determined through Nihydrin colorimetry in accordance with the national standard (GB8314-87). The content of reducing sugar was evaluated through Fijian Lin reagent colorimetry (Chen, 2009).

Data analysis

The obtained data were subjected to ANOVA with SPSS16.0 and Excel. A p-value less than 0.05 was considered to indicate statistical significance. In addition, the LSD test at a 0.05 probability level was used to compare the means.

RESULTS

Effect of ECM on root colonization

Microscopic examination showed that the roots treated with ECMF for 60 d reached above 50% colonization (Table 1). Treatments 3 and 4 (*S. areolatum* inoculation) showed the highest infection rates of 75 and 79%, respectively which significantly differed from the values in the other treatments ($p < 0.01$). The effect of the treatments with single ECMF was better than that of the treatments with mixed fungal inoculation. No root infection was found in the non-inoculated controls.

Effect of ECM on seedling growth

In the present study, inoculation with ECMF had positive effect on the height, stem base and fresh weight of the seedlings. The highest seedling height (162.33 cm) was observed in Treatment 20 (ECMF ① + ③) (Figure 1).

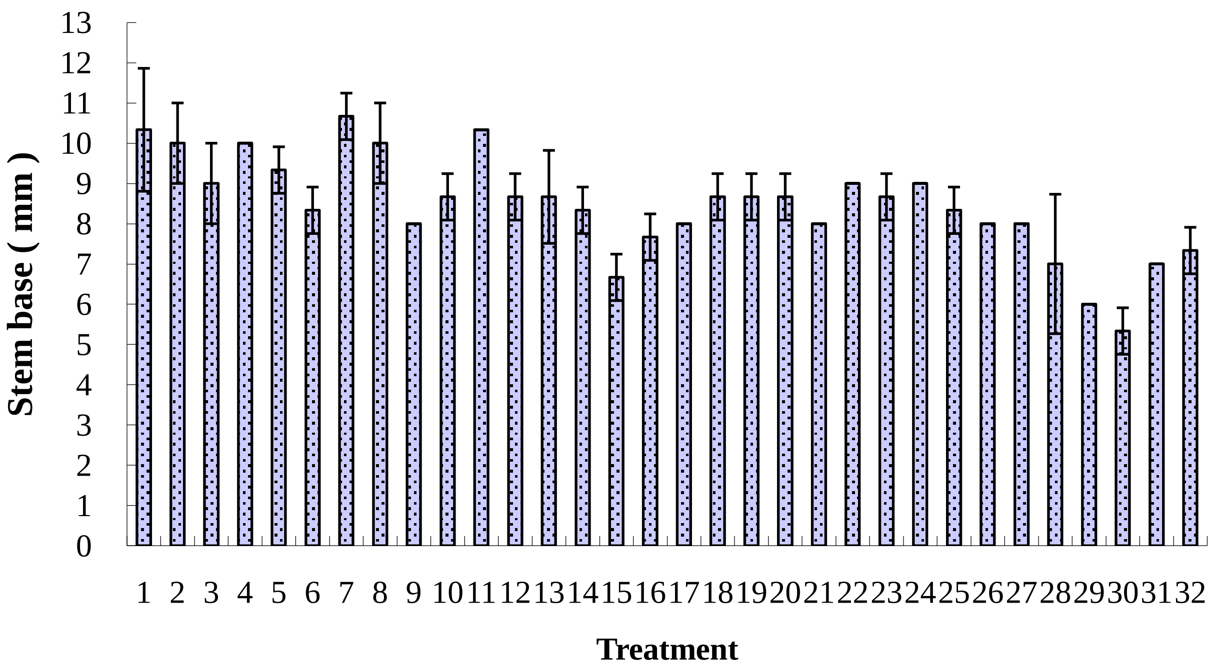

Figure 2: Stem bases of *E. grandis* seedlings under different treatments. Each value is mean of triplicate samples ± standard deviation (SD).

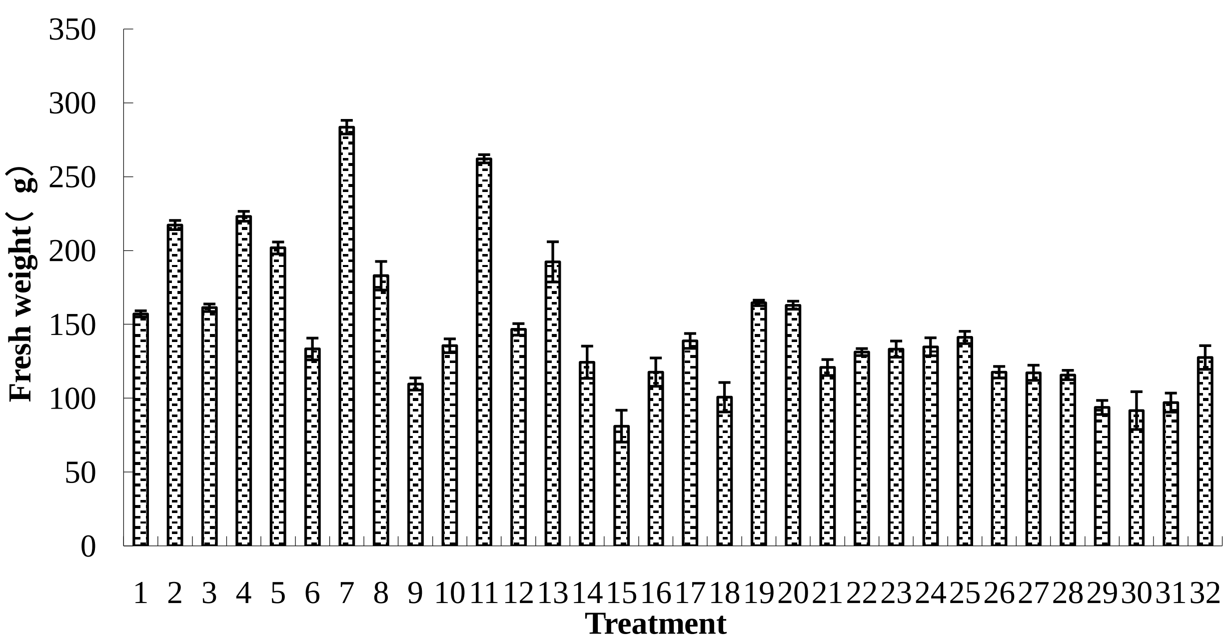

Figure 3: Total fresh weight of *E. grandis* seedlings under different treatments. Each value is mean of triplicate samples ± standard deviation (SD).

Meanwhile, Treatment 9 induced the lowest seedling height (100.33 cm), suggesting that the overlength of cutting root may affect seedling growth under the non-inoculated treatment. The result indicated that Treatments 7, 11, and 20 exerted positive effects with significant differences with other treatments ($p < 0.01$). The stem base showed the highest value (10.67 mm) in Treatment 7 (Figure 2), which was obviously different with other treatments ($p < 0.01$). Treatments 7 and 11 (ECMF ② and ③, respectively) significantly increased the fresh weight of seedlings (Figure 3). However, the seedling fresh weight was the lightest in Treatment 15 (ECMF ① + ②), indicating that the seedlings

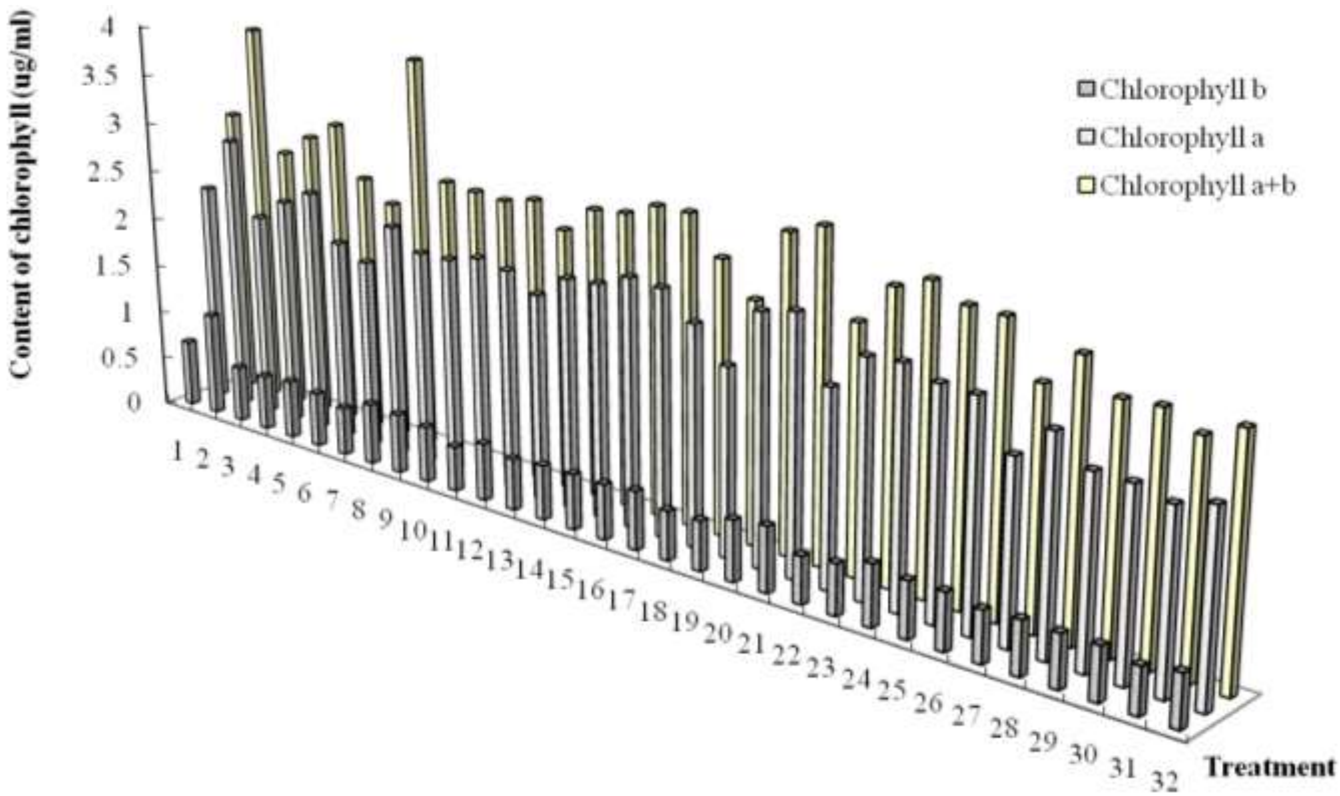

Figure 4: Content of chlorophyll under different treatments. Each value is mean of triplicate samples.

were seriously damaged by the overlength of cutting root. In general, the seedling height and stem base in the ECMF treatments were higher than those in the controls, while the seedling fresh weight was higher in the single ECMF treatments than in the other treatments.

Effect of ECM on chlorophyll content

The different treatments exerted different effects on leaf Chl a, b, and a + b (Figure 4). Treatment 31 significantly decreased Chl a and a + b contents ($p < 0.01$) as compared with the other treatments. In Treatment 2, Chl a, b and a + b concentration (2.80, 1.04 and 3.88 μg/mL) were highest and significantly different as compared with the other treatments ($p < 0.01$). Besides, no significant difference in Chl b content was detected except Treatments 2 ($p < 0.01$ and $p < 0.05$). Therefore, Treatment 2 was the best combination of three factors orthogonal design.

Effect of ECM on photosynthesis

In different months, Pn, E, Gs, and Ci in each treatment were changed and the extreme values showed in different treatments significantly differed as compared with the other treatments ($p < 0.01$) (Table 2). In May, July, and September (after 60, 120, and 180 d inoculation), the highest Pn was observed in Treatments 10, 24, and 20, respectively, and the highest E was detected in Treatments 1, 25, and 12, respectively. In the same corresponding periods, the lowest Pn was observed in Treatments 22, 1, and 2, and the lowest E was detected in Treatments 20, 1, and 10. Moreover, the highest Gs in the three months was found in Treatments 23, 4, and 3, respectively, whereas the lowest Gs was obtained in Treatments 7, 28, and 26, respectively. The highest Ci in the three months was achieved in Treatments 22, 1, and 9, respectively, whereas the lowest Ci was observed in Treatments 1, 28, and 26, respectively. All these treatments were significantly different as compared with all other treatments ($p < 0.05$)

The correlation of photosynthetic indexes under different treatments is shown in Table 3. Pn and Gs negatively correlated in May and then positively correlated afterward ($p < 0.01$). Pn and Ci significantly and negatively correlated, whereas Pn and E, Gs and Ci, and Gs and E significantly and positively correlated though with different variation trends ($p < 0.01$).

Effect of ECM on inorganic nutrient

The concentrations of inorganic nutrients differed under the various treatments (Table 4). Generally, N and P

Table 2: Photosynthetic rate and transpiration rate of E. grandis seedlings under different treatments.

S/N	Photosynthetic rate (μmol CO2 m^{-2} s^{-1})			Transpiration rate (mmol H2O m^{-2} s^{-1})			Stomatal conductance (mol H2O m^{-2} s^{-1})			Interstitial density of CO_2 (μmol CO_2 mol^{-1})		
	May	**July**	**September**	**May**	**July**	**September**	**May**	**July**	**September**	**May**	**July**	**September**
1	14.90±0.566	10.29 ±0.750^{a}	12.80 ±0.000	8.31±0.170^{a}	4.61±0. 408^{a}	5.34±0.267	0.57 ±0.027	0.58±0.065	0.37±0.021	313.6±1.265^{a}	329.9±2.025^{a}	290.6±4.858
2	14.88±0.162	13.17 ±0.779	9.38 ±0.161^{a}	7.46±0.323	5.32 ±0.274	4.41±0.051^{a}	0.59±0.008	0.64±0.104	0.26±0.021	318.8±0.422	315.2±8.842	281.7±7.034
3	13.98±2.061	15.61 ±1.239	14.23 ±0.125	7.56±0.223	5.76 ±0.241	5.94±0.189	0.55±0.062	0.68±0.039	0.57±0.015^{a}	319.4±12.572	304.3±4.398	296.4±0.966
4	14.39±0.032	12.60 ±0.618	9.84 ±0.283	7.80±0.124	5.84 ±0.023	5.34±0.000	0.61±0.013	0.83±0.082^{a}	0.34±0.000	322.2±0.789	318.1±2.132	292.5±1.080
5	14.04±0.593	12.34 ±0.486	13.18 ±0. 424	7.45±0.708	5.64 ±0.091	5.33±0.021	0.62±0.040	0.64±0.078	0.43±0.021	326.1±2.283	311.0±6.325	286.5±4.743
6	14.73±0.389	15.80 ±0.716	14.71 ±0.307	7.44±0.135	6.10 ±0.135	6.08±0.278	0.59±0.018	0.65±0.023	0.40±0.067	322.0±1.054	296.0±4.522	270.5±10.014
7	11.35±0.053	12.75 ±0.474	12.96 ±0.486	6.66±0.441	5.70 ±0.131	5.62±0.185	0.51±0.087^{a}	0.62±0.072	0.47±0.062	333.0±7.439	308.6±5.910	290.7±3.917
8	12.43±0.245	15.20 ±0.548	16.06 ±1.469	7.22±0.137	6.18 ±0.162	6.72±1.238	0.59±0.058	0.63±0.028	0.54±0.135	338.5±2.635	301.2±4.050	281.2±8.651
9	14.64±0.740	15.15 ±0.896	11.18 ±0.148	7.09±0.047	5.85 ±0.422	6.56±0.170	0.57±0.033	0.59±0.070	0.55±0.003^{a}	327.7±0.483	300.5±0.527	304.0±0.667^{a}
10	15.07±0.371^{a}	19.65 ±0.685	11.83 ±0.876	7.30±0.067	6.95 ±0.264	6.82±0.018	0.64±0.021	0.75±0.148	0.55±0.016^{a}	332.8±3.393	291.5±11.068	300.1±5.174
11	13.45±0.053	20.88 ±1.678	11.17 ±1.350	7.22±0.183	7.68 ±0.545	6.28±0.402	0.59±0.017	0.83±0.058^{a}	0.38±0.154	337.1±2.234	293.9±6.118	282.2±9.283
12	11.86±0.070	18.86 ±1.223	14.76 ±0.740	6.92±0.396	7.17 ±0.068	8.56±0.142^{a}	0.55±0.081	0.65±0.021	0.49±0.003	343.9±4.841	289.4±2.547	277.5±3.689
13	11.94±0.084	16.03 ±1.385	12.60 ±1.500	6.78±0.399	7.80 ±0.211	7.43±0.778^{a}	0.59±0.069	0.73±0.049	0.42±0.027	345.3±4.968	298.5±4.428	280.4±13.100
14	11.84±0.341	18.58 ±1.186	11.89 ±0.868	6.84±0.206	8.10 ±0.773	5.25±0.654	0.60±0.036	0.64±0.082	0.27±0.059	338.6±1.350	278.3±4.473	258.6±1.265
15	10.82±0.655	20.03 ±0.948	9.69 ±0.920^{a}	6.59±0.072	8.75 ±0.664	4.48±0.448^{a}	0.55±0.060	0.70±0.077	0.22±0.071	333.8±0.422	272.8±8.108	252.4±18.775
16	10.30±0.105	21.42 ±1.684	11.87 ±0.095	6.66±0.139	10.01 ±0.439	5.86±0.063	0.58±0.025	0.78±0.066	0.29±0.038	326.9±3.281	272.7±3.057	258.2±9.942
17	9.97±0.545	18.49 ±0.647	12.86 ±0.255	6.23±0.237	9.78 ±0.382	6.72±0.212	0.59±0.005	0.71±0.031	0.48±0.002	324.8±0.422	283.6±3.718	281.6±1.506
18	8.60±0.069	20.51 ±1.426	14.75 ±0.481	5.54±0.660^{a}	10.72 ±0.394	6.03±0.206	0.53±0.008	0.71±0.124	0.37±0.116	325.8±2.974	275.5±8.810	248.8±17.410
19	7.99±0.726	20.12 ±1.259	14.46 ±1.469	5.91±0.011	9.98 ±0.411	6.46±0.544	0.57±0.072	0.74±0.101	0.40±0.073	332.0±6.325	279.5±4.905	259.1±24.995
20	8.03±0.246	22.08 ±1.170	16.35 ±0.654^{a}	5.48±0.611^{a}	9.92 ±0.713	6.99±0.278	0.51±0.017	0.63±0.093	0.52±0.030	332.5±4.743	268.4±9.419	266.8±5.493
21	7.54±0.084	19.30 ±1.687	11.02 ±0.673	5.63±0.224	10.60 ±0.632	6.01±0.679	0.61±0.007	0.73±0.002	0.38±0.094	348.6±1.350	296.0±5.270	274.1±12.060
22	7.00±0.092^{a}	21.79 ±0.690	14.75 ±0.369	5.99±0.094	9.90 ±0.395	7.24±0.732	0.65±0.005	0.66±0.135	0.40±0.021	354.5±0.527^{a}	272.9±11.318	257.7±0.483
23	8.16±0.573	21.28 ±1.848	13.19 ±1.335	5.60±0.190^{a}	10.57 ±0.362	6.88±0.187	0.67±0.054^{a}	0.75±0.096	0.30±0.010	352.0±4.110	279.4±12.721	251.8±7.757
24	9.27±0.656	23.56 ±1.082^{a}	13.78 ±1.086	5.50±0.196^{a}	11.81 ±0.515	6.53±0.300	0.56±0.006	0.79±0.056	0.30±0.030	343.8±1.932	269.2±9.531	248.2±15.237
25	9.23±0.377	21.03 ±0.542	11.22 ±1.181	5.70±0.155	13.33 ±0.952^{a}	4.69±0.605	0.59±0.043	0.72±0.027	0.22±0.038	346.1±3.725	273.1±2.885	244.1±18.211
26	8.31±0.876	19.77 ±0.353	11.39 ±0833	5.95±0.112	11.05 ±0.737	5.06±0.759	0.63±0.019	0.76±0.197	0.17±0.032^{a}	346.3±5.870	289.5±8.960	213.2±11.603^{a}
27	10.93±0.236	16.10 ±0.527	14.93 ±1.583	5.93±0.160	9.75 ±0.369	6.56±1.240	0.58±0.024	0.59±0.048	0.30±0.050	327.3±1.418	289.5±5.798	238.6±7.152
28	10.66±0.989	17.97 ±0.566	14.50 ±0.105	6.07±0.113	7.23 ±0.465	7.33±0.276	0.64±0.002	0.45±0.082^{a}	0.36±0.039	331.7±3.917	265.6±10.069^{a}	254.8±6.546
29	13.08±0.256	13.02 ±0.439	9.52 ±0.371	5.91±0.438	7.95 ±0.665	7.23±0.245	0.60±0.042	0.470±167	0.53±0.017	325.4±1.846	286.2±9.778	300.5±6.876
30	14.88±0.162	13.17 ±0.779	9.38 ±0.161^{a}	5.91±0.424	8.26 ±1.228	7.22±0.088	0.59±0.071	0.49±0.093^{a}	0.53±0.010	326.4±1.506	290.2±10.758	302.3±7.973
31	10.15±0.900	15.60 ±0.211	11.25 ±1.002	5.91±0.028	9.27 ±0.137	6.00±0.085	0.61±0.030	0.61±0.044	0.36±0.022	334.2±3.393	291.5±3.689	278.1±9.597
32	11.44±0.659	18.80 ±0.527	12.26 ±0.746	5.64±0.109	10.01 ±0.100	6.22±0.076	0.56±0.029	0.69±0.045	0.26±0.048	327.9±4.332	280.0±5.270	244.6±12.112

The lowercase a means highest or lowest significant at the 0.05 level.

Table 3: Correlation of photosynthesis under different treatments in period of growth.

Factors	**May**				**July**				**September**			
	Pn	***Gs***	***Ci***	***E***	***Pn***	***Gs***	***Ci***	***E***	***Pn***	***Gs***	***Ci***	***E***
Pn	1	-0.082	-0.634^{a}	0.803^{a}	1	0.334^{a}	-0.833^{a}	0.735^{a}	1	0.385^{a}	-0.223^{a}	0.582^{a}
Gs		1	0.428^{a}	0.140^{a}		1	0.137^{b}	0.370^{a}		1	0.733^{a}	0.569^{a}
Ci			1	-0.441^{a}			1	-0.637^{a}			1	0.131^{b}
E				1				1				1

The lowercase a and b means that correlation is significant at the 0.01 level and 0.05 level (two-tailed), respectively.

Table 4: Inorganic nutrient content of *E. grandis* seedlings under different treatments.

No	Total N (%)	Total P (%)	K (%)	Ca (ppm)	Mg (ppm)
1	0.50 ±0.060^{ab}	0.26 ±0.000^{ab}	2.43 ±0.021^{bcd}	0.70 ±0.028^{cdefghi}	0.24 ±0.007^{bcde}
2	0.82 ±0.041^{fgh}	0.35 ±0.023^{j}	2.56 ±0.035^{cd}	0.92 ±0.021^{j}	0.29 ±0.035^{defgh}
3	0.99 ±0.001^{j}	0.32 ±0.007^{fghij}	2.44 ±0.007^{bcd}	0.56 ±0.078^{abcdef}	0.36 ±0.028^{ij}
4	0.88 ±0.003^{ghi}	0.34 ±0.011^{ij}	2.45 ±0.007^{bcd}	0.74 ±0.064^{efghij}	0.30 ±0.014^{efghi}
5	0.96 ±0.041^{ij}	0.32 ±0.003^{efghij}	2.41 ±0.057^{bcd}	0.53 ±0.042^{abcde}	0.31 ±0.014^{ghij}
6	0.84 ±0.022^{fgh}	0.32 ±0.001^{efghij}	2.35 ±0.085^{bcd}	0.93 ±0.014^{j}	0.31 ±0.007^{fghij}
7	1.10 ±0.001^{k}	0.25 ±0.011^{a}	2.57 ±0.184^{cd}	0.80 ±0.057^{ghij}	0.35 ±0.042^{ij}
8	0.67 ±0.022^{de}	0.28 ±0.018^{abcdef}	2.29 ±0.014^{bcd}	0.66 ±0.134^{bcdefgh}	0.25 ±0.035^{bcdefg}
9	0.81 ±0.020^{fgh}	0.30 ±0.015^{bcdefghi}	2.31 ±0.057^{bcd}	0.65 ±0.071^{bcdefgh}	0.22 ±0.000^{bcd}
10	0.84 ±0.016^{fgh}	0.31 ±0.010^{defghij}	2.43 ±0.049 ^{bcd}	0.57 ±0.042^{abcdef}	0.29 ±0.007^{defgh}
11	0.79 ±0.115^{fg}	0.25 ±0.004^{a}	2.59 ±0.014^{d}	0.61 ±0.078^{abcdefg}	0.23 ±0.035^{bcd}
12	0.70 ±0.022^{de}	0.33 ±0.017^{hij}	2.57 ±0.021^{cd}	0.83 ±0.014^{hij}	0.23 ±0.007^{bcd}
13	0.64 ±0.021^{cde}	0.29 ±0.000^{abcdefgh}	2.53 ±0.064^{cd}	0.62 ±0.113^{abcdefgh}	0.28 ±0.007^{defgh}
14	0.63 ±0.001^{cd}	0.27 ±0.010^{abc}	2.47 ±0.007^{bcd}	0.54 ±0.064^{abcde}	0.29 ±0.007^{defgh}
15	0.67 ±0.021^{de}	0.27 ±0.011^{abcd}	2.48 ±0.028^{bcd}	0.50 ±0.198^{abcd}	0.25 ±0.014^{bcdefg}
16	0.74±0.040^{ef}	0.27 ±0.012^{ab}	2.33 ±0.028^{bcd}	0.63±0.021^{abcdefgh}	0.27 ±0.000^{cdefgh}
17	0.82 ±0.041fgh	0.27 ±0.000abcd	2.22 ±0.014bc	0.88 ±0.085ij	0.33 ±0.049hij
18	0.56 ±0.021bc	0.31 ±0.012cdefghij	2.48 ±0.035bcd	0.77 ±0.212fghij	0.26 ±0.007bcdefg
19	0.60 ±0.042bcd	0.26 ±0.029ab	2.38 ±0.078bcd	0.49 ±0.028abc	0.27 ±0.014cdefgh
20	0.64 ±0.018cde	0.33 ±0.002ghij	2.49 ±0.007bcd	0.69 ±0.141cdefghi	0.30 ±0.014efghi
21	0.74 ±0.002ef	0.29 ±0.013abcdefg	2.45 ±0.134bcd	0.56 ±0.014abcdef	0.30 ±0.014efghi
22	0.82 ±0.083fgh	0.36 ±0.032j	1.85 ±0.735a	0.83 ±0.049hij	0.37 ±0.000j
23	0.90 ±0.078hij	0.35 ±0.030j	2.39 ±0.014bcd	0.51 ±0.064abcd	0.27 ±0.014cdefgh
24	0.81 ±0.015fgh	0.26 ±0.021ab	2.32 ±0.028bcd	0.41 ±0.014a	0.26 ±0.049bcdefg
25	0.63 ±0.079cd	0.30 ±0.017bcdefghi	2.40 ±0.078bcd	0.44 ±0.071ab	0.26 ±0.035bcdefg
26	0.70 ±0.018de	0.29 ±0.057abcdefgh	2.55 ±0.085cd	0.62 ±0.057abcdefgh	0.25 ±0.000bcdefg
27	0.57 ±0.079bc	0.26 ±0.002ab	2.64 ±0.007d	0.53 ±0.014abcde	0.24 ±0.014bcdef
28	0.52 ±0.001ab	0.27 ±0.003ab	2.58 ±0.078cd	0.55 ±0.021abcde	0.15 ±0.064a
29	0.45 ±0.020a	0.28 ±0.002abcde	2.22 ±0.014bc	0.62 ±0.057abcdefgh	0.22 ±0.000bcd
30	0.45 ±0.020a	0.35 ±0.025j	2.16 ±0.007b	0.61 ±0.219abcdefg	0.20 ±0.007ab
31	0.45 ±0.017a	0.28 ±0.002abcde	2.47 ±0.028bcd	0.72 ±0.099defghij	0.21 ±0.064bc
32	0.70 ±0.019de	0.32 ±0.019efghij	2.66 ±0.014d	0.55 ±0.000abcde	0.25 ±0.000bcdefg

Different lowercase letters mean significant at the 0.05 level and ppm means parts per million.

contents were higher in ECMF treatments than in sterile treatments. The accumulations of N and P were the highest in Treatments 7 and 22, and inoculated with *S. areolatum* Ehrenb. and *S. areolatum* Ehrenb. + *P. tinctorius* (Pers.) Coker & Couch., respectively. However, ECMF exerted no considerable effect on K accumulation, and the highest content was observed in sterile treatments (Treatment 32). The inoculation treatments increased the accumulation of Ca and Mg as follows: ECMF treatment > compound fertilizer treatment > sterile treatment. Besides, the single ECMF treatments were superior to the mixed ECMF treatments for Ca content.

Effect of ECM on organic nutrient

The seedlings inoculated with ECMF accumulated a high content of soluble sugar (Figure 5). Treatment 16 (ECMF ① + ②) significantly increased ($p < 0.05$) soluble sugar concentration. However, no significant difference ($p < 0.05$) in reducing sugar content was detected among the different treatments (Figure 6). This result suggests that the inoculation with ECMF only slightly affected the reducing sugar content in the seedlings. The highest content of free amino acid was found in Treatment 17 and the lowest content was observed in Treatments 1 and 13 (Figure 8), which significantly differed from the non-inoculated treatments ($p < 0.05$). The total protein was highest in Treatment 1 and lowest in Treatment 4 significantly ($p < 0.05$). However, no significant difference was observed among all other treatments, indicating no obvious correlation between ECMF inoculation and total protein content (Figure 7).

DISCUSSION

Over 50% *E. grandis* were infected with ECMF inoculation. This result is line with the findings of Pagano and Scotti (2008) and Chen et al. (2006).Furthermore, better colonization was detected in the treatments with single ECMF than in treatments with mixed ECMF. This result may be attributed to the fact that competition between two ECMF during the establishment of a symbiotic relationship

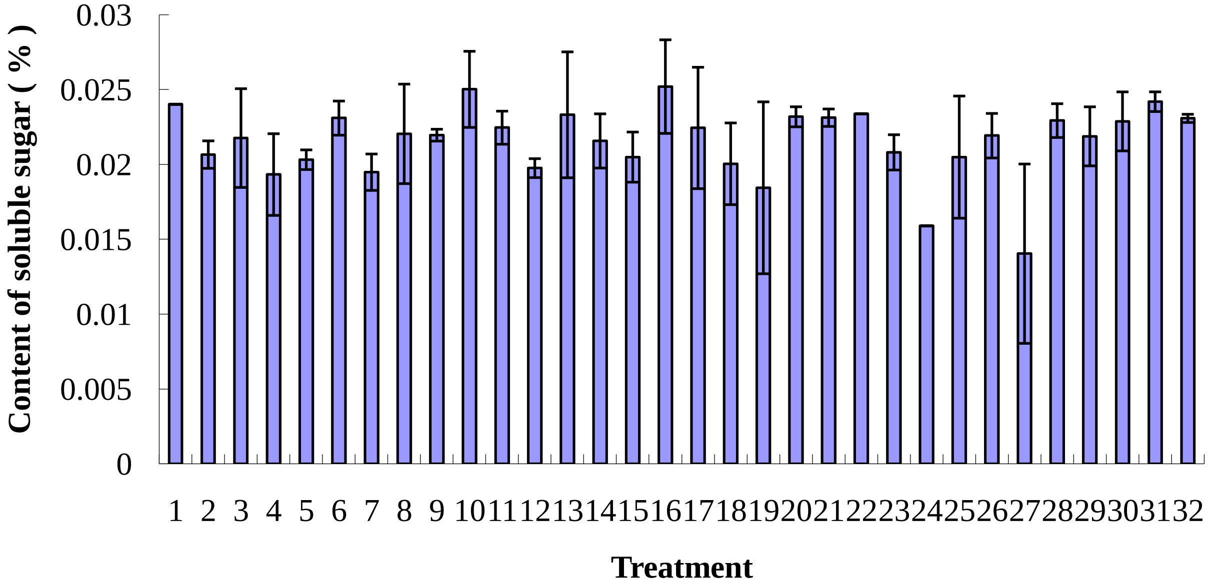

Figure 5: Content of soluble sugar under different treatments. Each value is mean of triplicate samples ± standard deviation (SD).

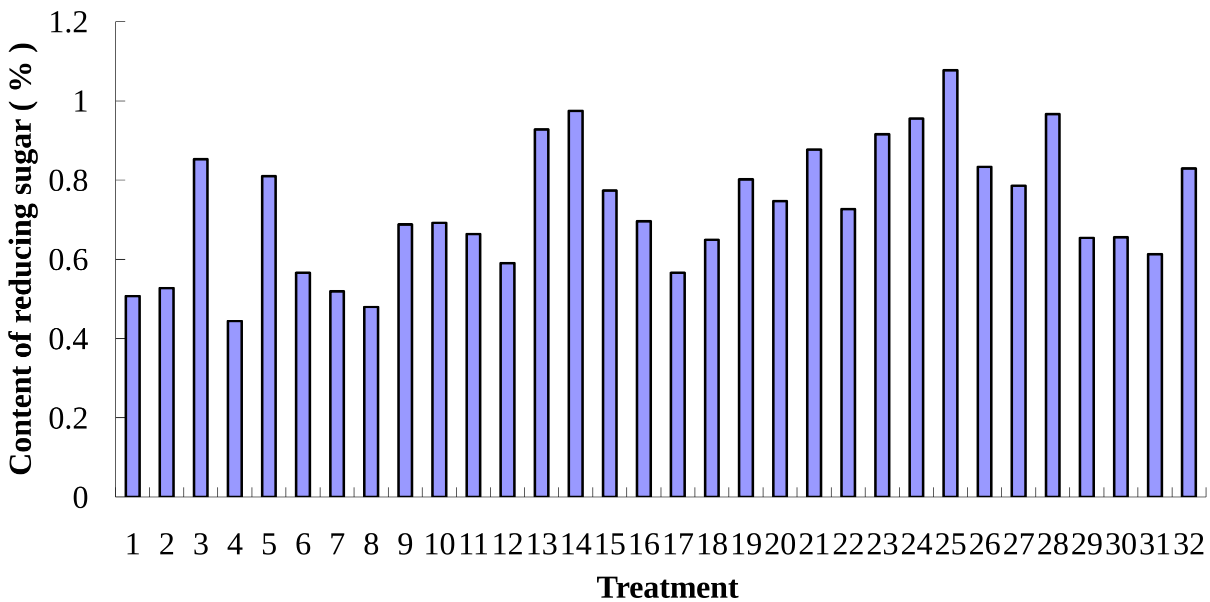

Figure 6: Content of reducing sugar under different treatments. Each value is mean of triplicate samples.

with seedlings can inhibit the formation of mycorrhiza. In the present study, inoculation with ECMF improved the growth of *E. grandis* seedlings. The seedling height and stem base in the inoculated treatments were higher than those in the compound fertilizer or sterile treatments. Moreover, the fresh weight was higher in the single ECMF than in the other treatments. Previous studies (Quoreshi et al., 2008; Gholamhoseini et al., 2013; Sukesh et al., 2013) confirmed that ECMF positively affect seedling growth. Mycorrhizal fungi promoted the nutrient uptake and enhanced the growth of host plants (Pagano and Scotti, 2008; Gholamhoseini et al., 2013). However, different species serve different functions. *Laccaria laccata* (Scop.:Fr) Berk. et Br., *Tricholoma matsutake* (Ito et Imai) Sing., and *Tuber melanosporum* Vittad. significantly enhanced the growth of *Castanopsis hystrix* A.DC seedlings (Chen et al., 2001), whereas *Suillus* sp. improved the heavy mental tolerance of host plants (Wei et al., 1989; Sarand et al., 1998). Moreover, *C. geophilum* Fr. increased drought resistance in host plants (Bai et al., 2006).

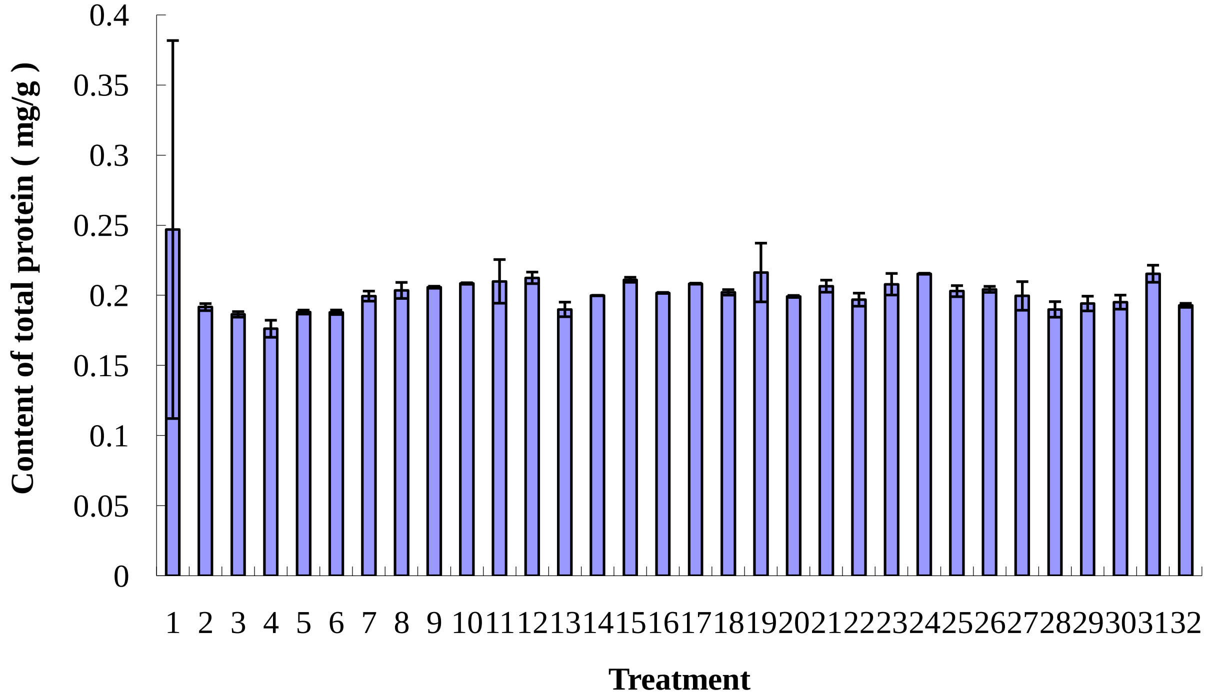

Figure 7: Content of total protein under different treatments. Each value is mean of triplicate samples ± standard deviation (SD).

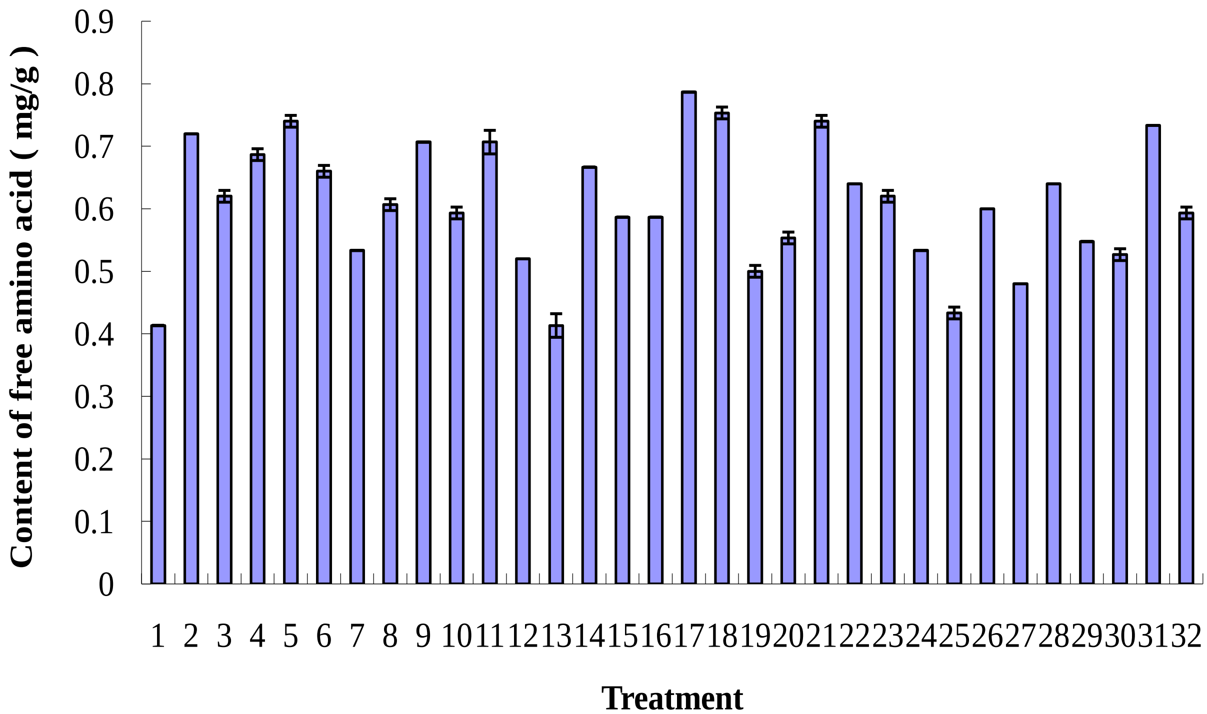

Figure 8: Content of free amino acid under different treatment. Each value is mean of triplicate samples ± standard deviation (SD).

According to Zhang et al. (2013), increasing Chl content improved the production efficiency of organic matter and the stress resistance of host plants. It has been found that the highest Chl a, b and a+b concentrations were obtained in Treatment 2, suggesting that *S. areolatum* can improve the production efficiency of organic matter in *E. grandis* seedlings. Meanwhile, in other treatments, the variation trend of Chl a, b and a+b concentrations in the same treatment were not fully coincident, which may have resulted from different combination levels and factors. In general, the variation trend of Chl content was as follows: ECMF treatment > compound fertilizer treatment > sterile treatment. Therefore, inoculation with ECMF can be used to increase the Chl content of *E. grandis* seedlings. Similarly, Chl content obviously increased in pine seedlings inoculated with *Boletus edulis*, *Boletus speciosus*, or *P. tinctorius* as compared with non-inoculated seedlings (Gao et al., 2010).

Photosynthesis can be influenced by various factors, such as temperature, soil moisture, and atmospheric CO_2 concentration. It was found that the photosynthesis of *E. grandis* was mainly affected by single or mixed inoculation ECMF treatments and growing periods. Pn was higher after single ECMF inoculation than after mixed fungal inoculation in the early period (May). However, no obvious improvement was observed in *E. grandis* after inoculation, contradicting with the findings of Chang et al. (2009) and Zhu et al. (2010). The possible reason was that, under the condition of multiple factors, seedlings showed inconsistent responses to the influence of different factors. Gs was relatively high under single ECMF inoculation treatment, especially in July. Ci was relatively high after mixed ECMF inoculation in May and after single fungal inoculation in July and September. Besides, the variation trend of Ci was as follows: ECMF treatment > compound fertilizer treatment > sterile treatment. Previous studies have found that Gs is proportional to Ci (Zhao et al., 1997; Liu et al., 2010). Similarly, the present results showed a negative correlation between Pn and Ci, a positive correlation between Pn and E, and a positive correlation among Gs, E, and Ci.

N, P and K are the most essential mineral elements during plant growth and development (Feng et al., 2010). N plays an important role in the physiology and metabolism of plants, whereas P promotes the growth and vitality of the root (Xie et al., 2014). Recent studies have focused on the mechanism by which mycorrhizal fungal inoculation, especially arbuscular mycorrhizal fungal (AMF) inoculation, affected the absorption and utilization of N and P in host plants (Shi et al., 2013). However, little was known about the nutrient variation of in *E. grandis* after ECMF inoculation. In our study, except for the highest content of P and Mg both presented in Treatment 22, the other highest and lowest accumulation of inorganic nutrients existed in different treatments. This result suggested that various factors affect nutrient accumulation. Moreover, N, P ,Ca and Mg contents were higher in ECMF treatments, demonstrating that ECMF exerts positive effects on the their uptake of *E. grandis* seedlings. Xie et al. (2014) confirmed that AMF inoculated on *Kandelia obovata* seedlings enhanced root vitality and increased plant N and P uptake. Similarly, Quoreshi et al. (2008) demonstrated that N and P contents in aspen seedlings significantly increased when co-inoculated with *Paxillus involutus* + *Burkholderia cepacia*. Meanwhile, Wei et al. (1989) reported that VA mycorrhizal fungi decreased the Ca and Mg contents in *Datura stramonium* L. This difference may be attributed to the different mechanisms and ecological niches between ECM and endotrophic mycorrhiza. However, ECMF exerted no considerable effect on K accumulation, and contradictory results were reported by Wang et al. (2002), showing that the different mycorrhizal fungi enhanced the N, P and K contents in tobacco seedlings. These scholars reasoned that the indigenous mycorrhizal fungi in non-sterile soil may form a positive symbiotic relationship with inoculated fungi and enhance the comprehensive effect of inoculation. Moreover, Ortas (2012) demonstrated that mycorrhizal inoculation induced higher P and Zn concentrations under non-fumigated conditions than under fumigated ones.

Many studies have investigated the effect of mycorrhizal fungi on colonization, seedling growth, photosynthesis, and nutrient accumulation of tree species for timber forest (Ciompi et al., 1996; Chen et al., 1999; Sun et al., 2007; Zhang et al., 2009; Gao et al., 2010). However, limited research focused on the impact of organic matter. The soluble sugar contents of *E. grandis* seedlings were higher in the ECMF treatments than in the compound fertilizer treatments. Besides, free amino acid content can also stabilized in high degree after inoculation with ECMF, while extreme values were observed in non-inoculated treatments. However, no significant difference in reducing sugar and total protein contents were found among the different treatments, suggesting that the association between the two contents and ECMF was not obviously. Our results are partly in accordance with previous studies. Lv et al. (2006) reported that the soluble sugar contents of cucumber fruit increased in treatment GV (Glomus versiforme), GV + GI (Glomus intraradices), and GI, amino acid contents increased by 47.66 and 23.19% in treatment GV and GM (Glomus mosseae) + GI, respectively and soluble protein content increased by 17.67 – 34.79% in all AMF treatments. Chen et al. (2010) also confirmed that AMF increases the contents of chlorophyll, soluble sugar, soluble protein, and mineral elements (N, P and K). Among these parameters, soluble protein was the most significantly affected by AMF.

Conclusion

ECMF improved root colonization, growth, Chl content, photosynthesis, and nutrient uptake of *E. grandis* seedlings. In general, the inoculated treatments reached above 50% colonization. After inoculation, the height, stem base, and fresh weight of *E. grandis* increased, and the Chl content and photosynthesis rate improved. The application of ECM could be vital in the cultivation of *E. grandis*. The ECMF treatments significantly affected N, P, Ca and Mg contents but not K content. The soluble sugar content increased and free amino acid content was stabilized to a high degree, after inoculation. Therefore, the application of ECMF could be useful in the cultivation of *E. grandis*.

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