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Full Length Research Paper

Using ectomycorrhizal inocula to increase slash pine (*Pinus elliottii*) growth in Southern China

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Bio-fertilizers are of great importance in forestry because they can positively affect plant growth and the environment. In this study, the seedlings of slash pine (*Pinus elliottii*) were planted in pots. One month later they were inoculated with 7 groups of ectomycorrhizal fungi including *Lactarius* sp., *Scleroderma polyrhizum* Pers., *Lactarius hatsudake* Tanaka, *Lycoperdon* sp., *Lycoperdon* sp./ *Lactarius* sp., *Lycoperdon* sp. / *Lactarius hatsudake* Tanaka and *Scleroderma polyrhizum* Pers./ *Lactarius* sp.. Plant growth variables were determined after three months. The results showed that: (1) all inocula significantly increased the ground diameter and plant height and strengthened the overall absorption of nitrogen, phosphorus and potassium; (2) the best inocula mixture was *Lycoperdon* sp. / *Lactarius hatsudake* Tanaka, likely due to synergistic interactions between the two partners and (3) mixed ectomycorrhizal fungi were not necessarily better than single fungus.

Key words: Slash pine (*Pinus elliottii*) growth, ectomycorrhizal inocula, bio-fertilizers, interaction, mixed ectomycorrhizal fungi.

INTRODUCTION

The mycorrhizal symbiosis is one of the most important mutualisms on earth since most vascular plant species are associated with either arbuscular or ectomycorrhizal fungi (Allen, 1991). The hyphae of ectomycorrhizal fungi can form closely interwoven mantles on the surface of feeder roots of host plants and Hartig nets between root cells. In the fungal-root symbiosis, carbons are transferred from the roots to the fungal symbiont, while the roots obtain minerals and water from the soil facilitated by the fungi (Allen, 1991; Deacon, 1997). The symbiotic nutrient exchange plays a crucial role in the resistance of plants against pathogens, heavy metals and drought stress etc. This interaction helps maintain and/or improve ecological balance (Müller et al., 2006).

Slash pine (*Pinus elliottii*), one of the most important commercial timber and plantation species, has played a vital role in afforestation in south China. Recently, problems such as degradation of soil structure, reduced water potential and increased soil erosion have negatively impacted plant growth. To increase wood supply, improve the quality of wood and promote the growth of the plant, many methods like the application of chemical and organic fertilizers have been adopted. Although those fertilizers can promote the growth of the tree in short time, they are not abiding and economically feasible. Using biological methods may be environmentally and economically helpful resulting in a sustainable forestry system (Miransari and Bahrami, 2007).

Since bio-fertilizer is of great concern in forestry, the development of mycorrhizal fertilizer is urgent. In this paper, the effect of ectomycorrhiza on slash pine (*P. elliottii)* is evaluated to identify the optimal ectomycorrhizal fertilizer for this tree species.

MATERIALS AND METHODS

Strain inocula production

The 4 strains of ectomycorrhizal fungi, named LGD-2, La-HZ3, LS3

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Table 1. Mean comparisons of ground diameter and plant height in different groups.

Group	Ground diameter(mm)	Plant height(cm)
C1	5.90±0.06[f]	25.23±0.32[cd]
C2	6.23±0.01[f]	26.13±0.34[bc]
C3	6.03±0.03[e]	26.37±0.27[b]
C4	7.89±0.10[a]	25.43±0.43[bcd]
C5	6.94±0.06[d]	27.73±0.35[a]
C6	6.68±0.02[c]	25.00±0.10[d]
C7	7.38±0.06[b]	28.30±0.42[a]
C8	6.54±0.08[d]	27.97±0.19[a]

C1:control,C2:La-HZ3,C3: LGD-2, C4: RHG1, C5:LS3,C6: LS3/La-HZ3,C7:LS3/RHG1, C8: LGD-2/La-HZ3 Values within the same column followed by different letter(s) are statistically different using protected Least Significant Difference (LSD) test at *P*=0.05.

and RHG1 identified as *Scleroderma polyrhizum* Pers., *Lactarius sp*., *Lycoperdon sp*. and *Lactarius hatsudake* Tanaka respectively，were cultured in modified PDA media (Potato 200 g, Glucose 20 g, Agar 20 g, $MgSO_4 \cdot 2H_2O$ 0.6 g KH_2PO_4 1.5 g, Water 1 L and Peptone 1 g) at 26 °C for 2 weeks. Five pieces of each fungal strain obtained with a sterilized 5 cm diameter puncher were put into 300 ml Erlenmeyer flask containing 100 ml of modified PDA media (without agar). Each of the four strains LGD-2, La-HZ3, LS3 and RHG1 had 5 replicates each. These cultures were maintained at 28 °C and 180 rpm for 2 weeks. Inocula solution was prepared after cultivation for 2-weeks.

Slash pine seedling treatment

The red soil was collected in Central South University of Forestry and Technology and sterilized at 121 °C for an hour with an autoclave and transferred to 10-kg round pots with a diameter and height of 20 cm. According to ground diameter and plant height, we selected similar seedlings (without ectomycorrhiza on their roots) from Zijinshan national forest farm of Hunan province and planted them in the pots. 24 seedlings were randomly selected for the experiment one month later.

Inoculation methods

By confront culture test, it was the three combinations LS3/RHG1, LGD-2/La-HZ3 and LS3/La-HZ3 that had synergistic interaction between them (Li et al., 2011). 100 ml fermentation broth of LGD-2, La-HZ3, LS3 and RHG1 were used and then placed in 5-cm depth under surface in the pots with 3 replicates, respectively. Concerning LS3/RHG1, LGD-2/La-HZ3 and LS3/La-HZ3, 50 ml of fermentation broth was used from each of the six strains to create the three combinations. They were then inoculated to the seedlings as before. Pots were watered to their maximum water holding capacity until the excess water started running out. The pots were kept in the greenhouse with natural light for 3 months.

Measurements of plant growth parameters

Plant height (cm) and ground diameter (mm) were determined at the beginning and end of the experiment. At harvest time, nitrogen (N) phosphorus (P) and potassium (K) in roots, stems and needles were measured. Nitrogen was measured by the Kjeldahl method (Nelson and Sommers, 1973). Phosphorus was determined using sodium bicarbonate extraction (Olsen, 1954). Potassium was measured by flame photometer (emission spectrophotometry) (Knudsen et al., 1982).

Statistical analysis

Using SPSS 17.0 data were analyzed and the statistical significances of the observed differences between treatments were obtained. We compared the means using the least significant difference (LSD) test at P = 0.05 (Steel and Torrie, 1980).

RESULTS

Effects of different inocula on ground diameter and plant height

At harvest, ground diameter and plant height of all groups increased. The biggest increase (Table 1) on ground diameter, as compared with C1 (without ectomycorrhizal fungi), was the C4 treatment (by 33.75%). Although treatment C7 was not the highest, its increase was comparatively high (by 25.20%). The plant height increased the most in treatment C7, by 12.15% over the control. The results showed that ectomycorrhizal fungi differentially affected the growth of slash pine trees.

Effects of different inocula on nitrogen, phosphorus and potassium

Compared with C1 (Table 2), all inocula significantly increased nitrogen content in roots, and the highest was treatment C7 (by 28.05%). The biggest increase was in stems, in treatments C3 and C7 (by16.19 and 15.71%, respectively). In needles, the respective increases over C1 for treatments C2, C3, C4, C5, C6, and C7 were 32.99%, 13.27%, 35.72%, 35.83%, 27.63% and 28.02%.

The highest phosphorus content in roots was found in treatment C3 (60.78% increase over C1), followed by C7 (56.86%).The phosphorus content in stems of treatment C7 was much higher (by 61.84% over C1) than others (Table 3). However, in needles, the highest increase was in treatment C5 (by 44.90%), followed by treatment C7 (by 36.73%). The lowest phosphorus content in roots was obtained in treatment C5 rather than in C1. In general, the treatments with ectomycorrhizal fungi strengthened the ability of slash pine to absorb phosphorus.

The ectomycorrhizal fungi can affect the absorption of potassium in roots, stems and needles.The potassium level (Table 4) in all treatments improved significantly over C1, with treatment C7 (by 32.22%) that resulted in the highest increase in roots. In stems, the highest content was in treatment C7 (by 36.63%), while in needles, the highest content was in C2 (by 35.29%), followed by C7 (by 31.53%).

Table 2. Mean comparisons of nitrogen by different inocula.

Group	N (%)		
	Root	Stem	Needle
C1	0.535±0.005^{g}	0.490±0.012^{d}	0.927±0.022^{e}
C2	0.614±0.008^{d}	0.559±0.017^{a}	1.232±0.011^{a}
C3	0.658±0.010^{b}	0.569±0.005^{a}	1.050±0.034^{d}
C4	0.635±0.016^{c}	0.520±0.009^{b}	1.258±0.014^{a}
C5	0.580±0.012^{e}	0.511±0.010^{b}	1.259±0.009^{a}
C6	0.560±0.013^{f}	0.521±0.008^{b}	1.183±0.004^{b}
C7	0.685±0.006^{a}	0.567±0.005^{a}	1.186±0.005^{b}
C8	0.559±0.008^{f}	0.505±0.008^{bc}	1.152±0.005^{c}

C1:control,C2:HZ3,C3: LGD-2,C4: RHG1,C5:LS3,C6: LS3/La-HZ3,C7:LS3/RHG1,C8: LGD-2/La-HZ3 Values within the same column followed by different letter(s) are statistically different using protected Least Significant Difference (LSD) test at P = 0.05.

Table 3. Mean comparisons of phosphorus by different inocula.

Group	P (%)		
	Root	Stem	Needle
C1	0.017±0.001^{bc}	0.253±0.002^{e}	0.033±0.002^{e}
C2	0.020±0.004^{b}	0.031±0.002^{cd}	0.039±0.003^{cd}
C3	0.027±0.002^{a}	0.030±0.003^{d}	0.042±0.003^{bc}
C4	0.015±0.001^{c}	0.037±0.002^{ab}	0.036±0.003^{de}
C5	0.019±0.004^{bc}	0.035±0.005^{bc}	0.047±0.002^{a}
C6	0.019±0.003^{bc}	0.016±0.001^{f}	0.034±0.003^{e}
C7	0.027±0.003^{a}	0.041±0.002^{a}	0.045±0.002^{ab}
C8	0.019±0.002^{bc}	0.034±0.003^{bcd}	0.036±0.002^{de}

C1:control,C2:HZ3,C3: LGD-2,C4: RHG1,C5:LS3,C6: LS3/La-HZ3,C7:LS3/RHG1,C8: LGD-2/La-HZ3 Values within the same column followed by different letter(s) are statistically different using protected Least Significant Difference (LSD) test at P = 0.05.

The best inoculum

Mixed strain inocula did not necessarily perform better than those with single strain inoculum (Table 1). For example, treatment C7 did not result in better performing plants as treatment C4 concerning ground diameter, and treatment C3 was superior to treatment C6 with respect to plant height. In general, treatment C7 was the ideal mixture for promoting the growth of slash pine.

DISCUSSION

Effects of ectomycorrhiza on growth

Different ectomycorrhizal fungi inocula can differently affect slash pine growth. These fungi can form external mycelia in soil and improve the roots' ability to water uptake and mineral nutrients, which is helpful to photosynthesis (Wiemken and Boller, 2002). Furthermore, ectomycorrhizal roots enhance nutrient uptake by increasing their absorptive area (Leyval and Berthelin, 1991). The best inocula combination was that of treatment C7, and the reason may be that the two ectomycorrhizal fungi act synergistically. The mechanisms involved in the interactions between the two ectomycorrhizal fungi are not well known yet. However, with the help of new techniques such as PCR, isotopic and molecular methods, the mechanism have become possibly understood (Hahn and Mendgen, 2001).

Nitrogen, phosphorus and potassium uptake and transfer in slash pine

Forest species are usually associated with ectomyc orrhizal fungi which can utilize any of the three N sources (ammonium, nitrate and amino acids) in pure culture (Abuzinadah and Read, 1988; Chalot and Brun, 1998; Finlay et al., 1992; Quoreshi et al., 1995;). Plants with no mycorrhiza may use a narrower range of N sources than those with mycorrhiza (Boukcim and Plassard, 2003).

The nitrogen content in roots, stems and needles, as compared with control, all increased, which has something to do with N transporters including nitrate, ammonium, amino acid, di - tri-peptide and oligopeptide transporters

Table 4. Mean comparisons of Potassium by different inocula.

Group	K (%)		
	Root	Stem	Needle
C1	0.787±0.069[f]	0．784±0.006[d]	1.015±0.004[c]
C2	0.861±0.014[d]	0.738±0.005[e]	1.374±0.010[a]
C3	0.862±0.010[cd]	0.968±0.003[b]	1.000±0.001[cd]
C4	0.847±0.018[d]	0.815±0.024[d]	0.986±0.005[d]
C5	0.884±0.006[bc]	0.965±0.003[b]	0.865±0.007[f]
C6	0.892±0.006[b]	0.729±0.032[e]	0.954±0.003[e]
C7	1.041±0.019[a]	1.071±0.009[a]	1.331±0.017[b]
C8	0.812±0.004[e]	0.855±0.003[c]	0.946±0.008[e]

C1: control, C2: La-HZ3, C3: LGD-2, C4: RHG1, C5: LS3,C6: LS3/La-HZ3,C7:LS3/RHG1, C8: LGD-2/La-HZ3; values within the same column followed by different letter(s) are statistically different using protected least significant difference (LSD) test at P=0.05.

with which plants can obtain N from ectomycorrhizal fungi (Müller et al., 2006). In this experiment, while treatment C7 mixed with LS3 and RHG1 may produce more N transporters that can help plant absorb N, treatments C6 (a mixture of LS3 and La-HZ3) and C8 (a mixture of LGD-2 and La-HZ3) may reduce some N transporters, presumably because of induction by each fungus. The mechanism that N transporters are induced by ectomycorrhizal fungi needs further study.

There exists much P in soil, but the majority of them are insoluble (Hagerberg et al., 2003). Plants can absorb soluble P quickly and completely from the soil around the root, thus generating an area without soluble P. However, the hypha can extend beyond the area to obtain soluble P. Moreover, ectomycorrhizal fungi may also excrete organic acids that can convert insoluble P into soluble P, facilitating the P absorption for plants (Unestam and Sun, 1995). Compared with non-mycorrhizal seedlings, the seedlings with ectomycorrhizal fungi can take up more K from biotite (Wallander, 2000b; Wallander and Wickman, 1999;). In this experiment, the ectomycorrhizal fungi may have enhanced P and K releases from the mineral. The increased P and K contents are consistent with the results in laboratory experiments (Wallander et al., 1997; Wallander, 2000a, b; Wallander and Wickman, 1999). When two fungi are mixed together, the results can be substantially different, generating patterns consistent with synergistic, antagonistic or additive interaction between them. With technology developing, the mechanism may be explained clearly in the coming years.

Conclusion

It is clear from our results that mixed ectomycorrhizal fungi do not always perform better than single one. However, appropriate mixing can promote slash pine growth, including increased nitrogen, phosphorus and potassium contents among different parts of plants. The LS3/RHG1 (*Lycoperdon* sp. /*Lactarius hatsudake* Tanaka) is the best inoculum among the tested, which should provide the basis for bio-fertilizer research to enhance slash pine growth in Southern China.

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