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Full Length Research Paper

The response of ectomycorrhizal (ECM) fungi under water stress induced by polyethylene glycol (PEG) 6000

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The growth models, diameter growth rates and biomass yield of three ectomycorrhizal (ECM) fungi, *Suillus tomentosus* (Kauff.) Sing. Snell and Dick, *Suillus laricinus* (Berk.in Hook.) O. Kuntze and *Aminita vaginata* (Bull.: Fr.) Vitt., were investigated under water stress induced by polyethylene glycol-6000 (PEG-6000) in pure culture. The results showed that the growth models of the three ectomycorrhizal fungi were not significantly affected by water stress, but the growth rates and biomass were changed. Lower water stress (-0.15 MPa) could stimulate the growth of three ectomycorrhizal fungi, but greater water stress restricted growth, and inhibitory effect became greater with the increasing water stress from -0.30 to -1.37 MPa. Their drought resistance was ordered by *S. laricinus*>*S. tomentosus*>*A. vaginata*. With the increasing water stress (-0.02 ~ -0.73 MPa), the contents of gibberellin (GA) and auxin (IAA) in mycelium reduced from 26.53 to 0 μg g^{-1} and 180.98 to 0 μg g^{-1} respectively. *S. laricinus* accumulated the most (184.36 μg g^{-1}) abscisic acid (ABA) at -0.30 MPa; when water stress was beyond the tolerance of *S. laricinus* (-0.49 MPa); however, it inhibited the accumulation of abscisic acid (ABA).

Key words: Abscisic acid, ectomycorrhizal fungus, phytohormone, water stress.

INTRODUCTION

Drought is considered one of the most important stressful environmental conditions that is a constrain to plant survival in arid and semi-arid regions (Chaves et al., 2003). Enhanced osmotic adjustment and leaf hydration of host plant and reduced oxidative damage or improved nutritional status have been proposed to explain the contribution of mycorrhizal fungi symbiosis to the drought resistance of host plants (Alguacil et al., 2003; Augé, 2001; Porcel and Ruiz-Lozano, 2004; Querejeta et al., 2003). Mycorrhiza improves the assimilation of water and nutrition for plants, and enhances resistant ability to drought, pathogens and heavy metals (Ahonen-Jonnarth, 2000). It promotes plant growth, increases crop yield and improves the tolerance to drought, saline and alkaline stress by strengthening nutrient absorption (Smith and Gianinazzi-Pearson, 1988).

Morphological and physiological characters of host plant roots are changed after symbiosis with mycorrhizal fungi, which improves plants survival under drought stress. Moreover, many mycorrhizal fungi can stand up to drought stress and high temperature (Han et al., 2006). A lot of ectomycorrhizal fungi have the maximal growth under water potential of -0.5 ~ -1.5 MPa, some species even grow well under water potential of -2.0 MPa (Zhao and Guo, 1989). For example, *Cenococcum geophilum* can form mycorrhiza with many trees under extreme drought condition, and endure low water potential of medium (Mosse, 1959). Besides, some ectomycorrhizal fungi excrete phytohormone to elevate drought resistance, which has a distinct function in dry areas (Han et al., 2006). Inoculation with mycorrhizal fungi could increase the content of auxin (IAA), gibberellin (GA) and cytokinin (CTK) in plants, but decrease abscisic acid (ABA) and ethylene, which are significant for water metabolism of plants (Cruz et al., 2000). In particular, plants inoculated with mycorrhizal fungi reduce the content of abscisic acid (ABA) to raise the utilization rate of water (Barker and Tagu, 2000; Ruiz-Lozano, 2003).

Osmotic solutions are used to impose water stress reproducibly in "*in vitro*" conditions (Pandey and Agarwal, 1998). Polyethylene glycol molecules with a Mr≥6000 (PEG-6000) are inert, non-ionic and virtually impermeable chains, that have frequently been used to induce water

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stress and maintain a uniform water potential throughout the experimental period (Berg and Zeng, 2006). Ectomycorrhizal fungi (ECMF) growth under drought stress is reported (Xu et al., 2008), but the report on phytohormone secretion of ECMF under different water stress *in vitro* is not available now. According to what has been discussed above, the objectives of this work, therefore, were:

(a) To determine the growth rate, biomass and growth models of the three species of ECMF (*Suillus tomentosus*, *Suillus laricinus* and *Aminata vaginata*) under water stress.
(b) To determine whether the most tolerant strain produces phytohormone, auxin, gibberellin, abscisic acid and cytokinin, to quantify the levels of phytohormone produced under different water stress in pure culture conditions.

MATERIALS AND METHODS

Fungal species

Three species of ectomycorrhizal fungi (ECMF), including *S. tomentosus* (Kauff.) Sing. Snell and Dick (Abbreviation for ST), *S. laricinus* (Berk. in Hook.) O. Kuntze (Abbreviation for SL) and *A. vaginata* (Bull.: Fr.) Vitt. (Abbreviation for AV), were used in the present study.

Effect of water stress on the growth rates of ECMF

The influence of drought stress on the diameter growth was investigated by cultivating the isolates in a modified Melin-Norkans medium (MMN) amended with different PEG-6000 concentrations (0, 10, 15, 20, 25, 30 and 35%), producing a corresponding water potential with -0.02, -0.15, -0.30, -0.49, -0.73, -1.03 and -1.37 MPa. MMN medium containing: glucose(10 g), agar(15 g), KH_2PO_4(300 mg), $(NH_4)_2HPO4$(500 mg), $MgSO_4 \cdot 7H_2O$(140 mg), NaCl (25 mg), $CaCl_2 \cdot 6H_2O$(50 mg), FeNaEDTA (12.5 mg), thiamine (0.01 mg), $ZnSO4 \cdot 7H_2O$(3 mg), streptomycin(15 mg), tetracycline(12 mg) and gentamycin(15 mg) per litre. MMN medium without PEG-6000 was set as control. 20 ml solidified modified, Melin-Norkans medium was poured into 9 cm diameter Petri dishes.

As it became cool and frozen, into it we poured 20 ml solution (sterilized at 121℃, 0.5 MPa for 20 min) with different concentrations of PEG-6000, and placed them in super clean bench for 48 h. Then, we spilled the PEG-6000 solution to get different concentrations of stress culture medium (Li et al., 2008). For each colony, radii were measured on two perpendicular axes bisecting the center of the colony and the growth rates and inhibitory efficiency were calculated, with inhibitory efficiency (I) = (V0−V/V0) × 100%, V0 representing the growth rate of control and V representing the growth rate under drought stress.

Effect of water stress on the biomass of ECMF

25 g sterile grit was paved on the button of each Petri dish, and then a piece of sterile filter paper was placed on the grit. 25 ml liquid MMN was poured into the Petri dish (with different water potential -0.02, -0.15, -0.30, -0.49, -0.73 and -1.03 MPa), which just made the filter paper submerged (Xu et al., 2006). Cultures were incubated at 25℃ in the dark for 10 days. Mycelial mats were removed from the liquid media, rinsed 3 times with distilled water, dried overnight at 80℃ and weighed. Inhibitory efficiency was calculated with (I) = (representing W0−W/W0) × 100%, W0 representing the biomass of control and W representing the biomass under water stress.

Effect of water stress on the phytohormone secretion

We chose the most tolerant strain (with the lowest inhibitory efficiency) under water stress, according to the results of effects of water stress on the growth of ECMF. 100 ml liquid MMN was poured into the 250 ml Erlenmeyer flasks (with the same water potential). Three discs of inoculums (5 mm diameter) for each erlenmeyer flask were inoculated into the liquid MMN for 30 days on a rotary shaker (25℃, 120 rpm) and each treatment was replicated three times. Vacuum filtration was used to get the mycelium cultivated in different water potential medium. The mycelium was rinsed with distilled water 3 to 5 times, and then the filter paper was used to absorb water. Mycelial mats were ground in a mortar with a pestle on ice containing 5 ml pre-chilled 80% methanol (v/v) for 10 min, and then transferred to the grown reagent bottles, all of which were carried out under dim light.

After adding 20 ml pre-chilled 80% methanol, the bottles were put at 0°C for 40 h, and were shaken for 5 min every 2 h. The mycelial mats were filtered and concentrated by evaporation under low pressure at 40℃ using a rotary evaporator, and the residue was dissolved in 3 ml mixed solution (Vacetonitrile:Vwater = 1:4). The solutions were adjusted to pH 3.0 with phosphoric acid, and then loaded onto C18 Sep-Pak Colum Ella primed with 4 ml methanol followed by 2 ml phosphoric acid at pH 3.0. After loading, the Sep-Paks were washed with 2 ml glacial acetic acid of pH 3.0 and eluted with 4 ml 40% methanol, containing 2% glacial acetic acid (Wang et al., 2008). The efflux was dried by freezing in a high vacuum system, later dissolved with 1 ml 35% methanol containing 0.15% (v/v) 0.1 $molL^{-1}$ phosphoric acid. Filtering through the 0.45 μm millipore filter, the solution was ready for high efficiency liquid chromatography. 6 bottles of mycelium were mixed together to determine the phytohormone for each water potential. Another 6 bottles of mycelium were dried to calculate the biomass of mycelium and inhibitory efficiency (Huang et al., 2008). Inhibitory efficiency was calculated:

(I) = (W0−W/W0) × 100%

Where, W0 representing the biomass of control and W the biomass under water stress.

Phytohormone determination

High efficiency liquid chromatography was employed to detect auxin (IAA), gibberellin (GA), zeatin (Z) and abscisic acid (ABA) in samples.

Data analysis

In this work, the results were statistically tested by two-way analyses of variance (ANOVAs) using SAS version 8.1 software package (SAS Institute Inc., Cary, NC, USA). Comparison between means was carried out using Duncan’s multiple range tests. Graphical work was carried out using Sigma Plot Version 10.0 software.

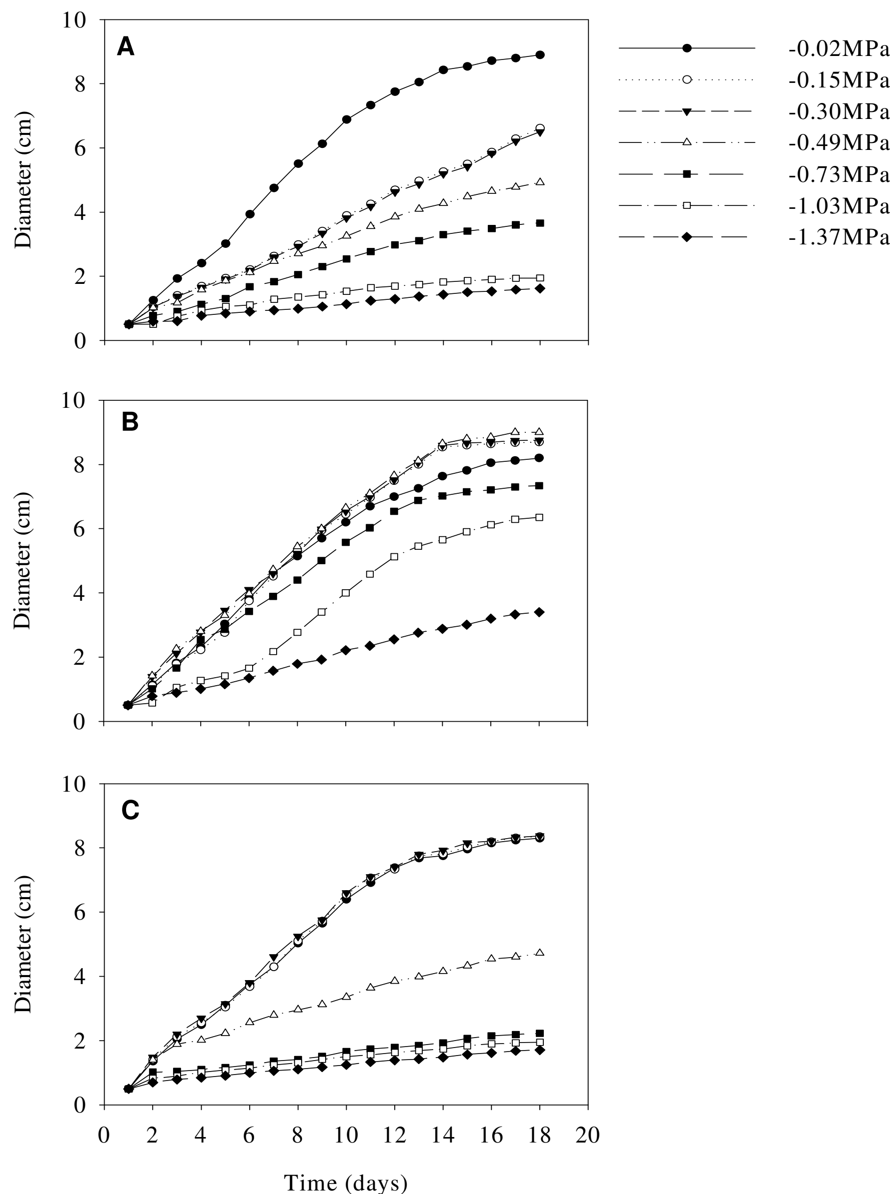

Figure 1. Dose-response curves for the growth models of three ECMF species under water stress (-0.02 ~ -1.37 MPa) solidified MMN medium during 18 days. (A) *S. tomentosus*, (B) *S. laricinus*, (C) *A. vaginata*.

RESULTS

Effect of water stress on the growth rates

The growth rates of all three species of ECMF were significantly different (P<0.05) from the control under water stress, but there was no significant difference between -0.15 and -0.30 MPa. The inhibitory efficiency of ST was from 25.7 to 81.8%. The growth rates of AV were not significantly different (P>0.05) from the control at -0.15 MPa, and the inhibitory efficiency was -0.6% (Figures 1 and 2). From the results, it showed that SL was the strongest resistance species.

Effect of water stress on the biomass

The biomass of ST was not significantly different (P>0.05) from the control at -0.15, -0.30 and -0.49 MPa respectively. There was a promotion to ST at -0.15 MPa. The biomass of ST was significantly different (P<0.05) from the control at -0.73 and -1.03 MPa. The biomass of SL was significantly different (P<0.05) from the control at -0.15 MPa, and the inhibitory efficiency was -7.8%; the

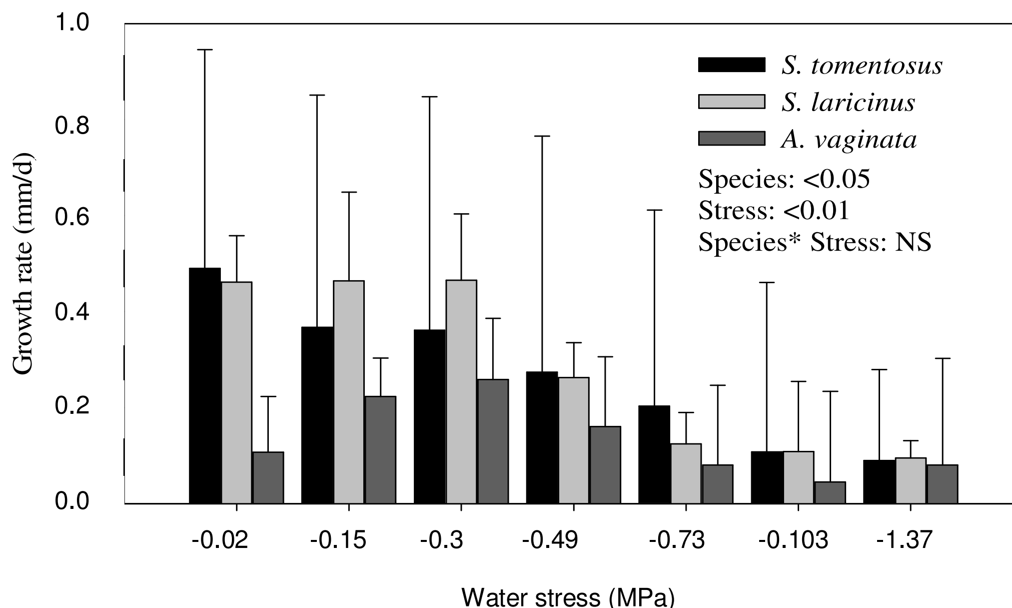

Figure 2. Growth rate of three ECMF growing under water stress solidified MMN medium (means ± SD, n=3) during 18 days. Values are means and SD for each water stress determined by Duncan's multiple-range test.

biomass was not significantly different (P>0.05) from the control at -0.30 and -0.49 MPa, and the inhibitory efficiency was -4.5 and -1.6%; the biomass of SL was significantly different (P<0.05) from the control at -0.73 and -1.03 MPa, and the inhibitory efficiency was 15.7 and 34.7%.

The biomass of AV was not significantly different (P>0.05) from the control at -0.15 MPa, besides there was a growth promotion and the inhibitory efficiency was -5.8%; the biomass of AV was significantly different (P<0.05) from the control at -0.30, -0.49, -0.73 and -1.03 MPa, and the inhibitory efficiency was 30.3, 65.9, 79 and 100% (Figures 3 and 4). From the results, their drought resistance was ordered: SL>ST>AV.

Effect of water stress on the growth model of ECMF

Non-linear fitting analysis was made for three species according to the effects of drought stress on the growth rates of ECMF (Figure 5). All ECMF species expressed typical exponential growth models at all water potential situations (Table 1), but the characters of growth were different. The growth slopes of the three species varied under different drought stress. The growth rates of three species decreased distinctly at greater drought stress, and their adaptive phase (from the beginning of inoculation to linear growth phase) extended.

Compared with the control at -0.15, -0.30, -0.49, -0.73, -1.03 and -1.37 MPa, the growth rates of ST were 74.9, 73.7, 55.8, 41.5, 22.0 and 18.4%, respectively, that of SL were 106.1, 106.7, 107.9, 89.5, 76.4 and 41.6% respectively, that of AV were 100.6, 100.8, 56.9, 26.9, 23.5 and 20.7%, respectively. It was not obvious to restrain the growth of SL and AV at -0.15 and -0.30 MPa. The growth rate of SL declined the least at -0.49 ~ -1.37 MPa, which revealed the highest adaptability to drought stress. The diameter of SL reached 3.40 cm after cultivating 18 days at -1.37 MPa, increasing about 6 times, but ST and AV were just 1.62 and 1.71 cm. Drought only changed the growth rates and biomass of three ECMF, but did not alter their growth model.

Effect of water stress on the phytohormone secretion

The ability of SL to produce phytohormone was different under drought stress (Table 2). At lower drought stress (-0.02 ~ -0.15 MPa), abscisic acid (ABA) was not determined, and auxin (IAA) had the maximal content (180.98 μg g^{-1}) at -0.02 MPa, gibberellin (GA) had the maximal content (26.53 μg g^{-1}) at -0.15 MPa. With the increasing drought stress, phytohormone in mycelium decreased (except ABA). Auxin (IAA) had the maximum content among all phytohormone at lower stress, showing the strain had a major function to promote plant growth.

The contents of gibberellin (GA) reduced from 26.53 to 0 μg g^{-1} in mycelium, and that of auxin (IAA) reduced from 180.98 to 0 μg g^{-1}. The contents of gibberellin (GA) and auxin (IAA) decreased by -18.44 and 34.30% compared with the control at -0.15 MPa; the contents of gibberellin (GA) and auxin (IAA) decreased by 4.60 and

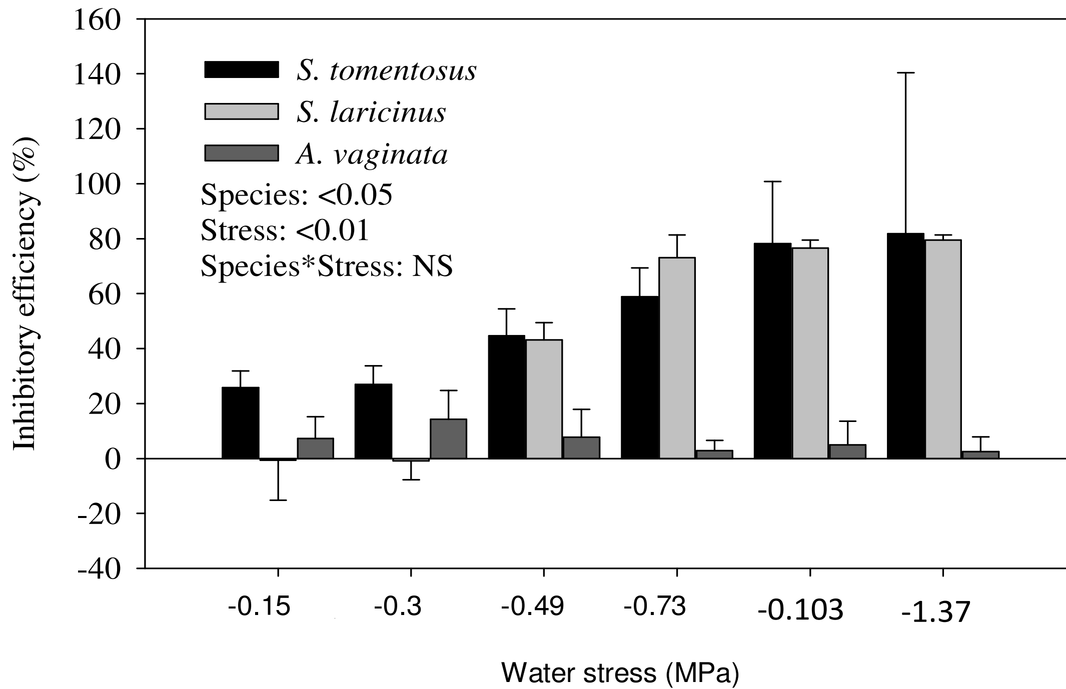

Figure 3. Inhibitory efficiency of three ECMF growing under water stress solidified MMN medium (means ± SD, n=3) during 18 d. Inhibitory efficiency (I) = (V0−V/V0) × 100%, V0 representing the growth rate of control and V representing the growth rate under drought stress.

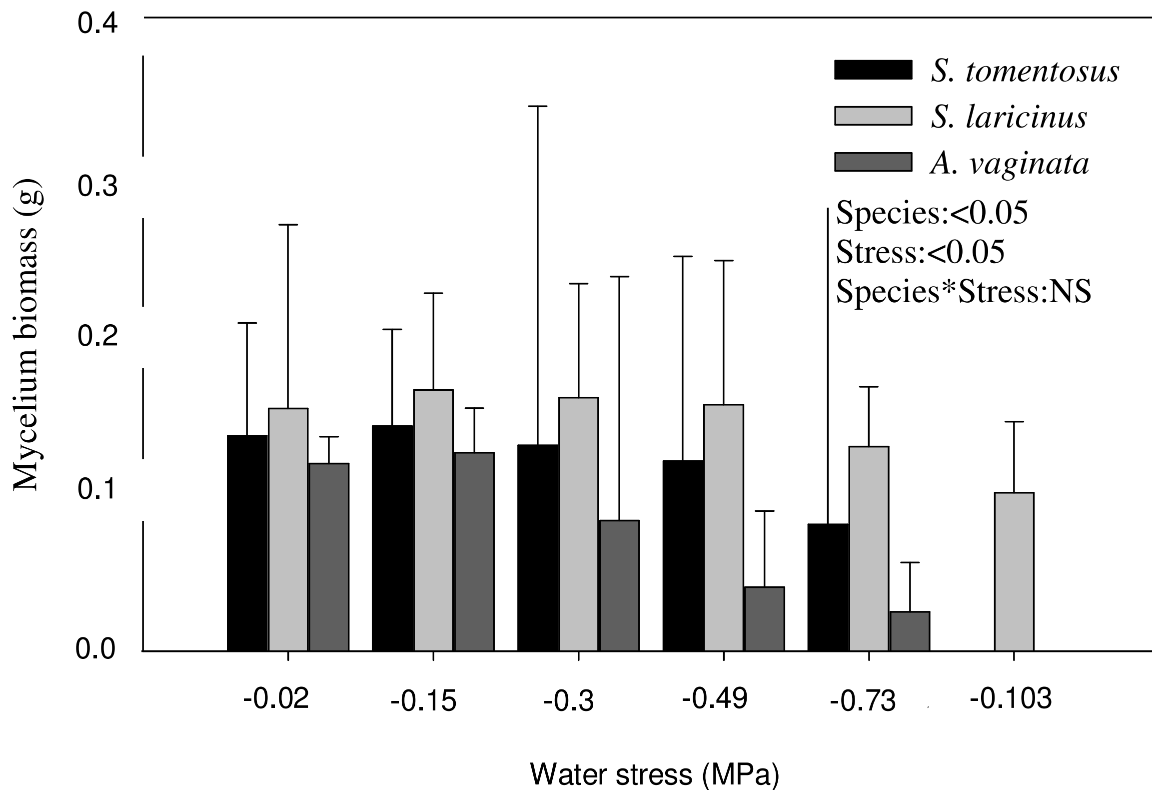

Figure 4. Mycelium biomass of three ECMF growing under water stress liquid modified MMN medium (means ± SD, n=3) during 10 d. Values are means and SD for each water stress determined by Duncan's multiple-range test.

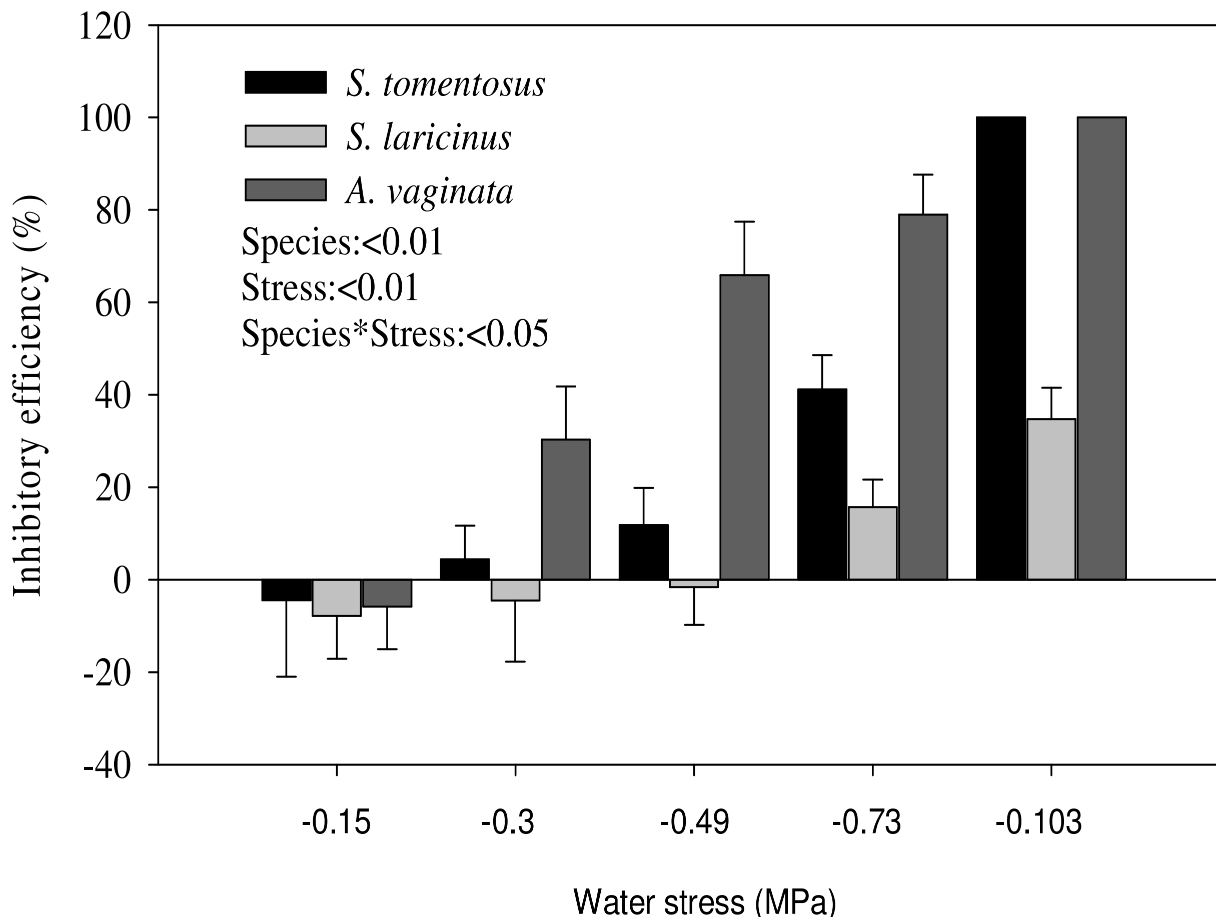

Figure 5. Inhibitory efficiency of three ECMF (ST, SL and AV) growing under water stress liquid modified MMN medium (means ± SD, n=3) during 10 days. Inhibitory efficiency (I) = (W0−W/W0) × 100%, W0 representing the biomass of control and W representing the biomass under water stress.

Table 1. Growth models of the three species of ECMF under water stress.

Water stress (MPa)	*S. tomentosus*	*S. laricinus*	*A. vaginata*
-0.02	D = -10.269/{1+exp[(t-6.11)/3.23]}+9.172, R2 = 0.999	D = -10.972/{1+exp[(t-4.992)/3.895]}+8.597, R^2 = 0.999	D = -9.774/{1+exp[(t-6.001)/3.464]}+8.676, R2 = 0.997
-0.15	D = -12.917/{1+exp[(t-9.083)/8.573]}+9.932, R2 = 0.998	D = -10.1/{1+exp[(t-6.552)/3.251]}+9.154, R2 = 0.998	D = -9.449/{1+exp[(t+6.222)/3.284]}+8.674, R2=0.997
-0.30	D = -12.472/{1+exp[(t-9.268)/8.368]}+9.723, R2 = 0.998	D = -12.201/{1+exp[(t-5.197)/4.27]}+9.596, R2 = 0.996	D = -10.015/{1+exp[(t+5.713)/3.494]}+8.763, R2=0.997
-0.49	D = -9.269/{1+exp[(t-4.153)/7.439]}+6.199, R2 = 0.999	D = -12.401/{1+exp[(t-5.394)/4.318]}+9.851, R2 = 0.997	D = -27.826/{1+exp[(t-15.545)/11.015]}+5.957, R2=0.987
-0.73	D = -4.3287/{1+exp[(t-6.9626)/4.3647]}+3.9917, R2 = 0.999	D = -9.576/{1+exp[(t-5.378)/3.87]}+7.837, R2 = 0.996	D = -9.88/{1+exp[(t-15.615)/16.356]}+3.357, R2 = 0.973
-1.03	D = -3.679/{1+exp[(t-0.01)/6.094]}+2.141, R2 = 0.995	D = -6.165/{1+exp[(t-9.058)/2.546]}+6.509, R2 = 0.998	D = -10.466/{1+exp[(t-17.95)/12.699]}+2.545, R2 = 0.989
-1.37	D=-3.233/{1+exp[(t-3.882)/10.96]}+2.335, R2 = 0.995	D = -4.352/{1+exp[(t-8.638)/5.401]}+4.07, R2 = 0.999	D = -10.873/{1+exp[(t-26.317)/19.557]}+2.733, R2 = 0.994

Growth model D = (A1-A2)/{1+exp[(t-t0)/td]}+ A2, D means colony diameter, A1 and A2 mean the original and the final diameter, t0 means the time to reach (A1-A2)/2, td means the duration of cultivating.

Table 2. Phytohormone contents of *S. laricinus* under water stress.

WT (MPa)	Phytohormone contents					
	Z	GA	IAA	ABA	DW	I (%)
-0.02	0	22.40	180.98	0	0.42	/
-0.15	0	26.53	118.91	0	0.37	11.90
-0.30	0	21.37	101.97	184.36	0.33	21.43
-0.49	0	2.38	11.90	24.82	0.09	78.57
-0.73	0	0	0	0	0.07	83.33
-1.03	/	/	/	/	0	100

WT represents water stress, DW represents dry weight of mycelium (g), / represents no mycelium grown in the culture filtrate, I= (W0−W/W0) × 100%, W0 representing the biomass of control and W representing the biomass under drought stress. Phytohormone contents means in 1 g dry mycelium (μg g^{-1}).

43.66% compared with the control at -0.30 MPa, and the content of abscisic acid (ABA) was 184.36 μg g^{-1}; the contents of gibberellin (GA) and auxin (IAA) decreased by 89.38 and 93.42% compared with the control at -0.49 MPa, and the content of abscisic acid (ABA) was 24.82 μg g^{-1}; gibberellin (GA) , auxin (IAA) and abscisic acid (ABA) were not determined at -0.73 MPa; the growth of SL was inhibited completely at -1.03 MPa. When drought stress was beyond the tolerance of the fungus (-0.49 MPa), it prohibited abscisic acid (ABA) accumulation.

DISCUSSION

In the present study, the results showed that all three species of ECMF have different responses to water stress in pure culture, indicating that it is feasible to choose the best strain for resisting drought by interspecies variations. PEG-6000 is considered as the best macromolecular compound to simulate drought stress, and it is widely used in water stress physiology and genetic mechanism for plants (Christmann et al., 2005; Verslues and Bray, 2004; 2006). The results show that lower drought stress (-0.15 ~ -0.30 MPa) has no negative influence on the growth of SL and AV, even has growth stimulation.

However, when it is at -1.03 MPa, the majorities of ST and AV are inhibited. Be it growth rate or biomass, SL could stand the highest drought stress, so it is an excellent drought-resistant strain. But the physiology of ECMF may be changed owing to the impact of plant roots, after the ECMF and plants formed mycorrhiza. As a result, it is necessary to make the ECMF infect plant roots and have a further study on their associating drought resistance capacity before applying SL to forestation in arid region. The growth response of ECMF to water potential shows two patterns. Type I, growth increases up to certain stress levels, beyond which growth decreases. Type II, growth does not exhibit a certain tendency (Xu et al., 2008). Gleason et al. (2006) predicated three basic patterns of growth response for fungi in different water potential. First, as water potential increases the fungus may not be affected until the response mechanisms are overwhelmed and growth ceases. Second, growth of the fungus may slow in response to increasing water potential. Finally, the fungus may be adapted to relatively high water potential, and growth will increase with increasing water potential until the response mechanisms are overwhelmed. The latter case may be exemplified by fungi that exist in arid environments.

In this study, ST agrees with the second prediction of Gleason, and SL and AV exhibit the type I pattern. Strategies for survival during periods of increasing drought stress have evolved in fungi. However, some fungi appear to be sensitive to drought stress, which may limit their distribution (Gleason et al., 2006). *In vitro* response to different water potential may vary among strains of the same species. Tolerance of higher water potential indicates that SL is more likely than ST and AV to be found in arid and semiarid environments. Drought could induce some stress proteins to cope with the environments, such as *BADH, OSM* and *LEA* genes (Wu and Xia, 2004). Whether SL could induce the stress proteins to resist drought is a worthy problem to discuss.

Mycorrhiza is a typical symbiotic connection between plant roots and mycorrhizal fungi. The phytohormone excreted by mycorrhizal fungi has a positive effect on the plant growth. The increased phytohormone plays an important role in water absorption, drought fighting and development for the host plant. It could increase the contents of zeatin (Z), gibberellin (GA) and auxin (IAA), and decrease the content of abscisic acid (ABA) for plant inoculated with mycorrhizal fungi under drought stress (Liu et al., 2000). The relativity between the content of ABA and stomatal resistance, represents that leaf water potential could be elevated in plants inoculated with mycorrhizal fungi by altering endogenesis hormone contents (Liu et al., 2000). ECMF could excrete different phytohormone *in vitro*. Active producers of IAA are found among pathogenic and mycorrhizal fungal species (Maor et al., 2004; Niemi et al., 2002; Yurekli et al., 2003). The ability to excrete phytohormone is different for different species of mycorrhizal fungi even for different strains with the same species (Wu and Ma, 2008). This would be

explained by the dependence on the symbiotic life style of the fungus and on the host plant (Tsavkelova and Bomke, 2008).

Phytohormone produced by mycorrhizal fungi could be released extracellularlly and they may expand into host plant tissue to play a role (Wang and Huang, 1997), but phytohormone produced by plants themselves is intracellular. Although, no traces of zeatin (Z) were detected in this experiment, it was the major CTK in plant and its synthesis position was root tip (Zhou et al., 2006). Root tip was an important area, in which the interaction between ECMF and host plant occurred. Therefore, mycorrhizal fungi may have a physiological effect on plant by accelerating plant phytohormone excretion or CTK to above ground part through root tip and xylem (Wu and Ma, 2008). ABA was examined under modest drought stress conditions, which indicated that mycorrhizal fungi could accumulate some ABA. But it was not detected under lower (-0.02 ~ -0.15 MPa) and higher (-0.73 MPa) drought stress, maybe ABA was secreted on the condition that appropriate drought stress occurred. ABA secretion was inhibited when the drought was beyond the tolerance of fungi. ABA secretion increased greatly at -0.30 MPa. It is necessary to detect the phytohormone secretion from -0.15 to -0.30 MPa. It was found that mycorrhizal fungi enhanced the contents of IAA, GA, CTK and ABA in stems and leaves, and this phytohormone may be the initiators of adverse gene expression which were able to induce new genes and proteins. The study conducted by Ma et al. (2009) also revealed that ABA played a direct role in the inducement of adverse proteins by studying the mutant of corn, and ABA had a control over the synthesis of many adverse proteins. Tsavkelova et al. (2008) also found some specific genes to induce phytohormone excretion in orchid-associated fungi. It is, therefore, essential to detect the phytohormone excretion gene in SL used in this work in the future.

The results showed that IAA and GA whose functions were to promote growth were excreted more than ABA whose function was to speed deterioration at lower water stress, which justified that myrorrhizal fungi could produce phytohormone to stimulate growth. With the increasing water stress, the contents of IAA and GA declined. SL accumulated ABA under water stress, showing that aridity restrained the growth of SL. At -0.49 MPa, the contents of all phytohormone were low because the growth of fungi was inhibited seriously. The present study was carried out in pure conditions; therefore, it is necessary to investigate the phytohormone contents in host plant inoculated with ECMF under water stress in the future.

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