



The effects of ectomycorrhizal inoculation on survival and growth of *Pinus thunbergii* seedlings planted in saline soil

Zhugui Wen^{1,2} · Jincheng Xing¹ · Chong Liu¹ · Xiaomei Zhu¹ · Baoquan Zhao¹ · Jing Dong¹ · Tingting He¹ · Xiaohui Zhao¹ · Lizhou Hong¹

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Abstract

Soil salinization is one of the important factors for agriculture and considered to be responsible for the loss of cultivated land worldwide. Ectomycorrhizal (ECM) fungi can improve the tolerance of their hosts to adverse conditions. Therefore, the objective of this study was to analyse the effects of three widespread and broad host range ECM fungi, *Laccaria amethystina* (La), *Pisolithus tinctorius* (Pt) and *Cenococcum geophilum* (Cg), inoculation on growth and nutrient uptake of *Pinus thunbergii* seedlings under salt stress. Results indicated that, under saline conditions, compared to non-ectomycorrhizal seedlings, ECM fungi inoculation, especially La, significantly increased seedling biomass, chlorophyll a, nutrient elements (like phosphorous, nitrogen, and potassium) in shoots, as well as maintained a low ratio of Na⁺/K⁺ in roots under salt stress. Inoculation with ECM fungi could assist the host to overcome salt stress, but the effectiveness of the symbiosis in salt conditions might depend on ECM species. In-depth study of the effects of different strains on the salt tolerance associated with different host plants will become an important direction for the research of subsequent ECM.

Keywords Salt stress · Ectomycorrhizal (ECM) fungi · Chlorophyll · Nutrient uptake · Reforestation

1 Introduction

Global water resources shortage, environmental pollution and soil and water salinization are the most pressing problems in the current century. And among them, soil salinization is a major limiting factor for agriculture, like crop productivity, in arid and semi-arid regions. Salinity is considered to be responsible for the loss of cultivated land worldwide, and the amount is increasing day by day (Chandrasekaran et al. 2014; Gupta and Huang 2014; Bencherif et al. 2015). It may directly or indirectly inhibit the growth, productivity and yield of the whole plant (James et al. 2011; Sinclair et al. 2014) by reducing its ability to uptake water and nutrients. One of the most harmful influences of salinity

stress to living plants planted in high Na⁺-content soil is the accumulation of Na⁺ in tissues, which leads to severe ionic imbalance and significant physiological disorders (Dagar and Tomar 2002; James et al. 2011; Gupta and Huang 2014).

Pinus plantations fail to establish without mycorrhizal fungi, especially the coevolved ECM fungi (pine-specific suilloid ECM fungi: *Suillus* and *Rhizopogon*) (Dickie et al. 2010), which was an important limitation to pine establishment and growth. *Pinus thunbergii* Parl., commonly known as Japanese black pine, grows naturally in Japan and Korea and has been introduced in China. It had been widely naturalized in sandy land owing to its feature of salinity and drought resistance, adapted to nutrient-poor environment with strong sand-fixing capacity. In addition, it can grow in difficult areas such as coastal zones (Kataoka et al. 2009) with no substitutes in the construction of warm temperate coastal shelterbelts. Additionally, like other pine species, *P. thunbergii* could be colonized by ectomycorrhizal (ECM) fungi easily and ECM symbiosis is necessary for pine trees. In the Tottori sand dune, Japan, Kataoka et al. (2008) found more than 90% of *P. thunbergii* seedling roots associated with ECM fungi, of which *C. geophilum* was most dominant species, indicating that ECM fungi, especially *C. geophilum*,

✉ Zhugui Wen
wenzhugui@163.com

✉ Lizhou Hong
ychonglz@163.com

¹ Jiangsu Coastal Area Institute of Agricultural Sciences, Yancheng 224002, Jiangsu, China

² Yancheng Teachers University, Yancheng 224007 Jiangsu, China

might play an important role in the growth of *P. thunbergii* planted in coastal areas. Besides, due to its melanin production, *C. geophilum*, was considered to be drought tolerant and abundant in water-stressed habitats (Fernandez and Koide, 2013).

Soil microbes are essential components of forest ecosystems, among which ECM fungal species are especially important for their beneficial ability of forming symbiotic association, ectomycorrhizae, with host plants. Recent researches showed that soil carbon sequestration (Clemmensen et al. 2013; Averill et al. 2014), tree population dynamics (Bennett et al. 2017) and mitigation of CO₂ fertilization (Terrer et al. 2016) have recently been linked to ectomycorrhizae, which play an important role in host plant growth and terrestrial ecosystems (Smith and Read 2008; Bennett et al. 2017). Estimates of ECM fungal species suggest that there are c. 20,000 ECM fungi associated with c. 6000 plant species (Rinaldi et al. 2008; Tedersoo et al. 2010, 2012). Therefore, ECM fungi are ubiquitous in forested soils, including saline ones (Taniguchi et al. 2007; Ishida et al. 2009; Matsuda et al. 2009b; Botnen et al. 2015). In addition, ECM fungi can acquire large amounts, which sometimes represent up to 80% of plant N and 75% of plant P (Simard et al. 2002; Read and Perez-Moreno 2003; Hobbie and Hobbie 2006), and can improve the survival and tolerance of their hosts to adverse conditions like biotic (e.g. pathogen attack) and abiotic (e.g. drought, salinity, heavy metals) stresses (Brazanti et al. 1999; Adriaensen et al. 2003; Sousa et al. 2012; Fernandez and Koide, 2013; Herzog et al. 2013; Ma et al. 2014; Chen et al. 2015).

Compared to some specific ones, many ECM fungi have a broad host range (Molina et al. 1992) including three ECM fungi, *Laccaria amethystina* (La), *Pisolithus tinctorius* (Pt) and *Cenococcum geophilum* (Cg). These three ECM fungal species are widespread, and among them, *L. amethystina* and *C. geophilum* could associate with a broad variety of hosts including *Quercus*, *Pinus*, *Populus*, *Betula* and *Castanea*, could associate with many *Pinus* species. Besides, the effects of them on host plant salinity tolerance have been confirmed in many studies (Carney and Chambers 1997; Ashkannejhad and Horton 2006; Taniguchi et al. 2007; Roy et al. 2008; Obase et al. 2009; Matsuda et al. 2009a, b; Vincenot et al. 2012). Additionally, under laboratory conditions, these species could form ectomycorrhizae with host plants easily

(Martin et al. 2002; Park et al. 2006; Douhan et al. 2007; Sebastiana et al. 2013; Chen et al. 2015; Shi et al. 2017; Wen et al. 2017; Zhang et al. 2019). Because of their ubiquity in forests, grow rapidly, wide geographic distribution, large host range, easily cultivated and readily form ECM associations, we intentionally selected three ECM fungal species as Shi et al. (2017) studied (Three ECM fungal isolates: *C. geophilum*, KY075873; *P. tinctorius*, KY075875 and *L. amethystina*, KY075878) to inoculate *P. thunbergii* to assess their potential in improving the growth of host seedlings planted in saline soil, expecting some common patterns that imply inoculation may have broad applications in forestry reconstruction under salt stress along coastal areas.

2 Materials and methods

2.1 Soil collection

Soils for the pot experiments were collected from Jinhai Farm (32°59'N, 120°49'E), located southeast of Dafeng, Yancheng (Jiangsu, China). We collected soil from the cultivation layer (0–20 cm) from saline and conventional (normal) soils located more than 10 km apart. The chemical properties of the two soil types for the pot experiments are summarized in Table 1.

Pot experiments.

Seedlings were prepared as Wen et al. (2017) described. Surface-sterilized Japanese black pine seeds (Obtained from College of Life Sciences, Nanjing Agricultural University) were soaked overnight in demineralized water and surface-sterilized in 30% H₂O₂ for 15 min, and were germinated in 500-mL pots containing a mixture of vermiculite, soil, and sand (v/v/v = 1/1/1), which had previously been autoclaved at 120 °C for 2 h. The pots were cultivated in a clean room with a filter system in a glass greenhouse at 25 °C/20 °C during the day/night under natural light conditions. And we generally followed the protocols described in Wen et al. (2017) for ECM fungi inoculation. The mycelial culture was prepared by pure cultivation of the fungus in 20 mL of MMN medium for one month at room temperature in the dark. Then, the mycelium was separated from the broth medium and washed several times with distilled water. The mycelium was placed in 250-mL flasks containing 50 mL

Table 1 Chemical characteristics of normal and saline soils

Soil	Number of survival seedlings				pH (H ₂ O)	Cond	OM	Available N	Available P	Available K
	NM	Pt	Cg	La						
Normal	9	8	10	9	6.98	0.59	1.98	85.41	19.38	1.02
Saline	8	7	7	9	8.19	1.27	0.56	38.32	42.52	3.35

Cond: Conductivity; OM: Organic Matter

of distilled water and blended to prepare a slurry. In total, 25 mL of slurry containing mycelium was added near the seedling roots. After 100 days inoculation, the seedlings for the pot experiments, based on inoculation rates and growth, were chosen. The percentage of ECM fungal colonization was determined by the proportion of root tips with ECM to total fine root tips per seedling. Except the control seedlings, the inoculation rate ranged from 55% to 82% (Data not shown) with no significant differences among the seedlings ($F = 0.235$, $p = 0.792$). They were approximately the same as the differences between the non-ectomycorrhizal (NM) control and the ECM-inoculated seedlings were negligible, so as to facilitate the follow-up study on the effects of ECM fungi inoculation and saline stress on the growth (height, weight, nutrients absorption etc.) of seedlings. In total, 80 (4 [*L. amethystine*, *P. tinctorius*, *C. geophilum*, NM] \times 10 [replicates] \times 2 [saline and normal soil]) pots were prepared. The seedlings were cultivated in a glass greenhouse at 25 °C/20 °C during the day/night under natural light conditions. The pots were watered as needed with tap water.

2.2 Sampling

One year and a half after transplanting, all roots of a survive seedling were washed carefully under tap water, examined and classified based on their surface color, texture, and emanating hyphae under a dissecting microscope. Due to the distinctive features of the fungi we studied (Pt: yellow, Cg: black, La: purple or translucent, Fig. 1, Supplemental Figs), it is possible for us to distinguish by their shape and color of the ectomycorrhizae using dissecting microscope.

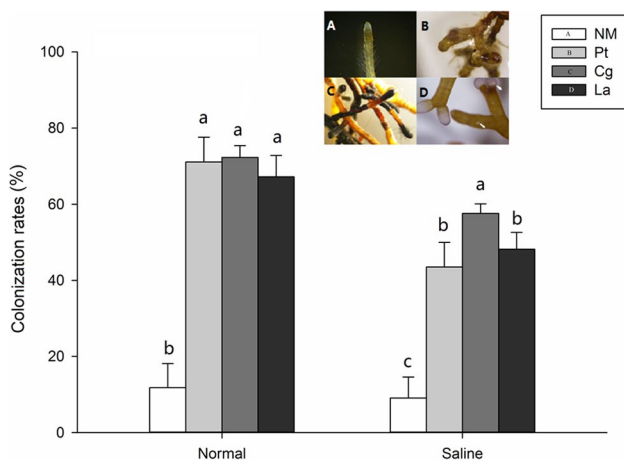


Fig. 1 Inoculation rates of *P. thunbergii* seedlings planted in normal and saline soils colonized by three ectomycorrhizal fungi (Pt, Cg, La) and the appearance of the Non-ectomycorrhizal (NM) and mycorrhizal (Pt, Cg, La) root-tips. Different letters above bars indicate significant differences based on Tukey test ($p < 0.05$). (Data are presented as mean \pm standard error of 3 replicates). A, Non-ectomycorrhizal; B, C and D, inoculated with Pt, Cg and La

After that, three seedlings of each treatment were randomly selected and used for chlorophyll and inoculation measurements. Another three were harvested and washed in distilled water to remove soil from the roots, then each individual plant sample was divided into shoots and roots subsamples, dried at 60 ± 2 °C for 72 h, weighed and milled for chemical analysis.

2.3 Chemical analysis

To measure chlorophyll content, cleaned and cut needles (0.2 g) were weighed, and then put in a test tube with a mixture of acetone and ethanol (v:v = 2:1). Tubes were kept in the dark at room temperature for 16 h for chlorophyll extraction. The light absorption was determined by UV spectrophotometry according to Hiscox and Israelstam (1979).

The milled pine materials were digested with H_2SO_4 according to Zhang et al. (2014) for total nitrogen (N) and total phosphorus (P) test. Total N was determined using the Kjeldahl method, and total P using the Vanado-Molybdate colorimetric method. To test the remaining macroelements (Na, K, Ca, Mg), flame-atomic absorption spectroscopy (Jena nov AA 400, Gernmen) was employed after the milled pine materials finished digesting with a mixture of spectrally pure concentrated HNO_3 and $HClO_4$ (v:v = 87:13) (Zhao et al. 1994).

2.4 Statistical analysis

Two factors, ECM type (i.e., NM seedlings and ECM seedlings) and soil type (i.e., normal and saline) were set up with three replicates per group. The data were analyzed using the SPSS software package for Mac (ver. 22.0, IBM, Armonk, NY, USA). Two- and one-way analyses of variance (ANOVA) with honest significant difference (HSD) of Tukey test at $p < 0.05$ was applied to determine significant differences in inoculation rates, growth and nutritional element concentrations in the seedlings.

3 Results

3.1 Ectomycorrhizal (ECM) formation

After cultivating for one year and a half, survival seedlings were counted and most of them (83.8%) were alive at the end of the experiment (Table 1). ECM colonization of the root tips of every inoculation seedling was examined by dissecting microscope based on their surface color (Pt: yellow, Cg: black, La: purple or translucent, Fig. 1) (Shi et al. 2017). Figure 1 showed the colonization of *P. thunbergii* seedlings by ECM fungi under different soil treatments. The roots of control seedlings were inoculated by native ECM

fungi. However, the inoculation rate was significantly lower than the ECM fungi treated ones, and ECM colonization was significantly decreased by soil saline stress. There was no difference among ECM fungi species colonization rates under the normal soil condition. And the colonization rate for pine seedlings planted in saline soil were lower than that in normal soil (Fig. 1).

3.2 Seedlings growth

The height of seedlings was significantly affected by soil condition ($F = 10.045$, $p = 0.006$) and ECM fungal species ($F = 80.226$, $p < 0.001$) in two-way ANOVA. There were no significant differences between Cg-seedlings and NM-seedlings, both in normal and saline soil conditions. But, the other two ECM fungi (*P. tinctorius* and *L. amethystina*)

improved the height of the host seedlings significantly in both soil conditions (Fig. 2A).

The biomass (Dry weight, DW here after) of seedlings was significantly affected by ECM inoculation, especially those planted in saline soil. Compared to the seedlings without inoculation (NM), the growth (DW) of seedlings was significantly increased (Normal: $F = 21.250$, $p < 0.001$; Saline: $F = 131.317$, $p < 0.001$, data not show) by ECM fungi inoculation, especially *L. amethystina* (Fig. 2B). Under saline soil conditions, ECM fungi improved the DW of the host significantly, but only *L. amethystina* affected the growth of seedlings under normal soil conditions. Except Cg-seedlings planted in normal soil, DW of shoots was significantly increased by ECM fungi inoculation in different soil conditions. DW of the roots was increased by ECM

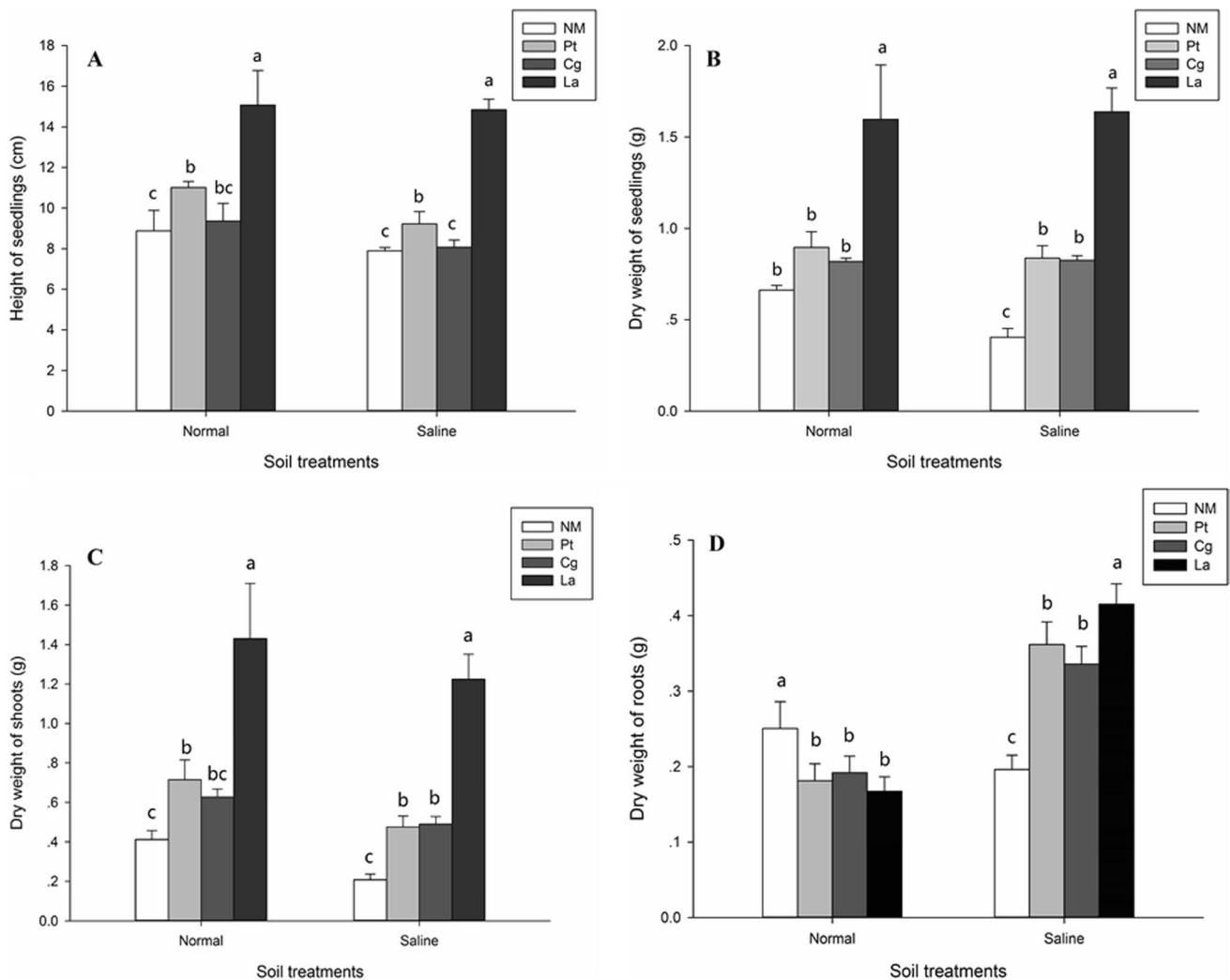


Fig. 2 Height and dry weight (Total seedlings, shoots, roots) of non-inoculated (NM) and inoculated (Pt, Cg, La) *P. thunbergii* seedlings planted in normal and saline soils. Different letters above bars indi-

cate significant differences based on Tukey test ($p < 0.05$). (Data are presented as mean \pm standard error of 3 replicates)

fungus inoculation planted in saline soil and decreased in normal soil (Fig. 2C, D).

3.3 Nutrients

In two-way analysis, both ECM inoculation and soil condition could impact nutrient absorption on host seedlings as shown in Table 2. In the present study, saline stress resulted in a significant decrease in nutrients, like Mg, Ca, N and K in shoot, in contrast, resulted in a significant increase in roots. But, saline stress increased Na contents both in shoot and root. There was no difference in the concentration of N in interaction effects of ECM inoculation and soil condition both in root and shoot in seedlings planted in saline soil (Tables 2, 3). Compared to NM and the other two ECM fungi, ECM fungus *L. amethystina* has the most positive effects in assisting host to absorb nutrients, like Mg, Ca and N. The ECM fungi, studied in our system, could maintain a low concentration of Na⁺ in roots to reduce the toxicity to plants when planted in saline soils (Table 2). The Na⁺/K⁺ ratio was affected by main treatments (ECM fungi and salt stress) and their interactions both in shoot and root (Table 3).

3.4 Chlorophyll

As shown in Fig. 3, in two-way ANOVA, the total chlorophyll (chlorophyll a and b) content of NM-seedling needles was significantly affected by ECM fungi inoculation ($F = 24.390$, $p < 0.001$), but not by soil condition ($F = 4.314$, $p = 0.054$). When planted in normal soil, except *C. geophilum*, *P. tinctorius*, and *L. amethystina* inoculation could improve the chlorophyll a and chlorophyll b content in needles significantly. But in saline soil, all three ECM fungi could improve the chlorophyll a content, but only *L. amethystina* could improve chlorophyll b in the needles of seedlings (Fig. 3A). In addition, *L. amethystina* inoculation could improve the chlorophyll a/b ratio of seedlings when planted in saline soil. In contrast, there was no significant difference in the chlorophyll a/b ratio of NM- and ECM-seedlings planted in normal soil (Fig. 3D). In two-way ANOVA, soil conditions affected chlorophyll a and chlorophyll a/b, and ECM species affected chlorophyll a, chlorophyll b and chlorophyll a and b, while their interactions affected chlorophyll a and chlorophyll a and b (Table 3).

To investigate the effects of ECM fungi and saline stress on the ability of seedlings to absorb minerals, we analyzed height, DW, macroelement (P, Mg, Ca, N, Na and K) levels in shoots and chlorophyll contents in needles (supported in Fig. 2; Table 2) using principal component analysis (PCA) based on individual replicates. In the PCA biplot (Fig. 4), the eigenvalues of PCA 1 and 2 were 8.14 and 2.46, respectively. The results revealed two main components, which explained

Table 2 Effects of ectomycorrhizal fungi (EMF) and saline stress on the macroelements (P, Mg, Ca, N, Na and K) concentrations of *P. thunbergii* seedlings

Part	Soil	Species	P g/kg	Mg g/kg	Ca g/kg	N g/kg	Na g/kg	K g/kg	Na/K
Shoot	Normal	NM	4.01±0.60b	5.39±0.28bc	4.66±0.63d	15.57±1.49c	1.86±0.11e	9.03±1.13c	0.21±0.04f
		Pt	4.33±0.54b	5.67±0.94b	5.82±0.70c	17.04±0.73bc	2.23±0.21e	12.23±1.25b	0.18±0.04f
		Cg	5.66±0.27a	5.43±0.44bc	6.70±0.19b	15.25±1.60c	3.48±0.41d	11.95±0.30b	0.29±0.03e
		La	5.74±0.55a	7.29±0.55a	7.39±0.43a	19.76±1.96a	4.32±0.28cd	14.93±0.66a	0.29±0.02e
	Saline	NM	2.10±0.17c	3.41±0.32d	1.80±0.23f	11.95±0.30d	4.69±0.86bc	5.78±0.72e	0.81±0.08b
		Pt	4.95±0.81ab	4.63±0.31c	2.98±0.13e	14.93±0.66c	7.04±0.99a	7.31±0.80d	0.96±0.04a
		Cg	2.26±0.11c	4.83±0.13bc	2.39±0.06ef	11.90±1.27d	5.56±0.55b	8.36±0.23cd	0.66±0.05c
		La	4.83±0.91ab	5.24±0.36bc	2.54±0.11e	18.79±1.05b	4.22±0.14cd	9.12±0.69c	0.46±0.02d
Root	Normal	NM	1.44±0.26b	0.91±0.08d	1.14±0.18e	5.60±0.53c	0.73±0.06de	3.78±0.48c	0.20±0.04c
		Pt	3.09±0.62a	1.02±0.16cd	1.66±0.20de	5.72±0.86c	0.50±0.08e	3.54±0.70c	0.14±0.02d
		Cg	1.54±0.21b	1.03±0.16cd	1.93±0.27d	5.54±0.48c	0.78±0.08d	3.23±0.67c	0.25±0.07bc
		La	3.00±0.21a	1.05±0.09cd	1.76±0.22d	8.29±1.01ab	0.86±0.06d	3.50±0.24c	0.25±0.01bc
	Saline	NM	2.49±0.37a	1.54±0.18bc	3.59±0.35c	7.79±0.75b	3.30±0.18a	7.74±0.25a	0.43±0.05a
		Pt	2.48±0.40a	1.56±0.47bc	4.39±0.43ab	7.98±0.92ab	1.57±0.09c	5.72±0.86b	0.28±0.02b
		Cg	2.42±0.45a	1.80±0.46b	3.97±0.55bc	7.16±0.38b	1.78±0.27bc	8.56±1.28a	0.21±0.02bc
		La	2.33±0.51a	2.91±0.38a	4.68±0.35a	9.23±0.24a	1.88±0.06b	8.82±0.60a	0.21±0.01bc

Different letter in same treatment indicate significant differences by Tukey test ($p < 0.05$). (Data are means ± standard error of 3 replicates)

Table 3 Significance of the main treatment effects (ECM fungi and soil conditions) and their interactions based on two-way ANOVA on tested variables (the macroelements and Chlorophyll a, b, a+b, and a/b) of seedlings

Variable		Main effects				Interaction effects	
		Soil		Species		Soil × Species	
		<i>F</i>	<i>p</i>	<i>F</i>	<i>p</i>	<i>F</i>	<i>p</i>
Shoot	P	36.854	< 0.001	17.151	< 0.001	13.514	< 0.001
	Mg	53.514	< 0.001	15.823	< 0.001	3.400	0.044
	Ca	563.158	< 0.001	22.484	< 0.001	10.653	< 0.001
	N	24.525	< 0.001	27.236	< 0.001	1.441	0.268
	Na	118.819	< 0.001	7.821	0.002	21.047	< 0.001
	K	183.359	< 0.001	34.263	< 0.001	3.353	0.045
	Na/K	749.22	< 0.001	21.514	< 0.001	53.957	< 0.001
Root	P	0.966	0.34	7.076	0.003	7.938	0.002
	Mg	64.613	< 0.001	8.414	0.001	6.761	0.004
	Ca	335.928	< 0.001	7.084	0.003	1.947	0.163
	N	38.024	< 0.001	14.638	< 0.001	1.162	0.355
	Na	566.158	< 0.001	49.487	< 0.001	42.149	< 0.001
	K	210.341	< 0.001	5.41	0.009	6.661	0.004
	Na/K	24.182	< 0.001	9.647	0.001	19.692	< 0.001
Chlorophyll a		11.007	0.004	30.863	< 0.001	4.263	0.022
Chlorophyll b		0.074	0.789	8.625	0.001	1.349	0.294
Chlorophyll a + b		4.314	0.054	24.39	< 0.001	3.471	0.041
Chlorophyll a/b		11.913	0.003	1.333	0.299	0.112	0.952

58.1% (PCA1) and 17.5% (PCA2) of the variation. PCA1 ($R = 0.658$) clearly separated the effects of ECM fungi, whereas PCA2 ($R = -0.702$) separated the effects of saline stress.

4 Discussion

ECM fungal colonization is sensitive to soil conditions, such as nutritional and pollution status (McAfee and Fortin 1989; Hryniewicz and Baum 2012; Averill et al. 2014; Chen et al. 2015; Hryniewicz et al. 2015; Wen et al. 2017). It is well known that soil salinity has a negative impact on the growth of fungal hyphae (Aggarwal et al. 2012; Hameed et al. 2014), and it has already been confirmed that the level of fungal colonization capacity decreased with soil salinity (Aggarwal et al. 2012; Hryniewicz et al. 2015). In the present study, even our investigation showed that *P. thunbergii* could form good symbiotic relationships with ECM fungi. However, the inoculation rates were reduced by saline stress as the results show. But, interestingly, Thiem et al. (2018) identified that soil salinity could only effect on fungal taxa significantly but not on fungal colonization rates. ECM root colonization varies with the ECM fungus, suggesting that some strains are able to grow and have the ability of colonize the host in some stressful condition such as saline stress as in our study system. Therefore, the mechanism by which ECM fungi colonize host under stress is not known, and further investigations are necessary to clarify this.

Salinity, as one of the most important abiotic stressors, can limit plant growth by affecting nutrient absorption (Ishida et al. 2009; Chen et al. 2014). Many studies (Langenfeld-Heysler et al. 2007; Hanin et al. 2016) have shown that the salinity tolerance of many plants is improved by symbiotic associations formed between ECM fungi and plant roots. In this study, we analyzed the impact of ECM fungi inoculation on the salt tolerance of *P. thunbergii* seedlings. One year and a half after transplanting, compared to NM-seedlings, inoculated ones, especially *P. tinctorius* and *L. amethystina* in this study, showed higher survival rate, height and dry weight, suggesting that pine seedlings inoculated with ECM fungi were well-adapted to saline stress. ECM fungal inoculation can reduce the salt impact to *P. thunbergii* seedlings and increase host plant tolerance to soil salinity stress by excluding salts and improving nutrient uptake. Considering the difficulty of forest restoration under such salt stress conditions, nursery inoculation with ECM fungi can be an advantage for improving seedling performance after transplanting in saline areas.

Firstly, many previous studies (Smith and Read 2008; Marschner 2012; Herzog et al. 2013; Wen et al. 2017) showed that pine seedlings developed more biomass when inoculated with ECM fungi, which was confirmed by our obtained results. The improvement in host growth could be explained by increasing mineral nutrient contents in response to ECM fungi inoculation, especially P and N (Chen et al. 2014; Shi et al. 2017). These results were

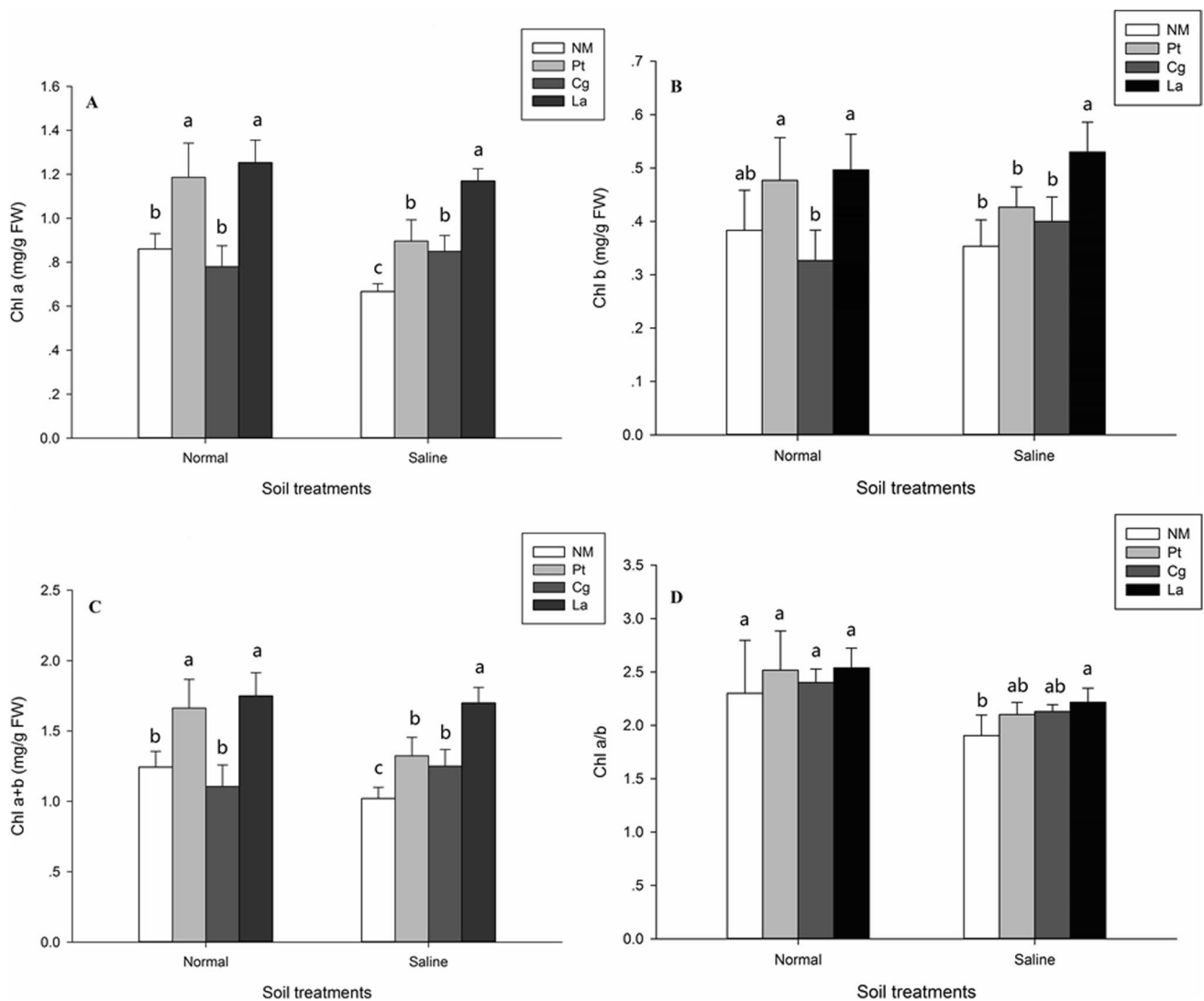


Fig. 3 Effects of ectomycorrhizal fungal inoculation on chlorophyll content and the chlorophyll a/b ratio in needles of *P. thunbergii* seedlings. Different letters above bars indicate significant differences

based on Tukey test ($p < 0.05$). (Data are presented as mean \pm standard error of 3 replicates)

confirmed by other studies on other soil conditions, like heavy-metal contaminated ones (Chen et al. 2015; Wen et al. 2017). Results showed that ECM fungal inoculation leads to a low concentration of Na^+ and affects Na^+/K^+ ratio of host seedlings. Na^+ and K^+ are the main inorganic metal ions involved in plant osmotic regulation, thus, maintaining a balance of intracellular Na^+/K^+ concentration is the key to keep normal physiological metabolism of plants under salt stress (James et al. 2011; Hasegawa 2013). Uptake of K^+ , an essential element for growth and development of plants, is always inhibited by high Na^+ concentration (James et al. 2011; Gupta and Huang 2014), resulting in lower productivity and possible death. Accordingly, plants always maintain the activity of cytoplasmic enzymes by accumulating K^+ under salt stress (Zhang and Shi 2013). Thus, uptake

K^+ enhanced by the ECM associations seems to be crucial for host plants adapt to salt stress environment (Wang et al. 2013; Guerrero-Galán et al. 2019).

Secondly, tests of the photosynthetic pigment composition revealed that a significantly higher concentration of chlorophyll (a and b), indicating more active light harvesting by the ECM fungi inoculated plants. And some previous findings (Song and Wu 2011; Shi et al. 2017) were consistent with our results. Guerrero-Galán et al. (2019) summarized that the inoculation of salt-tolerant ECM fungi could prevent Na^+ from flowing into the photosynthetic tissues, which would decrease the photosynthetic rate, the Hill reaction rate and ATPase activity in chloroplast. Moreover, chlorophyll a and b contents in seedlings inoculated with *L. amethystina* were significantly higher than with *P. tinctorius* or

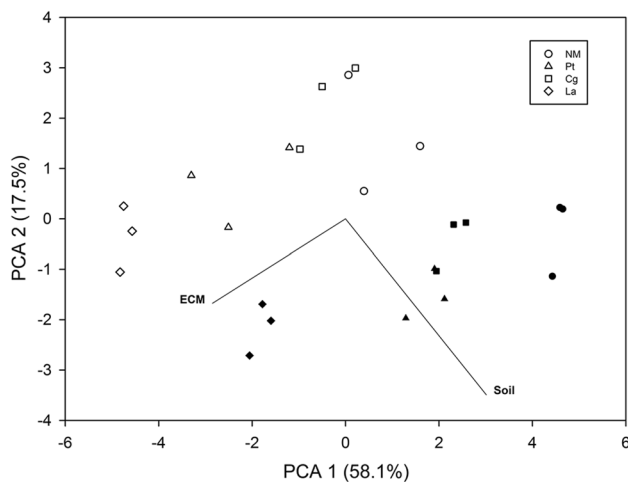


Fig. 4 Principal component analysis (PCA) plots of height, DW, macroelements (P, Mg, Ca, N, Na and K) levels in shoots and chlorophyll contents in needles in non-ectomycorrhizal (NM) or mycorrhizal (Pt, Cg, La) *P. thunbergii* exposed to normal (open symbols) or saline (filled symbols) soil with 3 individual replicates. The mean data of height, DW, macroelements and chlorophyll contents were presented in Table 2 and Fig. 2

C. geophilum when planted in saline soil (Fig. 3C). Plants exhibit greater shade-tolerance by increasing in chlorophyll content to enhance the absorption and utilization of light energy (Huang et al. 2011; Shi et al. 2017). Thus, ECM fungi may improve the shade tolerance of *P. thunbergii* seedlings via this way, and help the pine seedlings survive in the gloom of the thick forest. Moreover, it was reported that chlorophyll content in conifers responded positively to increased canopy openness (Sun et al. 2016).

Lastly, we highlighted the fact that the benefits of ECM inoculation are usually better evidenced under stressful conditions, salt stress for instance in our study system. Compared to host *P. thunbergii* seedlings planted in normal soil conditions, ECM inoculation showed a better promotion effect in host seedlings planted in saline soils based on the increased ratios of DW (shoot, root and total), chlorophyll a and chlorophyll a and b (Figs. 2, 3). In addition, our results suggested that among the three ECM fungi, *L. amethystina* showed the most significant promoting effect on *P. thunbergii* seedling growth when planted under salt stress. But, in the vitro pure culture experiment, *C. geophilum* exhibited the highest salt tolerance among these three ECM fungi (Under-review). Besides, along peatland–forest gradient, the abundance of *C. geophilum* on the roots of pines was similar while the medium and long distance exploration types of ectomycorrhizae shift with the water content increase (Aučina et al. 2019). We supposed that there is no correlation between in vitro salt tolerance of ECM strains and their effectiveness in association with plants under salt-stressed conditions based on our results. Thus, positive facilitation

of ECM symbioses to saline stress is primary dependent on the salt tolerance of the host plant and the interaction effects between ECM strains and hosts. In our study system, studies were carried out under controlled greenhouse condition. Accordingly, we recommend further investigations in the field environment to fully understand the competitive mechanisms of ECM fungal species in improving host tolerance to salt stress.

In summary, we tested the effects of three ECM fungi in *P. thunbergii* seedlings. Our results indicated that the ECM fungi studied here had beneficial effects in pine seedlings transplanted in saline soil through the enhancement of excluding salts, improving nutrient uptake (like, P and N in shoot of seedlings inoculated with *P. tinctorius* and *L. amethystina*), maintaining the balance of Na^+/K^+ concentration, and increasing chlorophyll contents. Moreover, according to our results, different ECM fungi had different effects, suggesting that the effectiveness of the symbiosis in salt conditions depends on ECM species. In-depth study of the effects of different strains (pine-specific suilloid ECM fungi, especially) on the salt tolerance associated with different host plants will become an important direction for the research of subsequent ECM, and it will provide a theoretical basis and technical support for saline soils reforestation and rehabilitation along sea areas.

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