Research paper

Ectomycorrhiza Enhanced the Cold-Acclimation Growth and Freeze Tolerance of Scots Pine (*Pinus sylvestris* L.)

Burenjargal Otgonsuren,[1,2] Ming-Jen Lee[3,4]

【Summary】

Scots pine (*Pinus sylvestris*) is an economically important source of timber in Mongolia and has been widely used in reforestation programs. Our earlier study showed that *Phialocephala fortinii* was capable of forming symbiotic ectomycorrhizal associations with Scots pine seedlings. In this study, *Phi. fortinii* inoculation significantly increased the growth, biomass, and mineral (P, K, Ca, Mg, Na, and N) contents in roots, stems, and needles of Scots pine seedlings under normal and cold-acclimation conditions. Furthermore, the proline content of inoculated Scots pine seedlings was significantly higher than that of non-inoculated ones after hardening and cold-acclimation treatments.

The inoculated and non-inoculated Scots pine seedlings were cold-acclimated and subsequently subjected to freezing tolerance tests at -12, -14, -16, -18, and -20℃ for 7 d, and then cultivated at 12±2℃ for 14 d. Values of the temperature for 50% mortality (LT_{50}) of needles of non-inoculated and inoculated Scots pine seedlings were -12 and -15℃, respectively. Consistently, respective LT_{50} values of seedlings of non-inoculated and inoculated pines were -14 and -18℃. In addition to *Phi. fortinii* effectively forming ectomycorrhiza with Scots pine seedlings, this study demonstrated that *Phi. fortinii* significantly improved the growth, nutrition acquisition, proline content, and freeze tolerance of Scots pine.

Key words: *Phialocephala fortinii*, *Pinus sylvestris*, ectomycorrhiza, freeze tolerance, proline.

Otgonsuren B, Lee MJ. 2013. Ectomycorrhiza enhanced the cold-acclimation growth and freeze tolerance of scots pine (*Pinus sylvestris* L.). Taiwan J For Sci 28(2):97-111.

[1] Graduate Institute of Agriculture, National Chiayi Univ., 300 Univ. Rd., Chiayi 60004, Taiwan. 國立嘉義大學農學研究所，60004嘉義市學府路300號。

[2] Department of Ecology, Mongolian State University of Agriculture, Zaisan 153, Khan-Uul District, Ulaanbaatar 210153, Mongolia. 蒙古國立農業大學生態學系，210153蒙古烏蘭巴托市汗烏拉區Zaisan街153號。

[3] Department of Forestry and Natural Resources, National Chiayi Univ., 300 Univ. Rd., Chiayi 60004, Taiwan. 國立嘉義大學森林暨自然資源學系，60004嘉義市學府路300號。

[4] Corresponding author, e-mail:mjlee@mail.ncyu.edu.tw 通訊作者。

Received April 2013, Accepted July 2013. 2013年4月送審 2013年7月通過。

研究報告

外生菌根增進歐洲赤松(*Pinus sylvestris*)的冷馴化生長和耐凍性

奧特根蘇仁布倫[1,2)] 李明仁[3,4)]

摘 要

歐洲赤松(*Pinus sylvestris*, Scots pine)為蒙古重要的經濟用材來源，且廣泛應用於人工造林計畫。吾等先前的研究曾證實*Phialocephala fortinii*可以和歐洲赤松苗木形成共生組合外生菌根。本研究顯示，接種*Phi. fortinii*顯著地增進正常及冷馴化處理的歐洲赤松苗木的生長、生物量、及其根、莖、葉中礦物質（磷、鉀、鈣、鎂、鈉、氮）的含量。此外，在健化及冷馴化處理下，接種*Phi. fortinii*的歐洲赤松苗木的脯胺酸含量顯著高於未接種者。

接種及未接種的歐洲赤松苗木經冷馴化處理，隨之分別於-12、-14、-16、-18及-20℃進行耐凍性試驗7天後，並培養於12±2℃生長箱14天。接種及未接種的歐洲赤松苗木針葉的半致死溫度分別為-12及-15℃。相一致地，接種及未接種的歐洲赤松苗木的半致死溫度分別為-14及-18℃。結果顯示*P. fortinii*不但可以和歐洲赤松苗木形成外生菌根，並能顯著地增進其生長、養分獲得、脯胺酸含量、及耐凍性。

關鍵詞：*Phialocephala fortinii*、*Pinus sylvestris*、外生菌根、耐凍性、脯胺酸。

奧特根蘇仁布倫、李明仁。2013。外生菌根增進歐洲赤松(*Pinus sylvestris*)的冷馴化生長和耐凍性。台灣林業科學28(2):97-111。

INTRODUCTION

Scots pine (*Pinus sylvestris* L.) is an economically important source of timber in Mongolia and has been widely used in reforestation programs.

Ectomycorrhizal (ECM) fungi are functionally important in temperate forest ecosystems and play influential roles in forest community dynamics (Smith and Read 1997). Trees with well-developed ECM root tips are more resistant to environmental stresses such as drought and biotic stresses like root pathogens.

Temperature is one of the most important factors affecting the survival and growth of forest trees in Mongolia. The ability to measure cold hardiness was necessary for successful production and establishment of forest tree seedlings (Warrington and Rook 1980, Glerum 1985). Late-spring frosts that coincide with the sensitive phases of bud break in conifers may cause severe problems for newly planted seedlings (Sakai and Larcher 1987). Furthermore, adaptation and acclimation to low temperatures during autumn are important factors affecting the survival of tree seedlings in Mongolia. Frost damage was reported in Norway spruce (*Picea abies* (L.) Karst.), when freezing temperatures (-16℃) occurred 1 wk following spring planting in southern Norway (Kohmann 1991). In a study on *Pin. sylvestris* and *Pic. abies* seedlings, Laiho and Mikola (1964) reported that only a very small part of the mycorrhizae died during the winter

freeze in the nursery, and the common reason for death was heaving caused by ground frost, which physically broke the long roots. Coutts and Nicholl (1990) indicated that ECM root tips survived in microcosms over the winter, while Alexander and Fairley (1983) found that up to 75% of ECM colonization was retained in roots of *Pic. sitchensis* in a Scottish plantation in September to December. In another study, *Pisolithus tinctorius* mycelium was shown to survive at soil temperatures of < 0℃ in nursery plots in the field in North America and subsequently colonized *Pin. taeda* seedlings (Marx and Bryan 1975).

The ability of plants to survive low temperatures can be estimated by evaluating damage after artificial freezing. In many direct trials, whole seedlings were frozen, transferred to favorable conditions, and examined after a few days for visible signs of frost damage (Nilsson and Andersson 1987). Visible injury to needles, buds, stems, and other tissues on whole plants caused by controlled freezing treatments can also be assessed. Several nondestructive methods for measuring frost hardiness in conifer seedlings were described, including changes in needle color in Scots pine (Toivonen et al. 1991), stem electrical impedance (Glerum 1973), and chlorophyll a fluorescence (Sundblad et al. 1990).

The aims of this study were to isolate and identify ECM fungi and assess the effects of isolated fungi on the growth and freeze tolerance of Mongolian pine seedlings through mycorrhizal colonization and a freezing test. We expect that the findings from this study will contribute to application of mycorrhizal techniques in the reforestation of Mongolian lands.

MATERIALS AND METHODS

Strains of mycorrhizae

Phialocephala fortinii was previously isolated from the roots of Scots pine from Bogd Mountain, Ulaanbaatar Province, Mongolia (107°06′65″E, 47°44′970″N, at an elevation of 2000 m) (Otgonsuren and Lee 2012). The isolated endophyte was deposited in the Bioresource Collection and Research Centre (BCRC) Hsintsu, Taiwan (*Phi. fortinii* sensu lato P2, BCRC 34985). Seeds of Scots pine were also collected from the same site.

Seedling culture

After cleaning the surface with running tap water, seeds of Scots pine were sterilized with a 10% sodium hypochlorite solution for 15 min, rinsed 3 times with sterile distilled water, and then germinated in a sterilized mixture of peat moss, vermiculite, and perlite (1: 1: 1, v/v). When the seedlings had attained 4 cm in height, they were transferred to pots filled with sterilized soil for ECM infection and colonization.

Inoculation of mycorrhizal fungus

Scots pine seedlings were inoculated with the isolated fungal strain of *Phi. fortinii*. The inocula consisted of 20-mm-diameter fungal plugs taken from the edge of actively growing colonies on MMN medium in Petri dishes and maintained in an incubator at 23±2℃ for 4 wk. All seedlings were cultured in a greenhouse at 20±3℃ with 1000±200 μmole photons m^{-2} s^{-1} of photosynthetic photon flux density (PPFD), and watered with deionized water as needed without supplemental fertilization. Seedlings were examined 6 mo after inoculation.

Observation of ECMs

Roots of Scots pine seedlings were sampled and cleaned with water in a supersonic oscillator (Upson et al. 2007). The morphology of mycorrhizae was observed with a stereomicroscope (Usuki and Narisawa 2005).

Absorbing roots were hand-sectioned and stained by modified methods published by Frohlich (1984) and Brundrett et al. (1988). Young absorbing roots of the pine were washed with running tap water to remove particles. Roots were placed between sheets of parafilm on a paraffin base and kept moist with water. Root tissues were cross-sectioned, and preserved in 50% ethanol overnight. Root sections were covered with 10% KOH for 15 min and then transferred to 3% H_2O_2 for 3 min, rinsed with distilled water 3 times, covered with 1% HCl for 5 min, stained with 0.01% Chlorazol black E solution in an autoclave for 15 min at 121℃, and mounted in glycerol under a cover glass. Then, the root tissues were examined with a stereomicroscope.

For light microscopy, pine roots were cut into small pieces, fixed in formalin: acetic acid: alcohol (FAA, 5: 5: 50, v/v) overnight, rinsed with distilled water 3 times, and dehydrated in 70% ethanol and a series of tert-butyl alcohol (TBA) concentrations of 20, 35, 55, 75, and 100%. Specimens were embedded in paraffin wax (with a melting point of 56℃). Transverse sections 10~12 μm thick were cut with a rotary microtome (Leica Reichert-Jung 820-II Histocut Microtome, Holly, MI, USA). Paraffin was removed with xylol, and sections were stained with Safranin and Fast green (Ruzin 1999).

For the ultrastructural study, root samples were fixed with 2.5% glutaraldehyde and 4% paraformaldehyde fixative in a phosphate-buffered solution (0.1 M, pH 7.0) for 4 h at room temperature, then rinsed with the phosphate-buffered solution 3 times each time for 15 min, followed by serial dehydration in 30, 50, 70, 80, 95, and 100% ethanol and 100% acetone, and finally dried in a critical-point dryer using liquid carbon dioxide. Dried materials were mounted on an aluminum stub with adhesives, coated with gold, and observed with a Hitachi S-3500N scanning electron microscope (Tokyo, Japan) (Brundrett et al. 1996).

Cold acclimation

Pine seedlings of 1.5 yr old were used for the freeze-tolerance test, and each treatment included 30 pots of seedlings (with 6 seedlings pot^{-1}). For non-stressed treatments, seedlings were inoculated with the isolated fungal strain (*Phi. fortinii*) or non-inoculated (control) and grown in a greenhouse at 20±3℃ until being harvested. For cold-acclimation treatments, seedlings were inoculated with the fungal strain (*Phi. fortinii*) or non-inoculated (control), hardened for 2 wk at 12℃ under a 10-h photoperiod, and then cold-acclimated at 2℃ under an 8-h photoperiod for 2 wk, 0℃ for 24 h, and 2℃ for 24 h (Pociecha et al. 2009). Cold-acclimated seedlings were used for the subsequent freeze test. The growth, biomass, and mineral contents were measured after cold acclimation. The proline content was measured after 2 wk of hardening at 12℃ and 2 wk of cold acclimation at 2℃.

Assessment of the freeze tolerance

After cold acclimation, 6 seedlings in each treatment were subjected to a freeze test at -12, -14, -16, -18, and -20℃ for 7 d. Then, the plants were transferred to a greenhouse at 12±2℃ and a PPFD of 1000±200 μmole photons m^{-2} s^{-1}, and watered with deionized water. The extent of frost injury was visually estimated by examining needles 7 d after the last freeze treatment. The temperature which damaged 50% of the needles was estimated. The assessment of visible frost damage on needles was modified from a previous study (Kohmann 1999) and was determined after 7 d as follows: 0, undamaged; 1, a little needle browning (10%); 2, needle browning of up

to 30% of the needle mass; 3, needle browning of 30~40% of the needle mass; 4, needle browning of 50% of the needle mass; 5, needle browning of > 50% of the needle mass; and 6, 100% of needles dead.

The temperature causing 50% mortality (LT_{50}) was determined by controlled freeze treatment followed by a visual rating of plant regrowth after 14 d at 12±2℃ (Palonen and Buszard 1998).

Proline analysis

The free proline content was determined according to Bates et al. (1973). Needle samples (0.5 g) from each plant were homogenized in 3% (w/v) sulfosalicylic acid, and the homogenate was filtered through filter paper. After the addition of acid ninhydrin and glacial acetic acid, the resulting mixture was heated at 100℃ for 1 h in a water bath. The reaction was stopped by immersion in an ice bath. The mixture was extracted with toluene, and the absorbance of the fraction with toluene was aspirated from the liquid phase and read at 520 nm. The proline concentration was determined using a calibration curve and is expressed as parts per million (ppm).

Growth and biomass measurements

After cold acclimation, 4 plants per treatment were harvested, and the height, root lengths, root collar diameter, fresh (FW) and dry weights (DW) of the needles, stems, and roots were determined. The DW was assessed after drying in an oven at 70±2℃ for 48 h.

Mineral concentration analysis

For the mineral concentration analysis, root, stem, and needle samples were oven-dried at 70±2℃ and digested with concentrated H_2SO_4 and H_2O_2. Nitrogen contents of the roots, stems, and needles were estimated by a micro-Kjeldahl method (MacDonald 1977). Phosphorus, potassium, calcium, sodium, and magnesium contents were estimated by inductively coupled plasma atomic emission spectrometry.

Statistical analysis

Statistical analyses were carried out with the software Statistical Package for the Social Science (SPSS 12.0, Chicago, IL, USA) for Windows. All data are presented as the mean of 4 separate experiments±standard error (n = 4). Differences in growth and physiological characteristic rates among treatments were analyzed by Tukey's multiple-range test at a $p \leq 0.05$ significance level.

RESULTS AND DISCUSSION

Morphology and ultrastructure of ECMs

At 6 mo after inoculation, ECMs had formed in the root systems of Scots pine seedlings inoculated with *Phi. fortinii*. The morphology and ultrastructure of the Scots pine seedling root clearly showed a mantle and Hartig net of ECM (Fig. 1). O'Dell et al. (1993) reported labyrinthine tissue (similar to Hartig net tissue) in roots of *Pin. contorta* when inoculated with *Phi. fortinii*. Fernando and Currah (1996) were the first to present an anatomical study showing that *Phi. fortinii* formed ECMs with *Salix glauc*a in an axenic resynthesis experiment. In another study, *Phi. fortinii* formed typical ECM on roots of birch seedlings, with a complete mantle and Hartig net (Hashimoto and Hyakumachi 2001). In a recent study, roots of *Pin. banksiana* and *Pin. strobes* seedlings inoculated with *Phi. fortinii* showed varying amounts of surface hyphae, obvious Hartig nets, and some intracellular hyphae (Peterson et al. 2008). In contrast, no hyphae, mantle, or Hartig net were present in roots of non-inoculated pine seedlings (Fig. 2).

Plant growth under cold acclimation

Phialocephala fortinii inoculation treatments significantly stimulated plant height, root collar diameter, and root length compared with the control in both normal and cold-acclimation conditions (Table 1). Under cold acclimation, respective enhancements in plant height, root length, and root collar diameter

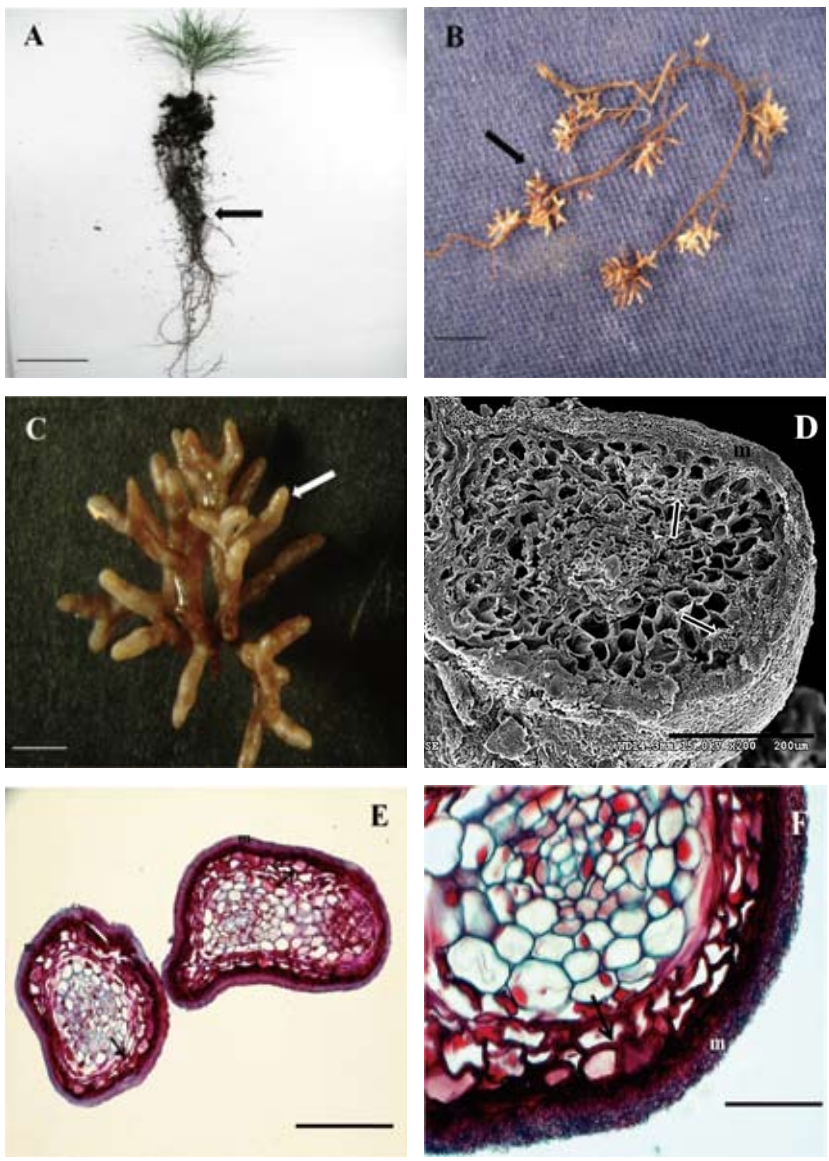

Fig. 1. Root of Scots pine (*Pinus sylvestris*) seedlings inoculated with *Phialocephala fortinii*. (A) Seedling of *Pin. sylvestris* (bar: 1 mm); (B, C) arrows indicate the root-fungus association (bars: 1 mm); (D) cross-section of pine ectomycorrhizal root (M, mantle; arrows, Hartig net hyphae; bar: 500 µm); (E, F) root stained with safranin and fast green (M, mantle; arrows, Hartig net hyphae; bars: E and F, 100 µm).

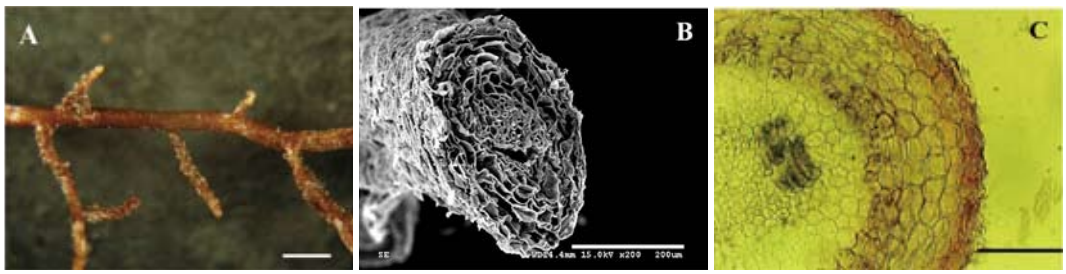

Fig. 2. Root morphology and cross-sections of non-inoculated Scots pine seedlings. (A) Morphology of the root (bar: 1 mm); (B) ultrastructure of the root (bar: 200 μm); (C) root stained with Chlorazol black E solution (CBE) (bar: 100 μm).

Table 1. Growth of inoculated and non-inoculated Scots pine seedlings under cold-acclimation treatment

Treatment	Net height growth (cm)	Net root length growth (cm)	Net root collar diameter growth (mm)
P2C	20.3±1.4[a]	64.0±5.8[a]	2.9±0.1[a]
CC	10.8±0.1[b]	23.5±8.7[b]	1.9±0.1[b]

All values are the mean±standard error of 4 replicates.
Values in the same column with different superscript letters significantly differ at 5% significance level.
P2C, pine seedlings inoculated with *Phialocephala fortinii* under cold acclimation; CC, non-inoculated control seedlings under cold acclimation.

were 88, 172, and 53%. These plant growth characteristics decreased with cold acclimation. Moreover, the FW and DW of roots, stems, and needles of inoculated plants were significantly higher than those non-inoculated seedlings under cold acclimation (Tables 2, 3). Under cold acclimation, respective increases in the fresh biomass of roots, stems, and needles of inoculated pine seedlings were 114, 109, and 128%, and corresponding values in DWs were 197, 141, and 265%.

Our study revealed that *Phi. fortinii* inoculation largely promoted the growth and biomass of pine seedlings under cold acclimation (Tables 1~3). These results agreed with previous studies showing the beneficial effects of *Phi. fortinii* of promoting the growth and biomass of host plants (Fernando and Currah 1996, Jumpponen et al. 1998, Jumpponen and Trappe 1998, Alberton et al. 2010, Newsham 2011). On the other hand, many studies documented the positive effects of ECM symbiosis on plant growth and biomass (Dixon et al. 1983, Parke et al. 1983, Kropp et al. 1987, Kropp and Fortin 1988, Kropp and Langlois 1990, Rao et. al. 1996). Similar findings were reported elsewhere for ponderosa pine seedlings (Theodorou and Bowen 1970, Le Tacon and Bouchard 1986, Stenstrőm 1990).

Proline concentration

The free proline concentrations of needles of both inoculated and non-inoculated Scots pine seedlings significantly increased after exposure to low temperatures (Table 4). *Phialocephala fortinii*-inoculated pine seedlings had significantly higher proline concentrations in the needles than non-inoculated

Table 2. Fresh biomass of inoculated and non-inoculated Scots pine seedlings under cold-acclimation treatment

Treatment	Fresh biomass (g)		
	Roots	Stems	Needles
P2C	2.01±0.10[a]	1.78±0.26[a]	2.73±0.22[a]
CC	0.94±0.29[b]	0.85±0.06[b]	1.20±0.14[b]

All values are the mean±standard error of 4 replicates.
Values in the same column with different superscript letters significantly differ at the 5% significance level. Treatment codes are defined in Table 1.

Table 3. Dry biomass of inoculated and non-inoculated Scots pine seedlings under cold-acclimation treatment

Treatment	Dry biomass (g)		
	Roots	Stems	Needles
P2C	1.10±0.12[a]	0.70±0.03[a]	1.13±0.16[a]
CC	0.37±0.09[b]	0.29±0.03[b]	0.31±0.06[b]

All values are the mean±standard error of 4 replicates.
Values in the same column with different superscript letters significantly differ at the 5% significance level. Treatment codes are defined in Table 1.

Table 4. Proline concentration of Scots pine needles under hardening and cold-acclimation treatments

Treatment	Proline concentration (ppm)
P2H	8.10±0.39[c]
P2C	22.96±0.54[a]
CH	5.02±0.77[d]
CC	14.64±1.86[b]

All values are the mean±standard error of 4 replicates.
Values in the same column with different superscript letters significantly differ at the 5% significance level.
P2H, *Phialocephala fortinii* + hardening; P2C, *Phi. fortinii* + cold acclimation; CH, control + hardening; CC, control + cold acclimation.

ones after hardening and cold acclimation (Table 4). However, under normal conditions, there was no significant difference in proline concentrations between inoculated and non-inoculated pine seedlings. Under cold acclimation and hardening, respective proline concentrations of pine seedlings inoculated with *Phi. fortinii* were 57 and 61% higher compared to non-inoculated pine seedlings. Our study indicated that the concentration of proline in needles of hardened non-inoculated pine seedlings and needles of hardened pine seedlings inoculated with *Phi. fortinii* both increased 3-fold after cold acclimation (Table 4). Sagisaka and Araki (1983) reported that in tissues of many plant species, including conifers, there are seasonal changes in the free amino acid contents, particularly proline and arginine. In a study of white spruce (*Pic. glauca*) needles, the content of arginine increased in late autumn and decreased in winter, whereas the content of proline decreased during late autumn and increased in winter (Durzan 1968). Odlum et al. (1993) reported that in 5℃ treatments of black spruce seedlings, the shoot proline content was more than 3-fold that observed in 25℃ treatments. In

contrast, elevated proline contents were correlated with frost hardiness in the apple (Benko 1968), and when proline was applied to cultured maize cells, freeze damage was reduced (Withers and King 1979). Thus, our results show that low temperatures and *Phi. fortinii* inoculation which induces proline accumulation significantly increased the cold tolerance of pine seedlings.

Mineral concentrations

Mineral concentrations in the roots, stems, and needles of Scots pine seedlings were significantly affected by *Phi. fortinii* inoculation under normal and cold-acclimation treatments (Tables 5~7). Concentrations of N, P, and K very prominently increased, and Ca, Mg, and Na were also elevated by *Phi. fortinii* inoculation. For example, under cold-acclimation conditions, Ca, K, Mg, Na, and P concentrations in roots were respectively enhanced by 104, 77, 110, 37, and 150% (Table 5). Corresponding values of mineral concentrations in stems increased by 90, 75, 90, 45, and 122%, while values of mineral concentrations in needle increased to 69, 92, 68, 69, and 98%, respectively (Tables 6, 7). Clearly, the most significant increase was for phosphate, particularly in roots and stems. Also, nitrogen concentrations in roots, stems,

Table 5. Nitrogen and mineral concentrations of roots of inoculated and non-inoculated Scots pine seedlings under cold-acclimation treatment

Treatment	N (%)	Ca (ppm)	K (ppm)	Mg (ppm)	Na (ppm)	P (ppm)
P2C	1.52 ± 0.26^{a}	898 ± 70^{a}	643 ± 75^{a}	103 ± 21^{a}	477 ± 59^{a}	4402 ± 1007^{a}
CC	0.57 ± 0.21^{b}	441 ± 38^{b}	364 ± 38^{b}	49 ± 8^{b}	348 ± 47^{b}	1758 ± 370^{b}

All values are the mean ± standard error of 4 replicates.
Values in the same column with different superscript letters significantly differ at the 5% significance level. Treatment codes are defined in Table 1.

Table 6. Nitrogen and mineral concentrations of stems of inoculated and non-inoculated Scots pine seedlings under cold-acclimation treatment

Treatment	N (%)	Ca (ppm)	K (ppm)	Mg (ppm)	Na (ppm)	P (ppm)
P2C	1.16 ± 0.28^{a}	881 ± 12^{a}	780 ± 26^{a}	114 ± 20^{a}	432 ± 46^{a}	4291 ± 460^{a}
CC	0.39 ± 0.11^{b}	464 ± 25^{b}	447 ± 28^{b}	60 ± 12^{b}	299 ± 52^{b}	1933 ± 34^{b}

All values are the mean ± standard error of 4 replicates.
Values in the same column with different superscript letters significantly differ at the 5% significance level. Treatment codes are defined in Table 1.

Table 7. Nitrogen and mineral concentrations of needles of inoculated and non-inoculated Scots pine seedlings under cold-acclimation treatment

Treatment	N (%)	Ca (ppm)	K (ppm)	Mg (ppm)	Na (ppm)	P (ppm)
P2C	1.92 ± 0.09^{a}	804 ± 47^{a}	908 ± 70^{a}	74 ± 13^{a}	423 ± 56^{a}	4774 ± 418^{a}
CC	0.76 ± 0.26^{b}	476 ± 32^{b}	472 ± 49^{b}	44 ± 15^{b}	250 ± 5^{b}	2411 ± 143^{b}

All values are the mean ± standard error of 4 replicates.
Values in the same column with different superscript letters significantly differ at the 5% significance level. Treatment codes are defined in Table 1.

and needles were significantly (p = 0.05) higher in *Phi. fortinii*-inoculated Scots pine seedlings than the controls (Tables 5~7), with respective enhancements being 167, 197, and 153% after cold acclimation.

In the present study, *Phi. fortinii* inoculation was found to significantly increase the acquisition of nitrogen (N) and minerals (P, K, Ca, Mg, and Na) in roots, stems, and needles of Scots pine seedlings under normal and cold acclimation (Tables 5~7). Presumably, the increased uptake of minerals stimulated the growth of the test plants (Table 1). Previous studies showed that beneficial effects of *Phi. fortinii* inoculation included improved nutrient uptake of the host plant, especially N and phosphorus (P) (Stoyke et al. 1992, Newsham 1999, 2011, Addy et al. 2005, Mandyam and Jumpponen 2005, Grünig et al. 2008, Peterson et al. 2008, Smith and Read 2008, Upson et al. 2009). Furthermore, many studies documented the effects of ECM colonization on plant growth and nutrient uptake, especially N and P (Harley and Smith 1983, Nelsen 1987, Read et al. 1989, Sarjala and Potila 2005). Lee (1990) reported that ECMs could play an important role in successful establishment of seedlings by increasing nutrient and water uptake by plants and their resistance to environmental stress. Our results confirm the significant effects of ECM on the nutritional status of Scots pine seedlings.

Freeze tolerance of Scots pine seedlings

Analysis of freeze tolerance revealed that needle mortality of non-inoculated Scots pine seedlings was significantly higher than that of inoculated ones; the needle LT_{50} of non-inoculated pine seedlings was -12℃, whereas the needle LT_{50} of inoculated ones was lower at -15℃ (Fig. 3A). Under sunlight, freeze-injured needles of Scots pine seedlings turned brown followed by desiccation. Needles of non-inoculated Scots pine seedlings were more sensitive than inoculated ones, whereas needles of inoculated pine seedlings were more tolerant to freezing than were non-inoculated ones (Fig. 3A).

The whole-plant mortality of non-inoculated pine seedlings was also significantly higher than that of inoculated ones; the plant LT_{50} of non-inoculated Scots pine seedlings was -14℃, while that of the inoculated ones was -18℃ (Fig. 3B).

For seedling regrowth after freezing treatment, inoculated Scots pine seedlings exhibited a higher freeze tolerance than the controls (Fig. 3). For example, after 7 d at -14℃, respective mortality rates for inoculated and non-inoculated Scots pine seedlings were 16.7±4.1 and 50.0±5.5%, representing an almost 3-fold increase in cold tolerance (Fig. 3). In this study, proline concentrations changed after hardening and cold acclimation, which points to the importance of this period for acquiring frost tolerance. On the other hand, some studies showed the benefits of improved mineral nutrition on cold hardiness. Timmis (1974) reported that Douglas-fir seedlings with low foliar N content (of 0.8%) had an LT_{50} of -13℃, and those with a higher content (of 1.6%) had an LT_{50} of -30℃. Gleason et al. (1990) showed that fall-fertilized ponderosa pine seedlings with an N content of 1.55% were more cold-hardy than control seedlings with 1.47% N. Fernandez et al. (2007) observed that N-fertilized plants with > 1.25% N better tolerated freezing than those that had < 1%. In another study, *Pic. abies* seedlings with poor autumn cold hardiness had lower (of 1.1%) compared to higher (of > 1.6%) foliar N contents (Luoranen et al. 2008). Also, Islam et al. (2009) showed that an increased fertilizer rate improved mineral nutrition (higher shoot N concentrations), and this was associated with greater

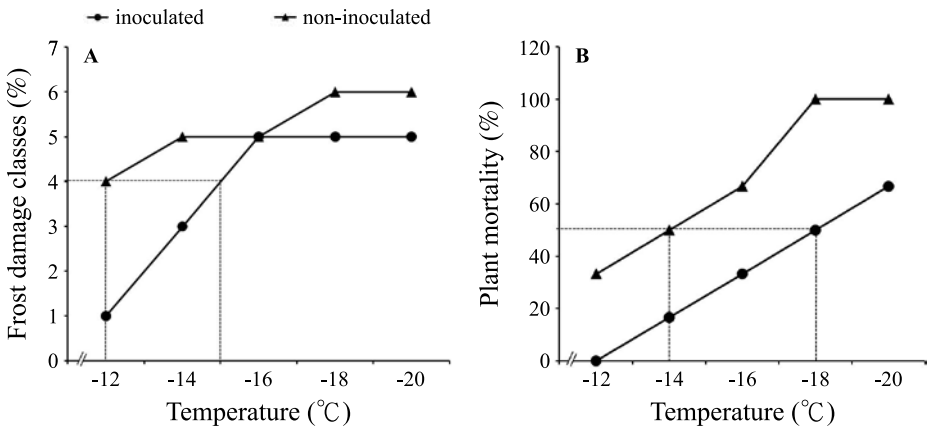

Fig. 3. Needle and plant mortality as a function of freezing temperatures. Needle (A) and whole plant (B) temperatures at which 50% mortality (LT_{50}) of inoculated and non-inoculated Scots pine seedlings occurred.

cold hardiness after the first growing season of *Pin. resinosa* seedlings. Also, our results showed that high N concentrations of Scots pine seedlings inoculated with ECM improved the freeze tolerance of pine seedlings (Tables 5~7). Overall, these results demonstrated that inoculation with ECM could improve the freeze tolerance of Scots pine seedlings.

CONCLUSIONS

In this study, *Phi. fortinii* effectively formed ECM in roots of pine seedlings. *Phialocephala fortinii* inoculation significantly promoted the growth and biomass accumulation of Scots pine seedlings. The enhancement in growth was reflected in increased plant height, root length, and net root collar diameter growth of *Phi. fortinii*-inoculated Scots pine seedlings. *Phialocephala fortinii* inoculation also significantly increased the nitrogen and mineral (P, K, Ca, Mg, and Na) contents in all tissues of Scots pine seedlings. Clearly, enhanced acquisition of P through ECM could stimulate root growth and subsequently promote absorption of other minerals and N to substantiate the higher growth rate and freeze tolerance. Furthermore, our results show that low temperatures and *Phi. fortinii* inoculation which induce proline accumulation increased the freeze tolerance of Scots pine seedlings. The needle and plant LT_{50} values of the inoculated Scots pine seedlings were significantly higher than those of non-inoculated plants. Inoculated Scots pine seedlings exhibited total survival at temperatures of as low as -18℃, while only 50% of non-inoculated seedlings survived at this freezing temperature. These results clearly demonstrated that *Phi. fortinii* could effectively form ECM with Scots pine seedlings and improve its growth and freeze tolerance.

ACKNOWLEDGEMENTS

We wish to thank Prof. Maurice S. B. Ku of the Department of Bioagricultural Science of National Chiayi Univ. and Prof. N. S. Talekar of the Department of Biological Resources of National Chiayi Univ. for reviewing the manuscript. Financial support from National Chiayi Univ. is also gratefully acknowledged.

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