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Effect of ectomycorrhizal infection on growth and photosynthetic characteristics of *Pinus densiflora* seedlings grown under elevated CO_2 concentrations

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Abstract

The effect of ectomycorrhizal *Pisolithus tinctorius* (*Pt*) infection was studied on the growth and photosynthetic characteristics of *Pinus densiflora* seedlings grown at ambient (360 µmol mol^{-1}, AC) and elevated (720 µmol mol^{-1}, EC) CO_2 concentrations. After 18 weeks, *Pt* inoculation had led to significantly increased dry mass and stem diameter of *P. densiflora* at both CO_2 concentrations, relative to non-inoculated seedlings. Moreover, EC significantly increased the ectomycorrhizal development. The phosphate content in needles inoculated with *Pt* was about three times higher than without inoculation at both CO_2 concentrations. The PAR saturated net photosynthetic rates (P_{sat}) of *P. densiflora* inoculated with *Pt* were clearly higher than for control seedlings at both CO_2 concentrations, and the maximum net photosynthetic rate (P_N) at saturated CO_2 concentration (P_{max}) was higher than in controls. Moreover, the carboxylation efficiency (CE) and RuBP regeneration rate of the P_N/C_i curve for *P. densiflora* inoculated with *Pt* were significantly higher than for non-inoculated seedlings at both CO_2 concentrations, especially at EC. The water use efficiency (WUE) of seedlings inoculated with *Pt* grown at EC was significantly raised. Allocation of photosynthates to roots was greater in *Pt* inoculated pine seedlings, because of the enhanced activity of ectomycorrhiza associated with seedlings at EC. Moreover, P_N of non-inoculated seedlings grown for 18 weeks at EC tended to be down regulated; in contrast, *Pt* inoculated seedlings showed no down-regulation at EC. The activity of ectomycorrhiza may therefore be enhanced physiological function related to water and phosphate absorption in *P. densiflora* seedlings at EC.

Additional key words: down-regulation; dry mass; phosphorus; *Pinus densiflora*; *Pisolithus tinctorius*; seedling diameter; stomatal limitation; water use efficiency.

Introduction

Red pine (*Pinus densiflora*) is the dominant conifer in infertile granite regions in the Korean peninsula and Japan. *P. densiflora* can survive and grow on the nutrient-poor ridges of mountains after disturbances. This species usually has a symbiotic interaction with ectomycorrhiza such as *Pisolithus tinctorius* (Pers.) Coker *et* Couch (*Pt*), allowing for vigorous growth. Where there have been several disturbances, *P. densiflora* was used for forest rehabilitation practices in nutrient-poor soils (*e.g*. Moroto *et al*. 1987), because they grow fast to mature trees in about 20 years.

The pattern and amount of precipitation at middle and high latitudes have recently been predicted to undergo great changes with the increase in atmospheric CO_2 concentration [CO_2] (IPCC 1996). Precipitation could decrease significantly. Moreover, photosynthetic adjustment is frequently observed in plants grown under high [CO_2] because of dilution effects of nutrient, enhanced accumulation of photosynthates in photosynthetic organs, and sink strength (Wardlaw 1990, Farrar and Williams 1991, Berntson and Bazzaz 1996).

Symbiotic microorganisms such as ectomycorrhiza usually act as an efficient root system for absorbing water and essential nutrients (nitrogen, phosphate) (Bolan 1991,

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Smith and Read 1997, Quoreshi *et al*. 2003). The activity of the host plant (*P. densiflora*) should be enhanced at high [CO_2] because symbiotic ectomycorrhiza plays an essential role in absorbing water and important elements such as nitrogen and phosphate, and also act as a large carbon sink (Ceulemans and Mousseau 1994, Smith and Read 1997). Therefore, no down-regulation of photosynthesis is predicted following *P. densiflora* inoculation with ectomycorrhiza at high [CO_2].

Several ectomycorrhizas form symbiotic interactions. Of these, *Pt* is the most common species in pine forests world-wide (Marx *et al*. 1984, Allen 1991). We studied the effect of *Pt* on the physiological responses of *P. densiflora* seedlings under high [CO_2]. Our aim was to estimate the effect of *Pt* development on the down-regulation of photosynthesis responses and the growth of *P. densiflora* at enhanced [CO_2] through water use efficiency and P concentration in needles.

Materials and methods

Plants: Seeds of red pine (*Pinus densiflora* Sieb. *et* Zucc.) were collected at the Yongmun Experimental Field of the Korea Forest Service (37.3°N, 127.2°E). These seeds were surface sterilized with 30 % H_2O_2 for 20 min and rinsed 4–5 times with sterile de-ionized water. They were then germinated on sterilized media, black sand : vermiculate : peat moss = 3 : 2 : 1 (volume), in a glasshouse at day/night temperatures 25/20 ºC with a 16-h photoperiod.

Ectomycorrhiza fungi, *Pisolithus tinctorius* (Pers.) Coker *et* Couch (*Pt*), were obtained from *Horticultural Alliance*, USA. The *Pt* spores were dissolved in distilled water, then inoculated directly to roots of *P. densiflora* seedlings, and mixed with prepared soil media (see below). The seedlings inoculated with *Pt* were naturalized in a rhizo-box (100×230×18 mm) filled with sterilized medium consisting of vermiculite : black sand : peat moss = 2 : 2 : 1 (volume).

Experimental design: Our experiments with *P. densiflora* were conducted at the Hokkaido Research Center, Forestry and Forest Products Research Institute (FFPRI), Sapporo, Japan in 2002. The seedlings were grown in a FFPRI phytotron with a natural sunlight, day/night temperature range of 26/16 ºC, and humidity range of 55–75 % during the study period lasting 18 weeks. Six cabinets were used, with two differing [CO_2]. The rhizo-boxes were allocated at random such that half of the seedlings experienced ambient [CO_2], AC (360 µmol mol^{-1}) and the other half experienced elevated [CO_2], EC (720 µmol mol^{-1}) (Koike 1995). At each CO_2 concentration (3 rooms of phytotron), half of the seedlings were *Pt* inoculated and the other half were not inoculated (control). The seedling number of each treatment in a room was sixteen. Each treatment was repeated in three rooms.

Measurement of photosynthesis: The net photosynthetic rate/intercellular CO_2 concentration (P_N/C_i) curves were examined using an open gas exchange system (*LI-6400*, *Li-Cor*, Lincoln, NE, USA) between 09:00 and 15:00 local time. From five to seven cm of above ground part was covered with a conifer chamber. The change in P_N was measured at saturating photosynthetic photon flux density (PPFD) of 1 000–1 200 µmol m^{-2} s^{-1} that was provided by a cool halogen lamp (*Walz*, Effeltrich, Germany). The leaf temperature was 25 ºC and the relative humidity was 50–70 %. Leaves were allowed to acclimate to their surroundings for 10 min prior to measurement, following which we began the determination with 150–1 500 µmol(CO_2) mol^{-1}.

The initial slope of the P_N/C_i curve is proportional to the carboxylation activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO), *i.e*. the carboxylation efficiency CE [µmol Pa^{-1}]. CE was calculated from the increment in C_i and P_N of the initial slope of the P_N/C_i curve (Terashima 1992). The maximum P_N at CO_2 saturation, P_{max} [µmol m^{-2} s^{-1}] was determined as 1 200 µmol(CO_2) mol^{-1}. Water use efficiency, WUE [µmol $mmol^{-1}$] was taken as the ratio of P_N to transpiration rate at PPFD saturation and constant water vapour pressure deficit (Field *et al*. 1983). The relative stomatal limitation of photosynthesis, l_s [%] was calculated as follows, based on P_N/C_i curves: $l_s = (P_0 - P_N)/P_0$, where P_0 is P_N when stomatal resistance to CO_2 diffusion is zero (Sharkey 1985a). After the gas exchange measurement, we measured the needle projection area with an image scanner (*FB636U*, *Canon*, Japan), and then calculated P_N per unit area.

Infection rate: We observed the root tips and counted both infected and non-infected roots (Quoreshi 2003). The inoculation rate of ectomycorrhiza (IRE) was determined according to the following formula: IRE [%] = ER/(ER+NR)×100, where ER and NR respectively denote the number of ectomycorrhizal and non-ectomycorrhizal roots.

Growth rate: Stem diameter of each seedling was measured immediately by digital caliper (*CD-15B*, *Mitutoyo Corp.*, Japan) take out from rhizo-box. Shoot and root dry masses of each seedling were measured after drying at 60 ºC for 1 week using an electronic balance (*HR-202*, *A&D*, Japan).

Phosphorus analysis: The dried samples were then ground to a fine powder in a vibrating sample mill (*Wonder Blender*, *Osaka Chemical Co.*, Osaka, Japan). To determine the content of P, the samples were digested by a microwave digestion system (*O·I Analytical*, College Station, TX, USA) and underwent ICP analysis.

Statistical methods: Mean values of the inoculation rate, the P content in needles, P_N, WUE, l_s, the shoot length, root growth, and stem diameter were examined by the *t*-test (Li 1964) using the *Stat View 5.0* software (*SAS Institute*, Cary, NC, USA).

Results

Dry mass and stem diameter: *Pt* inoculation significantly increased the shoot dry mass relative to non-inoculated seedlings grown at AC, from 187.7 to 293.7 ($p<0.05$). Moreover, the shoot dry mass of inoculated seedlings tended to increase at EC from 280.0 to 293.3 mg. The root dry mass also increased significantly relative to non-inoculated seedlings, from 169.3 to 437.0 mg at AC and from 276 to 464 mg at EC ($p<0.05$) (Fig. 1). Moreover, the stem diameter was significantly greater in seedlings infected with *Pt* at both CO_2 concentrations ($p<0.05$) (Fig. 1). *Pt* inoculation increased all growth parameters for *P. densiflora* seedlings, *i.e.* shoot and root dry mass and stem diameter, especially at AC. The dry masses and stem diameters of control seedlings grown at EC were significantly greater than at AC.

Mycorrhizal development and P content in needles (Fig. 1): After 18 weeks the ectomycorrhizal development was significantly affected by the CO_2 concentration ($p<0.05$). The inoculation rates with *Pt* grown at AC and EC were 55.1 and 66.5 %, respectively. We did not find *Pt* or any other ectomycorrhiza development in the non-inoculated seedlings grown at both CO_2 concentrations. The P content in needles inoculated with *Pt* was about 3 times higher than without inoculation at both CO_2 concentrations ($p<0.001$).

Photosynthetic response (Fig. 2): P_N of *P. densiflora* inoculated with *Pt* was significantly higher than for control seedlings in both [CO_2] treatments. The maximum P_N at saturating CO_2 concentration (P_{max}) of seedlings inoculated with *Pt* was higher than for controls ($p<0.05$). Moreover, CE and ribulose-1,5-bisphosphate (RuBP) regeneration rate of the P_N/C_i curve for *P. densiflora* inoculated with *Pt* were significantly higher than for non-inoculated seedlings at both CO_2 concentrations, and especially at EC (Table 1, $p<0.05$).

Table 1. Parameter estimates as functions of the P_N/C_i curve. Maximum net photosynthetic rate at saturated CO_2 concentration (P_{max}), carboxylation efficiency (CE), and RuBP regeneration rate of photosynthesis in the needles of *P. densiflora* seedlings grown at ambient (AC) or enhanced (EC) CO_2 concentrations. Means±SE, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. (*Pt* means seedlings infected with *Pt*).

		CE	RuBP regeneration	P_{max}
AC	Control	0.21±0.11	14.33± 5.42	7.86±1.37
	Pt	$0.43\pm0.12^{*}$	$54.94\pm 8.05^{**}$	$11.78\pm0.65^{*}$
EC	Control	0.17±0.02	20.18± 8.36	6.55±0.74
	Pt	$0.44\pm0.06^{***}$	$60.80\pm13.12^{**}$	$13.22\pm1.93^{**}$

WUE and stomatal limitation: WUE of *Pt* inoculated seedlings at EC was significantly higher than for uninoculated seedlings ($p<0.05$). However, no difference in WUE of seedlings at AC was found with *Pt* infection. In contrast, the WUE of non-inoculated seedlings grown at EC was significantly decreased, but in *Pt* inoculated seedlings the increase was not significant. Moreover, *P. densiflora* seedlings inoculated with *Pt* had lower stomatal limitation in photosynthesis (l_s) than non-inoculated seedlings. This tendency was clearer in the *Pt* inoculated seedlings at EC ($p<0.05$) (Fig. 1). The stomatal limitation in photosynthesis of *Pt* inoculated seedlings grown at EC was significantly decreased (Table 1).

Discussion

Inoculation with *Pt* significantly increased the dry mass and stem diameter of seedlings grown at both CO_2 concentrations after 18 weeks (Fig. 1). Previous research on the effect of ectomycorrhiza and EC on the biomass growth of forest tree seedlings revealed a varied response (Tissue *et al*. 1996, Gavito *et al*. 2000). Growth improvement in ectomycorrhizal plants has been found in several species (Allen 1992, Mousseau *et al*. 1996, Norby *et al*. 1996, Tissue *et al*. 1996, Smith and Read 1997, Rouhier and Read 1998, 1999). However, even at EC the dry mass of the shoot of seedlings inoculated with *Pt* was similar to that for non-inoculated seedlings. This growth pattern suggests that more photosynthates were allocated to those roots for increased activity of root cells and ectomycorrhiza, and pool accumulation of saccharides (*e.g.* Norby *et al*. 1987, Wright *et al*. 2000); this could also increase the production of fine roots and ectomycorrhiza.

The infection rate of *Pt* in *P. densiflora* seedlings grown at EC was significantly higher than at AC. The significantly enhanced P content in needles of *Pt*-inoculated seedlings was therefore due to the development of ectomycorrhiza (Fig. 1). Previous studies have also found that ectomycorrhizal development in seedlings of several tree species at EC was greater than at AC (Seegmüller and Rennenberg 1994, Ineichen *et al*. 1995, Rey and Jarvis 1997, Runion *et al*. 1997, Rouhier and

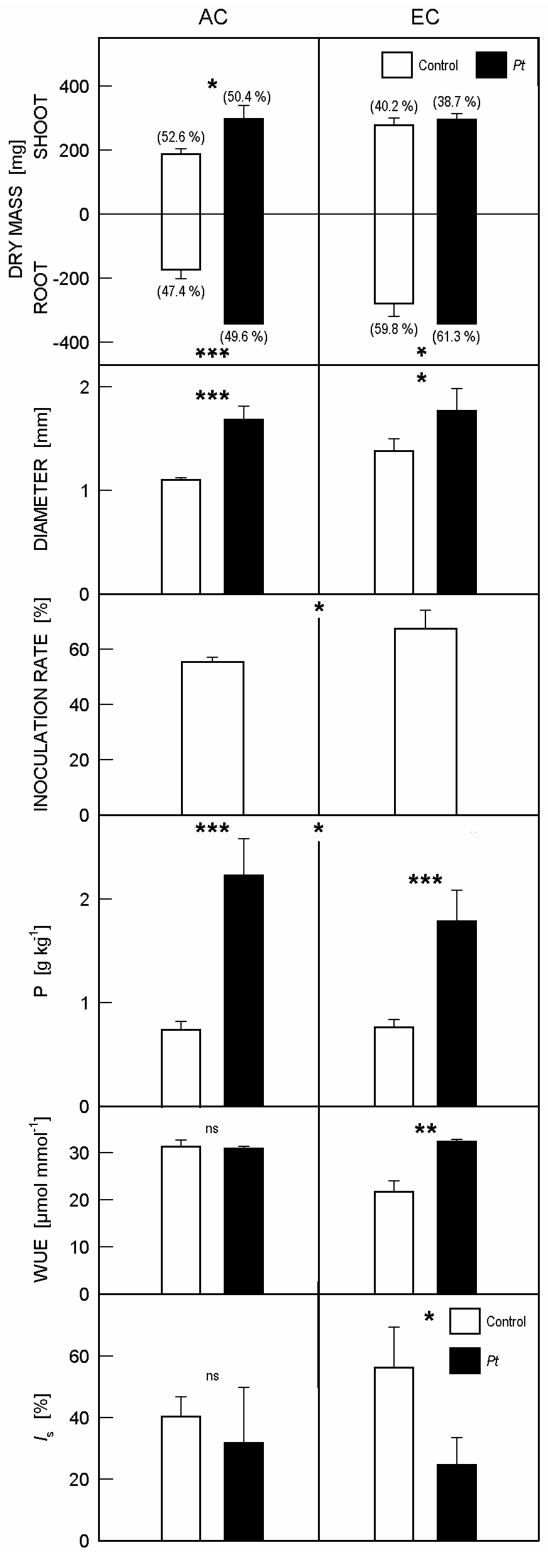

Fig. 1. Effect of ectomycorrhizal inoculation by *Pt* on the shoot and root dry mass, diameter, inoculation rate, P content, water use efficiency (WUE), and stomatal limitation (l_s) of *P. densiflora* seedlings grown at 360 μmol mol^{-1} (AC) or 720 μmol mol^{-1} (EC). % values give partition rate. Means±SE, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

Read 1998). Ectomycorrhizal development enlarges the absorptive surface of the root, with widely ramified hyphae allowing the release of phosphatases, which enhance the availability of organic phosphate and exude organic acids. These interactions between host plant and ectomycorrhiza increase the use efficiency of limited soluble phosphate and organic N in soil (Smith and Read 1997, Lambers *et al.* 1998). The P_N, CE, RuBP regeneration rate, and P_{max} of *Pt* inoculated *P. densiflora* seedlings grown at both CO_2 concentrations were significantly higher than those of non-inoculated seedlings, and the P content in needles was also significantly greater (see Table 1, Figs. 1 and 2). These photosynthetic changes are regulated by the capacity of RuBPCO to fix CO_2 and the capacity to regenerate RuBP (Farquhar *et al.* 1980, Farquhar and Sharkey 1982). Under some conditions, phosphate (P_i) may limit photosynthesis if P_i utilization during CO_2 assimilation and RuBP regeneration exceeds the capacity for P_i release during starch and sucrose synthesis (Sharkey 1985b, Lambers *et al.* 1998).

Ectomycorrhizal plants enhance the rate of photosynthesis over non-mycorrhizal plants as a result of improved plant nutritional status, including N and P (Reid *et al.* 1983, Bolan 1991, Finlay 1992, Smith and Read 1997, Quoreshi 2003, Quoreshi *et al.* 2003), even if the P content is the same in needles (Rousseau and Reid 1990). Moreover, ectomycorrhiza improves WUE (Guehl *et al.* 1990, Duñabeitia *et al.* 2004) by enlarging the soil volume exploited, *via* widely distributed hyphae (Smith and Read 1997, Lambers *et al.* 1998). In general, the P content in needles affects photosynthesis through RuBP regeneration (Sharkey 1985a, Fredeen *et al.* 1990, Kirschbaum and Tompkins 1990, Lewis *et al.* 1994), through thylakoids (Conroy *et al.* 1986, Lambers *et al.* 1998), and through the peak carboxylation velocity or peak capacity of electron transport (Harley and Sharkey 1991). We conclude that ectomycorrhiza development in inoculated seedlings and the increased P content in needles enhance the P_N and WUE and reduce stomatal limitation relative to non-inoculated seedlings (Smith and Read 1997, Hensen *et al.* 1998) (Table 1, Figs. 1 and 2).

P_N of non-inoculated seedlings grown at EC for 18 weeks tends to be down regulated, *i.e.* the seedlings exert reduced CE, P_{max}, and P_N/C_i curve. In contrast, *Pt* inoculated seedlings showed no down-regulation at EC. This phenomenon has been reported in other studies with adequate soil N fertility (Idso and Kimball 1991), with N_2 fixing species even when grown in poor soils (Vogel and Curtis 1995), or with mycorrhizal plants (Staddon *et al.* 1999). Phosphorus or nitrogen limitation (Conroy *et al.* 1986, Jach and Ceulemans 2000, Koike *et al.* 2000, Eguchi *et al.* 2004) and reduced sink strength (*e.g.* Stitt 1991, Rogers *et al.* 1998, Griffin *et al.* 2000) have both been proposed as mechanisms inducing down-regulation, *via* reduction of RuBP regeneration capacity and reduced RuBP carboxylase/oxygenase activity (Sharkey *et al.* 1994, Adam *et al.* 2004). We therefore suggest that *Pt*

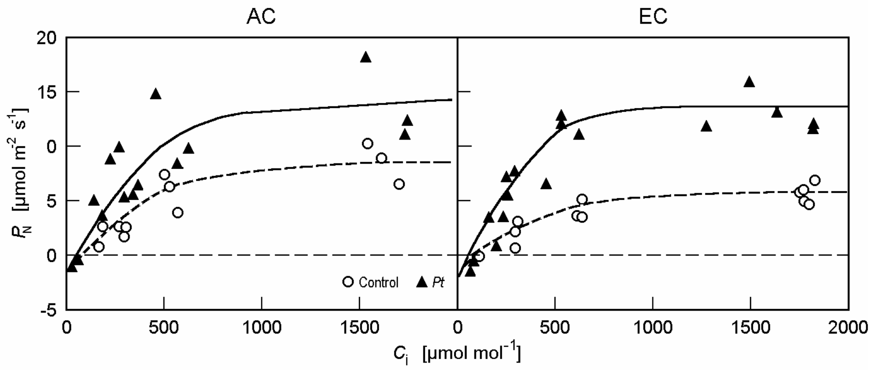

Fig. 2. Effect of ectomycorrhiza inoculation by *Pt* on the net photosynthetic rate (P_N) response curve to intercellular CO_2 concentration (C_i) of *P. densiflora* seedlings grown at 360 μmol mol^{-1} (AC) or 720 μmol mol^{-1} (EC). ▲ = inoculated *Pt* seedlings, ○ = control. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

inoculated seedlings have increased nutrient and water uptake, leading to improved plant nutritional status and giving rise to more vigorous physiological response, in particular photosynthetic activity; and that these responses delay down-regulation at EC.

Our present results indicate that ectomycorrhiza inoculation increases uptake of nutrients and water, and the resulting vigorous physiological response significantly increases the dry mass and stem diameter of seedlings grown at AC and EC. Moreover, ectomycorrhizal seedlings moderated down-regulation at EC. Ectomycorrhiza therefore might have strong positive effects on *P. densiflora* seedlings at both CO_2 concentrations.

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