Mycorrhiza (2009) 19:425–434
DOI 10.1007/s00572-009-0245-2

ORIGINAL PAPER

Ostryopsis davidiana seedlings inoculated with ectomycorrhizal fungi facilitate formation of mycorrhizae on *Pinus tabulaeformis* seedlings

Shu-Lan Bai · Guo-Lei Li · Yong Liu · R. Kasten Dumroese · Rui-Heng Lv

Received: 27 October 2008 / Accepted: 2 April 2009 / Published online: 28 April 2009

Abstract Reforestation in China is important for reversing anthropogenic activities that degrade the environment. *Pinus tabulaeformis* is desired for these activities, but survival and growth of seedlings can be hampered by lack of ectomycorrhizae. When outplanted in association with *Ostryopsis davidiana* plants on reforestation sites, *P. tabulaeformis* seedlings become mycorrhizal and survival and growth are enhanced; without *O. davidiana*, pines often remain without mycorrhizae and performance is poorer. To better understand this relationship, we initiated an experiment using rhizoboxes that restricted root and tested the hypothesis that *O. davidiana* seedlings facilitated ectomycorrhizae formation on *P. tabulaeformis* seedlings through hyphal contact. We found that without *O. davidiana* seedlings, inocula of five indigenous ectomycorrhizal fungi were unable to grow and associate with *P. tabulaeformis* seedlings. Inocula placed alongside *O. davidiana* seedlings, however, resulted in enhanced growth and nutritional status of *O. davidiana* and *P. tabulaeformis* seedlings, and also altered rhizosphere pH and phosphatase activity. We speculate that these species form a common mycorrhizal network and this association enhances outplanting performance of *P. tabulaeformis* seedlings used for forest restoration.

Keywords Reforestation · Common mycorrhizal networks · Phosphatase · Mycorrhizal infection facilitation · Rhizobox

S.-L. Bai
College of Forestry, Inner Mongolia Agricultural University, Hohhot 010019, China

G.-L. Li (✉) · Y. Liu · R.-H. Lv
Key Laboratory for Silviculture and Conservation, Ministry of Education, Beijing Forestry University, Beijing 100083, China
e-mail: glli226@163.com

R. Kasten Dumroese
USDA Forest Service, Rocky Mountain Research Station, Moscow, ID 83843-4211, USA

Introduction

Past and present anthropogenic activities in China have led to widespread soil erosion, flooding, and dust storms (Wang 2004). Because these maladies are linked in part to forest degradation, forest restoration has become an important issue. The Chinese government has infused large amounts of capital into reforesting and afforesting the country to reduce these problems, as well as retain biodiversity, restore ecological function, and enhance rural community welfare (Li 2004). Daqing Mountain, within the Yinshan Mountain Range of Inner Mongolia north of the cities of Hohhot and Baotou, is an area where forest restoration is underway. Although this mountainous area 400 km west of Beijing acts as a buffer against dust storms, anthropogenic degradation of vegetation remains severe on Daqing Mountain.

In some areas, the soils of Daqing Mountain are protected by the Chinese endemic *Ostryopsis davidiana* Decaisne. (Betulaceae) (syn = *Corylus davidiana* (Decaisne.) Baillon). This shrubby (<3 m tall) species is found throughout north-central China, forming sparse forests or thickets (Li and Skvortsov 1999). *Pinus tabulaeformis* Carr. (Pinaceae), another Chinese endemic, is also found across north-central China; its range overlaps with *O. davidiana* in Sichuan, Hebei, and Inner Mongolia provinces (Ren 2002). Widely used in northern China for afforestation (Wu and Nioh 1997), this conifer is desired for forest restoration on Daqing

Mountain. Unfortunately, in the absence of mycorrhizae, survival after outplanting is poor (Gong et al. 1997). Compared to colonized seedlings, non-mycorrhizal seedlings lacked drought tolerance (Boyle and Hellenbrand 1990) and reduced access to soil nutrients (Owusu-Bennoah and Wild 1980; Pfeffer et al. 2001). Bai et al. (2006) found, however, that survival of outplanted *P. tabulaeformis* seedlings was more than 85% and trees grew well if they were outplanted in association with *O. davidiana*, even on extremely harsh (sunny and dry) sites. Moreover, colonization of *P. tabulaeformis* by ectomycorrhizal fungi (ECM) was greater than 50% when the pine and *O. davidiana* formed a mixed forest. On similar sites that lacked *O. davidiana*, *P. tabulaeformis* remained without ECM associations and survival and growth were much poorer. This suggests the possibility that *O. davidiana* may facilitate mycorrhizal colonization of *P. tabulaeformis*, and/or that these two species may share a common mycorrhizal network (CMN).

CMNs are characterized by hyphae of ECM physically connecting roots of plants from similar or diverse species, genera, or families that make up various plant communities (Wu et al. 2001; Kennedy et al. 2003; He et al. 2006). Nutrients, water, and photosynthates move through these connections (Wu et al. 2001; He et al. 2006; Plamboeck et al. 2007). Therefore, outplanted pine seedlings that readily become part of an existing mycorrhizal network associated with *O. davidiana* plants could more rapidly benefit from ECM, and this may explain the enhanced survival and growth of *P. tabulaeformis* seedlings outplanted in association with *O. davidiana* observed by Bai et al. (2006).

In this study, we hypothesized that *O. davidiana* seedlings inoculated with common ECM from Daqing Mountain facilitate ECM formation on *P. tabulaeformis* seedlings via hyphal contact. We grew *O. davidiana*, with and without viable ECM inoculant, alongside *P. tabulaeformis* seedlings in rhizoboxes that allowed hyphal contact between plants but prevented root-to-root contact. We predicted that hyphae from *O. davidiana* mycorrhizae would contact *P. tabulaeformis* roots and thereby cause ECM colonization of the pines.

Materials and methods

Seed and fungi collection

Daqing Mountain is located in central Inner Mongolia within the Yinshan Mountain Range (Long 109°46′ E to 113°04′ E, Lat 40°34′ N to 41°18′ N). Seeds of *O. davidiana* were collected from healthy trees on Daqing Mountain (1,600 m) whereas seeds of *P. tabulaeformis* were purchased from a commercial seed company in Inner Mongolia. ECM fruiting bodies were collected underneath *O. davidiana* plants growing in mixture with *Larix gmelinii* var. *principis-rupprechtii* (Mayr) Pilger (Pinaceae) (Fu et al. 1999) on Daqing Mountain in August 2004. They were identified, according to fruiting body morphology as described by Mao (1998), as *Leucocortinarius bulbiger* (Alb. et Schw.) Sing., *Rhizopogon luteolus* Fr. et Nordg., *Suillus grevillei* (Klotzsch) Sing., *Tricholoma fulvum* DC.: Fr.Rea., and *Tricholoma terreum* (Schaeff.:Fr.) Kummer.

Mycorrhizal inoculant

Each fungus was aseptically isolated onto agar to produce pure culture isolates following the techniques of Brundrett et al. (1996). We prepared inoculant by mixing 1,000 ml of liquid modified Melin Norkans medium (MMN; Marx 1969) with 450 g vermiculite into a glass container, autoclaving the mixture 60 min at 121°C, and then adding ten pieces (each 5×5×5 mm) of each pure culture isolate growing on agar. Once added, the fungi were allowed to grow inside a dark growth chamber (25°C) for about 25 days to colonize the MMN–vermiculite matrix (Bai et al. 2004).

Seedling cultivation

Seeds of both species were sterilized by soaking 20 min in a 2% NaClO solution, rinsed three times with aseptic water and placed inside an aseptic glass container for germination. Germinated seeds were transplanted into aseptic clay containers (20 cm tall and 25 cm in diameter). Containers were filled with two parts soil collected from Daqing Mountain, sieved with a 5-mm screen, autoclaved 90 min at 121°C, and mixed with one part vermiculite (*w*/*w*). The substrate had 0.028% total N, 4.56 mg kg^{-1} P (Olsen-P), 61.01 mg kg^{-1} NH_4Ac-K, 601.11 mg kg^{-1} HNO_3-K, and a pH of 8.2. Each container received ten transplants of a single species, and full containers were placed inside a growth chamber with a 14-h light/10-h dark photoperiod providing 370 μmol s^{-1} m^{-2} and temperatures of 27°C/20°C. Seedlings were irrigated once every 5 days (500 ml $container^{-1}$) and fertilized once every 15 days (200 ml $container^{-1}$) with a 10% Hoagland nutrient solution (Liu and Li 2000). Seedlings were ready for experimentation after 3 months of growth; *Ostryopsis* and pine seedlings were about 52 and 46 mm tall, respectively.

Rhizobox construction

To test our hypothesis, we used Plexiglas rhizoboxes constructed following the basic style of Faber et al. (1991). Each box was 30 cm long, 10 cm wide, and 12 cm deep, with a piece of nylon net (30-μm mesh to restrict plant root growth but allow hyphae passage; see Warren et al. 2008; Withington

et al. 2006) inserted vertically to divide each rhizobox into two 15 cm long compartments. Each compartment had three 5-mm drainage holes.

2006 pilot seedling–inoculant treatments

In 2006, we tested the six levels of ECM fungi (five species and a control) three times (three rhizoboxes per each level of ECM). We placed a 3-cm deep layer of the same autoclaved vermiculite–forest soil substrate described above into each half (compartment) of the rhizobox. We then carefully removed an *O. davidiana* seedling from the growth chamber and transplanted it into one compartment of the rhizobox. As the compartment was filled with more vermiculite–soil substrate, we placed about 5 g of the fungal inoculum from one species proximal (0 to 1 cm distant to) the root system at a depth of about 10 cm. This rate was based on earlier successful work (Bai et al. 2004; Han et al. 2005). Similarly, a *P. tabulaeformis* seedling from the growth chamber was transplanted into the other compartment filled with the same vermiculite–soil substrate, so that the distance between the seedlings was about 15 cm. For the control (no viable inoculum), we took 1 g of inoculum from each fungus, mixed them, autoclaved this composite 60 min at 121°C, and added it to the *O. davidiana* side as described above. We also tested the effect of adding autoclaved inoculum in the absence of an *O. davidiana* seedling. All rhizoboxes were randomly placed inside a growth chamber with the same conditions described for seedling cultivation.

2007 seedling–inoculant treatments

Because of observations from 2006, we repeated the experiment but instead evaluated two levels of *O. davidiana* seedlings (present or absent) in combination with six levels of ECM fungi (five species and a control). Each *O. davidiana*–ECM combination constituted a treatment, and each treatment was replicated three times (three rhizoboxes per treatment). The resulting 2 *O. davidiana* (present or absent)×6 ECM (including the autoclaved control)×3 replications completely randomized design required 36 rhizoboxes.

Measurements

For both years, 3 months after transplanting into the rhizoboxes, seedlings were gently removed from the rhizoboxes and their root systems carefully washed free of the substrate. ECM colonization rate was quantified using the gridline interaction methods of Brundrett et al. (1996) and mycorrhizae characterized as described below. Height was measured from the root collar to the most acropetal point on the main stem. Stem diameter was measured at the root collar. Biomass was determined after drying at 60°C to constant weight. We determined total nitrogen (N) concentration of seedlings using an HR-500 Auto-Nitrogen-Meter (Huarui Instrument Company, Shanghai, China) and total phosphorus (P) colorimetrically using an Ultro-spectrophotometer (Daojing U-1800, Hitachi High-technologies Corporation, Tokyo, Japan) following the methods of Cui (1998).

In 2007, we collected about 50 g soil (about 5 cm from the main taproot of each seedling) to determine pH and assay phosphatase activity. For pH, we thoroughly mixed 25 ml distilled water with 10 g of soil and after 30 min measured reactivity with a pHs-3C acidity meter. For the acid and alkaline phosphatase assay, we placed 0.5 g of soil in a 50-ml Erlenmeyer flask and added 0.2 ml methylbenzene, 5 ml *p*-nitrophenyl phosphate, and a buffer (0.2 M acetic acid to adjust the sample to pH 5.0 or 0.5 M $NaHCO_3$ to adjust to pH 8.5). The flasks were stoppered and placed in a stable temperature oven at 30°C. After 1 h, we added 4 ml of 0.5 M NaOH to each flask to stop the reaction followed by 1 ml of 0.5 M $CaCl_2$. After mixing well, the soil suspension was filtered through filter paper and the filtrate analyzed in an Ultro-spectrophotometer (Daojing U-1800) at 410 nm using distilled water as the control (Guan 1986). The amount of *p*-nitrophenyl phosphate (milligram per gram) in the sample was calculated against a standard calibration curve.

Mycorrhizae characterization

Washed roots were observed at ×10–45 magnification with a stereomicroscope (Motic SMZ-168, Hong Kong, China). We described mycorrhizae (unbranched or dichotomous), mantle color, morphology of extraradicular hyphae, and measured rhizomorph length and diameter with a micrometer. A small portion of a hypha was extracted with a dissecting needle, wet-mounted on a slide, and observed at ×400–640 with an Olympus BHS-312 (Center Valley, PA, USA) to observe clamp connections. Finally, we made paraffin cross sections of mycorrhizal root tips. Root sections were washed and then fixed by soaking 24 h in formalin-aceto-alcohol (FAA; 50 ml 95% alcohol, 5 ml glacial acetic acid, 5 ml 37–40% formalin, and 35 ml distilled water). After soaking, sections were dried by immersion in a sequence of 85%, 95%, and 100% alcohol for 2–4 h at each concentration and then soaked in paraffin. Sections were sliced and dyed with 1% safranine (50% alcohol) for 6–12 h, washed to remove the color, and dyed with 0.5% fast green FCF (95% alcohol) for 10–60 s. Sections were washed with acetone, treated with phenol and dimethylbenzene, and washed three times with dimethylbenzene. Slides were placed under ×200–1,000

(Olympus BHS-312) to observe Hartig nets and measure mantle thickness with a micrometer.

Statistical analysis

For the 2006 experiment, two-way analysis of variance (ANOVA; SAS Institute, Cary, NC, USA) was used to determine if our predictor variable (fungal species) significantly affected our response variables (ECM infection rate, height, stem diameter, and dry mass) of *O. davidiana* seedlings using SAS PROC GLM (alpha=0.05). The process was repeated for pine seedlings. For each analysis, treatment means within main effects were compared using Bonferroni tests.

For the 2007 experiment, we again used two-way ANOVA PROC GLM to determine if our predictor variable (fungal species) significantly affected our response variables (ECM infection rate; seedling height, stem diameter, dry mass, and N and P concentrations; soil pH and acid and alkaline phosphatase activity) at alpha=0.05. To test the effects of the different ECM fungi on *P. tabulaeformis*, we used a 2 *O. davidiana* (present or absent)×6 ECM (including the autoclaved control)×3 replications completely randomized design. We used ANOVA and alpha=0.05 to determine if our predictor variables (fungal species, presence or absence of *O. davidiana*, and the fungi × *O. davidiana* interaction) significantly (alpha=0.05) affected the same response variables as described for the *O. davidiana* analysis. For each analysis, treatment means within main effects were compared using Bonferroni tests.

Results

Mycorrhizae descriptions used to confirm fungi identity

For each isolate, we observed unique mycorrhizae morphology. Moreover, for each isolate that formed mycorrhizae on *O. davidiana* and subsequently on *P. tabulaeformis*, we found similar mycorrhizae morphology (except for root tip branching pattern) as described herewith.

L. bulbiger Mycorrhizal root tips were 100% unbranched on *O. davidiana*; 31% unbranched and 69% dichotomous on *P. tabulaeformis*. On both species, mantles were milky white and 40 to 50 μm thick, Hartig net in outer cortex cells; extraradicular hyphae had obvious clamp connections and were white, hairy, somewhat sparse and very long, twining together; rhizomorphs had color a bit darker than the extraradicular hyphae and were 300 to 400 μm in diameter and 20 to 30 mm long, easily observed with the naked eye.

R. luteolus Mycorrhizal root tips were 100% unbranched on *O. davidiana*; 27% unbranched and 73% dichotomous on *P. tabulaeformis*. On both species, mantles were milky white and 40 to 50 μm thick, Hartig net in outer cortex cells; extraradicular hyphae had less obvious clamp connections and were white, cotton fiber-like, somewhat dense, and 3 to 5 mm long; rhizomorphs were absent.

S. grevillei No mycorrhizae were observed on *O. davidiana*, 100% dichotomous root tips on *P. tabulaeformis*. Mantles were milky white and 40 to 50 μm thick; extraradicular hyphae had obvious clamp connections and were white, very fine, hairy-like, and 2 to 3 mm long; rhizomorphs were absent.

T. fulvum No mycorrhizae were observed on *O. davidiana*; 29% unbranched and 71% dichotomous on *P. tabulaeformis*. Mantles were milky white and 20 to 30 μm thick, Hartig net in outer cortex cells; extraradicular hyphae had obvious clamp connections and were white, very fine, hairy-like, sparse, and closely attached to the root surface; rhizomorphs were absent.

T. terreum Mycorrhizal root tips were 100% unbranched on *O. davidiana*; 28% unbranched and 72% dichotomous on *P. tabulaeformis*. On both species, mantles were milky white and 40 to 50 μm thick, Hartig net in outer cortex cells; extraradicular hyphae had obvious clamp connections and were white, hairy, sparse and very long, twining together; rhizomorphs had color a bit darker than the extraradicular hyphae and were 300 to 500 μm in diameter and 30 to 40 mm long, easily observed with the naked eye.

2006 experiment

We observed that only *L. bulbiger*, *R. luteolus*, and *T. terreum* readily formed mycorrhizal associations with *O. davidiana*, with a colonization of about 70% (Table 1). Colonization significantly increased *O. davidiana* height, stem diameter, and dry mass on average 114%, 322%, and 114%, respectively, compared to non-mycorrhizal plants and the control (Table 1). All five fungi formed associations with *P. tabulaeformis*; root tip colonization averaged 48% but was zero for the *O. davidiana* + autoclaved inoculum and the no *O. davidiana* + autoclaved inoculum treatments (Table 2). In general, about 70% of the mycorrhizal root tips on the pine were dichotomously branched. As with *O. davidiana*, ECM colonization significantly increased height (64%) and dry mass (93%) compared to the autoclaved inoculum controls (Table 2). Stem diameter, however, was unaffected (P=0.993; data not shown) by ECM colonization and averaged 4.5 mm across treatments.

Table 1 Means±1 SE (n=3) for *Ostryopsis davidiana* seedling parameters after inoculation with five ectomycorrhizal fungi (*Leucocortinarius bulbiger*, *Rhizopogon luteolus*, *Suillus grevillei*, *Tricholoma fulvum*, and *Tricholoma terreum*) and a control (autoclaved mixture of inocula)

	ECM colonization rate (%)		Height (mm)		Stem diameter (mm)		Dry mass (g)	
	2006	2007	2006	2007	2006	2007	2006	2007
Control	0±0 b	0±0 c	80±2 c	78±2 d	1.5±0.1 d	1.2±0.1 c	0.90±0.1 c	0.94±0.04 cd
L. bulbiger	70.1±1.6 a	86.4±0.7 ab	255±3 a	285±9 a	16.4±0.3 a	17.3±0.4 a	2.46±0.06 a	2.56±0.09 a
R. luteolus	69.3±0.6 a	88.0±1.8 a	255±7 a	260±5 ab	15.8±0.2 a	10.2±0.2 c	2.48±0.05 a	2.40±0.06 a
S. grevillei	0±0 b	0±0 c	165±3 b	166±3 c	5.5±0.4 c	4.1±0.1 d	1.26±0.04 b	1.23±0.05 bc
T. fulvum	0±0 b	0±0 c	100±2 c	110±5 d	4.0±0.1 c	4.1±0.3 d	0.87±0.05 c	0.85±0.06 d
T. terreum	70.0±0.1 a	80.5±2.8 b	236±6 a	236±10 b	13.4±0.5 b	14.6±0.4 b	1.38±0.04 b	1.48±0.01 b
P value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0006	<0.0001	0.0022

Different letters within each response variable column and year column indicate statistically different values using analysis of variance (alpha=0.05) and Bonferroni groupings to separate means

2007 experiment

O. davidiana

Although the addition of ECM significantly ($P \leq 0.0022$) affected every response variable measured for *O. davidiana* seedlings, we again observed that only *L. bulbiger*, *R. luteolus*, and *T. terreum* readily formed mycorrhizal associations (Tables 1 and 3). Colonization by ECM resulted in greater seedling height, stem diameter, and dry mass (Table 1). In addition, colonization by *L. bulbiger*, *R. luteolus*, and *T. terreum* also increased seedling N and P concentrations, lowered rhizosphere pH, and increased phosphatase activity compared to the control (Table 3). Although we were unable to observe ectomycorrhizae when seedlings were inoculated with *T. fulvum* or *S. grevillei*, compared to the control, these seedlings had greater concentrations of N, tended toward more P, had larger stem diameters, and for *S. grevillei*, greater height as well (Table 3).

P. tabulaeformis

O. davidiana significantly affected pine seedling morphology (except stem diameter) and nutrient concentration, as well as rhizosphere chemistry (Table 4). Without *O. davidiana* seedlings, pine seedlings lacked mycorrhizae but with *O. davidiana* seedlings, ECM colonization rate was 58% with most (70%) colonized root tips being dichotomous. Mycorrhizal pine seedlings were 49% taller with 59% more biomass and had 82% and 98% greater N and P concentrations, respectively (data not shown). Moreover, rhizosphere soil from mycorrhizal pine seedlings had, on average, 78% and 60% greater acid and alkaline, respectively, phosphatase activity as well as a reduction in soil pH from 7.9 to 7.5 (data not shown).

ECM fungi significantly affected pine seedling morphology (except stem diameter) and nutrient concentration, as well as rhizosphere phosphatase activity (Table 4). Rhizosphere pH was unaffected. As with *O. davidiana* seedlings, *L. bulbiger*, *R. luteolus*, and *T. terreum* showed the greatest

Table 2 Means±1 SE (n=3) for *Pinus tabulaeformis* seedling parameters after inoculation of *O. davidiana* with five ectomycorrhizal fungi (*Leucocortinarius bulbiger*, *Rhizopogon luteolus*, *Suillus grevillei*, *Tricholoma fulvum*, and *Tricholoma terreum*) and two autoclaved inocula controls in the 2006 experiment

	ECM colonization rate (%)	Height (mm)	Dry mass (g)
Autoclaved inoculum	0±0 c	50±2 b	0.48±b
O. davidiana + autoclaved inoculum	0±0 c	58±5 b	0.54±b
L. bulbiger	53.1±1.8 ab	99±2 a	1.00±a
R. luteolus	54.1±2.3 a	99±6 a	1.09±a
S. grevillei	43.0±1.3 b	95±3 a	0.93±a
T. fulvum	48.2±1.3 ab	93±1 a	0.92±a
T. terreum	43.0±1.9 b	88±1 a	0.98±a
P value	<0.0001	<0.0001	<0.0001

Table 3 Means±1 SE (n=3) for *Ostryopsis davidiana* seedling nitrogen and phosphorus concentration and soil rhizosphere chemistry after inoculation with five ectomycorrhizal fungi (*Leucocortinarius bulbiger*, *Rhizopogon luteolus*, *Suillus grevillei*, *Tricholoma fulvum*, and *Tricholoma terreum*) and a control (autoclaved mixture of inocula) in the 2007 experiment

	Seedling		Rhizosphere		
	N concentration (mg g^{-1})	P concentration (mg g^{-1})	pH	Acid phosphatase[a] (mg g^{-1})	Alkaline phosphatase (mg g^{-1})
Control	12.5±0.4 b	1.9±0.1 b	7.93±0.05 a	6.37±0.62 c	4.46±0.12 c
L. bulbiger	23.2±0.6 a	3.0±0.2 a	7.12±0.06 b	12.28±0.38 a	8.47±0.18 a
R. luteolus	22.3±0.3 a	3.1±0.2 a	7.20±0.01 b	10.36±0.55	5.46±0.29 b
S. grevillei	19.6±0.3 a	2.2±0.1 ab	7.70±0.09 a	8.36±0.23 bc	5.46±0.09 b
T. fulvum	20.4±0.9 a	2.5±0.2 ab	7.18±0.09 b	7.37±0.16 c	4.62±0.15 bc
T. terreum	23.4±0.8 a	3.1±0.2 a	7.14±0.04 b	10.37±0.65 ab	5.46±0.12 b
P value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

Different letters within each response variable column indicate statistically different values using analysis of variance (alpha=0.05) and Bonferroni groupings to separate means

[a] *p*-nitrophenyl phosphate

(>40%) colonization rates, but pine seedlings were also colonized by *T. fulvum* or *S. grevillei* albeit it at a significantly lower (26%) rate, which was significantly greater than the control seedlings (0%). Despite these differences in ECM colonization rates, seedling morphology (height, dry mass) and N concentration and phosphatase activity were similar among the five ECM fungi and significantly greater than the control (data not shown).

The interaction of *O. davidiana* and ECM significantly affected pine seedling morphology (except stem diameter) and N concentration, as well as phosphatase activity (Table 4). Seedling P concentration and rhizosphere pH were unaffected. Co-placement of ECM inoculum and an *O. davidiana* seedling was more effective in increasing mycorrhizae formation, seedling size and nutrient concentration (Fig. 1), and phosphatase activity (Fig. 2) than either inoculum alone or *O. davidiana* seedlings alone. Although not significant, when *O. davidiana* and ECM were present, P concentration showed a trend toward higher levels (Fig. 1) whereas soil pH trended toward a lower value (Fig. 2) than for non-mycorrhizal seedlings.

Discussion

Our results show that *O. davidiana* seedlings colonized with ECM fungi grew better than non-mycorrhizal seedlings. Both colonized and non-colonized *O. davidiana* plants facilitated subsequent mycorrhizae formation on *P. tabulaeformis* seedlings, manifested by production of dichotomous root tips on the pine root systems and improved seedling growth. Because root-to-root contact was prevented, this facilitation was through hyphal contact. In the absence of *O. davidiana* seedlings, ECM inoculum was unable to grow and reach the root systems of *P. tabulaeformis* seedlings. These results support our hypothesis.

Without mycorrhizae, our *P. tabulaeformis* seedlings grew poorer than their mycorrhizal cohorts, suggesting, at least in the short-term, that this pine is mycorrhizal responsive rather than dependent in the soil we used (Janos 2007). We observed enhanced *P. tabulaeformis* growth and nutritional status after colonization by ECM, which concurs with others for this pine (Wu and Nioh 1997; Wu et al. 1999) and with others for various species (Harley and

Table 4 *P* values for the effects of the predictor variables (ECM and *O. davidiana*) and their interaction on the nine response variables for *Pinus tabulaeformis* in the 2007 experiment using two-way analysis of variance (alpha=0.05)

Predictor variables	Seedling						Rhizosphere		
	ECM colonization rate	Height	Stem diameter	Dry mass	N concentration	P concentration	pH	Acid phosphatase	Alkaline phosphatase
O. davidiana (O)	<0.0001	<0.0001	0.3899	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
ECM (E)	<0.0001	<0.0001	0.9807	<0.0001	<0.0001	0.0420	0.8673	<0.0001	0.0003
O × E	<0.0001	<0.0001	0.9905	<0.0001	0.0014	0.1119	0.1855	<0.0001	0.0209

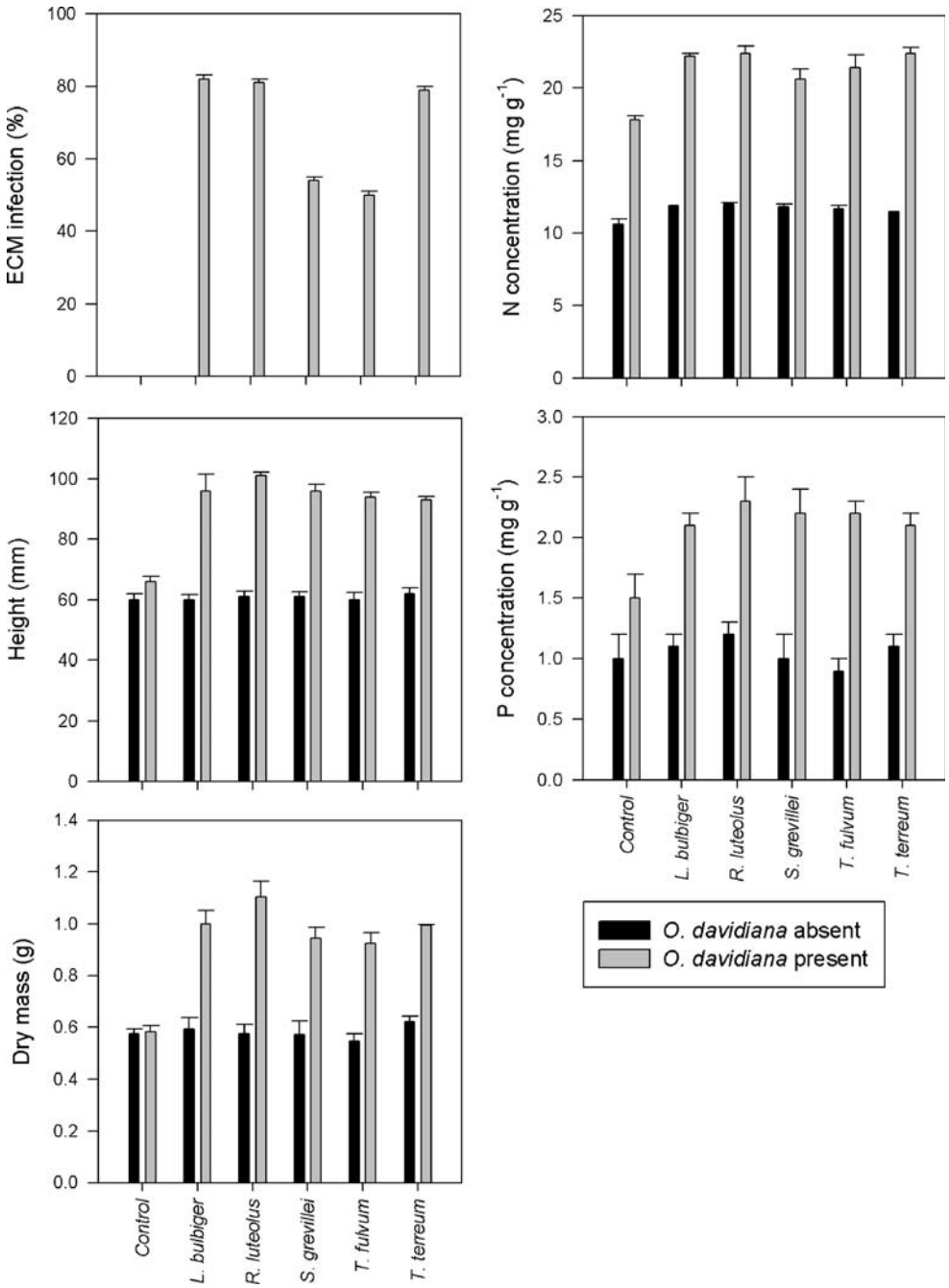

Fig. 1 Interaction of ectomycorrhizal fungi (*Leucocortinarius bulbiger*, *Rhizopogon luteolus*, *Suillus grevillei*, *Tricholoma fulvum*, *Tricholoma terreum*, and a control [autoclaved mixture of all inocula]) and *Ostryopsis davidiana* on *Pinus tabulaeformis* colonization, height, biomass, and N and P concentrations. *Error bars* are 1 SE, n=3

Smith 1983; Gerlitz and Werk 1994). Improved nutrient status may be a function of the increased root surface area provided by mycorrhizal hyphae, uptake ability of the ECM (Wallander et al. 1999), as well as changes to rhizosphere chemistry. We noted increases in phosphatase activity in the rhizosphere soil of mycorrhizal seedlings; phosphatase activity is associated with improved seedling access to inorganic phosphorus (Hua 1995; Vazquez et al. 2000). In addition, ECM are known to produce compounds such as oxalic acid that weather mineral forms of P, such as apatite, allowing them to be used by plants (Wallander 2000). We also noted decreases in soil pH after formation of mycorrhizae; in general, pine seedlings grow better at lower pH, and, most nutrients have higher availability as well (Landis et al. 1989).

Although studies indicate that ECM have low host specificity between canopy trees (Cullings et al. 2000) and between canopy trees and understory plants (Dickie et al. 2006; Visser 1995), only three of the five ECM fungi formed mycorrhizae with *O. davidiana*. Despite this, all five ECM fungi in this study, when partnered with *O. davidiana*, were able to move across the nylon barrier and associate with *P. tabulaeformis*. For ECM that did not appear to form mycorrhizae with *O. davidiana* (i.e.,

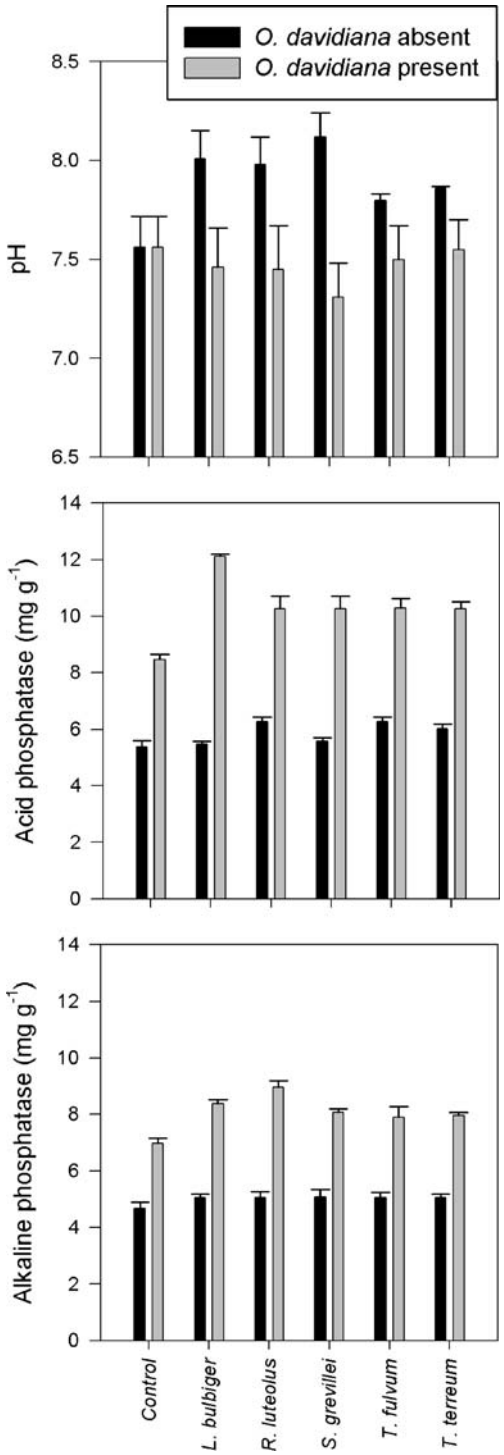

Fig. 2 Interaction of ectomycorrhizal fungi (*Leucocortinarius bulbiger*, *Rhizopogon luteolus*, *Suillus grevillei*, *Tricholoma fulvum*, *Tricholoma terreum*, and a control [autoclaved mixture of all inocula]) and *Ostryopsis davidiana* on pH and acid and alkaline phosphatase (*p*-nitrophenyl phosphate) activity in the rhizosphere of *Pinus tabulaeformis* seedlings. *Error bars* are 1 SE, $n=3$

T. fulvum and *S. grevillei*), it may be that the fungi used exudates from *O. davidiana* roots to grow to *P. tabulaeformis* roots (Fries et al. 1985; Vierheilig et al. 1998), but for the ECM that form mycorrhizae on *O. davidiana*, it appears those hyphae grew across the barrier and colonized pine roots.

Of special interest in this study is *S. grevillei* and its mycorrhizae-like associations with *P. tabulaeformis*. Although it did not form mycorrhizae on *O. davidiana*, the extraradicular hyphae we observed on *P. tabulaeformis* were fine, hairy like and relatively short, similar to the mycorrhizae that form on *L. gmelinii* (Gong et al. 1997). *S. grevillei* is a well-known mycorrhizae of *Larix* and is known to form mycorrhizae on *Pseudotusga menziesii* (Mirb.) Franco (Molina and Trappe 1982). With *Pinus ponderosa* C. Lawson and *Pinus contorta* Douglas ex Louden, however, Molina and Trappe (1982) found that although *S. grevillei* caused dichotomous branching and was observed to penetrate between root cells, formation of regular, uniform Hartig nets was absent. Based on this, we cannot ascertain conclusively whether *S. grevillei* formed mycorrhizae with *P. tabulaeformis*, but regardless, we can state that its association with *O. davidiana* or *P. tabulaeformis* resulted in positive growth increases in the trees compared to their non-inoculated cohorts.

Afforestation with *P. tabulaeformis* on arid and poor sites is limited (Wang 1981), most likely because seedlings experience high levels of stress. Immediately after outplanting conifer seedlings for either afforestation or reforestation, rapid initiation of new roots is essential to alleviate seedling stresses (Grossnickle 2005; Nambiar and Sands 1993), usually a function of limited water availability (Burdett 1990) due to the restricted root volume of seedlings. Unfortunately, this lack of available water reduces photosynthesis and new photosynthates are required for production of new roots (van den Driessche 1987). For *P. tabulaeformis* under water stress conditions, however, mycorrhizal associations have been shown to improve net photosynthesis rate, needle water potential, and water use efficiency (Wu and Nioh 1997) and nitrogen absorption (Wu et al. 1999) compared to non-mycorrhizal seedlings. These attributes should mitigate post-planting stress and thereby enhance survival and growth, particularly on harsh sites. Moreover, *P. tabulaeformis* seedlings with mycorrhizae also have higher net photosynthesis rates when soil moisture levels are favorable (Wu and Nioh 1997), inferring a competitive advantage across a wider range of environmental conditions than their non-mycorrhizal cohorts, but ECM associations and subsequent improvement in seedling–water relations could improve outplanting results.

Although *O. davidiana* plants may simply be a source of inocula for *P. tabulaeformis* (similar to a relationship found with other Pinaceae; Hubert and Gehring 2008) and our

ECM fungi followed root exudates to travel to pine roots, we speculate that the hyphae from mycorrhizae on *O. davidiana* penetrated the nylon screen and colonized the pine roots, thus linking the two species through a CMN. Mycorrhizal fungi are known to form CMNs among a diverse cadre of species, effectively connecting forest species (He et al. 2006; Kennedy et al. 2003). Such CMNs are instrumental in sharing carbon, nitrogen, phosphorus, and water (Brownlee et al. 1983; He et al. 2003; Plamboeck et al. 2007). A CMN between *O. davidiana* and *P. tabulaeformis*, likely because our results show they share several ECM (similar to results with other Pinaceae; Hubert and Gehring 2008), would have significant implications. Outplanted *P. tabulaeformis* seedlings rapidly colonized by hyphae connected to established *O. davidiana* plants may have more immediate access to water and nutrients, thereby alleviating outplanting stress sooner through enhanced photosynthesis and new root growth. This relationship may explain the improved survival and growth of *P. tabulaeformis* associated with *O. davidiana* after outplanting (Bai et al. 2006). Future work using isotopes (e.g., Wu et al. 1999; He et al. 2003) should seek to ascertain if such a CMN occurs.

Acknowledgments We thank Xiaolan Mao, senior researcher at the Chinese Academy of Sciences, Institute of Microorganisms, for identifying the fungal fruiting bodies, Amy Ross-Davis for additional statistical analysis, Drs. Deborah Page-Dumroese and Michael Castellano for their review of an earlier draft, and the insightful comments from two anonymous reviewers and Dr. Randy Molina. This work was supported by the National Natural Science Foundation of China (Nos. 30560122 and 30471380).

References

Bai SL, Bai YE, Fang L, Liu Y (2004) Mycorrhiza of *Cenococcum geophilum* formed on *Ostryopsis davidiana* and mycorrhizal affection on the growth of *Ostryopsis davidiana*. Scientia Silvae Sinicae 40:194–196, in Chinese

Bai SL, Liu Y, Zhou J, Dong Z, Fan R (2006) Resources investigation and ecological study on ectomycorrhizal fungi in Daqingshan Mountains, Inner Mongolia. Acta Ecol Sin 26:837–841, in Chinese

Boyle CD, Hellenbrand KE (1990) Assessment of the effect of mycorrhizal fungi on drought tolerance of conifer seedlings. Can J Bot 69:1764–1771. doi:10.1139/b91-224

Brownlee C, Duddridge JA, Malibari A, Read DJ (1983) The structure and function of mycelial systems of ectomycorrhizal roots with special reference to their role in forming inter-plant connections and providing pathways for assimilate and water transport. Plant Soil 71:433–443. doi:10.1007/BF02182684

Brundrett M, Bougher N, Dell B, Grove T, Malajczuk N (1996) Working with mycorrhizas in forestry and agriculture. Canberra: ACIAR Monograph. pp 120–290

Burdett AN (1990) Physiological processes in plantation establishment and the development of specifications for forest planting stock. Can J Res 20:415–427. doi:10.1139/x90-059

Cui XY (1998) Modern experimental analysis technology for forestry soil. Northeast Forestry University Press, Harbin, pp 73–99

Cullings KW, Vogler DR, Parker VT, Finley SK (2000) Ectomycorrhizal specificity patterns in a mixed *Pinus contorta* and *Picea engelmannii* forest in Yellowstone National Park. Appl Environ Microb 66:4988–4991. doi:0099-2240/00/$04.0010

Dickie IA, Oleksyn J, Reich PB, Karolewski P, Zytkowiak R, Jagodzinski AM, Turzanska E (2006) Soil modification by different tree species influences the extent of seedling ectomycorrhizal infection. Mycorrhiza 16:73–79. doi:10.1007/s00572-005-0013-x

Faber BA, Zasoski RJ, Munns DN, Shackel K (1991) A method for measuring hyphal nutrient and water uptake in mycorrhizal plants. Can J Bot 69:87–94

Fries N, Bardet M, Serck-Hanssen K (1985) Growth of ectomycorrhizal fungi stimulated by lipids from a pine root exudate. Plant Soil 86:287–290. doi:10.1007/BF02182906

Fu L, Nan L, Mill RR (1999) Pinaceae, vol 4. In: Wu Z-Y, Raven PH et al (eds) Flora of China. Missouri Botanical Garden Press, St. Louis, pp 11–52

Gerlitz TGB, Werk WB (1994) Investigations on phosphate uptake and polyphosphate metabolism by mycorrhized and non-mycorrhized roots of beech and pine as investigated by in vivo ^{31}P-NMR. Mycorrhiza 4:207–214. doi:10.1007/BF00206782

Gong MQ, Chen YL, Zhong CL (1997) Mycorrhizal research and application. China Forestry Press, Beijing, pp 17–32

Grossnickle SC (2005) Importance of root growth in overcoming planting stress. New For 30:273–294. doi:10.1007/s11056-004-8303-2

Guan SY (1986) Soil enzyme and its research method. Beijing Agric Press, Beijing, pp 1–376

Han XL, Fang L, Zhou J, Bai SL (2005) The search, synthesizing, and screening out of outstanding *Ostryopsis davidiana* ectomycorrhiza. Acta Agric Boreali-Sinica 20:101–104, in Chinese

Harley JL, Smith SE (1983) Mycorrhizal symbiosis. Academic, Cambridge, pp 1–99

He X-H, Critchley C, Bledsoe C (2003) Nitrogen transfer within and between plants through common mycorrhizal networks (CMNs). Crit Rev Plant Sci 22:531–567. doi:10.1080/07352680390253520

He X-H, Bledsoe CS, Zasoski RJ, Southworth D, Horwath WR (2006) Rapid nitrogen transfer from ectomycorrhizal pines to adjacent ectomycorrhizal and arbuscular mycorrhizal plants in a California oak woodland. New Phytol 170:143–151. doi:10.1111/j.1469-8137.2006.01648.x

Hua XM (1995) Introduction to mycorrhiza. In: Hua M (ed) Studies on mycorrhiza of forest trees. Chinese Sci Tech Press, Beijing, pp 1–20

Hubert NA, Gehring CA (2008) Neighboring trees affect ectomycorrhizal fungal community composition in a woodland-forest ecotone. Mycorrhiza 18:363–374. doi:10.1007/s00572-008-0185-2

Janos DP (2007) Plant responsiveness to mycorrhizas differs from dependence upon mycorrhizas. Mycorrhiza 17:75–91. doi:10.1007/s00572-006-0094-1

Kennedy PG, Izzo AD, Bruns TD (2003) There is high potential for the formation of common mycorrhizal networks between understorey and canopy trees in a mixed evergreen forest. J Ecol 91:1071–1080. doi:10.1046/j.1365-2745.2003.00829.x

Landis TD, Tinus RW, McDonald SE, Barnett JP (1989) Seedling nutrition and irrigation. The Container Tree Nursery Manual, Volume 4. US Dept Agric, Washington DC, Agric Handbk 674

Li P-C, Skvortsov AK (1999) Betulaceae, vol 4. In: Wu Z-Y, Raven PH et al (eds) Flora of China. Science Press, Beijing, pp 286–313

Li W (2004) Degradation and restoration of forest ecosystems in China. For Ecol Manage 201:33–41. doi:10.1016/j.foreco.2004.06.010

Liu RJ, Li XL (2000) Arbuscular Mycorrhizae and application. Science Press, Beijing

Marx DH (1969) The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections I. Antagonism of

mycorrhizal fungi to root pathogenic fungi and soil bacteria. Phytopathology 59:153–163

Mao XL (1998) Economic fungi in China (in Chinese). Science Press, Beijing

Molina R, Trappe JM (1982) Patterns of ectomycorrhizal host specificity and potential among Pacific northwest conifers and fungi. For Sci 28:423–458

Nambiar EKS, Sands R (1993) Competition for water and nutrients in forests. Can J Res 23:1955–1968. doi:10.1139/x93-247

Owusu-Bennoah E, Wild A (1980) Effects of vesicular–arbuscular mycorrhiza on the labile pool of soil phosphate. Plant Soil 54:233–242. doi:10.1007/BF02181849

Pfeffer PE, Bago B, Shachar-Hill Y (2001) Exploring mycorrhizal function with NMR spectroscopy. New Phytol 150:543–553. doi:10.1046/j.1469-8137.2001.00139.x

Plamboeck AH, Dawson TE, Egerton-Warburton LM, North M, Bruns TD, Querejeta JI (2007) Water transfer via ectomycorrhizal fungal hyphae to conifer seedlings. Mycorrhiza 17:439–447. doi:10.1007/s00572-007-0119-4

Ren XW (2002) Dendrology (northern edition). China Forest Press, Beijing, pp 63–201

van den Driessche R (1987) Importance of current photosynthates to new root growth in planted conifer seedlings. Can J Res 17:776–782. doi:10.1139/x87-124

Vazquez MM, Cesar S, Azcon R, Barea JM (2000) Interactions between arbuscular mycorrhizal fungi and other microbial inoculants (*Azospirillum, Pseudomonas, Trichoderma*) and their effects on microbial population and enzyme activities in the rhizosphere of maize plants. Appl Soil Ecol 15:261–272. doi:10.1016/S0929-1393(00)00075-5

Vierheilig H, Alt-Hug M, Engel-Streitwolf R, Mäder P, Wiemken A (1998) Studies on the attractional effect of root exudates on hyphal growth of an arbuscular mycorrhizal fungus in a soil compartment-membrane system. Plant Soil 203:137–144. doi:10.1023/A:1004329919005

Visser S (1995) Ectomycorrhizal fungal succession in jack pine stands following wildfire. New Phytol 129:389–401. doi:10.1111/j.1469-8137.1995.tb04309.x

Wallander H (2000) Uptake of P from apatite by *Pinus sylvestris* seedlings colonized by different ectomycorrhizal fungi. Plant Soil 218:249–256. doi:10.1023/A:1014936217105

Wallander H, Arnebrant K, Dahlberg A (1999) Relationships between fungal uptake of ammonium, fungal growth and nitrogen availability in ectomycorrhizal *Pinus sylvestris* seedlings. Mycorrhiza 8:215–223. doi:10.1007/s005720050237

Wang JL (1981) Studies on drought tolerance of trees in Beijing western mountain area. Beijing For 2:10–21, in Chinese

Wang Y (2004) Environmental degradation and environmental threats in China. Environ Monit Assess 90:161–169. doi:10.1023/B:EMAS.0000003576.36834.c9

Warren JM, Brooks R, Meinzer FC, Eberhart JL (2008) Hydraulic redistribution of water from *Pinus ponderosa* trees to seedlings: evidence for an ectomycorrhizal pathway. New Phytol 178:382–394. doi:10.1111/j.1469-8137.2008.02377.x

Withington JM, Reich PB, Oleksyn J, Eissenstat DM (2006) Comparisons of structure and life span in roots and leaves among temperate trees. Ecol Monogr 76:381–397. doi:10.1890/0012-9615(2006)076[0381:COSALS]2.0.CO;2

Wu B, Nioh I (1997) Growth and water relations of *P. tabulaeformis* seedlings inoculated with ectomycorrhizal fungi. Microbes Environ 12:69–74

Wu B, Watanabe I, Hayatsu M, Nioh I (1999) Effect of ectomycorrhizae on the growth and uptake and transport of ^{15}N-labeled compounds by *Pinus tabulaeformis* seedlings under water stressed-conditions. Biol Fertil Soils 28:136–138. doi:10.1007/s003740050474

Wu B, Nara K, Hogetsu T (2001) Can C^{14}-labelled photosynthetic products move between *Pinus densiflora* seedling linked by ectomycorrhizal mycelia? New Phytol 149:137–147. doi:10.1046/j.1469-8137.2001.00010.x