

## Ectomycorrhizal Effect on Physiological Activities of Water-Stressed Nodulated *Alnus rubra* Seedlings<sup>1</sup>

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外生菌根이 水分缺乏된 루브라 오리나무 苗木의  
生理活動에 미치는 影響<sup>1</sup>

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### ABSTRACT

Red alder (*Alnus rubra* Bong.) seedlings inoculated with *Frankia* pure cultures were grown in a walk-in growth chamber for sixteen weeks. Half were inoculated with the spores of the ectomycorrhizal fungus *Alpova diplophloeus* (Zeller & Dodge) Trappe & Smith. The mycorrhizal seedlings were significantly larger than nonmycorrhizal plants in diameter, and nodule and shoot dry weight by 6 to 16% when their heights were very similar.

The mycorrhizal effects on water relations of red alder seedlings were explored in a 30 hours water stress. Mycorrhizal and nonmycorrhizal seedlings did not significantly differ in leaf water potentials, CO<sub>2</sub> exchange rates or N<sub>2</sub>-fixation rates during the drought. Our results suggests that *A. diplophloeus* mycorrhizas increased red alder seedling growth under well-watered conditions but do not affect water relations of the plant under water-stress.

### 要 約

루브라 오리나무 (*Alnus rubra* Bong.) 묘목에 질소고정균, *Frankia*를 접종하고, 이 묘목의 반을 외생균근균, *Alpva diplophloeus* 포자로 접종하여 인공생장상에서 16주 동안 길렀다. 그 결과, 외생균근 형성묘는 비균근묘보다 직경생장, 질고정뿌리혹 형성 그리고 지상부 생체량에서 6~16% 더 컸다.

이들 묘목에 대하여 30시간 동안 수분결핍 처리를 하여 균근이 루브라 오리나무의 수분생리에 미치는 영향을 조사하였다. 이 수분 결핍동안 균근은 잎의 수분 포텐셜, 광합성, 그리고 질소고정 활동에 영향을 미치지 않았다. 이 결과는 외생균근균 *A. diplophloeus*는 수분이 부족하지 않은 경우에는 루브라오리나무 묘목의 생장을 촉진시키지만 수분결핍 상태에서는 생리활동에 영향을 미치지 않음을 나타낸다.

**Key Words** : Ectomycorrhizas, *Alnus rubra*, Physiological activity, Water stress, *Frankia*, *Alpova diplophloeus*.

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## INTRODUCTION

Red alder (*Alnus rubra* Bong.) is a tripartite symbiont composed of the plant, N<sub>2</sub>-fixing microorganism, *Frankia* and mycorrhizal fungi (Gardner and Barrueco, 1999; Jha *et al.*, 1993; Koo *et al.*, 1995). It is an ecologically and economically important tree species in Pacific Northwest forests. It significantly enhances soil fertility through large inputs of N symbiotically fixed by *Frankia* actinomycetes in root nodules (actinorrhizae). Red alder also forms ectomycorrhizas (Miller *et al.*, 1991; Molina, 1981) as well as arbuscular mycorrhizas (Rose, 1980) that likely function in nutrient uptake. Of the mycorrhizal fungi of alders *A. diplophloeus* is currently the best known ectomycorrhizal species (Gardner and Barrueco, 1999).

Red alder is widespread in disturbed mesic habitats of the coastal and Cascade mountains, and is often the dominant tree in riparian zones. Not surprisingly, its range is limited by severe moisture stress. Moisture stress is an important limiting factor for N<sub>2</sub>-fixation in legumes, directly affecting N<sub>2</sub>-fixation by reducing gas diffusion through shrinking nodules (Weisz *et al.*, 1985), or indirectly by affecting photosynthetic activity of host plants through stomatal closure (Huang *et al.*, 1975b).

Mycorrhizas can affect plant water relations, but such interactions with alder are unexplored. Safir *et al.* (1971) presented the following four hypotheses to explain improved plant water relations by arbuscular mycorrhizas: (1) increase in total soil absorptive surface area by external hyphae, (2) low-resistance water pathway to the endodermis by hyphal penetration into root cortex, (3) decrease in water transport resistance within the roots by nutrient uptake enhancement, and (4) larger root systems of mycorrhizal plants. Several studies show that mycorrhizas can increase a plant's drought tolerance (Ming and Hui, 1999), increase plant growth under water stress (Wu *et al.*, 1999), and even improve plant survival in the field after short periods of exposure to drought (Allen and Boosalis, 1983). Mycorrhizas can also

lower stomatal resistance through a lower level of abscisic acid (Lambers *et al.*, 1998).

Ectomycorrhizal *Pinus radiata* seedlings resist summer drought better than non-mycorrhizal seedlings (Theodorou and Bowen, 1970). Increased absorptive surface of external mycelium and water transport through vessel hyphae in rhizomorphs or hyphal strands have been well supported by the enhancement of <sup>15</sup>NO<sub>3</sub>-uptake by ectomycorrhizas under water stress conditions (Wu *et al.*, 1999; Tobar *et al.*, 1994) and by arbuscular mycorrhizas (Subramanian and Charest, 1999; Koo, 1997). Mudge *et al.* (1987) also proposed that ectomycorrhizal fungi can affect plant water relations by fungal osmoregulation of water uptake.

The interactions of ectomycorrhizas, actinorrhizas and host plants are relatively unexplored compared to the legume-mycorrhizas-*Rhizobium* complex. Actinorrhizal nodules differ strongly from legume nodules because they are perennial and their endophytes are *Frankia* sp. with vesicles for N<sub>2</sub>-fixation. Similarly, ectomycorrhizas differ strongly from arbuscular mycorrhizas in form and functions. It is likely that the tripartite symbioses of alder may operate differently from that in the legume symbioses. The objective of this study, therefore, was to examine the effect of ectomycorrhizas on physiological activities, photosynthesis and N<sub>2</sub>-fixation rate of moisture stressed nodulated red alder seedlings.

## MATERIALS AND METHODS

### 1. Biological materials

Red alder seeds (Brown Seed Company, Vancouver, Washington) were surface-sterilized with 30% H<sub>2</sub>O<sub>2</sub> for 15 min. prior to planting. *Frankia* was isolated by filtration method (Benson, 1982) from nodules on 1-year-old red alder seedlings collected in the Oregon coast range and cultured on N-free BAP liquid medium (Murray *et al.*, 1984) for one month. Sporocarps of *Alpova diplophloeus* (Zeller & Dodge) Trappe & Smith, a hypogeous ectomycorrhizal fungus specific to alder (Molina, 1981) were collected

under young alder trees at the Oregon coastal range in autumn and stored at  $-18^{\circ}\text{C}$  until the spores were used.

## 2. Growth conditions

Surface-sterilized seeds were planted into 3.2 cm diameter  $\times$  20 cm long leach tube containers filled with a 2 : 1 : 1 mixture of sandy loam soil : sphagnum peat moss : coarse vermiculite and misted daily. After seed germination, 2 ml of water-diluted *Frankia* inoculum containing ca.  $1\mu\text{l}$  packed cell volume were inoculated into each leach tube. Ten million *A. diplophloeus* spores suspended in 5 ml were similarly inoculated into each leach tube. The nonmycorrhizal treatment received the same amount of autoclaved spores. The alder seedlings were grown in a walk-in growth chamber with day/night regimes of 14/10 hr light period, 25/17 $^{\circ}\text{C}$  temperature and 60/80% relative humidity, with photosynthetic photon flux density of ca. 570  $\mu\text{mol}/\text{m}^2/\text{s}$  measured at soil surface. Seedlings were irrigated daily with tap water to saturation and never fertilized. Fifteen weeks after the inoculation, 21 seedlings with similar height were selected from each mycorrhizal and nonmycorrhizal treatments taken.

## 3. Experimental design and data collection

This was a  $2 \times 7$  factorial experiment arranged in a completely randomized design with three replications measured twice at a three day interval. A total of 42 seedling (14 treatments  $\times$  3 seedlings/treatment) were randomly distributed within holding trays. The first factor was fungus inoculation at two levels : *A. diplophloeus* spore inoculation and no inoculation. The second factor was water stress measured at seven time intervals four hours apart beginning two hours after the final morning watering (seven measurements during a 30-hour dry cycle). Water stress treatment was generated by withholding water until the seedlings wilted.

## 4. Physiological parameters

Nitrogenase activity, photosynthetic activity,

stomatal conductance and leaf water potential were measured for three seedlings from each treatment. Nitrogenase activity was measured by acetylene reduction assay of intact root systems entirely enclosed in a plastic tube 5.2 cm diameter  $\times$  25 cm deep; stems and leaves extended above the tube (Koo et al., 1995). Photosynthetic rate and stomatal conductance were measured on the middle  $16\text{cm}^2$  of the 4th leaf of each seedling with a portable LI6000 photosystem (LI-COR, Inc, Lincoln, Nebraska). Leaf water potential was measured on the 5th or 6th leaf by a pressure bomb. At the end of the dry down cycle (30 hours duration), seedlings were watered daily to saturation for three days. A second identical dry down cycle was then conducted and the same parameters measured.

After this second replication, growth parameters; i. e. height and root collar diameter, were also measured in addition to the physiological parameters. Soil water contents were also calculated from the dry weight of about one-third volume of the pot substrate. All nodules were collected from whole root systems and dried at  $65^{\circ}\text{C}$  to constant weight and measured. Degree of ectomycorrhizal development was calculated from three root subsamples collected at 2-5, 7-10 and 12-15 cm depth of each root mass and expressed as percentage of total short roots colonized. Data were analyzed for the physiological parameters using the means based on three seedlings from each sampling time. Mean values for the nonmycorrhizal and mycorrhizal seedlings were tested individually using Tukey's test ( $p < 0.05$ ).

## RESULTS

### 1. Growth

Nodulated *A. diplophloeus* mycorrhizal seedlings were significantly larger than nodulated non-mycorrhizal ones in diameter, shoot dry weight and nodule dry weight when they were similar in height (Table 1).

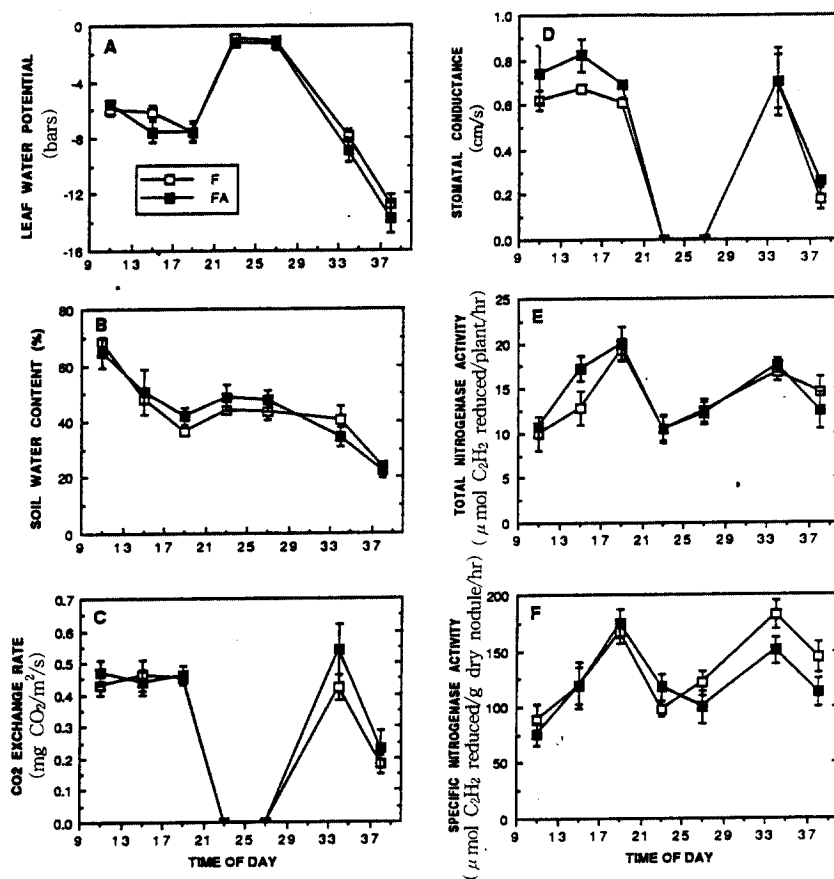
**Table 1.** Mean growth of 16-week-old nodulated *Alnus rubra* Bong. seedlings grown in a walk-in growth chamber, inoculated with *Alpova diplophloeus* spores.

Parameter	<i>Frankia</i>	<i>Frankia</i> + <i>A. diplophloeus</i>
Height (cm)	52.1 ± 1.4a	53.8 ± 1.3a
Diameter (mm)	5.2 ± 0.1a	5.5 ± 0.1b
Shoot dry weight (g)	2.4 ± 0.1a	2.8 ± 0.1b
<i>A. diplophloeus</i> mycorrhizas (%)	0.0a	51.0 ± 5.0b
Nodule dry weight (mg)	105 ± 5a	123 ± 7b

Values are means of 21 seedlings ± stand error. Values within a row followed by a different letter are significantly different at the  $p < 0.05$  level according to Tukey's test.

## 2. Physiological response to rapid drought stress

In general, *A. diplophloeus* mycorrhizal seedlings did not differ significantly from non-mycorrhizal ones in physiological activity during rapid drought stress (Fig. 1). Leaf water potentials of both mycorrhizal and non-mycorrhizal seedlings decreased slightly from ca. -6.0 bars two hours after watering in the morning to -8.0 to -9.0 bars in the next morning, then rapidly dropped to wilting point around -14.0 bars in the next afternoon (Fig. 1A) as soil water content dropped from ca. 65 through 40 to 22% (Fig. 1B). During the night leaf water potentials were kept at ca. -1.0 bars. Most mycorrhizal seedlings started to wilt ca. 30 hours after



**Fig. 1.** Physiological activity changes during 30-hr drought cycle of 6-month-old nodulated non-mycorrhizal (F) and *Alpova diplophloeus* mycorrhizal (FA) *Alnus rubra* seedlings grown in a walk-in growth chamber. Each point is a mean of two replications of three seedlings each. Standard errors of treatments are shown on each point. A : leaf water potential; B : soil water content; C : CO<sub>2</sub> exchange rate; D : stomatal conductance; E : total nitrogenase activity; F : specific nitrogenase activity.

the last watering when the water potential of the seedlings was -14.2 bars, at which time non-mycorrhizal ones were at ca. -13.5 bars and had not begun to wilt.

CO<sub>2</sub> exchange rates (CER) of both mycorrhizal and non-mycorrhizal plants were constantly maintained at ca. 0.45 mg CO<sub>2</sub>/m<sup>2</sup>/s until the next morning, then rapidly decreased to ca. 0.20 mg CO<sub>2</sub>/m<sup>2</sup>/s when water stress developed during the afternoon (Fig. 1C). Stomatal conductance of both mycorrhizal and non-mycorrhizal plants also remained stable at ca. 0.7 cm/s until the next morning, although the rates of mycorrhizal plants were higher than non-mycorrhizal at the first three measurements, and then rapidly dropped to ca. 0.3cm/s (Fig. 1D). This pattern was very similar to that of CER except for the first three measurements.

Total nitrogenase activity (TNA) continuously increased from ca. 10.2 in the morning to 19.5 μmol C<sub>2</sub>H<sub>2</sub> reduced/plant/hr in the evening, rapidly dropped to ca. 10.5 μmol C<sub>2</sub>H<sub>2</sub> reduced/plant/hr during the night, and then again increased to pre-evening rate in the following morning (Fig. 1E). N<sub>2</sub>-fixation rates also dropped to ca. 13.5 μmol C<sub>2</sub>H<sub>2</sub> reduced/plant/hr during afternoon water stress. The changes in TNA were not directly related to CER and stomatal conductance during the first three measurements, i.e. TNA gradually increased without changes in CER. TNA remained relatively high during the night, and decreased less under water stress (by 22%) compared to the other physiological activities (by 55% in CER and 57% in stomatal conductance). Specific nitrogenase activity in mycorrhizal plants were significantly lower than the nonmycorrhizal at the last three measurements (Fig. 1F).

## DISCUSSION

This experiment showed that ectomycorrhiza formation with *A. diplophloeus* can increase red alder seedling growth in some aspects, such as diameter, nodule dry weight and tissue dry weight

when well-watered. But the ectomycorrhizas neither affected the water relations of red alder nor influenced leaf water potential, photosynthesis, stomatal conductance, transpiration and N<sub>2</sub>-fixation under this rapidly developed 30 hours water-stress cycle. These findings support previous conclusions that mycorrhizas may not affect water relations of the host plant under certain drought-stressed conditions (Graham et al., 1987; Fitter, 1987) and that not all fungi confer drought tolerance (Parke et al., 1983). Rather *A. diplophloeus* mycorrhizal plants were water-stressed earlier than the non-mycorrhizal in this study. This may mean mycorrhizal hyphae absorbed soil water more quickly than root itself did.

Several studies shown that plant responses and mycorrhizal development under water stress are often inconsistent and depend on plant species, fungal species and growth conditions, For example, greenhouse corn inoculated with *Glomus mosseae*, did not show benefit from mycorrhizas when exposed to cyclic drought stress, even though root colonization under drought was greater than that for adequately watered plants at reduced soil phosphorus levels (Hetrick et al., 1984). Conversely, *G. fasciculatum* enhanced soybean drought tolerance, even though mycorrhizal colonization was significantly reduced by water stress (Busse and Ellis, 1985). In another soybean study, *G. mosseae* increased N<sub>2</sub>-fixation, transpiration, leaf conductance, tissue dry weight, and P nutrition of host plants without changing root colonization under cyclic water stress (Bethlenfalvay et al., 1987).

Fewer examples are available for ectomycorrhizal interactions with water. However, in one important study of Douglas-fir seedlings grown in a greenhouse, an unidentified ectomycorrhizal fungus and *Rhizopogon vinicolor* improved plant water relations under cyclic water stress but other tested fungi did not (Parke et al., 1983). Unfortunately, no root colonization rates were reported in that study after the drought stress treatment.

The failure of plants to respond to mycorrhizal

fungus inoculation under water stress has often been attributed to the rapid and severe water stress that develops when large plants are confined to a small volume of potting substrate in a greenhouse (Hetrick et al., 1984; Graham and Sylvertsen, 1984). Even in large pots, however, drought stress can develop rapidly and mycorrhizal effects are still not evident. For example, water stress developed in 10 days on mycorrhizal citrus grown in large pots and no mycorrhizal benefit occurred (Graham et al., 1987). Similarly, severity of stress, plant size, or soil type could not explain the lack of mycorrhizal benefit in corn and Sudan grass grown in large containers (Hetrick et al., 1987).

Lack of mycorrhizal benefit during water stress also draws attention to the direct effect of water stress on plant physiological activity and mycorrhizal dependence on host carbohydrate. Photosynthate translocation, measured with  $^{14}\text{C}$ , from source leaves to roots decreased as water stress level increased in loblolly pine seedlings (Khuns and Gzerstad, 1988). Under high stress conditions sugars can be used as substrates for increased respiration (Kramer, 1983), or as solutes for osmotic adjustment in leaves and roots (Osonubi and Davies, 1978). Clearly specific studies on changes in root sugar content of plants under water stress and consequent effects on mycorrhiza development is needed.

Although water stress also directly affects  $\text{N}_2$ -fixation, our studies indicate that alder nodules may be less sensitive to water stress than legume nodules. The  $\text{N}_2$ -fixation process in legumes is thought to be more sensitive to water stress than photosynthesis, because the shrinkage of nodules due to water deficit reduces oxygen transport to ATP production sites (Sprent, 1976). In support of this hypothesis, Bennett and Albrecht (1984) found that  $\text{N}_2$ -fixation was closely correlated with nodule water potential, which was more sensitive to drought stress than leaf water potential or diffusive conductance. A reduction in  $\text{N}_2$ -fixation was also reported for nodules water-stressed on a separated root system without decreasing supply of photosynthate

(Khanna-Chopra et al., 1984). Thus, reduced  $\text{N}_2$ -fixation under water stress can be directly caused by a decreased in nodule gas permeability, that is, limited oxygen flux, followed by a decrease in nodule surface area (Weisz et al., 1985).

Several observations by researchers show that actinorhizas have adaptable mechanisms to oxygen problems. For example, *Casuarina* nodule cell walls *Frankia* infected become impregnated with hydrophobic suberin-like structural compounds (Berg, 1983) and the *Frankia* endophyte can alter the wall thickness of vesicles, the site of  $\text{N}_2$ -fixation, in response to an  $\text{O}_2$  concentration ( $\text{Po}_2$ ) of ambient (Parsons et al., 1987). Silvester et al. (1988) found that  $\text{N}_2$ -fixation in alder nodules also has a wide optimum range of 10 to 21  $\text{Po}_2$ , when the nodules were grown at 21  $\text{Po}_2$ . Low  $\text{Po}_2$  in the range may mean oxygen deficiency by low nodule gas permeability due to water flooding or water stress.

On the other hand, our data indirectly show that  $\text{N}_2$ -fixation in alder nodules is reduced by low gas permeability due to flooding.  $\text{N}_2$ -fixation increased with decreasing soil water content at the beginning of the water stress treatment, but leaf water potential, photosynthesis, stomatal conductance and transpiration remained relatively unchanged or slightly decreased, i.e.  $\text{N}_2$ -fixation was lowest immediately after the substrate was saturated and increased as the substrate dried until the water stress became limiting. Huang et al., (1975a) also observed this increase in soybean at various times after water was withheld. They found that acetylene reduction decreased when more water was added to the soil of a well-watered plant and increased by draining the water without changing photosynthesis or transpiration. Schwintzer (1985) also reported this negative flooding effect on  $\text{N}_2$ -fixation in actinorhizal *Myrica gale*.

In summary, although we did find *A. diplophloeus* mycorrhizas able to enhance growth of red alder seedlings, it did not influence on plant water stress under our experimental conditions. *A. diplophloeus*

mycorrhizas apparently ceased functioning under drought stress. More detailed field analysis of red alder root growth patterns are needed to define natural adaptations to soil moisture status. For example Koo (unpublished data) has observed red alder seedlings growing on exposed slopes in clearcuts to produce deeper tap root systems than seedlings growing in riparian habitats. As moisture decreased in the clearcut soil, the fibrous root system including ectomycorrhizas is present in the deep soil profile. Future investigations need to consider natural root dynamics to understand water stress adaptations by red alder.

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