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Effects of Light and Inoculation of *Frankia* and *Alpova diplophloeus* on the Tripartite Symbioses Development in *Alnus rubra* Bong. Seedlings.*

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光度와 *Frankia* 窒素固定菌 및 *Alpova diplophloeus* 外生菌根菌의 接種이 루브라 오리나무 苗木內 三者共生關係 發達에 미치는 影響*

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ABSTRACT

To investigate the effect of low light intensities and the inoculation of *Frankia* and/or *Alpova diplophloeus* on the symbioses development and their host growth, red alder(*Alnus rubra* Bong.) seedlings were grown in an air - filtered walk - in growth chamber with either N_2 - fixing *Frankia* inoculation or N - fertilization and live or dead spore inoculation of the ectomycorrhizal fungus *A. diplophloeus*(Zeller & Dodge) Trappe & Smith. When they were 20 weeks old, the seedlings were grown under three levels of light intensities of 680, 320 and 220μmol/㎡/s PPFD(photosynthetic photon flux density) for three weeks. PPFD of 220 significantly decreased the development of *A. diplophloeus* mycorrhizae and nodules, the rates of N_2 - fixation and CO_2 exchange, and the growth of the seedlings. PPFD 320 significantly decreased the CO_2 exchange rate only. *Frankia* inoculation significantly increased mycorrhiza formation and seedling growth. *Alpova* inoculation significantly increased seedling growth but not nodule development and N_2 fixation. None of the symbionts affected CO_2 exchange rates. *Frankia* was more critical for seedling growth and mycorrhizal development than the mycorrhizal fungus for seedling growth and nodule development.

Key words : Alnus rubra, *ectomycorrhiza, N2 - fixation, shading,* Frankia, Alpova diplophloeus

要 約

光缺乏과 *Frankia* 窒素固定菌, 그리고 *Alpova diplophloeus* 外生菌根菌이 루브라 오리나무(*Alnus rubra* Bong.) 묘목의 생장, 空中窒素固定, 菌根發達에 미치는 영향을 究明하기 위하여, 播種苗에 *Frankia* 窒素固定菌을 접종하거나 窒素를 施肥하고, 또는 *A. diplophloeus* 外生菌根菌 胞子를 접종하여 여과된 공기로 순환시키는 인공생장실에서 길렀다. 20주가 지난 묘목을 3 수준의 光度 680, 320, 220μmol/㎡/s PPFD(photosynthetic photon flux density)에서 3주일 동안 기른 결과, 光度

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220 PPFD에서는 菌根과 뿌리혹의 발달, 光合成 및 窒素固定속도 그리고 식물생장량이 감소하였고, 320 PPFD에서는 光合成 속도만이 감소하였다. *Frankia*로 접종된 묘목은 窒素가 施肥된 묘목보다 菌根形成率과 묘목생장율이 높았다. *A. diplophloeus* 胞子로 접종된 묘목은 비접종묘보다 생장율은 높았으나 뿌리혹 발달과 窒素固定量에서는 차이가 없었다. 두 共生菌은 모두 光合成속도에는 영향을 미치지 않았다. *A. diplophloeus* 菌根菌이 묘목의 생장과 뿌리혹 발달에 기여하는 것 보다는 *Frankia* 窒素固定菌이 묘목의 생장과 菌根발달에 더 크게 기여하였다.

INTRODUCTION

To maximize crop yield per unit land area with minimum use of N-fertilizer intercropping systems with N-fixing plants have been intensively researched in agriculture(Ofori and Stern, 1987). With this in mind, researchers have been interested in the development of practical procedures to facilitate and utilize the benefits to forest productivity resulting from effective symbiotic associations that lead to improve N and P utilization from the environment(Torrey, 1992).

Red alder(*Alnus rubra* Bong.) is an important N_2-fixing tree species in Pacific Northwest forests. It forms a tripartite symbiosis among roots, nitrogen-fixing actinomycetes in root nodules(actinorhizae) and mycorrhizal fungi(Molina et al., 1994). In addition to being a dominant riparian tree, it rapidly colonizes exposed mineral soil recently disturbed by logging or land slide. Because it has less economic value than Douglas-fir(*Pseudotsuga menziesii*(Mirb Franco) or western hemlock(*Tsuga heterophylla*(Raf.) Sarg.) and competes with conifers, alder has often been treated more as a weed than as a manageable or a profitable species for forest productivity. However, the importance of red alder in Pacific Northwest forestry is gradually increasing in annual sawtimber production(Resch, 1988), in use to improve soil fertility by adding N and organic matter into the forest ecosystem(Bormann and DeBell, 1981), and in as an alternative species in sites infected with *Phellinus weirii* root rot of conifers(Hansen, 1979).

Photosynthesis is recognized as a key factor in N_2-fixing plant growth(Hardy and Havelka, 1976), because the N_2-fixing process demands high energy input. Thus, most N_2-fixing plants, particularly pioneering species, are not shade tolerant. Shading generally reduces both nodule number and size in legumes(Trang and Giddens, 1980 ; Antoniw and Sprent, 1978), and nodule formation and nitrogenase activity in European alder(*Alnus glutinosa*)(Gordon and Wheeler, 1978).

In legume symbioses, vesicular arbuscular mycorrhizae(VAM) are essential for the N_2-fixing plants(Bethlenfalvay, 1992), because the plants need extraordinarily high P supply to energize the N_2-fixing process ; VAM enhance P uptake of N_2 fixing plants(Barea et al., 1987 ; Bethlenfalvay et al., 1987 ; Dixon *et al.*, 1993). In addition, VAM can increase CO_2 fixation per unit shoot weight(Kucey and Paul, 1982) of host plants by increasing sink strength (Koch and Johnson, 1984), or increase CO_2 fixation per unit leaf P content(Brown and Behtlenfalvay, 1987 ; 1988). Ectomycorrhizae are also vital in P uptake of the host plant(Harley and Smith, 1983). Mejstrik and Benecke(1969) found enhanced P uptake in excised ectomycorrhizae of *Alnus veridis*. Ectomycorrhizae in *Alnus rubra*(Molina, 1981 ; Molina et al., 1994 ; Miller et al., 1992) probably function similary to influence N_2-fixation and photosynthesis.

Light generally limits tripartite symbioses in the same manner as dual symbioses. Plant growth response to VAM infection generally decreases when photon irradiation is reduced (Bethlenfalvay and Pacovsky, 1983 ; Tester et al., 1985 ; Son and Smith, 1988 ; Borges and Chaney, 1993). However, the responses of three symbionts can vary tremendously depending on the kinds and degrees of the stress. For example, in alfalfa short day length(5hr) produces more small nodules and slightly increased N_2 fixation rate/plant, whereas mycorrhizal colonization and plant growth decrease(Daft and El-

Giahmi, 1978). On the other hand, limiting photosynthate availability by defoliation severely reduces nodulation and nodule activity of soybean, but root colonization by the VAM fungus in the tripartite symbiosis is less affected (Bayne *et al.*, 1984). Bayne *et al.*(1984), therefore, concluded that the fungal endophyte was more competitive than the N_2-fixing endophyte for host carbohydrate as photosynthetic products became limiting.

Actinorhizal alders have been reported to form both VAM and ectomycorrhizae(Rose and Youngberg, 1981). VAM effects have been highly significant for their alder host growth only in a certain soil phosphorus regimes(Jha et al, 1993; Russo, 1989), or only when alder plants were inoculated with *Frankia* first (Lumini, et al., 1994). However, ectomycorrhizal effects on the alder growth and N_2-fixation are still poorly known compared to VAM effects on legume plants.

Mycorrhizal fungi have significant role in the translocation of fixed atmospheric nitrogen from an alder seedling to a pine seedling through sharing hyphae, but the direction and extent of such movement will be influenced by soil nutrient status, light intensity and the presence of symbiotic microorganisms(Arnebrant *et al.*, 1993). Our objective, therefore, was to examine the responses of red alder seedlings to shading, in terms of photosynthetic rates, plant growth, *Frankia* and ectomycorrhizal fungus interaction, nodule and mycorrhiza formation and N_2-fixation.

MATERIALS AND METHODS

Biological materials : Red alder seeds(seed zone 251, Brown Seed Company(12101 N.E. 28th St. Vancouver, Washington)) were selected for uniform size by dry sieving. *Frankia* was isolated by filtration method(Benson, 1982) from nodules of one-year-old red alder seedlings collected at the U.S. Forest Service Cascade Head Experimental Forest near the Oregon coast. The *Frankia* isolates were cultured for one month on N-free BAP liquid medium(Murry et al., 1984). Sporocarps of *A. diplophloeus*(Zeller & Dodge) Trappe & Smith, a hypogeous ectomycorrhizal fungus specific to alder(Molina, 1981), were collected under young red alder trees at the Cascade Head Experimental Forest and stored at -18℃ until used.

Seedling growth conditions : Red alder seeds were surface-sterilized with 30% H_2O_2 for 15 min, planted in a tray with fine-granule vermiculite, covered with autoclaved coarse sand 0.2㎝ deep, and mist-irrigated. The tray was then covered with a clear plastic to maintain moist conditions until germination was complete. When the seedlings produced their first real leaves in two weeks, they were transplanted to Conetainer super cell plastic tubes, 3.4cm diameter·20㎝ long(165ml capacity). Potting substrate was a 1:1:2 mixture of peatmoss, vermiculite and sandy loam soil collected at Willamette Valley in Oregon. The soil mixture was autoclaved for 120 min. Nutrients in the mixture were 0.068% N, 620ppm total P, 8ppm available P, 583ppm K, 1799ppm Ca, and 430ppm Mg after autoclaving. Plants were grown at a day/night temperature of 25/17℃, 14/10hr light regime, and 60/95% relative humidity. Photosynthetic photon flux density(PPFD) was 680±26μmol/㎡/s as measured with a LICOR quantum radiometer/photometer located at 50㎝ above the surface of the growth tubes.

Experimental design : The 2·2·3 experimental design included two levels of *Frankia* colonization, two levels of mycorrhizal colonization and three levels of light intensities. The first factor was N-fertilization vs. *Frankia* inoculation. Because plant growth was stunted without *Frankia* inoculation, non-nodulated red alder seedlings received 10ml of 10mM NH_4NO_3 every other day. The second factor was live spore vs. autoclaved spore inoculation of *A. diplophloeus*(Zeller & Dodge) Trappe & Smith. Ten million spores suspended in 5ml sterilized distilled water were irrigated into each seedling. The third factor was light at three respective intensities : PPFD of 680±26, 320±25 and 220±15μmol/㎡/s measured at 50㎝ height from the tube surface. These intensities were obtained by

Table 1. Growth data of 20-week-old red alder seedlings grown in a walk-in growth chamber before shading treatment with and without *Frankia* and *Alpova diplophloeus* spore inoculation.

Parameter	N	NA	F	FA
Mycorrhizae(%)	0a	2±2a	0a	23±7b
Nodule dry weight(mg)	0a	0a	85±14b	78±8b
Total nitrogenase activity (μmol C_2H_2 reduced/plant/hr)	0a	0a	14.1±1.8b	14.0±2.2b
Specific nitrogenase activity (μmol C_2H_2 reduced/g dry nodule/hr)	0a	0a	186±39b	176±18b
Height(cm)	43.6±0.9ab	41.5±0.7a	43.0±1.7ab	46.0±0.8b
Diameter(mm)	4.2±0.1a	4.5±0.2a	5.1±0.2b	5.3±0.2b
Leaf dry weight(g)	1.02±0.07a	1.11±0.05ab	1.41±0.13bc	1.60±0.15c
Shoot dry weight(g)	1.78±0.12a	1.91±0.06a	2.48±0.25b	2.81±0.25b
Root dry weight(g)	0.43±0.05	0.42±0.04a	0.79±0.12b	0.80±0.08b
Shoot/root ratio	4.41±0.39a	4.70±0.34a	3.14±0.17b	3.50±0.27b

Values are the means of eight samples ± standard error.
N=N-fertilized only ; NA=N-fertilized and *Alpova diplophloeus* spore inoculated ; F=*Frankia* inoculated only ; FA=*Frankia* and *Alpova diplophloeus* spore inoculated.
Different letters within a row show significant difference between treatments at $p<0.05$ by Duncan's multiple range test.

top and side shading with grey colored window shield set at 90cm height from the tube surface. Shading treatments started at the 20th week (when seedlings had formed mycorrhizae with *A. diplophloeus*) and were continued for three weeks before harvesting. Seedling growth at 20th week is shown in Table 1.

A total of 16 seedlings(four inoculation treatments with four replicated seedlings each) were randomly arranged within the shade rack(20×60cm for 98 super cells). The non-shade treatment had a total of 32 seedlings(four inoculation treatments with eight replicated seedlings each). The seedlings were randomly rearranged in the racks repositioned every week to even out location effects.

Data collection : At the 20th week, before shading, and then three weeks after shading began, photosynthetic rates, nitrogenase activity, seedling growth, and symbiosis development were measured for each seedling. Apparent photosynthetic rate was measured as CO_2 exchange rates with a LI6000 portable photosynthesis system(LICOR, Inc, Lincoln, Nebraska) for 10cm² leaf area on the 4th leaf from the top under each shading treatment. During the measurements a breathing mask connected to vacuum was used to remove CO_2 input from the investigator.

To measure *in situ* acetylene reduction rates, we used the closed system diagrammed in Fig. 1. An entire seedling root system was placed inside a 5.2cm inside diameter × 27cm long PVC tube. A hole was made on the PVC tube 5cm below the top to remove air, inject acetylene and collect gas samples. A rubber tube 0.5cm diameter × 10cm long was connected to the hole and a syringe directly attached at the other tube end to expedite gas injection and sampling. Gas flow into the tube was controlled with a spring clip. A split #9 holed rubber plug was used to seal the seedling growth tube in the PVC tube. Space around the seedling stem was sealed with #2 degree Roma Italian Plastilina. The air volume in the closed PVC tube after seedling base insertion was calculated to be ca. 400ml. After removing 40ml of air(10% of the air volume) from the sealed PVC tube with a 60ml syringe, an equal amount of acetylene gas was injected into the tube and the syringe was pumped ten times to mix the gas. The rubber tube was then clamped with the spring clip.

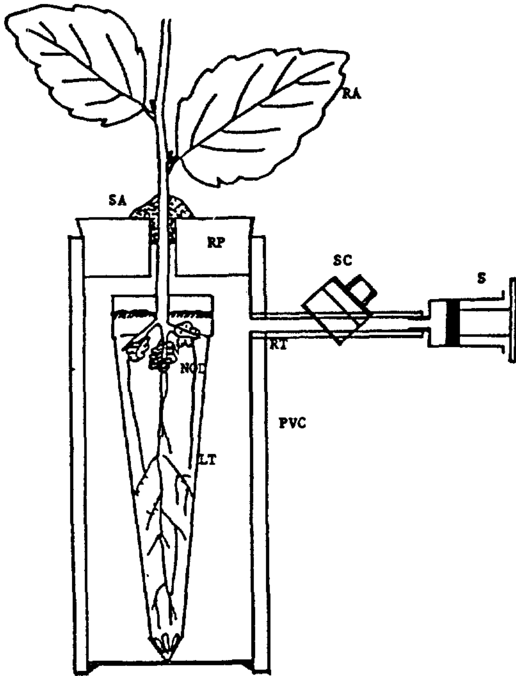

Fig. 1. A simple apparatus for determining nitrogenase activity of intact red alder seedling soil system.
RA=red alder seedling ; SA=sealing agent, Roma Italian Pastilina #2 grade ; RP=120° splitted #9 holed rubber plug ; RT=rubber tubing 0.5×10cm ; SC=spring clip ; S=syringes for injecting and sampling gas ; Nod=nodules ; LT=leach tube, 3.2×20cm, 165ml ; PVC=5.2×27cm, 573ml PVC tube.

After one hour incubation under the shading treatment, a gas sample was collected in a two ml vacutainer with a five ml syringe through the rubber tube after pumping 15 times to mix the inside air. The gas sample was analyzed for acetylene and ethylene with a gas chromatograph (Hewlett Packard, Model HP5830A) equipped with a hydrogen flame ionization detector and a Porapak R(80-100 mesh) filled column(1.8m along・2mm inside diameter) by injecting 0.2ml sample gas with 1cc tuberculin syringe. The oven temperature was adjusted to 70℃. The temperature of injection and detection was adjusted to 70℃. Flow rate of N carrier was 40ml/min. Acetylene reduction rates were calculated as a percent value of produced ethylene to injected acetylene per plant and per unit nodule dry weight. The acetylene reduction assay with the intact root nodules showed that the amount of the reduction had a linear relationship with incubation time up to four hours.

Height, diameter, dry weight of shoot and root, area of the ten top leaves, and dry weight of the leaves were determined for each seedling. Dry weight was obtained after drying the tissues at 65℃ to constant weight. Leaf area was measured with LI3100 Area meter(LI-COR, Inc, Lincoln, Nebraska). Specific leaf dry weight was calculated by dividing the leaf dry weight by the leaf area of each plant. A mean value of mycorrhiza formation for each plant was calculated from three root subsamples collected at 2.5 to 5cm, 7.5 to 10cm, and 12.5 to 15cm deep along the lenght of the root plug. From each subsample 50 to 100 root tips were counted as mycorrhizal or non mycorrhizal and mycorrhiza formation was calculated as percentage.

Data analysis : Data were analyzed by the general linear models(GLM) procedures in SAS (SAS Institute Inc, Cary, North Carolina) to test the treatment effects for each parameter.

RESULTS

Frankia inoculated seedlings grew significay better than the non-inoculated N-fertilized seedlings. *Frankia*+*A. diplophloeus* inoculated seedlings performed the best in growth parameters(Figs 2, 3 and 4, and Tables 1, 2). *Frankia* inoculation and shading have affected mycorrhiza formation(Fig. 2A). *Frankia* inoculation significantly increased *A. diplophloeus* mycorrhiza percentage at all light levels. Of the light levels PPFD of 220μmol/m²/s significantly decreased mycorrhiza formation ; *A. diplophloeus* formed more ectomycorrhizae at 680 or 320 PPFD (65%) than at 220 PPFD(25%). At full light *Frankia* inoculated seedlings formed six times as many mycorrhizae as non-*Frankia* N-fertilized ones. Seedling in N-fertilized+*A. diplophloeus* treatment formed no mycorrhiza at 220 PPFD.

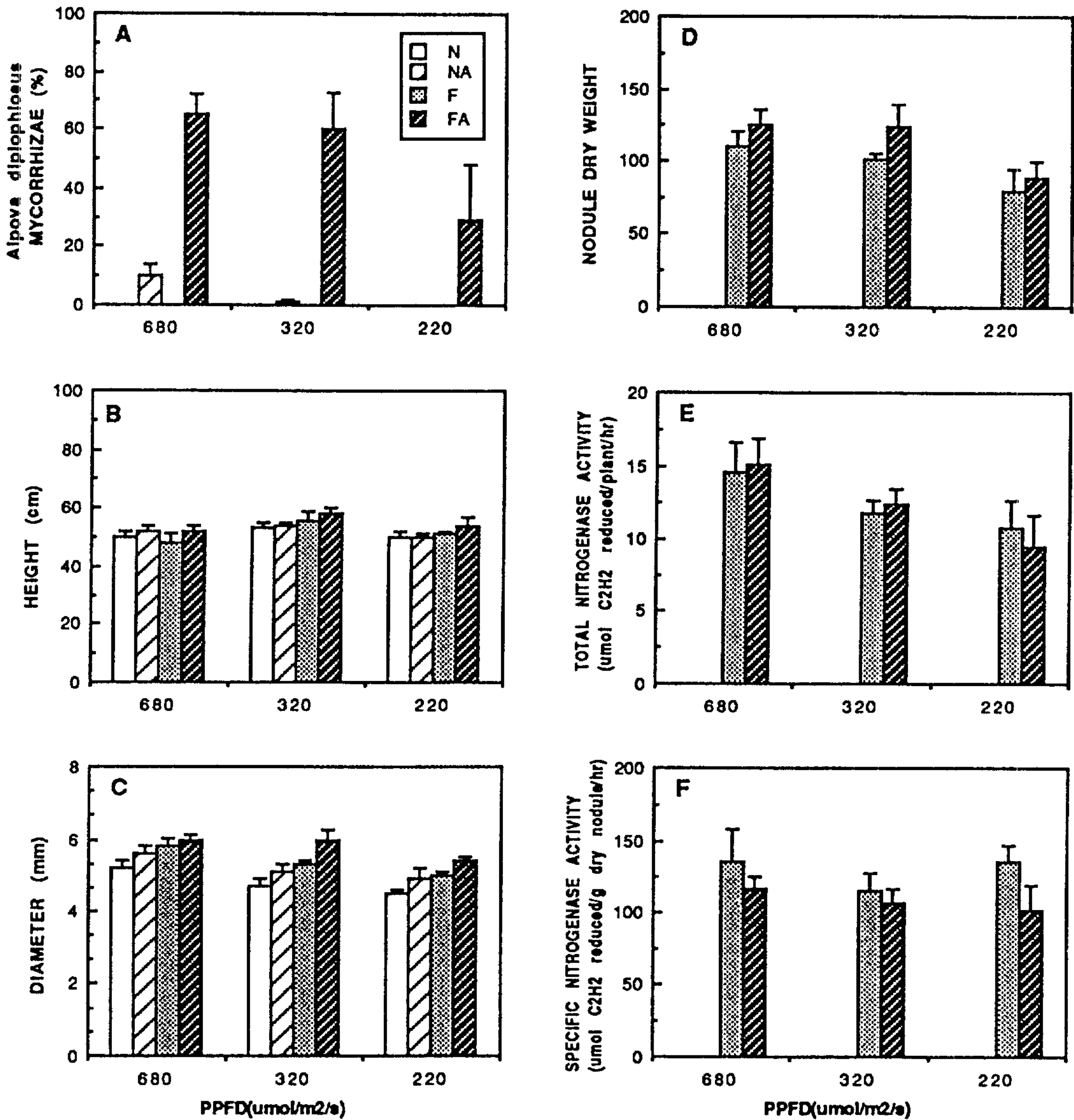

Fig. 2. Three-week shading effects on mycorrhizal development, growth, and N fixation of 23 week-old red alder seedlings grown in a walk-in growth chamber. N=N-fertilized only, NA=N-fertilized and *Alpova diplophloeus* spore inoculated, F=*Frankia* alone, and FA=*Frankia* and *A. diplophloeus* spore inoculated. PPED= Photosynthetic photon flux density. Standard error bar shows on each treatment.

Height growth of N-fertilized seedlings resembled the nodulated ones and, overall, shading did not significantly affect height growth(Fig. 2B). *A. diplophloeus* treated seedlings were consistently taller than those treated with killed spores at 320 and 220 PPFD. Height was significantly increased by the *Frankia*+*A. diplophloeus* inoculation(Table 2). Although diameter growth was significantly reduced by shading under 320 PPFD, *Frankia*−*A. diplophloeus* significantly enhanced diameter growth over all other treatments(Fig. 2C).

Nodule formation measured as dry weight was significantly decreased only at 220 PPFD and ranged from 80 to 110㎎ for non-mycorrhizal seedlings and from 89 to 125㎎ on the mycorrhizal

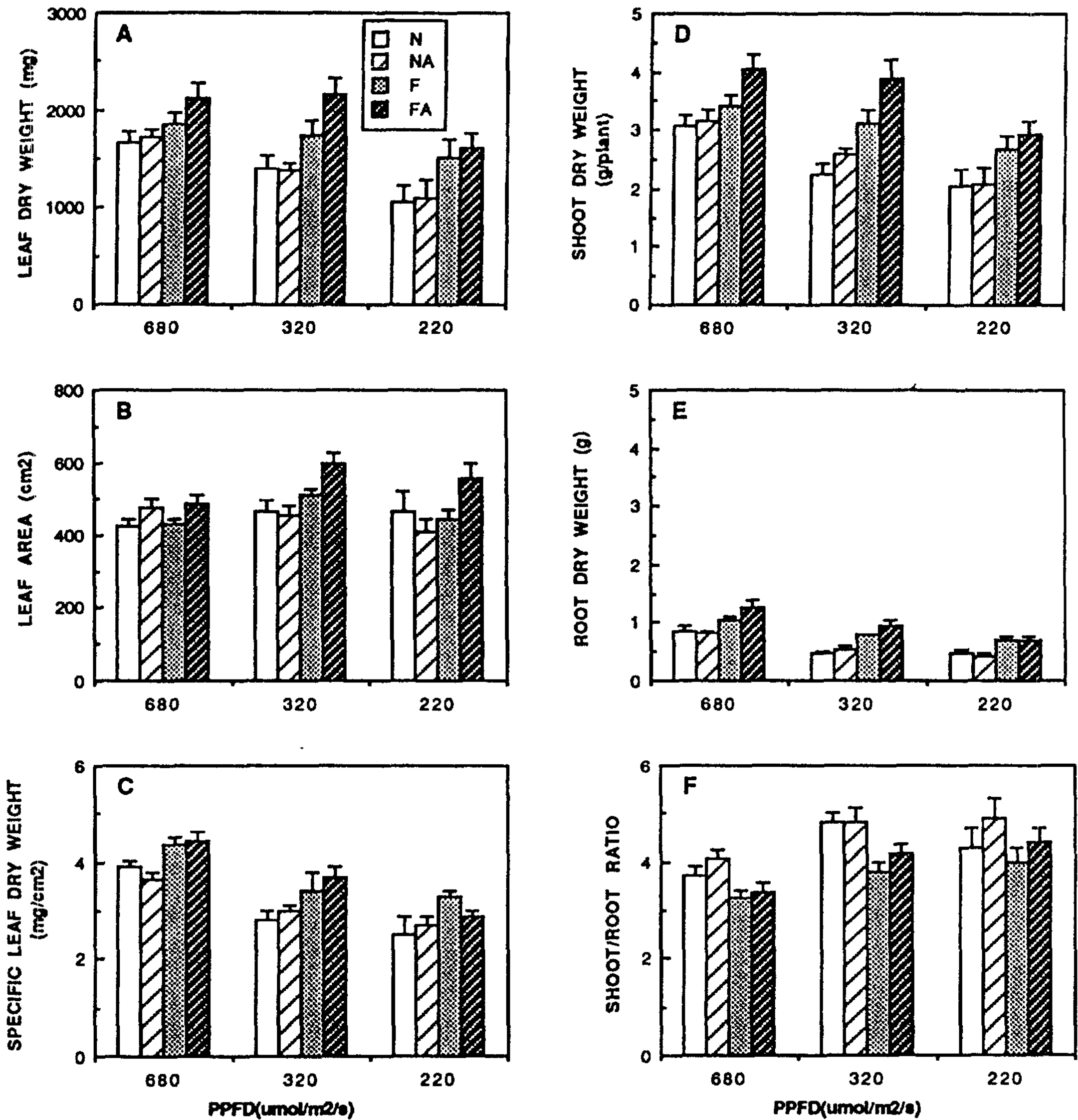

Fig. 3. Three week shading effects on the growth of 23 - week - old red alder seedlings grown in a walk - in growth chamber. N=N - fertilized only, NA=N-fertilized and *Alpova diplophloeus* spore inoculated, F=*Frankia* alone, and FA=*Frankia* and *A. diplophloeus* spore inoculated. PPED=Photosynthetic photon flux density. Standard error bar is shown on each treatment.

plants(Fig. 2D). *Frankia*+*A. diplophloeus* did not significantly increase nodule formation over the *Frankia* alone at any light level. Total nitrogenase activity per plant was also significantly decreased by shading but unaffected by *A. diplophloeus* treatment(Fig. 2E). Specific nitrogenase activity per unit nodule dry weight was not affected by shading(Fig. 2F).

Frankia and *Frankia*+*A. diplophloeus* treatments increased leaf dry weight, leaf area and specific leaf dry weight(Fig. 3A to F). Shading decreased leaf dry weight and specific leaf dry weight and, less so, leaf area, but the interaction of shading with *Frankia*+*A. diplophloeus* increased leaf dry weight and leaf area over N - fertilized or either symbiont inoculation treatment (Fig. 3A, B and C, and Table 2). Despite significant interactions between light and inocu-

Table 2. Comparison of treatment by analysis of variance of data from Figs 2, 3 and 4.

Parameter	Shade(S)	*Frankia*(F)	*Alpova*(A)	SxF	SxA	FxA	SxFxA
Alpova diplophloeus ectomycorrhizae	**	**	**	-	**	**	
Height	-		*		-	*	*
Diameter	**	**	**				
Nodule dry weight	**	**		**	-	-	-
Total nitrobenase activity	*	**	-		-		
Specific nitrogenase activity	-			-	-		
Leaf dry weight	**	**	*	-	-	*	*
Leaf area	-		**	-	-	**	*
Specific leaf dry weight	**	**		-	-		
Shoot dry weight	**	**	**		*	*	*
Root dry weight	**	**		-	-		
Shoor/root ratio	**	**	**		-		
CO_2 exchange rate	**		-	-	-	-	-

* and ** are significant at $p<0.05$ and $p<0.01$, respectively.
- means not significant at $p<0.05$.

lation treatments, shading overall decreased shoot dry weight(Fig. 3D and Table 2); *Frankia*+*A. diplophloeus* increased shoot dry weight, particularly at 320 PPFD(Fig. 3D). Root dry weight was similarly enhanced by *Frankia*+*A. diplophloeus* and decreased overall by shading(Fig. 3E). Conversely, the shoot/root ratio was generally decreased by *Frankia* alone and *Frankia*+*A. diplophloeus* treatments compared to N-fertilized seedlings(Fig. 3F). Apparent photosynthetic rate measured as CO_2 exchange rate significantly decreased with shading but there was little difference between inoculation treatments(Fig. 4)

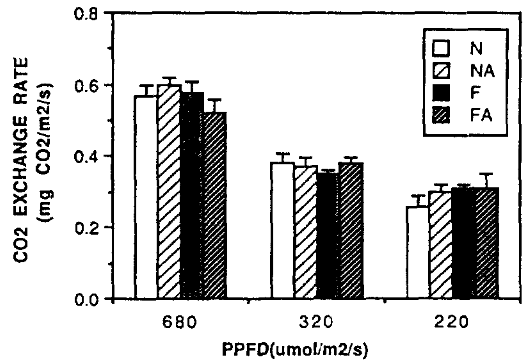

Fig. 4. Three week shading effects on the CO_2 exchange rate of 23 week old red alder seedlings grown in a walk in growth chamber. N=N fertilized only, NA=N fertilized and *Alpova diplophloeus* spore inoculated, F=*Frankia* alone, and FA=*Frankia* and *A. diplophloeus* spore inoculated.
PPED=Photosynthetic photon flux density. Standard error bar is shown on each treatment.

DISCUSSION

Mycorrhizae formed with *A. diplophloeus* significantly benefited red alder growth in this experiment but the effect was strongly overshadowed by the impact of the N_2-fixing symbiosis. Overall, nodulated *A. diplophloeus* mycorrhizal seedlings grew better than most other treated seedlings, particularly compared to the non nodulated, nonmycorrhizal seedlings. However, the extra growth benefit from *A. diplophloeus* mycorrhiza formation was small compared to *Frankia* nodulated seedlings, and only significantly different in increasing shoot dry weight. This small benefit occurred in spite of the fact that approximately 65% of the feeder roots were mycorrhizal with *A. diplophloeus*. For non nodulated seedlings, 10% *A. diplophloeus* mycorrhizae on inoculated seedlings provided no benefit to seedling growth. Our results support Lumini et al.(1994) who found that *Frankia* inoculation effect on the alder host growth was remarkable

but VAM inoculation effect was significant only when the host was inoculated with *Frankia* first.

The two microbial symbionts did interact significantly. *Frankia* inoculation increased *A. diplophloeus* mycorrhiza formation from 10% on N-fertilized seedllings to 65% on nodulated seedlings ; the seedlings were similar in height and stem diameter but nodulated seedlings had greater leaf, shoot and root dry weight(Fig. 2). This result immediately raises the question of whether such differences in mycorrhiza formation were due to the presence of *Frankia*, either via a direct interaction between microorganisms or as mediated through the nodulated root system, or an inhibition by the N-fertilization. Koo (1989) found that high N fertilization(5 times higher than this experiment) did not inhibit *Alpova* mycorrhiza formation when the seedlings were nodulated. Koo also presented evidence that early nodule formation on red alder seedling promoted more rapid and abundant ectomycorrhiza formation than did delayed nodule formation. Such a pattern of root symbiosis development also follows the natural sequence on wild red alder seedlings. Koo(unpublished data) followed the development of nodules and ectomycorrhizae on red alder seedlings in a variety of disturbed forest soils and observed that nodulation always preceded mycorrhiza development.

However, Ba et al.(1994) suggested that in ectomycorrhizal *Acacia holosericea* seedlings fungal hyphae could modify directly and/or indirectly the recognition factors leading to nodule meristem initiation and infection thread development. They found that prior inoculation with *Bradyrhizobium* sp. did not inhibit ectomycorrhizal colonization in root segments adjacent to nodules and that simultaneous inoculation with both microorganisms inhibited infection thread development of the bacteria. On the other hand non-N_2-fixing lucerne genotypes produced very few mycorrhizas in their roots(Bradbury, et al., 1993). This sequence raises the possibility that *Frankia* nodulation enhances the receptivity of roots to mycorrhizal fungi, perhaps through biochemical or morphological changes in the tissue or release of attractant root exudates. Such helper microbial interactions have been hypothesized in other root symbiosis and merit further investigation in the alder *Frankia* mycorrhiza interaction.

Further work is also needed to elucidate the symbiotic role of mycorrhizal fungi for N_2 fixation of red alder. One major difficulty we encountered in these experiments and preliminary trials was in separating effects of the two root symbionts. Red alder germinants remained stunted and chlorotic when *Frankia* inoculation was withheld ; when inoculated with *A. diplophloeus* without *Frankia*, few or no mycorrhizae formed and the seedlings remained stunted. When non-nodulated seedlings were fertilized with N, they grew normally but mycorrhiza development remained low. When mycorrhiza enhancement of seedling growth occurred in combination with nodulation, it was rarely over 20% of what nodulation alone provided and never affected N_2 fixation rates. These results contrast sharply with mycorrhizal benefits seen in VAM rhizobial legumes(Asimi et al., 1980 ; Bethlenfalvay and Yoder, 1981 ; Piccini et al., 1988), and in VAM-actinorhizal snowbrush(Rose and Youngberg, 1981) and aldet al., 1993). In those tripartite symbioses VAM significantly increased N_2-fixtion, N and P nutrition, and plant growth by 50% to several times. Koo(1989) found that *A. diplophloeus* mycorrhizal inoculation did increase P concentration in leaves at full light over non-inoculated but mycorrhizal(*Thelephora* + brown type) seedlings. Regretably, however, we did not record P tissue contents in this experiment to compare mycorrhizal and non-mycorrhizal seedlings.

The supply of photosynthate from the host is important for nodule and mycorrhiza formation. Reducing photosynthate by shortening photoperiod or shading reduced the formation of nodules(Sprent, 1973 ; Chu and Robertson, 1974) and mycorrhizae(Hayman, 1974 ; Bjorkman, 1970). The growth of the both symbionts on root was in balance with the shoot growth of the host, irrespective of climatic conditions(Saito and Kato, 1994). However, this generalization is not

fully supported by our results. Because nodule formation and total nitrogenase activity significantly decreased under the heaviest shading, and mycorrhiza formation was also significantly reduced after three weeks of heaviest shading (Fig. 2A). It is important to note that nodulated mycorrhizal seedlings had about 23% of mycorrhiza formation at 20th week just prior to initiating shade treatments and that the percentage increased to ca. 60% for both 680 and 320 PPFD but remained unchanged in 220 PPFD.

On the other hand, plants may need comparable amounts of N for photosynthetic enzyme formation and cell division under shading as in full light. This may explain why total nitrogenase activity was only reduced by 30% and specific nitrogenase remained unaffected under the heaviest shading even though CO_2 exchange rate was significantly and proportionally decreased (59 to 75%) by shading. These minimum effects of shading on specific nitrogenase activity agree well with results from legumes by Sprent(1973) and Eriksen and Whitney(1984).

Shading remarkably changed seedling growth pattern in terms of morphology, i.e. reduced root growth, increased leaf area and shoot/root ratio. It is important to realize how these structural changes may operate in a natural forest setting and how they may be useful in a management context. The pattern of structural adaptation under shading can make the plant vulnerable to environmental stress, especially water stress, due to high shoot/root ratio and fresh to dry weight ratio(Son and Smith, 1988 ; Tester et al., 1986). Plants producing more shoot than root under heavy shade easily lose their large leaves during water stress period. This sets up a negative feedback loop wherein ; when water stress is eased, plants must struggle with less photosynthesis for recovery. One result is decreased root production. The shaded trees will gradually weaken and be unable to accumulate enough carbohydrate for regrowth after the stress damage or for the following year.

In conclusion, *Frankia* was more critical for seedling growth and mycorrhiza development than a mycorrhizal fungus for seedling growth and nodule formation. Nodulated mycorrhizal plants were consistently larger than nodulated nonmycorrhizal plants in growth parameters, and they were significantly different. The role of *A. diplophloeus* mycorrhizae in physiological activities such as CO_2 exchange rate and nitrogenase activity was not significant, and further experimentation with *A. diplophloeus* and other alder related mycorrhizal fungi is needed to address mycorrhizal mediation of mineral uptake, particularly under P limited conditions. Such experimentation is needed to shed further light on possible mycorrhizal effects on N_2 fixation demanding high P nutrition. Although VAM have had large positive effects on N_2 fixation of legumes, no similar ectomycorrhizal effects were evident in our alder studies.

Shade can be an overwhelming environmental factor influencing growth of N_2 fixing plants by reducing photosynthesis. In our three weeks long shading treatment study, shaded plants adapted their structure by increasing shoot/root ratio. Specific N_2 fixation rates were not significantly changed but nodule development and total nitrogenase activity decreased with shading. Even though plant photosynthate is a key influence on both microsymbionts, we found no evidence that the symbionts significantly increased photosynthetic rate per unit leaf area. For balanced red alder seedling growth PPFD of 220μmol/m/s was not sufficient. To improve intercropping forest management with shaded alder, we need to further understand how these plants adapt their morphology and biochemistry, and still maintain normal symbioses and growth under reduced light intensity.

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