



Tripartite associations in an alder: effects of *Frankia* and *Alpova diplophloeus* on the growth, nitrogen fixation and mineral acquisition of *Alnus tenuifolia*

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Abstract

The role of tripartite associations among *Frankia*, *Alpova diplophloeus* (an ectomycorrhizal fungus) and *Alnus tenuifolia* in growth, nitrogen fixation, ectomycorrhizal formation, and mineral acquisition of *A. tenuifolia* was investigated. Seedlings of *A. tenuifolia* were planted in pots containing a mixture of ground basalt–perlite, or perlite alone, which served as the control. The seedlings were inoculated with *Frankia* isolated from root nodules of alder, followed by spores of *A. diplophloeus* and grown for 5 months in a greenhouse. The seedlings grown in the pots with a mixture of ground basalt–perlite after dual inoculation with *Frankia* and *A. diplophloeus* had the heaviest shoots and root nodules in dry weight, and showed the greatest nitrogen-fixing ability measured by acetylene reduction. Ectomycorrhizae formed with *A. diplophloeus* increased when this fungus was inoculated together with *Frankia*. The mineral composition (P, K, Ca, Fe, Mg, Mn, Na, Si and Al) in the seedlings was also determined. The results of these experiments showed that the tripartite associations could improve the growth, nitrogen fixation and mineral acquisition (rock solubilization) of *A. tenuifolia*.

Introduction

Actinorhizal plants fix atmospheric nitrogen in root nodules symbiotically formed by an actinomycete *Frankia*. Owing to their capacity for nitrogen fixation, nodulated species can grow and improve soil fertility in disturbed sites, and are used in the recolonization and reclamation of eroded areas, sand dunes, screes, moraines, area of industrial waste and road cuts, and are planted following fire, volcanic eruption and logging (Hibbs and Cromack, 1990; Wheeler and Miller, 1990).

Many of the actinorhizal plants are capable of sustaining a mycorrhizal association as well, thus forming a tripartite symbiosis and enhancing the success of

these plants under poor soil conditions (Chatarpaul et al., 1989; Rose and Youngberg, 1981). Ectomycorrhizae (EM), arbuscular mycorrhizae (AM) or both have been found in actinorhizal plants (Rose, 1980). Actinorhizal alder species form EM with comparatively few fungus species (Molina, 1979). Less than 50 species of fungi form ectomycorrhizae in the entire genus *Alnus* (Brunner et al., 1990; Miller et al., 1991), while about 2000 species of EM fungi associate with Douglas fir (*Pseudotsuga mensiezii*) (Trappe, 1977).

Mycorrhizas are well known for improving phosphorus nutrition of host plants (Jakobsen, 1999). Mejsstrik and Benecke (1969) found that ectomycorrhizal *Alnus viridis* absorbed phosphorus five times more rapidly than non-mycorrhizal *A. viridis*. The extraradical mycelia of the associated fungi may act as extensions of the root systems (Rousseau et al., 1994), or the fungi have a high phosphorus solubilizing and

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mobilizing potential (Lapeyrie et al., 1991). Organic acids produced by EM fungi can probably accelerate rock solubilization, thereby enhancing nutrient availability for uptake by plants (Cairney and Ashford, 1991; Paris et al., 1995; Watteau and Berthelin, 1990). Plant roots also release organic acids into the rhizosphere and induce weathering processes of rock minerals (Hoffland et al., 1989; Krafczyk et al., 1984). Thus, combined weathering processes by plants and soil microbes may be important for the survival and establishment of these plants in the nutrient-limited soil ecosystem, thereby improving forest productivity (Bormann et al., 1998; Crawford et al., 2000; Li and Strzelczyk, 2000).

The objective of the present study was to determine whether the ability of alder to nodulate, fix N and solubilize rock minerals could be enhanced by dual inoculation with *Frankia* and EM fungus in pot culture under a greenhouse condition. Ectomycorrhizal fungi are expected to improve nodulation and nitrogen fixation of the actinorhizal plants, perhaps by solubilizing rock minerals, and by transferring them to the plants. Therefore, this benefit of mycorrhizal fungi should be observed when these plants are cultivated in pots containing ground basalt, as the source of mineral nutrients.

Materials and methods

Soil preparation

Basalt rocks were collected from Hebo Ranger District in Siuslaw National Forest, Oregon, USA. The composition of basalt in this area was described by Snavely et al., (1973). The collected rocks were washed and air-dried and then ground and screened to obtain particles of 1–2 mm in diameter (LA) and those 0.2–0.6 mm in diam. (SM) fractions. Twenty-five grams of these rock particles were mixed with ground perlite and put into a Ray Leach tube (164 mL; SC-10; Stuewe & Sons Inc., OR, U.S.A.). The bottom of the tube was cut and sealed with a 50- μ m mesh nylon screen to prevent the particles of perlite and the ground basalt rock from dropping out of the tube.

Seedlings

Seeds of *Alnus tenuifolia* Nutt. were surface-sterilized in 30% H₂O₂ containing a drop of Tween 20 for 20 min, and then washed several times with steril-

ized distilled water. After sterilization, the seeds were placed on a moist filter paper in a petri-dish, and exposed to light at room temperature until they germinated. The seedlings were transplanted into the soil prepared as described above.

A *Frankia* isolate (AS-2) used in the present study was obtained from root nodules of *Alnus sieboldiana* Matsumura, which is a Japanese actinorhizal alder. The isolate was cultured on N-free BAP liquid medium for *Frankia* (Murry et al., 1984) in darkness at 24 °C for 4–6 weeks. All but controls were inoculated by pouring 1 mL of a well-fragmented inoculum suspension prepared by homogenation, equivalent to 0.01 mL packed cell volume (3000 rpm, 20 min), near the base of the seedling.

Three weeks after *Frankia* inoculation, the seedlings were inoculated with *Alpova diplophloeus*. Sporocarps of *A. diplophloeus* were collected from a red alder (*Alnus rubra* Bong.) forest near Florence, Oregon. Spore suspension was prepared by homogenizing the sporocarps in distilled water with a Waring blender at a high-speed for about 3 min. One mL spore suspension containing 1×10^6 spores, determined by haematocytometry, was inoculated at the base of the seedling.

Ten to 12 replicates were prepared for each treatment. Seedlings were grown in a greenhouse for 5 months with a 24–18 °C (day-night) regime and under a 16–8 h photoperiod under the light from sodium-vapor lamps at 11 000 lx, and watered every 2 days. The pots receiving different inoculation treatments were systematically rotated to different bench positions once a week to minimize differences due to the location in the greenhouse.

Data collection

We measured the nitrogen-fixing activity of *A. tenuifolia* using the acetylene reduction (AR) technique. The root of each seedling was rinsed in distilled water and placed into a 27-mL test tube. After the tube was sealed with a rubber serum cap, acetylene gas was injected into the tube through a plastic syringe to constitute 10% of the total gas volume. After a 2-h incubation at 24 °C, a 0.1-mL gaseous sample from each tube was collected, and analyzed for C₂H₄ and C₂H₂ with a Hewlett-Packard 5830A gas chromatograph fitted with a flame-ionization detector (FID) and a 2.0 m \times 2.1 mm stainless steel column packed with Porapak R on 80–100 mesh on chromosorb W. The oven temperature was adjusted to 70 °C; injection and FID

Table 1. Effect of *Frankia* and *Alpova diplophloeus* on growth, acetylene reduction and ectomycorrhizal formation of *Alnus tenuifolia*. Values with a different letter within columns are significantly different at $P < 0.05$

Inoculation type	Soil treatment	Shoot		Root	Nodule		Acetylene reduction ($\mu\text{mol C}_2\text{H}_2$ /plant/h)	Ectomycorrhiza formation ^a
		Height (cm)	Dry weight (g)	Dry weight (g)	No. of lobe	Dry weight (g)		
Control	perlite	0.9a	0.00a	0.01a	0.0	0.00	0.00	–
	+SM ^b	1.0a	0.00a	0.02a	0.0	0.00	0.00	–
	+LA ^c	1.0a	0.02a	0.08a	0.0	0.00	0.00	–
<i>Alpova</i>	perlite	1.0a	0.00a	0.01a	0.0	0.00	0.00	–
	+SM	1.2a	0.04a	0.01a	0.0	0.00	0.00	–
	+LA	1.1a	0.03a	0.05a	0.0	0.00	0.00	–
<i>Frankia</i>	perlite	1.3a	0.02a	0.03a	10.2a	0.00a	0.04a	–
	+SM	11.9c	1.10c	1.36c	85.7d	0.05c	3.21d	–
	+LA	6.8b	0.59d	0.52b	36.2b	0.02b	1.32b	–
<i>Frankia</i> + <i>Alpova</i>	perlite	1.2a	0.01a	0.02a	9.0a	0.00a	0.01a	+
	+SM	14.8d	1.48d	1.82d	88.0d	0.07d	2.29cd	+++
	+LA	10.3c	0.99b	1.21c	60.7c	0.04c	1.86bc	+++

^a Approximate degrees of ectomycorrhizal formation of the roots: +++, 75–100%; ++, 40–75%; +, 10–40%; \pm , <10%; –, 0%.

^b SM, small-particle (0.2–0.6 mm) basalt.

^c LA, large-particle (1–2 mm) basalt.

temperatures were adjusted to 100 °C. The nitrogen carrier gas flow rate was adjusted to 40 mL min⁻¹.

The formation of ectomycorrhiza was observed with a stereomicroscope. The ectomycorrhiza formed by *A. diplophloeus* was recognized after Godbout and Fortin (1983), and its relative abundance was measured.

For the determinations of seedling biomass, shoot height was measured, and the dry weights of shoot, nodule and root were determined after oven drying at 50 °C to a constant weight.

The dried shoot was ground in a mill, and its mineral composition was analyzed by the Central Analytic Laboratory, Department of Crop and Soil Science, Oregon State University, Corvallis, Oregon. The methods included digestion of 0.25 g sample in 5.0 mL HNO₃ and 1.0 mL HF acids with 2.0 mL distilled water in a pressure controlled microwave unit. Elements were determined by inductively coupled plasma (ICP) emission spectroscopy.

Data analyses

Two-way analysis of variance and contrast test were used to determine the significance of mean differences

between treatments of rock and inoculation. Kendall's rank correlation coefficient and its significance level were also calculated for the statistical analysis of the relationship between total and nodule biomass, and nitrogen fixation.

Results

Growth

As seen in Figure 1, *Frankia* inoculation under cultivation in a ground basalt–perlite mixture resulted in visible larger and healthier seedlings when compared to the seedlings without *Frankia*, or to those with *Frankia* in perlite only. Shoot height, dry weight of shoot, root and nodule in the seedling grown in a basalt–perlite mixture were significantly higher when inoculated with *Frankia* and *A. diplophloeus* together than with *Frankia* alone (Table 1). Cultivation in small-particle basalt resulted in better growth of the seedling with *Frankia* than that in large-particle basalt, presumably due to an increase of the surface area of rock per a weight basis whereby enhancing the rate of solubilization of rock. Very low nodulation was



Figure 1. *Alnus tenuifolia* seedlings with different treatments. Seedlings in a mixture of large-particle ground basalt and perlite (a), in a mixture of small-particle ground basalt and perlite (b), or in perlite alone (c). The seedlings on the left are controls; the seedlings next to the controls are treated, from left to right, with *Alpova diplophloeus*, *Frankia*, and *A. diplophloeus* + *Frankia*.

Table 2. Kendall's rank correlation coefficients (τ) and their significance between total and nodule biomass, and AR

Inoculation type	Soil treatment	No. sample	Total biomass vs. nodule biomass	Total biomass vs. AR	Nodule biomass vs. AR
<i>Frankia</i>	+SM ^a	10	0.600*	0.200	0.244
	+LA ^b	10	0.778**	0.377	0.511*
<i>Frankia</i>	+SM	11	0.564*	-0.055	0.091
+ <i>Alpova</i>	+LA	12	0.485*	0.121	0.212

^aSM, small-particle (0.2–0.6 mm) basalt.

^bLA, large-particle (1–2 mm) basalt.

* $P < 0.05$, ** $P < 0.01$.

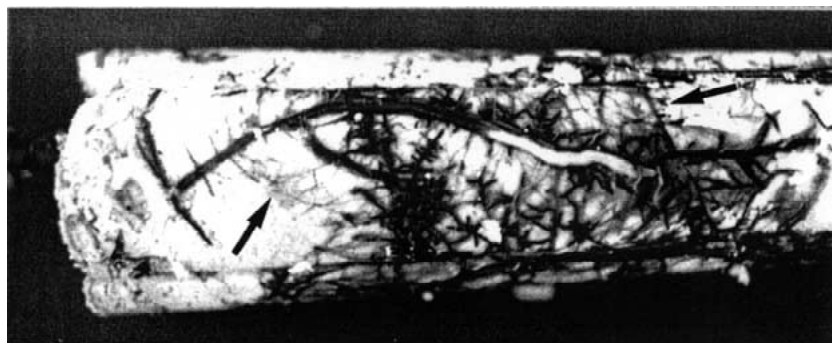


Figure 2. Formation of ectomycorrhizae of *A. tenuifolia* by *Alpova diplophloeus*. The seedling inoculated with *Frankia* and *A. diplophloeus* was grown in a mixture of ground basalt (large particle) and perlite. Note that extrametrical hyphae (arrows) of *A. diplophloeus*.

observed in a seedling without *Frankia* inoculation, perhaps due to contamination by splashes of water during watering.

Nitrogen fixation

Acetylene-reducing (AR) activity, an indirect measurement of the nodule ability to fix atmospheric nitrogen, was observed when the seedlings were inoculated with *Frankia* (Table 1). The AR rates were higher in the mixture of basalt–perlite than in perlite alone, indicating that minerals taken up promoted nitrogen fixation. Seedlings with *Frankia* in small-particle basalt and perlite reduced more acetylene than seedlings with *Frankia* in large-particle basalt and perlite; however, dual inoculation of *Frankia* and *A. diplophloeus* negated the effect of the size of basalt on AR rate.

Correlation between the factors investigated

Kendall's rank correlation coefficients between total biomass, nodule biomass and AR rate are shown in Table 2. Because nodulation was not observed without *Frankia*, the investigated factors in the treatment without *Frankia* were not included in the correlation

calculations. When seedlings of *A. tenuifolia* were inoculated with *Frankia* and *A. diplophloeus* together or *Frankia* alone, and cultivated in perlite only, the seedling were small, and their AR rate was not detected in some of the samples, the correlation analyses were not calculated for these treatments.

A significant correlation was found between total and nodule biomass in all treatments. In the seedlings cultivated in a mixture of large-particle basalt and perlite, a significant correlation was observed between nodule biomass and AR rate.

Ectomycorrhiza formation

Ectomycorrhizal formation by *A. diplophloeus* was observed after inoculation of this fungus together with *Frankia* (Table 1). In the mixture of basalt–perlite ectomycorrhizae were formed abundantly, and vegetative hyphae of this fungus were visibly developed (Figure 2). Ectomycorrhizae of other types were not observed.

Table 3. Effect of *Frankia* and *A. diplophloeus* on nutrient content (mg/plant) of shoot of *Alnus tenuifolia*. Sufficient sample for analysis of nutrient content were obtained only in four treatments. Values with a different letter within columns are significantly different at $P < 0.05$

Inoculation type	Soil treatment	P	K	Ca	Mg	Mn	Al	Fe	Na	Si
<i>Frankia</i>	+SM ^a	1.10	5.23	12.2	3.46b	1.32	0.33b	0.72b	0.30	0.30
	+LA ^b	0.63	3.75	7.90	1.85a	0.70	0.17a	0.19a	0.28	0.26
<i>Frankia</i> + <i>Alpova</i>	+SM	0.78	4.87	9.91	2.56ab	0.98	0.25ab	0.56b	0.31	0.44
	+LA	0.97	5.52	11.1	2.69ab	1.15	0.39b	0.48b	0.65	0.45

^aSM, small-particle (0.2–0.6 mm) basalt.

^bLA, large-particle (1–2 mm) basalt.

Mineral content of the shoot of *A. tenuifolia* seedlings

Sufficient samples of *A. tenuifolia* for analyses of mineral composition were obtained only after the seedlings were inoculated with *Frankia* alone or with *Frankia* and *A. diplophloeus* together, and cultivated in the mixture of basalt and perlite (Table 3). Without mycorrhizal inoculation, *Alnus tenuifolia* seedlings grown in the small-particle basalt had significantly higher levels of magnesium, aluminum and iron than those grown in the large-particle basalt (Table 3). However, these differences were not clear after dual inoculation with *Frankia* and *A. diplophloeus*.

Discussion

Tripartite symbiosis

The present results showed that the growth and nodulation of *A. tenuifolia* significantly increased after dual inoculation of *Frankia* and *A. diplophloeus* compared to single inoculation of *Frankia* (Table 1). Ectomycorrhizal formation by *A. diplophloeus* was abundant when the seedlings were inoculated with this fungus together with *Frankia*, and then cultivated in a mixed ground basalt and perlite. The association with *A. diplophloeus* seemed to be dependent on the nodulation by *Frankia*, as stated by Koo (1989). Besides, basalt, that is, the source of mineral nutrients, is necessary for the EM development as well as seedling growth and nodulation after *Frankia* inoculation.

Actinorrhizal plants are associated with *Frankia* and mycorrhizal fungi simultaneously (Rose, 1980). Several studies have been reported that a dual inoculation with *Frankia* and AM fungi resulted in a better growth of actinorrhizal plants, compared to single inoculation with *Frankia* (Jha et al., 1993; Rose and Youngberg, 1981). Arbuscular mycorrhizal fungi im-

prove the P supply to the host by means of hyphal extension and ramifications through the soil although they do not solubilize phosphorus (Mosse et al., 1976). Arbuscular mycorrhizal fungi, thus, improve the nutrient balance and, in concert with *Frankia*, stimulate the growth, nodulation and the AR rate of actinorrhizal plants (Rose and Youngberg, 1981).

The role of EM fungi in the tripartite symbiosis has also been studied. *Alpova diplophloeus*, used for the present study, is well known as an alder-specific EM species developing hypogenous sporocarps (Arora, 1986). The EM formed by this species are the most common and abundant type developed in the bioassay test from forest soils in the Pacific Northwest of the United States, indicating the ecological importance of this host specific fungus of alder in this area (Miller et al., 1992). The formation of EM structures by *A. diplophloeus* on young root nodules of *Alnus crispa*, representing a 'hypersymbiosis' (Godbout and Fortin, 1983), may show a lack of competitive interactions between *Frankia* and EM. Koo (1989) found that EM formation by *A. diplophloeus* improved the growth and phosphorus status of the seedlings of red alder. His study emphasized the importance of actinorrhizal development and function in influencing on red alder growth and EM development; actinorrhizae appear the dominant root symbiosis in the tripartite symbiosis during seedling stage (Molina et al., 1994).

Dual inoculation of *Frankia* and *A. diplophloeus* negated the effect of the size of basalt on AR activity in single inoculation with *Frankia* (Table 1), indicating the supplemental effect of ectomycorrhizal fungi to solubilize minerals and gather them for nitrogen fixation. In tripartite associations of *Frankia*, AM fungi and actinorrhizal plants, the AR rate was higher in dual inoculation of *Frankia* and AM fungi than in single inoculation of *Frankia* (Jha et al., 1993; Rose and Youngberg, 1981).

Chatarpaul et al. (1989) reported the tetrapartite associations of *Alnus incana*, AM fungus, EM fungus and *Frankia* on the growth of *A. incana*. In their study the growth and nodulation of the seedling and mycorrhizal infection were highest when the seedlings were inoculated with all the three symbionts together, suggesting that the presence of a multiple symbiosis could have added benefits to the actinorhizal plants.

Nodule biomass correlated positively with seedling biomass (Table 2). Rojas et al. (2001, 2002) also obtained a positive correlation between seedling biomass and nodule biomass in red alder and snowbrush (*Ceanothus velutinus*). They also reported positive correlations between seedling biomass and AR rate, and between nodule biomass and AR rate in these plants. Such correlations have been obtained in many studies, and confirmed the view that symbiotic N₂ fixation is dependent on host plant photosynthesis (Arnone and Gordon, 1990).

In the present study, basalt rock from Oregon was used without sterilization. However, this rock is not a source of bacterial and fungal contamination because nodules and mycorrhizae were not formed without the inoculation of *Frankia* and *A. diplophloeus*, an EM fungus.

Weathering

The present study showed that nodulated *A. tenuifolia* mobilized abundant mineral elements from basalt and took them up as nutrients (Figure 1; Tables 1 and 3). Supplementary functions of *A. diplophloeus* in rock solubilization were also observed (Table 3).

Bormann et al. (1994) reported a process how alder and its microbial associates interact with their environment to bring about the changes in the productive potential of the land. The process might be supported by the results of the present study. Seedlings of *Alnus tenuifolia* grow better by supply of nitrogen fixed by *Frankia*, extend its roots into the soil and release organic acids capable of mobilizing mineral elements from basalt. Enhanced growth of *A. tenuifolia* also provides the energy for forming mycorrhizal associations with *A. diplophloeus* and spreads its mycelial network into the soil, thereby accelerating the weathering process. Weathering and redistribution make cations and phosphorus more available, which in turn stimulate the fixation of atmospheric nitrogen. In this way, on young, nitrogen-poor soils with abundant weatherable minerals, a positive feedback is likely (Bormann et al., 1994). Bormann et al. (1998)

demonstrated the potential for weathering rates that are about 10 times faster; at least under conditions where primary minerals are abundant, soil have been disturbed, and plants are growing rapidly.

The growth of red alder (*Alnus rubra*) was also improved by *Frankia* inoculation and cultivation in a mixed ground basalt–perlite, compared to their poor growth in perlite only (Li, C.Y., personal communication).

The formation of root nodules by *Frankia* and of EM by *A. diplophloeus* accelerates mineral solubilization, and is essential for the survival and establishment of actinorhizal plants in nutrient-poor degraded sites. These associations between plants and its symbiotic rhizosphere microbes can therefore have significant impact on belowground terrestrial processes, such as mobility and cycling of nutrients in nutrient-limited forest ecosystems. The presence of actinorhizal plants such as alder enhances soil fertility and productivity of forest ecosystems.

Beside mycorrhizal fungi, a variety of soil microorganisms colonize in the rhizosphere of actinorhizal plants, and should affect actinorhizal interactions. Some actinomycetes isolated from surfaces of nodules and roots of red alder, or its surrounding soil, influence the growth, nodulation and AR activity of seedlings of red alder (Rojas et al., 1992). *Pseudomonas cepacia*, a common root-associated microbe, improved nodulation of red alder (Knowlton and Dawson, 1983). *Pseudomonas* isolates produce strong Fe₃⁺ chelators, hydroxymate siderophores (Torres et al., 1986), and could accelerate solubilization of rock minerals (Li and Strzelczyk, 2000). Further studies on the effect of these rhizosphere microbes on multiple symbioses of actinorhizal plants, *Frankia* and mycorrhizal fungi and on the weathering process are necessary.

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