Alnus acuminata in dual symbiosis with Frankia and two different ectomycorrhizal fungi (Alpova austroalnicola and Alpova diplophloeus) growing in soilless growth medium

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Abstract
In this study we investigated the capacity of Andean alder (Alnus acuminata Kunth), inoculated with Frankia and two ectomycorrhizal fungi (Alpova austroalnicola Dominguez and Alpova diplophloeus ([Zeller and Dodge] Trappe and Smith), for nodulation and growth in pots of a soilless medium that contained vermiculite or a mixture of ground basalt rock and vermiculite. The seedlings were inoculated with Frankia suspensions prepared from root nodules of A. acuminata, followed by inoculation with spores of either one of the two Alpova species. The seedlings were grown in a greenhouse for 12 months. The seedlings grown in the vermiculite-based growth medium containing large (1-3 mm) basalt particles and Alpova austroalnicola or medium-sized (0.5-1 mm) basalt particles and A. diplophloeus had the heaviest shoot and root node dry weights and abundant ectomycorrhizal colonization. Ectomycorrhizas formed by A. acuminata with Alpova austroalnicola is described here for the first time. Growth of Alnus acuminata inoculated with ectomycorrhizal fungi and Frankia in the soilless primary minerals indicates that Andean alder can alter resource supply by tapping an otherwise unavailable nutrient source.

Keywords: Alpova, Alnus acuminata, ectomycorrhiza, Frankia

1. Introduction

Alnus acuminata Kunth (Andean alder), a member of the Betulaceae, is distributed along the Andes from Venezuela to latitude 28°S in northwestern Argentina (Grau, 1985). Andean alder is mainly harvested for firewood, pulp, and timber. It is an important species recommended for management in land reclamation, watershed protection, agroforestry, and erosion control (National Academy of Sciences, 1984). A. acuminata is tolerant to infertile soils given its ability to form ectomycorrhizal (ECM), arbuscular mycorrhizal and actinorhizal relationships (Cervantes and Rodríguez Barrueco, 1992).

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The abilities of different ECM fungi to mobilize inorganic nutrients might be species specific (Lapeyrie et al., 1991). Actinorhizal alders such as *Alnus tenuifolia* and *A. rubra* nodulated by *Frankia* solubilize basalt rocks, thereby releasing base-elements for uptake by plants and rapidly increasing soil organic matter (Li and Borman, 2003; Yamanaka et al., 2003). Ectomycorrhizal fungi are expected to improve nodulation of actinorhizal plants, perhaps by solubilizing rock minerals, and thus improving nutrient acquisition by the plants (Yamanaka et al., 2003). These combined processes may be important for the survival and establishment of these plants in degraded, nutrient-limited soil ecosystems, thereby improving forest productivity (Bormann et al., 1998; Li and Strzelczyk, 2000).

From studies on ECM of alder species in North America, Europe and South America, it is known that ectomycorrhizal symbionts on *Alnus* spp. roots are dominant (Becerra et al., 2002, 2003a,b,c; Miller et al., 1991; Pritsch 1996; Pritsch et al., 1997). *Alnus acuminata* is associated with a number of ECM fungi belonging to the genera *Russula*, *Lactarius*, *Inocybe*, *Laccaria*, *Corinarius*, *Gyrophorus* and *Naucoria* (Singer and Morcillo, 1961; Raithelhuber, 1988; Moscr, 2001).

Mycorrhizae of *Alpova diplophloeus* (Zeller and Dodge) Trappe and Smith and *Alnus* spp. (except *A. acuminata*) have been described in detail (Godbout and Fortin, 1983; Mussicotte et al., 1986; Molina, 1979; Wiedner and Sein-Friet, 2001). *Alpova astrosalnicola* Dominguez is a new fungal species of *Alpova* recently collected and described under *A. acuminata* in the Yungas district of Argentina (Nouhra et al., 2005). The mycorrhizae on *A. acuminata* formed with *Alpova astrosalnicola* were never characterized.

The aims of the present study were: 1) determine the capacity of *Alnus acuminata* to nodulate and to growth in nutrient poor substrates when inoculated with *Frankia* and two ectomycorrhizal fungi under controlled conditions, and 2) characterize the ectomycorrhizae formed by *Alpova astrosalnicola* and *A. diplophloeus* synthesized with *A. acuminata*.

### 2. Materials and Methods

#### Soil preparation

Basalt rocks were collected from La Calera district (31°21′10.3″S, 64°21′26.6″W), Córdoba, Argentina. The rocks were ground and wet sieved to a grain size of between 250 μm to 3 mm. In order to remove fine dust, the minerals were washed in 0.1 M HCl, followed by demineralized water for several days until the effluent reached the pH of the demineralized water. The treatments were pots containing: 1) rocks of 1-3 mm in diameter for L (large size), 2) rocks of 500 μm – 1 mm in diameter for M (medium size), 3) rocks of 250–500 μm in diameter for S (small size), and 4) sterilized vermiculite served as control. Vermiculite is a light, porous silicate mineral that serves as a base medium; it retains moisture and gives an aerated root environment. Fifty grams of each rock particle size were mixed with vermiculite (1:1) and placed into tubes (25 cm deep, 7 cm in diameter), the bottom of which was sealed with a 50 μm mesh nylon screen to prevent the particles of vermiculite and the ground basalt rock from falling out of the tube. The mixed rock-vermiculite substrates as well as pure vermiculite were sterilized by autoclaving for 1 h at 121°C. The chemical composition of basalt rock and vermiculite was analyzed using the Cameca SX50 electron microprobe and are shown in Table 1.

#### Seedlings

Seeds of *Alnus acuminata* were surface-sterilized in 30% H2O2 for 20 min and washed several times with sterilized 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 emerging seedlings were transplanted into the pots described above. Fresh nodules (clusters of modified lateral roots) with *Frankia* were harvested during fall from an *A. acuminata* forest in the Yungas, Argentina. The nodules were kept in a refrigerator for about one month before they were used as an inoculum. About 5 g of root nodules were gently crushed in a mortar and pestle. All treatments and controls were inoculated by pouring 1 ml of a well-fragmented inoculum suspension (approximately 30 mg fresh weight nodules) near the base of the seedlings.

Three weeks after *Frankia* inoculation, the seedlings were inoculated with spores of *Alpova astrosalnicola* or *A. diplophloeus* depending on the treatments. The treatments received dual inoculation with *Frankia* and *A. astrosalnicola* or *Frankia* and *A. diplophloeus*.

#### Table 1. Chemical composition of the basalt-rock and vermiculite (% of dry weight) used as substrates in the experiment.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Basalt-rock</th>
<th>Vermiculite</th>
</tr>
</thead>
<tbody>
<tr>
<td>SiO2</td>
<td>72</td>
<td>33-36.5</td>
</tr>
<tr>
<td>Al2O3</td>
<td>14</td>
<td>11-17</td>
</tr>
<tr>
<td>Fe2O3</td>
<td>3</td>
<td>4-11</td>
</tr>
<tr>
<td>MgO</td>
<td>1</td>
<td>18-27</td>
</tr>
<tr>
<td>Na2O</td>
<td>3</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>K2O</td>
<td>4</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>CaO</td>
<td>1</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>TiO2</td>
<td>0.3</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>MnO</td>
<td>0.05</td>
<td>Not detected</td>
</tr>
<tr>
<td>P2O5</td>
<td>0.16</td>
<td>Not detected</td>
</tr>
</tbody>
</table>

*Mean of 5 samples.*
Table 2. Growth of *Alnus acuminata* inoculated with *Frankia* and *Alpowa australnicola* and grown in soilless substrates.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Height (cm)</th>
<th>Shoot dry weight (g)</th>
<th>Root length (cm)</th>
<th>Root dry weight (g)</th>
<th>Number of nodule lobes / seeding</th>
<th>Nodule lobe dry weight (g)</th>
<th>ECM (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.57 ± 0.42d</td>
<td>0.013 ± 0.02c</td>
<td>17.80 ± 3.20b</td>
<td>0.031 ± 0.037b</td>
<td>3.57 ± 3.17c</td>
<td>5.210⁻⁵ ± 8.08 10⁻⁴c</td>
<td>-</td>
</tr>
<tr>
<td>S</td>
<td>3.76 ± 2.04c</td>
<td>1.05 ± 0.864b</td>
<td>21.43 ± 1.43a</td>
<td>0.42 ± 0.33b</td>
<td>94.05 ± 52.43b</td>
<td>0.08 ± 0.03b</td>
<td>+/-</td>
</tr>
<tr>
<td>M</td>
<td>17.70 ± 1.41b</td>
<td>1.77 ± 0.82b</td>
<td>21.85 ± 0.85a</td>
<td>2.08 ± 0.86a</td>
<td>123.00 ± 41.14b</td>
<td>0.14 ± 0.08b</td>
<td>+/-</td>
</tr>
<tr>
<td>L</td>
<td>18.90 ± 2.49a</td>
<td>2.68 ± 0.41a</td>
<td>22.22 ± 1.12a</td>
<td>2.67 ± 0.61a</td>
<td>178.40 ± 26.41a</td>
<td>0.48 ± 0.08a</td>
<td>+++</td>
</tr>
</tbody>
</table>

Treatments: Control with vermiculite only; small rock particles (S, 250–500 μm); medium-sized rock particles (M, 500 μm – 1 mm); large rock particles (L, 1–3 mm). ECM measured as percentage of colonization on 100 roots (- 0%; +/- <10%; + 10–40%; ++ 40–75%; +++ 75–100%), nodulation measured as number of nodules lobes. Values in each column followed by different letters differ at P<0.05 according to a Tukey test. Values are mean of 10 seedlings ± standard error for each treatment.

Table 3. Growth of *Alnus acuminata* inoculated with *Frankia* and *Alpowa diplophloeus* and grown in soilless substrates.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Height (cm)</th>
<th>Shoot dry weight (g)</th>
<th>Root length (cm)</th>
<th>Root dry weight (g)</th>
<th>Number of nodule lobes / seeding</th>
<th>Nodule lobe dry weight (g)</th>
<th>ECM (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.51 ± 0.29d</td>
<td>5.810⁻³ ± 8.9 10⁻⁴d</td>
<td>12.48 ± 4.58b</td>
<td>0.011 ± 2.10⁻³⁵b</td>
<td>3.52 ± 1.84c</td>
<td>2.10⁻⁴ ± 1.9 10⁻⁴c</td>
<td>-</td>
</tr>
<tr>
<td>S</td>
<td>7.1 ± 3.8c</td>
<td>1.15 ± 0.81c</td>
<td>22.41 ± 1.78a</td>
<td>1.95 ± 1.41a</td>
<td>142.4 ± 55.03b</td>
<td>0.35 ± 0.03b</td>
<td>+</td>
</tr>
<tr>
<td>M</td>
<td>17.4 ± 1.95a</td>
<td>3 ± 0.39a</td>
<td>22.53 ± 0.94a</td>
<td>2.50 ± 1.75a</td>
<td>283.2 ± 95.16a</td>
<td>0.59 ± 0.23a</td>
<td>++</td>
</tr>
<tr>
<td>L</td>
<td>13.24 ± 0.73b</td>
<td>2 ± 0.44b</td>
<td>22.96 ± 2.89a</td>
<td>2.1 ± 0.56a</td>
<td>194.00 ± 40.48b</td>
<td>0.40 ± 0.11b</td>
<td>+++</td>
</tr>
</tbody>
</table>

Treatments: Control with vermiculite only; small rock particles (S, 250–500 μm); medium-sized rock particles (M, 500 μm – 1 mm); large rock particles (L, 1–3 mm). ECM measured as percentage of colonization on 100 roots (- 0%; +/- <10%; + 10–40%; ++ 40–75%; +++ 75–100%), nodulation measured as number of nodules lobes. Values in each column followed by different letters differ at P<0.05 according to a Tukey test. Values are mean of 10 seedlings ± standard error for each treatment.

Sporocarps of *A. australnicola* were collected from an Andean alder forest in the Yungas (Nouhra et al., 2005); sporocarps of *A. diplophloeus* were collected from a red alder (*A. rubra* Bong.) forest near Florence, Oregon, USA. For each fungus, a spore suspension was prepared by homogenizing the sporocarps in distilled water in a Kenwood blender at a high-speed for about 3 min. One ml of spore suspension containing 1 x 10⁸ spores, determined by haemacytometry, was inoculated at the base of the seedlings.

Ten replicates were prepared for each treatment. Seedlings were grown in a greenhouse for 12 months with a 24/16°C (day/night) regime under a 16/8 h photoperiod, and watered every day. Water content of the growth substrates was kept constant by addition of water. No fertilization was provided to the seedlings. The pots from different treatments were systematically rotated to different bench positions once a week to minimize differences due to the location in the greenhouse.

**Data collection**

The formation of ECM was observed with a stereomicroscope. Description of the ECM followed the methodology of Agerer (1991, 1999), for describing color, mantle layers, branching pattern, emanating hyphae, characteristics of rhizomorphs and cystidia. Mantle views were examined and photographed with a Zeiss Axioshot light microscope at 200–1,000x magnification. Characterization of the Hartig net followed the nomenclature by Godbout and Fortin (1983). The quantification of the ECM colonization was estimated visually and characterized using five classes of mycorrhizal root colonization: - (0%); +/- (<10%); + (10–40%); ++ (40–75%); +++ (75–100%) (Schenk, 1982).

Seedling growth was measured as shoot height, root length (main root and first lateral roots) and as shoot dry weight and root dry weight after drying at 50°C to a constant weight. The capacity for nodulation was measured as number of nodule lobes and dry weights of nodule lobes after drying as above.

**Data analyses**

The variables shoot height, root length, number of nodule lobes and dry weights of shoot, roots and nodule lobes were analyzed by two-way analysis of variance and contrast test (Tukey, P<0.05) to determine the significance of mean differences between treatments of basal-rock and inoculation. Statistical analyses were performed with Infostat (2001).
3. Results

Growth

*Alnus acuminata* seedlings with dual *Frankia-Alpova austroalnicola* or *Frankia-A. diplophloeus* inoculation in vermiculite-basalt growth medium had significantly higher shoot height, dry weights of shoot and nodule lobes, and number of nodule lobes than the seedlings in vermiculite only (Fig. 1A, B; Tables 2 and 3). With *Frankia* and *A. austroalnicola* inoculations, the seedlings cultivated in vermiculite mixed with large basalt particles had significantly higher shoot height, shoot dry weight, number of nodule lobes, and nodule lobe dry weight than in the treatments with small or medium-sized basalt particles (Fig. 1A, Table 2). However, *A. acuminata* seedlings inoculated with *Frankia* and *Alpova diplophloeus* and grown in the medium-sized basalt particles mixed with vermiculite produced significantly higher shoot dry weight, number of nodule lobes, and nodule lobes dry weight than in the treatments with small or large basalt particles (Fig. 1B, Table 3).

A very low nodulation was observed in controls grown in vermiculite only as compared to all other treatments. This could be due to available phosphorus released from breakdown of basalt, by not from vermiculite (Table 1), by Andean alder.

**Ectomycorrhizal formation**

Ectomycorrhizal formation by *Alpova austroalnicola* and *A. diplophloeus* on *Alnus acuminata* were observed in all treatments except in the control. Abundant ECM in the mixture of large basalt particles and vermiculite were observed with both fungi (Tables 2 and 3; Fig. 2A and 3A). No other ECM types were seen on the plants.

**Description of the morphotypes**

*Alpova austroalnicola-Alnus acuminata.* Monopodial pinnate to irregular pinnate (Fig. 2A), root tips straight, bent to tortuous, up to 10 mm long and 0.3–0.6 mm in diameter, yellowish brown to brown. Root tips are acute to cylindrical. Emanating hyphae: abundant, irregularly sinuous, membrane-like yellow to brown, branched, thin walled, 2–5 μm in diameter, regularly septate, clamped, surface rough with crystals; hyphal ends simple or inflated (Fig. 2B). Hyphae with simple anastomoses without septa (Agerer 1991, Type A). Rhizomorphs: hyaline to pale yellow, straight, up to 20 μm in diameter (Fig. 2C). Mycorrhiza mantle (plane view): mantle usually continuous over the root apex. Outer mantle layer plecithrophalous (Fig. 2D), with bundles of hyphae growing in parallel and forming a distinct ring-like pattern (Agerer and Rambold, 1998); surface rough, with few mineral particles and isodiametric to elliptic crystals; hyphae membrane-like yellowish, thin-walled, septa always with clamps, hyphae forming terminally globose inflations, 3–6 μm in diameter. Middle mantle layer plecithrophalous (Fig. 2E), hyphae arranged without pattern, 2–4 μm in diameter, cell walls thin. Inner mantle layer plecithrophalous (Fig. 2F), parallel hyphae running in parallel to root axis, 2–4 μm in diameter, clamps not observed. Mycorrhiza (cross section): mantle 50–90 μm thick (Fig. 2G), differentiated into a plecithrophalous outer layer of tangentially arranged hyphae 2–7 μm in diameter, a middle plecithrophalous layer of globose cells 2–5 μm in diameter, and a plecithrophalous inner layer of globose cells 1.2–5 μm in diameter; parapidermical Hartig net, hyphae lined up in one row between epidermal cells 2–8 μm in diameter. Voucher specimen: ectomycorrhizas under *A. acuminata,* in herbarium A. Becerra AB 21 (CORD).

*Alpova diplophloeus-Alnus acuminata.* Monopodial pinnate (Fig. 3A), root tips straight, bent to tortuous, up to 15 mm long and 0.4–0.9 mm in diameter, yellowish brown to brown; older mycorrhizae brown to dark brown. Root tips are cylindrical. Emanating hyphae: abundant,
irregularly sinuous, membranaceous yellow to brown, branched, thin walled, 2–6 μm in diameter, regularly septate, clamped, surface rough with crystals (Fig. 3B), hyphal ends simple or inflated (Fig. 3C). Hyphae with simple anastomoses without septa (Agerer, 1991, Type A). Rhizomorphs: abundant, pale yellow, straight, up to 60 μm in diameter (Fig. 3D). Mycorrhiza mantle (plane view): mantle usually continuous over the root apex. Plecenchymatous outer mantle layer (Fig. 3E), membranaceous yellowish; surface rough, with few mineral particles and isodiametric to elliptic crystals; with bundles of hyphae growing in parallel and forming a distinct ring-like pattern (Agerer and Rambold, 1998), thin-walled, clamped septa, hyphae forming terminally globular inflations, 3–9 μm in diameter. Middle mantle layer plecenchymatous (Fig. 3F), hyphae arranged without pattern, 2–5 μm in diameter, cell walls thin. Inner mantle layer plecenchymatous (Fig. 3G), parallel hyphae running in parallel to root axis, 2–4 μm in diameter, clamps not observed. Mycorrhiza (cross section): mantle 40–90 μm thick (Fig. 3H), differentiated into a plecenchymatous outer layer of tangentially arranged hyphae 2–10 μm in diameter, a middle plecenchymatous layer of globose cells 2–7 μm in diameter, and a plecenchymatous inner layer of globose cells 2–7 μm in diameter; parapapillar Hartig net, hyphae lined up in one row between epidermal cells 2–8 μm in diameter. Voucher specimen: ectomycorrhizas under *A. acuminata*, in herbarium A. Becerra AB 22 (CORD).
4. Discussion

The present study showed that *Alnus acuminata* grew, nodulated and formed ectomycorrhizas in soilless growth medium of vermiculite and ground basalt rocks. *Alnus acuminata* seedlings grew better in pots with large or medium-sized basalt particles than in pots with small particles. On the other hand, seedling growth of other *Alnus* species was significantly better in pots containing small particles size (0.2–0.6 mm) of basalt rock (Li and Bormann, 2003; Yamanaka et al., 2003). The potting mixtures used in the present study was basalt rock and vermiculite while Li and Bormann (2003) and Yamanaka et al. (2003) used basalt rock and perlite. Vermiculite is essentially a member of the phyllosilicate group of minerals, resembling mica in appearance. Mycorrhizal fungi or mycorrhizas are silicate-solubilizers, involved in nutrient cycling from relatively insoluble minerals (Luyvai et al., 1990; Watteau and Berthelin, 1990; Paris et al., 1995, 1996; Wallander et al., 2006; Balogh-Brunstad et al., 2008) due to their ability to release siderophores, oxalic acid and other non-volatile organic acids, which could enhance nutrient availability through solubilization and chelation of exchangeable or stable mineral resources. In this study, *Alnus acuminata* mycorrhizal plants were able to grow in a basalt rock-vermiculite mixture. The nutrients released from both primary minerals in pots of basalt-vermiculite mixture would enhance more seedling growth than the seedlings grown in pots with vermiculite alone. The vermiculite effects could shift preference of *A. acuminata* to large or medium basalt particles sizes, inducing a rapid and deep depletion of K in basalt and releasing other nutrients (Hinsinger et al., 1992).

Ectomycorrhizal formation by *Alpova australchnicola* or *A. diplophloeus* were abundant in the large basalt particle treatment when inoculated together with *Frankia* and resulted in larger and healthier *Alnus acuminata* plants (Tables 2 and 3). This result is in accordance with the findings by Yamanaka et al. (2003).

In natural environments *Alnus acuminata* can reach a height of 25 m (average 10–15 m) with a stem diameter at breast height of 45 cm. Its annual height growth averages 1 m (Aceñolaza, 1995). Meanwhile, under laboratory conditions and in soilless substrates, *A. acuminata* seedlings grew up to 20 cm in a year. This is in agreement with other reports (Balza Viloria, 1959; Rojas et al., 1978).

The results of more nodule lobes in the bigger seedlings are in accordance with the findings by Rojas et al. (2001, 2002) in *A. rubra* and *Ceanothus velutinus* Dougl, which showed a positive correlation between seedling biomass and nodule biomass. One possible explanation could be the presence of phosphorus in the basalt-rock substrates, although in low amounts (Table 1). It has been shown that severe phosphate deficiency markedly impairs both host plant growth and symbiotic nitrogen fixation (Israel, 1987). In this study, an increase of nodule lobes was observed in taller seedlings (which also showed longer and heavier roots). Another possible explanation for the results obtained is simply that N$_2$ fixation in the root nodules could be the only N source and that nodule formation and plant growth are interrelated.

*Alnus acuminata* formed ectomycorrhizas with both *Alpova australchnicola* and *A. diplophloeus*. In general, the *A. australchnicola* ectomycorrhiza showed similar features to that of *A. diplophloeus*. The most conspicuous differences between the two were the mycorrhizal system and the differentiated rhizomorphs. In this study, the mantle and emanating hyphae of both ectomycorrhizae contained crystals. In contrast, Wiedmer and Senn-Irlelt (2001) did not observe crystals in the mantle and emanating hyphae of *A. diplophloeus* ectomycorrhiza in *Alnus viridis*. *Alpova australchnicola* and *A. diplophloeus* have earlier been found to be associated with *Alnus acuminata* in the Costa Rica and Yunga forests (Halling and Mueller, 2004; Nohra et al., 2005) while the *Alpova australchnicola-Alnus acuminata* mycorrhizas are characterized for the first time in this study. As far as we know, *Alpova diplophloeus-Alnus acuminata* ectomycorrhizae have not been described (De Román et al., 2005) until this study. Their morphological characteristics were relatively similar to the ectomycorrhizae on *A. acuminata* and other *Alnus* species formed with *A. diplophloeus* (Brunner et al., 1990; Nohra et al., 2003; Wiedmer and Senn-Irlelt, 2001).

The results presented in this study showed that actinorhizal *Alnus acuminata* is able to grow in primary mineral substrates, indicating its capacity to breakdown primary minerals such as basalt rocks, thereby releasing nutrients available for uptake by the plant. It thus is able to survive and establish on highly degraded, nutrient-poor soil ecosystems (Li and Strzelczyk, 2000; Yamanaka et al., 2003). Its association with symbiotic rhizosphere microbes such as *Frankia* and mycorrhizal fungi can therefore have significant impact on belowground terrestrial processes involved in mobility and cycling of nutrients in nutrient-limited forest ecosystems. The presence of actinorhizal and mycorrhizal plants such as *A. acuminata* can, therefore, have significant impacts on soil fertility and productivity of forest ecosystems.

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