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ORIGINAL ARTICLE

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Growth, nitrogen fixation and mineral acquisition of *Alnus sieboldiana* after inoculation of *Frankia* together with *Gigaspora margarita* and *Pseudomonas putida*

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Abstract The role of tetrapartite associations among *Frankia*, *Gigaspora margarita* (an arbuscular mycorrhizal fungus), *Pseudomonas putida* (rhizobacterium), and *Alnus sieboldiana* in growth, nitrogen fixation, and mineral acquisition of *A. sieboldiana* was investigated. Seedlings of *A. sieboldiana* were inoculated with *Frankia* isolated from root nodules of alder, followed by inoculation of *G. margarita* and *P. putida*, and were grown for 5 months in a greenhouse. The seedlings inoculated with *Frankia* and *G. margarita* together produced the highest biomass of shoots and root nodules. Nitrogen-fixation activity, measured by acetylene reduction assay, was observed when *Frankia* was inoculated. The activity, on a per-nodule gram basis, decreased after *G. margarita* inoculation, but on a per-plant basis there was no significant difference in the activity among inoculation treatments. The mineral content in the seedlings changed after inoculation with *Frankia*, but not after inoculation with *P. putida* and/or *G. margarita*. The results showed a synergistic interaction among *Frankia*, the mycorrhizal fungus, and the rhizobacterium on the growth of *A. sieboldiana*.

Key words *Alnus* · *Frankia* · *Gigaspora* · *Pseudomonas* · Tetrapartite symbiosis

Introduction

Some dicotyledonous plants fix atmospheric nitrogen in root nodules symbiotically formed by an actinomycete, *Frankia*. These plants are called actinorhizal plants (Baker and Schwintzer 1990). Five genera of actinorhizal plants (*Alnus*, *Coriaria*, *Elaeagnus*, *Myrica*, and *Dryas*) are native to Japan. Because of their capacity for nitrogen fixation, nodulated species can grow and improve soil fertility in disturbed sites (Hibbs and Cromack 1990; Wheeler and Miller 1990). Actinorhizal plants also form a symbiotic association with specialized soil fungi (mycorrhizal fungi). Mycorrhizal fungi spread their vegetative hyphae intensively into soil, and improve nutrition of the plants by their ability to take up nutrients and water in soil (Smith et al. 2003). Thus, formation of a multipartite symbiosis among actinorhizal plants, *Frankia*, and mycorrhizal fungi could increase plant survival under poor soil conditions (Rose and Youngberg 1981; Chatarpaul et al. 1989; Yamanaka et al. 2003). In addition to mycorrhizal fungi, a variety of soil microorganisms colonize the rhizosphere of actinorhizal plants. Some actinomycetes isolated from the surface of nodules and roots of red alder (*A. rubra*) or its surrounding soil, affected the growth, nodulation and nitrogen-fixing activity of seedlings of red alder (Rojas et al. 1992). *Pseudomonas cepacia*, a root-associated microbe, improved the nodulation of red alder by root-hair deformation (Knowlton and Dawson 1983). In addition, *Pseudomonas* spp. are able to solubilize rock minerals such as iron and phosphate (Bar-Ness et al. 1992; Li and Strzelczyk 2000), rendering nutrients available for uptake by plants.

Alnus sieboldiana Matsumura is a warm temperate actinorhizal plant distributed in upland sites along the Pacific coast in central Japan (Kitamura and Murata 1979). This tree has been harvested as fuelwood and a food source for domestic stock (Kitamura and Murata 1979); its strobili (seed catkins) are used to make a dye. *Alnus sieboldiana* grows well on nutrient-poor sites through its ability to participate in both mycorrhizal and actinorhizal symbiosis (Yamanaka and Okabe 2003), and therefore has been used widely for land stabilization in Japan. Thus, this tree is an important species in land reclamation, erosion control, and sand stabilization (Murai 1963).

The objective of this study was to determine whether the growth, nodulation, and nitrogen fixation of *A. sieboldiana*

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could be enhanced by inoculation of *Glomus margarita* (an arbuscular mycorrhizal (AM) fungus) and *P. putida* (a rhizobacterium), together with *Frankia*, in pot cultures under greenhouse conditions.

Materials and methods

Plant, microorganisms and inoculation procedures

Seeds of *A. sieboldiana* were soaked in running water for several days, and surface-sterilized in 95% ethanol for 1 min, then in 1% sodium hypochlorite solution for 5 min. They were then rinsed five times in sterilized distilled water and placed aseptically in Petri dishes, which contained 0.9% water agar medium. The Petri dishes were sealed with Parafilm and placed in a growth chamber maintained at 28°C under continuous light of 10000 lux. After germination, uncontaminated seedlings were transplanted onto sterilized growth medium of a mixture of volcano ash–diatomaceous earth (3:2 (v:v)) in a Ray Leach tube (164 ml, SC-10, Stuewe & Sons, Oregon, USA), and cultivated in a greenhouse.

The *Frankia* isolate (AS-2) used in the present study was obtained from root nodules of *A. sieboldiana*. The isolate was cultured in an N-free BAP liquid medium (Murry et al. 1984) in darkness at 24°C for 4–6 weeks. *Frankia* cultures were homogenized (10000 rpm, 10 s) with an Ultra-Turrax (TP 18/10S4, IKA, Staufen, Germany), and washed with sterilized distilled water three times. One milliliter of inoculum suspension of *Frankia*, equivalent to 0.01 ml packed cell volume (2320 *g*, 20 min), was poured near the base of 6-week old seedling of *A. sieboldiana*.

Two weeks after *Frankia* inoculation, *G. margarita* and *P. putida* were inoculated. *Gigaspora margarita* were isolated by wet-sieving and decanting (Gerdemann and Nicolson 1963) from a natural soil at the Forestry and Forest Products Research Institute, Tsukuba, Japan, and maintained in pot cultures containing a mixture of volcano ash–diatomaceous earth (3:2 (v:v)) where alfalfa (*Medicago sativa*) was the host plant. Twenty spores of *G. margarita* for each seedling were placed near the seedling about 1 cm below the soil surface. *Pseudomonas putida* (SC-83) was isolated from scoriae accumulated in Miyake-jima, Tokyo, Japan (34°03′N, 139°30′E) (Yamanaka and Okabe 2003), by taking a colony producing fluorescent substances observed under UV light radiation on King's B medium (King et al. 1954), and identified using Biolog plates (Biolog, California, USA) for analyzing carbon substrate utilization. We observed that this isolate had the potential to solubilize insoluble phosphate by formation of a clear zone around a colony growing on agar medium containing insoluble phosphate (Henderson and Duff 1963). The isolate was cultured on Tryptic Soy Agar (Difco, Michigan, USA) for 2 days, and bacterial cells were harvested from a colony on this medium. The cells were suspended in sterilized distilled water and centrifuged. One milliliter of bacterial suspension adjusted to 1.8×10^7 cells, determined by counting the cell after staining with 4′.6-diamino-2-phenyl indole (DAPI, Sigma, Missouri, USA), was inoculated at the base of the seedling.

Twelve replicates of seedling were prepared for each inoculation treatment. Seedlings were grown in the greenhouse for 5 months (June to November) and watered every other day.

Measurement

Nitrogen fixation of *A. sieboldiana* was measured using the acetylene reduction (AR) technique. The excised roots of each seedling were rinsed in distilled water and placed into a 23-ml test tube. After the tube was sealed with a silicon cap, acetylene gas was injected into the tube through a plastic syringe to constitute 10% of the total gas volume. After 5 h incubation at 30°C, a 0.2-ml gaseous sample from each tube was taken and analyzed for C_2H_2 and C_2H_4 with a gas chromatograph (GC-14B, Shimadzu, Kyoto, Japan) equipped with a flame-ionization detector (FID) and a 2 m × 2.1 mm stainless steel column packed with Porapak N (Shimadzu GLC, Tokyo, Japan). The oven temperature was adjusted at 70°C; injection and FID temperatures were adjusted at 200°C. The nitrogen carrier gas flow rate was adjusted to 40 ml/min. The same sample without acetylene addition was prepared as a control to check endogenous ethylene production.

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

The chemical composition of *A. sieboldiana* shoot was analyzed using seven replicates for each inoculation combination. Shoot samples, obtained from the seedlings without *Frankia* inoculation, were combined together for chemical analysis, because sufficient materials could not be obtained, due to the poor growth of *A. sieboldiana* seedlings without *Frankia*. The dried shoot was ground in a mill. Total carbon and total nitrogen in the ground samples were determined using automatic gas chromatography (C-N Corder; Yanagimoto, Kyoto, Japan). Mineral composition was measured by inductively coupled plasma (ICP) emission spectroscopy after wet acid digestion of a 0.05-g sample in 2.5 ml conc $HClO_4$ and 5.0 ml conc HNO_3 with 2.5 ml distilled water.

Data analyses

In the present study, *Frankia*, *G. margarita*, and *P. putida* were inoculated in combination or alone. A three-way ANOVA was used to examine the effects of these inoculation treatments on the growth of *A. sieboldiana*. Nodulation and AR activity were observed only after *Frankia* inoculation, and the effects of *G. margarita* and *P. putida* inoculation on these data and mineral composition of *Frankia*-inoculated *A. sieboldiana* shoots were analyzed by two-way ANOVA. A Mann-Whitney *U*-test was used to examine the effects of *Frankia* inoculation on mineral com-

position of 32 samples (28 samples of *Frankia*-inoculated *A. sieboldiana* and four uninoculated *A. sieboldiana*).

Results

Growth

Frankia inoculation resulted in significantly higher biomass production of *A. sieboldiana* than without *Frankia* inoculation; dry mass of shoots and roots was significantly higher when *G. margarita* was inoculated (Tables 1 and 2). Root nodules were formed in the seedlings inoculated with *Frankia*, and their dry mass was significantly higher when *G. margarita* was inoculated as well. Interactions of the two factors (*Frankia* inoculation and *Gigaspora* inoculation) were observed in the dry mass of *A. sieboldiana* seedlings. *Pseudomonas putida* inoculation did not change biomass production of *A. sieboldiana*.

Nitrogen fixation

Acetylene reduction (AR) activity, an indirect measure of the ability of nodules to fix atmospheric nitrogen, was observed when the seedlings were inoculated with *Frankia* (Table 1). The AR rate, on a per-nodule gram basis, was significantly lower in the nodulated seedlings inoculated with *G. margarita* than those without *G. margarita* (Tables 1 and 2). On a per-plant basis, however, there was no significant difference in AR rate among inoculation combinations.

Mineral composition of the shoot of *A. sieboldiana* seedlings

Frankia inoculation increased the content of N and K, and decreased Zn, Mg, and Ca contents in the shoot of *A. sieboldiana* (Table 3; Mann-Whitney *U*-test, $P < 0.05$). Inoculation of *G. margarita* and/or *P. putida* did not affect the mineral concentrations of the shoot (Table 3; two-way ANOVA, $P < 0.05$).

Discussion

Several investigators have reported that dual inoculation with *Frankia* and AM fungi resulted in a better growth of actinorhizal plants, compared with single inoculation with *Frankia* (Rose and Youngberg 1981; Jha et al. 1993; Tian et al. 2002). The results of the present study (Tables 1 and 2) agreed with those reports. Arbuscular mycorrhizal fungi improve the nutrient balance of the host through P supply by hyphal extension and ramifications in the soil (Sanders et al. 1977) and, in concert with *Frankia*, stimulate the growth, nodulation, and nitrogen-fixing activity of actinorhizal plants (Rose and Youngberg 1981). In the present study, however, *G. margarita* did not improve nitrogen-fixing activity or P nutrition of nodulated *A. sieboldiana* (Tables 1

Table 1. Effect of *Frankia*, *Gigaspora margarita* and *Pseudomonas putida* on growth, nitrogen fixation of *Alnus sieboldiana*

Inoculation type	Shoot		Root Dry weight (mg)	S/R	Nodule		Nitrogen fixation	
	Height (mm)	Dry weight (mg)			Number of lobe	Dry weight (mg)	(μmol C_2H_4 /g nodule/h)	(μmol C_2H_4 /plant/day)
No *Frankia*								
Control	14.0	30	51	0.59	0	0	0	0
+*P. putida*	15.2	35	53	0.69	0	0	0	0
+*G. margarita*	17.4	41	56	0.75	0	0	0	0
+*P. putida* and *G. margarita*	14.6	37	52	0.73	0	0	0	0
Frankia								
Control	15.1	120	105	1.16	14.3	5.0	23.1	2.6
+*P. putida*	17.3	105	89	1.23	14.9	4.0	30.0	2.3
+*G. margarita*	19.5	141	120	1.18	17.0	5.8	16.0	2.0
+*P. putida* and *G. margarita*	17.9	150	116	1.32	18.7	6.3	14.4	2.0

Table 2. Results of ANOVA with corresponding *F*-ratio

Inoculation type	Shoot		Root Dry weight	S/R	Nodule		Nitrogen fixation	
	Height	Dry weight			Number of lobe	Dry	(per nodule weight	(per plant) weight)
Frankia	4.68*	305.4***	126.9***	213.5***				
P. putida	0.06	0.05	1.40	3.98*	0.23	0.18	0.50	0.15
G. margarita	3.83	13.7***	5.26*	4.74*	1.79	6.62*	9.13**	1.91
Frankia × *P. putida*	0.34	0.11	1.00	0.89				
Frankia × *G. margarita*	0.29	6.11*	4.02*	0.49				
P. putida × *G. margarita*	3.83	0.49	0.10	0.09	0.04	1.71	1.30	0.19
Frankia × *P. putida* × *G. margarita*	0.002	2.43	0.80	1.96				

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Table 3. Nutrient content (mg/g) of shoot of *Alnus sieboldiana* after inoculation of *Frankia*, *Gigaspora margarita* and *Pseudomonas putida*

Inoculation type	C (%)	N (%)	P (%)	Ca (%)	K (%)	Mg (%)	Mn (%)	Na (ppm)	Al (ppm)	Fe (ppm)	Zn (ppm)
No *Frankia**											
Control	45.7	0.71	0.12	2.75	0.77	0.45	0.23	738	155	72	51
+*P. putida*	45.1	0.68	0.08	2.76	0.73	0.52	0.29	848	173	107	77
+*G. margarita*	44.8	0.66	0.07	2.85	0.80	0.47	0.25	584	136	84	55
+*P. putida* and *G. margarita*	45.0	0.70	0.07	2.71	0.83	0.43	0.25	884	167	127	54
Frankia											
Control	50.4	1.92	0.08	1.69	1.20	0.23	0.23	860	172	127	36
+*P. putida*	45.6	1.87	0.09	1.77	1.30	0.29	0.25	910	211	121	41
+*G. margarita*	47.3	1.75	0.09	1.66	1.17	0.25	0.20	712	233	147	49
+*P. putida* and *G. margarita*	46.3	1.67	0.09	1.81	1.14	0.26	0.23	783	210	151	33

*Sufficient samples for analysis of nutrient content were obtained only in 4 treatments of *Frankia* inoculation. Without *Frankia* inoculation, treatment replicates were combined and analyzed

and 3). Improved growth of *A. sieboldiana* after AM infection may result from plant hormones produced by AM fungi (Barea and Azcón-Aguilar 1982) or induced by AM fungi (Allen et al. 1982).

Arbuscular mycorrhizal fungi do not solubilize P (Mosse et al. 1976), and inoculation of phosphorus-solubilizing bacteria (PSB) alone or with AM fungi should promote the growth of nodulated plants. We observed that *P. putida* used in the present study is capable of solubilizing P as well as producing siderophores (Watteau and Berthelin 1994). Phosphorus is well known as being one of the most important minerals for growth and nodulation of actinorhizal plants (Huss-Danell 1997) as well as legumes. Iron is essential for nitrogen-fixing microorganisms as a component in the Fe- and MoFe-proteins of nitrogenase and in the haem groups of cytochromes. Inoculation of this *Pseudomonas* was, therefore, expected to enhance the growth of the actinorhizal plants by improved availability of minerals in their rhizosphere.

In the present study, *P. putida* inoculation did not affect the growth or nitrogen fixation of the nodulated *A. sieboldiana* (Tables 1 and 2). Rojas et al. (2002) reported that fluorescent *Pseudomonas* did not affect the nitrogen fixation or plant biomass of red alder and snowbrush (*Ceanothus velutinus*). Meyer and Linderman (1986a) reported the effect of dual inoculation of AM fungi and *P. putida* on the growth and nodulation of subterranean clover (*Trifolium subterraneum*) in non-sterile soil. Shoot growth and nodulation of plants with AM fungi and *P. putida* were greater than with *P. putida* alone, AM fungi alone, or uninoculated controls. Grimes and Mount (1984) reported that dual inoculation of *Rhizobium phaseoli* and *P. putida* increased nodulation of *Phaseolus vulgaris* compared with *R. phaseoli* alone. Thus, fluorescent pseudomonads can stimulate or have no effect the growth of its associated plants. Meyer and Linderman (1986a) emphasized that pseudomonads in the rhizosphere are a diverse group of bacteria, and their interactions in the rhizosphere with plants and with mycorrhizal fungi would differ between strains of pseudomonads. Further investigations on the distribution of the pseudomonads in the rhizosphere of the plant and their physiological characteristics are needed to clarify the effects of the pseudomonads on plant growth.

Pseudomonas putida alone did not improve the growth of nodulated *A. sieboldiana*, but *P. putida* together with *G. margarita* resulted in better growth of *A. sieboldiana* (Tables 1 and 2). The interaction of *P. putida* with *G. margarita* and *A. sieboldiana* might be explained as follows. Pseudomonads in the rhizoplane might compete with plant roots for nutrients. However, pseudomonads can increase the susceptibility to AM infection by changing the cell-wall plasticity of the root (Mosse 1962), producing a plant growth regulator (Azcón et al. 1978), or removing pathogenic microorganisms (Howell and Stipanovic 1980). Co-inoculation of pseudomonads and AM fungi likely results in more rapid formation of mycorrhizae, thereby increasing the physiological benefit of mycorrhizal formation.

Mineral composition of *A. sieboldiana* is affected by *Frankia* inoculation (Table 3). The increased content of N produced by *Frankia* may affect the contents of other minerals in *A. sieboldiana*. Decreases in Zn, Mg, and Ca contents and an increase in K content were observed with increasing N content. Prégent and Camiré (1985) reported the same tendency in the mineral composition of *Alnus* spp. In their study, decreases in concentration of Ca and Mg with increasing N and P concentration were observed in *A. crispa* and *A. glutinosa* cultivated under various nutrient conditions. Improved plant biomass of nodulated plants may result in a dilution effect of Ca and Mg, as reported by Prégent and Camiré (1985). Effects of mineral concentration on nodulation and nitrogen fixation of nodulated actinorhizal plants have been focused on N and P (Huss-Danell 1997), while the effects of Fe, Mo, Co, Ca, and Na have also been studied.

Gigaspora margarita and *P. putida* did not change the P concentration of nodulated *A. sieboldiana* (Table 3). Plants inoculated with PSB are expected to improve P concentration in the plants by increasing the soluble P in soil; however, this effect appears to be variable. Barea et al. (1975) showed that maize (*Zea mays*) inoculated with both AM fungi and PSB took up more P than those with AM fungi or bacteria separately, although this effect was statistically significant only in one out of two soil types. Meyer and Linderman (1986a) also reported that P concentrations in subterranean clover inoculated with AM fungi and PSB were slightly higher than those inoculated with AM fungi or

PSB alone, although these data were not statistically analyzed. Azcón et al. (1976) explained that the improved P uptake of mycorrhizal plants must have resulted from increased accessibility of a sparingly soluble form of P to the plant by mycorrhizal association, and was not directly related to phosphate solubilization by PSB. Phosphate-solubilizing bacteria cannot transfer soluble P into the roots as mycorrhizal fungi can. Therefore, association of PSB with AM fungal hyphae in soil may take this into consideration. Barea et al. (1975) showed that inoculation with AM fungi favored the early maintenance of PSB introduced around the roots of maize. Meyer and Linderman (1986b) reported that fluorescent pseudomonads increased in the rhizoplane of sweetcorn and subterranean clover with AM fungi compared with that without AM fungi. On the other hand, rhizosphere soil around these mycorrhizal plants had fewer fluorescent pseudomonads than that around non-mycorrhizal plants. Similarly in the symbiosis in actinorhizal plants, the effect of colonization of AM fungi on growth of *P. putida* should also be studied.

The present study did not show additive or synergistic effects of rhizobacterium on multipartite symbiosis among this alder, *Frankia*, and AM fungi. However, extrapolation from our data to other plants or other microorganisms may not be possible without more extensive research. Additional work with the multipartite symbiosis in various environmental conditions will be necessary to better understand the role of multipartite symbiosis in nutrient cycling and productivity of forest ecosystems.

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