INTERACTION BETWEEN ACTINORRHIZAL AND MYCORRHIZAL ASSOCIATIONS ON *Alnus acuminata* ssp. *glabrata*

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ABSTRACT

Actinorrhizal and mycorrhizal symbiosis was studied in *Alnus acuminata* ssp *glabrata* growing under greenhouse conditions. In their natural habitat root nodules and both types of mycorrhizae are common in all alder plants. However, when inoculated in the greenhouse, nodules were observed only in 3 treatments out of 4 inoculated with the nitrogen-fixing *Frankia*. Alder plants were successfully inoculated with the endomycorrhizal fungus *Glomus versiforme* and the ectomycorrhizal *Pisolithus tinctorius*. After 18 weeks, inoculated plants with the combination *Frankia+P. tinctorius*, showed shoot volume and dry mass approximately 6 and 2 times greater respectively than those from the control treatment; nodule numbers increased 2.5 times in relation to the *Frankia* treatment. The same measured variables when plants were inoculated with *Frankia+G.versiforme* were approximately 4.5 and 2 times greater than the uninoculated plants. Number of nodules did not increase with this combination of microorganisms. No relationship between the number of nodules produced and the shoot dry mass was found. This study shows the potential of the tripartite association, either *P. tinctorius* or *G. versiforme* combined with the native *Frankia* 1801H on *A. acuminata* plants, resulting in a strong interrelationship leading to a greater host plant biomass yield.

Key words: alders, actinorrhizal, mycorrhizal, *Frankia*, host plant biomass

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RESUMEN

Las simbiosis tanto actinorrícica como micorrícica del aile, *Alnus acuminata* ssp *glabrata* fue estudiada en condiciones de invernadero. En su hábitat natural, este árbol muestra usualmente en sus raíces tanto nódulos fijadores de nitrógeno (formados por *Frankia*), como ectomicorriza y endomicorriza. Sin embargo, cuando se inoculó en el invernadero con la misma bacteria, se formaron nódulos sólo en 3 de los 4 tratamientos ensayados. La inoculación con hongos micorrícicos se logró exitosamente, tanto con el hongo endomicorrícico *Glomus versiforme*, como con el ectomicorríocico *Pisolithus tinctorius*. Después de 18 semanas, las plantas tratadas con la combinación *Frankia-P. tinctorius*, mostraron incrementos en el volumen y peso seco de la parte aérea de las plantas de 6 y 2 veces más, respectivamente, a las plantas del testigo; con este mismo tratamiento se incrementó en 2.5 veces el número de nódulos, respecto a las plantas tratadas solo con la bacteria. Las mismas variables se incrementaron aproximádamente 4.5 y 2 veces más con la combinación de microorganismos *Frankia-G. versiforme*; con este tratamiento no se incrementó el número de nódulos. No se encontró relación entre el número de nódulos y la biomasa de la parte aéreade las plantas. Este estudio muestra el potencial de la asociación tripartita entre *Alnus acuminata* y *Frankia* más un hongo micorrícico; interelación conducente a una mayor producción del árbol.

Palabras clave: ailes, actinorriza, micorriza, *Frankia*, biomasa de la planta hospedera

INTRODUCTION

The actinorrhizal plants are woody angiosperms that usually inhabit soils with marginal fertility. The ecological significance of the use of these plants have a great potential for reforestation of extreme environments of different ecosystems due to their ability to grow in very diverse habitats and sites of different geographical origin (Baker and Schwintzer, 1990)[2].

The genus *Alnus*, among the actinorhizal group of plants, is by far the most intensively studied. Species of this genus are reported to be responsible for amounts of N accretion ranging from 40 to 360 kg ha^{-1} $year^{-1}$ in North American ecosystems (Tarrant, 1983)[3], to stimulate the growth of intercropped species (Dawson, 1986)[4], and to improve soil N mineralization (Paschke *et al.*, 1989)[5]. Its use as a nitrogen-fixing woody nurse crop has

[2] Baker, D. D. and C. R. Schwintzer. 1990. Introduction. *In:* The Biology of *Frankia* and Actinorhizal Plants. pp 3-11.

[3] Tarrant, R. F. 1983. Nitrogen fixation in North American forestry: Research and Application. *In:* Biological Nitrogen Fixation in Forest Ecosystems: Foundations and Applications. pp 261-277.

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shown to be highly beneficial apart of improving nitrogen fertility; some of its additional benefits are: wind protection, weed control, faster height growth in crop trees, and also represents an additional source of wood fiber (Van Sambeek *et al.*, 1985)[6].

Some species of alders are native to Mexico (Furlow, 1979 a, b)[7,8], however, no reports on its biology or economical importance in this country are yet available. In other words, this tree is one more of the group of unexploited actinorhizal trees (Baker, 1990)[9]. This tree, known by its native name: *elite* or *ilite*, is or was intensively used in agroforestry practices by the inhabitant groups of the Valley of Mexico City many years ago (Anaya *et al.*, 1987)[10]. These practices are still observed among a few farmers in the State of Tlaxcala.

Alnus species form different root symbiosis. Its ability to meliorate the soil N fertility is related to the root-nodule nitrogen-fixing endosymbiont, *Frankia*. The root of alders, as well as other actinorhizal species, have been found to support ectomycorrhizae and/or endomycorrhizae, in addition to the nitrogen-fixing nodules (Trappe, 1979[11]; Rose and Youngberg, 1981[12]; Cruz-Cisneros and Valdés, 1990[13]). In reviewing Daft *et al.* (1985)[14] the symbiosis of actinorrhizal plants with endomycorrhizae concluded that arbuscular mycorrhizae enhance nodulation and nitrogen fixation as well as plant growth and phosphate uptake. In relation to the benefit of ectomycorrhizae, it was found that nodulated *A.viridis* absorbed phosphorous five times more quickly than nodulated roots only (Mejstrik and Benecke, 1969)[15]. Fraga-Beddiar and Le Tacon (1990)[16] working on the symbiosis of *A. glutinosa* showed that the arbuscular mycorrhizal symbiont must be established before host plant roots become colonized by *Frankia*; feeder roots colonized by ectomycorrhizal fungi develop later.

It would be expected that increases in phosphorous uptake, enhanced by the activity of either ecto- and endomycorrhizal fungi, might stimulate both, the number of nodule and

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[7] Furlow, J. J. 1979a. The systematics of the American species of *Alnus* (Betulaceae) I. Rhodora 81: 1-121.

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[15] Mejstrik, V. and V. Benecke. 1969. The ectotrophic mycorrhizae of *Alnus viridis* (Chaix) D.C. and their significance in respect to phosphorous uptake. New Phytol. 68: 141-149.

[16] Fraga-Beddiar, A. and F. Le Tacon. 1990. Interactions between a VA mycorrhizal fungus and *Frankia* associated with alder (*Alnus glutinosa* (L.) Gaetn.). Symbiosis 9: 247-248.

nitrogen fixation. However no reports are available on the comparative effects of an endomycorrhizal versus an ectomycorrhizal fungus on nodulated actinorhizal plants. The purpose of this study was to compare the interaction between a *Frankia* strain and two different arbuscular fungi, as well as an ectomycorrhizal fungus.

METHODS AND MATERIALS

There are no seed banks of actinorhizal plants in Mexico and they are not commercially available. Seeds of *Alnus acuminata* ssp. *glabrata* (Furlow) were collected from a sole tree in an alder population growing along the river Magú, Tepotzotlán, in the State of México. Seeds were surface sterilized using 30% H_2O_2 for 10 min, and were then soaked in a 50% ethanol solution for 2 min and rinsed with pollipored sterile water. Seeds were sown in containers filled with sterile quartz sand. Four weeks later, 2 uniform size seedlings were transplanted into each 185 mL-pots (Unicel®) containing an autoclaved soil – quartz mixture (1:1). Soil was collected in a pine- oak - alder forest and had a sandy texture (Bouyoucos), a 9.1% organic matter content, with a pH 5.1, a total N of 0.45% (Kjeldahl digestion) and 1.59 µg/g of available P (vanadomolibdo-phosphoric complex).

Seedlings were inoculated with a mycorrhizal fungus and a strain of *Frankia* sp. no. 1801H). The ectomycorrhizal fungus was a Mexican isolate of *Pisolithus tinctorius* (Pers.), Coker and the arbuscular fungi were *Glomus versiforme* (Karst.), Berch and *Glomus* sp. *Frankia* sp. no. 1801H is a native strain which was isolated from fresh, young nodules of *A. acuminata* seedlings inoculated with desinfected and crushed nodules collected in the field (Valdés *et al.*, 1991)[17]. *A. acuminata* seedlings were maintened in a growth chamber. The fruiting body of the ectomycorrhizal fungus was collected in a oak-pine forest. Mycelium from the internal part of the carpophore was isolated and grown on MNM agar medium. The endomycorrhizal fungus, *G. versiforme*, was obtained from the collection of Dr. V. Furlan (Agriculture and Agri-Food Canada Research Centre, Quebec).

Endomycorrhizal inoculum was obtained by propagating the fungus in sorgum plants in the greenhouse. The ectomycorrhizal inoculum was obtained by growing the vegetative mycelium in mason jars filled with a mixture of vermiculite, peat-moss, and MNM nutrient solution (Marx and Kenney, 1982)[18]. The content of mason jars was washed several times with tap water and 15 g of the washed mixture, containing the inoculum,

[17] Valdés, M.; H. Ramírez-Saad; G. Guillén; R. Del Valle y N. Serna. 1991. Algunas características morfológicas y fisiológicas de *Frankia* aislada de *Alnus* y *Casuarina*. Rev. Latinoam. Microbiol. 33: 271-277.

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and then placed into the root zone of the seedlings. All fungi were inoculated individually at transplanting time. *Frankia* inoculum, being added one week later, was obtained by growing the bacteria for 2 months and subculturing every 10 days in L/2 culture medium (Lechevalier and Lechevalier, 1990)[19]. Packed cells produced were diluted in a saline solution (1:1). One mL of the suspension was injected into the rooting zone of each seedling to be inoculated. Control seedlings did not receive any inoculum. No mineral fertilizer was added. There were four pots with two plants each per treatment.

Pots were randomized on a greenhouse bench. During the experiment, the temperature was variable from day to day ranging from 14 to 30° C. Daylight was extended to 12 h using daylight (40 W) fluorescent tubes over the pots. Every other day, distilled water was added to each pot at field capacity.

Eighteen weeks after the inoculation all seedlings were harvested. Plant volume was determined according to the formula HD^2 (Ruehle *et al.*, 1984[20]; Hatchell *et al.*, 1985[21]) and the shoot dry mass registered as soon as a constant mass was obtained after an oven dry period at 80°C for 48 hours.

The root colonization level by arbuscular mycorrhizae was assessed on 1-cm root segments following the technique of clearing and staining described by Phillips and Hayman (1970)[22]. Stained root segments were mounted in lactophenol and observed under a photomicroscope for the presence or absence of fungal propagules. Percent of ectomycorrhizal feeder roots was determined after taking a photocopy of the whole radical system, previously washed with tap water to remove all soil particles (Marx *et al.*, 1991)[23]. The total number of short roots was determined on each seedling and the percentage of ectomycorrhizal roots calculated. In addition, thin sections of short roots, stained with cotton blue, were examined under a photomicroscope to verify the presence of ectomycorrhizae.

The data were subjected to analysis of variance (ANOVA). Percent data were divided by 100 and multiplied by the arcsine to be analyzed using the repeated measures analyses of the General Linear Model procedure (SAS Institute, 1982)[24].

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[19] Lechevalier, M. P. and H.A. Lechevalier, 1990. Systematics, Isolation, and Culture of Frankia. *In:* The Biology of *Frankia* and Actinorhizal Plants. pp. 35-60.

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RESULTS AND DISCUSSION

Seedlings of *A. acuminata* ssp. *glabrata* were successfully icolonized with the native *Frankia* strain no. 1801H, the indigenous ectomycorrhizal fungus, *P. tinctorius*, and two different endomycorrhizal fungi, *G. versiforme* and *Glomus* sp. Figure N° 1a and 1b illustrate the presence of arbuscular mycorrhiza produced by *G. versiforme* and Figure N° 2 shows the ectomycorrhizal mantle and the Hartig net produced by *P. tinctorius*.

The *Frankia* strain, from the nodules of *A. jorullensis*, induced the formation of normal root nodules on *A. acuminata* which indicates a compatibility between the microendosymbiont and the host plant. It also confirms the **diagnostic** feature of the bacterial genus to induce the formation of nitrogen-fixing nodules. Root colonization (28 to 90%) by the endomycorrhizal fungi used demonstrates that this fungal symbiont is compatible with *A. acuminata* host plants. The presence of ectomycorrhizal root tips and of a stained fungal mantle state the ability of *P. tinctorius* to colonize alder roots. Control plant roots were free of nodule and mycorrhiza. These results support the fact that either ecto- or endomycorrhizal fungi can colonize alder tree roots under natural conditions. Sometimes, both type of mycorrhizal fungi are found to cohabit the same root (Cruz-Cisneros and Valdés, *op. cit.*).

Table N° 1 compares the data for shoot volume and dry mass, the relative growth enhancement compared to the control, the number of nodules, and the root colonization by either one of the mycorrhizal fungi, ecto- or endo-, of alder seedlings inoculated with *Frankia*. Shoot volume was significantly greater ($P = 0.0002$) when seedlings were inoculated with either *P. tinctorius* or *G. versiforme* combined with the *Frankia* strain no. 1801H and compared to all other treatments. Single symbiont inoculation, except with *Glomus* sp., produced a greater shoot volume compared to the control.

Plant growth performance in terms of shoot dry mass was significantly greater ($P = 0.002$) when host plants were inoculated with the *Frankia* strain and either one of the mycorrhizal fungi *P. tinctorius* or *G. versiforme* (Table N° 1). Seedlings inoculated with single symbionts, except with *Glomus* sp., had a shoot dry mass significantly greater compared to the control. In terms of shoot biomass productivity, Visser *et al.* (1990)[25] reported a yield increase by a factor of 4 and 5 for *Shepherdia canadensis* and *Elaeagnus communata*, respectively, compared to uninoculated treatments.

[25] Visser, S.; R. M. Danielson and D. Parkinson. 1990. Field performance of *Elaeagnus communata* and *Shepherdia canadensis* (Elaeagnaceae) inoculated with soil containig *Frankia* and vesicular-arbuscular mycorrhizal fungi. Can. J. Bot. 69: 1321-1328.

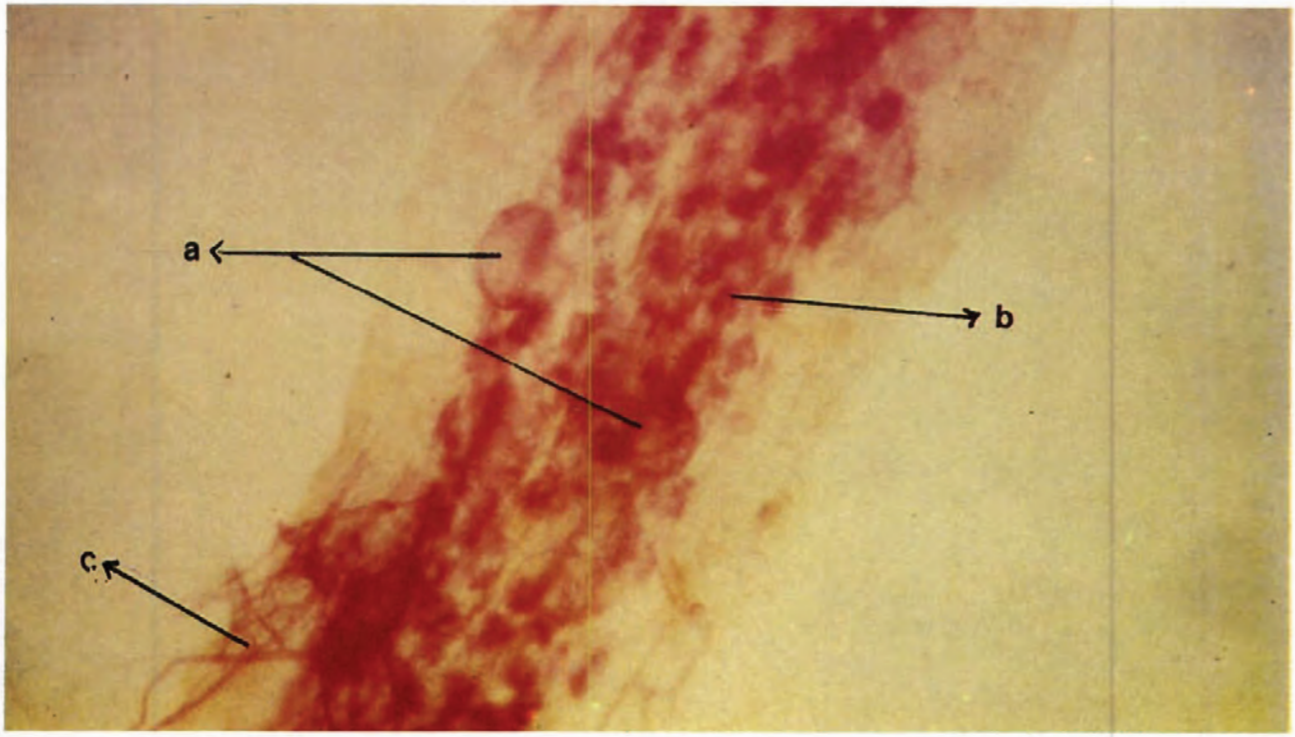

Figure N° 1a. Roots of Alnus acuminata ssp. *Glabrata colonized* by *Glomus versiforme* stained with fucsine acid. a) vesicles, b) arbuscules, c) hyphae. 10X. a) arbuscule. 100X. Fuscine acid dye.

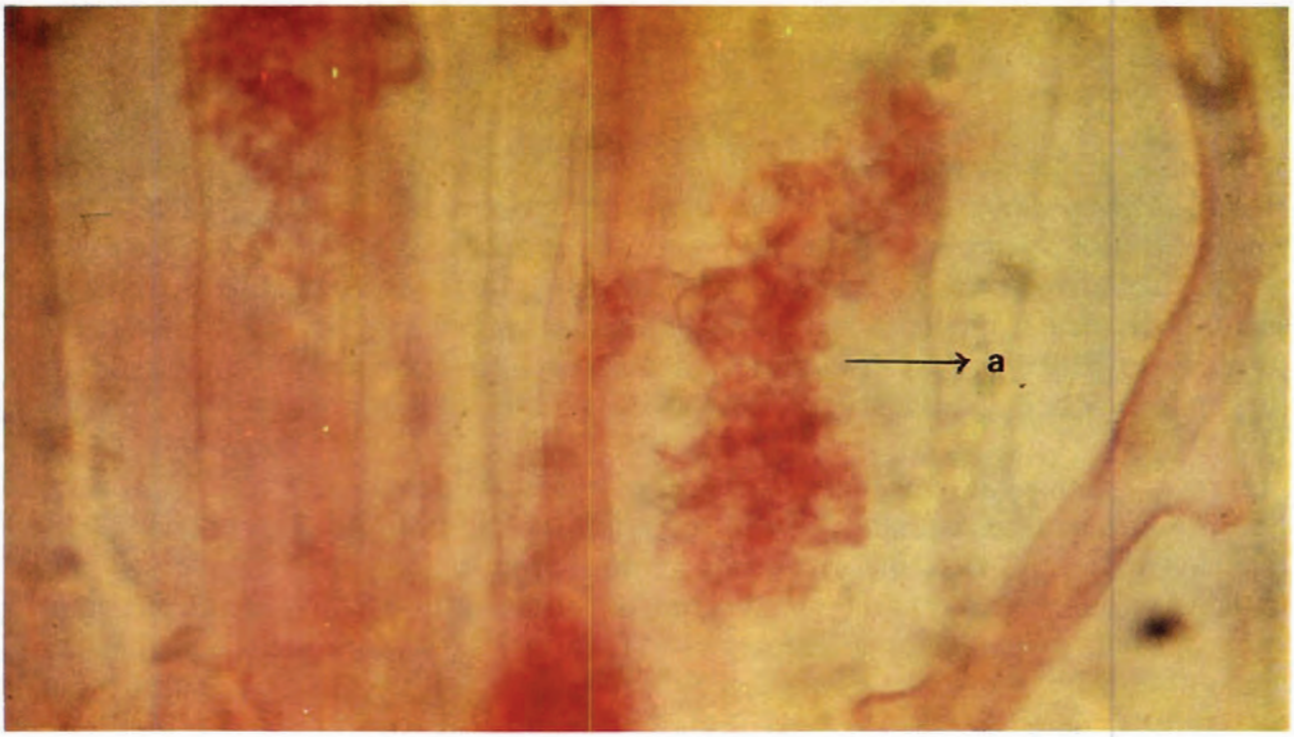

Figure N° 1b. Roots of Alnus acuminata ssp. *Glabrata colonized* by *Glomus versiforme* stained with fucsine acid. a) arbuscule. 100X. Fuscine acid dye.

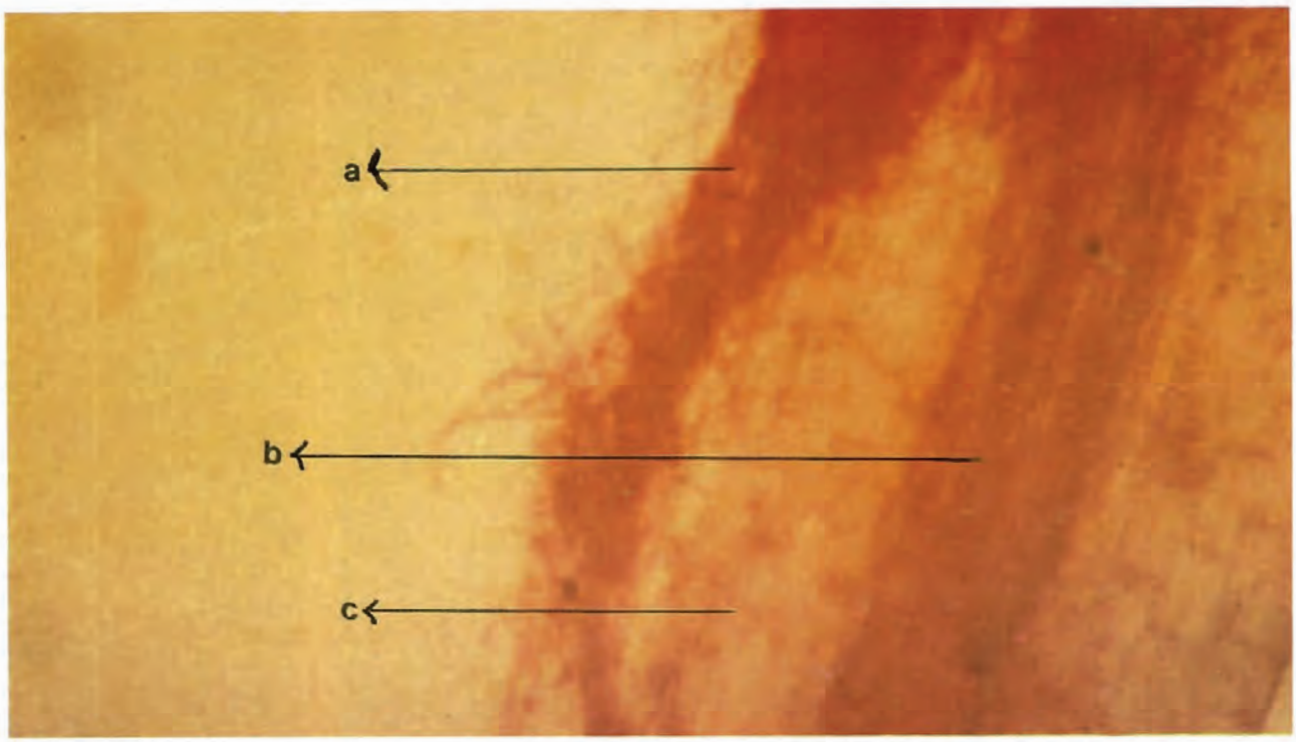

Figure N° 2. Feeder roots of *Alnus acuminata* ssp *glabrata* colonized by *Pisolithus tinctorius* stained with fucsine acid. a) fungal mantle, b) vascular tissue, c) cortical cells. 40X.

Nodules were observed in 3 treatments out of 4 inoculated. The greater number being obtained in presence of the *P. tinctorius* ectomycorrhizal fungus (Table N° 1). Meanwhile, *G. versiforme* did not have any effect on the number of nodules formed on *Alnus accuminata* root system. In the present study, there is no relation between the number of nodules produced and the shoot dry mass registered. However, Visser *et al.* (*op. cit.*) have observed a strong correlation between the actinorhizal shrubs yield and the number of nodule formed.

This study shows the potential of the tripartite association, either *P. tinctorius* or *G. versiforme* combined with the *Frankia* strain no. 1801H on *A. acuminata* plants. Plant biomass increase due to the synergism resulting from this double symbiosis is quite striking when compared to the relative growth enhancement of *Alnus* plants inoculated with a single symbiont or to the control (Table N° 1). Meanwhile, the indigenous endomycorrhizal fungus *Glomus* sp. did not respond as well as the *G. versiforme* one, very likely due to a lack of compatibility with *A. acuminata* host plants. This emphasizes the necessity of comparing the effect of different fungal symbionts when testing new host plants for reforestation or land reclamation.

TREATMENT	SHOOT			NODULE	ROOT
	Volume (cm^3)	Dry mass (g)	RGE*	Number	Colonization (%)
Control	0.344a	0.206a	—	0	0
Frankia no. 1801H	0.980b	0.329b	+ 60%	4b	0
Pisolithus tinctorius	1.001b	0.319b	+ 55%	0	46
Glomus versiforme	0.899b	0.338b	+ 64%	0	66
Glomus sp.	0.659ab	0.248a	+ 20%	0	30
Frankia no. 1801H + *Pisolithus tinctorius*	2.576d	0.601c	+ 192%	14c	55
Frankia no. 1801H + *Glomus versiforme*	1.911c	0.580c	+ 182%	3b	90
Frankia no. 1801H + *Glomus* sp.	0.468a	0.214a	+ 4%	0	28

* RGE = Relative growth enhancement compared to the control.
Means within a column followed by the same letter are not significantly different.

Table N° 1. Response of *Alnus acuminata* ssp. *glabrata* to different treatments.

The relative contribution of the mycorrhizal fungi and *Frankia* on host plant growth is merely explained by the fact that a strong tripartite interrelationship occurred which resulted in a greater biomass yield. Significant relationship between nodulation and plant biomass has been observed in actinorhizal plants (Diem and Gauthier, 1982[26]; Huss-Danell, 1980[27]; Sougoufara *et al.*, 1989[28]; this study) suggesting that *Frankia* nodules are highly beneficial to plant N nutrition. On the other hand, according to the summarized studies in Daft *et al* (*op. cit.*), ecto and endomycorrhiza could enhance the actinorhizal nodulation and nitrogen fixation by satisfying the high phosphorous demand required for these processes.

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