Evaluating Alder-Endophyte (*Alnus acuminata-Frankia-Mycorrhizae*)
Interactions:
Growth Response
of *Alnus acuminata* Seedlings
to Inoculation with *Frankia* Strain ArI3 and *Glomus intra-radices*,
Under Three Phosphorus Levels

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**ABSTRACT.** The objective of this study was to compare the interaction between *Frankia* and vesicular-arbuscular mycorrhizae (VAM) on the growth response of *Alnus acuminata* H.B.K. seedlings under three different phosphorus levels. *Alnus acuminata* seedlings grown in sterile vermiculite, were inoculated with *Frankia* strain HFPArI3 and/or VAM (as *Glomus intra-radices*) under three
phosphorus levels (10, 50 and 100 µg g⁻¹). After 120 days differences in growth were observed at the 50 µg g⁻¹ P level between nodulated and non-nodulated plants; whether inoculated with Frankia + VAM or just with VAM. Interaction between Frankia and VAM was positive with respect to nodule weight at 50 µg g⁻¹ P level. The data suggest that the presence of VAM can compete with the nodules as a carbohydrate sink. At increasing levels of P the presence of Frankia alone statistically increased growth over that of the control.

**INTRODUCTION**

*Alnus acuminata* H.B.K. (Furlow, 1977) is an actinorhizal tree, commonly associated with pastures on upland areas of Costa Rica, Colombia and Venezuela. The species is host to the nitrogen-fixing actinomycete *Frankia*. Its roots are also infected by endomycorrhizal fungi, resulting in a tripartite symbiotic association among *Alnus, Frankia*, and vesicular-arbuscular mycorrhizae (VAM) (Russo, 1990). The existence of tripartite symbiotic associations among actinorhizal plants, *Frankia*, and VAM has been previously reported by Rose and Trappe (1980) for *Alnus* spp., by Rose and Youngberg (1981) for *Ceanothus velutinus*, by Gauthier, Diem and Dommergues (1983) for *Casuarina equisetifolia*, and by Gardner, Clelland and Scott (1984) for *Hippophae rhamnoides*. However, the interaction between *Frankia* and VAM and the effects that such interaction may have on the host initial growth is still little known. It is expected that increased phosphorus uptake due to the action of VAM might stimulate nodule growth of the host plant. This leads to the hypothesis that simultaneous inoculation with *Frankia* and VAM would increase nodule formation. Thus, an increase in plant biomass should occur because of greater amounts of nitrogen and phosphorus resources available to the host. Carbon allocation and nutrient content of *A. acuminata* seedlings should also be modified. If the plants are well supplied with nutrient resources and water, allocation to roots and the root-shoot ratio should decrease. In order to test these hypotheses, the operational phase of this study was to compare effects of inoculation with *Frankia* and VAM on the growth response of *Alnus acuminata* seedlings under three different phosphorus levels.

**MATERIALS AND METHODS**

*Alnus acuminata* H.B.K. seeds (collection #2407) from the Latin American Forest Seed Bank at CATIE, Turrialba, Costa Rica were sowed on
August 20, 1986 in germination trays containing sterile sand, in the Greeley greenhouse at the Yale School of Forestry and Environmental Studies, New Haven, Connecticut. On October 10, 1986, when seedlings were around 2 cm in height, they were transplanted to 600 cc capacity green plastic pots. The vermiculite substrate used was previously oven sterilized for 30 minutes at 82°C (Shurtleff, Edwards, Courter & Randell, 1983). The temperature in the greenhouse on a daily basis ranged from 10°C to 25°C. Lowest temperatures occurred around 4:00 to 8:00 a.m. and highest around 12:00 to 4:00 p.m. Maximum photosynthetically active photon flux density (PPFD) was ca. 1200 μmol m⁻² s⁻¹. Daylength was extended to 12 hours by fluorescent lamps mounted horizontally 0.6 meter over the pots.

Seedlings received the following treatments: (1) *Frankia* Inoculation, F; (2) VAM Inoculation, V; (3) F + V Inoculation; (4) No inoculation (C). Three different phosphorus levels P10, P50, P100 (10, 50, and 100 μg g⁻¹, equivalent to 0.32 mmol, 1.61 mmol and 3.22 mmol respectively) were also supplied to the aforementioned treatments. Twelve replications were originally established for each treatment for each P-level, considering each pot as an experimental unit. The 144 pots were randomly arranged on the southside of a greenhouse bench. But at 120 days only ten replicates were harvested because a subset of two plants per treatment was left for further investigations.

*Frankia* inoculum (HFPArI3) was obtained from Harvard Forest cultures. *Glomus intraradices* (Nutri-Link Research Grade, lot # 8602) was used as VAM inoculum. All inoculations were made simultaneously with transplanting. Every seedling was watered daily with distilled water and treated twice a week with a nutrient solution (Hoagland with 20 mg N/liter), and with three different levels of phosphorus (P10, P50, and P100, μg g⁻¹) during the 120-day growth period. Variables measured were plant height (cm), number of leaves, leaf dry weight (mg), leaf area (cm²), stem dry weight (mg), root dry weight (mg), nodule dry weight (mg), and percentage of VAM infection in roots (%). Dry weights were obtained on an oven-dry (70°C) basis. Leaf area was measured with a LICOR portable area meter model LI-3000. Nitrogenase activity of these plants was reported in a previous paper (Russo, 1989). For assessment of mycorrhizal infection fine root segments, around 1 cm in length, were excised from the harvested plants, bleached in 10% KOH for 24 hours at room temperature and stained with 0.05% trypan blue in lactoglycerol following a modification of the procedure of Phillips and Hayman (1970). Fifty root segments from each treatment were mounted on microscope slides in clear lactoglycerol and examined microscopically for presence or absence of VAM structures. Differences between treatments were tested by analysis of variance using the statistical package STATVIEW (Abacus Concepts,
Infection percentage values were transformed with the arcsine transformation before analysis.

RESULTS AND DISCUSSION

_Growth in height_—Total height of seedlings was the first variable evaluated 50 days after inoculation. At that time no statistically significant differences were observed between treatments, but the means of V at P10 and F at P50 were higher than the other height means.

By the end of the experiment the best height performance at P10 was shown by F and V, but this changed at P50 where the nodulated plants (F and F + V) were significantly taller than C and V; and at P100 only F maintained its positive effect; F + V fell to where it was equal to C, but both C and F + V were higher than V, which showed very poor growth at P100 (Figure 1). It is evident that F alone can play a decisive role in height growth when P is adequately available. Of the three levels of phosphorus supplied it appears that P50 was closest to the optimum level while P10 was well below optimum and P100 above optimum for height growth.

_Shoot dry weight_—At P10 there was no difference between the C and the F + V treatment for total shoot dry weight, or for either leaf or stem dry weight (Figures 2, 3, and 4).

Plants inoculated with F or V both showed slight but significantly higher (P < 0.05) leaf dry weight than plants inoculated with C or F + V. At P50 there was a significant improvement of the F + V and the F treatment over that of C and V for total shoot dry weight, leaf and stem dry weight. Another change occurred at P100 where only F resulted in increased leaf dry weight whereas V was inhibitory to leaf dry weight. V + F gave essentially the same results as C (Figures 2, 3, and 4). The higher shoot weight of F treated plants at P100 could be attributed to an increase in leaf dry weight. The stem weight actually decreased significantly by 0.3 fold.

_Root weight_—At P10 the highest root weight values were reached by V inoculated plants (299.2 mg). These values (Figure 5) were significantly different from all other treatments (ranging 136.5 to 211.2 mg). At P50 there were no statistically significant differences among treatments, but the highest mean value occurred in F inoculated plants (292.1 mg) and the lowest one in the control (214.2 mg). At P100, the mean root dry weight of F inoculated plants was clearly and significantly (P < 0.01) higher (297.3 mg) than all the other treatments (less than 200 mg). There were
Figure 1. Growth in height of *Alnus acuminata* inoculated with *Frankia* and VAM at different phosphorus levels.
Figure 2. Leaf dry weight of *Alnus acuminata* inoculated with *Frankia* and VAM at different phosphorus levels.
Figure 3. Stem dry weight of Alnus incana inoculated with Frankia and VAM at different phosphorus levels.
Figure 4. Shoot dry weight of *Alnus acuminata* inoculated with *Frankia* and VAM at different phosphorus levels.
no statistically significant differences among the other treatments but V had the lowest values and the values for F + V were also less than that of C. This continues the trend that as phosphorus level increases above a certain level the presence of V can result in less root biomass. Thus the carbon cost of V becomes greater than the gain, and the presence of F, in addition to V, cannot entirely compensate for this carbon cost despite the fact that only F as compared to all other treatments showed greatly enhanced root biomass over that of the control.

Summarizing, at P50 there is a significant improvement of the above-ground variables for F and the F + V treatments over that of C and V. The V treatment did not result in increased leaf dry weight. Another dramatic change occurs at P100 where only F alone results in increased leaf dry weight whereas V alone is inhibitory to leaf dry weight while V + F gives essentially the same results as C. While not statistically significant, the double inoculation treatment V + F was slightly less promotive of this parameter than the control.

After 120 days, at P10 there was a difference in favor of the V treatment in total plant weight, shoot, stem and leaf weight. However, F alone surpassed V in promoting leaf area at P10. This enhanced growth at P10 with V inoculation is considered as supporting the concept that at low levels of phosphorus mycorrhizae can enhance phosphorus uptake. Nevertheless, the magnitude of a number of V-enhanced parameters at P10 is below the mean values found at higher P-levels with other treatments. For example V treated plants at P10 had a mean total dry weight (Figure 6) of 1029 mg while the maximum total weight occurred at P50 in F + V treated plants (1417.5 mg) and the F treated plants (1415.8 mg).

At P50 there were larger values of the measured above ground attributes (total plant, leaf, and stem weight, and plant height) for the nodulated plants (F and F + V) than for the non-nodulated plants (V and C). This is especially striking in the case of plant height and leaf area (Figure 7) where nodulated plants are clearly taller and have higher leaf area, in particular F + V had the highest values of these parameters. Interestingly, the effect of F + V drops precipitously at P100, suggesting that there is a restricted window of environmental conditions and growth parameters where the benefits of double inoculation outweigh the costs. This is a common pattern in these data. At P100 only the F-treatment showed higher means than the control in: plant height, leaf dry weight, leaf area, and shoot weight and total weight. F + V was the same as C, but the lowest means were that of the V treatment. It seems that VAM causes depression in growth even when compared with the non-inoculated control. A similar pattern was shown in all shoot related variables (plant height, number of
Figure 5. Root dry weight of *Alnus acuminata* inoculated with *Frankia* and VAM at different phosphorus levels.
Figure 6. Total dry weight of *Alnus acuminata* inoculated with *Frankia* and VAM at different phosphorus levels.
leaves, leaf dry weight, and leaf and shoot biomass) while both groups of
nodulated plants showed greater above ground growth at P50, only F
inoculated plants grew better at P100. It is speculated that at P100 the
VAM became parasitic. However, it is possible that the seedlings were
harvested too soon to allow the tripartite association the optimum time to
reach its full potential. As discussed by Gardner (1986) the allocation of
plant photosynthates by the two microsymbionts is competitive and could
be critical in the early stage of plant development. It has been suggested
that application of inocula at different times might alleviate this problem
(see Gardner, 1986).

Gauthier, Diem and Dommergues (1983) reported preliminary results
of a research on Frankia and Glomus mosseae associated with Casuarina
equisetifolia, finding that total dry weight was more than 80% greater in
seedlings simultaneously inoculated with Frankia sp. and Glomus mosseae
than in those inoculated with Frankia sp., and no differences between the
control and the VAM inoculated plants were found. These authors worked
with 10 μg g⁻¹ of P for all the treatments with the exception of one in
which inoculation was by crushed nodules and 90 μg g⁻¹ P was applied
to the plants.

Carbon allocation—The allocation of carbon to different plant parts was
significantly affected by treatment but not by P level. However, there
were some slight changes in the allocation patterns with differences in P-
level. Carbohydrates allocated to leaves were significantly higher (P <
0.05) in F and F + V treatments followed by the control. The allocation
to leaves in V was significantly lower at all P levels. Allocation to stem
showed only differences at P 50 in favor of F alone and was significantly
lower in V alone treatment. The larger differences were observed in root
allocation. They were in favor of the V treatment at all P levels. Thus, for
example, the V treated plants were smaller and had less leaf and root
biomass at P100, but the percentage allocation to roots was greater. How-
ever, at P10 the leaf and root biomass were the largest with the V-treat-
ment and the percentage allocation to roots was still larger than for all the
other treatments including the control. The allocation percentage to roots
in V treated plants did not change very much with P-level (29% to 31%)
despite the fact that the biomass of all the plant organs including the roots
changed greatly over these P-levels. Leaf Weight Ratio (LWR) was signif-
icantly lower in the V than in the other treatments regardless P level.

These changes in carbon allocation between shoot and root cause a
change on the S/R ratio, which decreases when V are present. However,
this effect tended to be overcome by the effect of F at increasing levels
of P.
Figure 7. Leaf area of *Alnus acuminata* inoculated with *Frankia* and VAM at different phosphorus levels.
Nodulation—Nodule dry weight (Figure 8) was not significantly different between double inoculation by F + V and V inoculation alone at P10. However, at P50 nodule dry weight per plant was significantly higher in double inoculated plants. At P100 there was again no significant difference between V and F + V. Thus we must reject our initial hypothesis that F + V inoculation would increase nodule formation in all conditions. Also it is evident that V was more effective at P50 whereas it was expected to be more effective at P10, especially since the V plants had the largest roots of any of the treatments at P10 and the roots decreased in biomass at P50. The mechanism by which V can enhance nodule dry weight at the middle P50, but not in the higher or lower phosphorus levels is not clear. The ratio of nitrogen and phosphorus in the nutrient solution may be involved. Specific activity of nitrogenase, as reported in a previous paper (Russo, 1989), showed no difference between F inoculated and F + V inoculated plants, with the exception of the plants double inoculated at P10 level where the ethylene production was 140% higher than the mean of the rest of the treatments. It now appears that this effect is due to smaller but more active nodules at P10. The results at P10 have relevance to the field conditions because in natural soil conditions where Alnus acuminata grows in association with pastures on volcanic soils in Coronado, Costa Rica, the P-level averages 5 µg g⁻¹ of soil. It is in such conditions that mycorrhizae may help to improve nitrogen fixation.

VAM infection—Analysis of variance of the means of percentage of V infection between V and F + V inoculation indicated that they were not significant at the 5% level, but were significant at the 8% level. However, the proportions of infected roots (between 9.7% and 18.7%, Figure 9) were low in general compared with those found in the field. These relatively low percentages of infection could be attributed to the fact that roots were grown in pure vermiculite and supplied with nutrients. Another explanation may be the fungal strain used; however, fungal strains involved in V are, in general, neither host specific nor geographically limited, although local differences correlated with soil characteristics and microbiota have been reported (Malloch, Pirozynski & Raven, 1980). While the infection percentages observed in these pot cultures inoculated with Glomus intra-radicis were somehow lower than that observed in Alnus VAM-infected roots collected from the field, mycelial growth, vesicles and arbuscules observed in the pots experiments were probably denser than those of the field. Visual vesicle counts were higher at the P10 level although no statistical analyses were performed. Hicks and Loynacham (1987) reported that phosphorus fertilization reduced V infection in soybean by an average of 80% and that vesicle counts were also reduced.
Figure 8. Nodule dry weight of *Alnus acuminata* inoculated with *Frankia* and VAM at different phosphorus levels.
Figure 9. Vesicular-arbuscular mycorrhizal infection in Alnus acuminata inoculated with Frankia and VAM under three P levels.
significantly. In this case the reduction due to P level averages 45% when comparing P10 with P100, and this difference is significant (P < 0.05) when comparing V infection at P10 against P100 in the double inoculated treatment. As in legumes, mycorrhizal symbiosis in actinorhizal plants functions by its well known P-uptake increasing effect, thereby increasing nodule number, nodule biomass, nitrogenase activity, and N, Ca, and P shoot content (Rose and Youngberg, 1981). On the other hand, it may be hypothesized, that at higher levels of exogenous P, as VAM are less functional, from the point of view of P uptake, and they may be parasitic in the sense that they compete for carbohydrate resources.

REFERENCES

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