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INTERACTIONS BETWEEN THE NITROGEN-FIXING SHRUB *ELAEAGNUS* AND MYCORRHIZAL FUNGI

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "INTERACTIONS BETWEEN THE NITROGEN-FIXING SHRUB ELAEAGNUS AND MYCORRHIZAL FUNGI", submitted by CAROLINE MATILDA MCBAIN in partial fulfillment of the requirements for the degree of Master of Science.

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ABSTRACT

The interactions between the partners of the symbiosis between Elaeagnus commutata, vesicular-arbuscular mycorrhizal (VAM) fungi and the nitrogen-fixing actinomycete Frankia, were studied. Two Frankia strains were isolated from E. commutata growing near Fort McMurray, Alberta. Two VAM species, Glomus aggregatum and G. etunicatum were used in the investigations. There was no difference in biomass production between mycorrhizal-only plants infected by either VAM species at two inoculum concentrations. However, the G. etunicatum 15% inoculum and G. aggregatum 30% inoculum treatments were smaller than control plants at the time of harvest.

Low and variable nodulation rates were observed when inoculating with the two Frankia strains; therefore, growing conditions were investigated. Seedlings nodulated at pH 7.0 but not at pH 6.5 or lower. There was no difference in nodulation between 6 and 15 week-old Frankia inoculum. Homogenizing the inoculum did not improve nodulation significantly. Mixing the inoculum into the soil or injecting the inoculum into the root zone were more effective than surface application or soaking seedling radicles in concentrated inoculum.

The growth response of Elaeagnus to combinations of Frankia strains and VAM species was determined. The two isolated strains (EANfmb and EANfm+), a strain donated by

M. Lalonde (EAN1), and a Frankia mixture purchased from Rhizotec Laboratories Inc. were used in combination with G. aggregatum and G. etunicatum. VAM infection levels were significantly lower than the controls in the presence of Rhizotec Frankia nodules, but not other strains. There was no significant difference in shoot weight amongst control plants and the various Frankia/mycorrhizal combinations with the exception of the EAN1/control treatment which weighed less than the no-nodule control. There were significant differences in nodule dry weights but not in acetylene reduction rates among treatments.

The effects on plant growth of inoculation with either Frankia, VAM fungi, or both were compared with uninfected control plants at limiting, sufficient and luxury levels of N and P. In the absence of nodules, VAM did not enhance plant growth relative to control plants, even at very low available P and N levels. However, VAM did enhance the growth of nodulated plants relative to controls under low available P conditions. Such growth enhancements were duplicated by the addition of P to non-mycorrhizal plants, indicating that VAM effects are due to an improvement in the plant nutrient status. There was an increase in nodule weight of VAM, nodulated seedlings over nodulated-only plants at low P and N levels. This did not appear to be duplicated by the addition of P to the nodulated-only plants.

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TABLE OF CONTENTS

TABLE OF CONTENTS (Concluded)

LIST OF TABLES

LIST OF TABLES (Concluded)

LIST OF FIGURES

LIST OF FIGURES (Concluded)

LIST OF APPENDICES

CHAPTER 1

1. GENERAL INTRODUCTION

Actinorhizal shrubs are nitrogen-fixers nodulated by the actinomycete, Frankia. In addition to the presence of Frankia nodules, Elaeagnus and Shepherdia also grow in association with vesicular-arbuscular mycorrhizal (VAM) fungi (Riffle 1977; Rose 1980). These shrubs are common in nutrient poor soils and disturbed areas (Torrey 1978; Rose 1980) and the success of these shrubs under stressful conditions has been related to their symbiotic status (Rose 1980). The ability to add nitrogen to the soil through N_2-fixation and survive under difficult conditions has led to the use of these shrubs in forestry interplanting (Dawson 1983), and land reclamation (Fessenden 1979; Wheeler et al. 1986).

The use of actinorhizal shrubs for reclamation purposes requires an understanding of the relationship between the host shrub and its root symbionts so that appropriately infected seedlings can be produced for outplanting. A number of questions are quickly raised about this three-way (tripartite) symbiosis. How interdependent are the partners of the symbiosis? By what mechanisms do they interact? Does each endophyte interact singly with the host plant or are there direct interactions between the endophytes? This investigation will try to define aspects of the interactions between Frankia, VAM

fungi and Elaeagnus commutata Bernh. ex Rybd. (Elaeagnaceae).

Elaeagnus is a native genus distributed throughout the southern half of Alberta. Its range extends to Alaska and the Yukon, parts of the Northwest Territories and south into the U.S.A. The shrub is somewhat drought resistant but generally grows where there is adequate moisture, in medium to coarse textured soils (Watson et al. 1980). Moore (1964) observed Elaeagnus on infertile erosion slopes in Alberta. It is common on valley slopes and the edges of aspen groves (Watson et al. 1980).

Earlier surveys established the mycorrhizal status of actinorhizal shrubs (Mejstrik 1971, Williams and Aldon 1976, Williams 1979). Riffle (1977) first described the VAM of Elaeagnus. Rose (1980) performed an extensive survey of the mycorrhizal status of a number of nitrogen-fixing shrubs and demonstrated that Shepherdia and Hippophae of the Elaeagnaceae were among those infected by VAM fungi as well as by Frankia. She felt that the tripartite status was necessary for successful invasion of stressed sites.

Few studies have investigated the interactions between Frankia, VAM fungi and the host shrub. Rose and Youngberg (1981), working with Ceanothus showed, that under low nutrient conditions, plants infected with both VAM and Frankia exhibited greater growth, nodulation, rates of

nitrogen fixation and higher shoot N, Ca and P levels than plants that were nodulated only. Gardner et al. (1984) published similar results for Hippophae, comparing mycorrhizal only, nodulated only, mycorrhizal/nodulated and control plants. Both of these studies used crushed nodules as Frankia inoculum.

The benefit of VAM fungi to their hosts has been well documented (Mosse 1973, Abbott and Robson 1984). Through the increased absorptive area afforded by hyphal exploration of the soil (Tinker 1975, Cooper 1984), increased uptake of P (Bowen et al. 1975), N (Ames et al. 1983), Zn and S (Cooper and Tinker 1978) have been demonstrated. There is also some evidence that absorption of K, Ca, Mg, Fe, Cu, Mn, Na and B ions may be higher in VAM plants (Cooper 1984). Mycorrhizae may also increase water uptake by plants (Nelson and Safir 1982). Cooper (1984) cites evidence that VAM infection can improve drought tolerance and decrease susceptibility to transplant shock. Bagyaraj (1984) discusses a number of studies where VAM infection conferred resistance to a number of fungal pathogens. However, there are also instances where VAM infection had either no effect or a detrimental effect on pathogen-infected plants (Ross 1972). Graham (1987) notes that increased water uptake, disease resistance and salt tolerance are well known physiological effects of improved P nutrition, therefore these effects may be due only to

improved P uptake and not due to any other effect of VAM on plant physiology. Increased nutrient uptake by mycorrhizal plants has implications for the ecosystem as higher shoot N and P contents (Stribley et al. 1980) may result in improved litter quality which in turn leads to greater soil fertility.

Frankia, as a nitrogen fixer, can help meet the nitrogen demands of the host plant (Becking 1970). Production of N enriched shoot, root and nodule litter, and leakage from nodules represents a significant nitrogen input into soils. Huss-Danell (1986) showed that shoot litter was the major such source of nitrogen. Examples of nitrogen accretion in mixed forestry plantings in which an actinorhizal shrub was involved include: Youngberg and Wollum (1976) working with Ceanothus, Pinus and Pseudotsuga; Coté and Camiré (1985) with Alnus and Populus; Friedrich and Dawson (1984) with Alnus, Elaeagnus, Robinia, Lespedeza and Juglans nigra; and Tarrant and Miller (1963) with Alnus and Pseudotsuga. Tarrant and Miller (1963) also recorded increased soil organic matter content in the mixed stand. Whysong and Bailey (1975) recorded higher nitrogen contents of mixed grasses growing under Elaeagnus than in an associated grassland. Nitrogen and organic matter inputs from actinorhizal shrubs would be especially important to nutrient deficient disturbed soils.

Tripartite symbioses, in which both a nitrogen fixing bacterium and a mycorrhizal fungus infect a host, have been extensively studied in legumes. Nitrogen fixation and nodulation have a high phosphorus demand (Bergersen 1971) and low levels of specific micronutrients can also limit growth (Daft and El-Giahmi 1974). Vesicular-arbuscular mycorrhizae appear to meet this demand through increased absorption of the soil solution as described above. Dually infected plants (VAM and nitrogen-fixer) usually exhibit greater biomass production, nodulation, rates of nitrogen fixation, shoot N and P and higher shoot/root ratios than nodulated only plants (Daft and El-Giahmi 1976, Smith and Daft 1977). There are also instances where VAM infection inhibited nodulation, nitrogenase activity and growth (Crush 1974; Bethlenfalvay et al. 1982a; Bethlenfalvay et al. 1982b). Possible reasons for growth reductions will be discussed later.

In addition to affecting the uptake of P, and micronutrients, VAM may also produce certain plant hormones or stimulate plant hormone production (Cooper 1984). Little research has been done in this area, and the possibility that differences in nutrient status between VAM and uninfected plants may be responsible for differences in hormone production has not been discounted (Abbott and Robson 1984; Graham 1987). It has also been proposed that VAM have a direct effect on Rhizobium in addition to the

improvement of host nutrition (Abbott and Robson 1977). Bayne et al. (1984) demonstrated that there appear to be no direct effects of P on nodules or bacteroids. Smith et al. (1979) detected no difference in the P concentration in the nodules of mycorrhizal and non-mycorrhizal legumes. Carling et al. (1978) and Powell (1980) showed that improved nodulation and nitrogen fixation of mycorrhizal vs. non-mycorrhizal clover could be duplicated by P amendments to non-mycorrhizal plants. Therefore, in legumes, it appears that VAM affect the host by improving its nutrient status, which results in more carbohydrates being available to the nitrogen fixer.

Infection by both symbionts poses a carbon cost to the host plant. Paul and Kucey (1981), using Vicia faba, demonstrated that the VAM fungus Glomus mosseae incorporated 1% of plant photosynthates and respired 3%, while the nodules utilized 7-12%. The legume was able to offset these costs with higher photosynthetic rates. Kucey and Paul (1982) recorded increases in CO_2 fixation of mycorrhizal vs. non-mycorrhizal plants of 8-17%. Such values vary depending on the stage of mycorrhizal infection. Cooper (1984) cites some unpublished data which showed that more ^{14}C was incorporated into mycorrhizal vs. non-mycorrhizal roots at early stages of infection. At later stages, similar amounts of ^{14}C were incorporated. Bethlenfalvay et al. (1982b) described growth inhibition of

VAM Glycine max relative to control plants up to 9 weeks of age. After 15 weeks, VAM plants had significantly greater biomass and shoot P than non-VAM plants. Hence, carbon costs to the plant may be higher during establishment of VAM or Rhizobium infection resulting in temporary growth depression relative to uninfected control plants. Therefore, when considering the effects of symbionts on plant growth, it is important to consider the stage of the symbiosis.

The response of a plant to these endophytes also appears to be greatly affected by soil nutrient levels. Soil P directly affects plant P concentration, which in turn appears to be the mechanism by which the plant regulates VAM infection (Menge et al. 1978). High tissue P levels preclude infection of the root, although the fungus is still present in the soil (Mosse 1973). Under conditions of low P availability, endophytic infection is usually beneficial to the plant. However, when P is abundant, there is often no difference in biomass between mycorrhizal and non-mycorrhizal plants (Abbott and Robson 1977, Robson et al. 1981). Bethlenfalvay (1983) described the growth of mycorrhizal and non-mycorrhizal Glycine max at a number of P levels. At very low and high P levels, mycorrhizal plants exhibited 20, 25 and 38% growth reductions relative to non-mycorrhizal P fertilized plants, with a significant growth enhancement (14%) at intermediate

P levels. The proposed explanation was that at very low soil P levels, the carbohydrate drain associated with mycorrhizal infection was not offset by the increased P uptake, thus mycorrhizal plants showed slower growth than non-mycorrhizal plants. At high P levels, the non-mycorrhizal plants absorbed P as effectively as mycorrhizal plants, without the associated carbon cost. Only at intermediate P levels, was the C cost offset by VAM mediated P uptake.

In addition to P, N has been shown to affect both VAM infection and nodulation. Chambers et al. (1980a,b) demonstrated that high levels of ammonium and nitrate ions could inhibit VAM infection. Trinchant and Rigaud (1984) described reduced nodule acetylene reduction activity and respiration with increasing nitrate concentrations. Since the interactions between the host plant and its VAM and nitrogen-fixing endophytes appear to vary with nutrient conditions, defining the effects of endophytes upon the host plant requires that the symbionts be studied at several different nutrient levels. To accomplish this, Abbott and Robson (1984) have discussed the use of response curves. Plants are grown at a series of nutrient levels such that the highest levels correspond to the maximum yield relative to that nutrient. Plants can be compared when they are at the same yield level and therefore likely the same physiological stage. Differences between infected

and uninfected plants would be reflected in the amount of nutrient required for a particular yield. In other words, mycorrhizal plants are compared to nutritionally equivalent non-mycorrhizal plants (Graham 1987; Brown et al. 1988). It can then be determined whether differences are due to nutrient supply alone or if VAM affect host physiology in some way. Also, if a complete growth curve is available, soil type and fertilization regimes are no longer important variables since the plant is grown to its maximum yield under specific environmental conditions.

In summary, associations of plants, nitrogen-fixing bacteria and VAM fungi are well adapted to disturbed conditions. The nitrogen-fixer provides a nitrogen input into nutrient deficient soils. The presence of the VAM fungus enhances nitrogen fixation through the increased uptake of P and perhaps micronutrients. For these reasons, actinorhizal shrubs (and legumes) are being used for reclamation and in mixed forestry planting to enhance the growth of commercial trees. Elaeagnus is a native nitrogen-fixing shrub normally infected with VAM fungi. However, little is known of the biology of these shrubs and how they interact with their endophytes. Although the legume-Rhizobium-VAM symbiosis has received considerable attention, the actinorhizal shrub-Frankia-VAM interaction

involves very different partners which may interact differently.

This project investigated the relative contributions of the nitrogen fixer, Frankia, and VAM fungi to the growth of Elaeagnus commutata (silverberry). A number of questions were addressed:

1) Different Frankia strains can be present in any particular locale and some of these strains are more effective symbionts to the host than others (Normand and Lalonde 1982). Of the strains available for Elaeagnus, and of the strains isolated from the Fort McMurray (FMM) region, which are the most effective in promoting nitrogen fixation by a nodulated-only plant?

2) VAM species can differ in their effects on host growth (Carling and Brown 1980). Glomus etunicatum is common in the Fort McMurray (FMM) area (based on spore extractions), and Glomus aggregatum is a common prairie species. Which of these two species is more effective in promoting the growth of mycorrhizal only Elaeagnus under nutrient poor conditions?

3) Are particular combinations of VAM species and Frankia strains more effective than others with respect to biomass production, nodule development,

rates of nitrogen fixation and shoot nutrient concentration?

4) What is the nature of the interaction between the two symbionts and their host? If there is increased growth, can this be explained by greater nutrient supply alone? At what nutrient levels is infection by these endophytes inhibited?

The following chapters will describe the experiments designed to address these questions.

CHAPTER 2

2. RESPONSE OF ELAEAGNUS TO NITROGEN AND PHOSPHORUS

2.1 PURPOSE

In order to study the interactions among the symbionts, it was first necessary to know the N and P levels required for maximum yield of Elaeagnus in the absence of symbionts. The resulting growth curves were then used to define the fertilizer levels necessary for subsequent experiments. As discussed in the introduction, high P and N levels can inhibit VAM infection and nodulation, respectively. Using the growth curves, P and N levels which allow for endophyte infection of the host and adequate plant growth can be determined.

2.2 METHODS

Elaeagnus commutata seeds (collected in 1985 near Bowness Park in Calgary, Alberta by S. Visser) were stratified under cold running water for 7 days and germinated on moist filter paper in petri dishes. The seedlings were planted into 150cc containers (Ray Leach Cone-tainer Nursery, Canby, Oregon) which had been filled with a mixture (pH 6.0) of one part autoclaved peat (220°C/60 min) to one part >2mm vermiculite. Seedlings were planted over a period of 7 days as the cotyledons emerged from the seed coats. Plants were then placed in a growth chamber with an 18 h photoperiod and day/night

temperatures of 25°C and 15°C respectively. Light levels were 300$\mu E \cdot m^2 \cdot sec^{-1}$. Seedlings were watered three times a week. After the first 1.5 weeks, plants received fertilizer each Monday and Friday.

A modified Crones solution (adapted from Lalonde 1979) was used to fertilize the seedlings. The basic solution contained 0.5 $g \cdot L^{-1}$ $MgSO_4 \cdot 2H_2O$, 0.75 $g \cdot L^{-1}$ KCl, 0.5 $g \cdot L^{-1}$ $CaSO_4 \cdot 2H_2O$, 1 $mL \cdot L^{-1}$ 1% ferric citrate and 1 $mL \cdot L^{-1}$ micronutrients (Allen and Arnon 1955). $Ca(NO_3)_2 \cdot 4H_2O$ and $Ca(H_2PO_4)_2 \cdot H_2O$ were then added in quantities sufficient to give the levels of N and P required for each treatment. Solutions were adjusted to pH 6.0 with 0.1 N NaOH.

The treatments were factorial combinations of four N (0, 50, 100, 200 ppm N) and four P levels (0, 30, 60, 120 ppm P). These treatments will subsequently be referred to as N0-N200 and P0-P120. Five Elaeagnus seedlings were grown at each of these 16 treatments.

Plants were harvested after 8 weeks. Plant height as well as leaf, stem and shoot dry weights were determined (80°C oven dry). Soil was shaken from the roots and saved for pH and conductivity determinations. The roots were then washed free of soil, oven dried at 80°C and weighed. Shoot material was ground in a Wiley mill (40 mesh) and a 0.25 g subsample was digested in H_2SO_4/H_2O_2. The digest

was analyzed for total N and P using a Technicon autoanalyzer II.

2.3 RESULTS AND DISCUSSION

Figure 1 shows the the shoot and root weights of Elaeagnus seedlings at each fertilizer level. Maximum shoot weights were reached in the range from 30 to 60 ppm P and 50 to 100 ppm N. Root weights were more variable in response to fertilization. Maximum root weights were attained from 30 to 60 ppm P at 100 ppm N. The highest phosphorus level (P 120) depressed shoot weights slightly, and roots weights considerably.

Shoot P and N concentrations are shown in Figure 2. At N0, P levels were higher than for any of the other N treatments. For the N50 and N100 treatments, shoot P concentration increased with increasing P amendment. The N200 treatment followed this pattern identically up to P120, where the concentration dropped sharply.

Shoot N concentrations followed a different pattern. Again, plants in the N0 treatment had very low levels of N. At P0, all three remaining N treatments showed very high N concentrations. This was probably a P deficiency effect. At P30 and higher, N levels stabilized. The only oddity was the N50 P30 treatment. The N concentration here was probably lower because this treatment demonstrated the same shoot production as treatments receiving more fertilizer N.

Figure 1: Mean shoot and root weights (±SE) of Elaeagnus commutata grown at four nitrogen and four phosphorus levels.

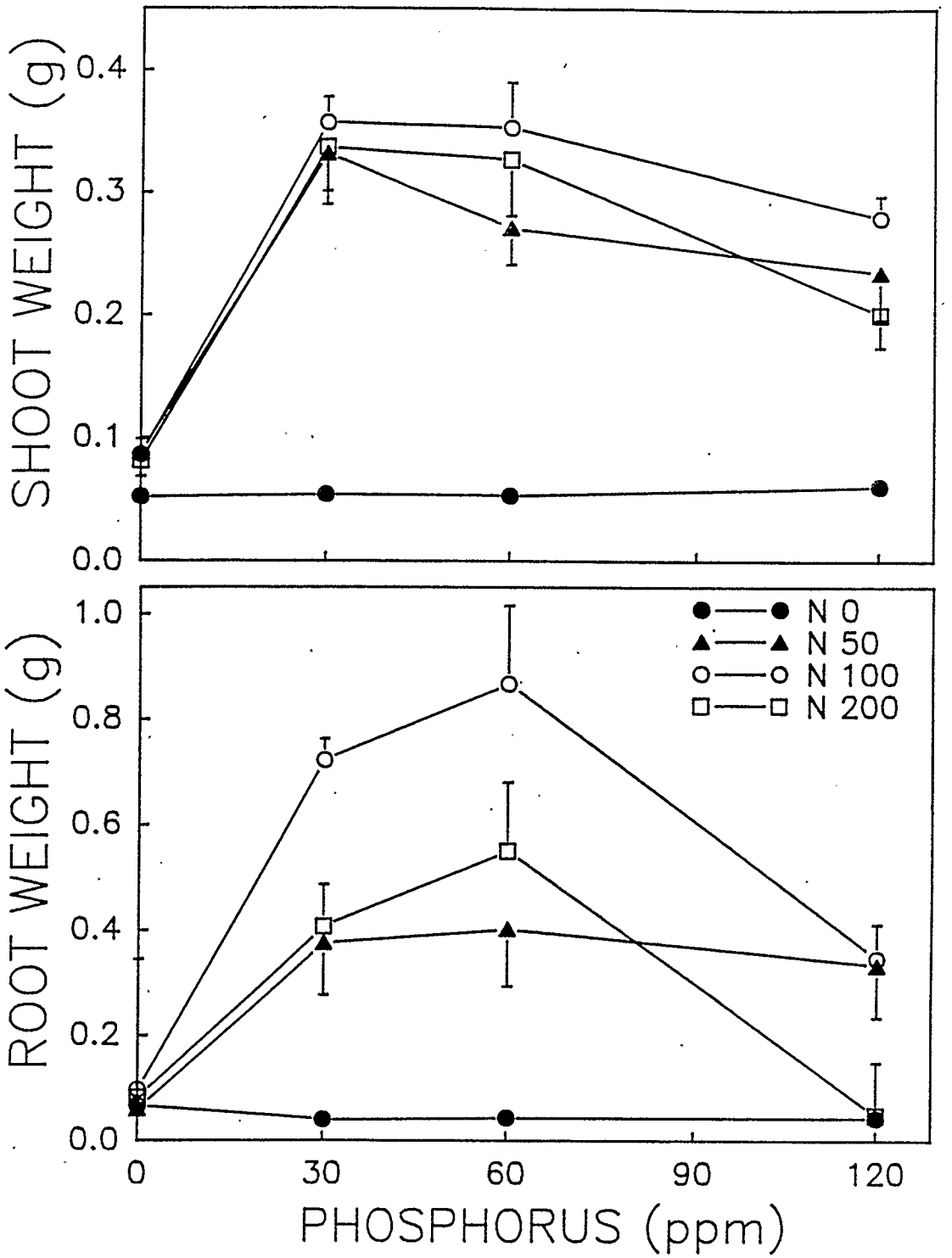
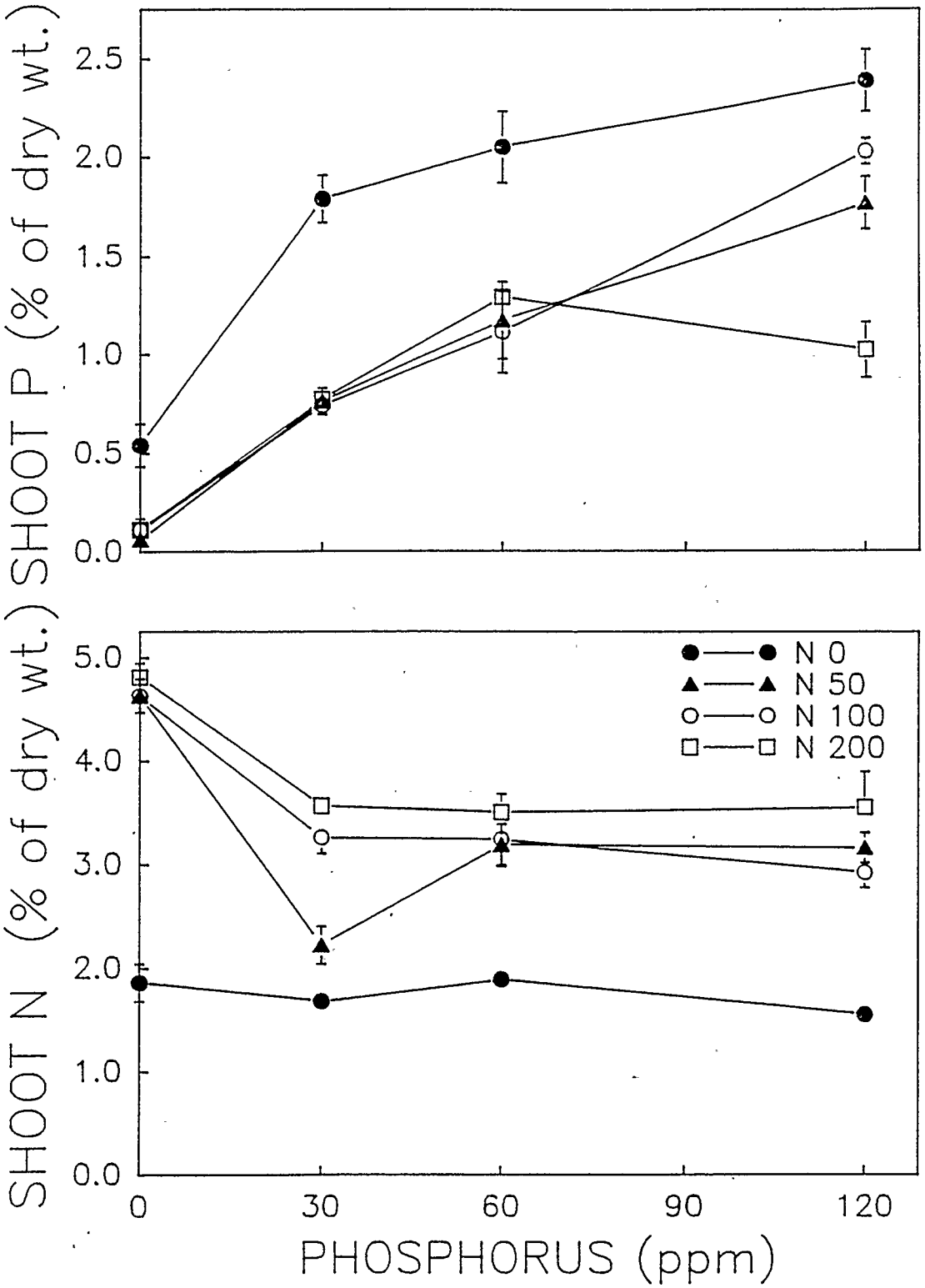

SHOOT WEIGHT (g)
0.0
0.1
0.2
0.3
0.4
ROOT WEIGHT (g)
0.0
0.2
0.4
0.6
0.8
1.0
N 0
N 50
N 100
N 200
0
30
60
90
120
PHOSPHORUS (ppm)

Figure 2: Mean shoot nitrogen and phosphorus concentrations (±SE) of Elaeagnus grown at four nitrogen and four phosphorus levels.


SHOOT P (% of dry wt.)
2.5
2.0
1.5
1.0
0.5
0.0
SHOOT N (% of dry wt.)
5.0
4.0
3.0
2.0
1.0
0.0
N 0
N 50
N 100
N 200
0
30
60
90
120
PHOSPHORUS (ppm)

Conductivity and pH measurements were made on the planting mixtures after harvest. Fertilization had a statistically significant effect on both soil pH (NxP $p<0.0001$) and conductivity (NxP $p=0.0049$) (the pH levels of the fertilizer solutions had been standardized to 6.0). The pH values ranged from 5.3 to 6.1 with a mean pH of 5.8. Most treatments were near this value. Only the treatments receiving high N and little or no P had low soil pH levels (<pH 5.5) The same pattern was observed in the conductivity measurements, which were extremely variable. Conductivity varied from 0.305 to 2.5 $mS \cdot m^{-1}$ with a mean 0.999 $mS \cdot m^{-1}$. Analysis of PO_4 levels in the fertilizer solutions demonstrated that the available P levels were very close to defined levels.

The plants in this experiment never looked healthy and older leaves turned brown at the tips, yellowed and fell off. It is possible that the poor health of the plants may have been due to K toxicity, especially considering the low N and P levels used. The K level recommended in the Crone's solution (600 ppm K) was considerably higher than that found in commercial fertilizers (eg. Plant Prod). However, it is difficult to differentiate between nutrient deficiency and toxicity symptoms in plants (Agrios 1969).

From these results decisions were made on the fertilizer levels for subsequent experiments. Considering the growth curves alone, maximum shoot production was

attained at P30 N100, with maximum root production at P60 N100. Although the P30 N50 treatment produced essentially the same shoot weights, considering the poor health of the plants, and that subsequent experiments would be grown under higher light intensities for longer durations, it was felt that the N100 and P60 fertilizer levels were more representative of the nutrient levels necessary to attain maximum growth rates. As well, previous fertilizer response experiments using Plant Prod 28-14-14 (Visser and Danielson 1988) had shown maximum growth rates for Elaeagnus at 400 $mg \cdot L^{-1}$, which was equivalent to 112 ppm N and 56 ppm P applied twice weekly.

CHAPTER 3

3. ISOLATION AND CULTURE OF FRANKIA STRAINS

3.1 PURPOSE

Elaeagnus commutata is one of the actinorrhizal shrubs being evaluated on oil sand reclamation plots in the Fort McMurray area. One of the goals of this project was to determine the relative dependence of *Elaeagnus* on each of its symbionts. To do this effectively for *Frankia*, it was felt that local strains should be considered as well as strains already available. Therefore, nodules from reclamation plots at the Syncrude Canada Ltd. oil sand extraction plant (Visser and Danielson 1988) were collected and endophyte isolations attempted. Strains were isolated and growth curves in liquid culture were produced. A strain donated by M. Lalonde (Univ. Laval) was also included in the investigations.

3.2 METHODS

Experiment 1. Strain Isolations

Frankia was isolated from nodules harvested in 1984 from *Elaeagnus* and *Shepherdia canadensis* (L.) Nutt. seedlings which had been planted at a Syncrude reclamation area (Visser and Danielson 1988).

The isolation method used was that of Lalonde *et al.* (1981) as modified by Diem and Dommergues (1983). Nodules were washed in running water and then blotted dry with

paper towels. For each isolation, approximately 100 hydrated, young and healthy looking nodule lobes were excised using a scalpel. The lobes were placed in gauze and immersed in 3% osmium tetraoxide for 4 min after which they were rinsed three times in sterile deionized water. Nodule lobes were then incubated in 2% polyvinylpyrolidone (PVP) in phosphate buffered saline (PBS) for 15 min. They were then crushed with a mortar and pestle in fresh buffer. Individual pieces of the nodules were transferred to culture tubes containing the culture medium QMOD (Lalonde and Calvert 1979) with 0.3% agar. Fifty tubes of nodule pieces from each of the inoculated and uninoculated treatments as well as pieces from Shepherdia nodules were sealed and incubated at 28°C. Eight weeks later, mycelial growth was observed on some nodule fragments.

Eleven of 50 tubes containing Elaeagnus nodule fragments from the inoculated treatment produced probable Frankia colonies. One tube was sampled to verify that the colonies were Frankia. Four of 50 tubes from the uninoculated treatment contained mycelial colonies. None of the tubes incubated with Shepherdia nodule pieces produced colonies, and most became contaminated.

Each colony was placed in a sterile 50 mL culture tube containing 20 mL of Baker and Torrey medium (Baker and Torrey 1979) and glass chips (Zak 1976). The glass chips were used to break up the colonies using a vortex mixer

every week. This resulted in faster mycelium growth. Frankia colonies were very tough, and homogenizing the cultures was difficult throughout the course of experiments.

The isolates from the uninoculated treatment (EANfma and EANfmb) grew quickly in Baker and Torrey broth. The EANfm+ isolate (from the inoculated treatment) grew very slowly and was therefore transferred to QMOD broth (Lalonde and Calvert 1979; Carpenter and Robertson 1983). This isolate grew just as slowly in this medium, therefore further culturing was done with Baker and Torrey broth. This is also a complete medium, but is much easier to prepare. The EANfm+ isolate seemed to be simply a very slow growing form.

Frankia cultures were maintained on yeast extract broth (Baker and Torrey 1979). Cultures were propagated by inoculating 200-250 mL broth with 10 mL of inoculum from an older culture. The flasks were incubated at 28°C and shaken by hand every few days.

As well as culturing Fort McMurray isolates, a culture (EAN1) donated by M. Lalonde (U. Laval) was also propagated as described above. This strain grew quite quickly on yeast extract broth.

Experiment 2. EAN1 Growth Curve

In order to determine the growth rates of Frankia strain EAN1, culture tubes containing 11 mL yeast extract broth were inoculated with 1 mL 8 week-old inoculum which had been cultured on the same medium. Four replicates were harvested immediately by washing three times with 0.15 N NaCl (5 min centrifuging at 5000 rpm, decanting between washes). After the final washing, the supernatant was removed leaving 1 mL in the graduated tube. The cells were then sonicated for 3 min at 200 W with a Braun Sonicator. The dye binding assay of Bradford (1976) was used to colorimetrically determine the soluble protein content of 0.2 to 0.4 mL subsamples of the sonicated cells. Bovine serum albumin was used as the standard. Further samples (four replicates each) were analyzed on days 3, 5, 7, 13 and 34.

Experiment 3. EANfma, EANfmb and EANfm+ Growth Curves

Culture tubes were set up as above and inoculated with 1 mL each of either EANfma, EANfmb or EANfm+ culture (10 weeks old). Cells were harvested weekly for 7 weeks as described above, except that sonication time had to be increased as the density of the cells increased (5 min on days 35 and 49).

3.3 RESULTS AND DISCUSSION

The growth curves for the strains studied are shown in Figures 3 and 4. EAN1 was in the lag stage of growth until at least day 15. Only one other sampling time was possible, and the protein content on day 34 seems to indicate that by this time, the culture was in the exponential growth phase (Figure 3). More samples would have been desirable to confirm the period of exponential growth. The length of time in the lag phase depended on the age and amount of inoculum used. However, it appeared that harvesting cultures at 4 to 6 weeks of age would ensure log-phase cells for inoculation. Not enough tubes were set up to complete the entire growth curve, but the goal of determining the period of log-phase growth was achieved.

Figure 4 displays the growth curves of EANfm+, EANfma and EANfmb. EANfma did not grow in the culture tubes although it had been growing in the culture flasks. EANfmb grew well and reached log-phase growth from weeks 3 to 5. EANfm+ grew much more slowly, with much less visible mycelium in the vials. However, protein contents were quite high. Log-phase growth seemed to occur from weeks 1 to 4. EAN1 had a doubling time of 7.5 days, while EANfmb and EANfm+ doubled in approximately 14 days. This is in contrast to the 48 h doubling times which can be achieved

Figure 3. Growth of *Frankia* strain EAN1 in yeast extract broth over time as measured by protein content (µg).

Each data point represents the mean±SE of four replicates. For most points the standard errors are very small.

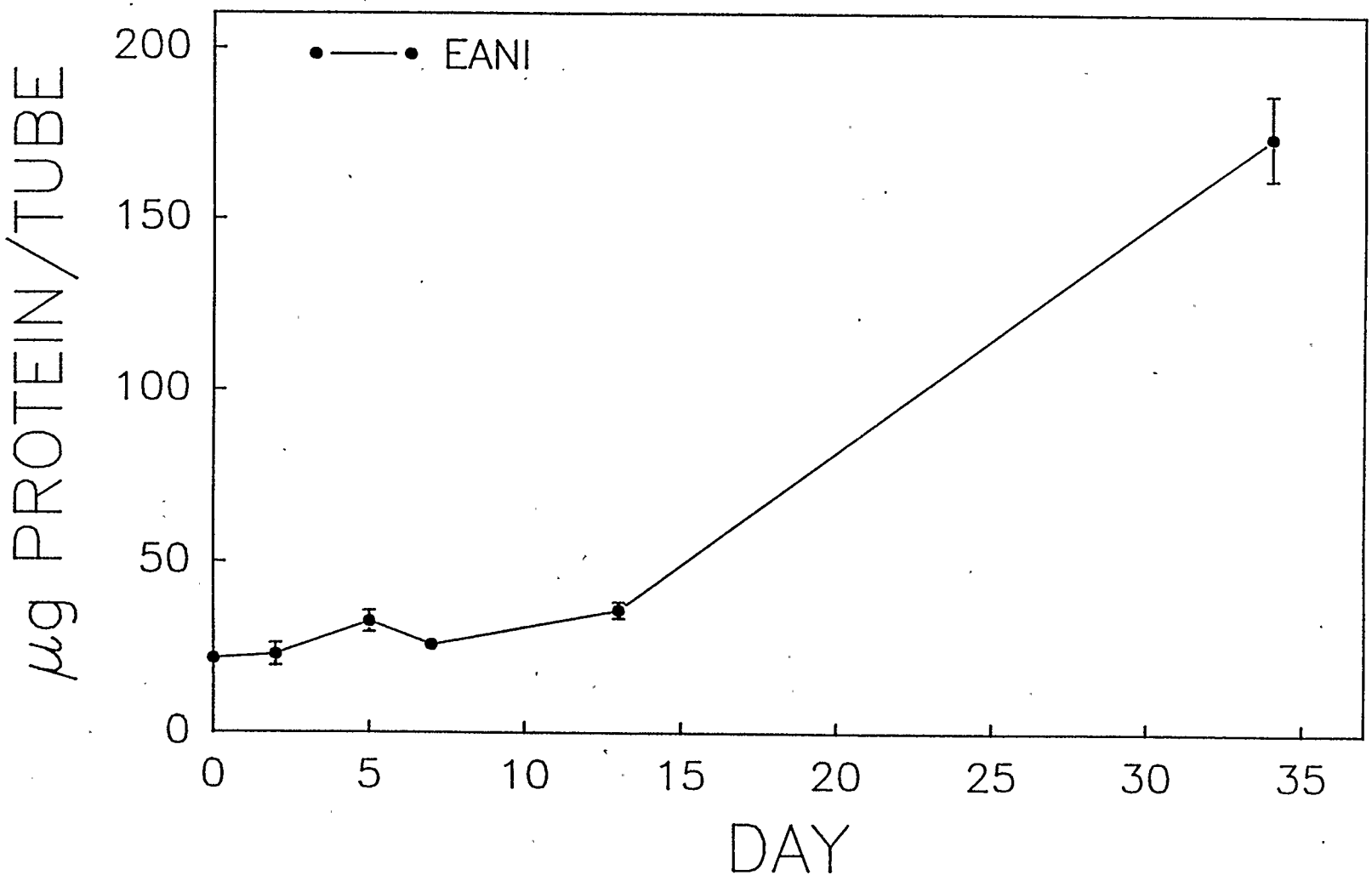
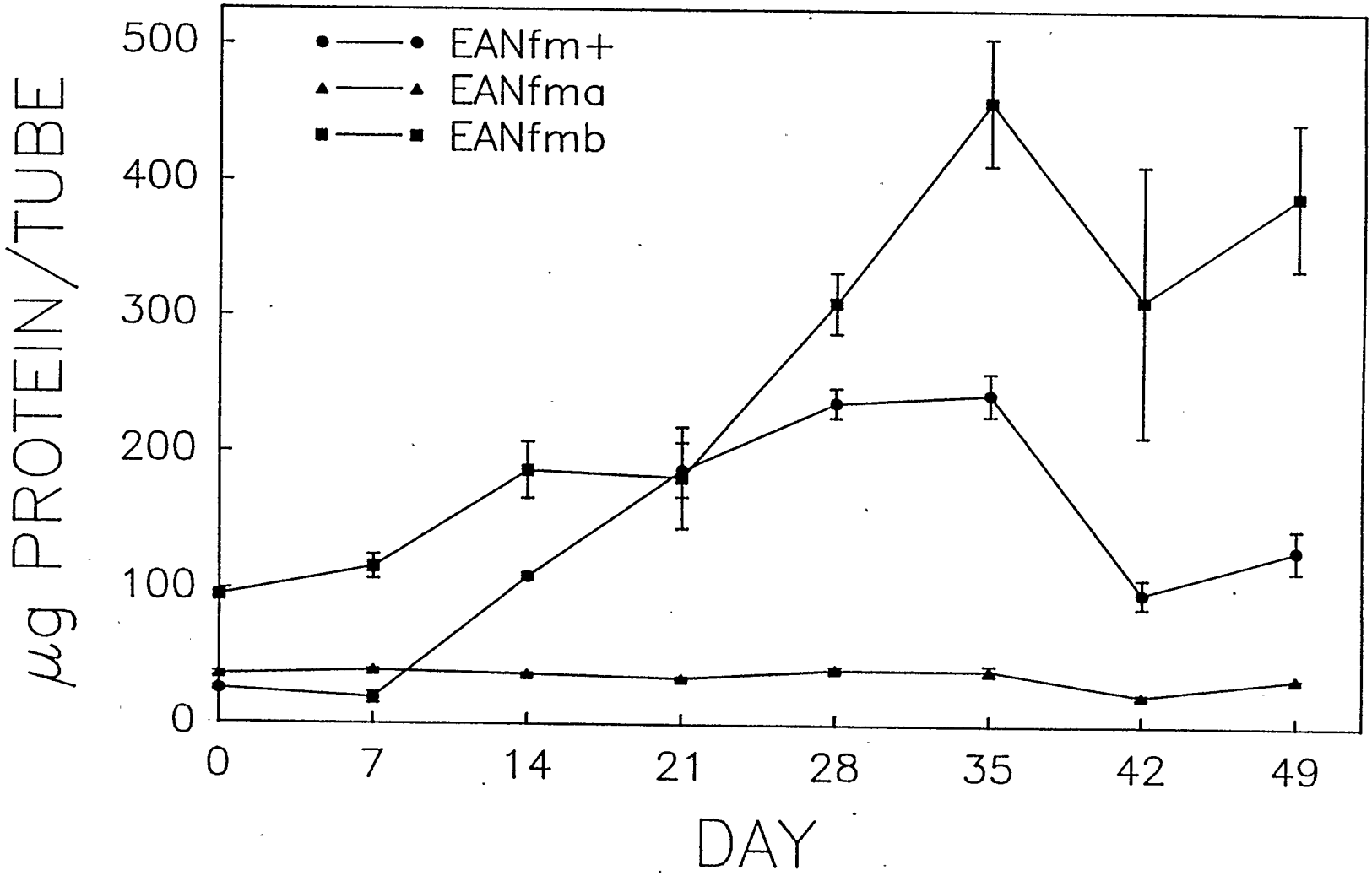

EANI
200
150
100
50
0
μg PROTEIN/TUBE
0
5
10
15
20
25
30
35
DAY

Figure 4. Growth of Frankia strains EANfm+, EANfma and EANfmb over time as measured by protein content (µg).

Each data point represents the mean±SE of four replicates.


EANfm+
EANfma
EANfmb
500
400
300
200
100
0
μg PROTEIN/TUBE
0
7
14
21
28
35
42
49
DAY

with an Alnus Frankia isolate using defined media in air-sparged vessels (Murry et al. 1984).

The lower protein values on day 42 were probably due to a 3 min sonication rather than the 5 min duration used on days 35 and 49. As well, the lower values on day 49 may not reflect a leveling off of the growth rate but may be due to insufficient sonication time. The cultures were extremely resistant to sonication; even after 5 min, were often still visible. Burggraaf and Shipton (1982) sonicated for only 1 min at 150 W. Murry et al. (1984) used a 15 sec sonication at 100W followed by 10 min digestion in 0.3 N NaOH. Sonicating for long periods of time heats the sample (even though cooled with an ice-water bath during the procedure) thereby risking loss of volume due to evaporation and denaturing of the proteins.

An additional problem was found with the protein assay. On performing the assay, the solution should have remained stable for approximately 1 h (Bradford 1976; Spector 1978); but this was not the case. Instead, absorbance measurements began to drop after about 5 min. This may have been due to the brand of dye used in the procedure. The assay was developed using Coomassie brilliant blue G-250. The original manufacturer stopped producing it, and similar dyes produced by other companies apparently exhibit different solubilities and stability in solution (Zaman and Verwilghen 1980). This may explain the

early precipitation of the assay solution. Other protein assays were not attempted since adequate growth curves had been produced.

Using the protein content to standardize inoculum was also questioned since there appeared to be little relationship between the amount of visible mycelium in the vials and the actual protein content. For example, very little EANfm+ mycelium was visible, yet protein contents were high. Since hyphal fragments and spores represent the infective particles, it was felt that protein content might not reflect propagule numbers.

CHAPTER 4

4. COMPARISON OF VAM SPECIES AND INOCULUM STANDARDIZATION

4.1 PURPOSE

The second question posed was whether VAM species differ in their effects on host growth. To investigate this, the effects of three VAM species on Elaeagnus growth were compared. Plants were grown at low P and adequate N levels in order to define plant response to VAM in the absence of Frankia. In addition, high and low VAM inoculum concentrations were compared in an attempt to standardize inoculum levels. Standardizing VAM inoculum is normally quite difficult as spores, root fragments and hyphae are all sources of infection. Daft and El-Giahmi (1974) and Wilson (1984) showed that the level of inoculum used affected the degree of mycorrhizal infection. Haas and Krikun (1985) found positive correlations between initial propagule numbers and plant height and weight. Therefore, to ensure that growth differences among plants infected by different VAM species were not due to differences in inoculum levels, it was necessary to ensure an overabundance of infective propagules.

4.2 METHODS

Glomus fasciculatum (Thaxter sensu Gerd.) Gerdemann and Trappe, G. etunicatum Becker and Gerdemann, and

G. aggregatum Schenck and Smith, were grown with Elaeagnus commutata at a number of inoculum levels. Mycorrhizal inoculum was produced by growing the fungus on the roots of either Agropyron trachycaulum (Link) Malte (slender wheatgrass) or Allium porrum L. (leek) in a pot culture. Pot cultures can be started using spores seived from field soil, roots and field soil, or root fragments and soil or spores from a previous pot culture. In the present study, G. aggregatum was isolated in pure pot culture from a prairie soil by John Zak; G. etunicatum was isolated by placing spores seived from a mixed culture onto leek roots; and the G. fasciculatum culture originated from infected roots which were washed to remove adhering soil. The G. fasciculatum culture appeared to be pure, although it was possible that more than one species was present. Pot cultures were kept in the greenhouse and watered three times a week. This was supplemented weekly with 5 ppm P Long-Ashton's fertilizer solution.

Pot cultures were harvested when five months old. The roots were finely chopped and well mixed with the rest of the culture soil containing hyphae and spores. Inoculum was mixed by volume with a planting mixture (pH 6.0) containing one part autoclaved peat (60 min) to one part >2mm vermiculite. Control treatments represented the corresponding percentage of autoclaved inoculum (20 min). For each treatment, 10 to 20 containers were each filled

with approximately 200 mL soil. The treatment combinations were as follows:

TREATMENT	INOCULUM CONCENTRATION		
	15%	30%	50%
G. fasciculatum	10 plants	20	10
G. fasc. control	10	20	10
G. aggregatum	10	20	-
G. etunicatum	10	20	-
G. etun. control	10	20	-

Two control treatments were necessary since G. fasciculatum and G. aggregatum had been pot cultured using slender wheatgrass, a plant with fine roots, as the host, while G. etunicatum was propagated on coarse rooted leek. This allowed identification of effects of different types of root on VAM infection and plant growth. A 50% inoculum treatment for G. fasciculatum was used because the infection levels of stained roots from these pot cultures were very low (<10%). In contrast, >75% of root pieces were infected in the G. aggregatum pot culture, with 25-50% infection for G. etunicatum cultures. These figures represent subjective estimates made using a dissecting microscope.

Elaeagnus seeds were stratified and germinated as described in Chapter 2 and planted into the treatment soils listed in the table above. After one week, the plants were moved to a growth chamber with a 16 h photoperiod and day/night temperatures of 25°C and 15°C respectively. Light levels were 300$\mu Em^{-2}sec^{-1}$.

Plants received deionized water each Monday and Wednesday, and fertilizer solution each Friday. Seedlings were fertilized after two weeks of age with a modified Crone's solution (adapted from Lalonde 1979) containing 0.5 $g \cdot L^{-1}$ $MgSO_4 \cdot 7H_2O$, 0.75 $g \cdot L^{-1}$ KCl, 0.5 $g \cdot L^{-1}$ $CaSO_4 \cdot 2H_2O$, 1 $mL \cdot L^{-1}$ ferric citrate, 1 $mL \cdot L^{-1}$ micronutrient (Allen and Arnon 1955) 0.0203 $g \cdot L^{-1}$ $Ca(H_2PO_4)_2$ (5 ppm. P) and 0.4215 $g \cdot L^{-1}$ $Ca(NO_3)_2 \cdot 4H_2O$ (50 ppm. N). This solution was adjusted to pH 6.0 with 1 N NaOH.

An initial assessment of VAM infection levels in the 30% inoculum treatment occurred at 6 weeks. There were two reasons for the initial sampling. First, it allowed for the detection of possible temporary plant growth depressions during establishment of the fungal biomass (Bethlenfalvay et al. 1982b). Secondly, a common method of estimating inoculum concentration involves sampling plants every few days in order to establish how quickly they become infected with VAM fungi. The six week sampling was a very abbreviated check to ensure that plants inoculated with all three VAM species became infected at approximately the same time. Up to 10 plants from each treatment at the 30% inoculum level were harvested. Severely stunted seedlings were discarded as they were not representative of treatment effects. Since there was evidence of fungus gnat infection, remaining seedlings were drenched with quarter strength Diazinon. This was repeated four weeks later.

Shoot condition, height and dry weight (80°C) were determined. Roots were washed free of soil and a subsample was taken for mycorrhizal assessment. The remaining roots were oven-dried at 80°C and weighed. Subsamples represented only young roots as suberized roots cannot be evaluated successfully for infection. Roots were cleared (10-15 min), stained (10 min) (Phillips and Hayman 1970) and then mounted on glass slides for VAM assessment (Zak and Parkinson 1982).

The final harvest occurred when seedlings were 12 weeks old. The previous procedure was followed except that the roots subsampled for mycorrhizal assessment were stored in FAA (formaldehyde, acetic acid and alcohol) for two weeks prior to staining. This was necessary due to time contraints. Shoot and root N and P contents were determined as described in chapter 2.

4.3 RESULTS AND DISCUSSION

Table 1 lists the shoot height and biomass production of seedlings harvested at 6 weeks at the 30% inoculum level. There were no significant differences in shoot heights or shoot and root weights. However, G. aggregatum and G. etunicatum inoculated plants showed somewhat lower shoot and root weights than the other treatments. This initial depression in biomass production

Table 1. Height (cm) and biomass (mg) of Elaeagnus commutata in response to two endomycorrhizal species at 30% inoculum at 6 weeks.

Treatment	n	Shoot Ht.	Shoot Wt.	Root Wt.
G. fasc.	10	6.9±0.5	212±30	110±23
G. fasc. control	10	6.6±0.6	209±20	120±16
G. etun.	6	5.9±0.6	190±18	70±13
G. etun., control	10	7.4±0.3	237±14	85± 9
G. agg.	7	5.6±0.5	140±15	68±12
Probability		0.1719	0.0530	0.1217

The data were analyzed by one-way ANOVA. The P values associated with the F value are given and show no significant differences among treatments. Means are given with their standard errors.

Table 2. Biomass (mg) of Elaeagnus commutata in response to two endomycorrhizal species at two inoculum levels at 12 weeks.

Treatment	n	Shoot Wt.	Stem Wt.	Root Wt.
Control	73	653±18 b	138± 5 b	499±16 a
G. etun. 15%	8	468±46 a	82±10 a	279±19 b
G. etun. 30%	10	559±40 ab	108±13 ab	379±28 b
G. agg. 15%	10	564±39 ab	119±13 ab	333±29 b
G. agg. 30%	10	503±46 a	96± 9 a	306±30 b

Values in each column labelled with the same letters represent treatments not significantly different by the Tukey multiple comparison test following one-way ANOVA. Means are given with their standard errors.

may be due to the carbon cost of establishing the fungal symbiont biomass (Bethlenfalvay et al. 1982b).

Biomass production after 12 weeks is given in Table 2. Since the two control treatments (different pot culture host species) did not differ significantly, and since the G. fasciculatum treatment did not become mycorrhizal, these seedlings were all combined to increase the number of control seedlings to 73. The G. etunicatum 15% and G. aggregatum 30% inoculum treatment groups had significantly smaller total shoot and stem weights than the control treatment plants, but such differences with other mycorrhizal treatments were not significant. All mycorrhizal seedlings had significantly smaller root systems than the controls. This is consistent with the fact that fungal hyphae increase the absorptive area in the soil making the plant less dependent on roots.

These data suggest that infection by neither G. aggregatum nor G. etunicatum resulted in increased plant growth over controls. There is instead a trend toward reduced biomass with mycorrhizal infection, although this was significant only for the G. etunicatum 15% and G. aggregatum 30% shoot weights, and the root weights. The lack of response to VAM infection is surprising considering the low P levels (5 ppm) and may have been due to container size effects (Baath and Hayman 1984). If the container size is too small, the plant root system can

explore the soil volume as effectively as a plant with mycorrhizal fungi without the associated carbon cost. Visser and Danielson (1988) compared the growth of mycorrhizal, nodulated Elaeagnus with non-symbiotic plants in 150 cc and 65 cc containers. They recorded a growth response to symbiont infection only in the 150 cc containers. This would indicate that container volume was probably not the reason for the lack of growth response in this experiment, where 150 cc containers were used.

The time of harvest is an important factor in whether or not a positive growth response is recorded (Sanders et al. 1977). Smith and Daft (1977) recommended time course studies when looking at tripartite symbioses. Bethlenfalvay et al. (1982a) detected growth inhibition of VAM, nodulated Glycine relative to control plants at 9 weeks of age, but positive growth responses after 15 weeks. Considering the slow growth rate of Elaeagnus relative to legumes, 12 weeks may have been too soon to harvest these seedlings, thus masking treatment effects. However, harvesting later would have risked the confounding effects of container volume.

Table 3 lists the shoot tissue P and N concentrations of the harvested seedlings. The G. etunicatum treatments and control showed significantly lower shoot P concentration than the G. aggregatum infected seedlings. All mycorrhizal seedlings had significantly

Table 3. Shoot tissue nutrient concentrations (% of dry weight) of Elaeagnus commutata in response to two endomycorrhizal species at two inoculum levels.

Treatment	n	Shoot P	Shoot N
Control	39	0.17±0.00 a	0.95±0.04 a
G. etun. 15%	5	0.18±0.01 a	1.22±0.07 b
G. etun. 30%	5	0.16±0.01 a	1.08±0.05 b
G. agg. 15%	5	0.25±0.02 b	1.18±0.06 b
G. agg. 30%	4	0.20±0.01 b	1.21±0.04 b

Values in each column labelled with the same letters represent treatments not significantly different at $p \leq 0.05$ by the Tukey test following one-way ANOVA. Means are given with their standard errors.

Table 4. Root tissue nutrient concentration (% of dry weight) of Elaeagnus commutata in response to two endomycorrhizal species at two inoculum levels.

Treatment	n	Root P	Root N
Control	39	0.18±0.01 a	1.14±0.05 a
G. etun. 15%	5	0.16±0.02 a	1.10±0.14 a
G. etun. 30%	4	0.18±0.03 a	1.32±0.14 ab
G. agg. 15%	5	0.28±0.02 b	1.48±0.10 b
G. agg. 30%	5	0.28±0.03 b	1.57±0.07 b

Values in each column labelled with the same letters represent treatments not significantly different at $p \leq 0.05$ by the Tukey test. Means are given with their standard errors.

higher shoot N concentrations than control seedlings. Root tissue nutrient concentrations are shown in Table 4. Again, the G. aggregatum treatments had significantly greater P concentrations than the control and G. etunicatum infected seedlings. A similar trend was observed for N, except for the G. etunicatum 30% treatment which displayed an intermediate value.

Mycorrhizal infection levels at the 6 and 12 week sample times are shown in Table 5. Glomus fasciculatum did not infect the seedlings. Glomus aggregatum showed significantly higher infection levels than G. etunicatum at 6 and 12 weeks.

Infection differences between the 15% and 30% inoculum levels were not significant for either species. Similarly, there were no significant differences in plant growth or tissue nutrient concentrations between the 15% and 30% inoculum concentrations. Therefore, the 15% inoculum level was sufficient to ensure maximum infection levels.

Although the two VAM species did not show differential effects on plant growth, there were apparent differences between the species. Glomus aggregatum infected Elaeagnus roots twice as extensively as G. etunicatum. Abbott and Robson (1981) showed that different fungal species can produce different amounts of infection. As well, G. aggregatum-infected seedlings

Table 5. Mycorrhizal infection (percent of root length) of *Elaeagnus commutata* in response to inoculation with two *Glomus* species at two inoculum levels.

Treatment	n	Hyphae	Arbusc.	Vesic.	Total
6 WEEK SAMPLING					
Control	20	-	-	-	-
G. *fasc.* 30%	10	-	-	-	-
G. *etun.* 30%	6	11±2	14±3	<1	26±5
G. *agg.* 30%	7	32±6	35±6	<1	68±9
MSE		0.0197	0.0241	0.0009	0.0520
P		0.0054	0.0116	0.1203	0.0033
12 WEEK SAMPLING					
Control	40	-	-	-	-
G. *agg.* 15%	10	41±2 a	37±2 a	4±1 a	81±3 a
G. *agg.* 30%	10	40±4 a	39±3 a	1±0 b	80±4 a
G. *etun.* 15%	8	14±3 b	29±5 a	1±0 b	44±7 b
G. *etun.* 30%	10	14±2 b	30±4 a	1±0 b	44±4 b
MSE		0.0130	0.0151	0.0030	0.0292
P		<0.0001	0.1201	0.0001	<0.0001

Values in each column labelled with the same letters represent treatments not significantly different at $p \leq 0.05$ by the Tukey test following one-way ANOVA. Data were transformed (arcsin(sqrt(x)) prior to analysis. Means are given with their standard errors.

showed higher shoot and root P and root N concentrations than G. etunicatum infected seedlings or control seedlings, even though there were no significant differences in biomass between plants infected by the two VAM species. The higher nutrient concentrations of the G. aggregatum tissues was probably related to greater fungal colonization by this species. The higher root N concentration, at a similar biomass to other treatments, is suggestive of nitrogen uptake by VAM.

CHAPTER 5

5. EXPERIMENTAL CONDITIONS NECESSARY FOR NODULATION

5.1 EXPERIMENT 1. FACTORS AFFECTING NODULATION

5.1.1 PURPOSE

Numerous attempts at performing Frankia inoculum concentration experiments and strain comparisons failed when experimental plants would not nodulate. In order to determine what factors were preventing the nodulation of test plants, an experiment was set up to investigate the effects of soil pH, inoculation method and inoculum age on nodulation.

In earlier experiments, the pH of the peat:vermiculite mixture was 6. This was adequate for both nodulation and VAM infection of test plants. However, subsequent batches of peat had a lower pH and plants grown at this pH did not nodulate.

Another reason for poor nodulation may have been the method of Frankia inoculation. In preliminary experiments, the inoculum had been applied to the soil surface as per Périnet et al. (1985), but the seedlings did not nodulate. There was a concern that the Frankia cells were not reaching the root system in adequate numbers. Therefore, it was felt that injecting the inoculum into the soil, where the cells would be closer to the young roots would be more effective than applying it to the surface.

Inoculum age was another factor that may have influenced nodulation success. It was expected that log phase cells would represent the most effective inoculum. However, there was a possibility that since Frankia is very slow growing, older cultures might still be viable. Therefore, 6 and 15 week cultures were compared with respect to their infectivity.

5.1.2 METHODS

Based on the foregoing comments, a 2x2x2 experiment was set up to assess the effects of soil pH, inoculation method and inoculum age on the nodulation success of test plants. A peat:vermiculite planting mixture was prepared as described previously. Half of this was amended with 5 $g \cdot L^{-1}$ CaCO3. Both treatment groups of soil were then autoclaved (20min). The pH level stabilized at 7.1 in the $CaCO_3$ treated soil and at pH 5.0 in the untreated soil after two weeks. Containers (150cc) were filled with the two soil mixtures into which two stratified Elaeagnus seeds/container were planted. After 18 days, most of the seeds in the pH 7.0 treatment had germinated, whereas only approximately 40% had germinated in the pH 5.0 treatment. Seedlings were transplanted from pH 7.0 to pH 5.0 soil to try to even out the number of replicates in each treatment. Seedlings were then taken to the greenhouse and inoculated with Frankia two days later. The inoculum consisted of

either 6 or 15 week old EAN1 cells cultured in a yeast extract broth (Baker and Torrey 1979). Cells of each age group were washed three times in phosphate buffered saline (PBS) (centrifuged 10,000 rpm for 10 min) and filtered onto 0.3 um millipore paper. Each type of inoculum was standardized by wet weight such that 1.36 g Frankia mycelium was dispersed in 300 mL PBS. The mixture was homogenized briefly in a virtis blender prior to seedling inoculation. Each seedling received the equivalent of 22.6 mg cells (wet weight) in 5 mL of PBS, applied either to the soil surface or injected into the root region using a syringe and canula. Seedlings were fertilized once a week with 100 $mg \cdot L^{-1}$ of 28-14-14 commercial fertilizer (Plant Prod).

Plants were harvested 6.5 weeks after inoculation and only the presence or absence of nodules was recorded. There were no apparent differences in shoot heights. The binomial data (# nodulated vs # not nodulated) were arranged in contingency tables and the Chi-square test used to test for treatment effects (Appendix I). It was assumed that there were no interactions amongst the different treatments.

5.1.3 RESULTS AND DISCUSSION

Nodulation of test plants occurred only in soil at pH 7.0 (Table 6). The actual pH range in which nodulation occurred is discussed later in this chapter.

There was a significant difference in nodulation success between inoculation by injection vs surface application when using 6 week old inoculum (Table 6, Appendix I). This difference was not significant with 15 week-old inoculum. Apparently, surface application at the rate used did not result in enough Frankia cells reaching the rhizosphere of test plants. Périnet et al. (1985) regularly use surface application of inoculum as this can be easily mechanized for greenhouse and commercial applications. In their study, nodulation success was 90% for E. commutata and 95% for E. angustifolia following application with an overhead greenhouse watering system.

Even though more plants became nodulated with 6 week-old inoculum than with 15 week-old inoculum, the difference was not significant for either the surface applied or injected inoculum (Table 6, Appendix I). Murry et al. (1984) observed that Frankia cells of strain $EaN1_{pec}$, isolated from Elaeagnus, can remain viable for up to a year in a rich organic medium when compared to a defined medium containing only specific carbon sources.

Table 6: Percent Elaeagnus seedlings nodulated after 6.5 weeks under conditions of variable pH, type of inoculation and age of inoculum.

Inoculum Age	Inoculation Method	n	pH 5.0	n	pH 7.0
6 weeks	surface	7	0	11	9.1
	injection	7	0	13	61.5
15 weeks	surface	8	0	13	15.4
	injection	7	0	13	30.8

5.2 EXPERIMENT 2. RESPONSE TO SOIL pH

5.2.1 PURPOSE

The previous experiment showed that Elaeagnus nodulated at pH 7.0 but not at pH 5.0. Therefore an experiment was set up to investigate in greater detail the effect of pH on nodulation over the pH 5.0 to 7.0 range.

5.2.2 METHODS

The peat:vermiculite mixture described previously was amended with $CaCO_3$ such that the treatment soils would stabilize at pH 5.0, 5.5, 6.0, 6.5 and 7.0. Stratified seeds were planted into the soils and inoculated by injecting unhomogenized inoculum 3 weeks after planting. This allowed sufficient time for seeds to germinate (14 days), and the pH to stabilize. Ten plants were grown at each pH level.

5.2.3 RESULTS AND DISCUSSION

The results of this experiment were required to allow initiation of subsequent experiments. Therefore, the 2 weeks normally allowed for the pH of the peat:vermiculite mixture to stabilize after $CaCO_3$ amendment were not taken. Since it takes two weeks for seedlings to germinate, and another few days before leaves emerge, it was felt that this was adequate time for the pH to reach the desired level before inoculating Frankia by injection. Table 7 lists the pH values over the course of the experiment.

Seedlings were injected on Jan. 26, 2.5 weeks after the soil was amended with $CaCO_3$. The pH readings of Feb. 12, 2.5 weeks later, were close to the desired treatment levels. Therefore, the pH levels were probably close to desired levels at the time that nodulation would be occurring. Also, the Feb. 12 pH measurements were taken from soil kept at 4°C. Soils in the greenhouse which were watered and given fertilizer (Plant Prod 28-14-14) weekly would have had higher pH values. This probably explains the higher pH values recorded at harvest (soil from containers, March 8). Table 7 shows that nodulation occurred only at pH 7.0. This may be due to the fact that the cultures were maintained at pH 7.0 and were therefore sensitive to lower soil pH levels. S. Visser (pers. comm.) achieved nodulation of E. commutata at pH 6.0 or higher when using soil as Frankia inoculum. Gardner (1958) recorded nodulation of E. angustifolia at pH 6.0 but not at pH 5.0. Griffiths and McCormick (1984) showed that Alnus glutinosa nodulated best from pH 5.5 to pH 7.0, while Smolander et al. (1988) found that optimum nodulation of A. glutinosa and A. incana occurred at a pH greater than 6.0. Elaeagnus is normally found in soils of a much higher pH than Alnus, and this could affect the pH range in which nodulation is optimal.

Table 7: Percentage nodulation of Elaeagnus seedlings at five pH levels.

Treatment pH	pH Jan.8 seeding	pH Feb.12 2 weeks after inoc.	pH Mar.8 harvest	n	Percent Nodulated
5.0	4.2	4.8	5.2	6	0
5.5	4.6	5.5	5.7	6	0
6.0	5.1	6.2	6.2	9	0
6.5	5.8	6.5	7.1	10	0
7.0	6.4	7.0	7.2	8	75

5.3 EXPERIMENT 3. INOCULATION METHOD AND HOMOGENIZATION

5.3.1 PURPOSE

Problems in achieving nodulation led to questions regarding the efficacy of watering the inoculum onto the soil surface as is commonly done. The results of experiment 1 showed that injecting the inoculum into the soil was quite effective. Visser and Danielson (1988) also had good success with mixing inoculum directly into the planting mixture prior to seeding. Some investigators have attempted Frankia inoculation by soaking germinated seedling radicles in a concentrated inoculum solution as is commonly done with Rhizobium. Therefore, this experiment was set up to compare mixing inoculum into the soil, soaking radicles in the inoculum solution, injection of inoculum into the soil and surface application.

Also, it was necessary to determine whether virtis blending the inoculum in order to get a homogeneous solution (similar numbers of infective units per milliliter) was affecting infectivity of the inoculum.

5.3.2 METHODS

The peat:vermiculite mixture was adjusted to pH 7.0 by adding $CaCO_3$. The inoculum used was 4 week old Frankia strain EAN1 described in chapter 3. For the injection treatment, a canula and syringe were used to place Frankia cells into the root zone of test plants at a rate of 3.5 ug protein per seedling. The seedlings in the surface

application treatment received the same quantity of inoculum watered onto the soil surface near the stem of each seedling. The mix-in treatment seedlings received the equivalent of 3.5 µg protein mixed into the soil prior to seeding. For the soak treatment, germinated seedlings were placed in a 0.44 µg/mL Frankia solution for 2 days prior to planting.

The effect of homogenizing the inoculum was investigated by comparing virtis blended and unblended inoculum. Seedlings were grown in the same soil as described previously. The inoculum was introduced into the planting mixture by initially mixing it into the soil and then by injecting it 2.5 weeks later.

5.3.3 RESULTS AND DISCUSSION

Table 8 shows the percentage of seedlings that nodulated under each of the six treatments described above. Blending the inoculum did not improve nodulation (X^2=0.605, p>0.25). Soaking the roots and watering the inoculum onto the soil surface were both ineffective as inoculation methods. This is in contrast to the data given by Périnet et al. (1985) who regularly spray inoculum onto seedlings. The protein content per seedling used in the present study was similar to that used by other investigators (eg. Hooker and Wheeler 1987), therefore low nodulation rates were probably not due to low total inoculum concentrations.

Table 8: Percentage nodulation of Elaeagnus seedlings at pH 7.0 using homogenized and un-homogenized inoculum and four inoculation methods.

Treatment	pH at harvest	n	Percent Nodulated
Blended (mix+inject)	7.4	10	30
Non-blended (")	7.3	12	8
Surface (Non-blended)		10	0
Mixed in (")		9	67
Soaked roots (")		7	0
Injected (")		8	25

The same soil was used for the inoculation treatments therefore the pH would be the same as recorded for the virtised/not virtised treatments.

Mixing the inoculum into the soil (67% nodulation) appeared to be more effective than injecting the inoculum (25%). However, plants given the blended/non-blended treatments had been inoculated by mixing-in followed by injection 2.5 weeks later, and nodulation rates did not reach 67%. As well, unblended inoculum was used in comparing the four inoculation methods. Therefore, it appears that these differences in nodulation success may be due to lack of homogeneous inoculum. Even after virtis blending, clumps of inoculum were still visible in the growth medium where they settled out quickly.

The lack of nodulation in the surface application treatment is in contrast to the results of Experiment 1 of this chapter (Table 6) where 15% and 9% nodulation were achieved using surface application. Perhaps inoculum levels were higher in Experiment 1. As well, Experiment 1 showed infection rates of 61% and 31% by injection while injection resulted in only 25% nodulation in Experiment 2. Therefore both injection and mixing appear to be effective inoculation methods, and differences in nodulation success between Experiments 1 and 3 were due either to differences in the total inoculum concentration used between the two experiments, or due to nonhomogeneous inoculum, resulting in seedlings receiving different quantities of Frankia. Mixing the inoculum into the soil may be advantageous as the inoculum is present when the radicle is emerging from

the seed coat. It is important that Frankia cells are placed in the vicinity of young, actively growing root tips since these are the apparent sites of infection (Diem et al. 1983), and Frankia cells do not appear to be mobile (Lalonde 1979).

Virtis blending does not appear to decrease infection rates and therefore is recommended as this procedure achieves a more homogeneous inoculum. Périnet et al. (1985) prepared their pure culture inoculum by sonicating to the point where the spores were freed from the sporangia and the hyphae were in 100 µm fragments. This procedure was not tested in these experiments, but should be considered as the number of infective units would be increased.

A number of factors are probably responsible for the variable nodulation rates observed in this series of experiments. The Frankia cultures used had few sporangia present, and spores remain viable longer than hyphal fragments (Lalonde and Calvert 1979). The low numbers of sporangia are characteristic of the group A strains (Elaeagnus host compatability group) which produce primarily vesicles in culture (Normand and Lalonde 1986). Murry et al. (1984) recorded no sporangial formation by strain EaN1$_{pec}$ when grown in air-sparged cultures in defined medium. The lack of spores in the inoculum would make it more important that the shorter-lived hyphal

fragments were placed in the vicinity of infectable roots.

A second factor causing variable nodulation rates was probably non-homogeneous inoculum. Akkermans and Houwers (1979) reported variable and unreproducible nodulation rates when using crushed nodule inoculum, possibly due to lack of homogeneity of the inoculum.

A third factor contributing to variable nodulation rates may have been high greenhouse temperatures, which resulted in maximum soil temperatures of 35° to 40°C. Périnet et al. (1985) observed that high temperatures could inhibit nodulation and growth. Visser and Danielson (1988) investigated the effects of temperature on nodulation, but the highest soil temperature tested was 26°C, at which nodulation and growth were optimal.

CHAPTER 6

6. GROWTH RESPONSE OF ELAEAGNUS TO TWO VAM SPECIES AND FOUR FRANKIA STRAINS

6.1 PURPOSE

The previous chapters described the experiments which determined effective levels and methods of inoculation for both Frankia and VAM fungi. This experiment investigated whether different Frankia strain/VAM species combinations would affect plant growth and nodulation differently. Four Frankia strains were grown in combination with two VAM fungal species. The effects of the symbionts, alone and in combination, on Elaeagnus growth, nodulation and rates of nitrogen fixation were determined.

6.2 METHODS

The VAM inoculum consisted of 5 month old G. etunicatum and G. aggregatum pot cultures which were grown and harvested as described in Chapter 4. For the control treatments, the inoculum was autoclaved (20 min). Inoculum (soil and roots) represented 15% by volume of the peat:vermiculite mixture. Samples of inoculum roots were stained and shown to be heavily mycorrhizal.

Previous experiments had shown extremely variable nodulation rates, therefore a more reliable inoculum was required in order to be able to observe VAM effects. The Frankia inoculum was purchased from Rhizotec Laboratories

Inc. (St. Chrysostom, Quebec). It contained 2 Frankia strains isolated from Elaeagnus angustifolia and E. umbellata. The other strains described in Chapter 3 (EAN1, EANfm+, EANfmb) had been isolated from E. commutata.

Frankia cells of each of the four strains were cultured on a yeast extract broth (Baker and Torrey 1979) for 4 weeks. Cells were then harvested by washing in 2% phosphate buffered saline (PBS) three times and collected onto 1.2 µm millipore paper. Mycelium wet weight was determined and the inoculum partially standardized on this basis. The amount of inoculum per plant is given below:

Strain	Wet Weight Mycelium
Rhizotec	3 mg/plant (recommended rate)
EAN1	12 mg/plant
EANfmb	12 mg/plant
EANfm+	not determined

The washed Frankia cells, were suspended in Crone's solution to which 0.5 $g \cdot L^{-1}$ yeast extract and 0.5 $g \cdot L^{-1}$ glucose had been added in case the cells needed a carbon source until they were able to infect the roots. The inoculum was mixed directly into the peat:vermiculite treatment soil which had been adjusted to pH 7.0 (Chapter 5). The treatments were arranged as follows:

	VAM Treatment			
Frankia treatment	Glomus aggreg.	autocl. G.agg.	Glomus etunic.	autocl. G.etun.
Control				
Rhizotec				
EAN1		16 seedlings/treatment		
EANfmb				
EANfm+				

The treated soil was packed into 150cc containers into which stratified (8 day) Elaeagnus seeds were sown. Developing seedlings were taken to the greenhouse 2 weeks later and received 200 $mg \cdot L^{-1}$ 28-14-14 Plant Prod Soilless Feed twice weekly. Light levels reached 500 $\mu Em^{-2}sec^{-1}$ on clear days and 156 $\mu Em^{-2}sec^{-1}$ on cloudy days, with a 20 h photoperiod (Visser and Danielson 1988).

The seedlings were harvested after 10.5 weeks. Due to the size of the experiment and the length of time required to assess the nitrogen fixation rates of each plant (acetylene reduction assay, Hardy et al. 1968; McNabb and Geist 1979), the experiment was harvested over a period of 4 days. Each day, 4 replicates of each treatment were assayed. Since acetylene reduction rates are highest at midday (Wheeler 1969), 2 replicates/treatment were incubated from 11:00 a.m. until 1 p.m. and the other two replicates/treatment from 11:30 a.m. until 1:30 p.m. A preliminary experiment which monitored the rate of acetylene reduction over time confirmed that a 2 h incubation was appropriate. Minchin et al. (1983) demonstrated a decline in the rate of ethylene formation after exposure to acetylene. They felt that the initial rates measured were the most accurate estimates of nitrogenase activity. Removing the shoot and shaking soil from the roots also reduced activity (Minchin et al. 1986); therefore, assays in these experiments were performed on

intact plants with undisturbed root balls. Tjepkema et al. (1988) monitored declines in acetylene reduction rates over time for five actinorrhizal species. Declines in acetylene reduction rates were noted within 5 minutes of the injection of acetylene. However, for most species, rates climbed again to within 30 to 90% of the maximum rate. These assays were conducted on plants grown in water culture. In this experiment, the presence of the soil core slowed the diffusion of acetylene to the nodules. Maximum acetylene reduction rates were not attained until 54 min after the addition of acetylene. Rates remained stable for 4 h.

For the acetylene reduction assay, plants were removed from their containers and placed intact in 1710 cc mason jars which were placed on the greenhouse bench in the same position in which the plants had been growing. The mason jars were closed with sealer caps that had been modified to hold a rubber septum. Plants and jars were allowed to equilibrate for 30 min. The temperature inside the jar was monitored and not allowed to exceed 30°C. When necessary, jars were cooled by misting with water. Hensley and Carpenter (1979) recorded maximum acetylene reduction rates for E. umbellata at 34 to 36°C. Rates measured are highly temperature-dependent, therefore day-to-day variations in temperature, as well as light levels, would increase the amount of variation in the measurements.

After equilibration, 170 cc of air was removed using a 500 mL gas-tight syringe. The air was replaced with 170 cc of pure acetylene. Plants were placed under the lights and the temperature carefully monitored. Jars were normally misted twice during the 2 h assay period. After 2 h, 7 mL samples were taken using a 10 mL plastic syringe. The gas samples were stored in 7 mL Vacutainers (Becton Dickson) until they could be analyzed by gas chromatography. This analysis was completed 2.5 weeks later on a Varian 3400 Gas Chromatograph, fitted with a Porapak R, 8/100 mesh column (G.C. parameters are given in Appendix III).

Once the nitrogen fixation rates of the seedlings had been determined, shoots were removed and oven dried (80°C). Soil was washed from the roots, nodules were counted, removed and dried, and root subsamples were taken for mycorrhizal assessment. The dry weights of the remaining roots were determined as described above. Root subsamples for mycorrhizal assessment were treated as described in Chapter 4. Mycorrhizal assessments were made using a dissecting microscope and the degree of mycorrhizal infection was ranked from 0 to 4 as follows:

0 - no infection
1 - <5% of root length infected
2 - 5-25% of root length infected
3 - 25-50% of root length infected
4 - >50% of root length infected

A second set of seedlings was also set up to test whether Frankia inoculum concentrations were adequate for consistent nodulation. These seedlings were inoculated at the same time as the previous set with Frankia concentrations of 0.01, 0.1, 1 and 10 times the concentration used for the combination experiment. Strains EAN1, EANfmb and Rhizotec were used in this test but there was not enough strain EANfm+ available to test.

The seedlings in the inoculum concentration test were harvested after 8 weeks. Shoot, root and nodule weights and nodule numbers were determined.

6.3 RESULTS AND DISCUSSION

6.3.1 FRANKIA INOCULUM CONCENTRATION

Before considering the data on Frankia/VAM combinations, it was important to ensure that the inoculum levels of each Frankia strain were not the source of any observed differences in nodulation rates. Table 9 shows that the Rhizotec inoculum infected 100% of the seedlings at the 1x and 10x concentrations. The EAN1 Frankia infected 60 and 40% of seedlings at the 0.1x and 1x rates, respectively. As there were only five replicates, this difference is not significant. The inoculum concentration ranged from 3 to 30 mg per seedling for the Rhizotec strains and 1.2 to 12 mg per seedling for EAN1 appeared to be optimal. No nodulation occurred in the 10x treatment

Table 9. Nodulation (percent) of *Elaeagnus* inoculated with three *Frankia* strains at three inoculum levels (n=5).

Frankia Strain	Inoculum Level x1/100	x1/10	x1	x10
Rhizotec	-	20	100	100
EAN1	-	60	40	0
EANfmb	0	0	0	0
EANfm+	(not enough inoculum to test)			

Table 10. Nodulation (percent) of *Elaeagnus* inoculated with three *Frankia* strains and two VA species (n=16).

Frankia Strain	Mycorrhizal Treatment Control	*G.agg.*	Control *G.etun.*	*G.etun.*
Control	0	6	0	0
Rhizotec	100	94	63	50
EAN1	88	63	69	13
EANfm+	0	19	0	13

with EAN1 (120 mg per seedling). EANfmb did not nodulate at any inoculum concentration although the mass of hyphae present in the inoculum was the same as for EAN1. EANfm+ could not be tested as this is a very slow growing strain and the full quantity of inoculum was required for the combination treatments.

6.3.2 NODULATION SUCCESS OF *FRANKIA*/VAM COMBINATION PLANTS

Table 10 shows the actual nodulation rates recorded in the combination experiment. The Rhizotec and EAN1 inoculum caused up to 100% and 88% nodulation, respectively, depending upon the VAM treatment. The EANfm+ strain did nodulate seedlings, but only at low levels in the treatments with active mycorrhizae. The EANfmb inoculated seedlings did not nodulate. A single control plant did nodulate (6% *G. aggregatum*) but this was a very small nodule which may have resulted from contamination caused by fungus gnats.

The Rhizotec inoculum contained two *Frankia* strains isolated from *E. umbellata* and *E. angustifolia*. The EAN1 strain had been isolated from *E. commutata*. Therefore, the strains did not appear to be species specific. The low nodulation rate achieved by EANfm+ was probably due to low inoculum concentration. EANfmb appears to be a non-infective strain. The isolation of non-infective *Frankia* strains is quite common (Lalonde *et al.* 1975; Hahn *et al.* 1988).

Both the Rhizotec and EAN1 strains produced nodules on significantly more plants in the G. aggregatum treated soils in comparison with the G. etunicatum treated soils (Table 10 and Appendix 2). Glomus aggregatum was cultured on slender wheatgrass while G. etunicatum was propagated on leek. Both types of pot cultures contained identical peat:vermiculite mixtures and were subjected to the same watering and fertilization regimes. Differences in effects on nodulation may be due to volatiles or leachates from the leek roots. Also, slender wheatgrass has a much more fibrous root system than leek which may have caused textural differences between the treatment soils.

Unlike the Rhizotec strain, EAN1 showed higher nodulation rates with non-mycorrhizal control plants than when plants were mycorrhizal (Appendix II). EANfm+ formed nodules only when mycorrhizal fungi were present, but because of the low nodulation rate, the VAM effect was not significant. There was no apparent difference in nodulation rates between plants infected by the two VAM species. The lower nodulation rate of EAN1 compared with Rhizotec was probably not related to inoculum concentration differences. Higher inoculum concentrations did not increase the nodulation rate (Table 9). Quispel (1954) showed an increase in nodulation with increased inoculum density using crushed nodules to inoculate Alnus. However, Lalonde (pers. comm.) feels that inoculum concentration is rarely

limiting to nodulation rates, a view which appears to be shared by other researchers, since inoculum levels are often not reported (Normand and Lalonde 1982; Prégent and Camiré 1985; St. Laurent and Lalonde 1987). When strain comparisons are being made, the inoculum may be standardized by optical density measurements (Carpenter et al. 1984; Dawson and Sun 1981), packed cell volume (Berry and Torrey 1985; Stowers and Smith 1985; Nesme et al. 1985), or protein content (Hooker and Wheeler 1987).

The data in Table 10 indicate that the presence of mycorrhizal fungi may have some effect on the nodulation success of different Frankia strains with Elaeagnus and that this effect is not necessarily detrimental, as demonstrated by EANfm+ where only VAM seedlings nodulated.

6.3.3 MYCORRHIZAL INFECTION LEVELS

The ranked VAM infection level data are presented in Table 11. Analysis of variance identified a significant difference in mycorrhizal infection levels between G. aggregatum and G. etunicatum seedlings. This difference is consistent with the results given in Chapter 4 (Table 5) and is related to the characteristics of each species. These infection levels were independent of the inoculum concentration and therefore were the maximum infection of Elaeagnus by each particular species under these experimental conditions.

Table 11. Mean of ranked mycorrhizal infection levels (±SE) of *Elaeagnus* seedlings grown with three *Frankia* strains and two VA species.

Frankia Strain	Mycorrhizal Treatment: Control	*G.agg.*	Control	*G.etun.*	Mean
Control	0	3.7±0.2	0	3.1±0.3	3.4±0.3 b
Rhizotec	0	3.1±0.4	0	2.1±0.4	2.6±0.4 a
EAN1	0	3.9±0.1	0	2.9±0.2	3.4±0.3 ab
EANfm+	0	3.8±0.2	0	2.3±0.4	3.0±0.4 ab
Mean	0	3.7 c	0	2.6 d	

Two-way analysis of variance showed a significant mycorrhizal effect as well as a significant *Frankia* effect. There were no interactions. Means followed by the same letter are not significantly different at $p \leq 0.05$ by Tukey test. MSE=0.6585, n=8.

The ANOVA also indicated a difference in VAM infection levels among seedlings infected with different Frankia strains. Rhizotec inoculated seedlings had lower infection levels than control plants, although this was not significantly different from that observed for the other Frankia strains tested. The infection rankings used compress a range of infection from 0 to 100% to a range from 1 to 4. Therefore, what were large differences in percent infection became very small differences in rank, and therefore not significantly different. As a result, the percentage of seedlings at each infection level was calculated in order to give a clearer picture of differences in infection levels among VAM treatments (Tables 12 and 13).

For G. etunicatum (Table 12), the control and EAN1 treatments showed similar patterns of infection with the majority of seedlings exhibiting 25% or more of the root length infected. The Rhizotec and EANfm+ treatments showed large numbers of seedlings with 5% or less infection. The EAN1 infected seedlings showed VAM infection levels as high as the controls, although nodulation rates were significantly lower. Conversely, the Rhizotec seedlings exhibited similar nodulation rates as the controls, while VAM infection levels were adversely affected. Therefore, there appears to be a difference between the Frankia strains in their ability to compete with VAM fungi, with

Table 12. Percent of G. etunicatum infected seedlings at each infection level for the four Frankia treatments (ranks described on page 62).

Frankia Strain	Ranks and corresponding range of % root infected 0 (0%)	1 (<5%)	2 (5-25%)	3 (25-50%)	4 (>50%)
Control	0	0	25.0	37.5	37.5
Rhizotec	12.5	12.5	25.0	50.0	0
EAN1	0	0	25.0	62.5	12.5
EANfm+	0	37.5	12.5	37.5	12.5

Table 13. Percent of G. aggregatum infected seedlings at each infection level for the four Frankia treatments.

Frankia Strain	Ranks and corresponding range of % root infected 0 (0%)	1 (<5%)	2 (5-25%)	3 (25-50%)	4 (>50%)
Control	0	0	0	25.0	75.0
Rhizotec	0	0	37.5	12.5	50.0
EAN1	0	0	0	12.5	87.5
EANfm+	0	0	0	25.0	75.0

nodulation by EAN1 being reduced in the presence of VAM fungi, while Rhizotec Frankia nodulates similarly in the presence or absence of VAM fungi, although the levels of VAM infection are affected (Table 10, 12 and 13). The EANfm+ treatment cannot be compared in this way since inoculum levels were probably limiting to nodulation.

A similar infection level breakdown is given for G. aggregatum seedlings in Table 13. Here too, the Rhizotec inoculated seedlings showed high nodulation rates and lower VAM infection levels. EAN1 seedlings again showed high VAM infection levels, with nodulation rates significantly lower in the presence of VAM. Although such effects have not been previously investigated in actinorhizal shrubs, Bethlenfalvay et al. (1985) described competitive interactions between a VAM fungus and Rhizobium on Glycine (soybean). Prior establishment of the fungus resulted in reduced nodulation. In a subsequent split-root study with Vigna (cowpea), Ames and Bethlenfalvay (1987) observed that the half-root without VAM fungal infection had less nodule activity than the mycorrhizal half-root. They interpreted this as evidence of a direct interaction between VAM and Rhizobium, unmediated by plant nutrition.

6.3.4 PLANT BIOMASS

In the following tables, seedlings which did not conform to the treatments described were eliminated. For example, only nodulated and mycorrhizal seedlings were

included in calculating mean shoot weight values for the G. etunicatum/EAN1 treatment.

Although there were differences in nodulation rates and degrees of VAM infection amongst treatments, these differences were not reflected in the shoot and root weight data (Tables 14 and 15). Only the G. aggregatum control/EAN1 treatment showed a significantly different (lower) shoot weight. There does not appear to be a good explanation for this. Tukey multiple comparisons between the root weight means showed no differences between treatment means, although the two-way ANOVA found significant VA, Frankia and interaction effects. The absence of growth effects in this study may have been due to the early harvest of the test plants (after only 10.5 weeks). It normally takes 8 weeks before nodules are are visible. No nitrogen fixation occurs until the nodules are established, and after this it would take some time before growth effects due to increased nitrogen availability became apparent. As well, plants were fertilized twice weekly with 200 $mg \cdot L^{-1}$ 28-14-14 which is the equivalent of 56 ppm N and 28 ppm P, and subsequent work (see Chapter 7) showed that this range would not permit significant biomass differences between VAM+Frankia and VAM only seedlings to be observed since P and N were not sufficiently limiting.

Table 14. Shoot weights (mg) ±SE of *Elaeagnus* grown with three *Frankia* strains and two VA species.

Frankia Strain	Mycorrhizal Treatment			
	Control		Control	
	G.agg.	*G.agg.*	*G.etun.*	*G.etun.*
Control	643±57 a	667±83 a	485±52 ab	728±68 a
Rhizotec	559±50 ab	641±32 ab	490±44 ab	568±80 ab*
EAN1	386±37 b	719±42 a	681±44 a	689±15 a
EANfm+	-	755±47 **	-	823±210 #

Data were analysed by two-way ANOVA. Means followed by the same letter are not significantly different at $p \leq 0.05$ by the Tukey multiple comparison test.
The EANfm+ treatment was not included in the analysis as there were only **3 and #2 replicates for the mycorrhizal treatments and none of the controls nodulated. MSE=0.02905, n=10 (*n=8).

Table 15. Root weights (mg) ±SE of *Elaeagnus* grown with three *Frankia* strains and two VA species.

Frankia Strain	Mycorrhizal Treatment			
	Control		Control	
	G.agg.	*G.agg.*	*G.etun.*	*G.etun.*
Control	198±24	270±42	171±17	219±29
Rhizotec	183±26	201±15	147±18	159±18 *
EAN1	140±21	244±26	288±27	216±30
EANfm+	-	285±65 **	-	229±85 #

Although the two-way ANOVA showed significant VA, *Frankia* and interaction effects, differences amongst means were not significant by the the Tukey multiple comparison test at $p \leq 0.05$. The analysis was performed on log transformed data. The EANfm+ treatment was not included in the analysis. MSE=0.03635, n=10 (*n=8, **n=3, #n=2).

6.3.5 NODULE DRY WEIGHT AND NUMBER

There were differences in nodule dry weights amongst treatments (Table 16). For Rhizotec inoculated seedlings, nodule weight and percent nodulation were not affected by the presence of either VAM species. However, EAN1 inoculated seedlings displayed greater nodule weights in the G. etunicatum treatment soils with no significant effect due to the presence of live VAM fungi. This indicates that in choosing effective strains, soil characteristics would be important.

Nodule number was also affected by VAM infection (Table 17). Glomus etunicatum-infected seedlings had fewer nodules than seedlings in other treatments, although the difference was significant only with G. aggregatum control seedlings. While there was a trend toward fewer nodules in the presence of VAM, there also appeared to be a trend for greater nodule numbers in the G. aggregatum vs the G. etunicatum soil. Although these differences are not significant, they support the nodulation rate data given in Table 10 where nodulation success was greater in the G. aggregatum soil, and where nodulation rates were lower for EAN1 in the presence of VAM fungal infection. Differences in nodule number are not reflected in the nodule weight data of Table 16.

Table 16. Mean nodule dry weights (mg) ±SE of *Elaeagnus* grown with three *Frankia* strains and two VA species.

Frankia Strain	Mycorrhizal Treatment: Control	*G.agg.*	Control	*G.etun.*
Control	0	0	0	0
Rhizotec	20±3 ab	18±3 b	18±3 b	11±3 b*
EAN1	12±2 b	21±4 ab	35±5 a	34±1 a#
EANfm+	0	25±6 **	0	14±4 #

Data were analysed by two-way ANOVA. Means followed by the same letter are not significantly different (Tukey) at $p \leq 0.05$. MSE=0.112, n=10 (*n=8, **n=3, #n=2). The EANfm+ treatment was not included in the analysis.

Table 17. Mean nodule numbers (±SE) of *Elaeagnus* grown with three *Frankia* strains and two VA species.

Frankia Strain	Mycorrhizal Treatment: Control	*G.agg.*	Control	*G.etun.*
Control	0	0	0	0
Rhizotec	3.1±1.0	2.4±0.4	2.6±0.8	1.4±0.3 *
EAN1	3.4±0.5	2.4±0.5	2.9±0.8	1.0±0.0 #
EANfm+	0	1.7±0.3 **	0	2.0±1.0 #
Mean	3.3±2.5 a	2.4±1.4 ab	2.8±2.5 ab	1.3±0.7 b

A two-way ANOVA was performed on log transformed data. The EANfm+ treatment was not included. Only VA effects were significant. Means followed by the same letter are not significantly different at $p \leq 0.05$ by the Tukey test. MSE=4.4544, n=10 (*n=8, **n=3, #n=2).

6.3.6 ACETYLENE REDUCTION RATES

Results on the efficiency of acetylene reduction (as an estimate of nitrogen fixation) are presented in Table 18. These data were extremely variable and no significant differences were apparent. Although there were differences in total ethylene produced, these were accounted for by differences in nodule weight. Therefore, there is no evidence here for VAM/*Frankia* interaction effects on nitrogen fixation rates.

6.3.7 CONCLUSIONS

The plants in this study may have been harvested before growth effects could be detected. This is demonstrated by the lack of correlation between shoot weight and nodule weight which has been observed in the field (Visser and Danielson 1988). As well, the fertilization regime was probably too high in P to allow for demonstration of mycorrhizal effects on the various *Frankia* strains. The experiment was harvested when shoot differences between treatments appeared to be present. As well, the plants were getting quite large and there was the danger that container size effects (Baath and Hayman 1984) would confound symbiont effects. However, the results of this experiment do indicate that nodulation and VAM infection rates may vary with the combination of *Frankia* strain and VAM treatment. In the first case (EAN1 treatment), the VAM infection remained high at the expense

Table 18. Efficiency of nitrogen fixation as measured by acetylene reduction assay (μmoles C_2H_2 produced/h/g nodule).

Frankia Strain	Control	Mycorrhizal Treatment *G. agg.*	Control	*G. etun.*
	G. agg.		*G. etun.*	
Control	0	0	0	0
Rhizotec	59.6±12	82.7±27	69.5±13	123.2±26 *
EAN1	96.7±17 ^	87.9±18	69.4±12	67.8±32 #
EANfm+	0	63.4±9 **	0	119.4±62 #

A two-way ANOVA showed none of the means to be significantly different at $p \leq 0.05$. The EANfm+ treatment was not included in the analysis. MSE=32082, n=10 (*n=8, ^n=9,**n=3, #n=2).

of nodulation, while in the other case (Rhizotec treatment), nodulation success was unchanged while VAM infection levels dropped. Further investigation of different VAM/Frankia combinations should be done in the field where the VAM are more likely to be beneficial, and where nutrient conditions would be more limiting to plant growth.

There were no differences in shoot and root weights among treatments. Research with Alnus glutinosa has indicated that ecotypes of the same species can differ in their nodulation capacity while Frankia strains can differ in their host plant specificity as well as nitrogen fixing activity, indicating that genotype/Frankia interactions are significant (Dawson and Gordon 1979; Dillon and Baker 1982; Hall et al. 1979). In this study, seeds were collected from within the same locale, but certainly represented great genetic diversity within the seedlings. M. Lalonde (pers. comm.) felt that since clonal seedlings were not used, the variation due to genetically different host material would be such that Frankia strain and Frankia/VAM interactions would be difficult to detect.

There were reasons for not using cloned seedlings for these experiments. When Elaeagnus is grown for reclamation purposes, it is grown from seed by commercial growers who do not yet use tissue culture technology. Also, it was possible that Frankia/VAM interaction effects

would exceed that amount of variation introduced by using seed grown plants. In this experiment, this was not the case with respect to biomass production. However, differences might have become apparent if seedlings had been planted in the field and grown over a longer time period.

CHAPTER 7

7. RESPONSE OF ELAEAGNUS TO VAM AND FRANKIA AT THREE NITROGEN AND THREE PHOSPHORUS LEVELS

7.1 PURPOSE

The relative importance of each endophyte to the host was investigated. As discussed in the introduction, the response of a plant to a particular symbiont varies with the nutrient levels at which it is grown, therefore it is necessary to study the symbioses under limiting, adequate and luxury nutrient conditions. Therefore, plants infected with either Frankia or Glomus etunicatum or with both symbionts, as well as uninoculated controls were grown at three P and three N levels as determined in Chapter 2. The highest level of application of each nutrient corresponded to that which allowed the maximum yield of an uninfected plant with respect to that nutrient.

Plant growth response is also very dependent on light levels as this directly affects the amount of photosynthate available to the endophyte (Hayman 1974; Daft and El-Giahmi 1978). The present experiment was set up in the spring (March) when light levels were low. However, mercury vapour lamps supplemented the natural light to give levels of 500$\mu Em^{-2}sec^{-1}$. The plant response measured will also vary with growth stage (Bethlenfalvay et al. 1982a). This was not investigated here. All plants were harvested at 12 weeks of age before container size effects started to

affect the results. The effects of mycorrhizal infection on growth decreases as pot size decreases (Baath and Hayman 1984; Kucey and Janzen 1987).

7.2 METHODS

Nine month old Glomus etunicatum pot cultures from a leek host plant were harvested. Glomus etunicatum inoculum was prepared by chopping the roots into 1-2 cm pieces and mixing these with the remaining pot culture soil. Half of the inoculum was autoclaved (20min). This was then allowed to air dry for one week to release some of the inhibitory substances produced during autoclaving (Rovira and Bowen 1966; Liegel 1986).

Frankia inoculum purchased from Rhizotec Laboratories was used to inoculate seedlings at the recommended rate. Controls received phosphate buffered saline (PBS) solution. Seedlings were inoculated by injecting the suspension into the soil core near the roots.

Four treatment combinations were set up:

Frankia	-500 mL Rhizotec inoculum -10% volume of soil mix autoclaved G. etunicatum pot culture soil -balance of soil = 1:1 pH 7.0 P:V as described previously
G. etun.	-500 mL PBS -10% volume of soil mix G. etunicatum pot culture soil -balance 1:1 P:V
both	-500 mL Frankia inoculum -10% volume of soil mix G. etunicatum pot culture soil -balance 1:1 P:V

control -500 mL PBS
-10% volume of soil mix autoclaved
G. etunicatum pot culture soil
-balance 1:1 P:V

For each treatment combination, 90 containers were planted with two *Elaeagnus commutata* seeds each (stratified for 12 days under cold running water until the radicles were visible). These 90 seedlings/endophyte treatment were divided into fertilizer treatments (10 seedlings/treatment) as follows:

		N LEVEL	
	25	50	100 ppm N
15	10 seedlings at each of four endophyte combinations		
P L E V E L 30			
60			

After the seedlings emerged, they were thinned to one seedling per container.

Nutrient levels were maintained by watering twice weekly with a modified Crone's solution (adapted from Lalonde 1979). As discussed in Chapter 2, it was felt that the Crone's solution was too high in K relative to the levels of other nutrients, therefore the amount of K in the fertilizer solution was reduced from 422 ppm to 200 ppm K. Also, some nitrogen-fixers apparently have a cobalt requirement for successful nodulation (Riley and Dilworth

1985). As Co was missing from the micronutrient solution used in the previous experiment (Chapter 2), it was added at this time. The lack of cobalt may have contributed to the poor nodulation observed in some previous experiments. The modified Crone's solution contained 0.324 $g \cdot L^{-1}$ KCl, 0.5 $g \cdot L^{-1}$ $CaSO_4 \cdot 2H_2O$; 0.5 $g \cdot L^{-1}$ $MgSO_4 \cdot 7H_2O$; 0.066 $g \cdot L^{-1}$ KH_2PO_4; 0.21 $g \cdot L^{-1}$ $Ca(NO_3)_4 \cdot 4H_2O$. This gives a base level of 15 ppm P and 25 ppm N to which $Ca(NO_3)_2 \cdot 4H_2O$ and $Ca(H_2PO_4)_2 \cdot H_2O$ were added to give the required N and P combinations. Also, micronutrients were added as follows: 1.81 $mg \cdot L^{-1}$ $MnCl_2 \cdot 4H_2O$; 0.018 $mg \cdot L^{-1}$ $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$; 0.22 $mg \cdot L^{-1}$ $ZnSO_4 \cdot 7H_2O$; 0.08 $mg \cdot L^{-1}$ $CuSO_4 \cdot 5H_2O$; 2.86 $mg \cdot L^{-1}$ H_3BO_3; 0.055 $mg \cdot L^{-1}$ $CoCl_2 \cdot 6H_2O$; 0.055 $mg \cdot L^{-1}$ $NiSO_4 \cdot 6H_2O$ (Allen and Arnon 1955). Ferric citrate (1 $mL \cdot L^{-1}$ of a 1% stock solution) was also included.

Seedlings were watered with this solution each Monday and Friday, and received deionized water each Wednesday. Seedlings were grown with a 20 h photoperiod, maximum and minimum temperatuures of 35°C and 20°C respectively, and light levels as described in Chapter 6.

The plants were harvested after 12 weeks of growth. The harvest procedure was identical to that described in Chapter 6.

7.3 RESULTS AND DISCUSSION

7.3.1 NODULATION SUCCESS

The percentage of test plants which nodulated is given in Table 19. Some of the control and Glomus-only plants nodulated and this may have been due to the transport of Frankia propagules by fungus gnats as discussed previously. The 50% nodulation rate of control plants in the N100 P15 treatment was due to accidental inoculation of some control seedlings with Frankia. Nodulation rates in the Frankia inoculated treatments were very good. Fewer plants nodulated at N100 as expected. Bond et al. (1954) demonstrated that nodulation is suppressed at high soil N levels, while Trinchant and Rigaud (1984) observed reduced acetylene reduction and respiration rates with increasing nitrate levels.

Since nodulation success was so variable, the number of replicates/treatment used in the statistical analysis varied. In the Frankia-inoculated treatments, only seedlings which actually nodulated were included in the treatment means. Similarly, control treatment means did not include plants which nodulated accidentally. The actual number of seedlings/treatment used in the analyses are given in Appendix IV.

7.3.2 MYCORRHIZAL INFECTION LEVELS

The mycorrhizal status of the seedlings was also determined (Table 20). Infection levels were in the range

Table 19. Nodulation (percent) of *Elaeagnus commutata* at three phosphorus and three nitrogen levels, with and without *Frankia* and *Glomus* inoculation.

N level (ppm)	Treatment	P level (ppm) 15	30	60
25	Control	10	0	10
	Frankia	100	100	100
	Glomus	10	10	0
	Frankia & *Glomus*	100	100	100
50	Control	0	20	10
	Frankia	80	100	100
	Glomus	0	20	10
	Frankia & *Glomus*	100	90	90
100	Control	50	10	0
	Frankia	80	50	20
	Glomus	0	0	0
	Frankia & *Glomus*	60	60	80

Table 20. Ranked mycorrhizal infection levels of Elaeagnus grown with and without Frankia at 9 fertilizer combinations (ranks described on page 62).

N Level (ppm)	Treatment	n	P Level (ppm) 15	30	60
25	Con.&Fr.	20	0.0	0.1±0.1	0.0
	Glomus	10	2.7±0.3 a	3.0±0.2 a	2.3±0.2 a
	Both	10	3.0±0.2 a	2.0±0.2 b	2.0±0.2 a
50	Con.&Fr.	20	0.2±0.1	0.1±0.1	0.0
	Glomus	10	2.3±0.2 a	2.5±0.2 a	2.9±0.3 a
	Both	10	2.6±0.2 a	2.2±0.3 a	1.8±0.3 b
100	Con.&Fr.	20	0.1±0.1	0.4±0.3	0.0
	Glomus	10	2.8±0.3 a	2.4±0.2 a	2.0±0.2 a
	Both	10	3.2±0.3 a	1.7±0.3 a	1.9±0.2 a

Within each of the nine fertilizer treatments, the two means of the VAM inoculated treatments were compared using a two-sample t-test. Means followed by the same letter are not significantly different at $p \leq 0.05$.

normally observed with Glomus etunicatum (<50% of the root length infected). Two-way analysis of variance of the entire data set showed a trend towards reduced infection with increasing P ($P<0.0001$, $MSE=0.4932$). There was no significant effect due to N. Differences in infection due to the presence of nodules were significant only in the N25 P30 and N50 P60 fertilizer treatments. At these levels, the amount of P relative to N was very high, and presumably the plant could absorb enough P from the soil to meet the requirements of N_2-fixation without relying on the mycorrhizal fungus (hence low infection levels, rank of 2=5-25% infection). Infection levels may have been higher in the Glomus only treatment at N25 P30 and N50 P60 because N was limiting (no fixer) and there is some evidence that VAM fungi may be able to absorb N from the soil (Ames et al. 1983). This difference was not seen at N25 P60, probably because infection levels were already minimal due to the inhibitory effect of high P (Mosse 1973; Mosse et al. 1981). Gardner et al. (1984) recorded an increase in mycorrhizal infection from 17% to 34% when plants were nodulated and mycorrhizal vs mycorrhizal only.

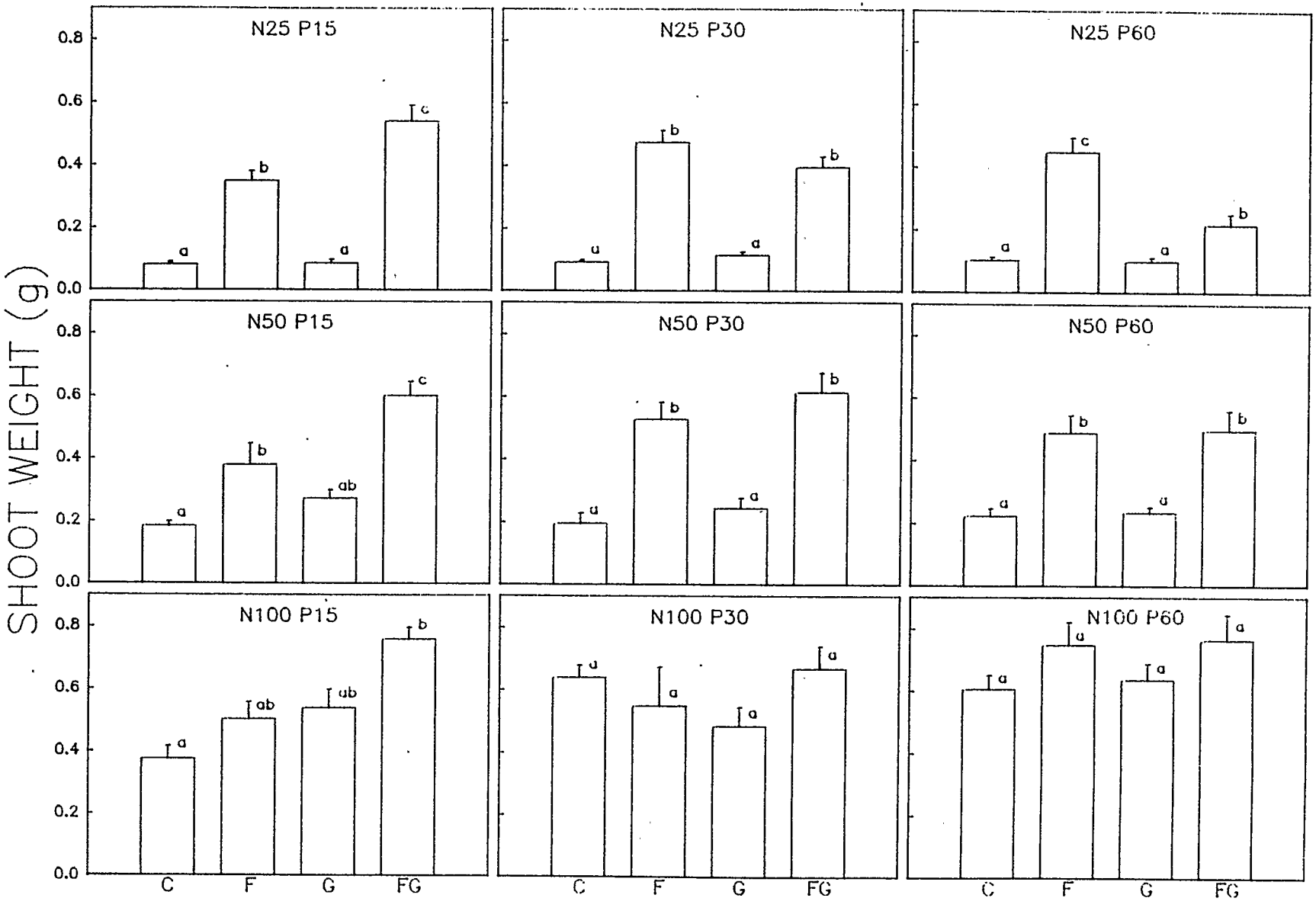
7.3.3 PLANT BIOMASS

Shoot weight data are presented in Figure 5. At all fertilizer levels (except N100 P15, N100 P30 and N100 P60), nodulation by Frankia resulted in increased plant growth when compared with the controls. Infection by Glomus alone

Figure 5. Shoot weight (g±SE) of Elaeagnus grown with and and without Glomus and Frankia at combinations of three nitrogen and three phosphorus levels.

Control (C), Frankia only (F), Glomus only (G), Frankia and Glomus (FG)

Within each of the nine graphs, means followed by the same letter are not significantly different from one another at $p \leq 0.05$ as determined by the Tukey multiple comparison test. The one-way ANOVA analysis was performed on square root transformed data to stabilize the variances and normalize the data.


SHOOT WEIGHT (g)
N25 P15
N25 P30
N25 P60
N50 P15
N50 P30
N50 P60
N100 P15
N100 P30
N100 P60
0.0
0.2
0.4
0.6
0.8
C
F
G
FG

never resulted in significantly greater biomass relative to control plants. Within the P15 treatment level, there was a trend toward greater shoot production in Glomus-infected seedlings relative to controls as N levels increased. This difference was almost significant at N100 P15. This lack of response to VAM fungi may have been due to the limited soil volume in the containers. Elaeagnus roots may have explored the core volume so efficiently that mycorrhizal hyphae were superfluous. Alternatively, this lack of response to VAM may simply be due to the fact that Elaeagnus is considerably more dependent upon its nitrogen fixing symbiont than on VAM fungi. Elaeagnus has not been found without nodules on its roots in any field surveys that we have done. Even at moderate N levels (50 ppm N), nodulated plants were larger than controls. Only at very high N levels with adequate P, would Elaeagnus survive well without Frankia. Soil nutrient levels are normally quite low in the soils in which Elaeagnus grows (Moore 1964).

However, when the shrubs were nodulated, VAM significantly enhanced growth relative to nodulated only and control plants where P was limiting with respect to available N (P15). Visser et al. (1984a) recorded nitrate levels of 3.8 $\mu g\ g^{-1}$ in an undisturbed Brown Chernozem from Bow City, Alberta. In a grasslands minespoil Visser et al. (1984b) recorded nitrate and extractable P levels of 6.4 $\mu g \cdot g^{-1}$ and 1.3 $\mu g \cdot g^{-1}$. Anlaysis of submontane mixed

grass prairie soils in southern Alberta yielded nitrate levels of 4.3 to 8.1 $\mu g \cdot g^{-1}$, ammonium levels of 9.9 to 10.0 $\mu g \cdot g^{-1}$, and phosphate levels of 1.9 to 2.7 $\mu g \cdot g^{-1}$ (Clapperton 1984). These figures can be considered estimates of the nutrient conditions found in areas where Elaeagnus normally grows. At these nutrient levels, VAM infection and nodulation would be beneficial to plant growth.

Similar results have been recorded in other studies. Rose and Youngberg (1981) recorded increased shoot and root weights, nodule weights, acetylene reduction rates and VAM colonization in VAM+Frankia infected seedlings when compared to nodulated only, VAM only or control plants. They did not observe VAM effects on shoot and root weights relative to controls in the absence of Frankia. Gardner et al. (1984) published similar results with Hippophae. The mean plant dry weight of nodulated/mycorrhizal plants was significantly greater than for nodulated only, mycorrhizal only or control plants. The latter three did not differ significantly, although there was a trend towards increased growth with VAM and then Frankia infection.

At the N100 P30 and N100 P60 treatments, neither N nor P were limiting to plant growth, and there were no differences in plant growth when the endophytes were present. Indeed, at these fertilizer levels, the rates of nodulation were so low that only two or three replicates

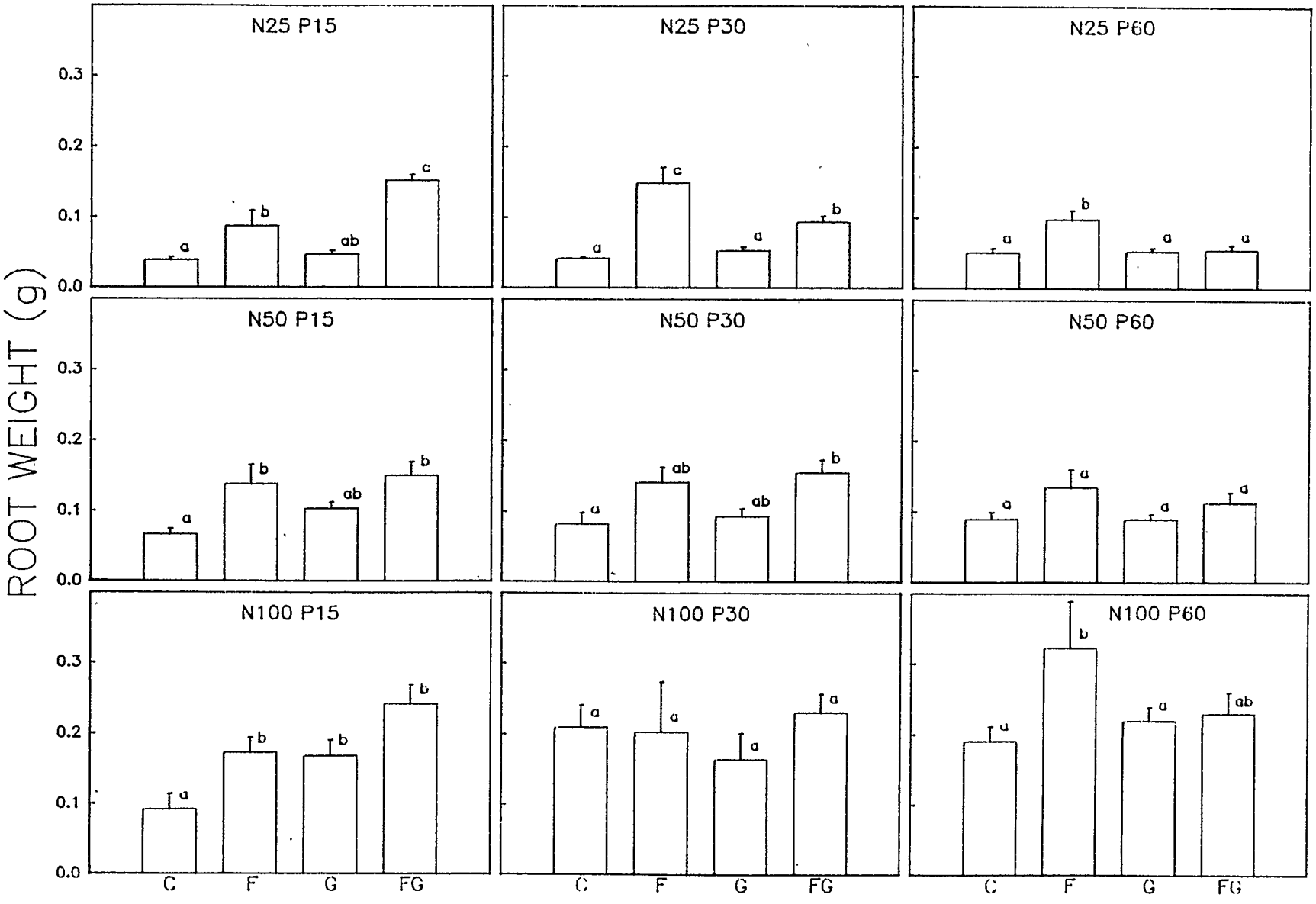
were present in the Frankia treatments (Appendix IV). As a result, plant response to the endophytes at these levels will not be discussed further.

The fertilizer treatment N25 P60 is somewhat anomalous. Again, nodulation resulted in increased growth relative to the controls. However, shoot production was lower in the Frankia+Glomus treatment vs Frankia alone. Since N levels were very low, the plant was very dependent upon its N_2-fixing symbiont. However, P levels were more than adequate relative to the amount of N, therefore, the presence of VAM fungi probably represented an unnecessary carbon drain. The only other possible explanation is a tissue P toxicity effect (Hall et al. 1977) but this is not supported by the shoot P content data which follow shortly.

The root weight data (Figure 6) followed a pattern similar to that of the shoot weights. Root weights were greater in the presence of Frankia except at the N50 P60 and N100 P30 fertilizer levels. At the N25 P15 level, root weights were greatest when both Frankia and Glomus were present. At the N100 P15 level, infection by Glomus alone resulted in root growth enhancement relative to the controls. The additional presence of Frankia at the N100 P15 fertilizer treatment did not significantly improve root growth.

Figure 6. Root weight (g*SE) of Elaeagnus grown with and without Glomus and Frankia at combinations of three nitrogen and three phosphorus levels.

Within each of the nine graphs, means followed by the same letter are not significantly different from one another at $p \leq 0.05$ (Tukey). The one-way ANOVA was perfomed on square root transformed data.


N25 P15
N25 P30
N25 P60
N50 P15
N50 P30
N50 P60
N100 P15
N100 P30
N100 P60
ROOT WEIGHT (g)
0.0
0.1
0.2
0.3
C
F
G
FG

The root weight response at N25 P30 is similar to that of shoot weight at N25 P60. At N25 P30, P levels were adequate relative to N availability and Glomus probably represents a carbon drain relative to nodulated-only seedlings. This pattern is even more pronounced at N25 P60, where the nodulated-only seedlings showed significantly greater root growth than all the other treatments.

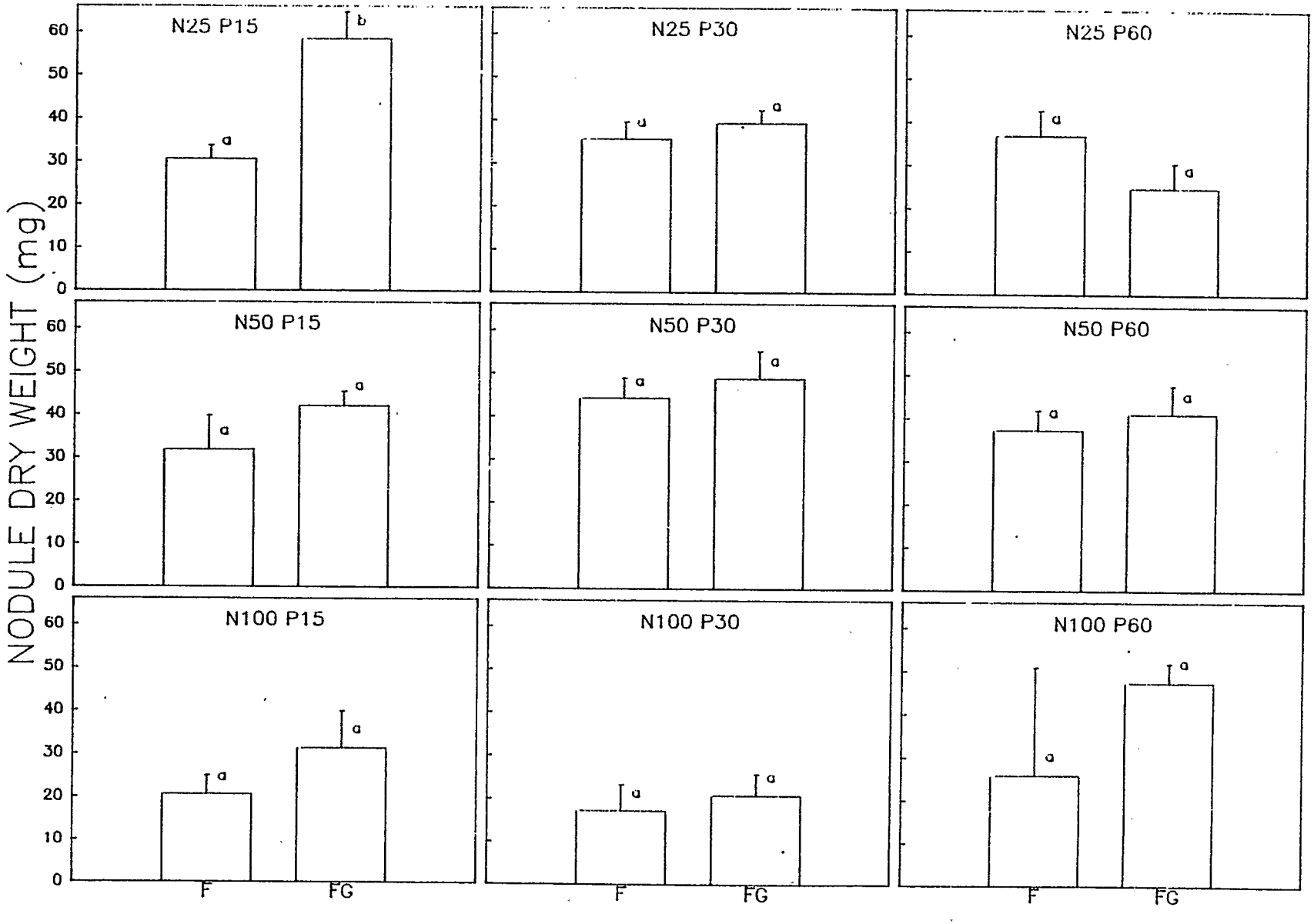
7.3.4 NODULE WEIGHT

Figure 7 gives the nodule dry weight data. Nodule weights were fairly consistent at the N25 and N50 treatment levels, but declined at N100. Only at N25 P15 did the presence of VAM significantly affect nodule weight. At this fertilizer combination, the presence of VAM resulted in a doubling of nodule weight over Frankia-only infected seedlings. This nodule weight was not achieved at any of the other fertilizer levels. Comparing this weight with the nodule weight in the Frankia-only treatment at the N25 P30 level, it appears that the improvement in nodule weight may not be due to increased P availability alone.

Analysis of variance on the entire nodule weight data set (treatment (Frankia vs Frankia+Glomus), N, P and all interactions, including 8 replicates) showed significant nitrogen ($p<0.0001$), nitrogen*phosphorus ($p=0.0004$), treatment ($p=0.0001$) and nitrogen*phosphorus*treatment effects

Figure 7. Total dry weight of nodules (mg±SE) on *Elaeagnus* grown with *Frankia*, with and without *Glomus* at nine fertilizer levels.

Means followed by the same letter are not significantly different at $p<0.05$. The data were analyzed by the rank sum two sample test, as the variances were not homogeneous.


NODULE DRY WEIGHT (mg)
N25 P15
N25 P30
N25 P60
N50 P15
N50 P30
N50 P60
N100 P15
N100 P30
N100 P60
0
10
20
30
40
50
60
a
b
F
FG

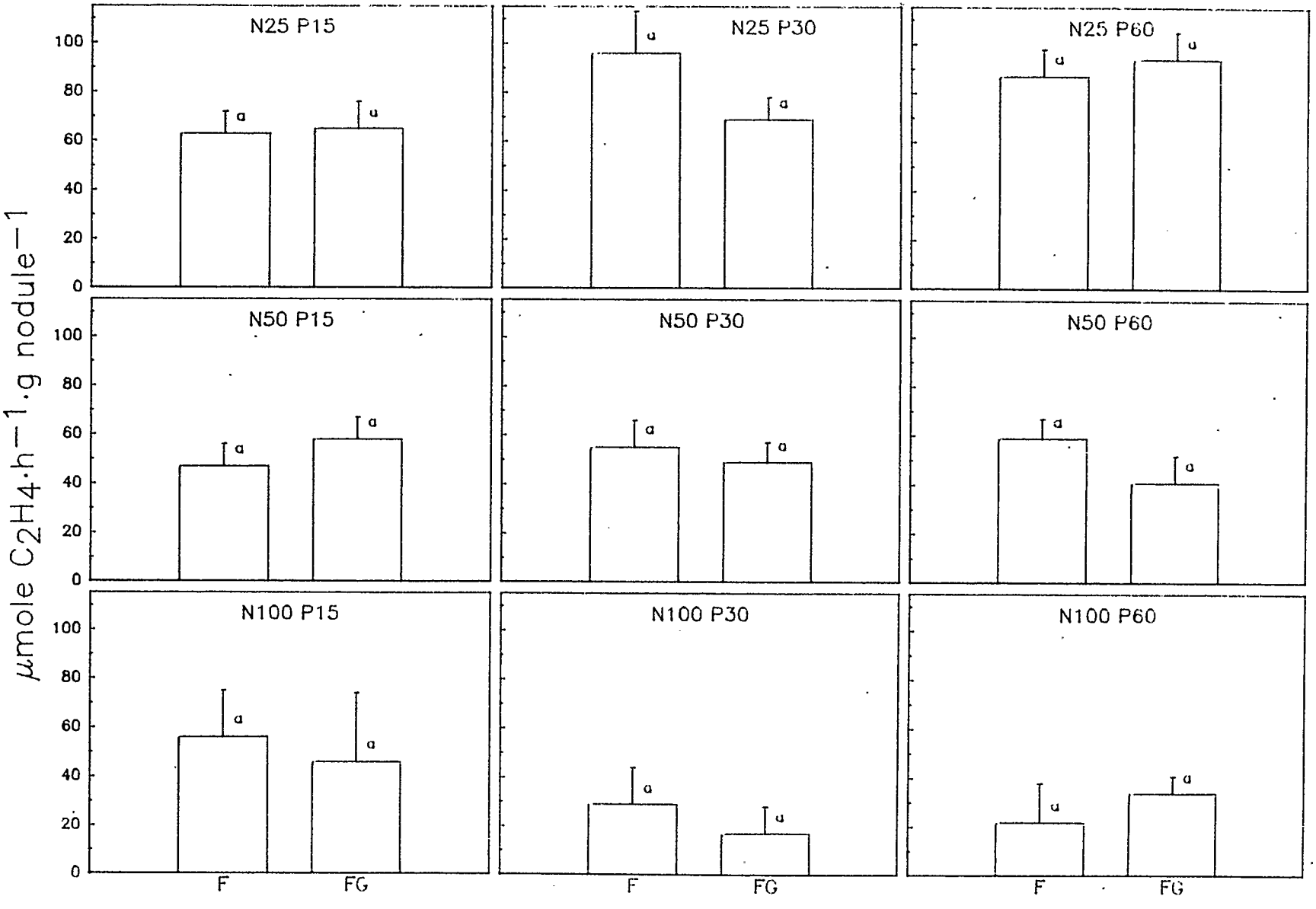
(p=0.0116, MSE=.0002151). The latter two effects are explained by the difference in nodule weight between Frankia and Frankia+Glomus treatments at the N25 P15 fertilizer level only. High soil nitrogen levels do appear to reduce nodule weight (Bond et al. 1954). The nitrogen*phosphorus interaction is difficult to explain since no clear pattern is visible in the data set (Figure 7).

7.3.5 ACETYLENE REDUCTION RATES

One explanation for the improved growth of Elaeagnus seedlings in the presence of both VAM fungi and Frankia nodules is that VAM fungi improve host nutrition in general, and therefore indirectly affect nodulation and nitrogen fixation rates. Another possible explanation is that there is direct movement of phosphorus to the nodules, which improves N_2-fixation by meeting the high P demand within the nodules, hence improving plant growth through greater N_2-fixation rates, hence N availability to the plant. If the latter explanation were true, one would expect to see greater N_2-fixation rates in the presence of VAM fungi. However, Figure 8 shows no significant difference in acetylene reduction rates when given on a nodule dry weight basis between seedlings infected with Frankia only or with Glomus+Frankia. Although the total rates measured differed, these differences were due only to variations in nodule weight. Since nodule tissue is

Figure 8. Rate of nitrogen fixation per gram nodule as estimated by the acetylene reduction assay (µmoles acetylene reduced to ethylene/h/g nodule). Rates are shown for Elaeagnus seedlings grown with Frankia only or with both Frankia and Glomus, at nine fertilizer combinations.

The data were analyzed by rank sum two sample test.


N25 P15
N25 P30
N25 P60
N50 P15
N50 P30
N50 P60
N100 P15
N100 P30
N100 P60
μmole C2H4·h−1·g nodule−1
0
20
40
60
80
100
a
F
FG

produced by the plant, it seems that the first explanation for the benefit of VAM fungi to nodulated plants is the more reasonable. However, Rose and Youngberg (1981) did observe a a doubling of the acetylene reduction rate per mg nodule in the presence of VAM+Frankia vs Frankia alone. These investigators performed their assays on rinsed, isolated root systems, therefore the values would not be directly comparable. As well, the rates recorded for Ceanothus are somewhat higher than the rates recorded for Elaeagnus in these experiments. Gardner et al. (1984) also recorded higher acetylene reduction rates in the presence of VAM vs Frankia alone, but they do not specify if these rates are given on a nodule weight basis. Actual fixation rates per g nodule do not have to be greater in the presence of VAM fungi in order to account for shoot weight differences. Greater nodule mass, fixing at the same rate per g nodule, will result in greater amounts of N being fixed, hence greater shoot growth.

Available N and P levels appear to influence acetylene reduction rates. Rates were highest within the N25 treatment, and increased with increasing P availability. Rates declined within the N50 and N100 treatments. Anlysis of variance using seven replicates per treatment, comparing Frankia to Frankia & Glomus inoculated plants over the three N and three P levels, showed a significant effect of available N on the efficiency of

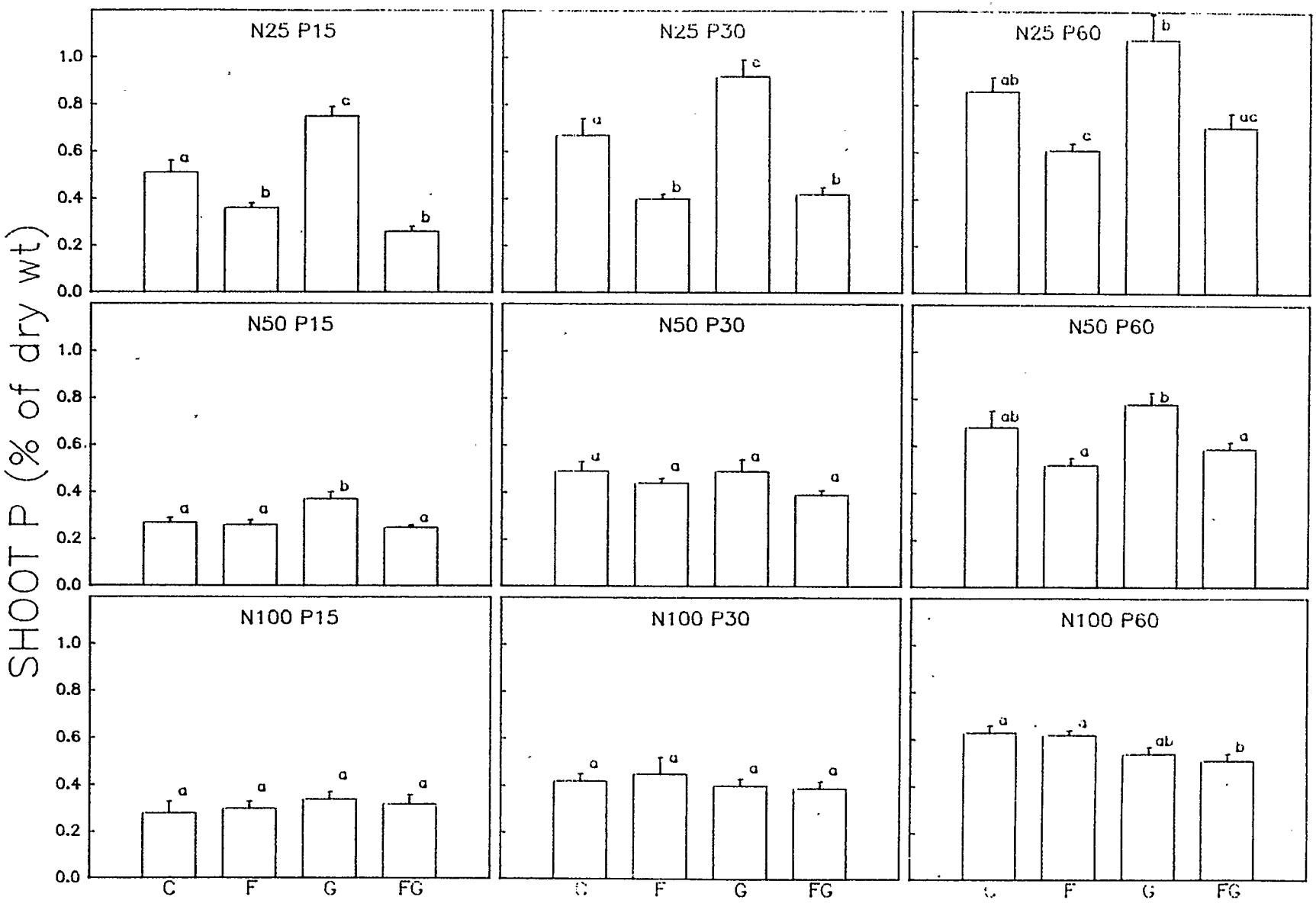
nitrogen fixation ($p<0.0001$) . Phosphorus availability did not have a significant effect alone. However, the PxN interaction was significant ($p=0.0015$, MSE=111840). This interaction may be due to the apparent trend toward increasing acetylene reduction rates with P at N25, but decreasing rates at N100. The inhibitory effect of available nitrogen on nitrogen fixation rates is well documented in the literature (Trinchant and Rigaud 1984).

7.3.6 SHOOT PHOSPHORUS CONTENT

In addition to endophyte effects on plant biomass production, changes in tissue nutrient contents (P, Zn, Cu) have also been recorded (Cooper 1984). Figure 9 gives the P concentration of the Elaeagnus shoots for each of the treatments. Within the N25 level, mycorrhizal seedlings had greater tissue P concentrations than controls or Frankia+Glomus seedlings. This effect was not apparent at higher N levels, presumably because N/P ratios were more favorable and seedlings were therefore larger and could make use of the P accumulated. This is demonstrated in Figure 10, which gives the total amount of P present in each plant. Higher tissue P concentrations in mycorrhizal and control plants than in nodulated plants (Figure 9), was due to these seedlings being smaller than nodulated plants [the dilution effect discussed by Jarrel and Beverly (1981)].

Figure 9. Phosphorus content (% of dry weight±SE) of Elaeagnus shoots grown with and without Frankia and Glomus at nine fertilizer combinations.

The data was transformed (ln(x)) prior to analysis to stabilize the variances and normalize the data. One-way ANOVA's were performed at each of the nine fertilizer combinations. Means followed by the same letter are not significantly different at $p<0.05$ (Tukey).


N25 P15
N25 P30
N25 P60
N50 P15
N50 P30
N50 P60
N100 P15
N100 P30
N100 P60
SHOOT P (% of dry wt)
0.0
0.2
0.4
0.6
0.8
1.0
C
F
G
FG

Rose and Youngberg (1981) recorded no difference in P concentration between control and VAM only Ceanothus, and between nodulated and nodulated/VAM plants, although the total P content between these two groups was significantly different. Gardner et al. (1984) however, did detect increases in plant P_2O_5 in VAM Hippophae, particularly when nodulated.

Total P content was higher in nodulated plants (Figure 10). Mycorrhizal effects can be observed in the total P content data. At N50 P15, mycorrhizal seedlings had higher total P contents than control plants and when both endophytes were present, the P content was higher than for all other treatments. The same trend was apparent at N100 P15, although the difference between Glomus and Frankia+Glomus treatments was not significant. Therefore, the increase in phosphorus content of plant tissues as a result of mycorrhizal infection as recorded in the literature (Cooper 1984) is supported by this data set.

Jarrel and Beverly (1981) showed that since the nutrient concentration in plant tissues is a function of both biomass production and total nutrient uptake, increases in either concentration or total uptake alone may not indicate direct uptake by mycorrhizae. Therefore, perhaps the easiest way to understand the effect of these varying phosphorus concentrations is to look at the mass of shoot produced per unit phosphorus taken up (Figure 11).

Figure 10. Phosphorus content (total mg/plant±SE) of *Elaeagnus* shoots grown with and without *Frankia* and *Glomus* at nine fertilizer combinations.

The data were transformed (ln(x)) prior to one-way analysis of variance within each of the nine fertilizer combinations. Means followed by the same letter are not significantly different at $p \leq 0.05$ (Tukey).

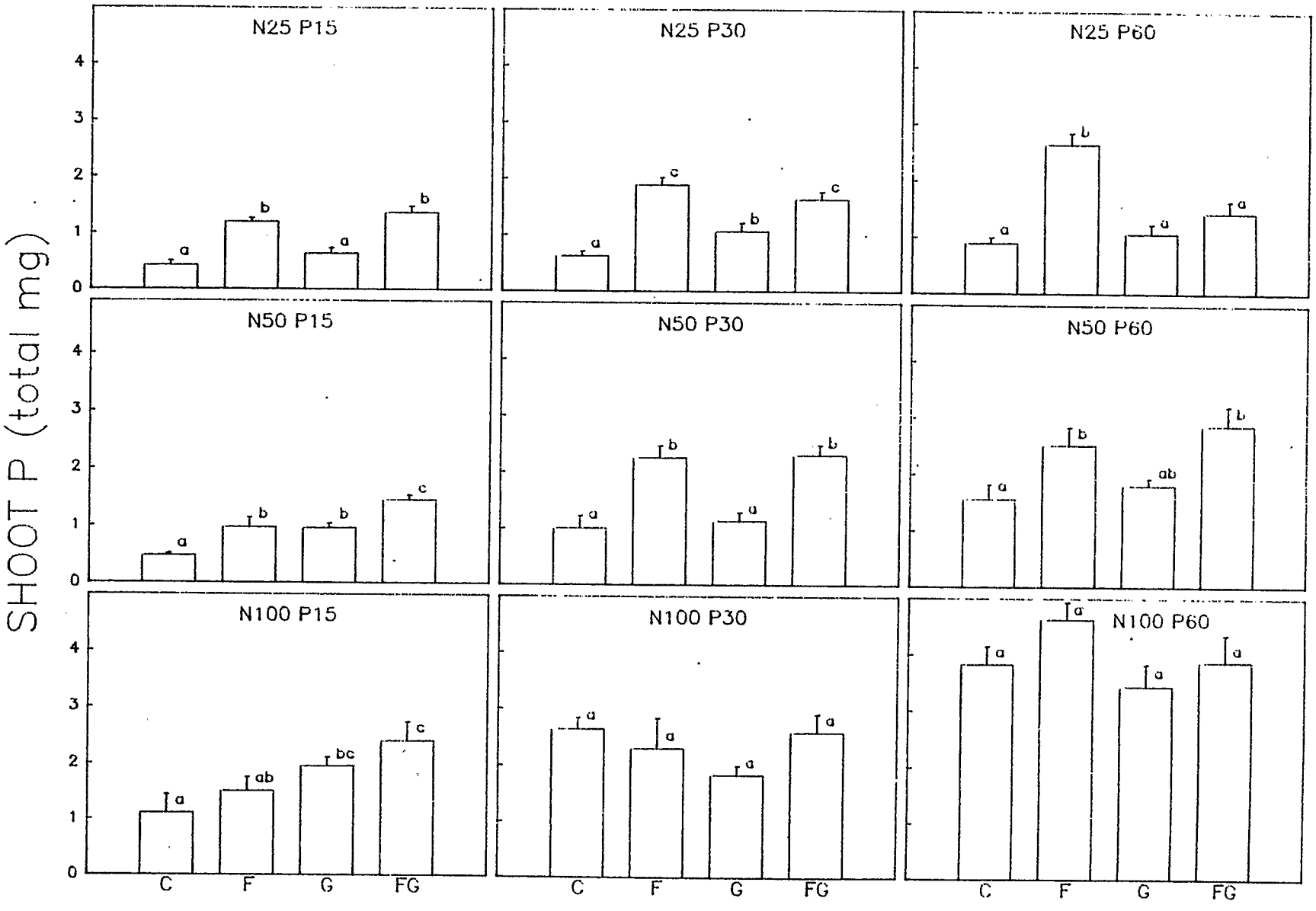
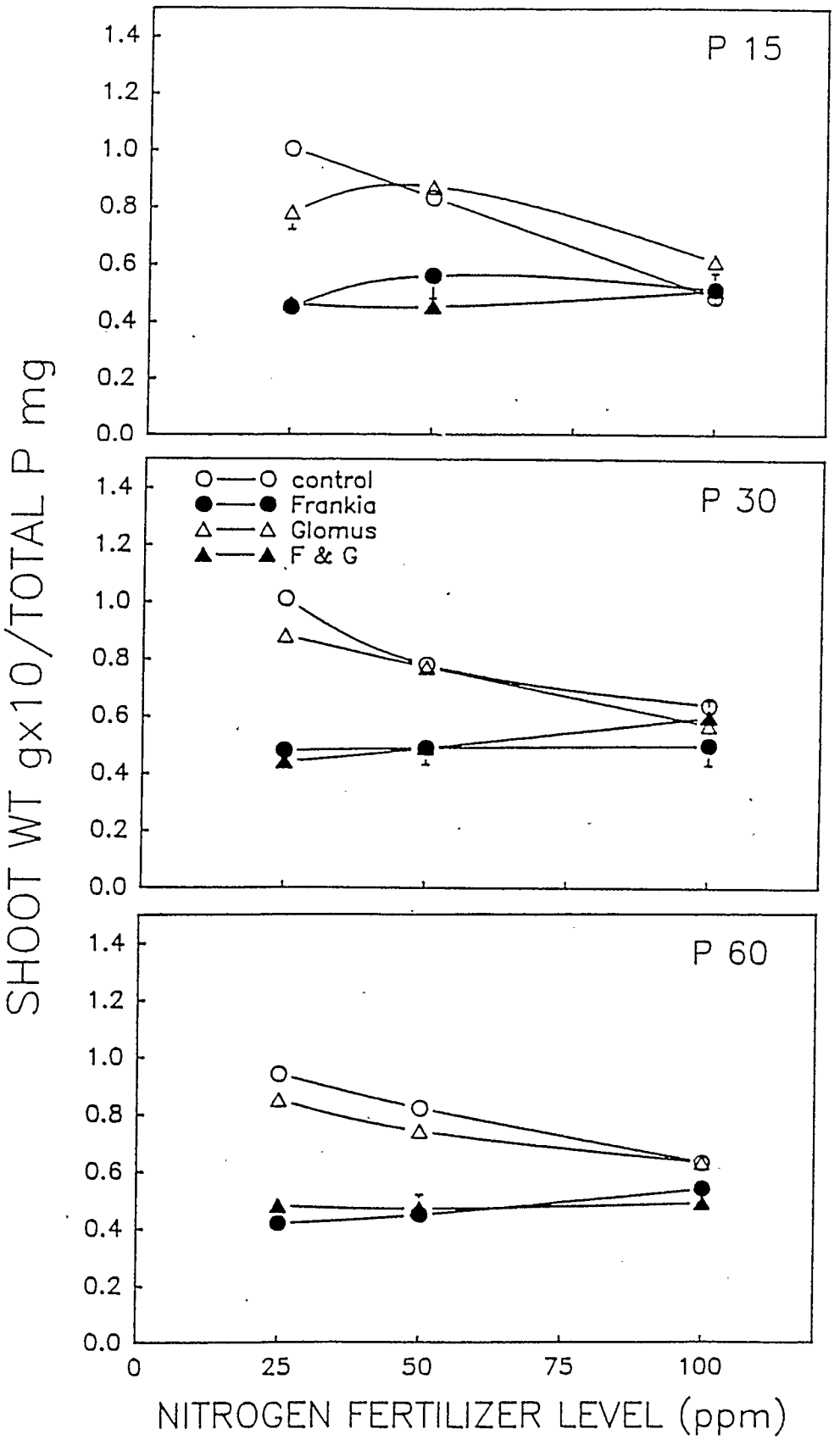

SHOOT P (total mg)
N25 P15
N25 P30
N25 P60
N50 P15
N50 P30
N50 P60
N100 P15
N100 P30
N100 P60
0
1
2
3
4
a
b
a
b
a
c
b
c
a
b
a
a
a
b
a
b
ab
b
a
b
b
c
a
a
a
a
a
a
a
a
a
a
a
a
a
ab
bc
c
C
F
G
FG

Figure 11. Phosphorus use efficiency (shoot production/total phosphorus absorbed) of *Elaeagnus* grown with and without *Frankia* and *Glomus* at nine fertilizer combinations. Means are given with their standard errors.


P 15
P 30
P 60
control
Frankia
Glomus
F & G
SHOOT WT gx10/TOTAL P mg
NITROGEN FERTILIZER LEVEL (ppm)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
0
25
50
75
100

Since previous analyses had shown a significant N effect, but no simple P effects, the data are presented along a N gradient, with a separate graph for each P level.

At the N25 P15 fertilizer treatment, all four data points are significantly different from one another (Tukey, $p \leq 0.05$). The Frankia+Glomus treatment showed the greatest phosphorus use efficiency, followed by the Frankia, control and Glomus treatments. At this fertilizer combination, where both N and P were very limiting, VAM resulted in greater biomass production per unit P absorbed in the presence of Frankia. However, when plants were not nodulated, control plants were more efficient than VAM plants. This was probably due to the carbon drain associated with VAM infection. Part of the VAM effect observed may have also been due to N uptake by the fungus, since at N50 P15, where P was even more limiting relative to available N, there was no longer a Frankia effect or Frankia+Glomus effect relative to control plants. These three treatments were still more efficient than VAM only seedlings, again probably due to the carbon cost of VAM infection. Differences at N100 P15, where P was very limiting relative to N are indistinct.

At the N25 P30 fertilizer level, there was no difference in efficiency between Frankia and Frankia+Glomus treatments. At this level, P was not limiting relative to N, therefore there was no advantage to VAM infection.

Nitrogen levels were still low enough that nodulation resulted in a significant growth effect. Control seedlings at this fertilizer level were also more efficient with respect to P use than mycorrhizal plants. As N levels increased, there was no longer any difference in efficiency among the treatments. Similarly, at P60, there were minimal differences in P use efficiency among the treatments and nitrogen levels. Nodulated seedlings were somewhat more efficient at N25, and perhaps at N50.

7.3.7 SHOOT NITROGEN CONTENT

The N content of Elaeagnus tissues can be evaluated similarly. Figure 12 gives the shoot N concentrations of the various treatment combinations. At N25 and N50, nitrogen concentrations were always higher in nodulated seedlings than control and mycorrhizal only plants. There was no apparent effect due to P. The total N content (Figure 13) showed similar trends, except that VAM effects were apparent. In all three low P treatments (P15), Frankia+Glomus plants had significantly greater total N contents than nodulated only or control plants. Mycorrhizal infection alone had no effect. The total N contents reflect identically the pattern observed in the shoot weight data.

Figure 14 gives the N use efficiency data for this experiment (shoot production per unit N present in the shoot). Nodulated plants, whether mycorrhizal or not,

Figure 12. Nitrogen content (% of dry weight ±SE) of *Elaeagnus* shoots grown with and without *Frankia* and *Glomus* at nine fertilizer combinations.

Data were transformed (ln(x)) prior to one-way ANOVA. Means followed by the same letter are not significantly different at $p \leq 0.05$ (Tukey).

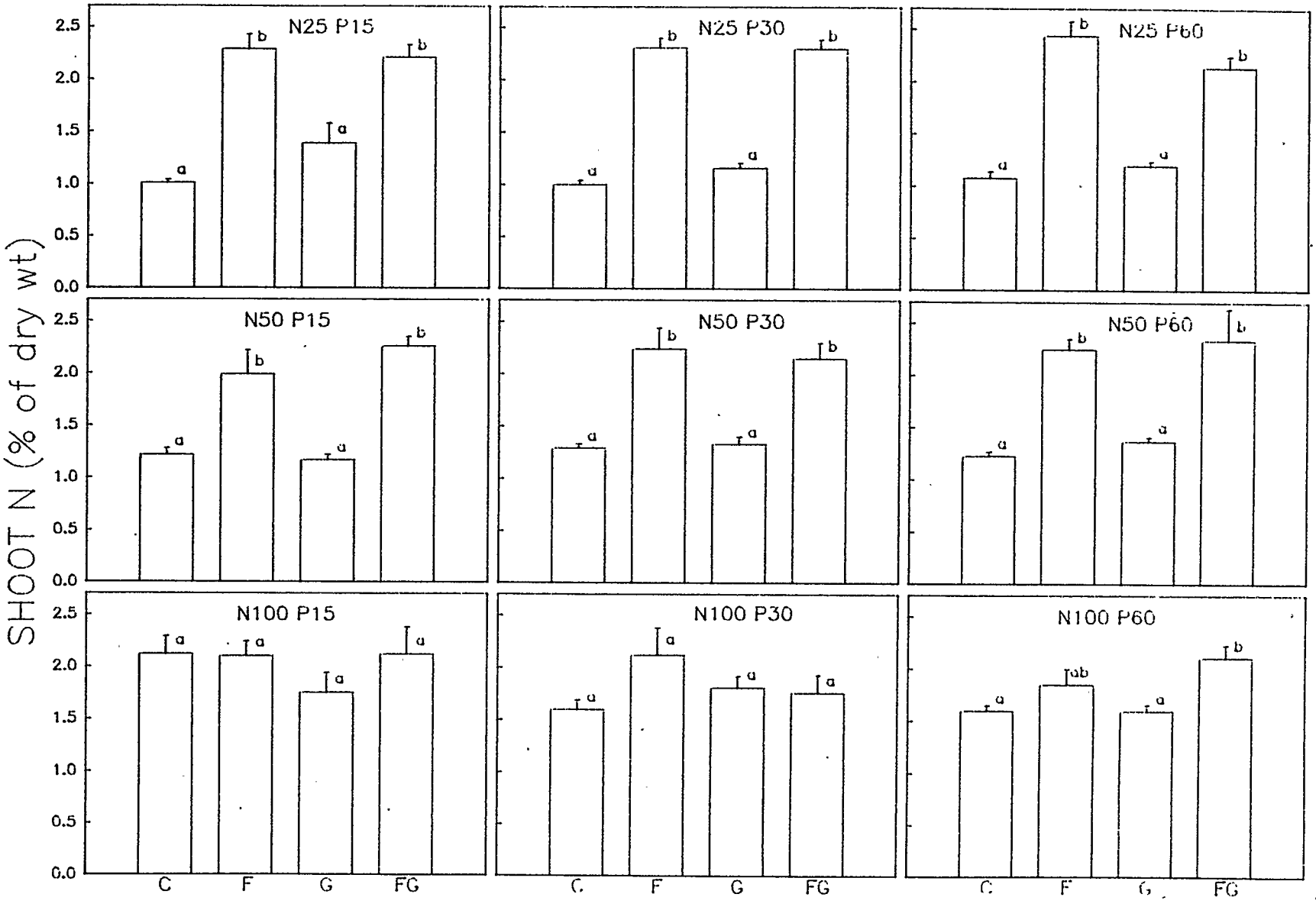

SHOOT N (% of dry wt)
N25 P15
N25 P30
N25 P60
N50 P15
N50 P30
N50 P60
N100 P15
N100 P30
N100 P60
0.0
0.5
1.0
1.5
2.0
2.5
C
F
G
FG

Figure 13. Nitrogen content (total mg/plant*SE) of Elaeagnus shoots grown with and without Frankia and Glomus at nine fertilizer combinations.

Data were transformed (ln(x)) prior to one-way ANOVA. Means followed by the same letter are not significantly different at $p \leq 0.05$ (Tukey).

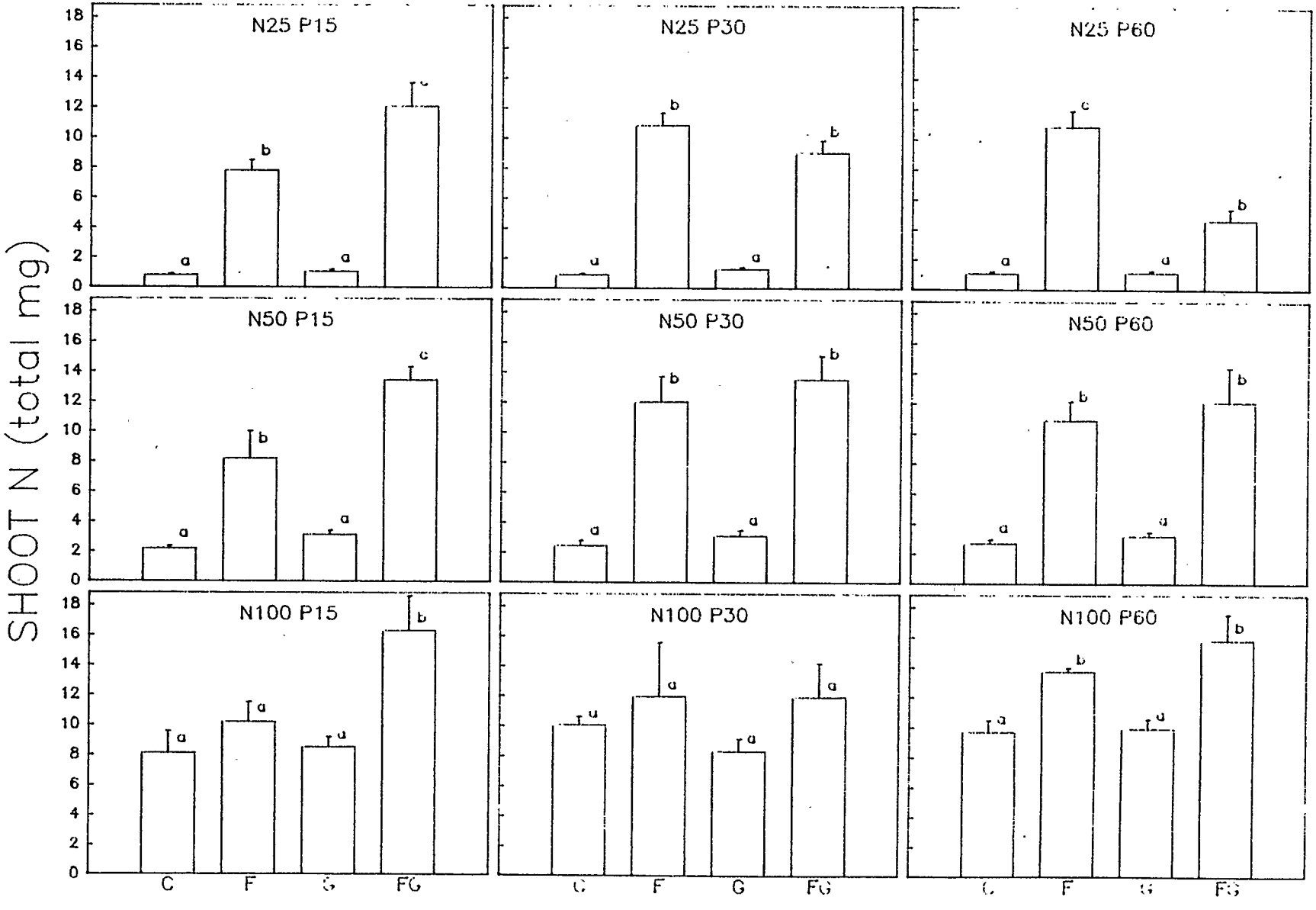
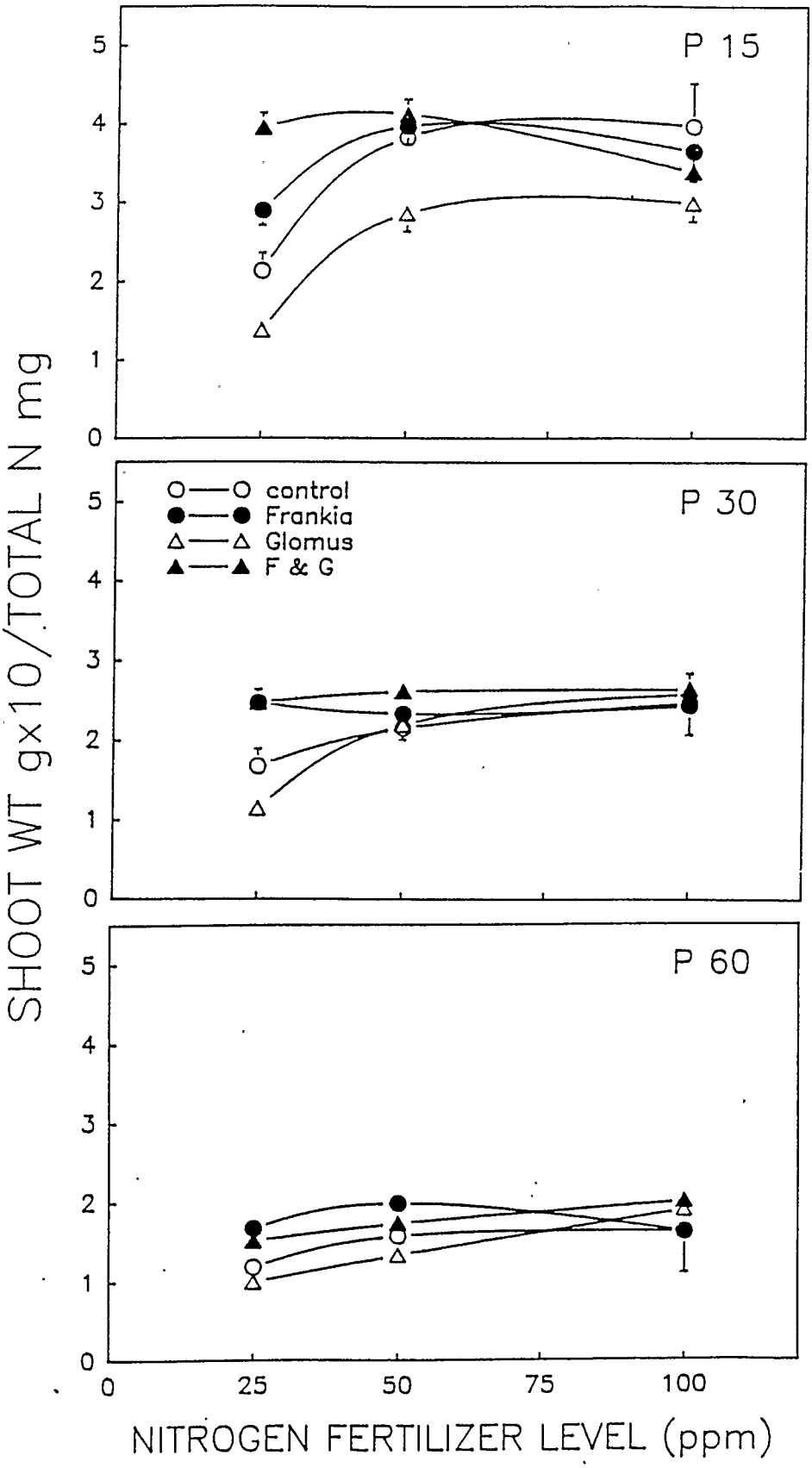

SHOOT N (total mg)
N25 P15
N25 P30
N25 P60
N50 P15
N50 P30
N50 P60
N100 P15
N100 P30
N100 P60
0
2
4
6
8
10
12
14
16
18
C
F
G
FG

Figure 14. Nitrogen use efficiency (shoot production/total nitrogen absorbed) of Elaeagnus grown with and without Frankia and Glomus at nine fertilizer combinations. Means are given with their standard errors.


P 15
P 30
P 60
control
Frankia
Glomus
F & G
SHOOT WT gx10/TOTAL N mg
NITROGEN FERTILIZER LEVEL (ppm)
0
25
50
75
100
1
2
3
4
5

showed the same N use efficiencies. These did not vary with either N or P level. The efficiencies did vary among treatments, with nodulated plants always showing significantly lower shoot production per unit N than control or mycorrhizal only plants (except at N100).

7.3.8 FRANKIA/VAM/ELAEAGNUS INTERACTIONS

In this experiment, Frankia increased plant growth at all fertilizer levels except N100 P30 and N100 P60. Mycorrhizal fungi alone had no significant effect on plant growth relative to control plants. However, at N25 P15 and N50 P15, VAM infection of nodulated plants resulted in increased biomass over all other treatments. These increases in plant biomass production relative to controls can be explained by improved P and N nutrition, as shown in Figure 11. In this Figure, mycorrhizal plants can be compared to non-mycorrhizal plants of equivalent nutrient status. Therefore, any VAM effects on plant growth other than P or N effects would be apparent. In this data set, at P15 N50 and P30 N25, Frankia+Glomus and Frankia-only infected plants had the same P use efficiency, indicating that improvements in growth due to VAM were due to differences in nutrition alone. At all the low P levels and at P30 N25, plants infected by VAM alone had significantly lower P use efficiencies than all other treatments. This is evidence of a carbon drain due to VAM infection in the absence of Frankia. Elaeagnus appears to

be considerably more dependent upon its N_2-fixing symbiont than upon VAM fungi.

Although Rose and Youngberg (1981) recorded higher N_2-fixation rates in the presence of VAM fungi, no such differences were observed in this experiment. However, there was a significant increase in nodule weight due to the presence of VAM at N25 P15 (Figure 7). This amount of nodule mass was not achieved in any of the other fertilizer treatments, indicating the possibility of a non-nutrient mediated effect of VAM on nodule weight.

CHAPTER 8

8. CONCLUSIONS

Frankia strains were easily isolated, but propagation in pure culture proved problematical. Since growth rates in complex media are very slow, it would be worthwhile determining the C sources used by each isolate and then growing the strains in a defined medium. Attempts should be made to induce sporangial formation, as this would ensure a more effective inoculum.

The soil conditions and inoculation procedures necessary for nodulation were also investigated. Nodulation occurred only at pH 7.0, but not at pH 6.5 or lower in the peat:vermiculite mixture used. Age of *Frankia* inoculum (6 vs 15 weeks) did not significantly affect nodulation. Homogenizing the inoculum with a Virtis blender did not significantly affect nodulation success, probably due to the very variable nodulation levels observed. Such a comparison should be repeated including a treatment with sonicated inoculum as used by Perinet *et al.* (1985). In a comparison of *Frankia* inoculation methods, both mixing the cells into the soil or injecting them proved effective, while surface application and soaking the seedling radicle in a *Frankia* slurry were ineffective. Here, too, nodulation success was extremely variable. It is possible that this variability was due to lack of homogeneity of the inoculum, low sporangial numbers or high

greenhouse temperatures. The effect of high soil temperatures on nodulation by Frankia should be investigated further.

The questions outlined in the introduction were addressed in the remaining experiments. The first question posed involved a comparison of the effects of different Frankia strains on Elaeagnus growth in the absence of mycorrhizal fungi. Three strains had been isolated from the Fort McMurray region. EANfma and EANfmb were non-infective strains, of which there are examples in the literature (eg. Lalonde et al. 1975). EANfm+ did nodulate seedlings, but inoculum levels were never high enough to ensure adequate replication for comparison with other strains. The strain donated by M. Lalonde (EAN1 isolated from E. commutata) nodulated effectively and was compared with the Rhizotec mixture of two strains isolated from E. angustifolia and E. umbellata. EANfm+ did not nodulate in the VAM control treatments which were being used here to compare Frankia strain effects on shrub growth. Since there were two VAM control soils (autoclaved slender wheatgrass and leek host plant pot cultures), plant response to Frankia can be compared in the two controls. In the slender wheatgrass source soil, plants nodulated by EAN1 showed significantly lower shoot weights than control plants, although the difference with Rhizotec nodulated seedlings was not significant. Differences between

nodulated and control plants in the leek source soil were not significant. The lack of response to Frankia was probably due to the early harvest time.

The second question dealt with the effects of different VAM species on Elaeagnus growth in the absence of Frankia. Mycorrhizal fungi alone did not promote plant growth relative to controls in any of the experiments. Actually, in two treatments, mycorrhizal plants were smaller than controls. Initially, it was felt that this was due to early plant harvest or container size effects, but subsequent results indicated that VAM fungi probably benefit Elaeagnus growth only in the presence of Frankia nodules.

The next question asked whether different VAM species/Frankia strain combinations affected host growth differently. There were differences in VAM infection levels and nodulation success among the combinations. Significant differences in nodule number but not nodule weight or acetylene reduction rates were observed. The treatment combination EAN1/no VAM had significantly lower shoot weight than the other treatments, which were not significantly different from one another. Differences may have been detected if clonal Elaeagnus seedlings had been used, if fertilizer levels had been lower, or perhaps if seedlings had been older. No conclusions about VAM species/Frankia strain interactions should be made from

these data, particularly when growth effects were not observed, and the possibility for such effects was demonstrated in the fertilizer response experiment.

The fourth question dealt with the nature of the interaction between the two symbionts and their host plant. The results of fertilizer response experiment indicated that Elaeagnus was more dependent upon Frankia than on the VAM fungus. Mycorrhizal fungi did benefit growth of nodulated shrubs under low P conditions. The growth enhancement due to VAM infection could be explained by improved P, and in some cases N nutrition. There is evidence for a non-nutritional effect of VAM on nodule weight at low available N and P levels, but this should be explored further.

The soil P and N levels at which VAM benefited nodulated Elaeagnus in these studies are similar to those found in soils in which the shrub grows. Disturbed soils often also have N and P concentrations in this range. When outplanting in disturbed areas, inoculation with both Frankia and VAM fungi would be beneficial, although Frankia appears to be the major contributor to improvements in plant growth over uninfected controls.

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10. APPENDICES

Appendix I: Contingency Table Analysis of Nodulation Experiment
(as per Cochran and Cox 1957, pp.103-105).

	Treatment				
	6 weeks		15 weeks		Total
	Surface	Inject	Surface	Inject	Total
Nodulated	1	8	2	4	15
Not-nod.	10	5	11	9	35
Total	11	13	13	13	50
Proportion Nodulated	0.0909	0.6154	0.1538	0.3077	0.3000

$$X^2=\frac{[1(.0909)+8(.6154)+2(.1538)+4(.3077)] - 15(0.3)}{0.3*0.7}$$

$=9.774$ vs $X^2_{0.05,3}=7.81$

Therefore, reject Ho: There is no difference between treatments.

Method of Inoculation (surface vs injected):

a) 6 weeks $X^2=6.84$ vs $X^2_{0.05,1}=3.84$
Therefore reject Ho: There is no difference between methods of inoculation.

b) 15 weeks $X^2=0.86$
Therefore do not reject Ho.

Age of Inoculum (6 weeks vs 15 weeks):

a) surface $X^2=0.22$
Therefore do not reject Ho: There is no difference between inoculum ages.

b) inject $X^2=2.46$
Therefore do not reject Ho:

Appendix II: Contingency table analysis of combination experiment nodulation rates (as per Cochrane and Cox 1957, pp.103-105).

	Strains EAN1 and Rhizotec				
	Slender wheatgrass		Leek		
	fi	Fi	fi	Fi	Total
Nodulated	58	45.5	33	45.5	91
Not nodulated	38	50.5	63	50.5	101
Total	96		96		192

$$X^2_c = \frac{(fi-Fi-0.5)^2}{Fi} = \frac{12^2}{45.5} + \frac{12^2}{50.5} + \frac{12^2}{45.5} + \frac{12^2}{50.5}$$

$X^2_c=12.03$ $X^2_{0.05,1}=3.84$ therefore reject Ho: There is no difference between inoculum sources.

	Strain EAN1				
	VAM		not VAM		
	fi	Fi	fi	Fi	Total
Nodulated	12	18.5	25	18.5	37
Not nodulated	20	13.5	7	13.5	27
Total	32		32		64

$X^2_c=9.23$ vs $X^2_{0.05,1}=3.84$ therefore reject Ho: There is no effect due to the presence of VAM.

Appendix II: (cont.)

	Strain Rhizotec				
	VAM		not VAM		
	fi	Fi	fi	Fi	Total
Nodulated	23	24.5	26	24.5	49
Not nodulated	9	7.5	6	7.5	15
Total	32		32		64

X^2_c=0.35 Therefore do not reject Ho: There is no effect due to the presence of VAM.

	Strain EANfm+				
	VAM		not VAM		
	fi	Fi	fi	Fi	Total
Nodulated	5	2.5	0	2.5	5
Not nodulated	27	29.5	32	29.5	59
Total	32		32		64

X^2_c=3.47 vs. $X^2_{0.05,1}$=3.84 Therefore do not reject Ho: There is no effect due to the presence of VAM.

Appendix III: Temperatures and flow rates used in gas chromatography.

Varian 3400 Gas Chromatograph

Column	Porapak R, 8/100 mesh, 6'x 1/8" O.D.
Column T	50°C
Injector T	50°C
Oven T	80°C
Detector T	100°C

Flow rates

		H_2	20mL/min
N_2	22mL/min		
air	200mL/min		

Acetylene peak	Attenuation 2 Range 10
Ethylene peak	Attenuation 16 Range 8

Standard Gas 100.6 ±2 ppm ethylene, balance N_2

Appendix IV: Number of replicates included in the calculation of treatment means.

Nitrogen Level (ppm)	Treatment	Phosphorus Level (ppm) 15	30	60
	Control	9	9	9
25	Frankia	10	9	9
	Glomus	9	9	10
	Both	10	10	10
	Control	10	8	9
50	Frankia	8	10	10
	Glomus	10	8	9
	Both	10	9	9
	Control	5	9	10
100	Frankia	8	5	2
	Glomus	10	10	10
	Both	6	6	8