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GREEN, THOMAS LOREN
  SYMBIOTIC RELATIONSHIPS AMONG ALNUS AND ITS
  MYCORRHIZAE AND ACTINORHIZAE.

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Symbiotic relationships among <u>Alnus</u>

and its mycorrhizae and actinorhizae

by

Thomas Loren Green

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INTRODUCTION

Inevitable world shortages in fixed nitrogen and fiber have generated renewed interest in nitrogen fixing plants and rapidly growing woody species. Several members of the genus Alnus Ehrh. have both attributes, efficient nitrogen fixation and rapid growth.

The ability of alder to fix nitrogen has enabled these woody plants to be a pioneer species on nutritionally poor sites. Alder has been extensively used for soil improvement on coal spoils, flood deposits, and landslide areas in Europe, Asia, and North America (Tarrant, 1968; Tarrant and Trappe, 1971). The nutritional enhancement to vegetation surrounding alder has long been recognized. Recent applications of this observation have employed the interplanting of alder with forest tree species as well as some high value species to enhance growth (Tarrant and Trappe, 1971). Substantial growth rates have been demonstrated by some Alnus species (Bajuk et al., 1978). In addition, some species have been found to have several desirable fiber qualities that could be used in the wood fiber industry (Vurdu and Bensend, 1978).

In the Pacific Northwest plantations of Douglas-fir appear to benefit from interplanted alder by the reduction of the incidence of a fungal root rot pathogen, Phellinus weirii (Murr.) Gilb. This biological control of the pathogen is believed to involve an increase in soil nitrogen which favors antagonistic soil microorganisms, and exudation of fungistatic fatty acid and phenolic compounds by alder roots (Li et al., 1970; Trappe et al., 1973).

In intensive forest management <u>Alnus</u> shows much promise. However, there are some problems inherent to production of seedlings for such intensive forestry. Two such problems involve seedling survival following outplanting, particularily on nutrient poor sites, and reduction or delayed establishment of seedlings following fertilization and fumigation practices used in nursery operations. Seedlings with mycorrhizae demonstrate much better growth than non-mycorrhizal seedlings growing in fumigated nursery beds (Marx et al., 1978). Survival rates for outplanted seedlings containing mycorrhizae was found to be substantially higher than non-mycorrhizal seedlings (Marx et al., 1977).

<u>Alnus</u> naturally establish mycorrhizae (fungus-root) and actinorhizae[1] (actinomycete-root) that enhance mineral nutrition and growth. It is desirable to exploit this natural growth stimulus by selecting the most efficient symbionts and encouraging timely symbiosis for maximum survival and growth. Most research involving tree microsymbionts often chooses one symbiont and compares results to a control. Such research with <u>Alnus</u> microsymbionts might produce misleading interpretations. In the natural ecosystem three known, separate microorganisms colonize <u>Alnus</u> roots, elicit some host response, influence rhizosphere micro-organisms, affect nutrient uptake and plant growth, and possibly interact with each other.

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[1]The term actinorhizae was adopted by the visiting scientists attending the Symbiotic Nitrogen Fixation in Actinomycete-Nodulated Plants Symposium, April 3-5, 1978, Harvard Forest, Petersham, Massachusetts to mean the root nodules formed as a result of a symbiotic association between certain actinomycetes and roots of susceptible hosts.

Very little is known about the occurrence of vesicular-arbuscular mycorrhizae (VAM) in Alnus. One general goal of this study was to determine the species range of VAM occurrence and to determine what influence, if any, VAM fungi had on the other Alnus symbionts.

The major objective of this study was the determination of the effect that the predominant symbionts, ectomycorrhizal fungi and nodule forming actinomycetes, have alone and in combination on the growth and mineral uptake in Alnus glutinosa (L.) Gaertn., and the effect these two symbionts have on each other. The information obtained should be useful in determining whether mycorrhizae and actinorhizae influence Alnus growth and mineral uptake in nursery-produced and containerized seedlings.

LITERATURE REVIEW

The Genus Alnus Ehrh.

The genus Alnus has been classified in the order Fagales, family Betulaceae, and subfamily Betuleae. Hutchinson (1959) has discussed the origin of the Fagales and its relationship to other orders. Alnus has been divided into three subgenera: Alnus, Alnobetula, and Clethropsis by Furlow (1974), and two subgenera: Alnaster and Gymnothyrsus by Murai (1964). The two taxonomists agree on seven genus sections: Clethropsis, Glutinosae, Japonicae, Fauriae, Cremastogyne, Alnobetula, and Bifurcatus.

There is some disagreement among Alnus taxonomists concerning the actual species number. Bond (1976) recognized 33 species. Willis (1966), cited by Bond and other authors, acknowledged 35 species. However, none of the 35 species were named or described thus, making Willis a questionable reference. Murai (1964) has detailed descriptions for 29 species. This work was written in Japanese, but there were sufficient sections in English to make this an essential reference for Alnus taxonomy. In 1968 Murai compared the species found in the northwestern United States to those found in Japan. Furlow (1974) studied the taxonomy of the North American Alnus species only. He has revised a few of Murai's new world species. Heebner (1977) combined the species of both taxonomists into 27 species (Table 1).

Table 1. _Alnus_ sections and species adapted from Murai (1964), Furlow (1974), and Heebner (1977)

Section	Species	Common Name	Ref[a]
Bifurcatus	_Alnus sieboldiana_ Matsum.	---	M, H
	A. firma Sieb. et Zucc.	Japanese green alder	M, H
	A. pendula Matsum.	Nodding alder	M, H
Alnobetula	_A. viridis_ (Vill.) Lan. & DC.	European green alder	F, H
	subsp. _viridis_ DC.		M, H
	var. _viridis_ DC.		M
	var. _brembana_ Rota		M
	subsp. _suaveolens_ Req.		M
	subsp. _crispa_ (Ait.) Req.	Green alder	F, H
	subsp. _sinuata_ (Req.) Love & Love	Sitka alder	F, H
	subsp. _mandshurica_ (Call.) Hara	---	M
	subsp. _maximowiczii_ (Call.) Love & Love	---	M
Cremastogyne	_A. cremastogyne_ Burk.	---	M, H
	A. lanata Duth.	Wooly leaf alder	M, H

Clethropsis	A. nepalensis D. Don	Nepal alder	M, H
	A. maritima (Marsh.) Nutt.	Sea-side alder	M, H
	A. nitida (Spach.) Steudel	Himalayan black alder	M, H
Japonicae	A. japonica (Thumb.) Steudel	Japanese alder	M, H
	var. formosana (Burk.) Call.		M
	var. japonica Steudel		M
	var. arguta (Reg.) Call.		M
	A. trabeculosa Hand.-Mazz.	---	M, H
	A. subcordata C.A. Mey.	Caucasian alder	M, H
	A. orientalis Dcne.	Oriental alder	M, H
	A. cordata (Lois.) Desf.	Italian alder	M, H
Fauriae	A. serrulatoides Call.	---	M, H
	A. Fauriei Levl. et Vant.	---	M, H

[a]References: M = Murai, F = Furlow, H = Heebner.

Table 1. (Continued)

Section	Species	Common Name	Ref
Glutinosae	A. rubra Bong.	Red alder	M, H
	A. Inokumae Murai et Kusaka	---	M, H
	A. Matsumarae Call.	Matsumarae alder	M, H
	A. hirsuta Turcz.	Manchurian alder	M, H
	var. hirsuta Turcz.		M
	var. tinctoria (Sarg.) Kudo		M
	A. glutinosa (L.) Gaertn.	European black alder	M, H
	var. glutinosa Gaertn.		M
	var. barbata (Mey.) Led.		M
	var. denticulata		M
	A. incana (L.) Moench	Gray alder	F, H
	subsp. incana Moench		F, H
	subsp. rugosa (D. Roi) Clau.	Speckled alder	F, H
	subsp. tenifolia (Nutt.) Breitung	Thinleaf alder	F, H
	A. serrulata (Air.) Willd.	Smooth alder	M, H

A. _oblongifolia_ Torr.	Lanceleaf alder	M, H
A. _rhombifolia_ Nutt.	White alder	M, H
A. _accuminata_ H.B.K.	Aile	F, H
A. _jorullensis_ H.B.K.	Aile	F, H

Vesicular-Arbuscular Mycorrhizae

The term mycorrhiza was coined by Frank in 1885. His original hypothesis that mycorrhizae were beneficial structures has been conclusively verified. Frank (1887) originally classified two types of mycorrhizae, ectotrophic and endotrophic. The terms ecto- and endomycorrhizae proposed by Peyronel et al. (1969) are preferred and widely accepted. However, some totally different mycorrhizal systems are included under the heading endomycorrhizae. The phycomycetous fungi that produce vesicles and/or arbuscules within cortex tissue are distinct from other endomycorrhizae and are referred to as vesicular-arbuscular mycorrhizae (VAM). Lewis (1975) presents an excellent discussion of the different classifications and their relationships for mycorrhizae.

Since the early 1960's the volume of research on VAM has grown at an exponential rate. This has been largely due to the development of techniques for pot culture propagation of VAM fungi with a variety of host plants and improved staining techniques of vesicules and arbuscules for determination (Mosse, 1973). Over 90% of the terrestrial plants form VAM. Their significance to the terrestrial plant kingdom has been recognized only recently. Phosphorus uptake has been found to be the most important function of VAM (Bieliski, 1973; Gerdemann, 1975; and Sanders et al., 1975). An obligate requirement for VAM establishment is indicated with some tree species. Sweet gum seedlings displaying phosphorus deficiency will not grow beyond the primary leaf stage during their first growing season without VAM (Kormanik et al., 1977).

There have been several excellent reviews and general discussions on VAM (Gerdemann, 1968, 1975; Harley, 1969; Kelley, 1950; Mosse, 1973; Nicolson, 1967; and Sanders et al., 1975).

Members of the families Pinaceae, Betulaceae, and Fagaceae are known to be almost always ectomycorrhizal. The Salicaceae, Juglandaceae, Tiliaceae, Myrtaceae, and Caesalpinaceae have species with both endomycorrhizal and ectomycorrhizal, often with both on the same plant (Gerdemann, 1968, 1975). The occurrence of VAM in Pinaceae, Betulaceae, and Fagaceae is rare.

Truszkowska (1953) studied the mycotrophic relationships with different alder forest associations. She found a higher incidence of mycorrhizal plants in areas that were drier and received more sunlight. One stand of Alnus glutinosa that was subject to frequent flooding had VAM only. Two other dry alder stands had ectomycorrhizae only. Willow growing in a wet site had both mycorrhizae with predominately VAM. Species usually mycotrophic with VAM fungi, Sorbus aucuparia L., Rhamnus frangula L., and Rubus suberectus G. Anderson, were reported to form ectomycorrhizae on dry sites. O'Malley (1974) found VAM in Quercus palustris Muenchh. located in low lying sandy sites that are frequently flooded. Ectomycorrhizal development occurred later in the drier part of the summer. Grand (1969) reported the occurrence of VAM on oak but did not give the time of year the roots were sampled or the edaphic conditions. Subsequent literature on Alnus VAM have been observations without discussion concerning site influence on their occurrence.

Mejstrik (1971) found VAM in A. glutinosa only in roots sampled in the spring from a depth of 10-30 cm. These alder trees were growing on lignite spoil banks. VAM were found in A. glutinosa and a number of other species growing on coal spoils (Daft et al., 1975; and Daft and Hacskaylo, 1976). Masui (1926) studied the ectomycorrhizae of Alnus and one of his photomicrographs is believed to show VAM. This figure was not discussed in the paper. Very little was known about VAM in 1926, and Masui possibly was unaware of their existence.

Ectomycorrhizae

Meyer (1973) estimated that approximately 3% of the phanerogams establish ectomycorrhizae. Yet, a very large portion of the mycorrhizal literature pertains to the ectomycorrhizae. There are several comprehensive reviews and texts on ectomycorrhizae (Kelley, 1950; Imshenetskii, 1955; Shemakhanova, 1962; Meyer, 1966; Harley, 1969; Hacskaylo, 1971; and Marks and Kozlowski, 1973).

Ectomycorrhizae are known to benefit their hosts by protecting the fine root system from attack by pathogens (Marx, 1973); increase the physiologically active surface area of roots; increase tolerance of tree roots to drought, high soil temperatures, soil toxins, and pH extremes (Marx and Bryan, 1973). However, the most important benefit of ectomycorrhizae is their increase in the efficiency of nutrient uptake, especially by plants growing under less than optimum soil nutrient conditions. A substantial portion of the ectomycorrhizal literature pertains to nutrient uptake and growth response. Increased

absorption of Ca, H_2PO_4, K, Rb, Cl, SO_4, Na, NO_3, NH_4, Mg, Fe, and Zn has been demonstrated with ectomycorrhizae or ectomycorrhizal fungi (Bowen, 1973). The fungal mantle is known to absorb and accumulate N, P, K, and Ca (Marx and Bryan, 1973). Increased absorption of N, P, and K in mycorrhizal plants is repeatedly correlated with increases in host growth (Bowen, 1973).

Alnus sp. with well developed ectomycorrhizae grew faster than plants with few mycorrhizae (Mishustin, 1955). Phosphate was absorbed five times faster in excised mycorrhizal roots of A. viridis than non-mycorrhizal roots (Mejstrik and Benecke, 1969). Three subtypes of ectomycorrhizae, sensu Dominik (1959), were described and were found to absorb phosphate at different rates. Nodules did not absorb phosphate.

Other studies involved with alder ectomycorrhizae have described the ectomycorrhizae, and the fungi and other microorganisms associated with alder forest communities. Frank (1888) was the first to report the occurrence of alder ectomycorrhizae. Masui (1926) described six types of ectomycorrhizae on three Japanese alder species. Klečka and Vukolov (1935) described the ectomycorrhizae of several tree species including A. incana. Neal et al. (1968a) presented detailed descriptions of two ectomycorrhizae found on A. rubra.

Until recently very little has been done to identify the fungal symbionts. Several fungal taxonomists have presented lists of fungi, mostly Hymenomycetes, associated with Alnus forest communities (Singer and Morello, 1960; Trappe, 1962; and Horak, 1963). Hall et al. (1979) have compiled a list of reported Alnus mycorrhizal associates or

possible associates. However, the presence of fungal fructifications in alder stands does not prove they are mycorrhizal symbionts.

Unless the fungal partner can be identified on the basis of its morphology, in which Cenococcum graniforme (Sow.) Ferd. & Winge is a rare exception, the procedures outlined by Zak (1971, 1973) must be followed for fungal symbiont identification. One of the procedures, perhaps the most reliable, is pure culture synthesis with isolates from known basidiocarps. Successful synthesis trials have shown that Paxillus filamentosus Fr. (Laiho, 1970), P. involutus (Batsch ex Fr.) Fr. (Laiho, 1970; Molina, 1979), Lactarius obscuratus (Lasch) Fr. (Froidevaux, 1973), Alpova diplophloeus (Zeller & Dodge) Trappe & Smith, Astraeus pteridis (Shear) Zeller and Scleroderma hypogaeum Zeller (Molina, 1979) are known Alnus mycorrhizal symbionts. These synthesis trials have involved several alder species. No studies have been conducted to see how many alder species will form ectomycorrhizae with each of these fungal species.

Actinorhizae

A large portion of the nitrogen fixation literature has been devoted to legume-Rhizobium symbiosis. In recent years a growing interest in non-legume nitrogen fixation has generated excellent reviews on the subject (McKee, 1962; Schaede, 1962; Bond, 1967, 1971; Quispel, 1974; Becking, 1975; and Nutman, 1976).

The observation that plants growing near alder derive benefits by this association has been found in the writings of Virgil (70-19 BC) and William Browne in 1630. The first description of alder nodules was made

in 1829 (Tarrant and Trappe, 1971). Woronin, 1866, as cited in Becking et al. (1964) was the first to associate the nodule formation with an endophyte that he believed to be a Hyphomycete. Experimental evidence that nodules were related to leaf growth and leaf nitrogen was presented by Dinger (1895). Hiltner (1896) and Nobbe and Hiltner (1904) presented evidence that atmospheric nitrogen was fixed in nodules.

Schaede, 1933, and von Plotho, 1941, as cited in Becking et al. (1964) provided evidence that the endophyte was an actinomycete. This was confirmed by Becking et al. (1964) with their electron microscope investigation of the alder endophyte. Becking (1974) classified this symbiont as Frankia alni Becking. Actinomycetes producing nodules in other genera are separate species with the species epithet based on the host genus. These may not be distinct species. Hall et al. (1979) has summarized the literature investigating intergeneric cross inoculation attempts. Nodules have been formed in A. glutinosa with crushed nodule inoculum from Myrica gale L., Elaeagnus angustifolia L., and Comptonia peregrina L. (=Myrica asplenifolia). Crushed nodule inoculum from A. incana, A. cordata, A. viridis, A. jorullensis, and A. rubra has produced nodules on A. glutinosa. Varying degrees of nitrogen fixation efficiency have been found in these inter- and intrageneric cross inoculations.

Apparently, Frankia alni has two strains based on whether nodules are produced with granula (+ strain) or without granula (- strain). Healthy nodules with granula (+) have been shown to be 100 to 1000 times more infective than healthy nodules without granula (-) (Akkermans

and van Dijk, 1976). Hall et al. (1979) showed different growth responses in A. glutinosa and A. rubra when inoculated with different strains of F. alni crushed inoculum; A. glutinosa (+), A. glutinosa (-), A. glutinosa (local strain), and A. rubra strain. In the same study different seed sources of A. glutinosa had different growth responses with each of the four F. alni strains. Dawson (1978) found different growth responses with the local A. glutinosa strain among different A. glutinosa clones. The results of these cross inoculation studies illustrate that there can be host genotype-strain specificity in the Alnus system just as there is in the legume-Rhizobium system. Further taxonomic clarification of the Frankia species awaits pure culture isolation of the actinomycete.

The Frankia strain and host interaction determine the nitrogen fixation efficiency. Various edaphic and climatic factors also influence efficiency as well as actinorhizae initiation. Nodulation and nitrogen fixation are affected by oxygen concentration; substrate pH; ionic form of soil nitrogen; concentration of soil nitrogen, cobalt, molybdenum, and copper; water; temperature; and light.

Oxygen is required for ATP production. Ferguson (1953, as cited in Dawson, 1978) found that actinorhizae have a higher requirement for oxygen than roots without actinomycete infection. However, anaerobic conditions inside the nodule are required for nitrogenase-catalyzed reactions (Becking, 1970). Bond (1961) determined that optimum nitrogen fixation in A. glutinosa occurred at a 12% oxygen concentration. All nitrogen fixation ceased at 40% oxygen.

The optimum pH of solution culture for the growth of nodulated
A. glutinosa was found to be between 4.2 and 5.4 (Bond et al., 1954).
At pH 3.3 nodulation was inhibited. However, plants with established
nodules could continue to grow at pH 3.3 (Ferguson, 1953, cited in
Dawson, 1978).

Low levels of ammonium-nitrogen (10 mg NH_4-N/1) was found to
increase actinorhizae weight in A. glutinosa. The nodule development
was depressed as the concentrations were increased to 50 and 100 mg/1
(MacConnell and Bond, 1957). Any concentration of nitrate-nitrogen
was found to inhibit nodulation (Quispel, 1958; Benecke, 1970).
Pizelle (1965, as cited in Dawson, 1978) separated an alder root system
into two compartments and found nodulation was suppressed in the compart-
ment not supplied with combined nitrogen. Therefore, the inhibitory
effect of the nitrate was not local.

The minor elements Mo, Co, and Cu have been found to be essential
for nodule formation. Becking (1961a,b) demonstrated that plants with
Mo were larger and had a 370% increase in nitrogen. Actinorhizae from
field plants contained six times more Mo than non-actinorhizae. The
Co requirement for nitrogen fixation was demonstrated in A. glutinosa
(Bond and Hewitt, 1962). Bond and Hewitt (1967) showed that growth of
A. glutinosa was stimulated by copper.

Nodule formation is inhibited when water is excessive or deficient.
McVean (1956) studied the root system of A. glutinosa and reported that
the shallow fibrous roots bearing actinorhizae and mycorrhizae did not
persist when subjected to flooding. Akkermans and van Dijk (1976)

discussed nodule formation in respect to moisture. On wet sites

nodules are not formed on roots below the water level. Moisture

stress also limits nodulation but to less of an extent than flooding.

Moisture stress caused a reduction in nitrogen fixation in legumes

(Sprent, 1972). More nodules occurred on Alnus roots adjacent to

drainage ditches and streams than on distal roots (Akkermans and van

Dijk, 1976).

Nitrogen fixation, fueled by ATP, shows a temperature sensitivity

similar to other biological processes fueled by ATP. A six times

higher rate of acetylene reduction was found at 25 C compared to 15 C

in A. glutinosa (Wheeler, 1971). Optimum nitrogen fixation occurred at

20 C in A. viridis (Benecke, 1970).

Carbohydrates produced from photosynthesis provide the energy for

nitrogen fixation. Within 24 hours after A. glutinosa plants were

placed in the dark nitrogen fixation nearly ceased (Wheeler, 1971).

Quispel (1954) found nodule formation was inhibited by low light levels

and shortened daylength. Diurnal fluctuations in nitrogen fixation

in first year A. glutinosa seedlings were found with peak rates

occurring at or just after midday (Wheeler, 1969). Diurnal fluct-

uations of photosynthate, particularly sucrose, corresponded to the

nitrogen fixation rates (Wheeler, 1971). However, Akkermans (1971,

as cited in Wheeler and Lawrie, 1976) was unable to find diurnal

fluctuations in fully grown alder.

Host-Symbiont Interaction

Healthy plant roots live harmoniously with a myriad of soil microorganisms. Many of these microorganisms are affected directly by the presence of the root. However, a few are capable of influencing the physiology of the root. There have been numerous studies on the effect of root pathogens on the physiological responses of root tissue. The mycorrhizal and actinorhizal symbionts elicit many of the same physiological responses as root pathogens in host root tissues with one extremely important exception. Root tissue destruction does not occur in roots associated with mutualistic symbionts as it does with pathogens.

Scannerini et al. (1975) and Kaspari (1975) discussed the response of the host following ingress by VAM fungi. Scannerini et al. (1975) compared the VAM fungi-host interaction to the host-pathogen relationships reviewed by Bracker and Littlefield (1973). Kaspari (1975) observed the hypertrophy of the host nucleus and an increase in host cytoplasm and organelles such as ER cisternae, mitochondria, and dictyosomes in response to the invasion of a VAM fungus into tobacco roots. This host cell reaction was similar to host response observations made in previous studies of orchid endomycorrhizae and roots attacked by pathogens.

There have been several ultrastructural studies of the Alnus-actinomycete association (Becking et al., 1964; Lalonde and Knowles, 1975a,b; and Gardner, 1976). Most of the attention has been paid to the actinomycete. However, Lalonde and Knowles (1975b) described a

hypertrophy and migration of the host nucleus and an increase in

dictyosomes (1975a) in response to penetration by the actinomycete.

Gardner (1976) described a rapid host cell response following entry

by the endophyte that included a decrease in starch grains and an

increase in mitochondria, ribosomes, Golgi bodies, and ER. The

pectic capsule formed around the endophyte during penetration was

considered to be a defense reaction by the host (Lalonde and Knowles,

1975a).

The host response to the invasion and intracellular development

of VAM fungi and the nodule forming actinomycetes is very similar.

The host response to the ectomycorrhizal fungi has some similarities.

True ectomycorrhizae lack intracellular hyphae. However, pine cortex

cells adjacent to the fungal hyphae, Hartig net, appeared to have

enlarged nuclei (Foster and Marks, 1966). They also found that host

cells within the Hartig net had smaller starch grains. Hofsten (1969)

reported an increase in mitochondria and plastids in host cells within

the Hartig net. Other investigations have found that the cortex

cells are unaffected by the presence of the fungus (Marks and Foster,

1973).

The three symbionts have different affects on host root morphology.

Most VAM fungi-host interactions have no observable effect on root

morphology. Nodules are composed of a proliferation of modified, often

dichotomously branched, roots that are arrested in growth. They

usually have a coralloid appearance (Becking, 1966). These modified

roots lack root hairs. The ectomycorrhizal fungi produce thickened

roots covered with a fungal mantle and lack root hairs. Ectomycorrhizae lack a functional epidermis and possess a tannin layer. Some angiosperms have elongated outer cortex cells (Harley, 1969; Marks and Foster, 1973). The ultrastructural and/or morphological host response to one or more of the three symbionts may have an effect on the other symbionts.

The fungal mantle of ectomycorrhizae presents a physical barrier (Zak, 1964; Marx, 1973) and in some cases an antibiotic barrier (Marx, 1969, 1973) to potential invading microorganisms. The tannin layer presents a biochemical barrier (Foster and Marks, 1966, 1967; and Marx, 1973). Marx (1973) in his review on mycorrhizae and feeder root diseases discusses these and additional host responses to ectomycorrhizal formation.

Various preformed phenolic compounds and other fungistatic compounds are produced to some extent by most plants. These compounds differ from plant to plant. Hillis et al. (1969) suggested that mycorrhizal formation could be hindered by the variety of toxic phenolic compounds encountered by an invading ectomycorrhizal symbiont. This conclusion was based on a comparison of two conifer hosts, their polyphenol production, and the number of symbionts.

An unidentified antibacterial compound was found to be produced by alder roots and nodules in the spring and summer but not in the winter. It was postulated that the compound was an antibiotic produced by the actinomycete, or an oxidized phenolic compound (Neal et al., 1968b). Fungistatic phenolic compounds were found in alder roots (Li et al., 1972). The actinorhizae produced similar phenolic compounds

(Li, 1974). A fungistatic fatty acid is known to be produced by alder roots (Li et al., 1970). The effect of these compounds on the establishment of the other symbionts is not known.

Becking (1966, 1970, 1975) and Angulo-Carmona et al. (1976) have described the ingression process of the actinomycete. Penetration occurs in the root hair tip. The endophyte propagules stimulate root hair curling and are encapsulated by an excretion produced by the root hair. This encapsulation seems to assist in the penetration of a deformed root hair (Lalonde, 1977). Following penetration the host cell wall invaginates and an infection thread is formed (Becking, 1970).

The VAM fungi also penetrate roots through root hairs as well as other epidermal cells. At the point of entry the fungal hyphae form appressoria (Nicolson, 1967).

Ectomycorrhizae are covered by the fungal mantle. Such structures lack the epidermal tissue required for actinomycete and VAM permeation and colonization. Therefore, in alder the development of actinorhizae and VAM could be inhibited proportionally with the percentage of fine roots that become ectomycorrhizae.

Synergistic interactions have been demonstrated between nitrogen fixing microorganisms and plants with mycorrhizae (Asai, 1944; Crush, 1974; and Daft and El-Giahmi 1975). Mycorrhizae increase mineral uptake, including nitrogen. Early mycorrhizal physiologists proposed that some mycorrhizal fungi may be capable of fixing nitrogen (Bowen, 1973). This has yet to be demonstrated.

Zak (1964) postulated that ectomycorrhizae create a favorable

rhizosphere for other microorganisms to compete with and repel pathogens. Richards and Voight (1964) showed that Pinus radiata D. Don with ectomycorrhizae fixed more nitrogen than those without. They presumed that the nitrogen fixation was accomplished by contaminating bacteria. The plants with mycorrhizae possibly created a more favorable environment for the nitrogen fixing microorganisms than the non-mycorrhizal trees.

A reverse phenomenon was reported by Vedenyapina (1955). He found that oak seedlings given free-living nitrogen fixing bacteria, Azotobacter, had a higher percentage of mycorrhizae than controls. Shemakhanova (1962) reviewed the work of several Russian microbiologists. They reported that microorganisms of the mycorrhizosphere including Pseudomonas sp., Azotobacter sp., and Trichoderma lignorum (Tode) Harz, stimulated the formation of ectomycorrhizae in oak seedlings. Azotobacter was reported to release thiamine that stimulated both the ectomycorrhizal fungi and the root.

A symbiotic interaction between nitrogen fixing bacteria, Rhizobium, and VAM fungi has been demonstrated in several legume hosts. Asai (1944) reported that legumes grew poorly and failed to nodulate in autoclaved soil unless they were mycorrhizal (VAM). Crush (1974) found that VAM fungi stimulated nodulation and growth of several legumes grown in phosphorus-deficient soil. Daft and El-Giahmi (1975) reported increased host growth, increased nodule number and weight, and higher acetylene reduction rates in legumes with VAM. Carling et al. (1978) demonstrated that in combination with Rhizobium, VAM fungi or phosphate fertilizers produced similar results in soybean growth responses, nodule weight,

nitrate reductase activity, and total nitrogen reductase. They did not find any direct interaction, though, between Rhizobium and VAM fungi. An increase in nodulation and nitrogen fixation had been reported in actinorhizal hosts containing VAM (Williams, 1978). The interaction among Alnus, its ectomycorrhizal fungi, and its actinorhizal symbiont has not been investigated. Very little is known about VAM formation in Alnus and nothing is known about their interaction with the other symbionts.

MATERIALS AND METHODS

Vesicular-Arbuscular Mycorrhizae

Roots of Alnus spp. growing under natural, plantation, and arboretum conditions were sampled to determine the occurrence of vesicular-arbuscular mycorrhizae (VAM). Root samples from natural stands of Alnus rugosa (=A. incana) were collected in the upper peninsula of Michigan, northern Wisconsin, and near St. Paul, Minnesota. Root samples from A. glutinosa plantations were obtained in southeast Ohio on coal spoils [selection numbers 28, 44, 46, and 58 cited by Funk (1973) were sampled], in central Georgia on kaolin spoils, and in central Iowa. Root samples from natural stands of A. serrulata were were obtained in western North Carolina. Root samples from A. glutinosa, A. hirsuta, A. incana, A. Inokumae, A. japonica, A. maritima, and A. rugosa (=A. incana) were collected at the Morton Arboretum in Lisle, Illinois.

The roots were washed and preserved in FAA solution. VAM were determined by microscopic examination of cleared and stained root material following a modified Phillips and Hayman (1970) procedure (Table A-1).

Ectomycorrhizal Synthesis Trials

Pisolithus tinctorius (Pers.) Coker and Couch, isolate 185, and Thelephora terristris (Ehrh.) Fr. were provided by D. H. Marx as potential mycorrhizal symbionts for the genus Alnus. P. tinctorius has a worldwide

distribution on a variety of sites and a broad host range including over 72 species within 10 genera of conifers and hardwoods (Marx, 1977). T. terristris is a common mycorrhizal fungus which has been used in pure culture synthesis trials.

Cultures of Paxillus involutus and Cenococcum graniforme, confirmed Alnus symbionts, were provided by J. M. Trappe. Cultures of all of these fungi were maintained on MMN agar medium (Table A-2).

Isolation of ectomycorrhizal fungi was attempted from fresh root samples of A. glutinosa obtained at the 4-H camping area near Madrid, Iowa. Root samples were placed in plastic bags and packed in ice until transported to the laboratory. The roots were washed in cold running tap water for a one-hour minimum period and rinsed with sterile deionized water. A dissecting microscope was used to select swollen ectomycorrhizal fine roots for surface sterilization.

Some of the fine roots were placed directly on MMN medium plates without surface sterilization. The remaining ectomycorrhizae were placed in perforated plastic vials and submerged in a solution of 100 ppm mercuric chloride for 2, 2.5, 3, and 4 min. The vials were transferred to four sterile water rinses for 5 min each. The vials were agitated every 30 sec in each rinse. The roots were removed aseptically from the vials, cut into small pieces, placed four per MMN medium plate, and incubated at 25 C in the dark. The MMN medium plates contained 0, 30, 50, and 80 ppm streptomycin sulfate. Fungal cultures containing hyphae with clamp connections were retained and considered to be mycorrhizal symbionts (Marx, Bryan, and Grand, 1970).

The fungal inocula were propagated in various media: a) Agar base; P. tinctorius (Pt), Paxillus involutus (Pi), and C. graniforme (Cg) were grown on MMN medium: a mycorrhizal isolate containing clamp connections, isolate A-3, was grown on potato dextrose medium (PDA); b) Vermiculite in jars; Pt, Pi, T. terrestris (Tt), and isolate A-3 were prepared by the method of Marx and Bryan (1975); c) Other; isolate A-3 was grown in potato dextrose broth (PDB), autoclaved millet, and autoclaved brown rice.

A. glutinosa, clone ISU 2-58, and a mixed seed collection from central Iowa, ISU 0910, were selected for synthesis trials. Clone ISU 2-58 included stock plants over 6 mo old and tip cuttings. Tip cuttings, approximately 10 cm long, were excised from the stock plants. The basal end of each cutting was dipped in a 6000 ppm solution of indobutric acid in 50% ethanol for 5 sec and inserted into saturated Jiffy-7 peat pellets. Plants were intermittently misted for 8 wk to allow for the establishment of roots. The interval of misting was gradually decreased during the 8 wk.

The A. glutinosa seed was washed with running tap water for 1 hr. Surface sterilization was obtained by agitating the seed for 10 min in 30% hydrogen peroxide (Neal et al., 1967). The seeds were rinsed in sterile water, and placed on moistened paper towels in a plastic crisper. The seedlings were transplanted after 10 da.

The mycorrhizal inocula were incorporated into various growth media at a 10-12.5% rate and mycorrhizal synthesis attempted (Table 2). The growth media included: a) Jiffy Mix, a commercial potting mix

Table 2. Fungal isolates, culture media, <u>Alnus</u> source, and potting substrate used in mycorrhizal synthesis trials

Fungal isolate	Culture media	Alnus[a] source	Potting substrate	Number of plants
Pt	vermiculite	2-58 stock	Jiffy Mix	2
	vermiculite	2-58 cutting	Soil[b]	48
	vermiculite	0910	MPB[c]	8
	vermiculite	0910	MPB + Ca(OH)	8
	vermiculite	0910	Peat-vermiculite	8
	MMN agar	0910	Soil[d]	8
Tt	vermiculite	0910	MPB	8
	vermiculite	0910	Peat-vermiculite	8
Cg	MMN agar	0910	MPB	8
	MMN agar	0910	Sand	8
	MMN agar	0910	Soil	8
Pi	MMN agar	0910	MPB	8
	MMN agar	0910	Sand	8
	MMN agar	0910	Soil	8
	vermiculite	0910	MPB	8
	vermiculite	0910	Peat-vermiculite	8
A-3	rice	2-58 cutting	Jiffy Mix	6
	PDB	2-58 cutting	Jiffy Mix	6
	PDA	2-58 cutting	Jiffy Mix	6
	PDA	0910	MPB	8
	PDA	0910	Sand	8
	rice	0910	Soil	4
	PDB	0910	Soil	4
	vermiculite	0910	Peat-vermiculite	2
	vermiculite	0910	MPB	2
	millet	0910	Peat-vermiculite	2
	millet	0910	MPB	2

[a] Iowa State University seed source number.

[b] Soil for 2-58 seed source was autoclaved topsoil-vermiculite-sand (1:1:1, v/v/v).

[c] MPB = milled pine bark.

[d] Soil for 0910 seed source was pasteurized loam-sand-peat (2:1:1, v/v/v).

supplimented with nutrients; b) Fumigated milled pine bark (MPB),

a portion of which was amended with 2.5 g $Ca(OH)_2/l$ to raise the pH;

c) Autoclaved sand; d) Autoclaved peat-vermiculite (P-V) (1:1, v/v);

and e) Steam pasteurized loam soil-sand-peat (2:1:1, v/v/v).

Nodule Synthesis Trials

The original source of the nodule inoculum was collected from one

A. glutinosa grown from seed in central Iowa and infected naturally.

This inoculum was propagated on hydroponic trees grown during a previous

study. Dawson (1978) found this nodule inoculum to be the spore-negative

strain.

Fresh root nodules were washed for 0.5-1.0 hr with running tap water

and surface sterilized by 5 min of agitation in a 5.25% NaOCl solution.

The nodules were rinsed five times with sterilized deionized water. For

two nodule synthesis trials the nodules were pulverized in a Waring

blender in deionized water. A 10% solution of inoculum was poured

directly onto the roots of two clone ISU 2-58 stock plants, and three

clone ISU 2-58 rooted cuttings. A 1% inoculum solution was applied also

to three clone ISU 2-58 rooted cuttings. Lalonde and Knowles (1975b)

procedure for inoculum preparation was followed for two other nodule

synthesis trials (Table A-3). Forty-eight clone ISU 2-58 rooted

cuttings were given 25 ml of a 1% inoculum solution. Three applications

were made at 2 da intervals. Sixteen clone ISU 0910 seedlings were

given 1 ml of 1% inoculum treatments. Three applications were given

at 2 da intervals to four seedlings each growing in milled pine bark,

milled pine bark containing $Ca(OH)_2$, peat-vermiculite, and pasteurized soil.

Isolate A-3 Characterization and Identification

Factors that stimulated optimum growth of isolate A-3 were determined for nutrient medium, temperature, pH, and light. Isolate A-3 was grown on agar and in broth of MMN, Hagem's, malt, V-8, oatmeal, Kaufman's, Singer's, Lutz, Czapek's, Fries, and potato dextrose media. Fungal plugs 5 mm in diameter were inoculated into six flasks and six plates of each medium and stored at room temperature (25-27 C) in the dark for 1 mo. PDA plates were inoculated with fungal plugs 5 mm in diameter and stored at 0, 5, 10, 15, 20, 25, 30, 35 C and room temperature in the dark for 2 wk. Fungal plugs 5 mm in diameter were inoculated into PDB with pH adjusted to 2.5, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and not adjusted. The pH values 2.5 to 6.0 were adjusted with HCl. The pH values 7.0 to 9.0 were adjusted with 1M Tris buffer (Trishydro-xymethylamino methane). The PDB was autoclaved (20 min at 15 p/si) following pH adjustment. Six replications of each pH were tested. Twelve plates of PDA inoculated with fungal plugs 5 mm in diameter were prepared for the effect of light. Six plates were placed under a continuous fluorescent light source, and six were kept in the dark. Both treatments were held at room temperature.

Identification of fungal symbionts isolated from mycorrhizae rather than sporophores or rhizomorphs is difficult. Sporocarps and rhizomorphs were not observed with the mycorrhizae that produce isolate

A-3. Therefore, Zak's (1973) key was followed in an attempt to characterize isolate A-3 (Table 3).

Table 3. Zak's (1973) proposed key for ectomycorrhizae identification

1. Tree species

2. Color of mycorrhiza (X 10)

3. Attached mycelium; presence or absence and gross (X 10-50) character

4. Attached rhizomorphs; presence or absence and gross (X 10-50) character

5. Surface texture of mantle (X 10-50)

6. Form or structure (X 5)

7. U-V light fluorescence (X 10)

8. Chemical reagent color reaction (X 10)

9. Attached mycelium microscope (X 100-1000) features

10. Attached rhizomorphs (X 100-1000) features

11. Form of individual elements

12. U-V light fluorescence of hyphae (X 100-1000)

13. Chemical reagent effect on hyphae (X 100-1000)

14. Character of mantle surface hyphae (X100-1000)

15. Mantle structure (X 100-1000)

16. Individual element diameter and uniformity in diameter (X 10-50)

17. Character of the Hartig net (X 100-1000)

18. Habitat

19. Taste and odor

Three-way Study

The Jiffy-7 substrate was removed on 8-wk-old A. glutinosa, clone ISU 2-58, cuttings. Twelve cuttings were randomly selected for each treatment. There were nine treatments. In each treatment live or dead inoculum of three suspected Alnus symbionts; VAM fungal chlamydospores, Pisolithus tinctorius, and ground nodules; was incorporated into autoclaved soil (topsoil: vermiculite: sand, 1:1:1, v/v/v). Each cutting was planted in a plastic pot.

The ectomycorrhizal fungus, P. tinctorius, was propagated in 2 1 mason jars containing 1450 cc vermiculite and 50 cc peat in a MMN solution (Marx and Bryan, 1975). After 2 mo for growth, half of the inoculum was autoclaved. The inoculum was removed from the jar, placed in nylon mesh, and rinsed with tap water for 45 sec. Six hundred cc of peat-vermiculite inoculum was mixed with approximately 5000 cc of sterile soil in eight of nine treatments.

VAM fungal chlamydospores were sieved and decanted (Gerdemann and Nicolson, 1963) from topsoil obtained beneath A. glutinosa trees growing in central Iowa. The spores were surface sterilized with 0.525 % NaOCl for 2 min followed with four rinses in sterile deionized water. The last rinse was filtered and retained as a leachate solution. This solution was used to standardize the microorganisms in the two treatments, without the VAM fungal spores (Bryan and Kormanik, 1977). VAM fungal spores were kept in Ringer's solution in the refrigerator until the cuttings were planted. The volume of the Ringer's solution was adjusted so that each ml contained approximately 20 VAM fungal spores. One ml

spore suspensions were added to 24 ml sterile deionized water. Twenty-five ml of water containing VAM fungal spores or VAM leachate was given to all trees in eight of nine treatments.

Fresh nodules were excised from stock plants. The inoculum was prepared followings Lalonde's procedure as previously described (Table A-3). One-half of a 4 l, 10% inoculum suspension was autoclaved. Twenty-five ml of nodule inoculum was applied to the root system of each of the cuttings in eight of nine treatments during planting.

Treatment 1; ectomycorrhizae (E); contained 600 cc of live P. tinctorius (Pt) inoculum, 25 ml of autoclaved nodule inoculum suspension, and 25 ml of VAM leachate. Treatment 2; VAM; contained 600 cc of autoclaved Pt inoculum, 25 ml of autoclaved nodule inoculum suspension, and 25·ml of live VAM inoculum suspension. Treatment 3; nodule (N); contained 600 cc of autoclaved Pt inoculum, 25 ml of live N inoculum suspension, and 25 ml of VAM leachate. Treatment 4; E + VAM; had live Pt and VAM plus dead N. Treatment 5; E + N; had live Pt and N plus VAM leachate. Treatment 6; VAM + N; had live VAM and N plus dead Pt. Treatment 7; E + VAM + N; had all three live symbionts. Treatment 8; control; had dead Pt and N plus VAM leachate. Treatment 9; check; had no added inocula. Pt inoculum was mixed with the sterile soil prior to plantings. N and VAM suspensions were added after the cuttings were planted. Height and basal stem diameter measurements were made on the cuttings following planting.

The cuttings were placed beneath greenhouse benches and arranged in a randomized block design with each treatment on a separate bench.

The plants were allowed to become established for 2 wk and then were placed on top of the benches. Because of the heat absorbing black plastic pots and excessive greenhouse temperatures 400 cc of perlite were added to the top of the soil and the pots were covered with aluminum foil. The trees were watered as needed.

Two-way Study

The failure of symbiont survival in the three-way study necessitated redesign. There was insufficient information concerning the VAM fungal species which infect Alnus and the conditions required for their establishment. Therefore, VAM treatments were eliminated from the new design. Pt failed to form ectomycorrhizae in all synthesis trials and was eliminated as the ectomycorrhizal symbiont. Fungal isolate A-3 was selected as the mycorrhizal symbiont after successful synthesis trials.

The two-way study was constructed to evaluate the effect of ectomycorrhizae alone, nodules alone, both symbionts together, and both lacking, on various growth parameters and mineral uptake of the host. The experimental design also handled possible symbiont interactions including the effect of one symbiont on the other after the other has been established for a period of 2 mo.

A 3 x 3 factorial design was used for the initial growth period of 2 mo. A. glutinosa seeds, clone ISU 0910, were surface sterilized with hydrogen peroxide (Neal et al., 1967) and placed in autoclaved pure quartz sand in a sterile crisper. The seeds were given Crone's

nitrogen-free nutrient solution supplemented with Hoagland's A-Z micronutrient solution (Hewitt, 1966). The crisper was placed in a growth chamber. Two-wk-old seedlings were planted in steam pasteurized loam soil-sand-peat (2:1:1, v/v/v) mixture. The seedlings were given nine different combinations of living, dead, or no mycorrhizal and nodule inocula (Figure 1).

The ectomycorrhizal fungus used in this experiment was isolate A-3, a known symbiont of A. glutinosa, determined in a previous synthesis trial. The fungus was propagated in PDB and grown at room temperature in the dark for 2 mo. Half of the inoculum was autoclaved. The fungal mats were removed from the broth and rinsed for 1 min with tap water. The mats were dried with paper towels to a point where water drops no longer formed as the mat was allowed to hang. This was the criteria for fresh weight. The washed mats were homogenized in tap water in a Waring blender. Thirty g of fungal tissue was mixed in 3 l of tap water. The 3 l, 1% fungal suspension was mixed with 10 l of soil. Thirty g of autoclaved fungal tissue was prepared in a similar manner.

The roots of the seedlings treated with live or dead fungi were dipped in a slurry of concentrated homogenized inoculum and were planted in the inoculated soil. One ml of a 1% fungal suspension was applied to the seedling at its stem base after planting.

The nodule inoculum used in this experiment was obtained from nodulated stock plants. Thirty-two g of nodules were rinsed, surface sterilized, and homogenized in a Waring blender as previously described. A 10% suspension was prepared in deionized water, and half was autoclaved.

Nodule Inoculum

	Live	Dead	None
Live	TRT 1	TRT 2	TRTS 5, 6
Dead	TRT 3	TRT 4	TRTS 7, 8
None	TRTS 9, 11	TRTS 10, 12	TRTS 13-16

Fungal Inoculum

Figure 1. Factorial (3 x 3) design for Alnus glutinosa seedling treatments with living, dead, or no mycorrhizal and nodule inocula for the first 2 mo

The nodule inoculum, minus 200 ml, was mixed with 10 l of soil. The remaining suspension was used as a seedling root dip prior to planting, and used for a 1 ml-inoculum application to the seedling stem base after planting.

The seedlings were planted in Rootrainers®. Each Rootrainer book held four seedlings which constituted one treatment. Each tray held eight books. Two trays held 16 books and made up one replication. There were five replications. The treatment sequence in each replication was selected at random. The seedlings were grown for 2 mo in a growth chamber (13 hr days, 11 hr nights, 29 C, 21 C respectively at approximately 350 μeinsteins/m^2/sec of daylight irradiance).

During the final 2.5 mo of the experiment the seedlings were given either live or dead mycorrhizal and nodule inocula except in treatment 17. Seedlings in treatment 17 did not receive inocula (Table 4). The 12 best seedlings of the 20 from the first 2 mo of the experiment were selected for transplanting into larger Rootrainers. Each Rootrainer had three seedlings per book, and each book represented one treatment. There was a total of 17 treatments with four replications. The treatment sequence was selected at random. Each book with seedlings was separated from the other treatments with an empty book to help reduce comtamination from splashing during watering. The trees were grown in the greenhouse for 2.5 mo and were watered as needed. No fertilizer was given to the seedlings during their entire 4.5 mo growth period.

Data collected for analysis included: height, basal stem diameter, root displacement, stem dry weight, leaf dry weight, acetylene reduction,

Table 4. Inocula treatments given to <u>Alnus glutinosa</u> seedlings for the first 2 mo and the next 2.5 mo; total growth period being 4.5 mo

Treatment	First 2 mo		Last 2.5 mo	
	Nodule inoculum	Fungal inoculum	Nodule inoculum	Fungal inoculum
1	L[a]	L	L	L
2	D[b]	L	D	L
3	L	D	L	D
4	D	D	D	D
5	-[c]	L	L	L
6	-	L	D	L
7	-	D	L	D
8	-	D	D	D
9	L	-	L	L
10	D	-	D	L
11	L	-	L	D
12	D	-	D	D
13	-	-	L	L
14	-	-	D	L
15	-	-	L	D
16	-	-	D	D
17	0[d]	0	-	-

[a]L = living inoculum.

[b]D = dead (autoclaved) inoculum.

[c]- = no inoculum.

[d]0 = Trt 17 seedlings were selected at random from Trts 13-16 for the final 2.5 mo.

and percentage of mycorrhizae. Dried leaf tissue from all four replications was chemically analyzed by the Soil Testing and Plant Analysis Laboratory, Extension Service, University of Georgia, Athens, Georgia. Soil phosphorus was determined by Iowa State University Soil Testing Laboratory, Ames, Iowa.

Becking (1975) stated that the acetylene reduction assay with gas chromatography for nitrogenase activity in actinorhizae is superior to other methods. Diurnal fluctuations in acetylene reduction have been demonstrated (Wheeler, 1969; Dawson, 1978). In order to eliminate this diurnal fluctuation the trees were placed in a growth chamber under continuous light 24 hr prior to acetylene reduction assay. The soil was washed free from the root system, and the stem was cut at the soil line. The roots were placed in a large test tube (100 ml), and root displacement data were obtained. Serum stoppers were placed over the test tubes. A 10% volume of air was withdrawn, and an equal volume of acetylene was injected into the tubes. After 1 hr of incubation at room temperature five 1 cc samples were withdrawn from each tube with syringes. The syringe needles were inserted in rubber stoppers to prevent the escape of gas from the samples while awaiting analysis.

Acetylene and ethylene concentrations were analyzed with the Varian model 2740 gas chromatograph equipped with two columns and flame ionization detectors. Five min were required to process each subsample through the stainless steel columns, 2.13 m long by 7.35 mm outside diameter and packed with Porapak R. Flow rates for the nitrogen carrier gas, hydrogen, and air were 40 ml/min, 40 ml/mim, and 400

ml/min respectively. Temperature settings for the column and detector were 60 C and 110 C respectively.

Standards with concentrations of 50, 100, 200, and 300 μl of ethylene were recorded prior to root sample analysis. Samples of air and of the 10% acetylene in air were taken to establish a background ethylene concentration. Ethylene evolution for each tree was recorded whenever three of the five subsamples yielded similar readings. Therefore, each replication (17 treatments with 51 trees) required over 13 hr to complete analysis.

Equations for calculating the concentrations of ethylene evolved were as follows: 1) Regression coefficient: Y (ethylene) = b (slope) times x (recorder readings) + a (Y-axis intercept). Ethylene standards were used to calculate b and a for each replication. One cc subsamples were injected into the gas chromatograph after a 1 hr minimum incubation period. The recorder readings were recorded, and this value times b plus a = Y (ethylene), E-1,/cc/time of incubation; 2) E-1 times total volume = total ethylene, E-2 in the tube/time of incubation; 3) E-2 divided by min of incubation = total ethylene, E-3, in the tube/min; E-3 was one of the parameters analysed statistically and was designated as CH in the data; 4) E-3 times 0.0446 = μmoles ethylene, μmoles C_2H_4/ min; and 5) μmoles C_2H_4/min divided by weight of nodules, g dry weight, = the specific acetylene reduction activity, μmoles C_2H_4/min/g nodule dry wieght; this was the second nitrogen fixation parameter analyzed statistically and was designated as MM in the data.

Statistical Analyses

Each tree in each treatment constituted a sampling unit. Each treatment constituted an experimental unit. The mean of the sampling units in each treatment was used to calculate the error mean square. Data were analyzed on the following growth variables: HTB (height in cm at 2 mo), HTF (height at the completion of the experiment), HTD (height difference = height for the last 2.5 mo), DIAB (basal stem diameter in mm at 2 mo), DIAF (diameter at completion of the experiment), DIAD (diameter difference = diameter for the last 2.5 mo), VOLB (volume in cc at 2 mo = HTB x $DIAB^2$), VOLF (completion volume = HTF x $DIAF^2$), VOLD (volume difference = VOLF - VOLB = volume for last 2.5 mo), ROOT (root displacement in cc), MM (μmoles C_2H_4/min/g nodule dry weight), CH (μl C H /plant/min), LV (leaf dry weight in g), STEM (stem dry weight in g), and MYCO (percentage of mycorrhizae). All mineral data were based on mineral concentrations (% and ppm) times the leaf dry weight and converted to mg. Data were analyzed on the following mineral variables: N (nitrogen), P (phosphorus), K (potassium), Cu (copper), Fe (iron), Al (aluminum), Ca (calcium), Mg (magnesium), B (boron), Sr (strontium), Ba (barium), and Na (sodium). The soil from the three seedlings in each treatment was consolidated for soil phosphorus samples, variable, SP in ppm.

A 3 x 3 factorial analysis of variance and Duncan's multiple range test were performed on the nine treatment means for each variable, HTB, DIAB, and VOLB. Data from these variables were based on averages of similar treatments within a replication. Remaining data

were collected after an additional 2.5 mo. Several trees that

were not given live nodule inoculum became contaminated during the final

2.5 mo. All such trees were eliminated for statistical analyses. This

resulted in the rejection of two treatments, treatment 4 from replication

2 and treatment 2 from replication 3. Two additional treatments, 12 and

16 from replication 1, were not analyzed for all leaf minerals by the

Georgia Soil Testing Laboratory. The least squares estimated of each

treatment was calculated in order to generate values for the missing

data and to maintain the complete factorial. However, as a result two

degrees of freedom were lost for the error term for variables HTD, HTF,

DIAD, DIAF, VOLD, VOLF, ROOT, LV, STEM, CH, MM, and MYCO. Four degrees

of freedom were lost for the error term for all leaf mineral variables

(Cochran and Cox, 1957). The analyses of all the variables were based on

four replications except for variable MYCO. Some of the roots of several

treatments in replication 1 were inadvertently discarded. Therefore,

statistical analyses were based on the three remaining complete repli-

cations. Analysis of variance with two sets of orthoginal comparisons,

C [among inocula at a time (Table 5)] and Z [among times within an

inoculum (Table 6)], and Duncan's multiple range test were performed on

the 17 treatments. All statistical analyses were conducted with SAS-76

(Barr et al., 1976).

Table 5. Orthoginal comparisons between treatments and designated
as C (among inocula at a time)

| C | Treatments | | Effect | Remarks |
	- Means	+ Means		
C1	3,4	1,2	Fungus	Both inocula present for entire experiment
C2	2,4	1,3	Nodule	"
C3	2,3	1,4	Interaction	"
C4	7,8	5,6	Fungus	Fungus at initial planting; nodule inoculum at 2 mo
C5	6,8	5,7	Nodule	"
C6	6,7	5,8	Interaction	"
C7	11,12	9,10	Fungus	Nodule inoculum at initial planting; fungus at 2 mo
C8	10,12	9,11	Nodule	"
C9	10,11	9,12	Interaction	"
C10	15,16	13,14	Fungus	Both inocula at 2 mo
C11	14,16	13,15	Nodule	"
C12	14,15	13,16	Interaction	"
C13	17	1-16	Control	Treatments with inocula compared to treatment without (17)
C14	1-4	13-16	Time	Trts with inocula from beginning compared to those amended at 2 mo
C15	5-8	9-12	Time	Trts with fungus first and nodule inoculum last compared to vice versa
C16	1-4, 13-16	5-12		Trts in C14 compared to Trts in C15

Table 6. Orthoginal comparisons within treatments and designated as Z (among times within an inoculum)

Z	Treatments		Effect	Inoculum[a]		Remarks
	− Means	+ Means		F	N	
Z1	9,13	1,5	Fungus	L	L	Live fungus at time 0 = +[b] Live fungus at 2 mo = -
Z2	5,13	1,9	Nodule	L	L	Live NI[c] at time 0 = + Live NI at 2 mo = -
Z3	5,9	1,13	Inter- action	L	L	
Z4	10,14	2,6	Fungus	L	D	Live fungus at time 0 = + Live fungus at 2 mo = -
Z5	6,14	2,10	Nodule	L	D	Dead NI at time 0 = + Dead NI at 2 mo = -
Z6	6,10	2,14	Inter- action	L	D	
Z7	11,15	3,7	Fungus	D	L	Dead fungus at time 0 = + Dead fungus at 2 mo = -
Z8	7,15	3,11	Nodule	D	L	Live NI at time 0 = + Live NI at 2 mo = -
Z9	7,11	3,15	Inter- action	D	L	
Z10	12,16	4,8	Fungus	D	D	Dead fungus at time 0 = + Dead fungus at 2 mo = -
Z11	8,16	4,12	Nodule	D	D	Dead NI at time 0 = + Dead NI at 2 mo = -
Z12	8,12	4,16	Inter- action	D	D	

[a] Inoculum: L = live, D = dead.

[b] + = + means, - = - means.

[c] NI = nodule inoculum.

Table 6. (Continued)

Z	Treatments		Effect	Remarks
	- Means	+ Means		
Z13	3,4,7,8, 11,12, 15,16	1,2,6,7, 9,10, 13,14	Fungus	Treatments with live fungus (+) compared to treatments with dead dead fungus (-)
Z14	2,4,6,8, 10,12, 14,16	1,3,5,7, 9,11, 13,15	Nodule	Treatments with live nodule inoculum (+) compared to those with dead nodule inoculum (-)
Z15	2,3,6,7, 10,11, 14,15	1,4,5,8, 9,12,13, 16	Inter- action	
Z16	17	1-16	Control	Same as C13 and omitted from subsequent tables

RESULTS

Vesicular-Arbuscular Mycorrhizae

Vesicles were observed in Alnus glutinosa roots collected in Georgia, Iowa, Ohio, and Illinois; A. rugosa (= A. incana) roots collected in Minnesota, Wisconsin, and Michigan; A. serrulata roots collected in North Carolina; and A. hirsuta collected in Illinois. Arbuscules were not observed in Alnus roots.

The frequency of vesicle occurrence was quite variable. The percentage of vesicular-arbuscular mycorrhizae (VAM) compared to non-VAM roots was the highest in A. rugosa from Wisconsin and Michigan, A. serrulata from North Carolina, and A. glutinosa from Georgia. The order of magnitude of vesicles in these roots ranged from less than 1 in 10 to 1 in 100. The order of magnitude of vesicles found in the remaining root samples was less than 1 in 100 to 1 in 1000.

Usually, the vesicles were clustered. One, two, or more adjacent fine roots would have numerous vesicles (Figure 2) while the remainder of the root sample containing 100 to 1000 roots often lacked them. Vesicles were equally present in the non-ectomycorrhizal non-woody mother roots and non-ectomycorrhizal fine roots to which they give rise. Vesicles occurred inter- and intracellularly and were confined to the cortex tissue. Often the vesicles took the shape and size of the enveloping cortex cell. However, the size and shape of the fungal structures varied considerably.

VAM chlamydospores were observed in close association with

A. rugosa from Michigan (Figure 3) and A. serrulata roots from North
Carolina. These spores were tentatively identified by Christopher
Walker as Glomus caledonius (Nicol. & Gerd.) Trappe & Gerd. and
G. fasciculatus (Thaxter sensu Gerd.) Gerd. & Trappe respectively.
The spores were immature, and positive species identification was
not possible. It was not possible to trace hyphae from the spores
to internal fungal structures following clearing and staining.

Ectomycorrhizal Synthesis Trials

The only successful ectomycorrhizal synthesis was obtained with
fungal isolated A-3 grown on PDA and three of four A. glutinosa, ISU
0910, seedlings grown in pasteurized soil. The fourth seedling died.
Dark brown ectomycorrhizae were observed after four weeks in the growth
chamber. The seedling mycorrhizae were similar to those from which
isolated A-3 was isolated. There was no evidence of ectomycorrhizal
development with the other fungi tested.

Nodule Synthesis Trials

No quantitative determinations of nodule formation were made with
any of the nodule synthesis trials. Only nodule presence or absence
was noted. There were two methods of nodule preparation, Lelonde's
(Table A-3) and homogenization. Excellent nodule formation was achieved
with both methods. Abundant nodule formation was observed with both
1% and 10% homogenized nodule suspensions. There was no difference
observed in nodule formation among the Alnus hosts, ISU clone 2-58

Figure 2. Vesicles within <u>A</u>. <u>glutinosa</u> cortex cells (bar = 100 µm)

Figure 3. Chlamydospores thought to be a <u>Glomus</u> sp. associated with <u>A</u>. <u>rugosa</u> roots; spores approximately 210 µm in diameter
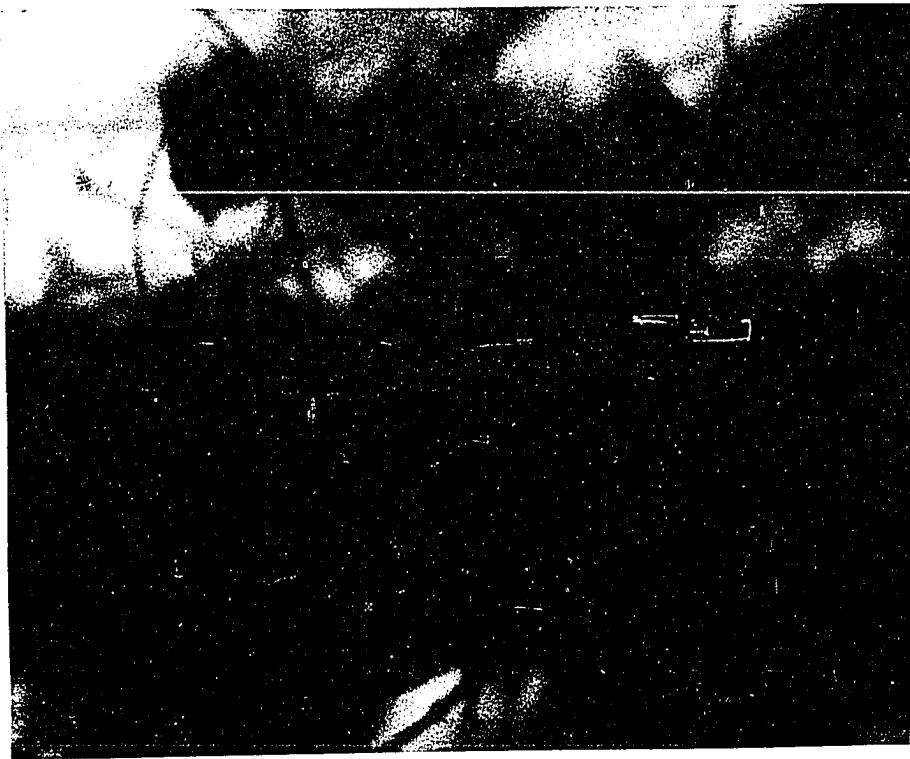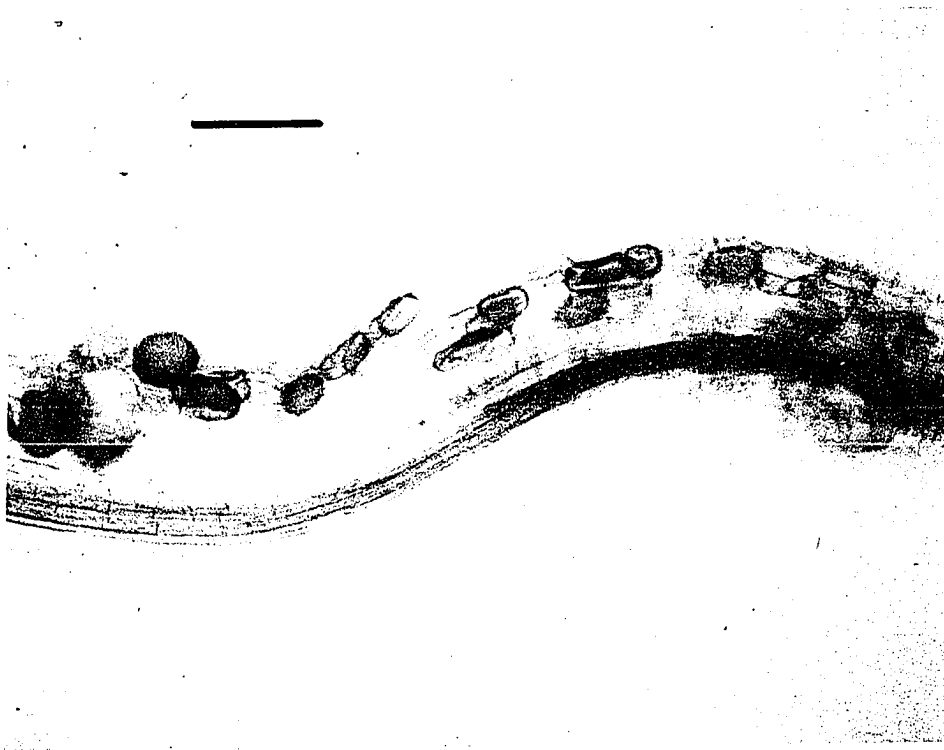
stock plants, 2-58 cuttings, and # 0910 seedlings. Likewise, nodules

were readily produced on <u>Alnus</u> trees grown in Jiffy Mix, milled pine

bark (MPB), Ca(OH$_2$) amended MPB, and peat-vermiculite (P-V). Nodules

were not observed in the sterilized controls of the above growth media

except MPB. Pasteurized soil and autoclaved sand were not used in

nodule synthesis trials. However, nodules were not observed as natural

comtaminants in the latter substrates which were used in the ectomycor-

rhizal synthesis trials.

Isolate A-3 Characterization and Identification

A total of 16 isolations with clamp connections were made from 190

mycorrhizal tips. Nine of the 16 isolates have survived in pure

subculture. The isolation of fungi containing clamp connections was best

achieved by emersing excised roots for 2.5 min in a solution of 100 ppm

HgCl$_2$ and placing them on MMN agar with 80 ppm streptomycin sulfate

(Table 7).

On MMN agar the growth of the white mycelium of all nine fungal

isolates was slow. When transferred to PDA and malt agar the growth

rate of all nine isolates increased, and each isolate produced a prune

brown pigment. This water soluble pigment was produced by the fungal

isolates growing in PDB, malt broth, and Moser's broth. The mycelium

had a viscid appearance during its initial growth on PDA and malt agar

giving it the appearance of having a bacterial contamination. The

bulk of the mycelium grew below the agar surface. Surface hyphae

became felt-like with age. There was no surface growth in broth media.

Table 7. Isolation of fungi from mycorrhizae on MMN agar with 0, 30, 50, and 80 ppm streptomycin sulfate and surface sterilization with $HgCl_2$ for different time periods

Streptomycin (ppm)	$HgCl_2$ Exposure (Min)				
	0	2	2.5	3	4
0	1 cc[a] 3 s[b] 4 nm[c]	11 s 1 nm	1 cc 22 s 2 nm	7 s 1 nm	8 s
30	8 nm	2 s 2 nm		4 s	4 s
50	8 nm	2 s 2 nm		4 s	4 s
80	6 cc 9 s 22 nm	1 cc 12 s 3 nm	7 cc 14 s 3 nm	4 s	4 s

[a] cc = fungal isolate with clamp connections.

[b] s = sterile, no fungal growth.

[c] nm = fungal isolates without clamp connections.

Hyphae were 3-4 μm in uniform diameter. Clamp connections, which were abundantly produced, occurred irregularly at septa.

The fungal isolate, designated A-3, showed the fastest growth of the nine isolates. Therefore, A-3 was selected to be propagated for the ectomycorrhizal synthesis trials and optimum growth requirements.

Fungal isolate A-3 grew best on PD media (Table 8). Growth on the other agar media was not always consistent with growth in corresponding broths. However, the production of pigment was consistent in PD, malt, and Moser's media.

The optimum temperature for A-3 was determined to be 25 C. Fungal growth rates were determined to be 0.89, 1.42, 1.39, and 1.03 mm/da at 20 C, 25 C, room temperature (25-27 C), and 30 C respectively. No growth occurred at 0, 5, 10, and 35 C. However, normal growth resumed in these latter temperatures after the cultures were stored at room temperature. Only slight fungal growth was detected at 15 C.

PDB was adjusted for the different pH values prior to autoclaving. As expected post-autoclaving pH values were changed. The pH readings of 2.5, 3.0, 4.0, 5.0, 6.0, and 7.0 increased to 2.60, 3.20, 4.18, 5.10, 6.10, and 7.21 respectively. The non-adjusted PDB remained unchanged at 6.46. Basic PDB solutions dropped from 8.0 and 9.0 to 7.53 and 8.08 respectively.

Optimum growth for isolate A-3 was determined to be in PDB adjusted to pH 5.0 (Figure 4). Nearly equal growth was achieved in non-adjusted PDB (N). Fungal growth was very good at pH 6.0 and good

Table 8. Growth and pigment production of isolate A-3 on various media

Media	Agar ratings[a]	Broth ratings[a]	Pigment production[b]
PD	4444	4444	+
Malt	333	333	+
V-8	333	0	-
MMN	333	22	-
Moser's	333	333	+
Kaufman's	333	0	-
Singer's	22	0	-
Haupt's	22	1	-
Lutz	22	0	-
Hagem's	1	1	-
Oatmeal	1	0	-
Fries	1	1	-
Czapek's	1	1	-

[a]Number value represents relative rate of growth.

[b]+ indicates pigment produced, - indicates no pigment.
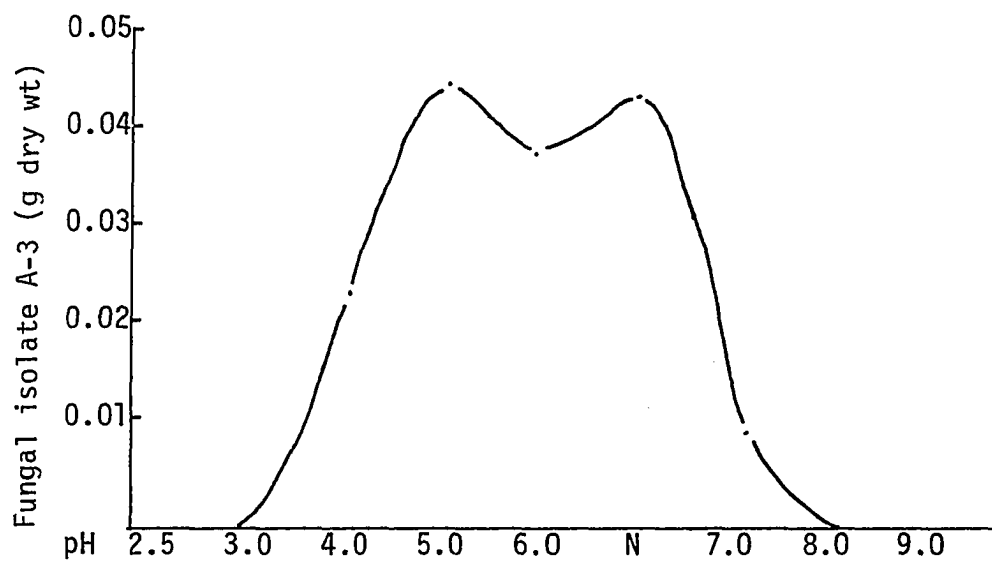
Figure 4. Effect of pH on growth of fungal isolate A-3 in PDB for 30 da
at room temperature; means of 3 replications

at 4.0. Isolate A-3 was capable of growing in pH 7.0 PDB but not in in PDB with pH >7.0 and <4.0. Mean dry weights for 30 days growth of isolate A-3 mycelium in pH 4.0, 5.0, 6.0, N, and 7.0 were 0.0230, 0.0432, 0.0362, 0.0427, and 0.0119 g respectively.

Most fungi grown in liquid media produce metabolites that change the pH as their concentrations increase. Isolate A-3 after 30 da of growth increased the post-autoclaved PDB pH of 4.18 to 4.21. The other post-pH values of 5.10, 6.10, and 7.21 decreased to 5.06, 6.02, and 7.02 respectively. The non-adjusted pH of PDB decreased from 6.46 to 6.37.

Pigment production in PDB was influenced by pH. The non-adjusted PDB was the first to initiate pigment production and produced the darkest color. Pigment was produced within 2 wk. Progressively reduced amounts of pigment were produced by A-3 in PDB at pH 6.0, 5.0, and 7.0 respectively. The pigment was more concentrated in the mycelial mats near the broth surface in pH 6.0 and 5.0 solutions.

Light was found to inhibit the growth of isolate A-3. The area of growth of isolate A-3 on medium in continuous light was one-half the area produced in the dark.

Isolate A-3-mycorrhizal roots were readily discernible from non-mycorrhizal roots and were easily counted using a dissecting microscope (Figure 5). The dark brown mantle covered the entire surface of most mycorrhizae. However, many roots had mycorrhizal tips and non-mycorrhizal bases. Others had mycorrhizal bases and non-mycorrhizal tips.

Cross sections of isolate A-3-mycorrhizae (Figure 6) showed a

smooth, pseudoparenchamous mantle lacking attached mycelium. The
mantle averaged 10-12 μm thick. The sinuous mantle hyphae gave a
mosaic appearance to tangential sections of the mycorrhizae. Outer
hyphae of the mantle retained typical hyphal construction including
clamp connections. The Hartig net rarely penetrated beyond the
first cortical cell layer. This was not considered typical of
most ectomycorrhizae.

The presence of the pigment in the mantle created problems in
differential staining. Safranine was taken up by the fungal mantle.
However, satisfactory staining was obtained with 1% aqueous safranine
and 0.5% fast-green in 100% ethanol for 6 hr and 1 min respectively.

Zak's key characters for identification of ectomycorrhizae formed
with isolate A-3 were as follows:

1. Tree species. Alnus glutinosa
2. Color of mycorrhizae (x 10). Prune brown
3. Attached mycelium (x 10-50). Absent
4. Attached rhizomorphs (x 10-50). Absent
5. Mantle surface texture (x 10-50). Smooth, uniform color
6. Form (x 5). Monopodial
7. U-V light fluorescence. None
8. Chemical reagent color reaction (x 10).
   a. Chlorovanillin   no change
   b. $FeSO_4$ (10%)   blackish-green
   c. H_2SO_4 (conc.)   no change
   d. KOH (15%)   no change
   e. Melzer   rust
   f. NH_4OH (conc.)   no change
   g. Sulfoformol   no change
   h. Guaicol   no change
9. Attached mycelium (x 100-1000). Absent
10. Attached rhizomorphs (x 100-1000). Absent
11. Form of individual elements. Straight
12. U-V light fluorescence of hyphae (x 100.) None
13. Chemical reagent color reaction on hyphae (x 100). Same as No 8
14. Mantle surface hyphae character (x 100). Sinuous hyphae
    resulting in a mosaic appearance
15. Mantle structure (x 100). Pseudoparenchymous hyphae, 10-12 μm

Figure 5. Mycorrhizae with dark isolate A-3 mantle (ruler scale = 1 mm)

Figure 6. Cross section of A. glutinosa - isolate A-3 ectomycorrhiza
           (bar = 20 μm)
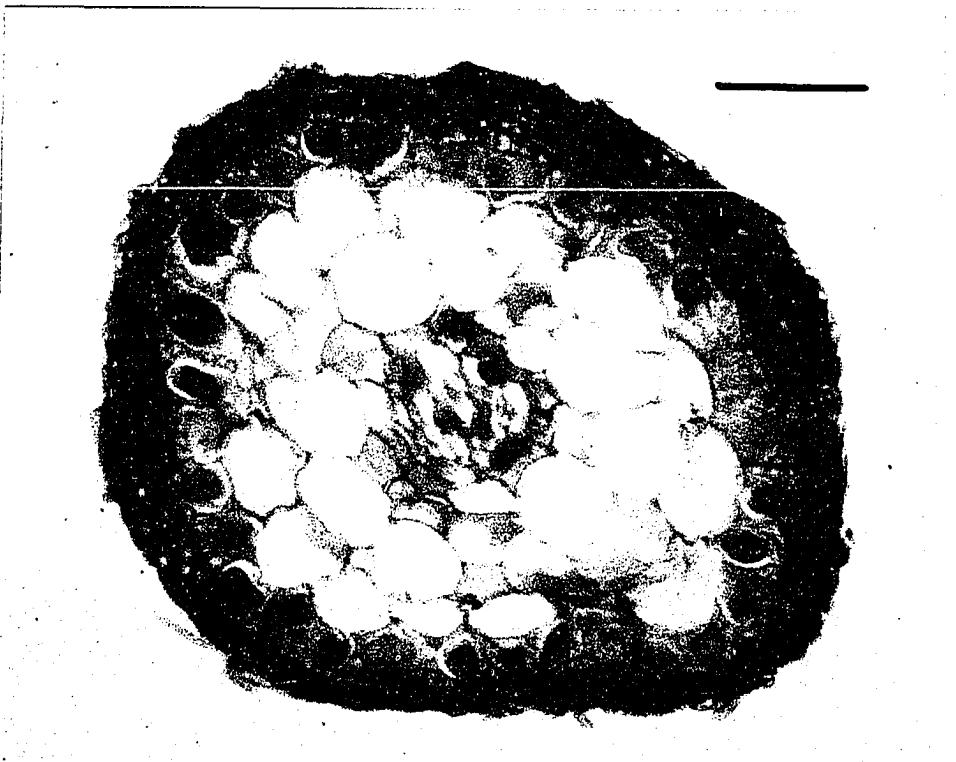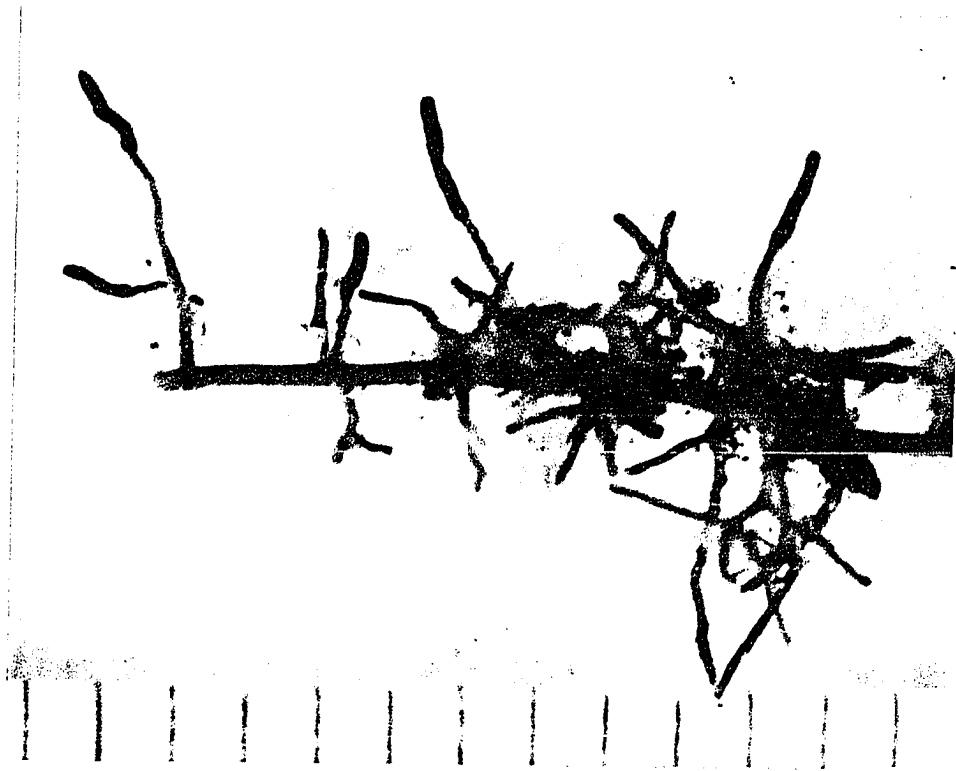
16. Individual element diameter and uniformity (x 10-50) .;H
    Hyphal diameter 3-4 µm, uniform; clamp connections
    common
17. Hartig net character (x 100). Penetration rarely deeper than
    second cortical cell layer
18. Habitat. Mycorrhizae collected from very well drained, sandy,
    loam topsoil
19. Taste and odor. Similar to commercial Agaricus

A. glutinosa - isolate A-3 ectomycorrhizae were keyed to genus Ff with

Dominik's (1959) key.

Three-way Study

During the week after the potted trees were placed on bench tops

daytime temperatures outside the greenhouse exceeded 38 C. Daytime

temperatures inside the greenhouse exceeded 40 C. The soil temperature

at 2.5 cm beneath the soil surface near the tree base was recorded at

45 C. Perlite was added to the soil surface, and pots were covered with

aluminum foil to reduce soil temperature.

Root samples were taken 1 mo after the high soil temperature

was recorded. The Alnus roots appeared healthy. However, no mycor-

rhizae or nodules were observed. This experiment was terminated after

2 mo when subsequent root samples lacked symbiotic microorganisms.

Because of the difficulty in handling specific VAM fungi, the apparent

lesser place VAM fungi have in natural alder stands, and the magnitude

of the statisical analyses, further three-way trials were discontinued.

Two-way Study

The analysis of variance for seedling height after 2 mo (HTB, Table

9) showed that height varied significantly among replications and was

highly significant among nodule inoculum treatments. There was a significant interaction between fungus and nodule inoculum. The live nodule inoculum treatment means were larger than treatment means with dead nodule inoculum or lacking nodule inoculum (Table 10).

After 2 mo both the average seedling diameter (DIAB, Table 11) and seedling volume (VOLB, Table 12) were highly significantly different among nodule inoculum treatments. The effect of fungal isolate A-3 was not significant for either variable. Additionally, no interaction was observed between nodule inoculum and fungal treatments. Trees grown with live nodule inoculum had the greatest mean stem diameter (Table 13) and volume (Table 14).

Although the effect of live nodule inoculum was found to be significant, a Duncan's multiple range test for height (Figure 7) showed that two dead nodule inoculum treatments were not significantly different from two live nodule inoculum treatments. For stem diameter (Figure 8) and stem volume (Figure 9) two dead nodule inoculum treatments were not significantly different from three live nodule inoculum treatments. For height there was not much range in growth after 2 mo. For diameter and volume only two of nine treatments were significantly different from each other for both variables.

Two wk prior to the scheduled final analysis trees on adjacent greenhouse benches were removed. These trees provided shade in the late afternoon. Their removal caused defoliation of 5 of 12 trees in treatment 1. A few individual trees in other treatments died. The trees in treatment 1 were allowed to refoliate for 4 wk. Acetylene

Table 9.   Analysis of variance of seedling height (cm) treatment
            means after 2 mo growth; HTB

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-value	Probability[a] F
Model	11	45.047	4.095	3.39	0.0060**
Error	24	28.986	1.208		
Total	35	74.033			
Rep	3	12.977		3.58	0.0286*
Fungus	2	1.767		0.73	0.4915
Nodule inoculum	2	15.928		6.59	0.0052**
Interaction	4	14.376		2.98	0.0396*

[a]Probability levels in all analysis of variance tables will be
denoted as follows:
  *p   0.050 = significant.
 **p   0.010 = highly significant.
***p   0.001 = very highly significant.

Table 10.   Factorial (3 x 3) treatment means for fungal and nodule
             inoculum for seedling height (cm) after 2 mo growth; HTB

Nodule inoculum	Fungal inoculum			Mean
	None	Dead	Live	
None	12.62	12.04	12.13	12.26
Dead	11.63	13.74	14.18	13.18
Live	14.19	13.75	12.72	13.55
Mean	12.81	13.18	13.01	

Table 11. Analysis of variance of seedling basal stem diameter (mm) treatment means at 2 mo; DIAB

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-value	Probability F
Model	11	0.28821	0.02620	2.08	0.0649
Error	24	0.30248	0.01260		
Total	35	0.59068			
Rep	3	0.00874		0.23	0.8737
Fungus	2	0.05760		2.29	0.1235
Nodule inoculum	2	0.20553		8.15	0.0020**
Interaction	4	0.01633		0.32	0.8591

Table 12. Analysis of variance of seedling stem volume (cc) treatment means at 2 mo; VOLB

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-value	Probability F
Model	11	0.06508	0.005916	2.01	0.0738
Error	24	0.07058	0.002941		
Total	35	0.13566			
Rep	3	0.00575		0.65	0.5894
Fungus	2	0.00509		0.87	0.4330
Nodule inoculum	2	0.04506		7.66	0.0027**
Interaction	4	0.00917		0.78	0.5495

Table 13. Factorial (3 x 3) treatment means for fungal and nodule inoculum of seedling basal stem diameter (mm) at 2 mo; DIAB

Nodule inoculum	Fungal inoculum			Mean
	None	Dead	Live	
None	1.13	1.04	1.18	1.12
Dead	1.24	1.17	1.27	1.23
Live	1.35	1.26	1.29	1.30
Mean	1.24	1.16	1.25	

Table 14. Factorial (3 x 3) treatment means for fungal and nodule inoculum of seedling stem volume (cc) at 2 mo; VOLB

Nodule inoculum	Fungal inoculum			Mean
	None	Dead	Live	
None	1.636	1.314	1.693	1.548
Dead	1.824	1.946	2.309	2.026
Live	2.722	2.226	2.290	2.413
Mean	2.061	1.829	2.097	

Figure legend: N+   F+   F-,0 N-,0

y-axis: Seedling height (cm) at 2 mo; HTB

y-axis values: 15, 14, 13, 12, 11, 10

x-axis (TRT^a):

| F+ N+ | F- N+ | F0 N+ | F+ N- | F- N- | F0 N- | F+ N0 | F- N0 | F0 N0 |

TRT^a			Mean
F	N	a[1]	
0	+	a	14.19 cm
+	-	ab	14.18
-	+	ab	13.75
-	-	abc	13.74
+	+	bc	12.72
0	0	bcd	12.62
+	0	cd	12.13
-	0	cd	12.04
0	-	d	11.63

^{a}Treatment terminology: F = fungal inoculum, N = nodule inoculum; + = live inoculum, - = dead inoculum, and 0 = no inoculum.

[1]Values in each vertical column followed by the same letters do not differ significantly at p = 0.05 by Duncan's multiple range test.

Figure 7. Histogram and treatment means for seedling height (cm) at 2 mo; HTB
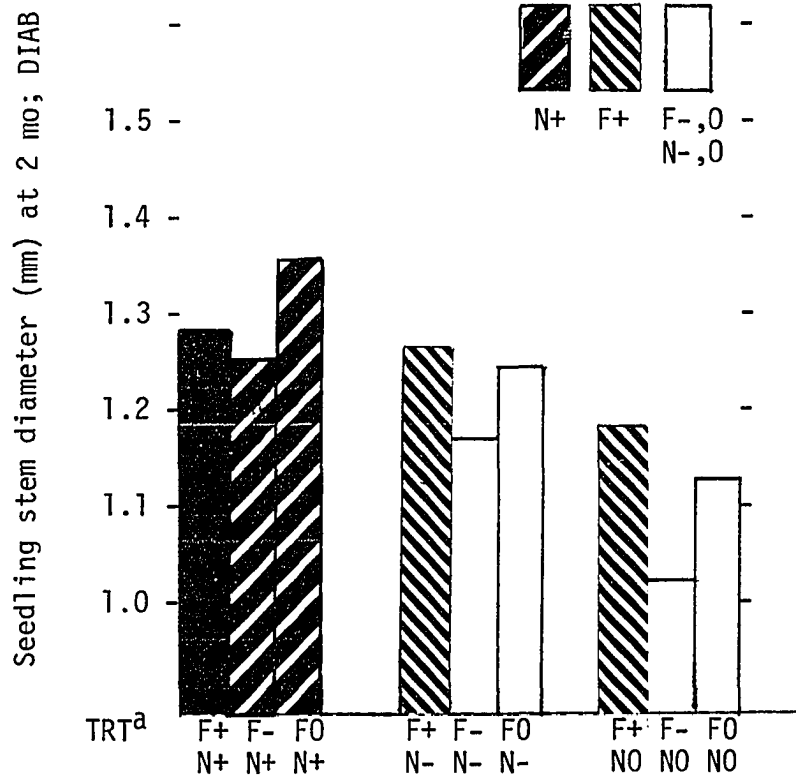
TRT[a]			Mean
F	N		
0	+	a[1]	1.35 mm
+	+	ab	1.29
+	-	ab	1.27
-	+	ab	1.26
0	-	ab	1.24
+	0	bc	1.18
-	-	bc	1.17
0	0	bc	1.13
-	0	c	1.04

[a]Treatment terminology: F = fungal inoculum, N = nodule inoculum; + = live inoculum, - = dead inoculum, and 0 = no inoculum.

[1]Values in each vertical column followed by the same letters do not differ significantly at p = 0.05 by Duncan's multiple range test.

Figure 8. Histogram and treatment means for seedling basal stem diameter (mm) at 2 mo; DIAB
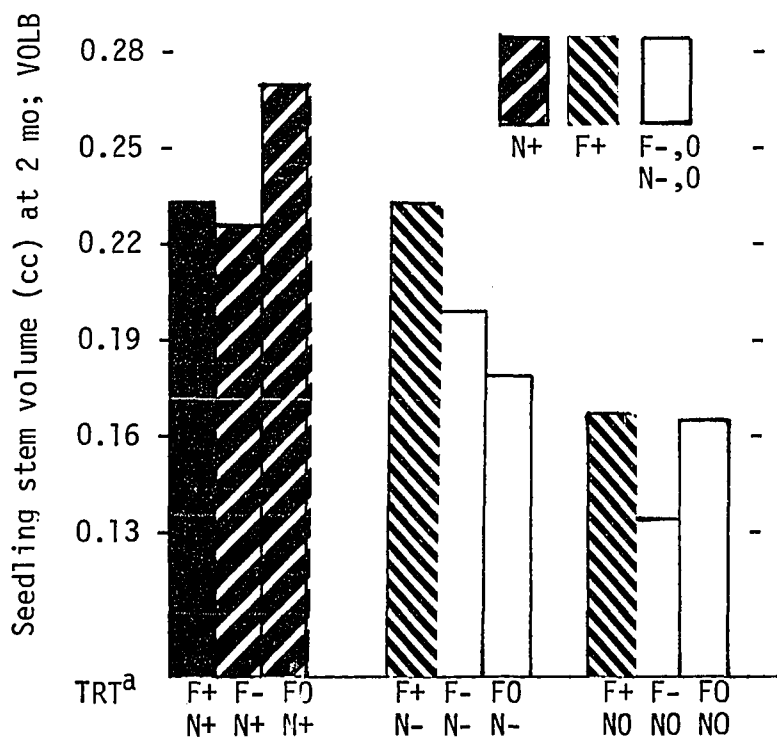
TRT[a]			Mean
F	N		
0	+	a[1]	0.272 cc
+	-	ab	0.231
+	+	ab	0.229
-	+	abc	0.223
-	-	abc	0.195
0	-	bc	0.182
+	0	bc	0.169
0	0	bc	0.164
-	0	c	0.131

[a]Treatment terminology: F = fungal inoculum, N = nodule inoculum; + = live inoculum, - = dead inoculum, and 0 = no inoculum.

[1]Values in each vertical column followed by the same letters do not differ significantly at p = 0.05 by Duncan's multiple range test.

Figure 9.   Histogram and treatment means for seedling stem volume (cc) at 2 mo; VOLB

reduction measurements rely on leaf photosynthesis and carbohydrate production. The refoliated leaves would not be likely to have the same dry weight, same mineral concentration, and same rate of photosynthesis as the original leaves. Therefore, results for variables, leaf dry weight (LV), total C_2H_4 evolved (CH), specific acetylene reduction activity (MM), and all leaf minerals for treatment 1 would most likely be affected and possibly influence levels of significance for the statistical analyses.

The analysis of variance for height growth during the last 2.5 mo (HTD, Table 15) showed that the effect of isolate A-3 was very highly significant when comparing initial-planting inoculations [Trts 1-4 (C1)], and significant when comparing nodule only delayed inoculations [Trts 5-8 (C4)]. However, in the latter case (Trts 5-8) the significance of isolate A-3 was due primarily to the value for delayed living nodule inoculum (Trt 5) rather than delayed dead nodule inoculum [Trt 6, (Figure 10)]. In both of the above comparisons (C1 and C4) treatments with live isolate A-3 had larger means than those with dead isolate A-3. The effect of nodule inocula was very highly significant when comparing initial-planting inoculations [Trts 1-4 (C2)]. The treatments with live nodule inoculum showed larger treatment means than those with dead nodule inoculum. An interaction between the two symbionts was found to be significant for nodule only delayed inoculations [Trts 5-8 (C6)]. All nodulated treatments had higher values than the non-nodulated treatments within each 2 x 2 factorial except for initial dead isolate A-3 inoculum and nodule only delayed, living inoculum [Trt 7, (Figure 10)].

Table 15. Statistical analyses for seedling height (cm) during the final 2.5 mo; HTD

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-value	Probability >F
Model	19	350.619	18.454	3.87	0.0001***
Error	46	219.177	4.765		
Total	67	569.797			

SV	Effect	– Mean[a]	+ Mean[a]	MSS	Probability
Rep				35.909	0.0614
C1	F=Fungus	6.540	10.645	67.404	0.0004***
C2	N=Nodule	6.104	11.081	99.102	0.0001***
C3	I=Interaction	8.386	8.799	0.681	0.7011
C4	F	6.340	9.036	29.079	0.0150*
C5	N	6.905	8.471	9.813	0.1492
C6	I	6.440	8.936	24.925	0.0237*
C7	F	7.681	8.274	1.404	0.5818
C8	N	7.314	8.641	7.049	0.2201
C9	I	8.000	7.955	0.008	0.9666
C10	F	6.979	6.218	2.318	0.4796
C11	N	6.316	6.880	1.271	0.6002
C12	I	6.788	6.409	0.574	0.7245
C13	Control	4.550	7.714	37.690	0.0060**
C14	1-4:13-16	8.593	6.598	31.820	0.0112*
C15	5-8:9-12	7.688	7.978	0.670	0.7034
C16	1-4,13-16:5-12	7.595	7.833	0.903	0.6586
Z1	F	7.613	12.204	84.318	0.0001***
Z2	N	8.689	11.128	23.790	0.0269*
Z3	I	9.991	9.825	0.111	0.8770
Z4	F	6.879	7.478	1.434	0.5778
Z5	N	6.565	7.791	6.015	0.2568
Z6	I	7.319	7.038	0.316	0.7935
Z7	F	7.909	7.349	1.254	0.6026
Z8	N	6.663	8.595	14.938	0.0768
Z9	I	7.121	8.136	4.121	0.3469
Z10	F	6.751	5.531	5.954	0.2592
Z11	N	6.656	5.626	4.244	0.3399
Z12	I	6.900	5.383	9.211	0.1620
Z13	F	6.885	8.543	43.990	0.0032**
Z14	N	6.660	8.768	71.149	0.0003***
Z15	I	7.403	8.025	6.175	0.2506

[a]Mean (+ and –) does not apply for comparisons C13–C16 (See Table 5).
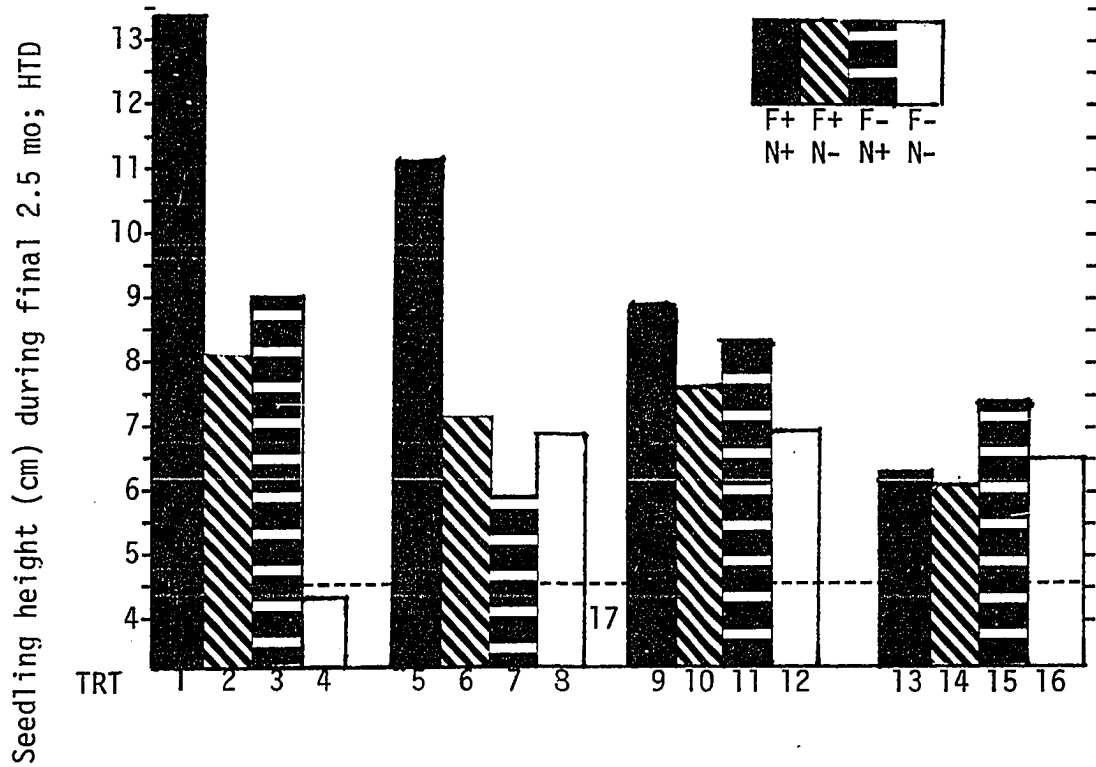
TRT [1]		Mean
1	a	13.34 cm
5	ab	11.07
9	bc	8.92
3	bc	8.82
11	bc	8.37
2	bcd	7.95
10	bcd	7.63
15	cd	7.45
6	cd	7.01
12	cd	6.99
8	cd	6.81
16	cd	6.51
13	cd	6.31
14	cd	6.13
7	cd	5.88
17	d	4.55
4	d	4.26

[1] Values in each vertical column followed by the same letters do not differ significantly at p = 0.05 by Duncan's multiple range test.

Figure 10. Histogram and treatment means for seedling height (cm) during the last 2.5 mo; HTD

This may have attributed to the significant interaction. The mean height of all treatments with inocula (Trts 1-16) was found to be significantly larger (C13) than the control (Trt 17). The effect of isolate A-3 was found to be very highly significant and the effect of nodule inocula was found to be significant when comparing treatments with both live inocula among inoculation times (Z1 and Z2). In both of these comparisons (Z1 and Z2) the trees in the treatments receiving live inocula initially showed the greatest height growth difference for the final 2.5 mo. The mean of all mycorrhizal treatments was highly significantly larger than all non-mycorrhizal treatments (Z13). The mean of all nodulated treatments was very highly significantly larger than all non-nodulated treatments (Z14).

Three of the four treatments containing both live inocula showed the greatest growth during the last 2.5 mo (Figure 10). Treatments with both live inocula where isolate A-3 was added initially were considerably larger than other treatment combinations. This accounted for the significant differences found in these four comparisons (C1, C4, Z1, and Z2).

Since few significant differences in average height were observed after the first 2 mo (Figure 7), most of the significant differences found in the final height (HTF, Table 16) reflected the growth differences found during the last 2.5 mo of the experiment. The effect of live nodule inoculum was significant for the first 2 mo of growth and also for the last 2.5 mo of growth. However, the effect of the fungus, isolate A-3, was found to significantly influence tree height only during the last 2.5 mo, especially, in the treatments containing live fungal

Table 16. Statistical analyses for final seedling height (cm); HTF

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-value	Probability >F
Model	19	471.902	24.837	4.12	0.0001***
Error	46	277.249	6.027		
Total	67	749.151			

SV	Effect	- Mean[a]	+ Mean[a]	MSS	Probability
Rep				24.261	0.2542
C1	F=Fungus	20.345	24.596	72.293	0.0009***
C2	N=Nodule	20.058	24.884	93.171	0.0002***
C3	I=Interaction	22.413	22.529	0.054	0.9233
C4	F	18.379	21.169	31.136	0.0245*
C5	N	18.502	21.045	25.857	0.0396*
C6	I	18.506	21.041	25.705	0.0401*
C7	F	21.049	20.731	0.403	0.7927
C8	N	18.945	22.835	60.528	0.0022**
C9	I	20.649	21.131	0.931	0.6898
C10	F	18.856	18.694	0.106	0.8930
C11	N	18.213	19.338	5.063	0.3539
C12	I	18.456	19.094	1.626	0.5982
C13	Control	18.833	20.477	10.185	0.1905
C14	1-4:13-16	22.471	18.775	109.261	0.0001***
C15	5-8:9-12	19.774	20.890	9.968	0.1952
C16	1-4,13-16:5-12	20.623	20.332	1.354	0.6304
Z1	F	21.246	25.388	68.261	0.0012**
Z2	N	21.641	24.993	44.923	0.0076**
Z3	I	23.313	23.321	0.001	0.9942
Z4	F	18.179	20.378	19.338	0.0735
Z5	N	18.221	20.335	17.872	0.0849
Z6	I	18.588	19.969	7.631	0.2561
Z7	F	20.926	20.541	0.593	0.7501
Z8	N	18.741	22.726	63.521	0.0017**
Z9	I	20.568	20.900	0.442	0.7832
Z10	F	18.979	18.183	2.536	0.5107
Z11	N	18.494	18.668	0.121	0.8856
Z12	I	18.860	18.301	1.249	0.6440
Z13	F	19.657	21.298	43.050	0.0088**
Z14	N	18.929	22.025	153.357	0.0001***
Z15	I	20.006	20.949	14.222	0.1232

[a]Mean (+ and -) does not apply for comparisons C13-C16 (See Table 5).

inoculum from the time of initial planting.

The analysis of variance for height at the completion of the experiment (HTF, Table 16) showed that the effect of isolate A-3 was very highly significant when comparing initial-planting inoculations [Trts 1-4 (C1)] and significant when comparing nodule only delayed inoculations [Trts 5-8 (C4)]. However, in the latter treatments (5-8) the significance shown for isolate A-3 was based primarily on only one of the two treatments with live isolate A-3, delayed living nodule inoculum (Trt 5). The treatments receiving live isolate A-3 in these comparisons (C1 and C4) had the largest treatment means for final seedling height.

The effect of nodule inocula was found to be highly significant when comparing initial-planting inoculations [Trts 1-4 (C2)], highly significant when comparing only fungus delayed inoculations [Trts 9-12 (C8)], and significant when comparing only nodule delayed inoculations [Trts 5-8 (C5)]. However, in only nodule delayed inoculations (Trts 5-8) the significance shown for nodule inoculum was based primarily on only one of the two treatments with live nodule inoculum, delayed living nodule inoculation (Trt 5). For these three comparisons (C2, C5, and C8) treatments with live nodule inoculum had larger treatment means for final seedling height than those with dead nodule inoculum. An interaction between the two symbionts was found to be significant for only nodule delayed inoculations [Trts 5-8 (C6)]. The height of the trees was significantly larger with initial-planting inoculations of both symbionts (Trts 1-4) than with delayed inoculations

of both symbionts [Trts 13-16 C14)].

The effects of nodule inocula and isolate A-3 were shown to be highly significant when comparing treatments with both live inocula (Z1 and Z2). Treatments with live nodule inoculum and dead isolate A-3 also were found to be highly significant (Z8). In all three of these comparisons treatments receiving live inocula at initial planting had the largest mean. Comparisons of all living fungal treatments to all dead fungal treatments (Z13) was found to be highly significant. Comparisons of all live nodule inoculum treatments to dead nodule inoculum treatments (Z14) was found to be very highly significant. In these two comparisons (Z13 and Z14) treatments with live inocula had larger means for final seedling height than those with dead inocula.

Initial-planting inoculations with live inocula of both symbionts (Trt 1) showed by far the greatest final height (Figure 11). Of the five treatments with the tallest trees three contained both live inocula and two contained live nodule inoculum and dead isolate A-3. Of the five tallest treatments four contained live nodule inoculum added at the time of initial planting. The tree height appeared to be influenced by the fungus when it was added at the initial planting time. The treatment with initial live fungus and dead nodule inoculations (Trt 2) was significantly taller than the treatment with initial inoculations of both dead symbionts (Trt 4); the latter can be considered a check. In the treatments with live nodule inoculum added after 2 months (Trts 5, 7, 13, and 15) tree height was significantly different from corresponding checks (Trts 8 and 16) in the treatment with only initial
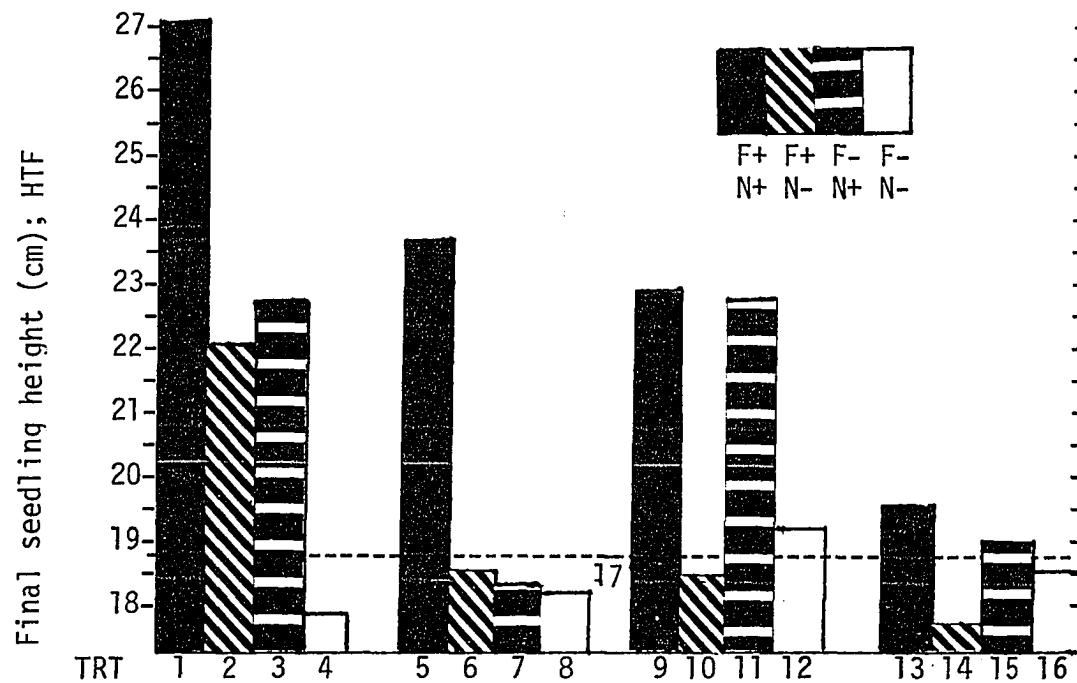
TRT		Mean	
1	a[1]	27.068	cm
5	ab	23.708	
9	bc	22.918	
11	bcd	22.753	
3	bcd	22.700	
2	bcde	22.125	
13	cdef	19.575	
12	cdef	19.345	
15	cdef	19.100	
17	def	18.833	
6	ef	18.630	
16	ef	18.613	
10	ef	18.545	
7	ef	18.383	
8	ef	18.375	
4	ef	17.990	
14	f	17.813	

[1] Values in each vertical column followed by the same letters do not differ significantly at p = 0.05 by Duncan's multiple range test.

Figure 11. Histogram and treatment means for final seedling height (cm); HTF

live fungus inoculum and delayed live nodule inoculum (Trt 5).
Therefore, it appeared that live nodule inoculum added after 2 mo
did not greatly influence tree height during the next 2.5 mo. The
treatments that were initially inoculated with living isolate A-3 and
delayed inoculations with either living or dead nodule inoculum were
difficult to interpret (Trts 5 and 6). Tree height when live nodule
inoculum was added two months after live fungus (Trt 5) appeared to be
mostly due to the presence of isolate A-3 added initially, and later in
combination with live nodule inoculum resulting in more growth than can
be accounted for by either inoculum separately.

A significant difference was found among replication means for the
basal stem diameter growth during the last 2.5 mo (DIAD, Table 17). This
difference accounted for the significant difference found among
replication means for the final basal stem diameter measurements (DIAF,
Table 18). The replication means of the basal stem diameter for the
first 2 mo were not significantly different. Because the other signif-
icant differences for DIAD and DIAF were either highly or very highly
significant, they are discussed in the succeeding paragraphs.

The significant differences found in the final basal stem diameter
measurements (DIAF) strongly reflected those found during the last 2.5
mo of growth (DIAD). The effect of nodule inoculum was found to be very
highly significant for both parameters when comparing initial-planting
inoculations [Trts 1-4 (C2)], initial nodule inoculations and delayed
fungas inoculations [Trts 9-12 (C8)], and treatments with live nodule
inoculum to those with dead nodule inoculum (Z14). When comparing the

Table 17. Statistical analyses for seedling basal stem diameter growth (mm) during the last 2.5 mo; DIAD

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-value	Probability >F
Model	19	5.2789	0.2778	3.35	0.0002***
Error	46	3.8157	0.0830		
Total	67	9.0946			

SV	Effect	- Mean[a]	+ Mean[a]	MSS	Probability
Rep				0.9821	0.0112*
C1	F=Fungus	1.096	0.994	0.0420	0.4707
C2	N=Nodule	0.715	1.375	1.7424	0.0001***
C3	I=Interaction	1.111	0.979	0.0702	0.3520
C4	F	0.975	0.983	0.0002	0.9578
C5	N	0.845	1.113	0.2862	0.0638
C6	I	0.945	1.013	0.0182	0.6342
C7	F	0.920	0.889	0.0039	0.8255
C8	N	0.634	1.175	1.1718	0.0004***
C9	I	0.884	0.925	0.0068	0.7711
C10	F	0.906	0.936	0.0036	0.8324
C11	N	0.708	1.135	0.7310	0.0039**
C12	I	0.961	0.881	0.0256	0.5730
C13	Control	0.973	0.962	0.0004	0.9446
C14	1-4:13-16	1.045	0.921	0.1225	0.2205
C15	5-8:9-12	0.979	0.904	0.0442	0.4592
C16	1-4,13-16:5-12	0.983	0.942	0.0276	0.5582
Z1	F	1.145	1.204	0.0138	0.6787
Z2	N	1.130	1.219	0.0315	0.5320
Z3	I	1.165	1.184	0.0014	0.8947
Z4	F	0.680	0.773	0.0342	0.5149
Z5	N	0.789	0.664	0.0626	0.3797
Z6	I	0.706	0.746	0.0064	0.7778
Z7	F	1.165	1.284	0.0564	0.4038
Z8	N	1.118	1.331	0.1828	0.1360
Z9	I	1.123	1.326	0.1661	0.1549
Z10	F	0.661	0.788	0.0638	0.3750
Z11	N	0.764	0.685	0.0248	0.5790
Z12	I	0.773	0.676	0.0371	0.4980
Z13	F	0.974	0.950	0.0093	0.7343
Z14	N	0.725	1.199	3.5958	0.0001***
Z15	I	0.975	0.949	0.0108	0.7145

[a]Mean (+ and -) does not apply for comparisons C13-C16 (See Table 5).

Table 18. Statistical analyses for final basal stem diameter (mm); DIAF

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-value	Probability >F
Model	19	6.7880	0.3573	3.37	0.0002***
Error	46	4.8812	0.1061		
Total	67	11.6692			

SV	Effect	$-$ Mean[a]	$+$ Mean[a]	MSS	Probability
Rep				0.9023	0.0416*
C1	F=Fungus	2.313	2.269	0.0077	0.7850
C2	N=Nodule	1.926	2.655	2.1243	0.0001***
C3	I=Interaction	2.379	2.203	0.1243	0.2745
C4	F	2.020	2.159	0.0770	0.3885
C5	N	1.971	2.208	0.2233	0.1450
C6	I	2.024	2.155	0.0689	0.4145
C7	F	2.235	2.158	0.0240	0.6291
C8	N	1.873	2.520	1.6770	0.0002***
C9	I	2.203	2.190	0.0006	0.9378
C10	F	1.975	2.036	0.0150	0.7026
C11	N	1.790	2.221	0.7439	0.0094**
C12	I	2.040	1.971	0.0189	0.6683
C13	Control	2.248	2.145	0.0392	0.5377
C14	1-4:13-16	2.290	2.006	0.6498	0.0148*
C15	5-8:9-12	2.089	2.196	0.0914	0.3479
C16	1-4,13-16:5-12	2.148	2.143	0.0005	0.9471
Z1	F	2.346	2.444	0.0380	0.5438
Z2	N	2.280	2.510	0.2116	0.1557
Z3	I	2.409	2.381	0.0030	0.8638
Z4	F	1.848	1.984	0.0743	0.3971
Z5	N	1.915	1.916	0.0001	0.9938
Z6	I	1.908	1.924	0.0011	0.9192
Z7	F	2.395	2.419	0.0023	0.8822
Z8	N	2.149	2.665	1.0661	0.0022**
Z9	I	2.319	2.495	0.1243	0.2745
Z10	F	1.815	1.914	0.0390	0.5386
Z11	N	1.846	1.883	0.0053	0.8211
Z12	I	1.936	1.793	0.0827	0.3718
Z13	F	2.136	2.155	0.0062	0.8060
Z14	N	1.890	2.401	4.1769	0.0001***
Z15	I	2.161	2.130	0.0159	0.6939

[a]Mean (+ and -) does not apply for comparisons C13-C16 (See Table 5).

delayed inoculations [Trts 13-16 (C11)] highly significant differences were observed. The treatments receiving live nodule inoculum had larger means for DIAF and DIAD than those receiving dead nodule inoculum. Treatments with live nodule inoculum produced significantly larger basal stem diameters during the first 2 mo (Figure 8). However, the range was not large, and seven of the nine treatments were not significantly different using Duncan's multiple range test. The effect of nodule inoculum for both the initial 2 mo growth and subsequent 2.5 mo growth combined to produce significant differences in the final measurements. For DIAF when comparing treatments with live nodule inoculum and dead isolate A-3 (Z8), the treatments receiving live nodule inoculum at initial planting were found to have significantly larger treatment means than those given live nodule inoculum after 2 mo. The comparison (C14) between initial-planting inoculations (Trts 1-4) and delayed inoculations (Trts 13-16) was found to be significant. The mean of the initial-planting inoculations (Trt 1-4), which contained live nodule inoculum from initial planting, was larger than the mean of delayed inoculations (Trts 13-16), which received live nodule inoculum after 2 mo. The effect of isolate A-3 was not found to be significant on stem diameter.

For DIAD Duncan's multiple range test showed that all treatments with live nodule inoculum were not significantly different from each other (Figure 12). Additionally, the treatments with dead nodule inoculum had relatively uniform basal stem growth which were not significantly different from each other. For DIAF the treatments containing live nodule inoculum from initial planting had the largest
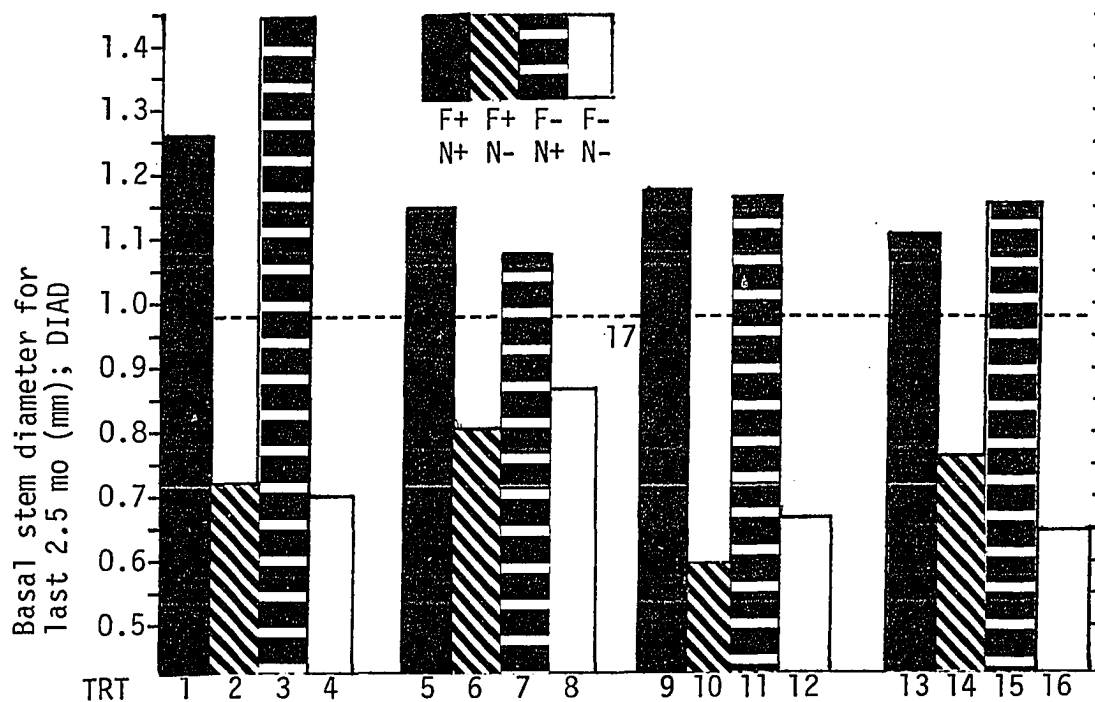
TRT[1]		Mean
3	a	1.493
1	ab	1.258
9	abc	1.180
11	abc	1.170
15	abc	1.160
5	abcd	1.150
13	abcde	1.110
7	abcdef	1.075
17	bcdef	0.973
8	bcdef	0.875
6	bcdef	0.815
14	cdef	0.763
2	cdef	0.730
4	def	0.700
12	ef	0.670
16	ef	0.653
10	f	0.598

[1] Values in each vertical column followed by the same letters do not differ significantly at p = 0.05 by Duncan's multiple range test.

Figure 12. Histogram and treatment means for basal stem diameter (mm) during the last 2.5 mo; DIAD

basal stem diameter (Figure 13). It was interesting to note that the treatment not receiving any inocula (Trt 17) had a greater basal stem diameter than all the dead nodule inoculum and some of the live nodule inoculum treatments, but this treatment mean was not significantly different from the largest or smallest means.

The stem volume for the final 2.5 mo (VOLD) was the difference of the final volume (VOLF) and the volume at 2 mo (VOLB). The effect of nodule inoculum was found to be very highly significant when comparing initial-planting inoculations [Trts 1-4 (C2)], initial nodule inoculations and delayed fungus inoculations [Trts 9-12 (C8)], and treatments with live nodule inoculum to those with dead nodule inoculum (Z14, Table 19). In these three comparisons (C2, C8, and Z14) treatments with live nodule inoculum had larger treatment means for volume difference than those with dead nodule inoculum. Significant difference for nodule inoculum also was shown comparing treatments with both live inocula (Z2), and treatments with live nodule inoculum and dead isolated A-3 (Z8) varied very highly significantly. In these latter comparisons (Z2 and Z8) treatments receiving live nodule inoculum initially had the largest seedling volume difference means. Seedlings with initial-planting inoculations (Trts 1-4) had significantly larger stem volume difference means than seedlings with delayed inoculations [Trts 13-16 (C14)].

Significant differences in the final stem volume (VOLF), product of HTF x $DIAF^2$, were associated only with the effect of nodule inoculum (Table 20). Comparison of initial-planting inoculations [Trts 1-4 (C2)], initial nodule inoculations and delayed fungus inoculations
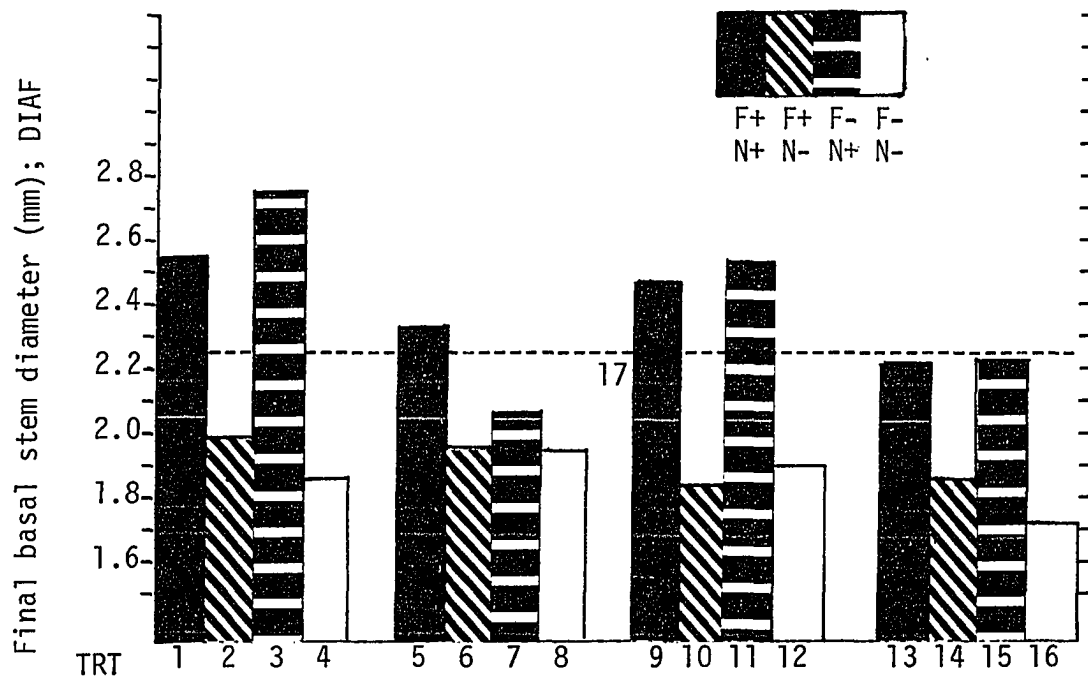
TRT		Mean	
3	a[1]	2.765	mm
11	ab	2.565	
1	ab	2.545	
9	abc	2.475	
5	abcd	2.343	
17	bcde	2.248	
15	bcde	2.225	
13	bcde	2.218	
7	bcde	2.073	
2	cde	1.993	
6	cde	1.975	
8	cde	1.968	
12	de	1.905	
4	de	1.860	
14	de	1.855	
10	de	1.840	
16	e	1.725	

[1] Values in each vertical column followed by the same letters do not differ significantly at p = 0.05 by Duncan's multiple range test.

Figure 13. Histogram and treatment means for final basal stem diameter (mm); DIAF

Table 19. Statistical analyses for stem volume growth (cc) during the last 2.5 mo; VOLD

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-value	Probability >F
Model	19	9.5723	0.5038	3.79	0.0001***
Error	46	6.3769	0.1329		
Total	67	15.9493			

SV	Effect	- Mean^a	+ Mean^a	MSS	Probability
Rep				0.7786	0.1336
C1	F=Fungus	0.9834	1.1116	0.0658	0.4850
C2	N=Nodule	0.5548	1.5402	3.8847	0.0001***
C3	I=Interaction	1.0953	0.9997	0.3660	0.6021
C4	F	0.6599	0.8571	0.1555	0.2846
C5	N	0.6167	0.9004	0.3221	0.1260
C6	I	0.6297	0.8874	0.2657	0.1638
C7	F	0.9279	0.8656	0.0155	0.7339
C8	N	0.5330	1.2604	2.1165	0.0002***
C9	I	0.8686	0.9249	0.0127	0.7587
C10	F	0.6517	0.6489	0.0001	0.9880
C11	N	0.4828	0.8177	0.4487	0.0723
C12	I	0.6474	0.6532	0.0001	0.9748
C13	Control	0.7257	0.8382	0.0477	0.5519
C14	1-4:13-16	1.0513	0.6503	1.2622	0.0034**
C15	5-8:9-12	0.7585	0.8967	0.1529	0.2888
C16	1-4,13-16:5-12	0.8508	0.8276	0.0072	0.8167
Z1	F	1.0383	1.3422	0.3693	0.1020
Z2	N	0.9735	1.4070	0.7515	0.0214*
Z3	I	1.1926	1.1879	0.0001	0.9792
Z4	F	0.4761	0.6266	0.0905	0.4133
Z5	N	0.5325	0.5702	0.0057	0.8367
Z6	I	0.5301	0.5726	0.0072	0.8164
Z7	F	1.0398	1.0984	0.0137	0.7492
Z8	N	0.7446	1.3937	1.6854	0.0008***
Z9	I	0.9682	1.1701	0.1630	0.2736
Z10	F	0.5397	0.5449	0.0001	0.9777
Z11	N	0.5670	0.5126	0.0098	0.7874
Z12	I	0.6196	0.4650	0.0957	0.4002
Z13	F	0.8057	0.8139	0.0678	0.4785
Z14	N	0.5468	1.1297	5.4361	0.0001***
Z15	I	0.8102	0.8683	0.0502	0.5415

^{a}Mean (+ and -) does not apply for comparisons C13-C16 (See Table 5).

Table 20. Statistical analyses for final seedling stem volume (cc); VOLF

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-value	Probalility >F
Model	19	11.1310	0.5858	3.77	0.0001***
Error	46	7.1485	0.1554		
Total	67	18.2795			

SV	Effect	- Mean[a]	+ Mean[a]	MSS	Probability
Rep				0.7971	0.1628
C1	F=Fungus	1.1919	1.3415	0.0895	0.4419
C2	N=Nodule	0.7675	1.7660	3.9879	0.0001***
C3	I=Interaction	1.3220	1.2115	0.0489	0.5693
C4	F	0.7913	1.0264	0.2212	0.2289
C5	N	0.7658	1.0519	0.3273	0.1447
C6	I	0.7715	1.0462	0.3018	0.1610
C7	F	1.1695	1.0785	0.0334	0.6391
C8	N	0.7154	1.5326	2.6714	0.0001***
C9	I	1.0974	1.1506	0.0113	0.7839
C10	F	0.7886	0.8011	0.0006	0.9488
C11	N	0.6233	0.9664	0.4707	0.0818
C12	I	0.7833	0.8064	0.0021	0.9050
C13	Control	0.9654	1.0236	0.0127	0.7711
C14	1-4:13-16	1.2668	0.7949	1.7814	0.0011**
C15	5-8:9-12	0.9088	1.1240	0.3704	0.1214
C16	1-4,13-16,5-12	1.0308	1.0164	0.0033	0.8820
Z1	F	1.2489	1.5461	0.3533	0.1301
Z2	N	1.1455	1.6496	1.0165	0.0120*
Z3	I	1.4102	1.1455	0.0026	0.8959
Z4	F	0.6306	0.8218	0.1462	0.3267
Z5	N	0.6820	0.7704	0.0313	0.6489
Z6	I	0.6946	0.7578	0.0159	0.7449
Z7	F	1.2501	1.2717	0.0019	0.9112
Z8	N	0.8728	1.6490	2.4102	0.0002***
Z9	I	1.1742	1.3475	0.1201	0.3736
Z10	F	0.7081	0.7115	0.0001	0.9861
Z11	N	0.7071	0.7125	0.0001	0.9780
Z12	I	0.7866	0.6331	0.0942	0.4302
Z13⁻	F	0.9853	1.0619	0.0937	0.4315
Z14	N	0.7180	1.3292	5.9771	0.0001***
Z15	I	0.9936	1.0537	0.0578	0.5361

[a] Mean (+ and -) does not apply for comparisons C13-C16 (See Table 5).

[Trts 9-12 (C8)], nodulated to non-nodulated (Z14), and those with live

nodule inoculum and dead isolate A-3 (Z8) were found to be very highly

significant. Significant difference was found for nodule inoculum

when comparing treatments with both live inocula (Z2). For the first

three comparisons (C2, C8, and Z14) treatments with live nodule inoculum

had greater final seedling stem volume means than those with dead

nodule inoculum. The latter two comparisons (Z2 and Z8) indicated

that the presence of the live nodule inoculum from initial planting

produced a greater final volume.

The stem volume for the first 2 mo growth showed little difference

among treatments, but three of the largest four treatment means were for

treatments with live nodule inoculum (Figure 9). During the final 2.5

mo the volume means showed considerable separation (Figure 14). The

final seedling volume exhibits a nearly identical separation of means

as the volume for the last 2.5 mo (Figure 15). The first six treatment

means have the same ranking for both variables. The four largest treat-

ment means received live nodule inoculum at the initial planting. The

strong relationship between VOLD and VOLF is shown by the partial

correlation comparison ($r = 0.99$, Table 21).

Significant difference was shown among replication means for root

volume (ROOT, Table 22). Root volume was significantly less in treat-

ments with live isolate A-3 when comparing initial-planted inoculations

[Trts 1-4 (C1)]. All mycorrhizal treatments when compared to non-mycor-

rhizal treatments had significantly less root volume (Z13). Likewise,

all nodulated treatments had significantly less root volume than

Figure 14. Histogram and treatment means for stem volume growth (cc) during the last 2.5 mo; VOLD
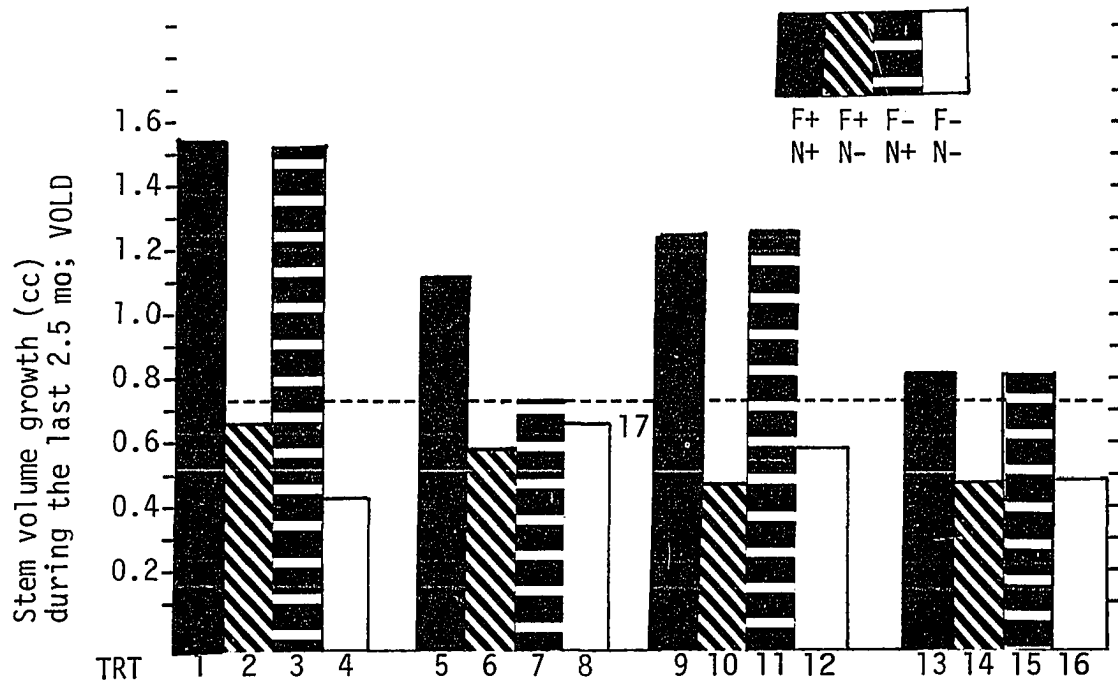
TRT		Mean	
1	a[1]	1.556	cc
3	a	1.524	
11	ab	1.263	
9	ab	1.257	
5	abc	1.128	
13	bcd	0.819	
15	bcd	0.816	
17	bcd	0.726	
7	bcd	0.723	
2	bcd	0.667	
8	cd	0.647	
12	cd	0.592	
6	cd	0.586	
16	d	0.487	
14	d	0.479	
10	d	0.474	
4	d	0.443	

[1] Values in each vertical column followed by the same letters do not differ significantly at p = 0.05 by Duncan's multiple range test.

Final seedling stem volume (cc); VOLF

1.7
1.5
1.3
1.1
0.9
0.7
0.5
0.3
0.1

F+   F+   F-   F-
N+   N-   N+   N-

17

TRT   1 2 3 4   5 6 7 8   9 10 11 12   13 14 15 16
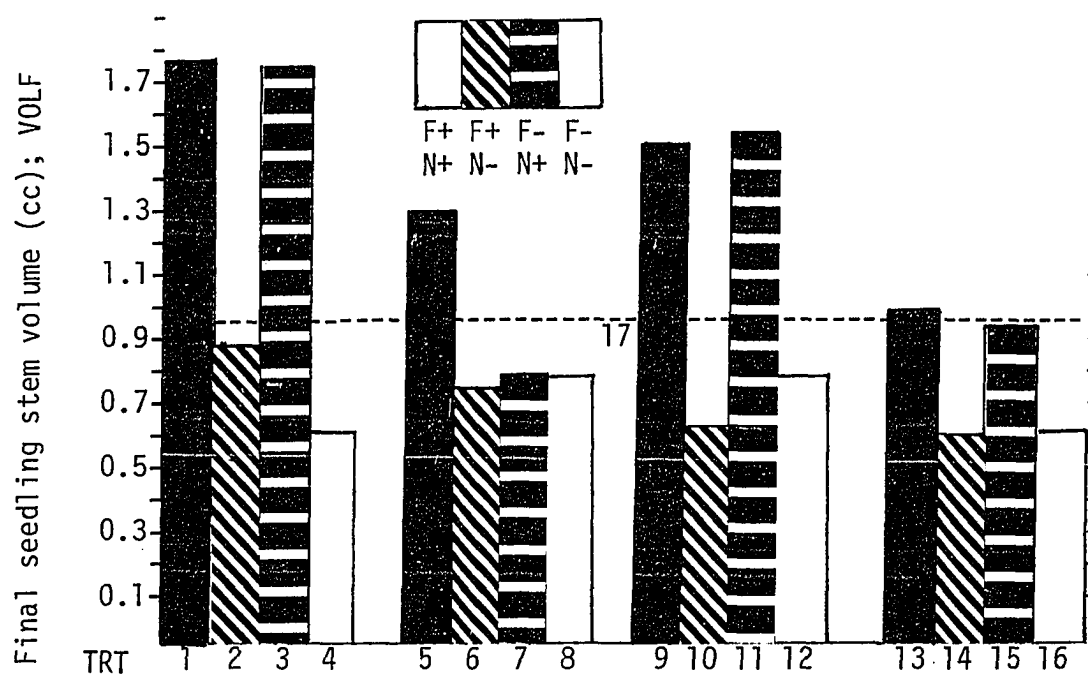
TRT[1]		Mean
1	a	1.786 cc
3	a	1.746
11	ab	1.551
9	abc	1.514
5	abcd	1.307
13	bcde	0.984
17	bcde	0.965
15	bcde	0.949
2	cde	0.898
7	de	0.797
12	de	0.788
8	de	0.786
6	de	0.746
10	e	0.643
4	e	0.637
16	e	0.629
14	e	0.618

[1]Values in each vertical column followed by the same letters do not differ significantly at $p = 0.05$ by Duncan's multiple range test.

Figure 15.   Histogram and treatment means for final seedling stem volume (cc); VOLF

Table 21. Partial correlation coefficients and probability levels for relationships between mean growth parameters determined from time of harvest data

Variable	VOLF	VOLD	DIAF	DIAD	HTF	HTD
VOLF	1.00^a	0.990 [b] 0.0001	0.912 0.0001	0.846 0.0001	0.798 0.0001	0.651 0.0001
VOLD		1.00	0.895 0.0001	0.879 0.0001	0.795 0.0001	0.706 0.0001
DIAF			1.00	0.929 0.0001	0.588 0.0001	0.438 0.0017
DIAD				1.00	0.555 0.0001	0.547 0.0001
HTF					1.00	0.852 0.0001
HTD						1.00
ROOT						
LV						
STEM						
MM						
CH						

[a] R value.

[b] Probability level.

Variable	ROOT	LV	STEM	MM	CH
VOLF	0.229	0.139	0.515	0.038	0.281
	0.1137	0.3419	0.0002	0.7913	0.0508
VOLD	0.220	0.135	0.511	0.068	0.335
	0.1287	0.3554	0.0002	0.6435	0.0185
DIAF	0.143	0.078	0.443	0.052	0.158
	0.3263	0.5958	0.0014	0.7206	0.2786
DIAD	0.217	0.139	0.406	0.138	0.292
	0.1351	0.3423	0.0038	0.3450	0.0418
HTF	0.167	0.158	0.549	0.061	0.440
	0.2493	0.2783	0.0001	0.6792	0.0015
HTD	0.156	0.144	0.447	0.216	0.616
	0.2852	0.3223	0.0013	0.1354	0.0001
ROOT	1.00	0.443	0.441	-0.136	0.079
		0.0014	0.0015	0.3518	0.5893
LV		1.00	0.789	-0.001	0.452
			0.0001	0.9972	0.0011
STEM			1.00	-0.142	0.447
				0.3309	0.0013
MM				1.00	0.505
					0.0002
CH					1.00

Table 22. Statistical analyses for seedling root volume (cc); ROOT

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-value	Probability >F
Model	19	545.599	28.716	1.92	0.0269*
Error	46	688.560	14.969		
Total	67	1234.159			

SV	Effect	- Mean[a]	+ Mean[a]	MSS	Probability
Rep				172.465	0.0126*
C1	F=Fungus	19.675	15.450	71.403	0.0304*
C2	N=Nodule	18.563	16.563	16.000	0.2962
C3	I=Interaction	18.588	16.538	16.810	0.2844
C4	F	13.375	13.588	0.181	0.9111
C5	N	14.288	12.675	10.401	0.3987
C6	I	13.788	13.175	1.501	0.7478
C7	F	16.713	14.725	15.801	0.2992
C8	N	16.288	15.150	5.176	0.5509
C9	I	15.750	15.688	0.016	0.9738
C10	F	15.725	12.650	37.823	0.1110
C11	N	15.713	12.663	37.210	0.1138
C12	I	14.488	13.888	1.440	0.7527
C13	Control	16.000	15.238	2.189	0.6978
C14	1-4:13-16	17.563	14.188	91.125	0.0151*
C15	5-8:9-12	13.481	15.719	40.051	0.1012
C16	1-4,13-16:5-12	15.875	14.600	26.010	0.1845
Z1	F	12.475	12.950	0.903	0.8030
Z2	N	11.650	13.775	18.063	0.2674
Z3	I	13.300	12.125	5.523	0.5379
Z4	F	14.900	16.088	5.641	0.5336
Z5	N	14.588	16.400	13.141	0.3433
Z6	I	15.013	15.975	3.706	0.6136
Z7	F	15.338	16.288	3.610	0.6182
Z8	N	13.688	17.938	72.250	0.0295*
Z9	I	14.525	17.100	26.523	0.1803
Z10	F	17.100	16.763	0.456	0.8593
Z11	N	15.413	18.450	36.906	0.1153
Z12	I	15.563	18.300	29.976	0.1548
Z13	F	16.372	14.103	82.356	0.0205*
Z14	N	16.213	14.263	60.840	0.0449*
Z15	I	15.653	14.822	11.056	0.6978

[a]Mean (+ and -) does not apply for comparisons C13-C16 (See Table 5).

non-nodulated treatments (Z14). The effect of nodule inoculum was found to be significant when comparing treatments with live nodule inoculum and dead isolate A-3 (Z8). Treatments receiving live nodule inoculum at initial planting had larger root volume means than those receiving nodule inoculum after 2 mo. The root volume mean for initial-planting inoculations (Trts 1-4) was significantly larger than the mean for delayed inoculations [Trts 13-16 (14)].

The effect of the inocula did not account for much significant root volume differences. Duncan's multiple range test showed that 14 means were not significantly different from each other (Figure 16). However, treatments given both live inocula were observed to have the lowest root volume within each 2 x 2 factorial. Treatments with both live inocula, Trts 1, 5, 9, and 13, were ranked 14th, 16th, 12th, and 17th respectively.

Dry weight analysis of leaf (LV) and stem (STEM) tissues (Tables 23 and 24) showed very highly significant differences for the effect of nodule inoculum for all treatment comparisons within the different inoculation time combinations [Trts 1-4 (C2), 5-8 (C5), 9-12 (C8), and 13-16 (C11)]. Comparison of nodulated to non-nodulated treatments (Z14) was very highly significant for both variables (LV and STEM). Treatments with live nodule inoculum for the above five comparisons (C2, C5, C8, C11, and Z14) had larger means than treatments with dead nodule inoculum. The effect of isolate A-3 for initial-planting inoculations [Trts 1-4 (C1)] was found to be significant for leaf dry weight but not stem dry weight. No interaction between inocula was
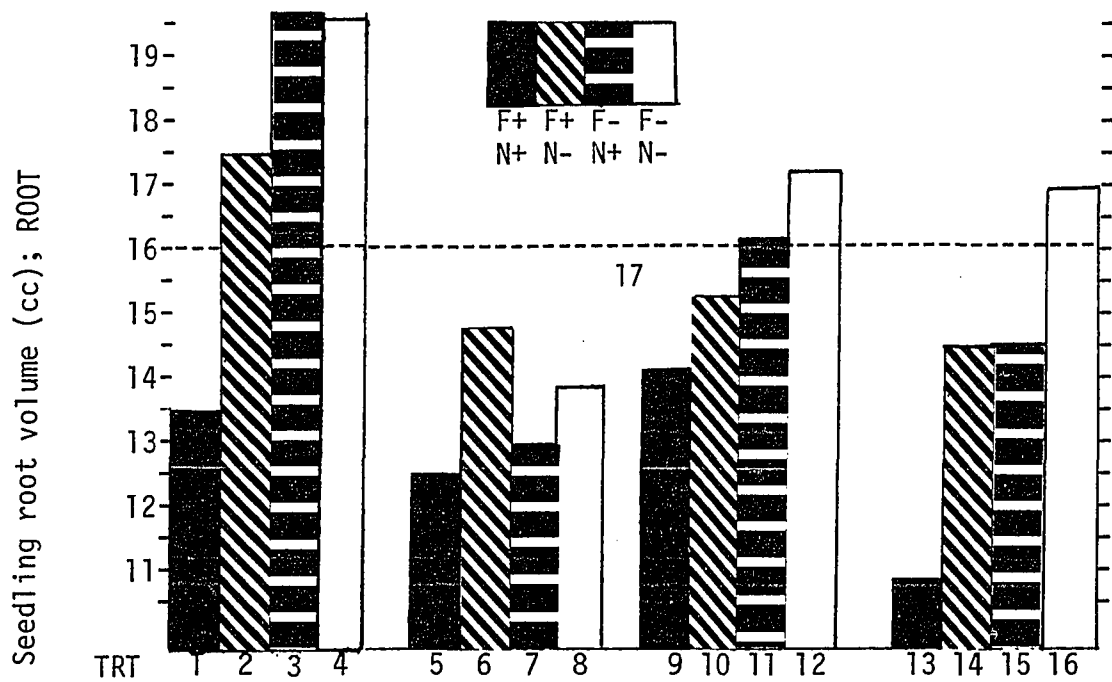
TRT		Mean
3	a[1]	19.70 cc
4	a	19.65
2	ab	17.48
12	abc	17.25
16	abc	16.95
11	abc	16.18
17	abc	16.00
10	abc	15.33
6	abc	14.70
15	abc	14.50
14	abc	14.48
9	abc	14.13
8	abc	13.88
1	abc	13.43
7	bc	12.88
5	c	12.48
13	c	10.83

[1] Values in each vertical column followed by the same letters do not differ significantly at p = 0.05 by Duncan's multiple range test.

Figure 16. Histogram and treatment means for seedling root volume (cc); ROOT

Table 23. Statistical analyses for seedling leaf dry weight (g); LV

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-value	Probability >F
Model	19	17.170	0.904	8.78	0.0001***
Error	46	4.733	0.103		
Total	67	21.903			

SV	Effect	- Mean^a	+ Mean^a	MSS	Probability
Rep				0.5037	0.1789
C1	F=Fungus	1.298	0.914	0.5891	0.0182*
C2	N=Nodule	0.643	2.655	3.4318	0.0001***
C3	I=Interaction	1.145	1.066	0.0248	0.6183
C4	F	1.065	1.140	0.0225	0.6350
C5	N	0.689	1.516	2.7390	0.0001***
C6	I	1.111	1.094	0.0012	0.9117
C7	F	1.244	1.238	0.0002	0.9684
C8	N	0.739	1.743	4.0301	0.0001***
C9	I	1.153	1.329	0.1243	0.2672
C10	F	1.313	1.118	0.1521	0.2203
C11	N	0.654	1.776	5.0400	0.0001***
C12	I	1.286	1.144	0.0812	0.3686
C13	Control	0.948	1.166	0.1796	0.1834
C14	1-4:13-16	1.106	1.215	0.0957	0.3295
C15	5-8:9-12	1.103	1.241	0.1526	0.2195
C16	1-4,13-16:5-12	1.160	1.172	0.0020	0.8866
Z1	F	1.718	1.441	0.3053	0.0849
Z2	N	1.576	1.583	0.0002	0.9684
Z3	I	1.686	1.473	0.1828	0.1797
Z4	F	0.638	0.613	0.0025	0.8742
Z5	N	0.681	0.569	0.0506	0.4771
Z6	I	0.691	0.559	0.0702	0.4029
Z7	F	1.801	1.644	0.0992	0.3208
Z8	N	1.716	1.729	0.0006	0.9369
Z9	I	1.573	1.873	0.3600	0.0620
Z10	F	0.755	0.719	0.0053	0.8184
Z11	N	0.661	0.813	0.0915	0.3402
Z12	I	0.736	0.738	0.0001	0.9937
Z13	F	1.229	1.102	0.2601	0.1109
Z14	N	0.681	1.651	15.0544	0.0001***
Z15	I	1.174	1.158	0.1796	0.1834

^{a}Mean (+ and -) does not apply for comparisons C13-C16 (See Table 5).

Table 24. Statistical analyses for seedling stem dry weight (g); STEM

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-value	Probability >F
Model	19	4.4707	0.2353	7.95	0.0001***
Error	46	1.3621	0.0296		
Total	67	5.8328			

SV	Effect	$-$ Mean[a]	$+$ Mean[a]	MSS	Probability
Rep				0.1697	0.1275
C1	F=Fungus	0.753	0.689	0.0163	0.4528
C2	N=Nodule	0.475	0.966	0.9653	0.0001***
C3	I=Interaction	0.710	0.731	0.0018	0.8019
C4	F	0.588	0.720	0.0702	0.1223
C5	N	0.426	0.881	0.8281	0.0001***
C6	I	0.624	0.684	0.0144	0.4797
C7	F	0.771	0.754	0.0012	0.8363
C8	N	0.474	1.051	1.3340	0.0001***
C9	I	0.705	0.820	0.0529	0.1785
C10	F	0.716	0.626	0.0324	0.2906
C11	N	0.453	0.890	0.7656	0.0001***
C12	I	0.714	0.629	0.0289	0.3180
C13	Control	0.563	0.702	0.0733	0.1146
C14	1-4:13-16	0.721	0.671	0.0195	0.4112
C15	5-8:9-12	0.654	0.763	0.0946	0.0741
C16	1-4,13-16:5-12	0.696	0.708	0.0024	0.7735
Z1	F	0.951	0.961	0.0004	0.9060
Z2	N	0.890	1.023	0.0702	0.1223
Z3	I	1.039	0.874	0.1089	0.0559
Z4	F	0.429	0.448	0.0014	0.8248
Z5	N	0.456	0.420	0.0053	0.6688
Z6	I	0.435	0.441	0.0002	0.9412
Z7	F	0.990	0.886	0.0431	0.2240
Z8	N	0.881	0.995	0.0518	0.1832
Z9	I	0.894	0.983	0.0315	0.2973
Z10	F	0.498	0.454	0.0077	0.6059
Z11	N	0.422	0.529	0.0452	0.2132
Z12	I	0.465	0.486	0.0018	0.8019
Z13	F	0.707	0.697	0.0015	0.8190
Z14	N	0.457	0.947	3.8465	0.0001***
Z15	I	0.688	0.716	0.0124	0.5121

[a]Mean (+ and -) does not apply for comparisons C13-C16 (See Table 5).

observed. Treatments given the same inocula at different times
(Z comparisons) were not significantly different from each other.

Duncan's multiple range tests for leaf and stem dry weights
(Figures 17 and 18) showed that live nodule inoculum greatly increased
leaf and stem dry weights compared to treatments with dead nodule
inoculum. For leaf dry weight treatments with live isolate A-3 (Trts
1 and 2) had considerably lower means than those with dead isolate A-3
(Trts 3 and 4) which accounts for the significant difference found in
comparison Cl. As previously reported some seedlings in treatment 1
defoliated. Therefore, the significant difference found in comparison
Cl may not be real.

For both total acetylene reduction (CH) and specific acetylene
reduction activity (MM) significant difference was found among replication
means (Tables 25 and 26). As expected for both parameters the effect of
nodule inoculum was found to be very highly significant when comparing
all treatments within the different inoculation time combinations [Trts
1-4 (C2), 5-8 (C5), 9-12 (C8), and 13-16 (Cll)], and those with nodulated
seedlings to those seedlings without nodules (Z14). The treatments with
live nodule inoculum had much larger treatment means in these comparisons
(C2, C5, C8, Cll, and Z14) than treatments with dead nodule inoculum.
For CH the overall interaction (Z15) was found to be significant.
Treatments with either all dead or all living inocula (Trts 1, 4, 5, 8,
9, 12, 13, and 16) had significantly higher values than the remaining
treatments, excluding the control (Trt 17). The values for all seedlings
without living nodule inoculum (even numbered Trts and Trt 17) were
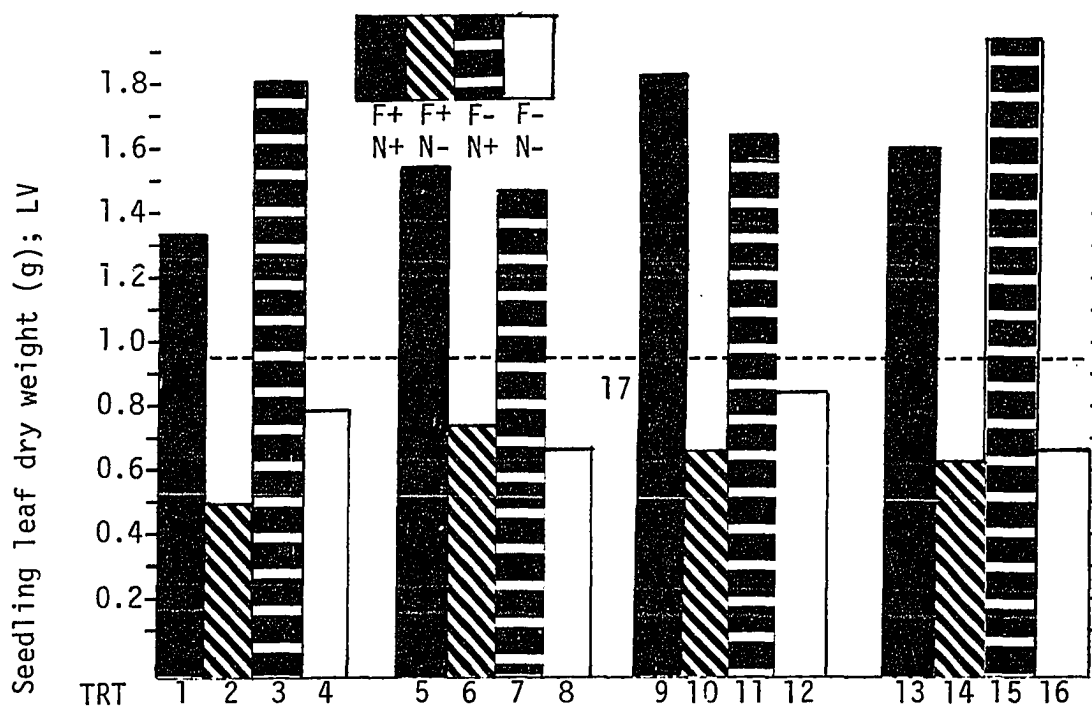
TRT[1]		Mean
15	a	1.945 g
9	ab	1.828
3	ab	1.800
11	ab	1.658
13	ab	1.608
5	ab	1.545
7	ab	1.488
1	bc	1.338
17	cd	0.948
12	d	0.830
4	d	0.795
6	d	0.735
16	d	0.680
10	d	0.648
8	d	0.643
14	d	0.628
2	d	0.490

[1] Values in each vertical column followed by the same letters do not differ significantly at p = 0.05 by Duncan's multiple range test.

Figure 17. Histogram and treatment means for seedling leaf dry weight (g); LV
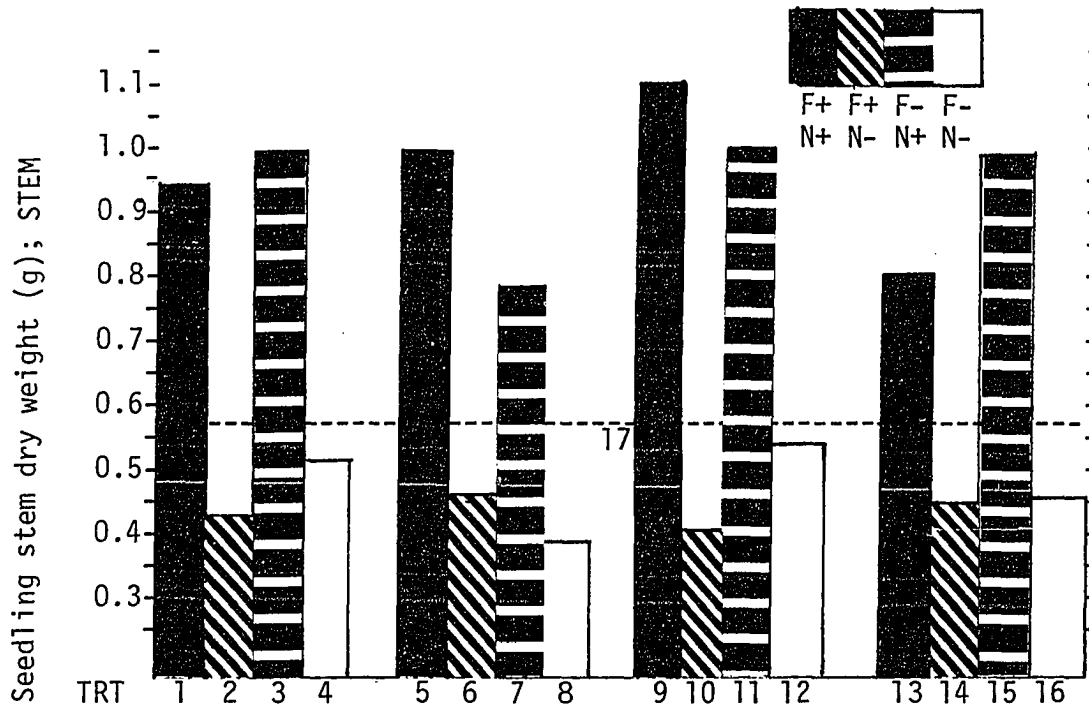
TRT		Mean
9	a[1]	1.100 g
11	ab	1.003
3	ab	0.988
5	ab	0.978
15	ab	0.978
1	ab	0.945
13	bc	0.803
7	bc	0.785
17	cd	0.563
12	cd	0.540
4	d	0.518
6	d	0.463
16	d	0.455
14	d	0.450
2	d	0.433
10	d	0.408
8	d	0.390

[1] Values in each vertical column followed by the same letters do not differ significantly at p = 0.05 by Duncan's multiple range test.

Figure 18. Histogram and treatment means for seedling stem dry weight (g); STEM

Table 25. Statistical analyses for total acetylene reduction
(μl C_2H_4/plant/min); CH

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-value	Probability >F
Model	19	108750421.62	5723706.40	14.27	0.0001***
Error	46	18456601.24	401230.45		
Total	67	127207022.86			

SV	Effect	- Mean[a]	+ Mean[a]	MSS	Probability
Rep				4881455.09	0.0098**
C1	F=Fungus	1339.71	1251.14	31382.12	0.7768
C2	N=Nodule	47.58	2543.27	24914073.96	0.0001***
C3	I=Interaction	1292.58	1298.71	172.92	0.9832
C4	F	953.18	1413.49	847550.39	0.1442
C5	N	0.00	2366.66	22404365.56	0.0001***
C6	I	953.18	1413.49	847550.39	0.1442
C7	F	856.86	1229.02	554019.71	0.2359
C8	N	0.00	2085.89	17403706.65	0.0001***
C9	I	856.86	1229.02	554019.71	0.2359
C10	F	1125.31	1545.20	705222.05	0.1820
C11	N	0.00	2670.51	28526548.05	0.0001***
C12	I	1125.31	1545.20	705222.05	0.1820
C13	Control	0.00	1214.24	5550593.89	0.0004***
C14	1-4:13-16	1295.42	1335.26	12692.23	0.8566
C15	5-8:9-12	1183.33	1042.94	157669.20	0.5250
C16	1-4,13-16:5-12	1315.34	1113.14	654177.66	0.1983
Z1	F	2774.22	2664.62	48048.65	0.7253
Z2	N	2953.69	2458.69	915944.70	0.1293
Z3	I	2642.51	2796.34	94648.52	0.6221
Z4	F	0.00	0.00	0.00	1.0000
Z5	N	0.00	0.00	0.00	1.0000
Z6	I	0.00	0.00	0.00	1.0000
Z7	F	1982.17	2245.31	276965.38	0.4003
Z8	N	2078.49	2149.00	19888.05	0.8211
Z9	I	1810.04	2417.45	1475799.78	0.0559
Z10	F	0.00	47.57	9053.52	0.8787
Z11	N	0.00	47.57	9053.52	0.8787
Z12	I	0.00	47.57	9053.52	0.8787
Z13	F	1068.77	1359.71	1354401.35	0.0666
Z14	N	11.89	2416.58	92520592.03	0.0001***
Z15	I	1056.87	1371.61	1584923.63	0.0479*

[a]Mean (+ and -) does not apply for comparisons C13-C16 (See Table 5).

Table 26. Statistical analyses for specific acetylene reduction
(μmoles C_2H_4/min/g nodule dry weight); MM

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-value	Probability $\geq$ F
Model	19	9382154.76	493797.62	17.83	0.0001***
Error	46	1273707.87	27689.30		
Total	67	10655862.63			

SV	Effect	− Mean[a]	+ Mean[a]	MSS	Probability
Rep				323600.54	0.0119*
C1	F=Fungus	255.73	239.07	1110.06	0.8388
C2	N=Nodule	12.95	481.85	879464.15	0.0001***
C3	I=Interaction	242.78	252.03	341.97	0.9101
C4	F	458.11	299.73	100336.90	0.0577
C5	N	0.00	757.84	2297255.55	0.0001***
C6	I	458.11	299.73	100336.90	0.0577
C7	F	298.01	294.94	37.67	0.9701
C8	N	0.00	592.95	1406352.88	0.0001***
C9	I	298.01	294.94	37.67	0.9701
C10	F	411.66	503.14	33474.36	0.2670
C11	N	0.00	914.81	3347472.75	0.0001***
C12	I	411.66	503.14	33474.36	0.2670
C13	Control	0.00	345.05	448221.57	0.0002***
C14	1-4:13-16	247.40	457.40	352802.10	0.0007***
C15	5-8:9-12	378.92	296.47	54374.95	0.1588
C16	1-4,13-16:5-12	352.40	337.70	3460.38	0.7196
Z1	F	798.08	538.80	268909.66	0.0026**
Z2	N	802.87	534.01	289137.42	0.0018**
Z3	I	594.67	742.22	87081.06	0.0763
Z4	F	0.00	0.00	0.00	1.0000
Z5	N	0.00	0.00	0.00	1.0000
Z6	I	0.00	0.00	0.00	1.0000
Z7	F	709.67	700.89	308.70	0.9146
Z8	N	869.77	540.79	432017.94	0.0002***
Z9	I	756.12	654.44	41351.22	0.2180
Z10	F	0.00	12.95	671.07	0.8743
Z11	N	0.00	12.95	671.07	0.8743
Z12	I	0.00	12.95	671.07	0.8743
Z13	F	355.88	334.22	7504.32	0.5973
Z14	N	3.24	686.86	7477421.89	0.0001***
Z15	I	353.64	337.46	3687.22	0.7110

[a]Mean (+ and −) does not apply for comparisons C13-C16 (See Table 5).

expected to be 0. This was true except for initial-planting inoculations with dead nodule and fungus inocula (Trt 4) which was an estimated value.

For MM the effect of isolate A-3 was found to be highly significant when comparing treatments with both live inocula (Z1, Table 26). Treatments receiving isolate A-3 at initial planting had lower treatment means than those receiving isolate A-3 after 2 mo. Highly significant differences for the effect of nodule inoculum was shown when comparing treatments with both live inocula (Z2); and comparison of treatments with live nodule inoculum and dead isolate A-3 varied very highly significantly (Z8). In both of these latter two comparisons (Z2 and Z8) treatments receiving nodule inoculum initially had lower treatment means than those receiving nodule inoculum after 2 mo.

For CH all inoculations with both symbionts living (Trts 1, 5, 9, and 13) had a mean of 2719.75 μl C_2H_4/plant/min. Inoculations with living nodule inoculum and dead fungus (Trts 3, 7, 11, and 15) had a mean of 2113.5 μl C_2H_4/plant/min. Using Duncan's multiple range test these means were not quite significantly different (Figure 19). However, there was a trend that for CH treatments with both live inocula reduced more acetylene than treatments with live nodule inoculum and dead isolate A-3. An opposing trend was indicated for MM (Figure 20). The mean for treatments with both live inocula, 668 μmoles C_2H_4/min/g nodule dry weight, was less than the mean for treatments with live nodule inoculum and dead isolate A-3, 705 μmoles C_2H_4/min/g nodule dry weight. For MM this trend would indicate that treatments with live nodule inoculum and dead isolate A-3 reduced more acetylene per g nodule dry weight than
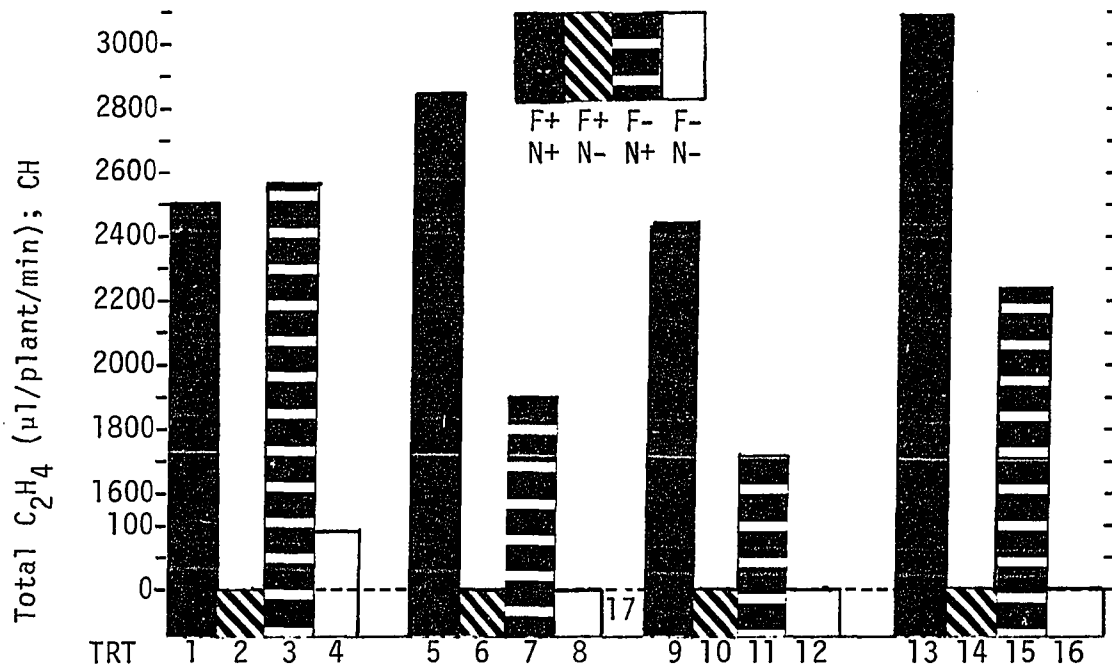
TRT[1]	Mean	
13 a	3090.400	μl C_2H_4 /plant/min
5 ab	2826.975	
3 abc	2584.275	
1 abc	2502.275	
9 abc	2458.050	
15 abc	2250.625	
7 bc	1906.350	
11 c	1713.725	
4 d	95.150	
2 d	0.000	
6 d	0.000	
8 d	0.000	
10 d	0.000	
12 d	0.000	
14 d	0.000	
16 d	0.000	
17 d	0.000	

[1] Values in each vertical column followed by the same letters do not differ significantly at p = 0.05 by Duncan's multiple range test.

Figure 19. Histogram and treatment means for total acetylene reduction (μl C_2H_4/plant/min); CH

Figure 20. Histogram and treatment means for specific acetylene reduction (μmoles C_2H_4/min/g nodule dry weight); MM
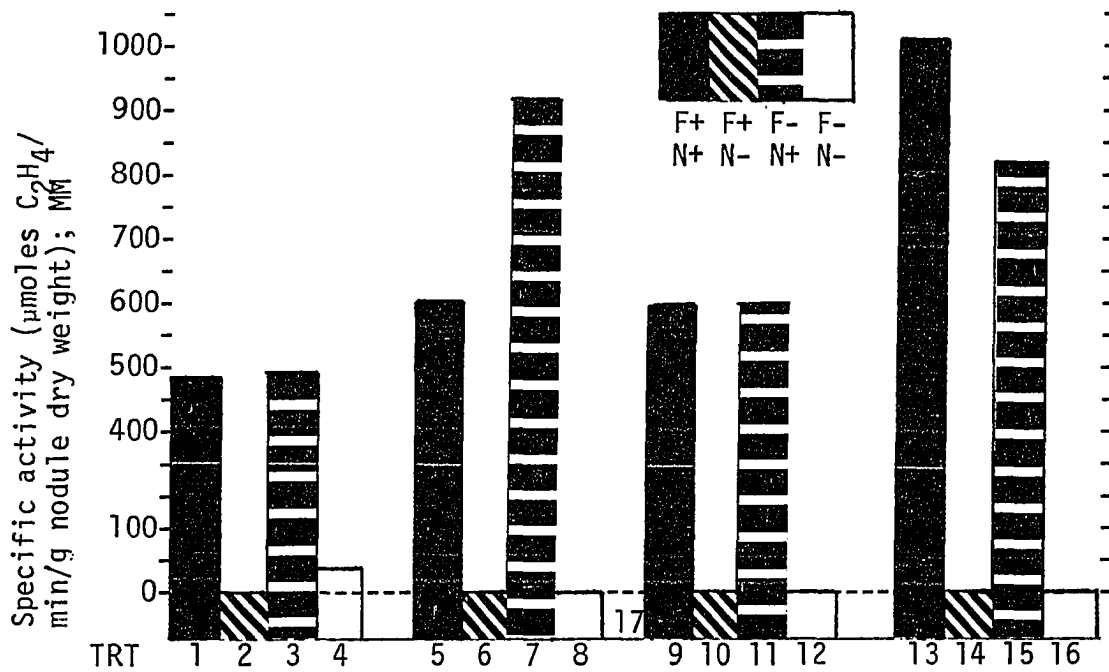
TRT		Mean	
T3	a[1]	1006.285	μmoles C_2H_4/min/
7	a	916.215	g nodule dry weight
15	ab	823.325	
5	bc	599.455	
11	bc	596.018	
9	bc	589.880	
3	c	485.558	
1	c	478.145	
4	d	25.905	
2	d	0.000	
6	d	0.000	
8	d	0.000	
10	d	0.000	
12	d	0.000	
14	d	0.000	
16	d	0.000	
17	d	0.000	

[1] Values in each vertical column followed by the same letters do not differ significantly at $p = 0.05$ by Duncan's multiple range test.

treatments with both live inocula. Therefore, without reaching the defined significance and with opposing trends, no conclusion can be made concerning the effects of isolate A-3 on nitrogen fixation rates.

For CH the two delayed live nodule inoculum treatments (Trts 5 and 13) gave the greatest acetylene reduction (Figure 19). For MM the treatments with the greatest specific acetylene reduction activity were not associated with the treatment effect, both live inocula vs live nodule inoculum and dead isolate A-3, but with the time the inocula was added. The four largest treatment means were treatments where live nodule inoculum was added to the seedlings after 2 mo (Figure 20).

The effect of isolate A-3 on the percentage of mycorrhizae (MYCO, Table 27) was found to be very highly significant when comparing treatments with initial-planting inoculations [Trts 1-4 (C1)], with delayed nodule inoculum and initial fungus inoculations [Trts 5-8 (C4)], with initial nodule inoculations and delayed fungus inoculations [Trts 9-12 (C7)], with mycorrhizae to those without (Z13), and highly significant when comparing treatments with delayed inoculations [Trts 13-16 (C10)]. In the above comparisons (C1, C4, C7, C10, and Z13) treatments with live isolate A-3 had larger percentage means than those with dead isolate A-3. Highly significant results for the effect of isolate A-3 were obtained when comparing treatments with live isolate A-3 and dead nodule inoculum (Z4). Significant results for the effect of isolate A-3 were obtained when comparing treatments with both live inocula (Z1). In both of these comparisons (Z1 and Z4) treatments receiving live isolate A-3 at initial planting had significantly larger percentages of

Table 27. Statistical analyses for percentage of mycorrhizae; MYCO

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-value	Probability >F
Model	18	35258.91	1958.83	12.58	0.0001***
Error	32	4984.67	155.77		
Total	50	40243.58			

SV	Effect	− Mean^a	+ Mean^a	MSS	Probability
Rep				309.47	0.3815
C1	F=Fungus	0.63	50.70	7520.01	0.0001***
C2	N=Nodule	11.05	40.34	2563.76	0.0003***
C3	I=Interaction	10.42	40.92	2790.75	0.0002***
C4	F	0.00	49.88	7465.04	0.0001***
C5	N	22.22	27.67	89.11	0.4550
C6	I	22.22	27.67	89.11	0.4550
C7	F	0.00	41.13	5075.85	0.0001***
C8	N	8.20	32.93	1835.21	0.0017**
C9	I	8.20	32.93	1835.21	0.0017**
C10	F	0.00	23.52	1659.10	0.0026**
C11	N	4.28	19.23	670.51	0.0461*
C12	I	4.28	19.23	670.51	0.0461*
C13	Control	0.00	20.73	1213.75	0.0088**
C14	1-4:13-16	25.67	11.76	1160.65	0.0102*
C15	5-8:9-12	24.94	20.57	114.84	0.3969
C16	1-4,13-16:5-12	18.71	22.76	196.02	0.2703
Z1	F	52.17	67.95	747.34	0.0359*
Z2	N	46.90	73.22	2077.70	0.0009***
Z3	I	59.52	60.60	3.52	0.8814
Z4	F	12.48	32.63	1218.07	0.0087**
Z5	N	26.50	18.62	186.44	0.2821
Z6	I	30.42	14.70	741.04	0.0366*
Z7	F	0.00	0.00	0.00	1.0000
Z8	N	0.00	0.00	0.00	1.0000
Z9	I	0.00	0.00	0.00	1.0000
Z10	F	0.00	0.63	1.20	0.9305
Z11	N	0.00	0.63	1.20	0.9305
Z12	I	0.00	0.63	1.20	0.9305
Z13	F	0.16	40.24	20319.87	0.0001***
Z14	N	11.44	30.03	4147.80	0.0001***
Z15	I	11.28	30.19	4290.30	0.0001***

^{a}Mean (+ and −) does not apply for comparisons C13-C16 (See Table 5).

mycorrhizae. The percentage of mycorrhizae for initial-planting inoculations (Trts 1-4) was significantly larger than the percentage of mycorrhizae for delayed inoculations [Trts 13-16 (C14)].

The effect of nodule inoculum on the percentage of mycorrhizae was found to be very highly significant when comparing initial-planting inoculations [Trts 1-4 (C2)], all treatments with live inocula [Trts 1, 5, 9, 13 (Z2)], and all nodulated treatments to all non-nodulated treatments (Z14). Highly significant results for the effect of nodule inoculum were obtained when comparing initial nodule inoculations and delayed fungus inoculations [Trts 9-12 (C8)], and significant results were obtained when comparing treatments receiving delayed inoculations [Trts 13-16 (C11)]. In C2, C8, C11, and Z14 comparisons treatments with live nodule inoculum had larger means than those with dead nodule inoculum. When comparing treatments with both live inocula (Z2) those receiving nodule inoculum at initial planting had larger percentage means than those receiving nodule inoculum after 2 mo.

The treatments receiving both live inocula had much larger mycorrrhizal percentage means than treatments receiving live isolate A-3 and dead nodule inoculum. This attributed to the significant interactions. Interaction between the two-inocula was found to be very highly significant when comparing initial-planting inoculations [Trts 1-4 (C3)], and treatments with either all dead or all living inocula in all time combinations (Trts 1, 4, 5, 8, 9, 12, 13, and 16) to remaining treatments (Z15). Highly significant interactions were found when comparing initial-planting nodule inoculations and delayed

fungus inoculations [Trts 9-12 (C9)], and significant interactions were observed when comparing delayed inoculations [Trts 13-16 (12)] and treatments with dead nodule inoculum and live fungus in all time combinations [Trts 2, 6, 10, 14 (Z6)]. The control (Trt 17), as expected, was significantly less than the mean of the 16 treatments (C13).

Each treatment containing both live inocula (Trts 1, 5, 9, and 13) had a higher percentage of mycorrhizae than its corresponding non-nodulated treatment (Trts 2, 6, 10, and 14) respectively (Figure 21). Large treatment mean differences were shown where the live nodule inoculum was added at initial planting compared to those given dead nodule inoculum initially (Trts 1:2 and 9:10). Initial-planting inoculation with live inocula (Trt 1) was significantly larger than initial-planting inoculation with live isolate A-3 and dead nodule inoculum (Trt 2). Initial-planting inoculation with live nodule inoculum and delayed live isolate A-3 (Trt 9) was significantly larger than initial-planting inoculation with dead nodule inoculum and delayed live isolate A-3 inoculation (Trt 10). A smaller difference was observed when live nodule inoculum was added after 2 mo. Initial-planting inoculations with live fungus and delayed inoculation with live nodule inoculum (Trt 5) was not significantly different from initial-planting inoculations with live fungus and delayed dead nodule inoculation (Trt 6). Delayed both live inoculation (Trt 13) was significantly larger than delayed live fungus and dead nodule inoculation (Trt 14), but their mean difference was much less than the difference of treatments 1:2 and 9:10. Treatments with both live inocula (1 and 9)
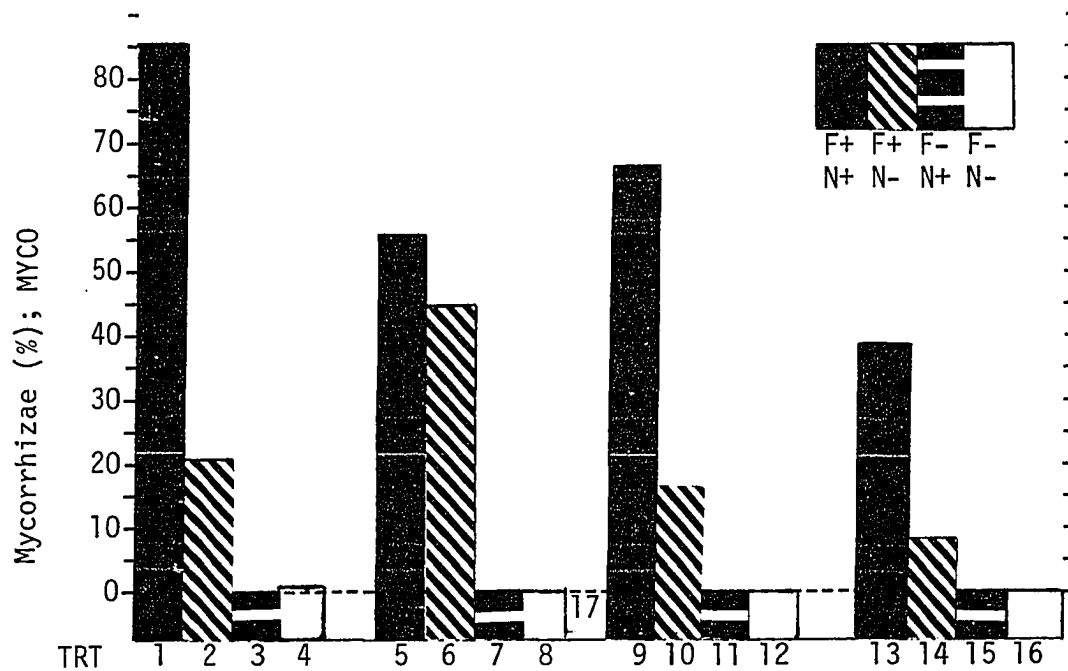
TRT [1]		Mean
1	a[1]	80.567 %
9	ab	65.867
5	bc	55.333
6	bc	44.433
13	cd	38.467
2	de	20.833
10	de	16.400
14	e	8.567
4	e	1.267
3	e	0.000
7	e	0.000
8	e	0.000
11	e	0.000
12	e	0.000
15	e	0.000
16	e	0.000
17	e	0.000

[1] Values in each vertical column followed by the same letters do not differ significantly at p = 0.05 by Duncan's multiple range test.

Figure 21. Histogram and treatment means for the percentage of mycorrhizae; MYCO

were not significantly different from each other even though live
isolate A-3 was added 2 mo later (Trt 9). Initial-planting inoculation
with live inocula (Trt 1) was significantly larger than treatment where
isolate A-3 was added at the same time but live nodule inoculum was
added 2 mo later (Trt 5). Initial planting nodule inoculation and
delayed fungus inoculation (Trt 9) was significantly larger than the
treatment where isolate A-3 and nodule inoculum were delayed 2 mo
after initial planting (Trt 13). The time of addition of live nodule
inoculum appeared to be more important than the time of live isolate
A-3 addition.

Leaf Mineral Analyses

The effect of nodule inoculum was found to be very highly sign-
ificant when comparing initial-planting inoculations [Trts 1-4 (C2)],
delayed nodule inoculations and initial fungus inoculations [Trts 5-8
(C5)], delayed inoculations [Trts 13-16 (C11)], and all nodulated
treatments to non-nodulated treatments (Z14) for the minerals nitrogen
(N, Table 28), phosphorus (P, Table 29), potassium (K, Table 30), and
copper (Cu, Table 31). All of these elements were found in larger
quantities in treatments with live nodule inoculum compared to those
with dead nodule inoculum. For N the effect of isolate A-3 was not
significant, and interaction between inocula was not observed. Duncan's
multiple range test for N showed that all nodulated treatments were
significantly larger than all the non-nodulated treatments (Figure 22).

Significances found for P and K in the comparisons among inocula

Table 28. Statistical analyses for leaf nitrogen (mg); N

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-value	Probability >F
Model	19	108.261	5.698	11.29	0.0001***
Error	44	22.211	0.505		
Total	67	130.472			

SV	Effect	- Mean[a]	+ Mean[a]	MSS	Probability
Rep				2.606	0.1460
C1	F=Fungus	2.278	1.804	0.896	0.1705
C2	N=Nodule	0.843	3.240	22.983	0.0001***
C3	I=Interaction	2.099	1.984	0.053	0.7365
C4	F	1.845	2.170	0.421	0.3450
C5	N	0.821	3.195	22.537	0.0001***
C6	I	1.919	2.096	0.126	0.6046
C7	F	2.186	2.482	0.351	0.3881
C8	N	1.054	3.614	26.216	0.0001***
C9	I	2.200	2.468	0.286⁻	0.4359
C10	F	2.477	2.016	0.849	0.1819
C11	N	0.910	3.582	28.565	0.0001***
C12	I	2.331	2.162	0.114	0.6225
C13	Control	1.627	2.157	1.059	0.1370
C14	1-4:13-16	2.041	2.246	0.337	0.3978
C15	5-8:9-12	2.008	2.334	0.852	0.1813
C16	1-4,13-16:5-12	2.144	2.171	0.012⁻	0.8740
Z1	F	3.582	3.195	0.597	0.2616
Z2	N	3.357	3.421	0.016	0.8516
Z3	I	3.671	3.107	1.272	0.1039
Z4	F	0.916	0.779	0.075	0.6883
Z5	N	0.829	0.866	0.005	0.9146
Z6	I	0.981	0.714	0.287	0.4353
Z7	F	3.615	3.239	0.565	0.2746
Z8	N	3.420	3.433	0.001	0.9706
Z9	I	3.138	3.715	1.334	0.0960
Z10	F	1.048	0.884	0.107	0.6332
Z11	N	0.902	1.031	0.066	0.7066
Z12	I	0.893	1.039	0.085	0.6706
Z13	F	2.196	2.118	0.098	0.6472
Z14	N	0.907	3.408	100.062	0.0001***
Z15	I	2.137	2.177	0.026	0.8141

[a]Mean (+ and -) does not apply for comparisons C13-C16 (See Table 5).

Table 29. Statistical analyses for leaf phosphorus (mg); P

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-value	Probability >F
Model	19	0.24433	0.01286	6.05	0.0001***
Error	44	0.09358	0.00213		
Total	67	0.33790			

SV	Effect	- Mean[a]	+ Mean[a]	MSS	Probability
Rep				0.00619	0.3754
C1	F=Fungus	0.205	0.167	0.00580	0.0910
C2	N=Nodule	0.126	0.246	0.05793	0.0001***
C3	I=Interaction	0.184	0.188	0.00010	0.8259
C4	F	0.163	0.158	0.00012	0.8071
C5	N	0.118	0.203	0.02870	0.0004***
C6	I	0.170	0.150	0.00157	0.3746
C7	F	0.158	0.186	0.00312	0.2118
C8	N	0.117	0.227	0.04846	0.0001***
C9	I	0.153	0.191	0.00578	0.0916
C10	F	0.196	0.169	0.00305	0.2170
C11	N	0.120	0.245	0.06236	0.0001***
C12	I	0.178	0.187	0.00034	0.6762
C13	Control	0.113	0.175	0.01445	0.0090**
C14	1-4:13-16	0.186	0.182	0.00011	0.8155
C15	5-8:9-12	0.160	0.172	0.00112	0.4519
C16	1-4,13-16:5-12	0.184	0.166	0.00513	0.1113
Z1	F	0.248	0.210	0.00579	0.0912
Z2	N	0.213	0.245	0.00411	0.1533
Z3	I	0.225	0.233	0.00022	0.7375
Z4	F	0.107	0.115	0.00025	0.7196
Z5	N	0.113	0.108	0.00010	0.8189
Z6	I	0.119	0.103	0.00099	0.4793
Z7	F	0.224	0.239	0.00089	0.5017
Z8	N	0.235	0.229	0.00015	0.7830
Z9	I	0.205	0.258	0.01148	0.0190*
Z10	F	0.130	0.129	0.00001	0.9564
Z11	N	0.125	0.135	0.00041	0.6473
Z12	I	0.117	0.143	0.00277	0.2388
Z13	F	0.181	0.170	0.00186	0.3330
Z14	N	0.120	0.230	0.19358	0.0001***
Z15	I	0.171	0.179	0.00105	0.4669

[a] Mean (+ and -) does not apply for comparisons C13-C16 (See Table 5).

Table 30. Statistical analyses for leaf potassium (mg); K

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-value	Probability >F
Model	19	17.9740	0.9460	13.16	0.0001***
Error	44	3.1628	0.7188		
Total	67	21.1368			

SV	Effect	- Mean[a]	+ Mean[a]	MSS	Probability
Rep				0.14987	0.5231
C1	F=Fungus	0.839	0.722	0.05477	0.3665
C2	N=Nodule	0.247	1.313	4.54596	0.0001***
C3	I=Interaction	0.793	0.768	0.00253	0.8455
C4	F	0.665	0.727	0.01529	0.6322
C5	N	0.303	1.090	2.48000	0.0001***
C6	I	0.677	0.715	0.00573	0.7693
C7	F	0.754	0.866	0.05023	0.3869
C8	N	0.304	1.315	4.08798	0.0001***
C9	I	0.706	0.914	0.17348	0.1112
C10	F	0.928	0.856	0.02086	0.5763
C11	N	0.290	1.493	5.78848	0.0001***
C12	I	0.939	0.845	0.03479	0.4710
C13	Control	0.536	0.795	0.25094	0.0568
C14	1-4:13-16	0.780	0.892	0.09966	0.2248
C15	5-8:9-12	0.696	0.809	0.10311	0.2170
C16	1-4,13-16:5-12	0.836	0.753	0.11035	0.2018
Z1	F	1.443	1.191	0.25404	0.0553
Z2	N	1.275	1.359	0.02796	0.5179
Z3	I	1.308	1.327	0.00142	0.8841
Z4	F	0.279	0.258	0.00171	0.8729
Z5	N	0.308	0.229	0.02495	0.5412
Z6	I	0.285	0.251	0.00470	0.7906
Z7	F	1.366	1.212	0.09406	0.2380
Z8	N	1.308	1.270	0.00589	0.7662
Z9	I	1.098	1.480	0.58661	0.0045**
Z10	F	0.316	0.292	0.00236	0.8508
Z11	N	0.285	0.323	0.00565	0.7710
Z12	I	0.322	0.287	0.00489	0.7864
Z13	F	0.797	0.793	0.00024	0.9526
Z14	N	0.286	1.303	16.54343	0.0001***
Z15	I	0.779	0.811	0.01631	0.6211

[a]Mean (+ and -) does not apply for comparisons C13-C16 (See Table 5).

Table 31. Statistical analyses for leaf copper (mg); Cu

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-value	Probability >F
Model	19	722.4660	38.0245	6.19	0.0001***
Error	44	270.1248	6.1392		
Total	67	992.5908			

SV	Effect	- Mean[a]	+ Mean[a]	MSS	Probability
Rep				18.2841	0.3653
C1	F=Fungus	10.422	7.920	25.0525	0.0401*
C2	N=Nodule	5.670	12.672	196.1470	0.0001***
C3	I=Interaction	9.662	8.680	3.8563	0.4119
C4	F	7.069	8.249	5.5684	0.3249
C5	N	5.229	10.089	94.5027	0.0002***
C6	I	7.841	7.477	0.5318	0.7599
C7	F	8.010	9.577	9.8235	0.1927
C8	N	5.625	11.961	160.6113	0.0001***
C9	I	8.159	9.428	6.4427	0.2900
C10	F	8.705	7.836	3.4427	0.4673
C11	N	5.127	11.414	158.0992	0.0001***
C12	I	7.918	8.623	1.9916	0.5547
C13	Control	6.296	8.473	17.8447	0.0813
C14	1-4:13-16	9.171	8.271	6.4854	0.2884
C15	5-8:9-12	7.659	8.793	10.2876	0.1827
C16	1-4,13-16:5-12	8.721	8.226	3.9155	0.4083
Z1	F	12.356	10.714	10.7896	0.1726
Z2	N	10.915	12.155	6.1516	0.3010
Z3	I	11.938	11.131	2.6058	0.4995
Z4	F	5.057	5.455	0.6348	0.7384
Z5	N	5.170	5.342	0.1171	0.8859
Z6	I	5.887	4.625	6.3794	0.2923
Z7	F	11.020	12.048	4.2322	0.3901
Z8	N	10.589	12.479	14.2903	0.1176
Z9	I	10.113	12.955	32.3221	0.0205*
Z10	F	5.695	5.443	0.2538	0.8327
Z11	N	5.186	5.953	2.3555	0.5207
Z12	I	4.966	6.172	5.8190	0.3143
Z13	F	8.552	8.395	0.3903	0.7934
Z14	N	5.413	11.534	599.6009	0.0001***
Z15	I	8.395	8.552	0.3947	0.7923

[a]Mean (+ and -) does not apply for comparisons C13-C16 (See Table 5).
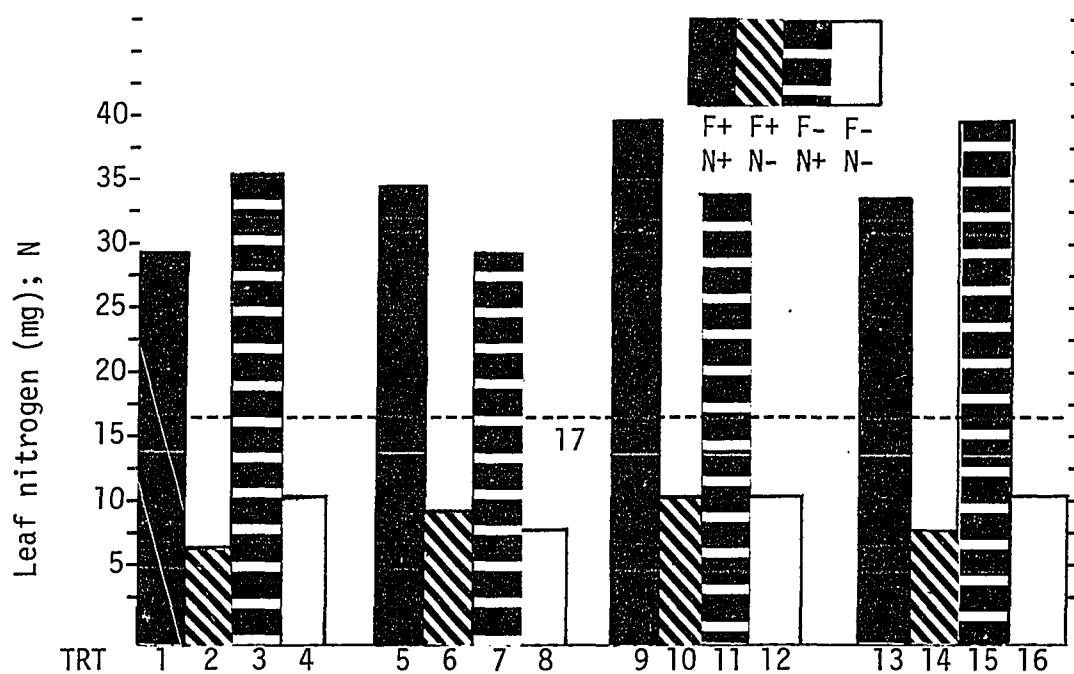
TRT [1]		Mean
15	a	38.971 mg
9	a	38.957
3	a	35.338
5	a	34.454
11	a	33.323
13	a	32.678
1	a	29.454
7	a	29.437
17	b	16.270
10	b	10.684
16	b	10.562
12	b	10.394
4	b	10.216
6	b	8.944
14	b	7.640
8	b	7.473
2	b	6.635

[1] Values in each vertical column followed by the same letters do not differ significantly at p = 0.05 by Duncan's multiple range test.

Figure 22. Histogram and treatment means for leaf nitrogen (mg); N

at a time and among times within an inoculum (C and Z) were similar with one exception. For P the control (Trt 17) had a significantly lower quantity than the rest of the treatments (C13, Table 29). In the Duncan's multiple range test the values of P in the checks (Trts 4, 8, 12, and 16) were not significantly different from the control (Trt 17) as would be expected (Figure 23). Therefore, the significance found in C13 was attributed to the influence of the nodulated treatments.

A significant interaction for P and K was found when comparing live nodule inoculum and dead isolate A-3 inoculations [Trts 3, 7, 11, 15 (Z9), Tables 29 and 30]. There was no apparent explanation for the significance found in this time of inoculation comparison.

There was considerable similarity between P and K in the C and Z comparisons. The Duncan's multiple range tests for P and K showed a similarity where treatments receiving live nodule inoculum had larger means than those receiving dead nodule inoculum (Figures 23 and 24). However, the rank of the treatment means for P and K were not compatible. Some of the live nodule inoculum treatment means for P did not differ significantly from some of the dead nodule inoculum treatment means. For K all treatments with live nodule inoculum were significantly larger than those with dead nodule inoculum.

The effect of fungal inoculum was found to be significant for Cu when comparing initial-planting inoculations [Trts 1-4 (C1), Table 31]. For Cu treatments with live isolate A-3 were significantly less than the treatments with dead isolate A-3. The Duncan's multiple range test for Cu showed that seven of the eight treatments with live nodule inoculum

TRT		Mean	
3	a[1]	2.628	mg
9	a	2.602	
15	a	2.540	
13	a	2.356	
1	a	2.296	
7	ab	2.153	
11	abc	1.942	
5	abc	1.901	
4	bcd	1.473	
16	cd	1.384	
6	cd	1.252	
12	cd	1.222	
17	d	1.133	
10	d	1.121	
8	d	1.108	
2	d	1.044	
14	d	1.015	

[1] Values in each vertical column followed by the same letters do not differ significantly at p = 0.05 by Duncan's multiple range test.

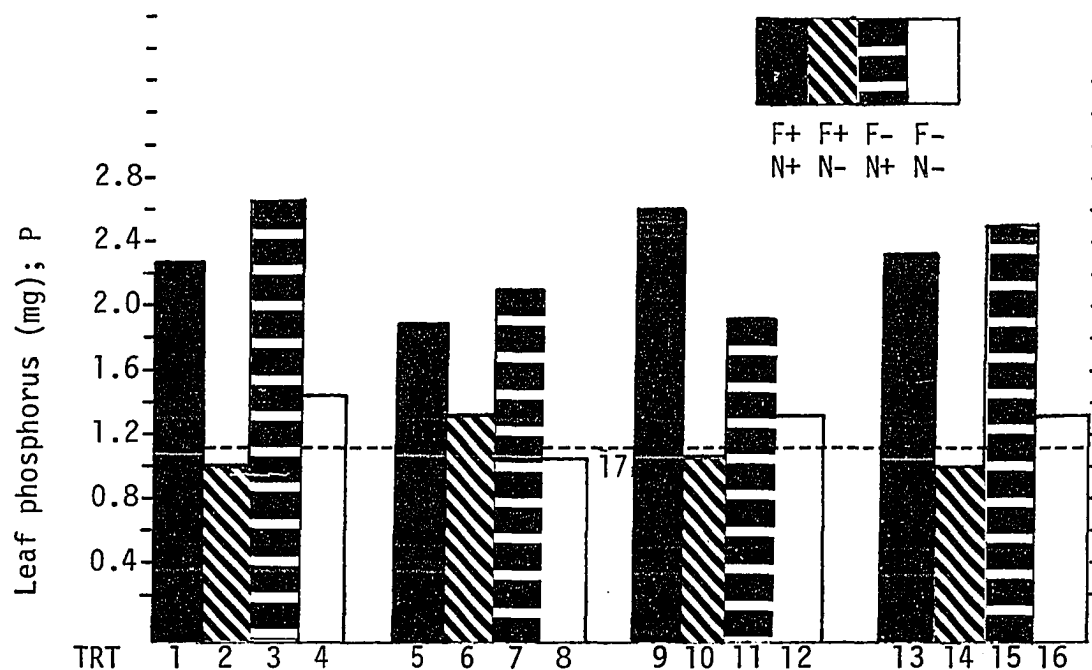Figure 23. Histogram and treatment means for leaf phosphorus (mg); P
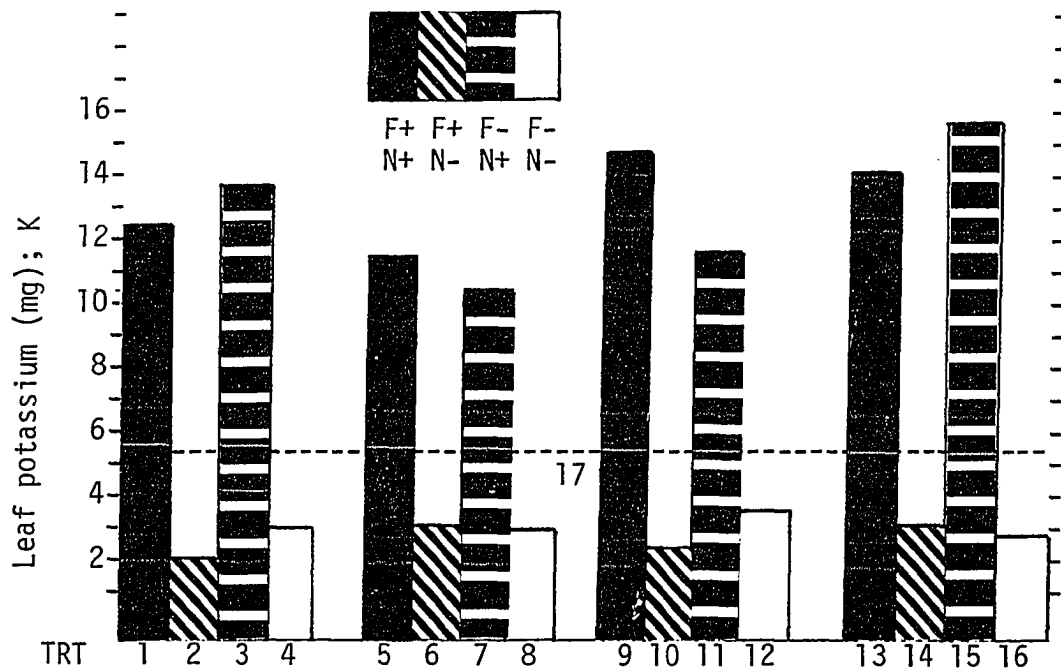
TRT	Mean
15 a[1]	15.762 mg
9 ab	14.755
13 ab	14.107
3 ab	13.845
1 ab	12.423
11 ab	11.552
5 ab	11.399
7 b	10.402
17 c	5.365
12 c	3.525
6 c	3.146
14 c	3.010
4 c	2.933
8 c	2.907
16 c	2.800
10 c	2.563
2 c	2.014

[1]Values in each vertical column followed by the same letters do not differ significantly at p = 0.05 by Duncan's multiple range test.

Figure 24. Histogram and treatment means for leaf potassium (mg); K

were significantly larger than treatments with dead nodule inoculum ·
(Figure 25).

Iron (Fe) and aluminum (Al), absorbed as trivalent cations, were
found to be extremely highly correlated (r = 0.98, Table 32). For both
minerals most of the comparisons that were significant were probably
so because of a single outlier value. A very high value for delayed
live nodule inoculum and dead isolate A-3 (Trt 15) produced very
highly significant difference when comparing delayed inoculation
treatments [Trts 13-16 (C11)] for both variables (Tables 33 and 34).
This high value also accounted for the significant interaction for
Al (C12). For Fe when comparing treatments with live nodule inoculum
and dead fungus in all time combinations (Trts 3, 7, 11, and 15)
the effects of isolate A-3 (Z7) and nodule inoculum (Z8) were found
to be significant, and the interaction (Z9) was found to be highly
significant. For Al comparisons Z7 and Z9 were found to be significant.
The significance for Z7 and probabily Z9 for both variables was
attributed to treatment 15. Duncan's multiple range tests for both
variables showed that treatment 15 mean was significantly larger than
all other treatments (Figures 26 and 27). Most nodulated treatments
had larger means than most non-nodulated treatments, but they were not
significantly different. The significance observed with nodulated
treatments compared to non-nodulated treatments (Z14) for both
minerals was again based on a high value for treatment 15.

For calcium (Ca) the effect of live isolate A-3 was found to be
very highly significant when comparing initial-planting inoculations

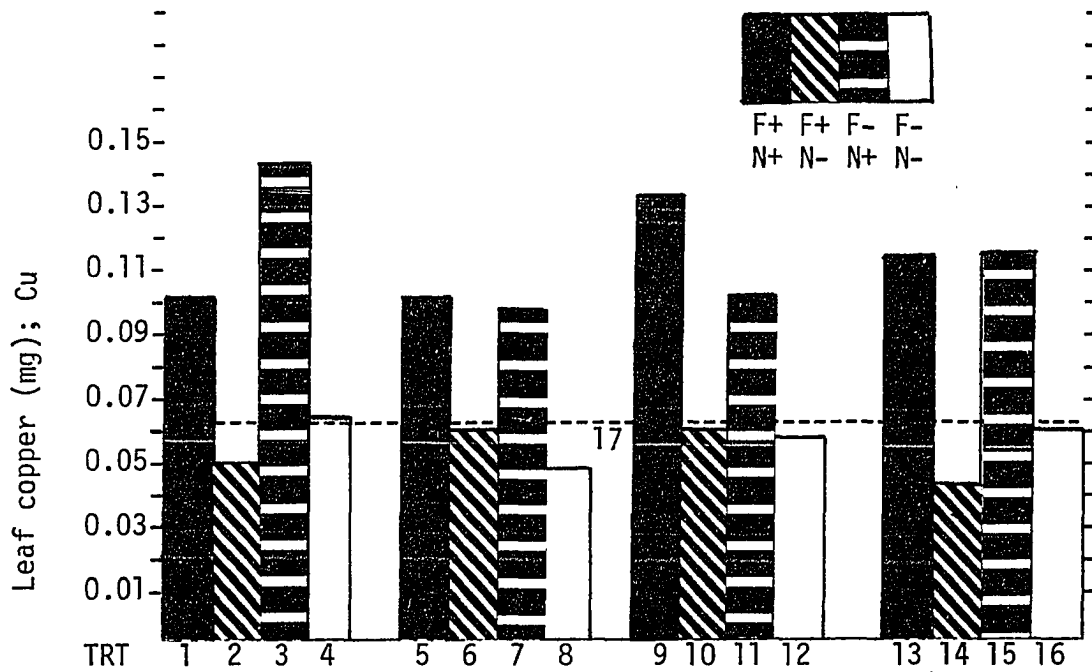| TRT		Mean	
3	a[1]	0.144	mg
9	ab	0.134	
15	ab	0.115	
13	ab	0.113	
1	ab	0.109	
11	ab	0.105	
5	ab	0.105	
7	bc	0.097	
4	cd	0.064	
17	cd	0.063	
6	cd	0.060	
16	cd	0.059	
10	cd	0.058	
12	d	0.055	
2	d	0.049	
8	d	0.046	
14	d	0.043	

[1] Values in each vertical column followed by the same letters do not differ significantly at p = 0.05 by Duncan's multiple range test.

Figure 25. Histogram and treatment means for leaf copper (mg); Cu

Table 32. Partial correlation coefficients and probability levels for relationships between mean mineral values determined from data collected at time of harvest

Variable	N	P	K	Ca	Mg	B
N	1.00[a]	0.684 0.0001[b]	0.865 0.0001	0.720 0.0001	0.642 0.0001	0.683 0.0001
P		1.00	0.705 0.0001	0.687 0.0001	0.624 0.0001	0.686 0.0001
K			1.00	0.678 0.0001	0.646 0.0001	0.675 0.0001
Ca				1.00	0.846 0.0001	0.766 0.0001
Mg					1.00	0.721 0.0001
B						1.00
Fe						
Al						
Cu						
Sr						
Ba						
Na						

[a]R value.

[b]Probability level.

Variable	Fe	Al	Cu	Sr	Ba	Na
N	0.391 0.0055	0.358 0.0116	0.728 0.0001	0.724 0.0001	0.739 0.0001	0.378 0.0075
P	0.313 0.0288	0.273 0.0570	0.606 0.0001	0.628 0.0001	0.690 0.0001	0.196 0.1767
K	0.381 0.0070	0.339 0.0171	0.723 0.0001	0.742 0.0001	0.743 0.0001	0.413 0.0031
Ca	0.299 0.0371	0.276 0.0546	0.591 0.0001	0.821 0.0001	0.763 0.0001	0.115 0.4303
Mg	0.236 0.1027	0.196 0.1770	0.491 0.0003	0.605 0.0001	0.639 0.0001	0.116 0.4261
B	0.199 0.1714	0.169 0.2459	0.614 0.0001	0.724 0.0001	0.705 0.0001	0.157 0.2799
Fe	1.00	0.982 0.0001	0.429 0.0021	0.351 0.0133	0.625 0.0001	0.378 0.0075
Al		1.00	0.403 0.0041	0.350 0.0138	0.615 0.0001	0.339 0.0173
Cu			1.00	0.742 0.0001	0.702 0.0001	0.289 0.0441
Sr				1.00	0.823 0.0001	0.327 0.0216
Ba					1.00	0.328 0.0213
Na						1.00

Table 33. Statistical analyses for leaf iron (mg); Fe

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-value	Probability >F
Model	19	867165.852	45640.308	2.18	0.0078**
Error	44	919560.521	20899.102		
Total	67	1786726.373			

SV	Effect	- Mean[a]	+ Mean[a]	MSS	Probability
Rep				45636.65	0.5032
C1	F=Fungus	247.784	223.424	2373.74	0.7264
C2	N=Nodule	182.414	288.794	45267.24	0.1308
C3	I=Interaction	245.556	225.652	1584.60	0.7749
C4	F	224.803	294.599	19486.21	0.3183
C5	N	237.280	282.122	8042.95	0.5201
C6	I	279.254	240.148	6117.27	0.5747
C7	F	231.904	256.325	2385.59	0.7257
C8	N	224.283	263.947	6293.09	0.5692
C9	I	258.755	229.475	3429.16	0.6741
C10	F	344.115	222.412	59247.21	0.0850
C11	N	95.247	471.280	565602.52	0.0001***
C12	I	350.341	216.186	71990.52	0.0585
C13	Control	207.631	255.671	8688.13	0.5039
C14	1-4:13-16	235.604	283.264	18171.33	0.3350
C15	5-8:9-12	259.701	244.115	1943.39	0.7515
C16	1-4,13-16:5-12	259.434	251.908	906.27	0.8287
Z1	F	302.434	282.064	1659.71	0.7698
Z2	N	320.408	264.090	12686.98	0.4198
Z3	I	279.492	305.006	2603.91	0.7140
Z4	F	176.303	235.959	14235.17	0.3930
Z5	N	196.602	215.659	1452.66	0.7842
Z6	I	271.432	140.829	68228.71	0.0652
Z7	F	432.793	288.851	82876.48	0.0429*
Z8	N	432.993	288.651	83338.02	0.0423*
Z9	I	266.576	455.068	142116.37	0.0090**
Z10	F	143.227	183.736	6563.84	0.5611
Z11	N	135.925	191.037	12149.44	0.4297
Z12	I	190.130	136.832	11362.92	0.4450
Z13	F	262.152	249.190	2688.06	0.7096
Z14	N	184.806	326.536	321397.15	0.0002***
Z15	I	283.476	227.865	49481.67	0.1146

[a]Mean (+ and -) does not apply for comparisons C13-C16 (See Table 5).

Table 34. Statistical analyses for leaf aluminum (mg); Al

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-value	Probability >F
Model	19	1103971.12	58103.74	1.98	0.0161*
Error	44	1290838.43	29337.24		
Total	67	2394809.54			

SV	Effect	- Mean[a]	+ Mean[a]	MSS	Probability
Rep				134632.02	0.1862
C1	F=Fungus	243.304	237.759	122.98	0.9464
C2	N=Nodule	190.457	290.605	40118.79	0.2279
C3	I=Interaction	244.457	236.606	246.56	0.9241
C4	F	204.388	281.465	23762.99	0.3519
C5	N	223.522	262.331	6024.55	0.6381
C6	I	259.349	226.504	4315.24	0.6905
C7	F	241.460	248.118	177.32	0.9356
C8	N	231.251	258.328	2932.66	0.7427
C9	I	266.458	223.120	7512.47	0.5996
C10	F	372.237	222.989	89100.16	0.0750
C11	N	100.481	494.746	621779.56	0.0001***
C12	I	384.406	210.820	120529.09	0.0395*
C13	Control	190.362	256.465	16450.04	0.4380
C14	1-4:13-16	240.531	297.613	26066.67	0.3298
C15	5-8:9-12	242.926	244.789	27.75	0.9745
C16	1-4,13-16:5-12	269.072	243.858	10172.26	0.5414
Z1	F	286.658	284.177	24.62	0.9760
Z2	N	308.887	261.948	8813.31	0.5697
Z3	I	262.217	308.618	8612.07	0.5741
Z4	F	184.449	235.046	10240.43	0.5401
Z5	N	195.566	223.929	3217.84	0.7309
Z6	I	267.365	152.130	53116.88	0.1663
Z7	F	466.415	268.759	156270.98	0.0198*
Z8	N	448.189	286.985	103946.76	0.0551
Z9	I	258.441	476.733	190605.37	0.0105*
Z10	F	147.282	178.933	4006.98	0.7012
Z11	N	128.436	197.778	19233.32	0.4019
Z12	I	187.407	138.808	9447.60	0.5562
Z13	F	265.347	247.583	5049.36	0.6667
Z14	N	186.428	326.502	313935.39	0.0013**
Z15	I	288.668	224.262	66368.13	0.1228

[a]Mean (+ and -) does not apply for comparisons C13-C16 (See Table 5).
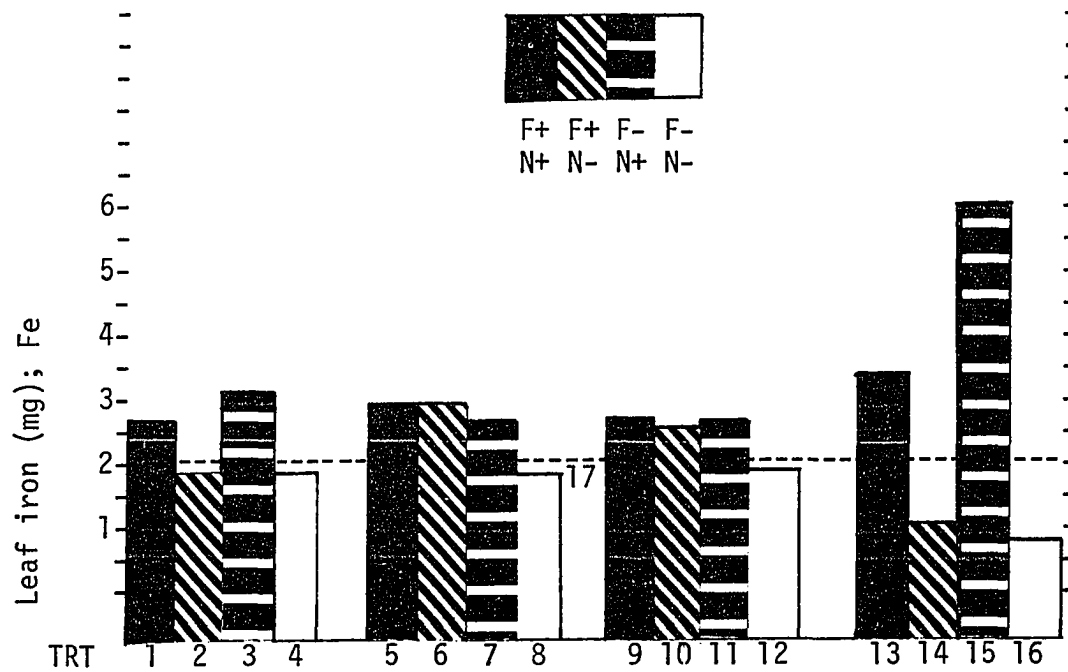
TRT[1]		Mean
15	a	5.992 mg
13	b	3.434
3	bc	3.109
5	bc	2.975
6	bc	2.917
7	bc	2.668
1	bc	2.667
11	bc	2.664
9	bc	2.615
10	bc	2.513
17	bc	2.076
12	bc	1.974
4	bc	1.846
8	bc	1.828
2	bc	1.802
14	bc	1.015
16	c	0.890

[1] Values in each vertical column followed by the same letters do not differ significantly at p = 0.05 by Duncan's multiple range test.

Figure 26. Histogram and treatment means for leaf iron (mg); Fe
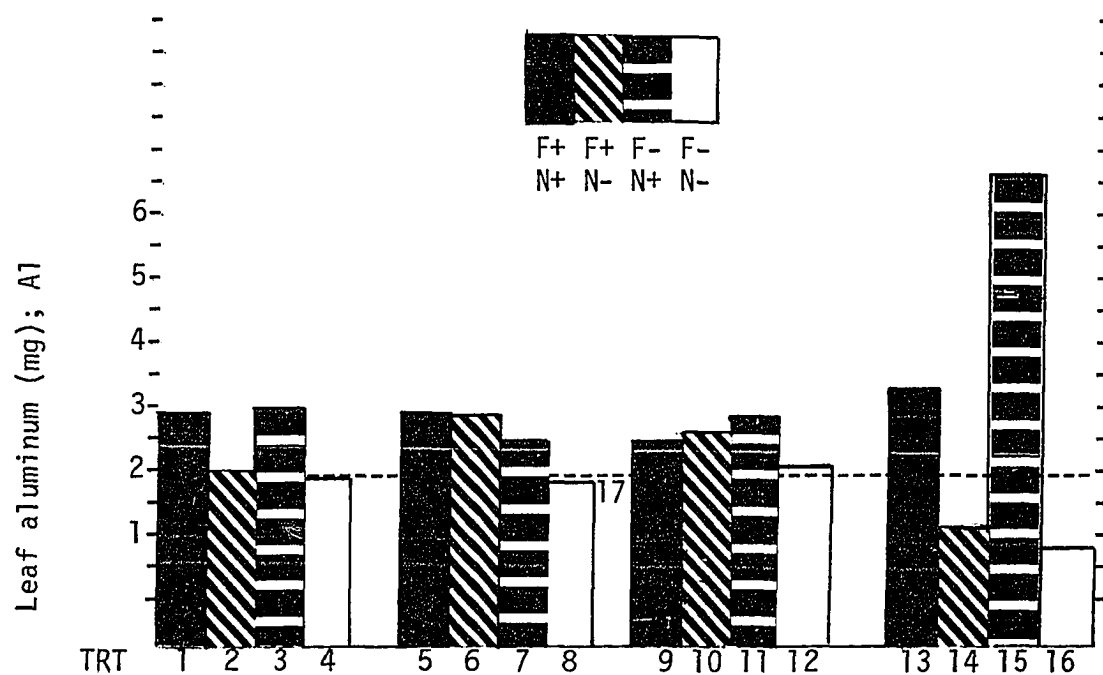
TRT	Mean
15 a[1]	6.562 mg
13 b	3.333
3 b	2.973
5 b	2.844
1 b	2.839
6 b	2.785
11 b	2.767
10 b	2.562
7 b	2.402
9 b	2.399
12 b	2.062
2 b	1.916
17 b	1.904
4 b	1.893
8 b	1.686
14 b	1.126
16 b	0.883

[1] Values in each vertical column followed by the same letters do not differ significantly at p = 0.05 by Duncan's multiple range test.

Figure 27. Histogram and treatment means for leaf aluminum (mg); Al

[Trts 1-4 (C1)] and all mycorrhizal to all non-mycorrhizal treatments (Z13, Table 35). Significant difference was observed when comparing delayed inoculations [Trts 13-16 (C10)]. In the C1, C10, and Z13 comparisons treatments with live isolate A-3 had significantly lower means than treatments with dead isolate A-3. When comparing treatments with both live inocula, treatments receiving delayed live isolate A-3 were significantly larger than those receiving the fungus at initial planting (Z1).

For Ca the effect of nodule inoculum was found to be very highly significant when comparing initial-planting inoculations [Trts 1-4 (C2)], delayed inoculations [Trts 13-16 (C11)], and all nodulated treatments to non-nodulated treatments (Z14). The effect of nodule inoculum was found to be highly significant when comparing initial-planting nodule inoculations and delayed fungal inoculations [Trts 9-12 (C8)]. In all the above comparisons (C2, C8, C11, and Z14) treatment means with live nodule inoculum were greater than those with dead nodule inoculum.

Highly significant interactions were observed when comparing initial-planting fungus inoculations and delayed nodule inoculations [Trts 5-8 (C6)] and treatments with either all dead or all living inocula (Trts 1, 4, 5, 8, 9, 12, 13, and 16) to the remaining treatments (Z15). Significant interactions were observed when comparing treatments with dead nodule inoculum and living fungus [Trts 2, 6, 10, 14 (Z6)] and treatments with living nodule inoculum and dead fungus [Trts 3, 7, 11, 15 (Z9)]. It was not known why the control (Trt 17) had a significantly higher value than the mean of the remaining treatments (C13).

Table 35. Statistical analyses for leaf calcium (mg); Ca

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-value	Probability >F
Model	19	24.1245	1.2697	5.92	0.0001***
Error	44	9.4313	0.2144		
Total	67	33.5559			

| SV	Effect	- Mean^a	+ Mean^a	MSS	Probability
Rep				1.4442	0.0749
C1	F=Fungus	2.145	1.232	3.3367	0.0001***
C2	N=Nodule	1.255	2.122	3.0079	0.0003***
C3	I=Interaction	1.908	1.470	0.7672	0.0539
C4	F	1.828	1.779	0.0102	0.8210
C5	N	1.581	2.027	0.7928	0.0502
C6	I	2.151	1.457	1.9226	0.0030**
C7	F	2.065	1.870	0.1524	0.3829
C8	N	1.582	2.353	2.3769	0.0011**
C9	I	1.938	1.998	0.0145	0.7873
C10	F	2.118	1.669	0.8081	0.0481*
C11	N	1.253	2.534	6.5621	0.0001***
C12	I	2.112	1.675	0.7620	0.0547
C13	Control	2.464	1.838	1.4731	0.0086**
C14	1-4:13-16	1.689	1.893	0.3352	0.1977
C15	5-8:9-12	1.804	1.968	0.2143	0.3016
C16	1-4,13-16:5-12	1.792	1.886	0.1444	0.3956
Z1	F	2.188	1.551	1.6257	0.0060**
Z2	N	1.873	1.866	0.0002	0.9761
Z3	I	1.970	1.769	0.1626	0.3675
Z4	F	1.351	1.460	0.0481	0.6232
Z5	N	1.575	1.236	0.4588	0.1331
Z6	I	1.679	1.132	1.1967	0.0172*
Z7	F	2.699	2.598	0.0404	0.6523
Z8	N	2.688	2.609	0.0246	0.7253
Z9	I	2.410	2.887	0.9125	0.0362*
Z10	F	1.485	1.376	0.0469	0.6274
Z11	N	1.260	1.601	0.4668	0.1298
Z12	I	1.485	1.376	0.0475	0.6251
Z13	F	2.040	1.637	2.5869	0.0007***
Z14	N	1.418	2.259	11.3167	0.0001***
Z15	I	2.027	1.650	2.2728	0.0014**

^{a}Mean (+ and -) does not apply for comparisons C13-C16 (See Table 5).

The treatments receiving live nodule inoculum and dead isolate A-3 had the highest treatment mean for Ca within each 2 x 2 factorial (Figure 28). Therefore, the effect of live nodule inoculum appeared to have enhanced total Ca uptake. However, there was an indication that the effect of the live isolated A-3 inhibited Ca uptake even in the presence of live nodule inoculum. This might have accounted for the significant interactions obtained. The connection between live isolate A-3 and decreased Ca uptake is not known.

For magnesium (Mg) the effect of isolate A-3 was found to be very highly significant when comparing initial-planting inoculations [Trts 1-4 (C1)] and significant when comparing treatments with all living inocula [Trts 1, 5, 9, 13 (Z1), Table 36]. Treatments receiving live isolate A-3 had a smaller treatment mean than those receiving dead isolate A-3 in the C1 comparison. In the Z1 comparison the treatments receiving live isolate A-3 initially produced a lower mean than those receiving isolate A-3 after 2 mo.

The effect of live nodule inoculum was found to be very highly significant when comparing initial-planting inoculations [Trts 1-4 (C2)], initial-planting nodule inoculations and delayed fungus inoculations [Trts 9-12 (C8)], both symbionts delayed inoculations [Trts 13-16 (C11)], and all nodulated treatments to all non-nodulated treatments (Z14). In all the above comparisons (C2, C8, C11, and Z14) the live nodule inoculum treatment means were larger than the dead nodule inoculum means,

Highly significant interaction was observed when comparing initial-planting fungal inoculations and delayed nodule inoculations [Trts 5-8
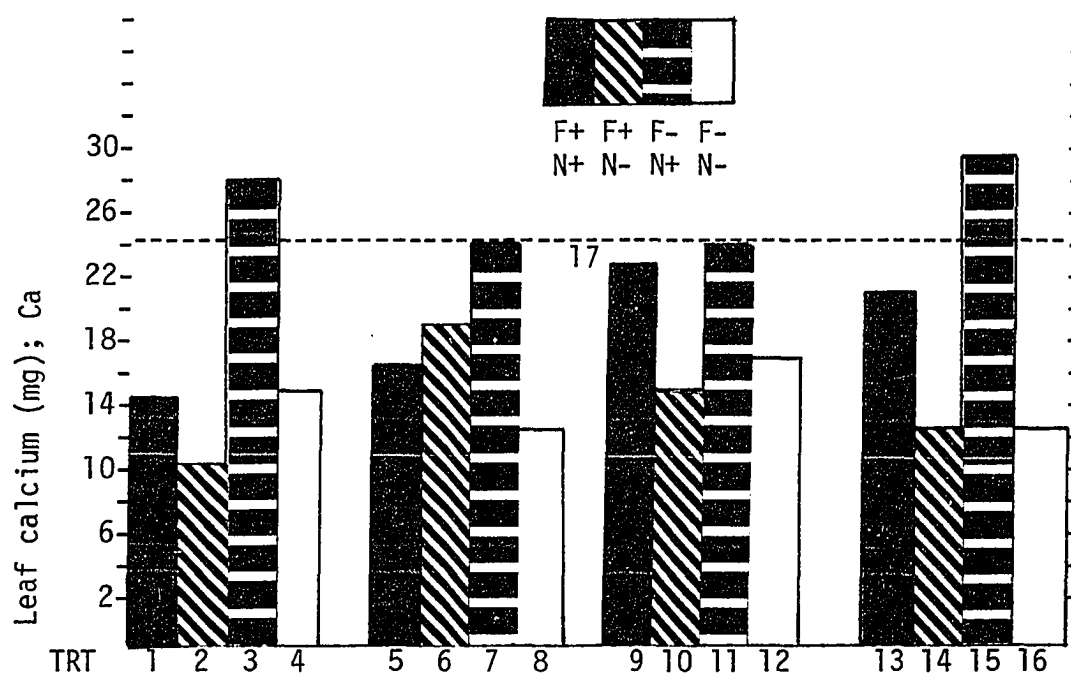
TRT [1]	Mean
15 a	29.767 mg
3 ab	27.979
17 abc	24.640
11 abc	24.207
7 abcd	23.986
9 abcd	22.857
13 bcde	20.908
6 cdef	19.030
12 cdefg	17.100
5 defg	16.549
4 efg	14.927
10 efg	14.547
1 efg	14.466
8 fg	12.601
16 fg	12.594
14 fg	12.464
2 g	10.174

[1] Values in each vertical column followed by the same letters do not differ significantly at p = 0.05 by Duncan's multiple range test.

Figure 28. Histogram and treatment means for leaf calcium (mg); Ca

Table 36. Statistical analyses for leaf magnesium (mg); Mg

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-value	Probability >F
Model	19	3.4382	0.1810	4.87	0.0001***
Error	44	1.6364	0.0372		
Total	67	5.0747			

| SV	Effect	- Mean[a]	+ Mean[a]	MSS	Probability
Rep				0.1917	0.1465
C1	F=Fungus	0.806	0.477	0.4316	0.0009***
C2	N=Nodule	0.457	0.826	0.5436	0.0002***
C3	I=Interaction	0.718	0.566	0.0923	0.1063
C4	F	0.642	0.728	0.0293	0.3582
C5	N	0.613	0.757	0.0833	0.1246
C6	I	0.829	0.541	0.3322	0.0030**
C7	F	0.646	0.652	0.0001	0.9488
C8	N	0.471	0.827	0.5089	0.0003***
C9	I	0.650	0.648	0.0001	0.9853
C10	F	0.732	0.615	0.0554	0.2082
C11	N	0.408	0.939	1.1261	0.0001***
C12	I	0.704	0.643	0.0150	0.5110
C13	Control	0.710	0.662	0.0086	0.6178
C14	1-4:13-16	0.642	0.673	0.0081	0.6284
C15	5-8:9-12	0.685	0.649	0.0104	0.5825
C16	1-4,13-16:5-12	0.658	0.667	0.0015	0.8371
Z1	F	0.839	0.621	0.1909	0.0220*
Z2	N	0.753	0.708	0.0081	0.6279
Z3	I	0.743	0.718	0.0025	0.7857
Z4	F	0.427	0.585	0.0990	0.0949
Z5	N	0.590	0.422	0.1130	0.0749
Z6	I	0.637	0.374	0.2762	0.0065**
Z7	F	0.927	0.962	0.0051	0.7010
Z8	N	0.943	0.946	0.0001	0.9799
Z9	I	0.842	1.047	0.1686	0.0309*
Z10	F	0.452	0.459	0.0047	0.7128
Z11	N	0.431	0.506	0.0225	0.4210
Z12	I	0.447	0.491	0.0079	0.6315
Z13	F	0.707	0.618	0.1258	0.0607
Z14	N	0.487	0.837	1.9607	0.0001***
Z15	I	0.725	0.599	0.2530	0.0090**

[a]Mean (+ and -) does not apply for comparisons C13-C16 (See Table 5).

(C6), treatments with dead nodule inoculum and live fungus in all time combinations [Trts 2, 6, 10, 14 (Z6)], and treatments with either all dead or all living inocula in all time combinations (Trts 1, 4, 5, 8, 9, 12, 13, and 16) to the remaining treatments (Z15). Significant interaction was observed when comparing treatments with living nodule and dead fungus inoculations in all time combinations [Trts 3, 7, 11, 15 (Z9)].

For Mg three of the four treatments receiving live nodule inoculum and dead isolate A-3 had the largest treatment means within each 2 x 2 factorial (Figure 29). Initial live nodule inoculum and delayed dead fungus inoculation (Trt 11) was slightly less than the companion treatment (9), where live isolate A-3 was added after 2 mo. The effect of live nodule inoculum enhanced Mg uptake. The effect of live isolate A-3 appeared to inhibit Mg uptake even in the presence of live nodule inoculum. This would account for the significant interactions observed in the Mg comparisons. The negative effect of live isolate A-3 is not known.

The effect of isolate A-3 for boron (B) was found to be highly significant when comparing initial-planting inoculations [Trts 1-4 (C1)] and significant when comparing all mycorrhizal treatments to all non-mycorrhizal treatments (Z13, Table 37). In both comparisons (C1 and Z13) the live isolate A-3 treatment means were lower than the dead isolate A-3 treatment means.

The effect of nodule inoculum for B was found to be very highly significant when comparing all nodulated treatments to non-nodulated
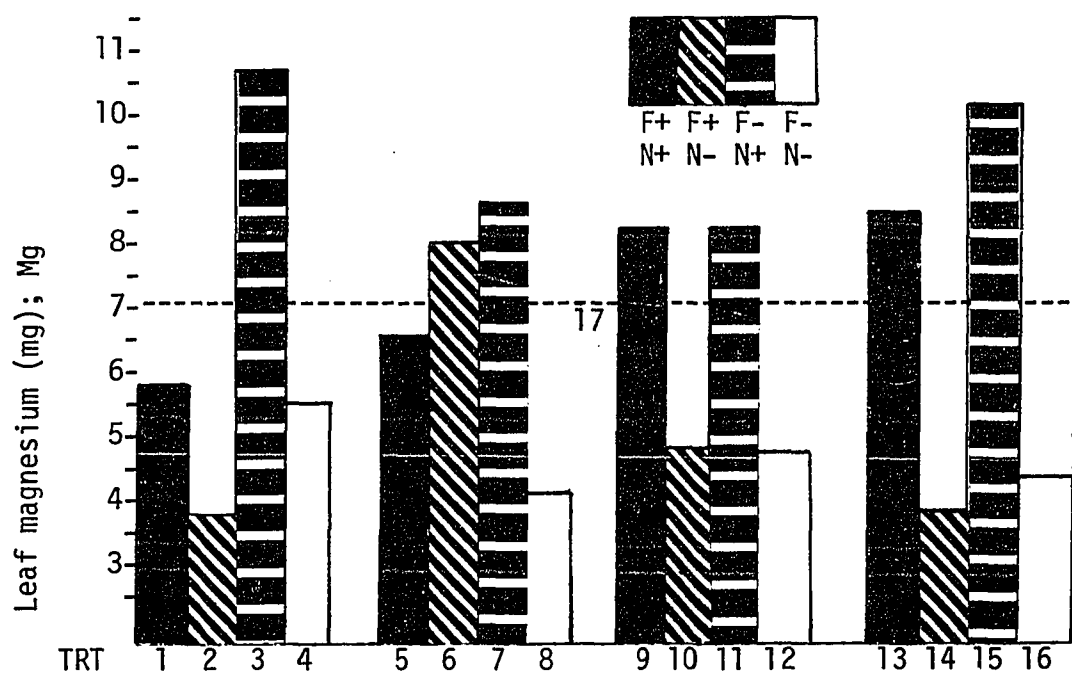
TRT		Mean
3	a[1]	10.662 mg
15	a	10.282
7	ab	8.586
13	ab	8.493
9	ab	8.295
11	ab	8.253
6	ab	8.000
17	bc	7.102
5	bcd	6.561
1	bcd	5.858
4	bcd	5.456
10	cd	4.746
12	cd	4.669
16	cd	4.365
8	cd	4.261
14	d	3.799
2	d	3.691

[1] Values in each vertical column followed by the same letters do not differ significantly at p = 0.05 by Duncan's multiple range test.

Figure 29. Histogram and treatment means for leaf magnesium (mg); Mg

Table 37. Statistical analyses for leaf boron (mg); B

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-value	Probability >F
Model	19	14139.039	744.160	1.73	0.0383*
Error	44	18887.812	429.264		
Total	67	33026.851			

SV	Effect	- Mean^a	+ Mean^a	MSS	Probability
Rep				736.655	0.6030
C1	F=Fungus	86.584	59.870	2854.578	0.0097**
C2	N=Nodule	62.460	83.993	1854.745	0.0349*
C3	I=Interaction	77.348	69.105	271.763	0.4101
C4	F	63.097	66.480	45.786	0.7345
C5	N	54.201	75.376	1793.480	0.0379*
C6	I	69.919	59.658	421.173	0.3061
C7	F	73.076	69.500	51.133	0.7201
C8	N	62.178	80.398	1327.928	0.0724
C9	I	66.122	76.455	427.197	0.3027
C10	F	76.083	62.290	760.918	0.1708
C11	N	55.833	82.541	2853.295	0.0097**
C12	I	71.656	66.717	97.590	0.6208
C13	Control	67.723	69.622	13.589	0.8534
C14	1-4:13-16	73.227	69.187	130.581	0.5673
C15	5-8:9-12	64.788	71.288	337.994	0.3587
C16	1-4,13-16:5-12	71.207	68.038	160.633	0.5259
Z1	F	78.476	69.226	342.287	0.3557
Z2	N	72.556	75.146	26.848	0.7951
Z3	I	77.857	69.845	256.875	0.4232
Z4	F	53.315	57.124	58.038	0.7026
Z5	N	56.215	54.224	15.854	0.8418
Z6	I	58.123	52.315	134.926	0.5609
Z7	F	84.463	90.143	129.072	0.5695
Z8	N	85.361	89.245	60.365	0.6970
Z9	I	77.917	96.689	1409.627	0.0644
Z10	F	64.696	59.537	106.451	0.6054
Z11	N	53.819	70.414	1101.642	0.1008
Z12	I	58.256	65.977	238.502	0.4401
Z13	F	74.710	64.535	1656.378	0.0457*
Z14	N	58.668	80.577	7680.178	0.0001***
Z15	I	71.261	67.984	171.843	0.5119

^{a}Mean (+ and -) does not apply for comparisons C13-C16 (See Table 5).

treatments (Z14), highly significant when comparing delayed inoculations [Trts 13-16 (C11)], and significant when comparing initial-planting inoculations [Trts 1-4 (C2)] and treatments with initial fungus and delayed nodule inoculations [Trts 5-8 (C5)]. In all of the above comparisons the live nodule inoculum treatment means were larger than the dead nodule inoculum treatment means.

The Duncan's multiple range test showed very little significant difference among individual treatment means (Figure 30). However, some trends were noted. Three of the four largest treatment means had live nodule inoculum and dead isolate A-3. Three of the four smallest means had live isolate A-3 and dead nodule inoculum.

The effect of isolate A-3 for strontium (Sr) was found to be very highly significant for comparisons C1 and Z13, highly singificant for comparisons C10 and Z1, and significant for comparisons C4 and C7 (Table 38). The effect of isolate A-3 for barium (Ba) was found to be significant for comparison C10, highly significant for comparisons Z1 and Z13, and very highly significant for comparison C1 (Table 39). For both minerals means of treatments for isolate A-3 were lower than means of treatments for dead isolate A-3.

The effect of nodule inoculum for both Sr and Ba was found to be very highly significant for comparisons C2, C11, and Z14, and highly significant for comparisons C5 and C8. All live nodule inoculum comparison means were larger than dead nodule inoculum means.

Interaction between the two inocula for Sr was found to be very highly significant for comparison Z15, highly significant for comparisons
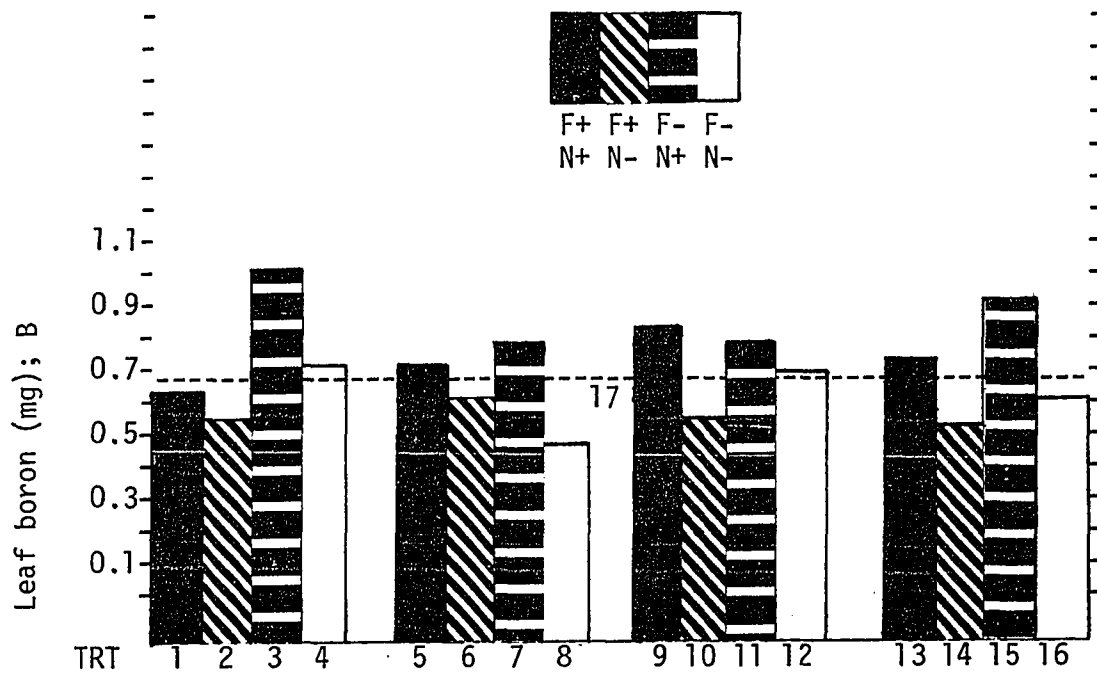
TRT		Mean
3	a[1]	1.015 mg
15	ab	0.919
9	abc	0.837
7	abcd	0.788
11	abcd	0.770
13	abcd	0.732
5	abcd	0.719
4	abcd	0.717
12	abcd	0.691
17	abcd	0.677
1	bcd	0.665
6	bcd	0.610
16	bcd	0.603
10	cd	0.552
2	cd	0.532
14	cd	0.514
8	d	0.474

[1] Values in each vertical column followed by the same letters do not differ significantly at p = 0.05 by Duncan's multiple range test.

Figure 30. Histogram and treatment means for leaf boron (mg); B

Table 38. Statistical analyses for leaf strontium (mg); Sr

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-value	Probability >F
Model	19	5800.440	305.286	7.37	0.0001***
Error	44	1823.577	41.455		
Total	67	7624.018			

SV	Effect	- Mean[a]	+ Mean[a]	MSS	Probability
Rep				99.797	0.4603
C1	F=Fungus	31.598	16.431	920.197	0.0001***
C2	N=Nodule	18.480	29.550	490.169	0.0008***
C3	I=Interaction	27.089	20.940	151.210	0.0517
C4	F	30.822	23.313	225.555	0.0186*
C5	N	21.702	32.433	460.639	0.0011**
C6	I	30.917	23.218	237.068	0.0160*
C7	F	32.504	26.169	160.542	0.0453*
C8	N	24.668	34.004	348.681	0.0039**
C9	I	30.178	28.494	11.337	0.5874
C10	F	34.186	24.988	338.450	0.0045**
C11	N	19.573	39.601	1604.483	0.0001***
C12	I	33.885	25.289	295.548	0.0076**
C13	Control	33.506	27.501	135.745	0.0648
C14	1-4:13-16	24.014	29.587	248.439	0.0138*
C15	5-8:9-12	27.067	29.336	41.182	0.3030
C16	1-4,13-16:5-12	26.801	28.202	31.401	0.3678
Z1	F	30.350	21.860	288.269	0.0083**
Z2	N	27.767	24.443	44.176	0.2863
Z3	I	27.412	24.798	27.337	0.4005
Z4	F	20.807	17.883	34.193	0.3475
Z5	N	20.534	18.156	22.620	0.4441
Z6	I	22.069	16.621	118.734	0.0834
Z7	F	43.256	40.122	39.288	0.3143
Z8	N	44.268	39.111	106.379	0.1008
Z9	I	39.025	44.353	113.529	0.0903
Z10	F	23.434	22.298	5.163	0.7140
Z11	N	20.741	24.991	72.271	0.1742
Z12	I	24.300	21.432	32.916	0.3566
Z13	F	32.278	22.725	1460.033	0.0001***
Z14	N	21.106	33.897	2617.921	0.0001***
Z15	I	30.517	24.486	582.070	0.0003***

[a]Mean (+ and-) does not apply for comparisons C13-C16 (See Table 5).

Table 39. Statistical analyses for leaf barium (mg); Ba

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-value	Probability >F
Model	19	2423.388	127.547	5.25	0.0001***
Error	44	1068.112	24.275		
Total	67	3491.500			

| SV	Effect	- Mean^a	+ Mean^a	MSS	Probability
Rep				25.785	0.7634
C1	F=Fungus	21.703	13.146	292.923	0.0007***
C2	N=Nodule	12.811	22.038	340.550	0.0003***
C3	I=Interaction	19.248	15.601	53.217	0.1286
C4	F	19.721	16.110	52.175	0.1323
C5	N	14.636	21.195	172.102	0.0077**
C6	I	20.829	15.002	135.810	0.0171*
C7	F˙	19.120	20.318	5.741	0.6138
C8	N	16.482	22.957	167.703	0.0085**
C9	I	19.030	20.409	7.607	0.5615
C10	F	22.260	17.407	94.206	0.0451*
C11	N	12.294	27.372	909.324	0.0001***
C12	I	22.203	17.463	89.861	0.0501*
C13	Control	19.669	18.723	3.367	0.6990
C14	1-4:13-16	17.424	19.833	46.421	0.1551
C15	5-8:9-12	17.916	19.719	26.026	0.2849
C16	1-4,13-16:5-12	18.629	18.817	0.570	0.8735
Z1	F	23.410	16.206	207.634	0.0037**
Z2	N	19.526	20.090	1.275	0.8119
Z3	I	20.361	19.255	4.886	0.6415
Z4	F	14.315	13.050	6.340	0.5942
Z5	N	13.991	13.374	1.523	0.7947
Z6	I	16.067	11.297	91.035	0.0487*
Z7	F	26.918	27.027	0.047	0.9633
Z8	N	29.041	24.904	68.463	0.0858
Z9	I	23.791	30.154	161.945	0.0096**
Z10	F	14.462	14.397	0.017	0.9783
Z11	N	12.940	15.919	35.507	0.2126
Z12	I	15.050	13.808	6.169	0.6009
Z13	F	20.701	16.745	250.403	0.0016**
Z14	N	14.056	23.390	1394.192	0.0001***
Z15	I	20.327	17.118	164.740	0.0090**

^{a}Mean (+ and -) does not apply for comparisons C13-C16 (See Table 5).

C6 and C12. Interaction between the two inocula for Ba was found to be very highly significant for comparisons Z9 and Z15 and significant for comparisons C6, C12, and Z6.

For Sr treatments with live nodule inoculum and dead isolate A-3 had the largest means (Figure 31). Three of the four treatments with live nodule inoculum and dead isolate A-3 for Ba had the largest means within each 2 x 2 factorial (Figure 32). The effect of live nodule inoculum appeared to have enhanced Sr and Ba uptake. For Sr three of the four treatments with live isolate A-3 and dead nodule inoculum had the lowest means within each 2 x 2 factorial. For Ba three of the four treatments with live isolate A-3 and dead nodule inoculum also had the lowest means within each 2 x 2 factorial. Therefore, there was an indication that live isolate A-3 inhibited Sr and Ba uptake even in the presence of live nodule inoculum. This might have accounted for the significant interactions observed.

For sodium (Na) the effect of nodule inoculum was found to be highly significant when comparing initial nodule inoculations and delayed fungus inoculations [Trts 9-12 (C8)] and all nodulated treatments to non-nodulated treatments (Z14, Table 39). Treatments receiving live nodule inoculum had higher means than those with dead nodule inoculum. Duncan's multiple range test for Na showed very little difference among treatments (Figure 33). However, some trends were noted. Treatments with live nodule inoculum and dead isolate A-3 had the largest means. The treatments with live isolate A-3 and dead nodule inoculum were among the lowest.
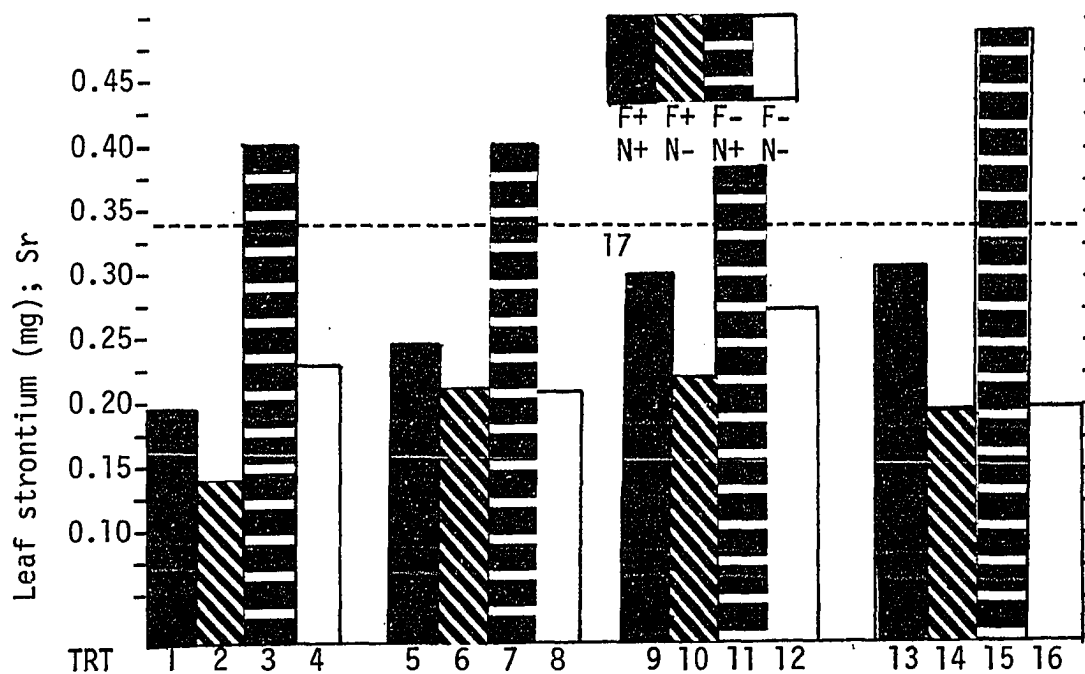
TRT [1]		Mean
15	a	0.485 mg
3	ab	0.402
7	ab	0.400
11	b	0.380
17	bc	0.335
13	bcd	0.307
9	bcde	0.300
12	cdef	0.270
5	cdef	0.248
4	defg	0.230
10	defg	0.223
6	defg	0.218
8	defg	0.216
16	efg	0.199
14	fg	0.193
1	fg	0.189
2	g	0.140

[1] Values in each vertical column followed by the same letters do not differ significantly at p = 0.05 by Duncan's multiple range test.

Figure 31. Histogram and treatment means for leaf strontium (mg); Sr

Figure 32. Histogram and treatment means for leaf barium (mg); Ba
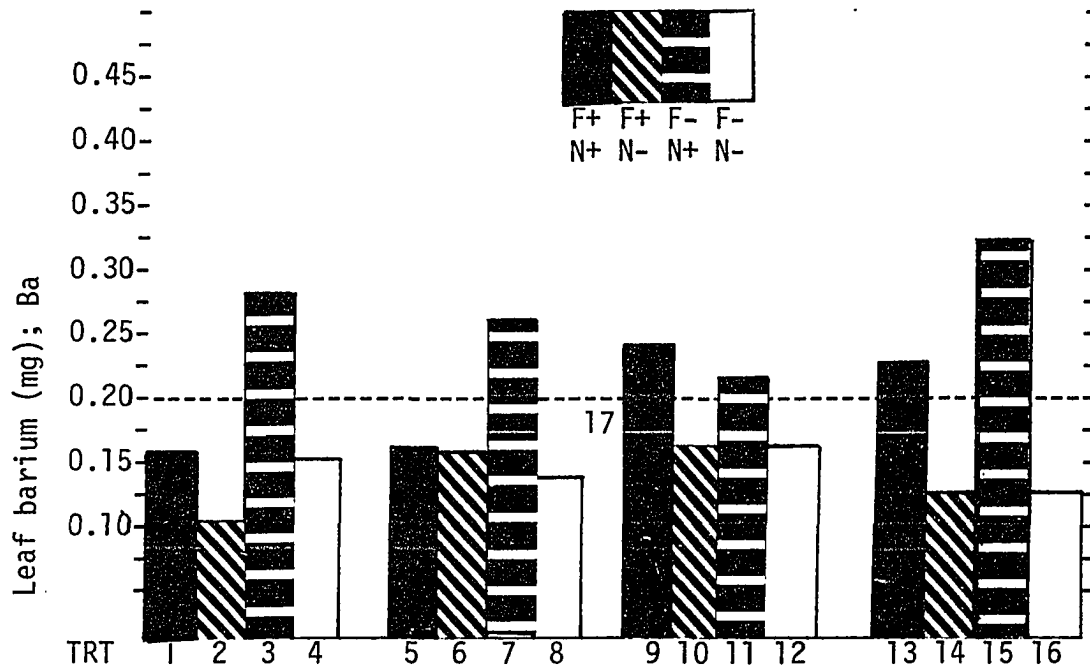
TRT[1]		Mean
15	a[1]	0.322 mg
3	ab	0.281
7	abc	0.259
9	bcd	0.242
13	bcde	0.226
11	bcdef	0.217
17	cdefg	0.197
12	defgh	0.166
5	defgh	0.165
10	defgh	0.164
1	efgh	0.159
6	efgh	0.157
4	efgh	0.153
8	fgh	0.135
16	gh	0.124
14	gh	0.122
2	h	0.104

[1] Values in each vertical column followed by the same letters do not differ significantly at p = 0.05 by Duncan's multiple range test.

Table 40. Statistical analyses for leaf sodium (mg); Na

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-value	Probability >F
Model	19	114943.229	6049.644	1.37	0.1306
Error	44	194245.906	4414.680		
Total	67	309189.136			

SV	Effect	$-$ Mean[a]	$+$ Mean[a]	MSS	Probability
Rep				5873.822	0.6951
C1	F=Fungus	129.725	67.821	15328.668	0.0575
C2	N=Nodule	76.563	120.983	7892.634	0.1690
C3	I=Interaction	118.784	78.762	6407.122	0.2144
C4	F	115.353	118.324	35.313	0.9260
C5	N	107.213	126.464	1482.327	0.5479
C6	I	130.440	103.237	2960.013	0.3967
C7	F	162.937	115.909	8846.484	0.1458
C8	N	93.655	185.190	33514.716	0.0060**
C9	I	152.651	126.196	2799.653	0.4097
C10	F	126.108	118.450	234.595	0.8108
C11	N	91.210	153.348	15445.021	0.0566
C12	I	121.702	122.856	5.334	0.9712
C13	Control	132.548	119.328	657.922	0.6886
C14	1-4:13-16	98.773	122.279	4420.350	0.3012
C15	5-8:9-12	116.839	139.423	4080.364	0.3203
C16	1-4,13-16:5-12	110.526	128.131	4958.888	0.2738
Z1	F	92.184	149.273	13036.559	0.0790
Z2	N	132.222	109.234	2113.862	0.4733
Z3	I	131.398	110.058	1821.604	0.5055
Z4	F	85.086	93.961	315.054	0.7814
Z5	N	104.552	74.495	3613.543	0.3494
Z6	I	102.835	76.212	2835.057	0.4067
Z7	F	189.266	155.263	4624.884	0.2904
Z8	N	147.590	196.939	9741.394	0.1273
Z9	I	180.256	164.273	1021.793	0.6176
Z10	F	99.779	89.815	397.096	0.7554
Z11	N	93.871	95.723	13.710	0.9538
Z12	I	98.034	91.560	167.644	0.8396
Z13	F	133.531	105.126	12909.362	0.0804
Z14	N	92.160	146.496	47238.795	0.0013**
Z15	I	130.894	107.762	657.922	0.6886

[a]Mean (+ and -) does not apply for comparisons C13-C16 (See Table 5).
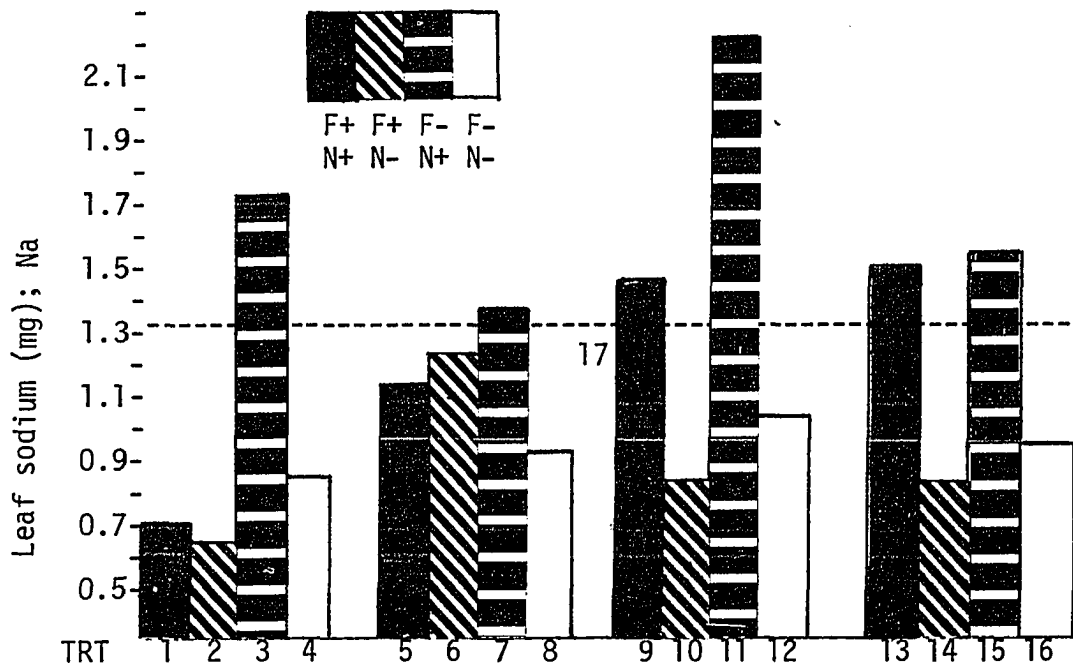
TRT		Mean
11	a[1]	2.219 mg
3	ab	1.719
15	ab	1.566
13	ab	1.501
9	ab	1.484
7	ab	1.386
17	ab	1.325
6	ab	1.223
5	ab	1.143
12	b	1.039
16	b	0.956
8	b	0.921
4	b	0.875
14	b	0.868
10	b	0.834
1	b	0.700
2	b	0.656

[1] Values in each vertical column followed by the same letters do not differ significantly at p = 0.05 by Duncan's multiple range test.

Figure 33. Histogram and treatment means for leaf sodium (mg); Na

Many of the minerals were very highly correlated to each other (Table 32). This explained the similar C and Z comparisons found in many of the mineral analyses. Correlations were considered to be very high with r values larger than 0.60. N, P, K, Ca, B, Cu, Sr, and Ba were very highly correlated to each other.

Soil analysis for phosphorus showed no significant difference among treatments. The seedlings and live inocula did not remove P from the soil at significantly different levels.

DISCUSSION

Vesicular-Arbuscular Mycorrhizae

It is not surprising to find vesicular-arbuscular mycorrhizal (VAM) fungi associated with those woody plants thought to be exclusively ectomycorrhizal. The VAM fungi have the capacity to colonize and establish mutualistic relationships with over 90% of the terrestrial plant species. They would be expected to have the same inherent capacity to colonize the 3% of land plants that form ectomycorrhizae.

Very little has been reported on the conditions condusive for the formation of VAM in the ectomycorrhizal species. The study done by Truszkowska (1953) provides the most supportive evidence that in plants with both mycorrhizal symbionts, VAM form when soil moisture is high and ectomycorrhizae form when soil moisture levels drop. O'Malley (1974) observed the same phenomenon in pin oak. The observation in this study that more VAM vesicles were found in Alnus spp. growing on wet sites than on dry sites provides evidence that soil moisture is implicated in VAM development.

The most heavily colonized plants with VAM fungi were two native species in their natural habitats, A. rugosa (= A. incana) from Michigan, Wisconsin, and Minnesota and A. serrulata from North Carolina; and A. glutinosa growing on a wet site in Georgia. Lower numbers of VAM were found in all other A. glutinosa samples and A. hirsuta from Illinois, but in all the latter cases the trees were growing on drier sites. With the finding of VAM in A. rubra, A. glutinosa, and A. incana

by Sharon Rose (personal communication, Forest Sciences Laboratory, Corvallis, Oregon, 1979) in Oregon it is believed that all <u>Alnus</u> spp. are capable of establishing VAM under conditions yet to be determined.

Truszkowska (1953) speculated that the wet sites did not have sufficient soil oxygen for the ectomycorrhizal fungi. In alder trees that are not readily accessible to high soil moisture VAM formation would most likely occur in the spring or during periods coinciding with the greatest amounts of rainfall. It is not known if the antibacterial substances found in root and nodule extracts have any influence of VAM fromation (Neal et al., 1968b). These substances are not present in the fall and winter, and VAM formation may occur during periods of low concentrations. High soil moisture may aid in diluting these inhibitory substances. In addition, these inhibitory compounds were found in mature trees. Their occurrence and concentration in seedlings is not known. The <u>Alnus</u> spp. capable of forming VAM, the identification of the VAM fungi, the environmental conditions condusive for VAM formation, and the effect of the inhibitory compounds have yet to be determined.

Ectomycorrhizal Synthesis Trials

Most of the fungi believed to be ectomycorrhizal symbionts of <u>Alnus</u> spp. are determined upon the presence of basidiocarps in alder stands. Pure culture synthesis is desirable for confirmation of ectomycorrhizal symbionts. However, in this study pure culture synthesis of ectomycorrhizae in <u>Alnus</u> <u>glutinosa</u> was found to be difficult. Molina (1979) found difficulty in pure culture synthesis with <u>A</u>. <u>rubra</u>. Only four of 28

fungi formed ectomycorrhizae. In this study only one of six fungi formed ectomycorrrhizae.

It appears that Alnus has a fewer number of ectomycorrhizal associates than most ectomycorrhizal genera. In addition to a restricted number of symbionts the Hartig net development is restricted to the outer cortex. This was shown for isolate A-3 and for various other ectomycorrhizal fungi (Masui, 1926; Neal et al., 1968a; Molina, personal communication, Forest Sciences Laboratory, Corvallis, Oregon, 1979). It is believed that the low symbiont number and limited Hartig net development with known symbionts is related to the inhibitory compounds; e.g., phenols, fatty acids; produced in alder roots (Neal et al., 1968b; Li et al., 1970; Li et al., 1972; and Li, 1974).

Although the identity of fungal isolate A-3 could not be determined, there should be sufficient information for comparison to isolates from known fungal species. The physical descriptions of the mycorrhizal anatomy based on Zak's key (1973) have some unique characteristics, especially for the production of pigment. Pigment production is influenced by media composition (Table 8). Pigment production may be involved in the ability of isolate A-3 to produce mycorrhizae in alder. Pigment was not produced when isolate A-3 was propagated on rice, millet, and vermiculite containing MMN culture media. Mycorrhizal synthesis trials with these isolate A-3 inocula were unsuccessful.

Three-Way Study

Additional basic information to what is now available is needed before the three-way study could be successfully evaluated. The species

of VAM fungi associated with <u>Alnus</u>, and the conditions necessary for colonization need to be known as a prerequisite for the three-way study. The establishment of ectomycorrhizae and VAM may not be possible under the same greenhouse conditions. In addition, experimentation during the summer in the greenhouse is not recommended.

Two-Way Study

Statistical analysis was complicated by this design. During the first 2 mo the seedlings were grown in a 3 x 3 factorial design. For the last 2.5 mo the seedlings were grown in an incomplete $(3 \times 3)^2$ factorial design. The 3 x 3 design produces nine treatments. The $(3 \times 3)^2$ design produces 81 treatments. However, because of space and time only 17 of the 81 treatments could be practically evaluated. Five treatments in the 3 x 3 design were duplicated in the $(3 \times 3)^2$ design (Trts 1, 2, 3, 4, and 17). The remaining treatments in the $(3 \times 3)^2$ design did not have complete treatment data for comparison purposes for the entire 4.5 mo. However, it was believed that these missing treatments would not contribute enough additional information to justify including them.

The growth differences produced in the growth chamber during the first 2 mo unfortunately could not be evaluated. The effect of position in the growth chamber on the seedling height would have shown significance.

Healthy trees, the tallest trees, were desired for optimum mycorrhizal and actinorhizal development. Therefore, the tallest trees were

chosen for the continuation of the study. The random selection of the 12 tallest trees when the trees were transplanted resulted in replication means being significantly different for height. Replication 4 was significantly smaller. At this point of development the range for height was not large, and the final seedling height means for replication was not significant. Replication means were not significantly different for the diameter and volume of the trees at the time of transplant. Therefore, this replication difference at 2 mo did not appear to bias the final results.

The seedlings should have been selected at random from the seedlings remaining after elimination of those considered to be unlikely host plants for the symbionts. The criteria for eliminating unlikely host plants should be the following: 1) dead trees, 2) twisted slow growing trees, and 3) trees with abnormal color. This would have left the 12 tallest seedlings for many treatments. However, there would have been 13, 14, 15, or possibly more trees from which to select 12 trees in many treatments. The selection of the 12 tallest seedlings decreased complete randomization.

Growth Responses

The addition of live nodule inoculum at the time of initial planting had the greatest influence on 4.5 mo seedling height. The addition of live nodule inoculum 2 mo after planting did not affect significantly the height of the 4.5 mo-old seedlings. The addition of live isolate A-3 at the time of initial planting did not influence 2 mo-old seedling

height but did enhance 4.5 mo-old seedling height. There was very strong evidence that both live inocula together (height mean of treatments with both live inocula = 22.87 cm) contributed to tree height more' than either inoculum alone (height mean for treatments with live isolate A-3 = 18.77 cm and for treatments with live nodule inoculum = 20.89 cm). The height of the trees containing live inocula at initial planting was significantly larger than the trees receiving live inocula after 2 mo (C16, Table 14). Therefore, in order to obtain maximum tree height with these symbionts both should be introduced at initial planting in containerized seedlings.

The significant growth difference among replications in basal stem diameter occurred in the greenhouse. It was possible that bench position somehow affected diameter growth. The effect of live nodule inoculum greatly increased basal stem diameter, and the earlier the inoculum was incorporated the greater the diameter growth response. Fungal isolate A-3 did not appear to affect diameter growth up to 4.5 mo.

Tree volume is one of the key growth parameters used in forest science for estimating productivity. Marx et al. (1978) used tree volume x percentage survival to derive a plot volume index (PVI). In large nurseries where large numbers of seedlings are planted the PVI may be a valuable economic parameter that can be used in estimating part of the overhead expenses concerning seedling loss and replanting. For this reason the seedling volume in this study was estimated using the height and (basal stem diameter)^2. It has not been determined whether this volume estimation is accurate or practical. However, the values

generated in this study are relative to each other and can be analysed statistically.

As has been shown with height and diameter parameters volume treatment means after 2 mo of growth did not show much range (Figure 9). Only treatments receiving live nodule inoculum had significantly larger seedling volumes, regardless of fungal inoculum treatment, than treatments with dead nodule inoculum (Table 14, Figure 9). Values for the volume for the last 2.5 mo (VOLD) and the final volume (VOLF) were very close, as shown be the high r^2 value (.99, Table 21). This was because the values for the volume for the initial 2 mo growth were quite small. Therefore, as expected the significant comparisons for VOLD and VOLF were similar. The significant effect of isolate A-3 on height was not enough to produce significance for volume comparisons.

During the first 2 mo the growth parameters (height, diameter, and volume) all showed that the treatments with live nodule inoculum had significantly larger means than the treatments with dead nodule inoculum. In all these parameters the treatment means range was not large. During the last 2.5 mo the confidence level of significant differences increased as did the treatment means ranges, especially in the treatments receiving live inocula initially. Fungal isolate A-3 was found to be significant for the last 2.5 mo for final height. The great increase in confidence level of significance and treatment mean range during the last 2.5 mo was the result of a delayed growth stimulation in the treatments given live inocula initally rather than the addition of live inocula at the second treatment. This is shown

with the C5, C11, and C14 comparisons. The C5 and C11 comparisons were treatments with delayed nodule inoculum. In comparison C5 no significant difference between treatments receiving live nodule inoculum and those receiving dead nodule inoculum was found for final volume (VOLF) and final diameter (DIAF). In comparison C11 no significant difference between treatments receiving live nodule inoculum and treatments receiving dead nodule inoculum was found for final volume (VOLF) and final height (HTF). The C2 and C8 comparisons were treatments receiving live nodule inoculum initially. All C2 and C8 comparisons were significant for final volume, final height, and final diameter. Treatments with initial planting inoculations (Trts 1-4) were significantly larger than treatments with delayed inoculations (Trts 13-16) for final height, stem diameter, and volume (C14, Tables 16, 18, and 20). If the experiment had been terminated after 6.5 mo rather than 4.5 mo significant difference between treatments with live nodule inoculum and those with dead nodule inoculum in the C5 and C11 comparisons would be expected. The C14 comparison may remain significant regardless of time. The seedlings in treatments 1-4 should maintain their growth superiority for an indefinite time period.

A satisfactory explanation was not determined for the significant difference found with the root volume measurements. The negative correlation of root volume to the presence of both live inocula seen in Figure 16 was not significant, but a trend was observed and deserves discussion. Ectomycorrhizal systems usually have larger volumes than non-mycorrhizal systems. More extensive root production and root

branching is the rule rather than the exception with ectomycorrhizae (Bowen, 1973; Slankis, 1973). However, in this study treatments with live isolate A-3 had less volume than those with dead isolate A-3 (C1, Z13, Table 22). Once the roots become mycorrhizal their root diameter remains constant without secondary growth. In the treatments with dead isolate A-3 the roots were not restricted in their secondary growth, and this may account for their larger volume. Isolate A-3 did not appear to induce lateral root production in A. glutinosa as some ectomycorrhizal fungi do in other genera.

The effect of the nodule inoculum had less of a negative correlation to root volume than isolate A-3. Treatments with live nodule inoculum might be expected to have less root volume than those with dead nodule inoculum based on shoot growth stimulation. Increases in seedling shoot to root ratios are easily obtained with nitrogen fertilization. The alder shoot to root ratios should likewise increase in seedlings receiving their nitrogen from actinorhizae. In non-actinorhizal seedlings the shoot would be less of a nutrient sink and more root growth may occur. A clear explanation for the effect of the live inocula on root volume cannot be given based on this study.

Leaf and stem dry weights were found to be very highly correlated ($r = .79$, Table 21). In all treatments with live nodule inoculum a substantial increase in dry weight was observed. The significant difference in leaf dry weight found for isolate A-3 may have possibly been attributed to a lower than expected value in treatment 1, both live symbionts initially inoculated. The lower value for treatment 1

was most likely attributed to the defoliation in five of 12 trees 1 mo

prior to termination of the experiment. The regenerated leaves would

probably not have attained the same dry weight as the original leaves.

Acetylene Reduction

The higher rate of acetylene reduction found in both total acetylene

reduction (CH) and specific activity (MM) in treatments receiving live

nodule inoculum after 2 mo was because these treatments were not growing

as fast as the treatments receiving live nodule inoculum from initial

planting. Larger nutrient sinks develop in the treatments receiving live

nodule inoculum from initial planting because of more rapid growth rates.

Ham et al. (1976) found a similar occurrence with soybean (Glycine max

L. Merr.) - Rhizobium symbiosis. They demonstrated that during pod

filling in soybeans a large photosynthate sink develops and nitrogen

fixation rates decline as a result on an inadequate supply of assimilate

to the nodules. The live nodule inoculum treatments (1, 3, 5, 7, 9, 11,

13, and 15) were averaged for the seven growth variables (DIAD, DIAF,

HTD, HTF, VOLD, VOLF, and STEM) and then ranked (Table 41). The

treatments found with the tallest seedlings, largest diameters, largest

volumes, and largest stem dry weights were those containing live nodule

inoculum from the initial planting (Trts 1, 3, 9, and 11). Therefore,

they had the greatest sink for photosynthates.

The expected ranking for nitrogen fixation (variable MM) in live

nodule inoculum treatments should have been exactly opposite of the rank

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inoculum from the initial planting (Trts 1, 3, 9, and 11). Therefore,

they had the greatest sink for photosynthates.

The expected ranking for nitrogen fixation (variable MM) in live

nodule inoculum treatments should have been exactly opposite of the rank

for growth. The actual ranking for nitrogen fixation almost perfectly

matches the expected ranking (Table 42). Therefore, the high rates of acetylene reduction (= nitrogen fixation) found in the treatments receiving delayed live nodule inoculum was because the rates of growth in these treatments were lower and there was less of a photosynthate sink than in treatments receiving live nodule inoculum at initial planting.

The values of specific activity (MM) were derived from the values of total acetylene reduction (CH). However, the partial correlation coefficients were not as high as expected (r = .51, Table 21). The specific activity values were based on nodule dry weight. Some nodules may have been lost during root preparation for acetylene reduction analysis. Some of the nodules may have been overlooked during nodule excision for dry weight determination. Some roots may have been fixing nitrogen prior to the formation of visible nodules.

The effect of the defoliation in five of 12 seedlings in treatment 1 may have affected the analysis of CH and MM. The rate of nitrogen fixation should have been reduced for both variables. The leaves that regenerated would not have had the same photosynthetic capacity as the original leaves, thereby, causing a reduction of photosynthate to the nodules. Also, to regenerate the leaves would have produced a larger than usual sink, again, causing a reduction of photosynthate to the nodules. Indeed, five of six of the lowest acetylene reduction values for variables MM and CH for treatment 1 were from the defoliated trees.

Table 41. Rank of treatments receiving live nodule inoculum for variables DIAD, DIAF, HTD, HTF, VOLD, VOLF, and STEM[a]

Treatment	1	3	5	7	9	11	13	15
Rank	1	2	5	8	3	4	7	6

[a]Average of seven variables was ranked 1-8.

Table 42. Expected and actual ranking of live nodule inoculum treatments for specific acetylene reduction activity; MM

Treatment	1	3	5	7	9	11	13	15
Expected Rank	8	7	4	1	6	5	2	3
Actual Rank	8	7	4	2	6	5	1	3

Mycotrophy

Mycorrhizal fungus isolate A-3 was shown not to affect nitrogen fixation. However live nodule inoculum profoundly affected ecto-mycorrhizal development. The treatments containing live fungal inoculum and dead nodule inoculum (Trts 2, 6, 10, and 14) had an average of 23% of their root systems with mycorrhizae (Figure 21). Within these treatments those with live isolate A-3 for the full 4.5 mo (Trts 2 and 6) had an average of 33% mycorrhizae compared to 13% in treatments given fungal inoculum the last 2.5 mo (Trts 10 and 14). Therefore, more mycorrhizae were produced when the fungal inoculum was added at initial planting. In either case these percentages (33 and 13) were lower than what would be desirable in containerized seedling production.

The treatments containing both live inocula (1, 5, 9, and 13) had significantly higher percentages of mycorrhizae with a 60.5% mean. Within these treatments those with live nodule inoculum for the full 4.5 mo (Trts 1 and 9) had an average of 74% mycorrhizae compared to 47% mycorrhizae in treatments given live nodule inoculum the last 2.5 mo (Trts 5 and 13).

Comparing the treatments with live inocula treatment 1, initial inoculation with both symbionts, had the most mycorrhizae (81%) and treatment 13, delayed inoculation with both symbionts, had the least (38%). Treatment 9, initial inoculation with nodule inoculum and delayed inoculation with isolate A-3 had a slightly higher percentage of mycorrhizae (66%) than treatment 5, initial inoculation with

isolate A-3 and delayed inoculation with nodule inoculum (55%).

Comparing treatments with initial live isolate A-3 inoculation and

delayed live nodule inoculation (5) to treatments with initial live

isolate A-3 and delayed dead nodule inoculum (6) showed that the live

nodule inoculum did not influence the percentages of mycorrhizae like

it does comparing treatment 1, initial inoculation with both

symbionts, to treatment 2, initial inoculation with live isolate A-3

and dead nodule inoculum, and treatment 9, initial inoculation with

live nodule inoculum and delayed inoculation with live isolate A-3,

to treatment 10, initial inoculation with dead nodule inoculum and

delayed inoculation with live isolate A-3. This would indicate that the

inoculation with live nodule inoculum produces a host response that

enhances ectomycorrhizal development for fungal isolate A-3. The

ectomycorrhizae develop rapidly in trees containing nodules regardless

of whether the fungal inoculum is added with the live nodule inoculum

initially or at a later point in time. However, if the live nodule

inoculum is added after ectomycorrhizal establishment the increase of

mycorrhizae is not significant from non-nodulated mycorrhizal seedlings.

The nitrogen fixing symbiont enhanced mycorrhizal development

with isolate A-3. Although this phenomenon was demonstrated for one

fungal symbiont, it should be applicable to most ectomycorrhizal

symbionts with alder. Molina (1979) had success with only four of

28 fungal isolates in non-actinorhizal alder. It is possible that

actinorhizal alder may enhance mycotrophy with additional fungal

isolates. Recommendations for establishing ectomycorrhizae and

actinorhizae in containerized alder seedlings would be to incorproate both inocula or at least live nodule inoculum at the time of planting. It would not be advised to establish ectomycorrhizae preceeding actinorhizae.

An additional reason for not establishing ectomycorrhizae prior to actinorhizae would be to ensure that there are sites for nodule inoculum penetration. Many ectomycorrhizal fungi will colonize virtually all roots produced within containers. This may not be true for Alnus as it is for other genera; e.g., Pinus. Most fine roots establishing ectomycorrhizae will have a fungal mantle in place of an epidermis and root hairs. Ectomycorrhizae could directly interfer with nodule development because of the elimination of the sites of nodule inoculum penetration, the root hairs. There was no proof of this in this study.

The benefit of the actinorhizal establishment coinciding with or preceding ectomycorrhizal establishment is believed to be due to an increase in the concentration of soluble sugars in the roots. The nodules are notable metabolic sinks for soluble carbohydrates. However, given sufficient light intensity excess carbohydrates accumulate in nodulated roots (Wheeler and Lawrie, 1976). Leaf area in nodulated plants was estimated to be several times the leaf area in non-nodulated plants (Dawson, 1978). The terminal photosynthesis rate, which is highly correlated to leaf area, was determined to be highly correlated to the nodule dry weight. Therefore, plants with nodules have more leaf area and a higher rate of photosynthesis that would produce more carbohydrates.

These carbohydrates, largely sucrose, promote mycorrhizal development. Marx et al. (1977) and Meyer (1974) have presented evidence showing the importance of sucrose on the establishment of ectomycorrhizae.

Mineral Uptake

For the total quantity (mg) of all leaf minerals all treatments with live nodule inoculum were highly significantly larger to very highly significantly larger than all treatments with dead nodule inoculum (Z14, Table 43). The effect of live nodule inoculum was found to produce significantly larger to very highly significantly larger concentrations of N, P, K, Cu, Sr, Ba, Ca, Mg, and B for most C comparisons. The nodulated seedlings have more leaf tissue, and this results in a larger total accumulation of leaf minerals.

Statistical analyses of the leaf mineral percentages showed entirely different results. Based on percentage only, nitrogen and potassium were found in larger concentrations in nodulated seedlings compared to non-nodulated seedlings. The concentrations of the other minerals were lower in the nodulated seedlings than the non-nodulated seedlings. This can lead to erroneous interpretations if growth increases more than nutrient uptake. The lower concentration is the result of a dilution effect in the rapidly growing tissue (Tamm, 1964). This dilution effect was apparent in this study. The lower mineral concentrations in nodulated seedlings when multiplied by leaf weight, which was greater in the nodulated seedlings, resulted in larger total mineral quantities in nodulated seedlings compared to

Table 43. Summary of the C and Z comparisons for leaf minerals and their level of confidence[a]

C & Z	N	P	K	Cu	Sr	Ba	Ca	Mg	Fe	Al	B	Na
C2	***	***	***	***	***	***	***	***			*	
C5	***	***	***	***	**	**					*	
C8	***	***	***	***	**	**	**	***				**
C11	***	***	***	***	***	***	***	***	***	***	**	
Z14	***	***	***	***	***	***	***	***	***	**	***	**
Z2												
Z5												
Z8									*			
Z11												
C1				*	***	***	***	***			**	
C4					*							
C7					*							
C10					*	*	*					
Z13					***	**	***				*	
Z1						*	**	*				
Z4												
Z7									*	*		
Z10												

(Nodule Inoculum: C2, C5, C8, C11, Z14, Z2, Z5, Z8, Z11)
(Fungal Inoculum: C1, C4, C7, C10, Z13, Z1, Z4, Z7, Z10)

[a]Probability confidence levels.
 * p = 0.050.
 ** p = 0.010.
 *** p = 0.001.

C & Z	N	P	K	Cu	Sr	Ba	Ca	Mg	Fe	Al	B	Na
C3												
C6					*	*	**	**				
C9												
C12					**							
Z15					***	***	***	**				
Z3												
Z6						*	*	**				
Z9		*	**				*	*	*	*		
Z12												
C13		**					**					
C14					*							
C15												

Interaction (vertical label at left)

non-nodulated seedlings.

The effect of live fungal isolate A-3 was shown to inhibit the uptake of the minerals Sr, Ba, Ca, B, and possibly Mg and Cu. All of these except Mg and Cu were found in lower total quantities in treatments given live isolate A-3 compared to treatments given dead isolate A-3 (Z13, Table 43). Mg was found to be very highly significant in comparison C1, initial planting with both inocula, with treatments given live fungal inoculum having much lower means than those given dead fungal inoculum. As previously discussed, treatment 1, initial planting with both live inocula, had five of 12 seedlings defoliated and leaf regeneration probably was not equal in weight or mineral concentration to what was lost. The significance found in C and Z comparisons involving treatment 1 may or may not be real. The signifiance found for Cu (p = 0.04, Table 31) would be the most likely to be erroneous. There are 15 growth and nitrogen fixation variables and 12 mineral variables each with 31 C and Z comparisons. This amounts to 837 comparisons in which there is a probability that 5%, 42 comparisons, may be wrong.

Why isolate A-3 would inhibit ion uptake of some minerals is not known. It is known that the fungal mantle of some ectomycorrhizae absorbs and accumulates ions (Bowen, 1973). It is possible that these minerals are bound by the fungus and less available to the host.

Most of the significant interactions involved the minerals where isolate A-3 inhibited mineral uptake; i.e., Sr, Ba, Ca, and Mg (Table 43). Significant individual interaction comparisons were found for comparison

Z9 with the minerals P, K, Fe, and Al.

Minerals Sr, Ba, Ca, and Mg are quite similar in their significant C and Z comparisons for nodule inoculum, isolate A-3, and interaction (Table 43). They are very highly correlated to each other (Table 32), and are also divalent cations.

Isolate A-3 does not appear to be a desirable ectomycorrhizal symbiont based on mineral uptake. Mineral uptake was not enhanced by the fungal symbiont. It even inhibited some mineral uptake. This contradicts what has been demonstrated in most other ectomycorrhizal nutrient uptake studies. Mejstrik and Benecke (1969) found an increase in the uptake of phosphorus in alder ectomycorrhizae. In their study they measured P levels in excised roots and not in leaf tissue. Bowen (1973) reported that P is one of the major minerals accumulated by the mantle. It is not known whether the alder leaves and other host tissues received any increase in P from the ectomycorrhizae in the study of Mejstrik and Benecke. This study did not include the mineral analysis of the roots. It would be interesting to see if any minerals, such as Sr, Ba, Ca, and/or Mg, are being accumulated in the mycorrhizae.

Since an increase in P uptake was not shown in mycorrhizal seedlings, an increase in nitrogen fixation would not necessarily be expected. An increase in the uptake of P by VAM has been shown to increase nitrogen fixation in several Rhizobium-legume studies and one Frankia-Cercocarpus study (Williams, 1978). Likewise, any fungal symbiont, VAM or ecto-mycorrhizal, that increased P uptake should increase nitrogen fixation in alder. The next logical study would be to find a P-fixing fungal

symbiont, including testing the ectomycorrhizal symbionts found by Molina (1979); Mejstrik and Benecke's fungal symbiont, if it can be isolated; and any available VAM fungi. If a P-fixing symbiont is found, then a 2 x 2 factorial experiment should help determine whether nitrogen fixation is increased in Alnus with the fungal symbiont as in the other nitrogen fixing systems.

Live fungal isolate A-3 in combination with live nodule inoculum added at initial planting did produce the tallest seedlings. The fungus is beneficial from this criteria. In addition, after the trees lost their afternoon shade and five of 12 trees in treatment 1 defoliated, all of these five trees refoliated. None of the trees in other treatments defoliated. However, four seedlings died without defoliating. It is believed that the ectomycorrhizae were involved in the protection of the seedlings from moisture stress.

In the studies with nitrogen fixing symbionts and VAM no effect on the VAM by the nitrogen fixing symbiont has been demonstrated. In this study the live nodule inoculum was found to greatly increase the percentage of ectomycorrhizae. It is possible that actinorhizae may be necessary for the establishment of some ectomycorrhizal species. It would be interesting to conduct synthesis trials with Molina's (1979) 28 fungal isolates in seedlings that have been inoculated with live nodule inoculum simultaneously. The number of ectomycorrhizal symbionts of alder is probably much lower than most ectomycorrhizal tree genera. However, alder can be planted on virtually any well-drained site in the northern hemisphere, and it will form nodules and

ectomycorrhizae. The nodule endophyte appears to be ubiquitous and
it may enhance ectomycorrhizal establishment with other fungal symbionts.

In conclusion nursery production of alder seedlings should
include the inoculation with an efficient nitrogen fixing nodule
inoculum and an ectomycorrhizal fungus, preferably one that is capable
of phosphorus uptake, at the time of seed germination. An alder
genotype selected for fast growth, disease resistance, and other
desirable characteristics could utilize a natural symbioses, efficient
nitrogen fixation and efficient nutrient uptake with ectomycorrhizae,
for obtaining an optimum fiber production and soil improvement.

SUMMARY

1. Vesicular-arbuscular mycorrhizae (VAM) were observed in Alnus hirsuta, A. glutinosa, A. rugosa (= A. incana), and A. serrulata.

2. A higher frequency of VAM vesicles was found in trees growing in wet sites.

3. An ectomycorrhizal basidiomycete, isolate A-3, was isolated from A. glutinosa ectomycorrhizae growing in central Iowa, and ecto-mycorrhizae were successfully synthesized with this fungal symbiont.

4. Nodule inoculum obtained from nodules growing on A. glutinosa in central Iowa was used to synthesize actinorhizae in A. glutinosa seedlings.

5. The nodule inoculum was found to increase seedling height, basal stem diameter, stem volume, stem dry weight, leaf dry weight, total C_2H_4 reduction, specific C_2H_4 activity, percentage of ectomycorrhizae, and total quantity of the minerals N, P, K, Cu, Sr, Ba, Mg, Ca, Fe, Al, B, and Na compared to seedlings with dead nodule inoculum.

6. Live fungal inoculum A-3 was found to increase seedling height; inhibit the uptake of Sr, Ba, Ca, and possibly Mg and Cu; but not influence rates of acetylene reduction.

7. Both symbionts appeared to produce greater seedling height together than either alone and may produce a smaller root volume together than either alone.

8. The greatest growth response for height, basal stem diameter, and stem volume was found in the treatments given live inocula initially, but most of the growth occurred in the final 2.5 mo.

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APPENDIX

Table A-1.   Vesicular-arbuscular mycorrhizal (VAM) clearing and
staining procedures

---

1. Wash the rootlets in fresh water to remove soil.

2. Cut roots into small (approx. 3 cm) lengths and place in vials
    containing 10% KOH; remaining roots may be preserved in FAA.

3. Autoclave for 7 min after closing waste valve.

4. Decant solution and wash roots in fresh 10% KOH for 1 min.

5. Bleach roots with 0.525% NaOCl (or 30% H_2O_2) until roots are
    a straw color.

6. Rinse roots with tapwater to remove bleaching agent.

7. Acidify roots with 1% HCl (quick rinse).

8. Stain roots with 0.05% Trypan Blue or Acid Fuchsin-Chloral Hydrate
    (900 ml H_2O, 100 g chloral hydrate, 0.5 g acid fuchsin); Trypan
    Blue stain produced best overall results.

9. Autoclave roots until pressure reaches 14 lbs/in^2 then turn autoclave
    off

10. Decant solution and destain in clear lactophenol (10 ml H_2O,
    10 ml phenol, 10 ml lactic acid, 20 ml glycerol).

Table A-2. Modified Melin-Norkrans (MMN) medium

$CaCl_2$	0.05 g
NaCl	0.025 g
KH_2PO_4	0.50 g
$(NH_4)_2HPO_4$	0.25 g
$MgSO_4 \cdot 7H_2O$	0.15 g
$FeCl_3$ (1% solution)	1.20 ml
Thiamine HCl	100 µg
Malt extract	3.00 g
Glucose	10.00 g
Agar	15.00 g
H_2O	1000.00 ml

Autoclave for 15 min at 121 C

pH 5.5 - 5.7

Table A-3. Lalonde and Knowles (1975b) nodule inoculum purification
procedures

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1. Wash 10 g fresh nodules in tap water to remove soil.

2. Surface sterilize nodules in 100 ml 5.25% NaOCl solution (straight
    bleach) in 250 ml flask by agitating for 3 min.

3. Rinse 3 times in 0.6% NaCl (100 ml/rinse).

4. Crush nodules in high speed blender for 3 minutes with enough NaCl
    solution to cover nodules (50-100 ml).

5. Pour crushed nodule slurry into 500 ml beaker and wash blender
    chamber into beaker with NaCl solution.

6. Add NaCl solution to bring total to 250 ml.

7. Allow beaker to stand until cell wall debris settles to bottom
    (5-10 min); an antifoam agent may be desired.

8. Pour supernatant into centrifuge tubes and discard cell wall debris.

9. Centrifuge for 5 min, discard supernatant and resuspend using
    phosphate buffer (Sorensen's) or 0.6% NaCl solution. Phosphate
    buffer might interfer with ectomycorrhizal synthesis. Centrifuge
    used is table top operated at its highest speed.

10. Repeat #9 three times or until supernatant is clear.

11. Bring total volume to 100 ml with buffer solution (phosphate or NaCl).

12. Store sealed under refrigeration until needed.