AN ABSTRACT OF THE THESIS OF

John Lawrence Kough for the degree of Doctor of Philosophy in Botany and Plant Pathology presented on June 1, 1984.

Title: RELATIONSHIP OF EXTRA-MATRICAL HYPHAE OF VESICULAR-ARBUSCULAR MYCORRHIZAL FUNGI TO PLANT GROWTH RESPONSE

Abstract approved: Redacted for Privacy

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The colonization of several species of plants by vesicular-arbuscular mycorrhizae (VAM) was studied to determine how host, soil, or symbiont factors interacted to enhance growth of the host plant.

Seedlings of two species of the Cuppressaceae and two of the Taxodiaceae formed VAM; colonization significantly improved growth. This response to VAM decreased with increasing seedling age and increased phosphorus availability. The initial growth enhancement by VAM was less in species with large seeds than those with small seeds. All three of the VAM fungi tested enhanced seedling growth, although isolates differed in this ability.

Nine different species and strains of VAM fungi were tested for ability to enhance legume growth and nitrogenase activity by Rhizobium nodules and for development of external mycelium (as estimated by sand aggregation) when colonizing Trifolium subterraneum and Lotus corniculatus hosts. The fungi varied in their ability to colonize roots and form external mycelium. Growth was most enhanced by isolates that formed abundant mycelium, but those isolates did not

necessarily colonize the roots extensively. Highest values for nitrogenase activity by Rhizobium nodules (per gm fresh weight) and development of external mycelium were measured at the first harvest before plant growth differed significantly. At later harvests, the treatments with the best growth enhancing isolates or with amendments of more phosphorus had the highest total nitrogenase activities per plant.

To monitor development of VAM hyphae in soil or on roots, an immunofluorescence assay was developed from cell wall extracts of Glomus epigaeum chlamydospores. Antiserum was specific for the genus Glomus with little detectable cross reaction with closely related genera of the endogonaceous fungi Acaulospora and Gigaspora, or other soil fungi. The immunofluorescent assay technique effectively distinguished Glomus species in the rhizosphere of colonized plants.

The density of VAM hyphae was examined in 3 different soil types in relation to the proximity of host root tissue, using the immunofluorescence assay and soil aggregation techniques. The soil aggregation method to measure extra-matrical hyphae was effective only in sandy soils. The immunofluorescence system worked in both sandy and silt loam soils but was not useful in peat soils. A higher density of VAM fungus hyphae occurred in soil near roots than in soil from which root growth was restricted by nylon screening. Root colonization by VAM fungi was greater in river sand than in silt loam or peat, more external hyphae developed in sandy soils than silt loams.

The magnitude of plant growth enhancement is a function of a three-way interaction between host, symbiont, and the soil, but the extra-matrical phase is most influenced by edaphic factors.

RELATIONSHIP OF EXTRA-MATRICAL HYPHAE OF VESICULAR-ARBUSCULAR MYCORRHIZAL FUNGI TO PLANT GROWTH RESPONSE

by

John Lawrence Kough

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Completed June 1, 1984

Commencement June 1985

APPROVED:

Redacted for Privacy

Professor of Botany and Plant Pathology in charge of major

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Date thesis is presented June 1, 1984

Typed by Nancy Kilburn Schoenemann for John Lawrence Kough

ACKNOWLEDGEMENTS

Many people have aided me in my long quest from bewilderment to semi-awareness; I want to express my appreciation for all their help.

I have worked with the personnel of the Horticultural Crops Research Laboratory through three name changes; whatever these people have been called they continued to be more than helpful. I esteem their camaraderie, cooperation and generosity during the time I have made use of their exceptional research facilities. Dr. James Trappe and the personnel at the Forestry Sciences Laboratory have likewise been helpful.

I am obliged to my wife, Nancy Jean Hoos, for her understanding, support, and patience during these investigations. Another Nancy, Nancy Schoenemann, is appreciated for her persistence in typing this thesis and other papers.

Dr. Robert Linderman has provided me with encouragement, support and stimulating discussions. I owe him my gratitude for revealing the complexity and beauty of soil ecology.

This thesis is dedicated to my parents: My father for his love of medicine and our walks in the woods, my mother for her love of plants and her confidence in my decisions.

CONTRIBUTIONS OF AUTHORS

R. G. Linderman and R. Molina helped design the experiments described in Chapter 1. Their suggestions and comments regarding the manuscript were greatly appreciated.

I thank both R. G. Linderman and N. Malajczuk for their assistance with the design of the experiments described in Chapter 3 and their suggestions while reviewing the manuscript. Dr. Malajczuk introduced me to the immunofluorescence methodology and assisted with interpretation of the study results.

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INTRODUCTION

Vesicular-arbuscular mycorrhizae (VAM) enhance growth of many plant species. This enhancement is attributed to the ability of the symbiotic fungus to alleviate nutrient stress, particularly for those nutrients, such as phosphorus, zinc, and copper, that are relatively immobile in the soil. The most convenient means to measure the activity of VAM fungi is to clear and stain roots (Phillips & Hayman, 1970). Examination of such root preparations reveals the presence of intraradical aseptate hyphae, hyphal coils, intracellular arbuscules and, in some species, vesicles that typify VAM colonization.

A VAM fungus has never been shown to benefit plant growth prior to root colonization. However, the capacity of a VAM fungus to colonize root tissue does not always correlate with its ability to enhance plant growth. Fungal isolates can thoroughly colonize roots without a corresponding increase in plant growth (Mosse, 1972; Graham et al., 1982). Other isolates significantly increase growth with only sparse root colonization (Sanders et al., 1977).

The VAM fungus is believed to obtain immobile nutrients to benefit plant growth by its soil mycelium (Mosse, 1959; Tinker, 1975). This mycelium proliferates into soil beyond the nutrient depletion zone of the root, reaching sources of immobile nutrients unavailable to the root and transporting these to the root. Isolates

of VAM fungi differ in ability to produce soil mycelium and, concurrently, in ability to enhance plant growth (Sanders et al., 1977; Graham et al., 1982).

Some VAM host species are more responsive to mycorrhizal symbiosis than others. Plants with thick roots that lack root hairs, so-called magnolioid roots, especially respond to mycorrhizal colonization with increased growth (Baylis, 1974; St. John, 1980). Many economically important tree species have this type of root system.

Mycorrhizal symbiosis aids growth and nitrogen fixation of leguminous species where plant growth is limited by both phosphorus and nitrogen deficiencies (Mosse et al., 1976; Abbott & Robson, 1977). Increased capacity for nitrogen fixation presumably reflects improved phosphorus nutrition resulting from the mycorrhizal symbiosis. Under such conditions, improved nitrogenase activity, measured by acetylene reduction, may serve as an indirect monitor of the phosphorus uptake effectiveness of the mycorrhizal fungus. If effectiveness were correlated well with the development of external mycelium, as suggested by Graham et al., (1982), then nitrogenase activity could become an indirect measure of external mycelium development in soils where the sand aggregation method (Sutton & Sheppard, 1976) does not work.

External hyphae of VAM have been laboriously removed from soils for quantitative analysis (Sanders et al., 1977; Kucey & Paul, 1982). However, it is difficult to distinguish VAM fungal hyphae from hyphae of non-mycorrhizal fungi. Immunofluorescence techniques

have been used successfully with other fungal species to distinguish the desired fungi from other background fungi (Malajczuk et al, 1975; MacDonald & Dunniway, 1979; Frankland et al., 1981). Similar procedures could be useful for VA mycorrhizal fungi. A major obstacle in producing sufficient VAM fungal material for use as an antigen can be overcome by use of a sporocarpic species such as Glomus epigaeum Daniels & Trappe [= Glomus versiforme (Karst) Berch].

The overall objective of the studies reported in this thesis was to develop effective means of monitoring the extra-matrical hyphae of VAM fungi in soil, and to test relationships of amounts of extra-matrical hyphae to observed growth responses of host plants. Experiments were designed to: (1) compare different species of VAM fungi on four woody plant species and two herbaceous legumes, and (2) develop means of observing and monitoring external hyphae in soil in relation to effectiveness of mycorrhizae in growth enhancement.

CHAPTER 1

Mycorrhizal Responsiveness of *Thuja*, *Calocedrus*, *Sequoia* and *Sequoiadendron* Species of Western North America

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SUMMARY

Four western conifers were grown in pasteurized soil noninoculated or inoculated with one of three species of vesicular-arbuscular mycorrhizal (VAM) fungi and maintained at low or moderate levels of phosphorus. VAM colonization increased growth on young more than older seedlings. At the early seedling stage, large seeded species responded less to phosphate addition or VAM colonization than small

seeded species. VAM seedlings at low P were always larger than noninoculated low P controls and comparable in size or larger than noninoculated controls at moderate P. VAM plants grew from 100% to 2000% more than noninoculated plants at low P, and from equality to 500% at moderate P. VAM fungi did not differ in plant growth enhancement and root colonization at any seedling age or phosphorus fertility. Tree species' responsiveness ranged as follows: Thuja plicata > Sequoia sempervirens > Calocedrus decurrens > Sequoiadendron giganteum. VAM enhanced seedling uniformity and size in all the tree species.

INTRODUCTION

Commercial production of tree seedlings routinely includes a biocidal treatment of the planting media for pest control. Such treatments also eliminate beneficial organisms such as mycorrhizal fungi which may be highly sensitive to biocides (Menge, 1982; Trappe et al., 1984).

Growth of plant species that depend on mycorrhizal symbiosis for nutrient absorption may be reduced or uneven in biocide treated soil, even if soil analysis reveals apparently adequate fertility. Woody plants with thick, magnolioid-type roots lacking root hairs depend especially on mycorrhizae for uptake of immobile nutrients such as phosphorus, copper and zinc (Baylis, 1974; St. John, 1980; Silberbush & Barber, 1983). The relative dependence of woody species on mycorrhizae in nature for nutrient uptake and completion of life cycle is hypothetical as nonmycorrhizal specimens of symbiotic

species are rare. However, plant growth responses to VAM colonization are easily quantified. Therefore responsiveness rather than dependency is used to describe the effects of VAM compared to noncolonized tree seedlings in this study. Such tree species, used for food and timber production and landscape plantings, are propagated in nurseries throughout the world. Most tree species appear nutrient deficient when grown in fumigated soil and respond by improved growth when inoculated with appropriate mycorrhizal fungi.

While VAM fungi have been noted by numerous researchers in many western coniferous species (Gerdemann & Trappe, 1974; Mejstrik & Kelley, 1979;), only Bärtschi et al. (1981) and Parke (1982) demonstrated response of any of these trees to VAM inoculation. Neither, however, examined the variation of the VAM response over time or the inoculation response with pure cultures of the VAM fungi.

Our studies were designed to determine the relative mycorrhizal responsiveness of four western tree species commonly grown in nurseries: Sequoia sempervirens (D. Don) Endl, Thuja plicata J. Donn ex D. Don, Calocedrus decurrens (Torr.) Florin, and Sequoiadendron giganteum (Lindl.) Buchh. The growth response was monitored over time after inoculation with three VAM fungi: Glomus deserticola Trappe and Bloss, G. epigaeum Daniels and Trappe [= Glomus versiforme (Karst) Berch], and Acaulospora trappei Ames and Linderman, at two levels of phosphorus fertility.

MATERIALS AND METHODS

Tree species

Seeds of the four tree species were stratified separately in plastic bags with moist paper towels at 5 °C for three weeks, then sown on a flat of 1:1:1 peat, sand and soil which had been pasteurized at 60 °C for 30 min. After two weeks growth, all seedlings had fully expanded cotyledons and were transplanted into plastic growth tubes (Leach "Super-cells", Ray Leach Cone-Tainer Nursery, Canby, OR) with approximately 160 cm^3 of soil mix.

Soil mix

The soil mix was a 1:1 mixture of pasteurized (60 °C/30 min) river sand and silt loam soil with the following characteristics: pH - 6.8, phosphorus - 11 ppm, potassium - 80 ppm, calcium - 8.4 meq/100 g, magnesium - 4.3 meq/100 g, sodium - 0.38 meq/100 g, total N - 0.06%, soluble salts - 0.30 meq/100 g. Twenty ml of VAM inoculum consisting of roots, soil and spores was layered approximately 5 cm below the soil surface in the VAM treatments. The noninoculated control treatments received 20 ml of pasteurized river sand, similarly placed, which had been inoculated with a 1 ml aliquot of a solution of microorganisms from a combination of the three VAM fungal inocula. This solution had passed through a 10 µm filter to exclude VAM propagules. Upon microscopic examination, this solution was seen to contain plant debris, soil protists, bacteria and spores of presumably non-VAM fungi.

VAM inoculum

Colonized roots, spores and river sand from Asparagus officinalis L. plant cultures of Glomus deserticola, G. epigaeum and Acaulospora trappei were chopped and diluted 1:1 with pasteurized river sand. Twenty ml of the mixture, containing spores and root pieces, was used as inoculum for each fungus. Three 20 ml aliquots of each VAM fungus inoculum were examined for spore number by sieving and decanting (Gerdemann & Nicolson, 1963) and percent root length colonized (Biermann & Linderman, 1981) after clearing and staining the roots with trypan blue (Phillips & Hayman, 1970). Twenty ml of inocula were characterized as follows: G. deserticola - 560 spores, root pieces 68% colonized; G. epigaeum - 417 spores, root pieces 31% colonized; A. trappei - 772 spores, root pieces 49% colonized.

Growth conditions

Plants were maintained in the glasshouse at 22/15 °C average day/night temperature with supplemental lighting for a 16 h photoperiod from high pressure sodium vapor lights at an average irradiance of 200 μEinsteins$\cdot sec^{-1} \cdot M^{-2}$. After one month, during which the seedlings were only watered, the plants were fertilized with 10 ml of Long Ashton's Nutrient Solution (LANS) (Hewitt, 1966) with either the full concentration of phosphate (43 ppm) or 1/4 strength (11 ppm). The plants were fertilized biweekly at first, then weekly as growth increased.

Harvest procedures

Emergence of secondary foliage was noted 60 and 75 days after transplanting and top height measured at 100 days. At 180 and 320 days after transplanting, seedlings were removed from the growth tubes, measured for top height and stem diameter, and fresh weight of tops and washed roots determined. The fine roots of ten root systems per tree species/fungus/P fertility regime were sampled at each harvest to determine the % root length with VAM colonization. The roots were cleared with 2 changes of 10% (w/v) KOH for 48 h at 70 °C, bleached for 4 h with 3% H_2O_2, then stained with trypan blue (Phillips & Hayman, 1970; Kormanik et al., 1980), and % root length with VAM colonization determined (Biermann & Linderman, 1981). The remaining root tissue and the shoots were dried to a constant weight at 70 °C. At each harvest, the dried foliage and root tissue for each tree species/fungus/P fertility regime were separately bulked for chemical analysis. Tissue was analyzed by the Plant Analysis Laboratory, Soil Science Department, Oregon State University, Corvallis, Oregon 97331, USA.

Data analysis

The experiment was a complete factorial design of 4 tree species X 4 inoculation treatments X 2 phosphorus fertility regimes in a completely randomized design. The data for each harvest are based on 30 single plant replications for each treatment in the S. sempervirens, S. giganteum, and T. plicata experiments, and 18 single plant replications for C. decurrens. Data were processed by an

analysis of variance with mean separation determined by Tukey's test (Steel & Torrie, 1960).

RESULTS

Sixty days after transplanting, all VAM inoculated seedlings had a higher percentage of individuals with secondary foliage than noninoculated seedlings. The percentages ranged from 89-91% for VAM seedlings at the lower phosphate (11 ppm) LANS compared to 21-47% for the noninoculated seedlings. At full strength phosphate (43 ppm) LANS, percentages of VAM seedlings with secondary foliage ranged from 83-91% compared to 38-66% for noninoculated seedlings. Two weeks later this developmental difference was less distinct at the higher phosphate LANS (98-100% for VAM seedlings compared to 78-97%) but persisted at the lower phosphate LANS especially in S. sempervirens (95% in VAM compared to 34%) and T. plicata (100% in VAM compared to 60%).

At 100 days after transplanting, the growth differential between VAM and non-VAM seedlings continued to be manifest. All the VAM-inoculated seedlings were significantly larger than the noninoculated seedlings irrespective of the phosphate fertility regime (Fig. 1-1). Noninoculated seedlings of S. giganteum and T. plicata were significantly taller with the higher phosphate regime than the lower, indicating that plants were already deficient for this nutrient in the lower phosphate regime (11 ppm). In addition, seedlings of T. plicata inoculated with G. deserticola and A. trappei had

significantly greater top height when grown at the higher phosphate LANS than when grown at lower phosphate LANS.

At 180 days after transplanting, noninoculated seedlings of all four of the tree species showed significantly increased height, stem diameter, fresh and dry weights in response to increased concentrations of phosphate; C. decurrens responsed less to increased phosphate. The growth increases in noninoculated seedlings with higher phosphate indicated that growth of all species had become phosphate-limited in the 1/4 phosphorus LANS (11 ppm) regime. Cortical cells in the roots of the VAM inoculated treatments were colonized by VAM fungi. Percentage root colonization by different symbionts did not differ significantly in relation to phosphorus fertility within the tree species, except that G. deserticola colonized roots more than A. trappei at lower phosphate in S. sempervirens (Table 1-2).

All VAM seedlings at 180 days were consistently larger than noninoculated seedlings in the measured growth indices regardless of phosphorus fertility (Tables 1-1, 1-2, 1-3, 1-4). Even the VAM seedlings with 1/4 phosphate LANS were larger than noninoculated seedlings at full strength LANS. However, regardless of the species of mycorrhizal fungus, there were few significant differences in growth response of VAM colonized seedlings resulting from the fertility regime or species of tree. In general for a given tree species, all VAM seedlings were of similar size.

At 320 days after transplanting, the growth differential between noninoculated seedlings and VAM seedlings at full strength phosphate

(43 ppm) LANS was reduced. This was particularly true for S. giganteum, where production of plant biomass by high phosphate LANS noninoculated seedlings equalled that of the VAM seedlings (Table 1-4). For other tree species, growth enhancement from VAM remained significant, although decreased in magnitude, compared to values obtained at 180 days (Table 1-2, 1-3). With T. plicata, biomass of VAM-colonized seedlings was increased in magnitude compared to noninoculated seedlings, which continued to grow very slowly (Table 1-1).

This variable response to VAM is shown by mycorrhizal reponsiveness values (Table 1-5), derived by comparing total dry matter produced by a VAM seedling at a given fertility level to that produced by a noninoculated seedlings at the same fertility regime. The response of the seedlings to VAM decreased with time except for the 1/4 phosphate LANS T. plicata seedlings. The magnitude of response was always lowered at the higher phosphate regime, reaching equality between noninoculated and VAM colonized seedlings of S. giganteum at 320 days. In general, T. plicata and S. sempervirens had greater growth enhancement than C. decurrens and S. giganteum under phosphate-limited conditions. C. decurrens was less responsive to VAM colonization than the other species at the first harvest.

Besides being larger than nonmycorrhizal seedlings, VAM seedlings were more uniform. This is indicated by the lower coefficients of variability for top height and stem diameter (Table 1-6). Regardless of their mycorrhizal status, S. sempervirens

seedlings were more variable than the other species in these dimensions.

While tree species differed significantly in their physical dimensions according to their mycorrhizal condition, the concentrations of nutrients in their foliage or roots were substantially the same for a given tree species (Table 1-7, 1-8, 1-9, 1-10). However, nitrogen, sulfur or phosphorus contents did differ, especially between noninoculated seedlings at different levels of fertility. The mineral concentrations for VAM seedlings and the full strength LANS noninoculated seedlings were essentially the same. VAM colonization increased sulfur uptake in S. giganteum. Manganese and nitrogen in the foliage of noninoculated seedlings was greater at 1/4 strength phosphate LANS.

Fungal colonization of the root tissue was erratic: large portions of cortical tissue were not colonized while other portions were heavily colonized. Vesicles were evident in roots of all VAM inoculated seedlings, especially those harvested at 320 days. Coiled hyphae, the most frequently observed evidence of fungal colonization, were scattered in the outer cortical cells just beneath the epidermal layer and rarely occurred in adjacent cells. Arbuscules were rarely observed. Characteristic vesicles and abundant external hyphae were seen on roots colonized by A. trappei. Root hairs were rare on either inoculated or noninoculated seedlings.

In general, seedlings colonized by G. deserticola were larger than seedlings colonized by the other two species. Occasionally, this difference was statistically significant. Trees colonized by A.

trappei or G. epigaeum sometimes had significantly greater biomass at the higher phosphate (43 ppm) LANS regime (Table 1-6). Higher phosphate VAM seedlings were sometimes smaller than the low phosphate VAM seedlings, but this decrease was rarely statistically significant (Table 1-2).

DISCUSSION

Response to VAM inoculation of the four conifers examined in this study was modified by phosphorus fertility, the tree species inoculated, and to a lesser extent, by the species of VAM fungus. VAM seedlings of all the tree species were always significantly larger than noninoculated controls when both were maintained with 1/4 strength phosphate LANS. These VAM seedlings were equal to or larger than noninoculated seedlings maintained at the full strength phosphate LANS. Growth enhancement from mycorrhizal fungus colonization generally decreased as seedlings aged.

Reduction in magnitude of growth enhancement over time and with high phosphate LANS which occurred in mycorrhizal S. giganteum, but not other conifer species could be explained by an inherent inefficiency in the roots of the latter species compared to S. giganteum. Weekly fertilization should have provided enough phosphate to saturate all phosphate binding sites in the soil so that added phosphate would be readily available to the roots (Marconi & Nelson, 1984). Even so, the higher level of phosphate was apparently inadequate for sufficient P uptake all species but S. giganteum.

Conversely, constricted root growth in VAM seedlings may have slowed their further development and allowed noninoculated seedlings to equal the VAM seedlings' growth by 320 days. This possibility is indicated by the fact that the root masses of S. giganteum seedlings at 320 days, unlike the three other species, did not differ significantly between noninoculated and VAM seedlings at the higher level of phosphate fertility. Finally, the difference in root physiology between these conifer species could reflect an ecological adaptation: S. giganteum characteristically occurs on drier sites than either T. plicata or S. sempervirens. However, it does coexist with C. decurrens suggesting that rooting strategy or phosphate utilization may differ between these two species.

Young seedlings clearly benefitted more from colonization by VAM fungi than older seedlings. From the time of emergence of the true foliage to the 320 day stage of growth, VAM seedlings developed faster than non-VAM seedlings regardless of phosphorus fertility. At the seedling stage of plant growth, phosphorus uptake is limited by the small volume of soil explored by the root system. Uptake of phosphorus by VAM hyphae exploring a greater soil volume could significantly aid seedling nutrition. Seedlings, especially those with magnolioid-type roots, are therefore more responsive to mycorrhizae colonization than older plants (Baylis, 1962; Harley, 1978). This does not imply older plants do not require or benefit from VAM but growth response to VAM differs with age. Critical work exploring the role of VAM compared to noncolonized older plants and trees has yet to be done.

While phosphorus is very mobile in plant tissue in most instances, the only phosphorus reserve in young seedlings comes from the seed itself. Species with large seed reserves might be expected to lag in VAM induced growth enhancement compared to plant species with smaller seeds. C. decurrens, which has the largest seeds of the species examined in this study, responded less to VAM under phosphorus-limiting conditions in the early growth stage. Kormanik et al. (1982) found hardwood tree species, such as Juglans nigra, were less responsive to VAM inoculation and surmised this could be due to the large seed reserves.

Although noninoculated seedlings maintained with 1/4 strength phosphate LANS had lower tissue phosphorus and higher nitrogen concentrations than VAM seedling, other minerals were similar within a given species despite significant growth differences resulting from VAM inoculation. Numerous accounts of phosphorus and other mineral concentrations have been reported in herbaceous species colonized with VAM fungi (Gerdemann, 1975). However, most reports on woody species confirm that no significant differences in nutrient concentrations occur, but rather more tissue is produced (Plenchette et al., 1981; Pope et al., 1982; Schultz et al., 1982).

Ecologically, tree species are considered to be tolerant of certain environmental stresses compared to herbaceous species (Grime, 1978). Tree species survive periods of stress as vegetative structures rather than dormant seeds or storage organs like herbaceous species. Due to their greater age at sexual maturity, tree species must also survive more of these stress periods than

herbs prior to completion of their life cycle. Stress tolerant species have lower growth rates under ideal growth conditions and display less morphologic plasticity under stress compared to herbaceous species. Characters such as tissue nutrient concentration and shoot to root ratios are believed to vary less in stress tolerant tree species than in herbaceous ruderal species (Chapin, 1983; St. John & Coleman, 1983). However, the tree species examined here do respond to the addition of phosphorus with both increased tissue mass and mineral concentration even without VAM colonization. In addition, it has been clearly shown here that these responses as well as growth rate can be significantly altered with colonization by VAM fungi indicating these assumptions should be reexamined.

Glomus epigaeum and G. deserticola have both been shown to be effective VAM fungi in studies on other tree hosts (Graham et al., 1982; Pope et al., 1982; Furlan et al., 1984). In the present study, these two species significantly increased growth compared to the noninoculated controls. G. deserticola tended to give better overall growth enhancement than the other fungi tested on the host plants examined here. Occasionally, this seedling response was significantly greater than that provided by either G. epigaeum or A. trappei colonized seedlings. G. deserticola colonized seedlings did not produce significantly greater plant biomass with higher phosphate addition whereas both A. trappei and G. epigaeum colonized seedlings did in numerous instances. The reasons for these different isolate responses to phosphorus are unclear.

Plant growth responses to colonization by these fungi differed significantly despite their similar level of colonization in cortical tissue of the root. These differences between VAM fungi in terms of plant growth enhancement, occurred with respect to phosphorus fertility, seedling age and tree species. Given the similar level of root colonization, the differences may indicate physiological variations among the fungi. One explanation may be that a species like G. deserticola, which does not respond to higher inputs of phosphorus, could have a lower K_m for phosphate. This would render this fungus unresponsive to increased phosphate, since it would be operating at peak efficiency at the lower phosphate level. Differences in the amount of extra-matrical hyphae produced by the different VAM fungal isolates could also account for the variable responses of the seedlings (Graham et al., 1982).

Growth of all four tree species responded significantly to VAM colonization. Considering the magnitude of the increase in growth, especially with T. plicata and S. sempervirens, it seems feasible to consider them to be VAM dependent hosts. However, all of these species also showed a significant growth response to increased phosphate fertility when not colonized with VAM fungi. This response to increased phosphorus indicates that the trees are responsive to mycorrhizae but not dependent (Gerdemann, 1975). These results also emphasize the fact that mycorrhizal responsiveness is a function of a three-way interaction: the host plant and the fungus endophyte mediated by physical and biological features of the soil.

VAM colonization increased seedling uniformity as well as size compared to the noninoculated controls. This effect of VAM inoculation has been reported on other hosts (Cooper, 1981; Janos, 1981; Biermann & Linderman, 1983) and indicates an additional economic factor in favor of VAM fungus inoculation of fumigated soil. Greater seedling size and uniformity with VAM inoculation means more seedlings acceptable for outplanting and less loss from culling. In relation to nursery management, the addition of phopshate increased crop uniformity in the noninoculated seedlings, while it slightly decreased seedling uniformity in VAM seedlings. This anomaly indicates the sensitivity of the VAM relationship to soil fertility, and suggests that nursery practices be examined for their effect on indigenous VAM activity. Since the world's supply of readily-mined phosphate is limited and, at present rates of use, will be exhausted in the next century, VAM savings in phosphate fertilizer are worthy of consideration even if the dollar savings at the moment are not important.

Table 1-1. Biomass and VAM colonization of *Thuja plicata* seedlings 180 and 320 days after inoculation.

VAM species fertility treatment*	180 days				320 days			
	Fresh weight (g)	Dry weight (g)	Shoot/ root ratio	% root length VAM	Fresh weight (g)	Dry weight (g)	Shoot/ root ratio	% root length VAM
NC 1/4 P	0.06d	0.05b	1.18c	0	1.44a	0.18a	1.43a	0
NC 1 P	0.32c	0.14b	1.44bc	0	8.74b	2.33b	1.65abc	0
GD 1/4 P	2.12a	0.86a	2.13a	30a	13.59c	3.84c	1.87bcd	28a
GD 1 P	2.07a	0.88a	1.88ab	30a	14.35cd	3.78c	1.80bcd	25a
GE 1/4 P	2.18a	0.94a	2.06a	38a	14.06cd	3.84c	1.57ab	31a
GE 1 P	2.04ab	0.80a	2.25a	32a	13.81cd	4.00c	1.93cd	26a
AT 1/4 P	1.80b	0.72a	1.94ab	39a	14.45cd	3.74c	1.60ab	35a
AT 1 P	1.95ab	0.81a	2.28a	40a	14.95d	4.05c	1.98d	34a

* VAM fungus treatments: NC - non-inoculated control; GD - *Glomus deserticola*; GE - *Glomus epigaeum*; AT - *Acaulospora trappei*. Fertilizer treatments: 1 P - full strength phosphate LANS; 1/4 P - 1/4-strength phosphate LANS. Means within columns not followed by the same letter are significantly different at $\alpha = 0.05\%$ by Tukey's HSD.

Table 1-2. Biomass and VAM colonization of Sequoia sempervirens seedlings 180 and 320 days after inoculation.

VAM species fertility treatment*	180 days				320 days			
	Fresh weight (g)	Dry weight (g)	Shoot/ root ratio	% root length VAM	Fresh weight (g)	Dry weight (g)	Shoot/ root ratio	% root length VAM
NC 1/4 P	0.97c	0.26d	1.15ab	0	6.05a	0.74a	1.23c	0
NC 1 P	3.28b	0.67c	1.46a	0	12.45bc	3.18b	1.10bc	0
GD 1/4 P	6.36a	1.35a	1.13ab	28a	14.97d	4.09cd	0.87ab	31a
GD 1 P	5.99a	1.28ab	1.14ab	26ab	14.22d	4.14d	0.90ab	30a
GE 1/4 P	5.64a	1.18a	0.96b	24ab	12.09bc	3.31b	0.81a	29a
GE 1 P	5.56a	1.04b	1.24ab	17ab	11.53b	3.38bc	0.91ab	28a
AT 1/4 P	6.32a	1.28ab	1.27ab	16b	13.59cd	3.53bcd	0.98abc	33a
AT 1 P	6.22a	1.30ab	1.25ab	25ab	12.21bc	3.82bcd	0.89ab	32a

* See Table 1-1 for abbreviations VAM fungus and fertilizer treatments. Means within columns not followed by the same letter are significantly different at $\alpha = 0.05\%$ by Tukey's HSD.

Table 1-3. Biomass and VAM colonization of Calocedrus decurrens seedlings 180 and 320 days after inoculation.

VAM species fertility treatment*	180 days				320 days			
	Fresh weight (g)	Dry weight (g)	Shoot/ root ratio	% root length VAM	Fresh weight (g)	Dry weight (g)	Shoot/ root ratio	% root length VAM
NC 1/4 P	1.92d	0.54b	1.04a	0	5.64c	2.03a	0.80a	0
NC 1 P	3.08cd	0.70b	1.10a	0	8.73b	2.79ab	0.89a	0
GD 1/4 P	5.86a	1.57a	1.06a	18a	12.44a	4.18cd	0.79a	31a
GD 1 P	5.34ab	1.47a	1.06a	28a	13.26a	4.76d	0.83a	35a
GE 1/4 P	5.60ab	1.37a	1.03a	23a	12.07a	3.80bcd	0.76a	34a
GE 1 P	5.68ab	1.57a	0.96a	27a	12.95a	4.04cd	0.92a	31a
AT 1/4 P	4.50b	1.49a	0.96a	17a	12.06a	3.64bc	0.88a	36a
AT 1 P	4.11bc	1.33a	1.07a	22a	12.78a	4.32cd	0.78a	35a

* See Table 1-1 for abbreviations of VAM fungus and fertilizer treatments. Means within columns not followed by the same letter are significantly different at $\alpha = 0.05\%$ by Tukey's HSD.

Table 1-4. Biomass and VAM colonization of Sequoiadendron giganteum seedlings 180 and 320 days after inoculation.

VAM species fertility treatment*	180 days Fresh weight (g)	180 days Dry weight (g)	180 days Shoot/ root ratio	180 days % root length VAM	320 days Fresh weight (g)	320 days Dry weight (g)	320 days Shoot/ root ratio	320 days % root length VAM
NC 1/4 P	2.12c	0.41d	1.04b	0	8.44a	1.78b	0.94a	0
NC 1 P	5.41b	1.02c	1.11b	0	15.81e	4.38a	0.91a	0
GD 1/4 P	5.86ab	1.35ab	1.14b	32a	12.69b	4.04a	1.03a	23bc
GD 1 P	5.98ab	1.27ab	1.18ab	36a	15.40de	4.45a	1.11a	21c
GE 1/4 P	6.20ab	1.30ab	1.32ab	34a	13.66bc	4.09a	0.93a	28ab
GE 1 P	6.02ab	1.14bc	1.48a	30a	15.83e	4.50a	0.98a	25abc
AT 1/4 P	5.85ab	1.42a	1.13b	25a	14.54cd	4.01a	0.97a	27ab
AT 1 P	6.42a	1.41a	1.29ab	25a	16.25e	4.55a	1.02a	30a

* See Table 1-1 for abbreviations of VAM fungus and fertilizer treatments. Means within columns not followed by the same letter are significantly different at $\alpha = 0.05\%$ by Tukey's HSD.

Table 1-5. Mycorrhizal responsiveness* of four western North America conifers† inoculated with three different VAM fungi‡ and maintained at two levels of phosphorus fertility‡.

	--- TP ---		--- SS ---		--- CD ---		--- SG ---	
	180 days	320 days	180 days	320 days	180 days	320 days	180 days	320 days
GD 1/4 P	17.2	21.3	5.2	2.9	2.9	2.1	3.3	2.3
GD 1 P	6.3	1.6	1.9	1.3	2.1	1.7	1.2	1.0
GE 1/4 P	18.8	21.4	4.5	2.3	2.5	1.9	3.2	2.3
GE 1 P	5.7	1.7	1.6	1.1	2.2	1.4	1.1	1.0
AT 1/4 P	14.4	20.8	4.9	2.6	2.8	1.8	3.5	2.3
AT 1 P	5.9	1.7	1.9	1.2	1.9	1.5	1.4	1.0

* Responsiveness values are the means of the total dry weight for each fungus/fertility treatment divided by the mean of the total dry weight of the corresponding noninoculated control at the same level of phosphate fertility.

† Conifers were Thuja plicata (TP), Sequoia sempervirens (SS), Calocedrus decurrens (CD), and Sequoiadendron giganteum (SG).

‡ See Table 1-1 for abbreviations of VAM fungus and fertility treatments.

Table 1-6. Effect of mycorrhizal colonization of 320-day-old seedlings on dimensions used for selection of stock suitable for outplanting.

	Thuja plicata				*Sequoia sempervirens*				*Calocedrus decurrens*				*Sequoiadendron giganteum*			
	Top height (cm)		Stem diameter (cm)		Top height (cm)		Stem diameter (cm)		Top height (cm)		Stem diameter (cm)		Top height (cm)		Stem diameter (cm)	
NC 1 P*	13.6b	(20.7)†	3.1b	(15.5)	8.4b	(18.1)	4.0b	(24.9)	10.9b	(17.9)	3.4b	(25.7)	8.4b	(17.9)	3.8b	(11.0)
NC 1/4 P	4.0a	(17.8)	1.1c	(15.4)	6.9a	(21.6)	2.8a	(23.6)	8.6a	(18.0)	2.8a	(25.7)	6.8a	(20.3)	2.8a	(10.4)
GD 1 P	16.1d	(12.7)	3.7a	(7.3)	10.9d	(13.8)	4.5bc	(11.3)	13.0bc	(9.4)	3.9c	(9.2)	8.4b	(11.3)	4.3d	(8.6)
GD 1/4 P	17.0d	(11.4)	3.8a	(9.0)	10.7d	(11.3)	4.7c	(11.1)	13.3c	(10.8)	3.9c	(9.7)	8.2b	(14.9)	4.0bc	(7.0)
GE 1 P	16.2d	(11.6)	3.7a	(8.7)	8.8bc	(22.5)	4.4bc	(16.4)	12.9bc	(7.4)	4.0c	(9.0)	8.3b	(12.5)	4.5d	(9.8)
GE 1/4 P	14.6bc	(10.8)	3.9a	(10.8)	8.8bc	(16.1)	4.5bc	(14.4)	12.3bc	(11.4)	3.8bc	(6.3)	7.8ab	(11.4)	4.2cd	(10.5)
AT 1 P	15.8cd	(9.4)	3.8a	(6.0)	10.0cd	(18.0)	4.6c	(11.5)	12.1b	(12.8)	3.8bc	(5.3)	7.8ab	(11.0)	4.2c	(10.0)
AT 1/4 P	14.3b	(8.4)	3.8a	(6.0)	9.3bc	(14.8)	4.5bc	(12.2)	12.8bc	(8.1)	3.9c	(7.4)	7.6ab	(17.8)	4.0bc	(9.0)

* See Table 1-1 for abbreviations of VAM fungus and fertility treatments.

† Means within a column not significantly different at $\alpha = .05$ by Tukey's HSD are followed by the same letter. Values in parentheses represent the coefficient of variability for each treatment derived from the treatment standard deviation divided by the treatment mean expressed as a percentage.

Table 1-7. Foliar mineral concentrations in mycorrhizal and non-mycorrhizal seedlings of *Sequoia sempervirens*.

VAM fungus treatment and harvest*	Mineral concentrations				
	N†	P	K	S	Mn
NC 1/4 P 1	1.55	.07	1.59	.16	104
2	1.09	.07	1.47	.12	77
NC 1 P 1	1.26	.15	1.69	.16	75
2	0.85	.12	1.04	.19	73
GD 1/4 P 1	1.03	.13	1.20	.20	71
2	0.97	.07	.96	.19	57
GD 1 P 1	1.09	.16	1.19	.20	73
2	1.03	.13	.94	.19	61
GE 1/4 P 1	1.38	.19	1.33	.22	71
2	1.00	.09	1.14	.20	54
GE 1 P 1	1.15	.18	1.33	.22	69
2	0.94	.15	1.17	.19	60
AT 1/4 P 1	1.15	.15	1.28	.21	71
2	1.00	.08	.82	.17	60
AT 1 P 1	1.15	.20	1.31	.21	64
2	1.00	.13	.88	.16	65

* VAM fungus treatments: NC - noninoculated; GD - *Glomus deserticola*; GE - *Glomus epigaeum*; AT - *Acaulospora trappei*. Fertility regimes: 1 P - full strength phosphate LANS; 1/4 P - 1/4 phosphate LANS. Harvests: 1 - 180 days; 2 - 320 days.

† N, P, K, and S values represent percentage dry weight mineral concentration values. Mn is expressed as parts per million.

Table 1-8. Foliar mineral concentrations in mycorrhizal and non-mycorrhizal seedlings of *Calocedrus decurrens*.

VAM fungus treatment and harvest*	Mineral concentrations				
	N†	P	K	S	Mn
NC 1/4 P 1	1.61	.12	1.15	.12	108
2	1.22	.09	1.04	.20	79
NC 1 P 1	1.44	.15	1.25	.14	100
2	1.15	.12	1.03	.11	74
GD 1/4 P 1	1.38	.15	1.25	.14	88
2	1.15	.09	1.08	.13	63
GD 1 P 1	1.32	.18	1.43	.15	101
2	1.09	.12	1.09	.12	64
GE 1/4 P 1	1.38	.15	1.31	.13	86
2	1.09	.09	1.05	.14	61
GE 1 P 1	1.38	.18	1.31	.14	85
2	1.09	.13	1.11	.14	62
AT 1/4 P 1	1.38	.15	1.22	.15	102
2	1.09	.11	1.02	.14	67
AT 1 P 1	1.32	.17	1.27	.15	101
2	1.03	.12	1.09	.12	64

* VAM fungus treatments: NC - noninoculated; GD - *Glomus deserticola*; GE - *Glomus epigaeum*; AT - *Acaulospora trappei*. Fertility regimes: 1 P - full strength phosphate LANS; 1/4 P - 1/4 phosphate LANS. Harvests: 1 - 180 days; 2 - 320 days.

† N, P, K, and S values represent percentage dry weight mineral concentration values. Mn is expressed as parts per million.

Table 1-9. Foliar mineral concentrations in mycorrhizal and non-mycorrhizal seedlings of *Sequoiadendron giganteum*.

VAM fungus treatment and harvest*	Mineral concentrations				
	N†	P	K	S	Mn
NC 1/4 P 1	2.06	.08	1.15	.28	139
2	1.36	.07	.98	.13	96
NC 1 P 1	1.40	.14	1.34	.19	130
2	0.94	.10	.61	.15	90
GD 1/4 P 1	1.32	.15	1.15	.22	79
2	1.00	.08	.74	.24	49
GD 1 P 1	1.32	.15	1.26	.23	75
2	0.94	.10	.74	.20	43
GE 1/4 P 1	1.09	.15	1.20	.25	97
2	1.00	.07	.71	.22	64
GE 1 P 1	1.55	.22	1.28	.29	108
2	1.00	.10	.63	.23	61
AT 1/4 P 1	1.34	.16	1.15	.28	85
2	1.06	.08	2.13	.23	57
AT 1 P 1	1.28	.18	1.03	.25	79
2	0.94	.10	.66	.23	62

* VAM fungus treatments: NC - noninoculated; GD - *Glomus deserticola*; GE - *Glomus epigaeum*; AT - *Acaulospora trappei*. Fertility regimes: 1 P - full strength phosphate LANS; 1/4 P - 1/4 phosphate LANS. Harvests: 1 - 180 days; 2 - 320 days.

† N, P, K, and S values represent percentage dry weight mineral concentration values. Mn is expressed as parts per million.

Table 1-10. Foliar mineral concentrations in mycorrhizal and non-mycorrhizal seedlings of Thuja plicata.

VAM fungus treatment and harvest*	Mineral concentrations				
	N†	P	K	S	Mn
NC 1/4 P 1	1.89	.09	--‡	--	--
2	1.48	.08	1.52	.13	120
NC 1 P 1	2.06	.14	1.55	.19	113
2	1.12	.17	1.36	.12	86
GD 1/4 P 1	0.61	.08	1.20	.12	60
2	1.12	.12	1.14	.11	64
GD 1 P 1	1.28	.21	1.24	.14	69
2	1.12	.16	1.22	.10	65
GE 1/4 P 1	1.22	.16	1.15	.12	62
2	1.06	.11	1.12	.12	56
GE 1 P 1	1.22	.19	1.19	.12	63
2	1.18	.17	1.15	.11	56
AT 1/4 P 1	1.40	.17	1.20	.16	71
2	1.12	.12	1.13	.12	58
AT 1 P 1	1.34	.21	1.24	.18	74
2	1.18	.17	1.22	.12	54

* VAM fungus treatments: NC - noninoculated; GD - Glomus deserticola; GE - Glomus epigaeum; AT - Acaulospora trappei. Fertility regimes: 1 P - full strength phosphate LANS; 1/4 P - 1/4 phosphate LANS. Harvests: 1 - 180 days; 2 - 320 days.

† N, P, K, and S values represent percentage dry weight mineral concentration values. Mn is expressed as parts per million.

‡ Insufficient tissue for analysis.

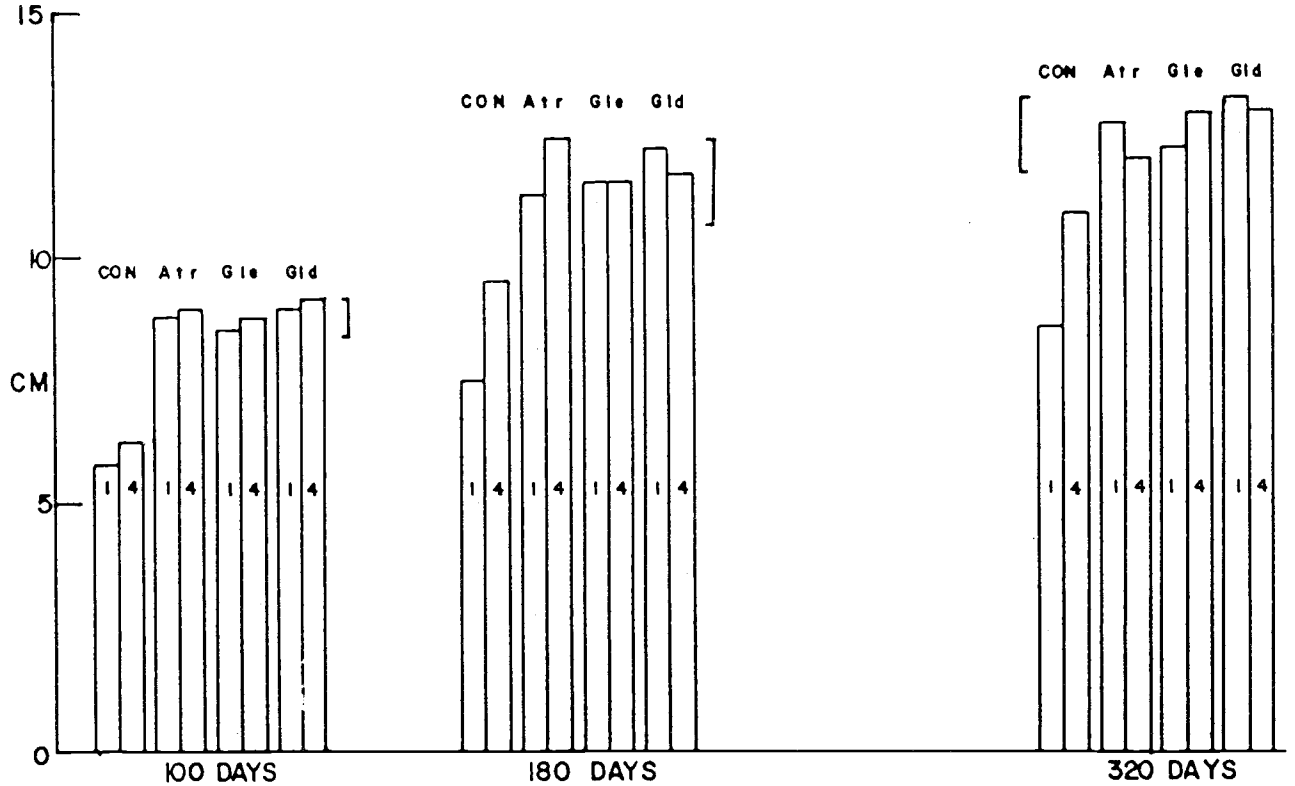

Figure 1-1. Effect of mycorrhizal colonization on top height of Calocedrus decurrens at three dates after inoculation.
(CON - control; Atr - A. trappei, Gle - G. epigaeum; Gld - G. deserticola; 1 - 1/4 P LANS; 4 - full strength P LANS. Brackets represent Honestly Significant Difference.)

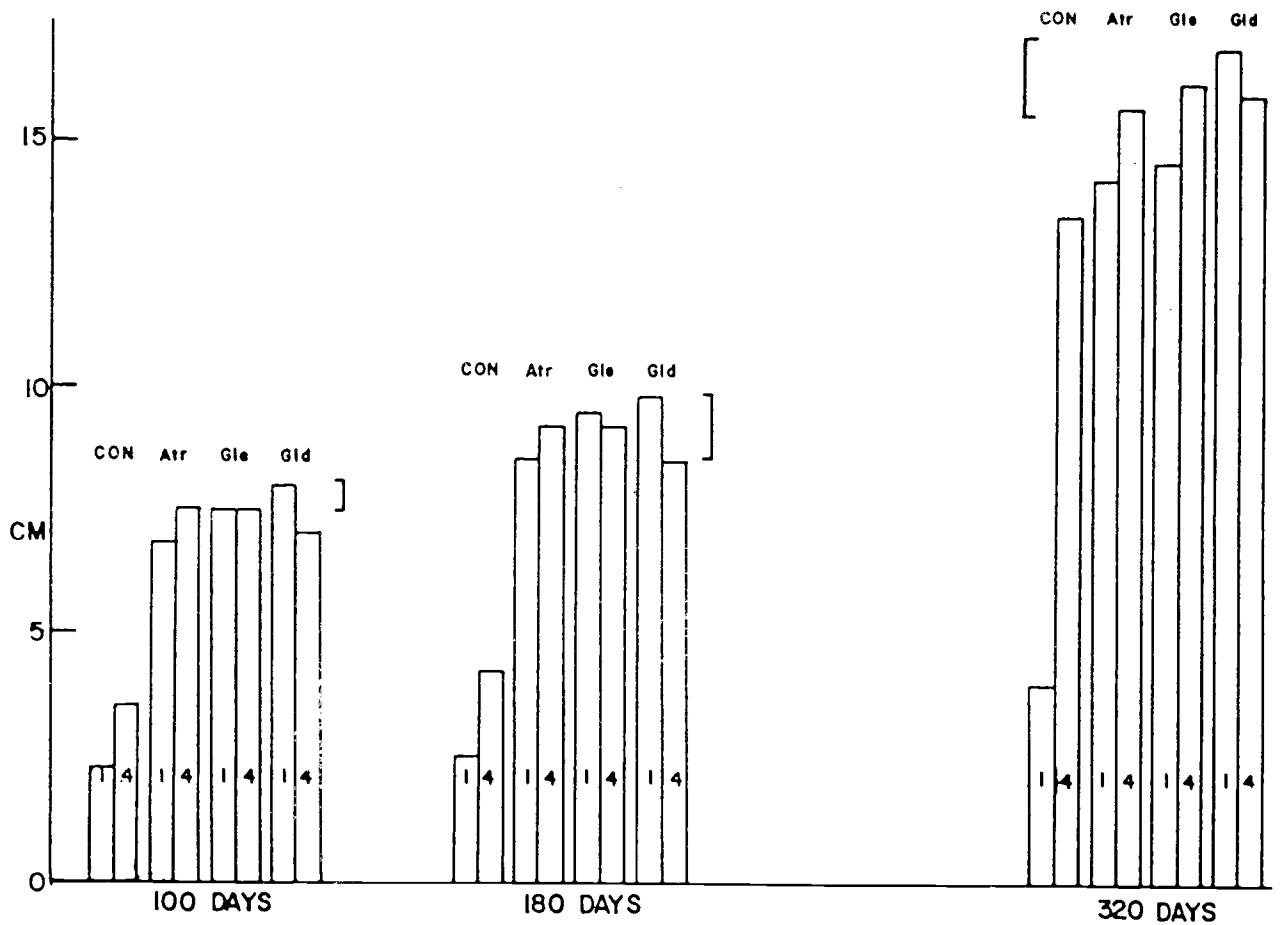

Figure 1-2. Effect of mycorrhizal colonization on top height of Thuja plicata at three dates after inoculation.

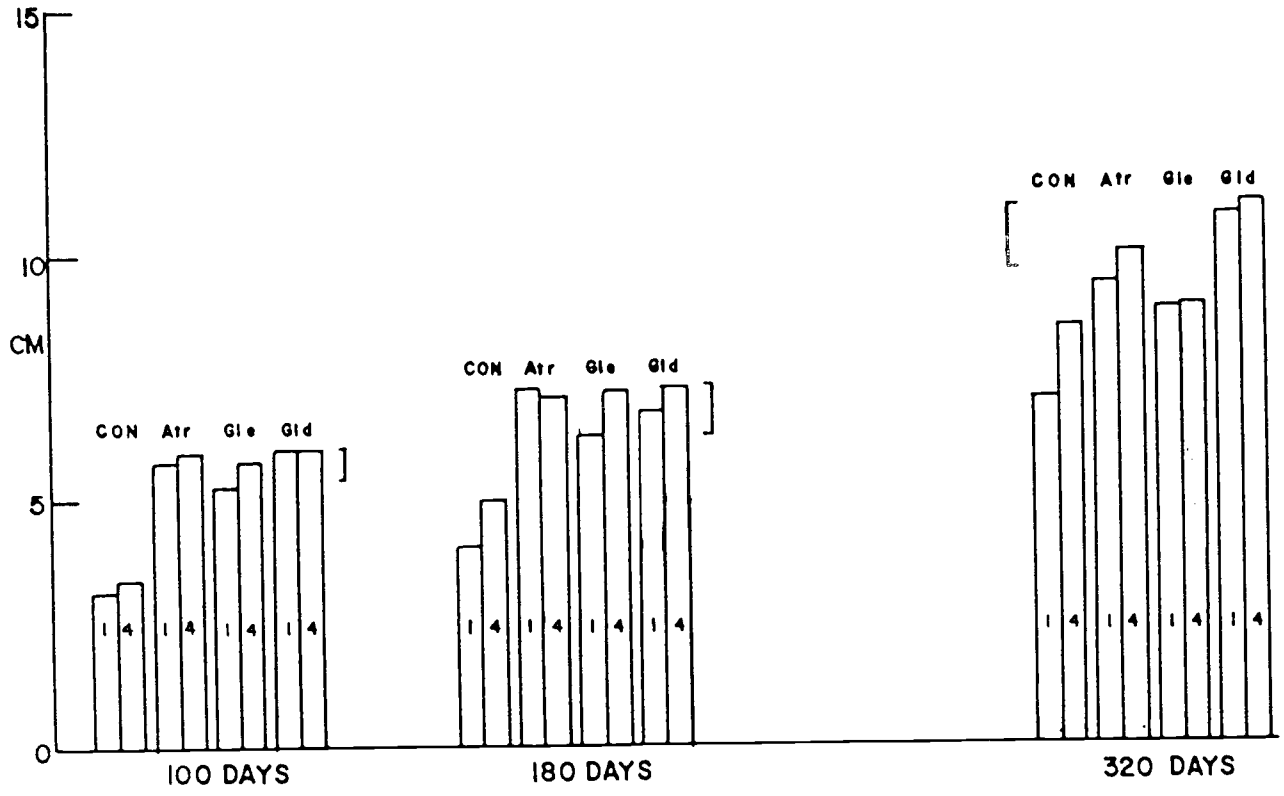

Figure 1-3. Effect of mycorrhizal colonization on top height of Sequoia sempervirens at three dates after inoculation.
(See Fig. 1-1 for abbreviations of VAM fungus and fertility treatments.)

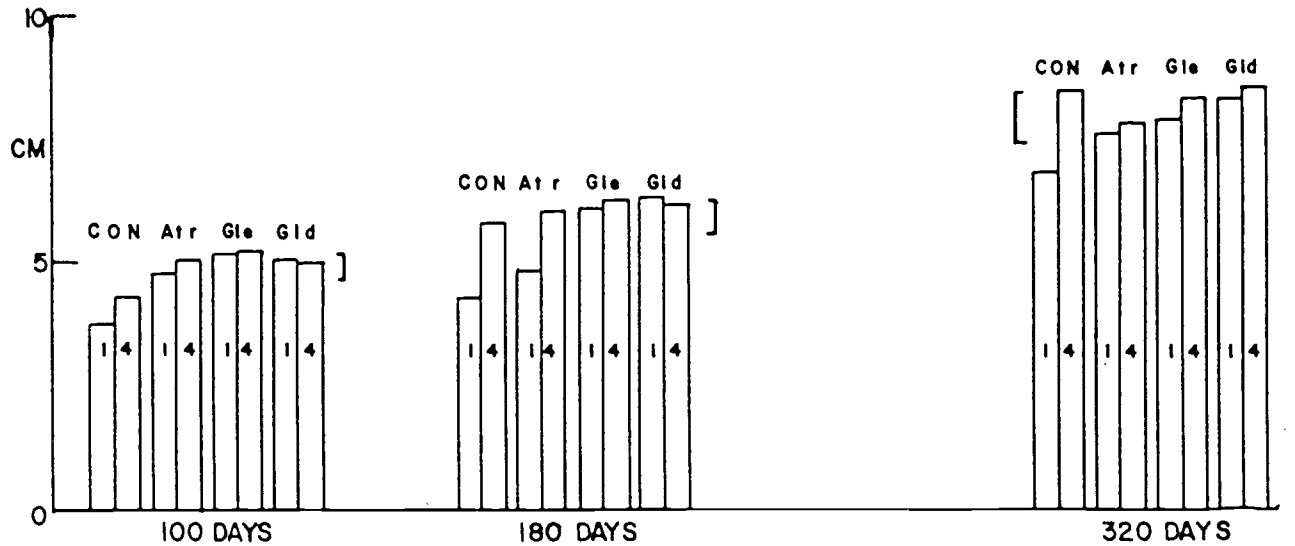

Figure 1-4. Effect of mycorrhizal colonization on top height of Sequoiadendron giganteum at three dates after inoculation.
(See Fig. 1-1 for abbreviations of VAM fungus and fertility treatments.)

REFERENCES

BÄRTSCHI, H., GIANINAZZI-PEARSON, V. & VEGH, I. (1981). Vesicular-arbuscular mycorrhiza formation and root rot disease (Phytophthora cinnamomi) development in Chamaecyparis lawsoniana. Phytopathologische Zeitschrift, **102**, 213-218.

BAYLIS, G. T. S. (1962). Rhizophagus, the catholic symbiont. Australian Journal of Science, **25**, 195-200.

BAYLIS, G. T. S. (1975). The magnolioid mycorrhiza and mycotrophy in root systems derived from it. In: Endomycorrhizas (Ed. by F. E. Sanders, Barbara Mosse & P. B. Tinker), pp. 149-174. Academic Press, New York & London.

BIERMANN, B. & LINDERMAN, R. G. (1981). Quantifying vesicular-arbuscular mycorrhizae: a proposed method towards standardization. New Phytologist, **87**, 63-67.

BIERMANN, B. & LINDERMAN, R. G. (1983). Increased geranium growth using pretransplant inoculation with a mycorrhizal fungus. Journal of the American Society of Horticulture, **108**, 972-976.

CHAPIN, F. S, III. (1983). Adaptation of selected trees and grasses to low availability of phosphorus. Plant and Soil, **72**, 283-287.

COOPER, K. M. (1981). The role of VA mycorrhizas in the development of a new commercial crop - tamarillo - in New Zealand. Proceedings of the 5th North American Conference on Mycorrhizae, Universite Laval, Quebec, p. 54 (Abstract).

FURLAN, V., FORTIN, J. A. & PLENCHETTE, C. (1983). Effects of different vesicular-arbuscular fungi on growth of Fraxinus americana. Canadian Journal of Forest Research, **13**, 589-593.

GERDEMANN, J. W. & NICOLSON, T. H. (1963). Spores of mycorrhizal Endogone species extracted from soil by wet sieving and decanting. Transactions of the British Mycological Society, **46**, 235-244.

GERDEMANN, J. W. & TRAPPE, J. M. (1974). The Endogonaceae in the Pacific Northwest. Mycologia Memoirs, **5**, 1-76.

GRAHAM, J. H., LINDERMAN, R. G. & MENGE, J. A. (1982). Development of external hyphae by different isolates of mycorrhizal Glomus spp. in relation to root colonization and growth of Troyer citrange. New Phytologist **91**, 183-189.

HARLEY, J. L. (1978). Ectomycorrhizas as nutrient absorbing organs. Proceedings of the Royal Society of London, Series B, **203**, 1-21.

GRIME, J. P. (1979). Plant Strategies and Vegetation Processes. John Wiley & Sons, Chichester and New York.

HEWITT, E. J. (1966). Sand and water culture methods used in the study of plant nutrition. In: Technical Communication No. 22, 2nd Edn, revised. Commonwealth Agricultural Bureaux, London.

JANOS, D. P. (1981). VA mycorrhizae increase productivity and diversity of tropical tree communities. Proceedings of the 5th North American Conference on Mycorrhizae, Universite Laval, Quebec, p. 18 (Abstract).

KORMANIK, P. P., BRYAN, W. C. & SCHULTZ, R. C. (1980). Procedures and equipment for staining large numbers of plant root samples for endomycorrhizal assay. Canadian Journal of Microbiology, **26**, 536-538.

KORMANIK, P. P., SCHULTZ, R. C. & BRYAN, W. C. (1982). The influence of vesicular-arbuscular mycorrhizae on the growth and development of eight hardwood tree species. Forest Science, **28**, 531-539.

MARCONI, D. J. & NELSON, P. V. (1984). Leaching of applied phosphorus in container media. Scientia Horticulturae, **22**, 275-285.

MEJSTRIK, V. & KELLEY, A. P. (1979). Mycorrhizae in Sequoia gigantea Lindl. et Gard. and Sequoia sempervirens Endl. Ceska Mykologie, **33**, 51-54.

MENGE, J. A. (1982). Effect of soil fumigants and fungicides on vesicular-arbuscular fungi. Phytopathology, **72**, 1125-1132.

PARKE, JENNIFER LEE. (1982). Factors Affecting the Inoculum Potential of VA and Ectomycorrhizal Fungi in Forest Soils of Southwest Oregon and Northern California. Ph.D. Thesis, Oregon State University, Corvallis.

PHILLIPS, J. M. & HAYMAN, D. S. (1970). Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Transactions of the British Mycological Society, **55**, 158-161.

PLENCHETTE, C., FURLAN, V. & FORTIN, J. A. (1981). Growth stimulation of apple trees in unsterilized soil under field conditions with VA mycorrhiza inoculation. Canadian Journal of Botany, **59**, 2003-2008.

POPE, P. E., CHANEY, W. R., RHODES, J. D. & WOODHEAD, S. H. (1983). The mycorrhizal dependency of four hardwood tree species. Canadian Journal of Botany, **61**, 412-417.

SCHULTZ, R. C., KORMANIK, P. P., BRYAN, W. C. & BRISTER, G. H. (1979). Vesicular-arbuscular mycorrhiza influence growth but not mineral concentrations in seedlings of eight sweetgum families. Canadian Journal of Forest Research, **9**, 218-223.

SILBERBUSH, M. & BARBER, S. A. (1983). Sensitivity of simulated phosphorus uptake to parameters used by a mechanistic-mathematical model. Plant and Soil, **74**, 93-100.

STEEL, R. G. D. & TORRIE, J. H. (1960). Principles and Procedures of Statistics. McGraw-Hill, New York.

ST. JOHN, T. V. (1979). Root size, root hairs and mycorrhizal infection: a re-examination of Baylis's hypothesis with tropical trees. New Phytologist, **84**, 483-487.

ST. JOHN, T. V. & COLEMAN, D. C. (1983). The role of mycorrhizae in plant ecology. Canadian Journal of Botany, **61**, 1005-1014.

CHAPTER 2

Correlation of Mycorrhizal Growth Enhancement with Development of External Hyphae, Root Colonization and Acetylene Reduction in *Lotus* and *Trifolium*

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SUMMARY

Plant growth enhancement by nine species and isolates of vesicular-arbuscular mycorrhizal (VAM) fungi was compared on *Trifolium subterraneum* and *Lotus corniculatus*. Two assays were incorporated as indicators of phosphorus uptake by VAM fungi prior to improved plant growth. One assay was the acetylene reduction method to follow nitrogenase activity, which responds to VAM colonization in phosphorus deficient soil. An assay for sand aggregation by extra-matrical hyphae of VAM fungi was also performed because proliferation of external mycelium into soil is a possible mechanism for VAM plant

growth enhancement. Values for acetylene reduction assay and external hypha development (sand aggregation assay) were highest at the first harvest before growth differed significantly among isolates. These activities did not always correlate with subsequent plant growth enhancement by VAM. When plant biomass differed significantly, they were correlated with total ethylene production per plant, nodule mass, and external hypha development by effective isolates. Some isolates gave plant growth enhancement without extensive of root colonization or development of external hyphae. In the latter cases, the levels of growth enhancement were correlated with ethylene production and nodule mass alone. VAM plant growth enhancement in legumes cannot be explained solely by the production of external mycelium by the fungus.

Key Words: Glomus, Gigaspora, Acaulospora, sand aggregation, nitrogen fixation.

INTRODUCTION

The ability of vesicular-arbuscular mycorrhizal (VAM) fungi to ameliorate plant growth is predominately a result of improved host plant phosphorus nutrition. The fungus increases growth of the colonized plant by making sources of immobile nutrients such as phosphorus, zinc, and copper available to the plant root. The absorption mechanism whereby the fungus obtains these nutrients is probably the extra-matrical mycelium proliferating beyond the nutrient depletion zone of the root (Mosse, 1959; Tinker, 1975). The

fungus may also have a higher affinity (lower apparent K_m) for phosphorus in solution than the root (Cress et al., 1979) or induce a change in the hormonal status of the plant which results in improved growth (Allen et al., 1980).

The current method for estimating the symbiotic activity of the VAM fungi in plant roots is to measure VAM root colonization. The capacity of a VAM fungus to colonize roots, however, does not always correlate with its beneficial effect on the host. Some fungal isolates can thoroughly colonize root cortical tissue without a corresponding increase in plant growth (Mosse, 1972; Graham et al., 1982). Conversely, plant growth may be significantly increased without extensive root colonization (Sanders et al., 1977; Ames, 1983; Davis et al., 1983).

Early root colonization probably characterizes an establishment or parasitic phase of the fungus during which its growth is primarily intraradical. Early growth depression noted with VAM plants may be due to energy drain associated with this parasitic phase of fungal growth prior to establishment of external mycelium (Bethlenfalvay et al., 1982; Kucey & Paul, 1982). Once the external mycelium becomes functional, plant growth reduction from carbohydrate loss to the fungus is offset by increased uptake of mineral nutrients. Therefore, development of external mycelium may be as critical a factor to monitor as root colonization to describe the dynamics of mycorrhizal symbiosis.

In legumes, nitrogen fixation by Rhizobium nodules increases with mycorrhizal colonization or increased phosphorus nutrition (McLachlan & Norman, 1961; Mosse et al., 1976; Abbott & Robson, 1977; Gates & Müller, 1979). When plant growth is limited by both phosphorus and nitrogen deficiencies, nitrogen fixation rates (as indicated by acetylene reduction) increase prior to any apparent increase in growth (Asimi et al., 1980). Measuring fungal symbiont phosphorus uptake activities indirectly with acetylene reduction in combination with external mycelium and root colonization may be easier than measuring fungal phosphate fluxes directly with radioisotopes.

Hypothetically, examination of these factors would provide a means of assessing the phosphorus uptake activity of the mycorrhizal symbiosis. If growth enhancement by VAM fungi relates to soil exploration by fungal mycelium, it may be more appropriate to examine soil interactions with these fungi rather than simply root colonization. Conversely, if isolates can be shown to enhance growth without extramatrical mycelium, these isolates may be active by mediating plant hormonal changes. The purpose of these studies was to determine, relative to phosphorus uptake, the correlation between level of nitrogen fixation, development of external hyphae, and root colonization, on growth enhancement of a number of VAM fungal species in two legume species.

MATERIALS AND METHODS

Two experiments were conducted. The first was with Trifolium subterraneum grown in plugged glass tubes under gnotobiotic conditions in a growth chamber. The gnotobiotic study showed that isolates varied with respect to plant growth enhancement, acetylene reduction activity and sand aggregation, but a question arose about the validity of this test. Since VAM activity is a dynamic process, it should be analyzed over a time course. In addition, the small volume of sand available in the tube system may limit root and hyphal proliferation. Therefore, a glasshouse study was designed to explore these additional factors.

In the glasshouse experiments, isolate reponse over time was measured at four harvests. To examine development of external hyphae with time, isolates known to produce little extra-matrical hyphae were used. In addition, Lotus corniculatus was included to probe the possibility of host plant differences being involved in isolate effects.

Plant species

Trifolium subterraneum L. cv. 'Mt. Barker' and Lotus corniculatus L. seeds were surface sterilized in 10% Chlorox for 30-45 min and rinsed in five changes of sterile deionized water (SDW). The seeds remained in the final rinse overnight to imbibe water before being placed on water agar containing 0.1% $CaCO_3$. When the radicles had emerged but were less than 1 cm long, they were dipped

in a supension (10^8 CFU/ml) of the appropriate Rhizobium strain immediately before planting.

Rhizobium strains

The Rhizobium trifolii strain C6DM, provided by C. Hagedorn, was a mutant derived from a strain originally isolated from subclover in southwest Oregon and exhibited resistance to streptomycin and erythromycin. The Rhizobium lotii strain was a mutant derived from an isolate in the nodules of naturally infected lotus plants grown in the glasshouse. The strain used was selected for resistance to rifampicin and streptomycin. The antibiotic resistance of the strains was used as a marker to facilitate their later recovery from the nodules. Both strains were grown on yeast mannitol agar (Vincent, 1970) for 4 days, then suspended and diluted in SDW for inoculation of radicles.

At the final harvest, isolations from 50 surface-sterilized nodules from roots of plants inoculated with VAM fungal species were made to determine if the marked strains used as inoculum were present in the nodules (Vincent, 1970).

VAM fungi

Spores were collected by wet sieving from host plant cultures of the fungi (Gerdemann & Nicholson, 1963) and placed in antibiotic solution of gentiamycin and streptomycin for two weeks prior to inoculation to disinfest them of microorganisms (Mertz, Heithaus & Bush, 1979). At the time of inoculation, spores were removed from

the antibiotic solution, rinsed twice and placed in SDW. Water from the final rinse in all spore treatments was combined. One ml of the suspension was placed in each planting location to inoculate each individual treatment in case organisms survived the antibiotic treatment. For each plant replicate, spores were dispensed in equal aliquots into the planting hole, approximately 3 cm below the soil surface. In the first study, there were 20 plants per fungus treatment per harvest; in the second, 16 plants per fungus treatment per harvest. Tables 2-1 and 2-2 describe characteristics of the inoculum for each experiment.

Controls

In the gnotobiotic experiment plants were grown in sterile soil in cotton-stoppered tubes. There were three control treatments; each was inoculated with Rhizobium. One was grown in sterile soil alone, another was inoculated with spore washing aliquots, and the third was inoculated with the spore washing aliquot but was also fertilized with full strength phosphate (43 ppm P) Long Ashton's Nutrient Solution (LANS) (Hewitt, 1966).

Control treatments in the glasshouse experiment were inoculated with the spore washing solutions, like all the VAM treatments, and planted with Rhizobium-inoculated seedlings, but were fertilized with LANS phosphate concentrations of 11, 43, and 172 ppm.

Plant growth conditions

In the first experiment, plants were grown in growth chambers in cotton-stoppered glass tubes (180 x 30 mm) containing 50 cm^3 of sterile river sand. The chamber was maintained at 22/16 °C average day/night temperatures with a 16 h photoperiod. The average irradiance value from the fluorescent and incandescent lights was 550 μ Einsteins$\cdot sec^{-1} \cdot M^{-2}$.

In the second study, the plants grew in open pots in a glasshouse maintained at an average day/night temperature of 22/16 °C with a 16 h photoperiod extended by supplemental lighting from high pressure sodium vapor lamps at an average irradiance of 200 μ Einsteins$\cdot sec^{-1} \cdot M^{-2}$.

Pots used in the second study ranged in size according to the harvest schedule: 700 cm^3 for the 5 week harvest, 1000 cm^3 for the 7 week harvest, and 1400 cm^3 for the 9 and 11 week harvests. Increasing soil volumes insured root growth was unrestricted. Two seeds of the same species were sown per pot separated by a 1 mm mesh nylon screen. The screen served to ease plant separation at harvest and prevent intergrowth of roots.

Both experiments were conducted in pasteurized (60 °C/30 min) river sand which had passed through a 2 mm sieve. Its characteristics before fertilization are shown in Table 2-3. In addition soil used in the first experiment was amended with 20 ml of LANS containing either 11 ppm or 43 ppm phosphate per 50 cm^3 soil sterilized by autoclaving prior to inoculation and planting.

Plants in the glasshouse experiment were watered every two weeks with 10 ml of sterile LANS with 11, 43, or 172 ppm phosphate concentrations. At harvest, the plant VAM treatments were split; half the replicates were assayed for acetylene reduction, the remaining replicates were used for the sand aggregation assay for external hyphae (Sutton & Sheppard, 1976; Graham et al., 1982).

Acetylene reduction assay

In the first study, the plant growth tubes were capped with serum plugs and injected with 5 ml of acetylene. After 1 h incubation, two 1 ml gas samples were removed from each tube. In the second study, one of the paired plants was removed from its pot, the adherent soil washed and the entire plant was placed in 140 cm^3 flasks and capped with serum plugs. Ten ml of acetylene were injected into the flask, and after 1 h incubation, two 1 ml gas samples were removed from each assay flask and analyzed for ethylene. All gas samples were analyzed in a Perkin-Elmer 3920 gas chromatograph equipped with a flame-ionization detector and a 2.4 m Porapak N column (80-100 mesh) operated isothermally at 70 °C. Ethylene was identified by cochromatography with an ethylene-in-air standard. Ethylene concentrations were quantified from peak height values using a standard curve generated with values ranging from 1.1 x 10^{-4} µMoles to .171 µMoles C_2H_4. The quantity of ethylene produced by acetylene reduction was calculated from the standard curve using mean peak height values.

Following the assays, tops and roots were weighed. Plant tops were dried, reweighed, and tissue analyzed for mineral concentration by the Plant Analysis Laboratory, Horticulture Department, Oregon State University, Corvallis, Oregon 97331, USA. The nodules were removed and weighed separately. Entire roots systems, free of nodules, were cleared and stained (Phillips & Hayman, 1970; Kormanik, 1980) and examined for VAM colonization. If VAM were present, the root system was chopped into segments ranging in size from 0.5 to 1.0 cm and the % root length with VAM determined (Biermann & Linderman, 1981).

Sand aggregation assay

Extent of external hypha development was estimated using the sand aggregation method described by Sutton & Shepard (1976) and modified by Graham et al. (1982). VAM-colonized and noninoculated root systems of clover and lotus were compared for their capacity to form stable aggregates of sand particles on or around the root, largely as a result of binding by sticky VAM hyphae. Plants not employed for acetylene reduction tests were assayed by this method. Briefly described, shoots of lotus and clover were removed and sand and roots were allowed to air dry for 4 to 5 days to stabilize the sand aggregate. Aggregates were collected on a 2 mm sieve after gentle agitation to remove loose soil. Roots with adherent soil and aggregates were washed, blotted and weighed (Graham et al., 1982). Total sand in aggregates collected from the screens or washed from the roots were combined. Excess water was decanted, and after drying

for 48 h at 70 °C the sand was weighed. The amount of sand in aggregates was expressed in gm sand/gm dry weight of root tissue, rather than gm sand/gm fresh weight of root tissue used by Graham et al. (1982) because the root tissue had partially dried prior to the washing process.

Data analysis

Data collected in the first experiment were analyzed as a completely randomized design. Due to significant differences between variances in the data for nitrogenase activity and sand aggregation assays, log transformations were done on these data prior to further analysis. Subsequent mean separations for each treatment were by the Student-Newman-Keuls' procedure (Steele & Torrie, 1960).

Data from the second experiment were analyzed as a split plot design with plant species, harvests and fungus treatments as main plots. As significant interactions were found in some plots, subsequent analysis was done separately for each plant species. Log transformations of the data were performed when significantly larger variances related to increasing mean values. Mean separations were determined by the Student-Newman-Keuls' procedure as above.

RESULTS

Plant growth

In the gnotobiotic experiment, VAM isolate differences were apparent in both shoot fresh and dry weights (Table 2-4). While VAM-

colonized plants had greater shoot fresh weight than noninoculated controls, these differences diminished in the dry weight values especially for G. mosseae and G. epigaeum [= G. versiforme (Karst) Berch]. Noninoculated controls had larger root fresh weight values than the VAM-colonized plants. In addition, root mass of the control plants decreased significantly with increased soil phosphorus.

These alterations in root and shoot allocations with VAM colonization are shown in the shoot to root ratios (Table 2-6). VAM infection produces plants capable of supporting more shoot tissue per unit mass of root tissue. Nodule mass was also altered by either added phosphorus or VAM colonization. However care should be taken in interpreting these parameters due to possible effects of limited soil volume on root growth.

The isolates that were superior in the gnotobiotic system, A. trappei and G. epigaeum, were also among the best in glasshouse studies. Plant growth increased significantly in each of the four successive harvests in all physical measurements made. However, effects of VAM fungal treatments or addition of phosphate in the controls were only significant in clover at the last two harvests, 9 and 11 weeks.

Lotus showed less growth increase than clover in response to inputs of additional phosphorus or VAM colonization. As there were no significant interactions between harvest date and treatment for lotus, all treatments were combined and examined across harvests to show overall treatment effects. Lotus is a non-responsive host and Gigaspora gigantea is an ineffective fungus for growth enhancement of

lotus, even though it readily colonized the roots and formed extramatrical hyphae (Table 2-6).

Subclover produced more biomass than lotus with phosphorus inputs when noninoculated or when colonized by VAM fungi (Fig. 2-3). Since there were statistically significant interactions between harvest and treatment in the analysis of variance, no overall treatment effects using data combined across harvests could be employed with the subclover data. At 9 and 11 weeks growth, G. epigaeum, A. trappei, and G. fasciculatum 624 gave the best growth enhancement of clover of the VAM isolates examined (Fig. 2-3). After 11 weeks, growth of plants colonized by these VAM fungi equalled growth of noninoculated controls with 4 times full strength phosphate (172 ppm) LANS soil amendments.

Acetylene reduction

Under gnotobiotic conditions, VAM fungus isolates differed in nitrogenase activities. Nitrogenase activity of all VAM-colonized plants was significantly greater than nonmycorrhizal controls; while total ethylene produced was similar (Table 2-4). In general, the nitrogenase activities of VAM colonized plants correlated (r^2 = 0.81 to 0.94) with growth parameters such as dry matter production and nodule weight.

In glasshouse studies, nitrogenase activity values differed significantly between VAM fungus treatments only at the earliest harvests: the 5 week harvest for lotus (Table 2-6) and 5 and 7 weeks for clover (Fig. 2-2). Lotus plants colonized by A. trappei had the

highest ethylene production values of all lotus VAM treatments, but were similar in biomass to noninoculated controls with 4 times full strength phosphate (172 ppm) LANS. While all VAM isolates except G. gigantea produced amounts of biomass similar to the controls in lotus, G. margarita and G. deserticola had higher nitrogenase activities than the other treatments at the 5 week harvest. Therefore, increased nitrogenase activities may not reflect subsequent biomass production by the host plant.

In subclover, a similar situation occurred. Nitrogenase activity at 5 and 7 weeks of the G. gigantea isolate did not differ significantly from G. epigaeum and A. trappei while plant growth enhancement did differ. After 7 weeks the activity values for nitrogenase reached a plateau ($\sim$0.1 $\mu M \cdot hr^{-1} \cdot gm^{-1}$) and no longer differed significantly between treatments (Fig. 2-2), however, the absolute amounts of ethylene produced per plant did differ significantly between treatments. With similar nitrogenase activities, the amount of plant nodule tissue became a critical factor and growth effectiveness of VAM isolates related directly to nodule weight (Fig. 2-1; Table 2-8).

Sand aggregation

VAM isolates varied in ability to form stable sand aggregates. Biomass production in some isolates such as A. trappei and G. gigantea correlated with nitrogenase activity and sand aggregation in the gnotobiotic study (Table 2-4). The relationship did not seem valid for the same isolates examined in the unrestricted root growth

conditions of the glasshouse experiment. G. gigantea-colonized plants still had substantial aggregation and high nitrogenase activities but subsequent plant biomass production did not differ significantly from the controls. In addition, isolate G. fasciculatum 624, which has been reported not to enhance growth or form extensive external mycelium (Graham et al., 1982), was effective at both activities in this soil system. As with the nitrogenase activity assay, the highest values of sand aggregate formation were obtained at the first harvests (Fig. 2-4). As root growth continued, the total amount of sand in aggregates increased, but the value per root weight decreased.

The sand aggregation values for the individual VAM isolates fluctuated more in lotus than in clover. The value of sand aggregation activity decreased steadily over time for clover but not in VAM treatments for lotus. This decrease was also reflected in the values of sand aggregation activity for the lotus or clover controls. Lotus also showed no effect of phosphate fertility on sand aggregation activity. The 4 times full strength phosphate (172 ppm) LANS clover control showed significantly lower sand aggregation at the 9 week harvest (Fig. 2-4).

Colonization by VAM fungi

In the gnotobiotic study infection by VAM fungi was extensive with the majority of the cortical tissue examined showing signs of fungal colonization (Table 2-4). In addition, the development of the extra-matrical mycelium through the soil could be visualized. In the

G. gigantea treatments, production of external auxillary cells and subsequent azygospore formation could be clearly seen.

Characteristic fungal colonization patterns were apparent after clearing and staining the root tissue. Both Gigaspora species produced abundant mycelium in the root tissue near the endodermis and arbuscules in cortical cells. Acaulospora trappei and the Glomus species produced vesicles in addition to intercellular hyphae and arbuscules. Acaulospora spinosa did not colonize roots of subclover after 8 weeks growth. However, spore inoculum from the same plant culture did successfully colonize and reproduce azygospores in subsequent plant cultures with subclover.

Spore inoculum is slower at germinating and colonizing roots than inoculum consisting of colonized root pieces (Abbott & Robson, 1982) and VAM spores have an obligate dormancy period (Tommerup, 1983). The A. spinosa inoculum used probably contained spores at all stages of maturity and quiescence. Tommerup (1983) has shown that of the three species she examined, the Acaulospora species had the longest dormancy period. An obligate dormancy period may account for the failure of A. spinosa to colonize roots within 8 wk. However, A. trappei spore inoculum colonized plants rapidly in both systems. The difference in rapidity of colonization between these two may relate to their longevity in soil. The small hyaline azygospores of A. trappei probably exhaust their nutrient reserves more quickly and are more susceptible to microbial attack than the large pigmented spores of A. spinosa.

Colonization values in the glasshouse study were lower than those in gnotobiotic conditions (Fig. 2-1 through 2-4) even for growth-effective VAM isolates G. epigaeum and A. trappei. At the 5 week harvest, germinating spores were still attached to cleared and stained roots. With isolates G. fasciculatum 624 and AB, colonization and production of abundant vesicles often occurred without apparent connecting hyphae. The Gigaspora species formed abundant intercellular mycelium, arbuscules, and clumps of external auxillary cells in stained root material as in the gnotobiotic studies. Glomus deserticola 0-1 colonized clover root tissue in the gnotobiotic study, however, it only sporadically colonized either clover or lotus in the glasshouse study. The low root colonization by G. deserticola treatments in the glasshouse study are a departure from its behavior in other experiments (see Chapter 1; Graham et al., 1982) and probably do not indicate its activity as a VAM symbiont.

Nodule occupancy

At the final harvest in both experiments, 50 of the larger nodules from each VAM fungus and control treatment were plated on both yeast mannitol agar and yeast mannitol agar amended with the marker antibiotics. High percentage recovery of the inoculated strains was obtained in both studies (Table 2-5). Nodule isolations occasionally yielded an unmarked strain with colony morphology similar to Rhizobium species. Rarely, isolations yielded no colonies, possibly due to excessive surface-sterilization of the nodules.

Foliar mineral concentrations

Leaf tissue from plants in the gnotobiotic study was analyzed for nitrogen and phosphorus (Table 2-4). Total foliar content (shoot weight X concentration) of these two minerals in plants was correlated ($r = .97$). Foliar mineral concentrations of phosphorus and nitrogen by themselves were not correlated ($r = .48$). For plants grown in the glasshouse experiments, total phosphorus and nitrogen content was found to be highly correlated in both lotus ($r = .97$) and subclover ($r = .94$). Again concentrations alone were not found to be correlated for either lotus ($r = .25$) or clover ($r = .07$). Phosphorus content correlating with nitrogen content under conditions of phosphorus and nitrogen deficiency indicates the amount of nitrogen symbiotically fixed by legumes depends directly on the plant's ability to absorb phosphorus and possibly other micronutrients.

Other minerals were examined in foliage of plants from glasshouse experiments at the 9 and 11 week harvests (Tables 2-9 & 2-10). As these determinations were not replicated, no statistical treatment of the data was possible and the values are only presented to indicate trends. In noninoculated controls, clover, but not lotus, showed an increased concentration of both nitrogen and phosphorus in response to increasing levels of phosphorus fertility. In these control plants, calcium, manganese and magnesium concentrations decreased with increasing tissue phosphorus concentrations, probably a tissue dilution effect.

Comparing noninoculated plants to VAM-colonized plants at the lowest level of phosphorus fertility, decreased tissue manganese and increased phosphorus and sulfur concentrations occurred consistently in VAM plants. Other mineral concentrations in VAM plants were similar to noninoculated controls regardless of phosphorus fertility. Few notable differences occurred between VAM isolates. Plants colonized by less effective isolates had tissue mineral concentrations similar to noninoculated controls maintained at the 1/4 strength phosphate (11 ppm) LANS.

DISCUSSION

Isolates of VAM fungi differed in plant growth enhancement. Fungal colonization of root tissue always coincided with increased plant growth. However, an isolate's ability to colonize cortical tissue did not always correspond to its plant growth enhancement. Sand aggregation and acetylene reduction assays clarified these root colonization and growth enhancement anomalies for some isolates Isolates less effective at plant growth enhancement often had lower total nitrogenase activities per plant. These less effective fungi, however, did not form significantly less external mycelium as measured by stable aggregate formation. It is not possible to determine if sand aggregation differences reflect spatial or physiological qualities which vary among VAM isolates. Sand aggregation differences among VAM isolates cannot presently be used as a reliable predictor of an isolate's capacity for plant growth

enhancement. There are, however, some relations that do arise with these measures.

Prior to any plant growth responses, both nitrogenase activities and sand aggregation values were high and differed significantly among VAM isolates. When plant biomass production by the isolates differed significantly, both of these indirect measurements had decreased. High initial nitrogenase activities were due to the nodule developmental state. Young nodules have larger proportions of bacteroid tissue, therefore higher concentrations of nitrogenase enzyme. As nodules mature, bacteroid mass decreases relative to the host support tissue and fixation activity per gram nodule tissue decreases and reaches a constant value. This does not imply activities of the nitrogenase enyzme itself varied with plant age, but reflect physical changes in nodule tissue causing a decrease in enzyme concentration or an apparent decrease in enzyme activity per gram fresh weight.

The decreasing value of sand aggregation with time probably represents a similar phenomenon. In young plants nutrient uptake from the soil is limited by root exploration. Harley (1978) and St. John & Coleman (1983) suggest that when mycorrhizae enhance plant growth, its activity is probably most noticeable in young plants. The decrease of sand aggregation activity with time reflects the host response to the initial nutrient input by VAM. The total amount of sand in aggregates increases with root growth since roots as well as VAM hyphae can induce sand aggregate formation. However as root biomass increases, the predominance of VAM induced aggregate

formation decreases relative to root biomass. As nutrient fluxes differ with certain physical characters of the absorbing organ which may have little bearing on its ability to aggregate sand, the aggregation phenomenon may reveal little about the relative nutrient input from root and VAM hyphae.

Root colonization by VAM fungi was considerably higher in the gnotobiotic study than in the glasshouse. Inoculum and root density characteristics could explain these discrepancies. In the gnotobiotic system, roots were confined to a small soil mass. Given similar amounts of fungal inoculum, the root colonization potential in the gnotobiotic system would be higher than in the glasshouse. The fungus would expend less energy growing to susceptible root tissue in a limited soil volume with high root density. Tissue near the root apex is 10 times more susceptible to VAM infection than tissue away from the apex (Smith & Walker, 1981). Root apices growing away from VAM inoculum in the glasshouse soil system, would reduce the probability of this highly susceptible tissue being in proximity for VAM colonization compared to the gnotobiotic system.

While small soil volumes do not reflect natural soil conditions, confined root growth may be advantageous to test isolate efficiency. Limited soil volumes with high root density cause faster development of overlapping depletion zones of immobile nutrients like phosphorus (Barber & Silberbush, 1984) especially in a soil where nutrient levels are not continually replenished. Since proliferation of VAM hyphae outside the root nutrient depletion zone is a primary mechanism of VAM activity, and maximum exchange between fungus and

host is provided by high root colonization levels, small soil volumes may maximize host response to VAM.

The relatively unrestricted top growth of the gnotobiotic system is much greater than the root growth, which results in high root to shoot ratios (Table 2-8). When the growth of roots is unrestricted, these ratios rarely approach unity. This calls to question the validity of experiments conducted in limited soil volumes as suggested by St. John & Coleman (1983).

The glasshouse study was designed to test the results of the gnotobiotic system and investigate the dynamics of soil exploration by the VAM hyphae. In the clover experiment, plant growth limited by phosphorus and nitrogen is confirmed by a phosphorus growth response in the noninoculated plants at the final harvest. Therefore, under conditions of nitrogen and phosphorus limitation and unrestricted root growth, results similar to the gnotobiotic system occurred for Acaulospora trappei, Glomus epigaeum and Gigaspora margarita. A. trappei and G. epigaeum enhanced growth and induced high nitrogenase activities. The isolates differed in sand aggregation activities at early harvests although values were similar at the final harvest. A. trappei had generally higher sand aggregation than G. epigaeum. These results were obtained in a situation of very limited root colonization by these VAM isolates. As in the gnotobiotic study, G. margarita was ineffective at growth enhancement with moderate sand aggregation and nitrogenase activities similar to the noninoculated controls except in sand aggregation. However, root colonization by

G. margarita was very similar between the gnotobiotic and glasshouse studies.

Some isolates had different activities when comparing the two studies or compared to previous reports. For example, Graham et al. (1982) found Glomus fasciculatum 624 did not form extensive mycelium in soil, yet heavily colonized the roots of Troyer citrange. In the present study, this same isolate formed extensive soil mycelium, assayed by the sand aggregation method, and was a growth promoting VAM fungus on clover. The formation of external hyphae by this isolate, and its effectiveness in growth enhancement adds further credence to the hypothesis that effective symbiosis only results from a compatible interaction between the host, fungus and soil system (Abbott & Robson, 1982).

Another example of altered efficiency occurred in the plants colonized by Gigaspora gigantea. This fungus had high levels of root colonization and sand aggregation, yet enhanced plant growth only in the gnotobiotic systems. There were indications that plants inoculated with G. gigantea were beginning to grow more rapidly at final harvest. Perhaps the symbiotic activity of the fungus was adversely affected by soil microbes, a phenomenon that deserves further study.

Table 2-1. Plant history and source of VAM fungal isolates employed in these studies.

Fungus	Plant host of original isolate	Source of original culture
Glomus epigaeum Daniels and Trappe*	*Araucaria*	B. A. Daniels
G. mosseae (Nicholson and Gerdemann) Gerdemann and Trappe	*Allium*	J. W. Gerdemann; J. M. Trappe
G. deserticola 0-1 Trappe and Bloss	*Citrus*	J. A. Menge; J. H. Graham
G. fasciculatum AB (Thaxter sensu Gerdemann) Gerdemann and Trappe	*Sorghum*	Abbott Labs, North Chicago, Illinois
G. fasciculatum 624 (Thaxter sensu Gerdemann) Gerdemann and Trappe	*Citrus*	N. C. Schenck; J. H. Graham
Acaulospora trappei Ames and Linderman	*Lilium*	R. N. Ames
A. spinosa Walker and Trappe	*Festuca*	R. N. Ames
Gigaspora margarita Becker and Hall	*Glycine*	J. W. Gerdemann
G. gigantea (Nicolson and Gerdemann) Gerdemann and Trappe	*Zea*	J. W. Gerdemann

* = *Glomus versiforme* (Karst) Berch

Table 2-2. Inoculum amounts and previous hosts of VAM fungi employed in these studies.

Fungus	1st study		2nd study	
	Previous plant culture host	# spores*	Previous plant culture host	# spores*
Glomus epigaeum	*Asparagus officinalis*	36	*A. officinalis*	48
G. mosseae	*A. officinalis*	32	X	X
G. deserticola 0-1	*A. officinalis*	58	*Trifolium subterraneum*/ *Festuca rubrum*	67
G. fasciculatum AB	X†	X	*T. subterraneum*/ *F. rubrum*	49
G. fasciculatum 624	X	X	*T. subterraneum*/ *F. rubrum*	54
Acaulospora trappei	*A. officinalis*	108	*T. subterraneum*/ *F. rubrum*	157
A. spinosa	*T. subterraneum*	51	X	X
Gigaspora margarita	*T. subterraneum*	26	*T. subterraneum*/ *F. rubrum*	16
Gig. gigantea	*A. officinalis*	28	*T. subterraneum* *F. rubrum*	23

* Spore number represents the mean of three sample aliquots used for inoculum.

† VAM fungus isolate not used.

Table 2-3. Mineral nutrient analysis of the river sand used for both experiments.

Organic matter - 0.27%; pH 7.2
Cation exchange capacity - 5.83 meqs/100 gm
Nitrate nitrogen - 0.43 ppm
Ammonium nitrogen - 1.19 ppm
Total nitrogen - 0.0004%
Phosphorus - 11 ppm
Potassium - 70.2 ppm
Calcium - 4 meq/100 gm
Magnesium - 1.6 meq/100 gm
Zinc - 0.84 ppm
Copper - 0.34 ppm
Manganese - 3.78 ppm

Table 2-4. Effect of mycorrhizal colonization on 56-day-old *Trifolium subterraneum* plants grown gnotobiotically.

VAM fungus treatment*	Shoot fresh weight (gm)	Shoot dry weight (gm)	Root fresh weight (gm)	Nodule weight (gm)	N_2ase activity†	Sand aggregation†	VAM % root length	% N	% P
Atr	.3120a‡	.0517a	.2058b	.0081a	.1940b	20.36a	75a	2.19	.16
Gle	.3329a	.0401c	.1336d	.0052cd	.0404c	9.66ab	84a	3.21	.27
Glm	.3192a	.0401c	.1506cd	.0064b	.0297c	28.69a	85a	2.48	.26
Gld	.2714bc	.0400c	.1625cd	.0049cd	.0125c	13.31ab	73a	3.21	.24
Gim	.2497c	.0433b	.2074b	.0060bcd	.0155c	5.58b	19b	2.19	.10
Gig	.2819b	.0460b	.1872bc	.0075ab	.6700a	35.11a	92a	2.20	.16
Asp	.1972d	.0387c	.2325b	.0044d	4.84^{-5}d	4.84b	0	2.54	.09
Rh1	.2163d	.0405c	.2104b	.0055bcd	2.38^{-4}d	5.44b	0	2.63	.11
Rh4	.2028d	.0402c	.1857c	.0052cd	2.89^{-4}d	6.02b	0	3.21	.14
Cont	.2226d	.0433b	.2823a	.0072ab	1.85^{-4}d	5.47b	0	2.98	.12

* Abbreviations for fungus treatments are Atr - *Acaulospora trappei*; Asp - *A. spinnosa*; Gle - *Glomus epigaeum*; Glm - *G. mosseae*; Gld - *G. deserticola*; Gim - *Gigaspora margarita*; Gig - *G. gigantea*; Rh1 - *Rhizobium trifolii* with spore washings; Rh4 - *Rhizobium trifoli* with spore washings at 43 ppm phosphorus; Cont - *Rhizobium trifolii* without spore washings.

† Nitrogenase activity is expressed as μM of C_2H_4 produced x nodule fresh wt gm^{-1} x hr^{-1}. Sand aggregation by VAM hyphae is expressed as sand aggregates (gm) x root dry wt (gm^{-1}).

‡ Means within columns not followed by the same letter are significantly different at $\alpha = .05\%$ using Student-Newman-Keuls' test.

Table 2-5. Percentage recovery of marked *Rhizobium* strains from nodules of inoculated plants.

VAM fungus treatment*	- Gnotobiotic -	------- Greenhouse -------	
	Trifolium subterraneum	*Trifolium subterraneum*	*Lotus corniculatus*
Gle	92†	88	96
Glm	94	--	--
Gld	90	80	82
Gfab	--‡	94	92
G624	--	96	86
Atr	96	98	84
Gim	84	76	94
Gig	96	92	70
Asp	88	--	--
Rh1	98	86	96
Rh4	94	90	86
Rh16	--	94	88
Cont	92	--	--

* Abbreviations for the treatments are as follows: Gle - *Glomus epigaeum*; Glm - *G. mosseae*; Gld - *G. deserticola*; Gfab - *G. fasciculatum*; G624 - *G. fasciculatum* 624; Atr - *Acaulospora trappei*; Asp - *Acaulospora spinosa*; Gim - *Gigaspora margarita*; Gig - *G. gigantea*; Rh1 - *Rhizobium* treatment with 1/4 P (11 ppm) LANS; Rh4 - *Rhizobium* treatment with full strength phosphate (43 ppm) LANS; Rh16 - *Rhizobium* treatment with 4x phosphate (172 ppm) LANS; Cont - control without spore washings.

† Values represent the percentage of 50 surface sterilized nodules from which the marked *Rhizobium* strains were recovered.

‡ Isolate not used in this study.

Table 2-6. Effect of mycorrhizal colonization on Lotus corniculatus plants grown in a glasshouse and averaged over four harvests.

VAM fungus treatment*	Shoot fresh weight (gm)	Shoot dry weight (gm)	Root fresh weight (gm)	Nodule weight (gm)	Total Moles C_2H_4 ($plant^{-1}$ hr^{-1})	N_2ase activity†	Sand aggregation†	% root length VAM	Shoot/ root ratio
Atr	.435a‡	.070a	.515b	.0465b	.0744c	.0412abc	9.030b	9.4bc	.935
Gle	.390a	.064a	.476b	.0350ab	.0531b	.0421abcd	8.656b	4.2ab	.819
Gfab	.395a	.061a	.409ab	.0339ab	.0524b	.0249ab	10.860b	13.1c	.966
G624	.370a	.060a	.487b	.0403ab	.0537b	.0373abc	17.538c	9.6bc	.760
Gld	.411a	.066a	.450b	.0393ab	.0502b	.0548cd	8.566b	0.5a	.913
Gig	.247b	.040b	.258a	.0237a	.0263a	.0227a	13.723bc	9.1bc	.957
Gim	.382a	.067a	.481b	.0383ab	.0473b	.0634d	11.947bc	13.4c	.794
Rh16	.428a	.063a	.532b	.0452b	.0554b	.0349abc	4.570a	0	.805
Rh4	.397a	.069a	.466b	.0445b	.0567bc	.0209a	4.835a	0	.852
Rh1	.397a	.067a	.468b	.0363ab	.0464b	.0462abcd	4.761a	0	.846

* Abbreviations for fungus treatments are described in Table 2-5.

† N_2ase activity for 5 week harvest only expressed as μM C_2H_4 produced x nodule fresh weight (gm^{-1}) x H^{-1}. Sand aggregation by VAM hyphae expressed as sand aggregate (gm) x root dry wt (gm^{-1}).

‡Means within columns not followed by the same letter are significantly different at $\alpha = .05\%$ using the Student-Newman-Keuls' test.

Table 2-7. Comparisons between shoot and root weights and shoot to root ratios for gnotobiotic plants (56 days old) and greenhouse plants (48 days old).

VAM fungus treatment*	Shoot fresh weight (gm)		Root fresh weight (gm)		Shoot/root ratio	
	Gnoto	Grnhs	Gnoto	Grnhs	Gnoto	Grnhs
Atr	.3120	.4445	.2058	.7146	1.52	.62
Gle	.3329	.3512	.1336	.5921	2.49	.59
Gld	.2714	.3595	.1625	.5820	1.67	.62
Gim	.2497	.3883	.2074	.5385	1.20	.72
Gig	.2819	.2743	.1872	.5016	1.50	.55
Rhl	.2163	.2575	.2104	.4977	1.03	.52
Rh4	.2028	.4474	.1857	.5589	1.09	.80

* Abbreviations for fungus treatments are Atr - Acaulospora trappei; Gle - Glomus epigaeum; Gld - G. deserticola; Gig - Gigaspora gigantea; Gim - G. margarita; Rh 4 - Rhizobium treatment with 43 ppm phosphate LANS; Rhl - Rhizobium treatment with 11 ppm phosphate LANS.

Table 2-8. Effect of VAM isolates on root and nodule biomass of Trifolium subterraneum grown in a glasshouse for 9 or 11 weeks.

VAM fungus treatment*	9 weeks		11 weeks	
	Root fresh weight (gm)	Nodule fresh weight (mg)	Root fresh weight (gm)	Nodule fresh weight (mg)
Atr	1.292ab†	43.0cd	4.867cd	128.3d
Gle	2.030b	69.7d	5.275d	105.8cd
Gfab	.987a	34.1cd	2.106ab	46.3ab
G624	2.051b	45.0cd	3.041abcd	68.7bcd
Gld	1.110ab	23.6abc	2.833bc	65.0bc
Gig	1.338ab	23.6abcd	3.011abcd	93.8
Gim	.904a	15.1ab	2.462abc	42.5ab
Rh16	2.086b	27.6abc	3.911bcd	64.0bcd
Rh4	1.005ab	18.3ab	3.151abcd	47.6ab
Rh1	.644a	11.5a	1.789a	31.5a

* Abbreviations for fungus treatments are described in Table 2-5.

† Means within columns not followed by the same letter are significantly different at $\alpha = .05\%$ using the Student-Newman-Keuls' test.

Table 2-9. Foliar mineral concentration for Lotus corniculatus plants nine and eleven weeks after inoculation with VA mycorrhizal fungi.

VAM fungus treatment*	N (%)		P (%)		Ca (%)		Mg (%)		S (%)		Mn (ppm)	
	9 wk	11 wk	9 wk	11 wk	9 wk	11 wk	9 wk	11 wk	9 wk	11 wk	9 wk	11 wk
Atr	2.94	3.04	.17	.20	2.21	1.98	.90	.77	.37	.36	228	204
Gle	3.08	3.12	.17	.22	2.44	2.51	1.00	1.05	.41	.40	234	303
Gfab	3.12	3.04	.20	.21	2.10	2.47	.83	.93	.49	.40	183	256
G624	3.14	3.04	.18	.21	1.76	2.29	.85	1.00	.36	.43	185	301
Gld	2.90	2.90	.16	.18	2.47	2.70	.99	1.01	.46	.36	246	296
Gig	2.86	3.06	.17	.22	2.21	2.62	.82	1.01	.43	.43	209	251
Gim	3.02	2.84	.16	.19	1.98	2.70	.85	1.05	.42	.36	212	289
Rh16	3.20	3.08	.16	.18	2.58	2.21	.93	.82	.40	.37	207	231
Rh4	2.92	2.70	.16	.14	2.43	2.54	.92	.92	.38	.31	217	259
Rhl	2.82	3.08	.16	.19	2.63	3.30	.94	1.12	.51	.39	248	318

* Abbreviations for fungus treatments are described in Table 2-5.

Table 2-10. Foliar mineral concentration for Trifolium subterraneum plants nine and eleven weeks after inoculation with VA mycorrhizal fungi.

VAM fungus treatment*	N (%)		P (%)		Ca (%)		Mg (%)		S (%)		Mn (ppm)	
	9 wk	11 wk	9 wk	11 wk	9 wk	11 wk	9 wk	11 wk	9 wk	11 wk	9 wk	11 wk
Atr	2.40	2.70	.26	.20	2.88	2.79	.53	.49	.82	.47	214	204
Gle	2.46	2.48	.18	.21	3.22	2.84	.63	.51	.44	.38	237	171
Gfab	3.22	2.76	.22	.25	3.03	2.97	.57	.60	.79	.63	170	193
G624	2.98	2.98	.25	.23	2.94	2.74	.50	.57	.45	.47	198	215
Gld	2.60	2.60	.18	.18	3.09	2.86	.63	.62	.44	.40	239	184
Gig	2.48	3.00	.20	.16	3.12	2.94	.63	.61	.55	.47	247	211
Gim	2.62	2.68	.24	.16	2.61	2.46	.52	.46	.49	.40	213	170
Rh16	2.90	3.14	.13	.21	2.53	2.23	.53	.52	.38	.53	188	196
Rh4	2.80	2.56	.13	.17	2.75	2.57	.59	.62	.46	.44	213	208
Rh1	2.56	2.64	.14	.18	3.04	2.65	.71	.64	.49	.43	272	206

* Abbreviations for fungus treatments are described in Table 2-5.

Figure 2-1. Total ethylene (μMoles) produced per plant of subclover as affected by mycorrhizal colonization at four harvests.

(Rh16 - *Rhizobium* control with 4x full strength phosphate (172 ppm) LANS. Rh4 - *Rhizobium* control with full strength phosphate (43 ppm) LANS. Rh1 - *Rhizobium* controls with 1/4 strength phosphate (11 ppm) LANS. Atr - *Acaulospora trappei*. Gld - *Glomus deserticola*. Gle - *G. epigaeum*. Gfab - *G. fasciculatum*. G624 - *G. fasciculatum* isolate 624. Gig - *Gigaspora gigantea*. Gim - *G. margarita*. The numbers following each identifying code are the % root length colonization by VAM at each harvest time. The error bar is the least significant difference between adjacent means from the Student-Newman-Keul's test for $\alpha = 0.05$.

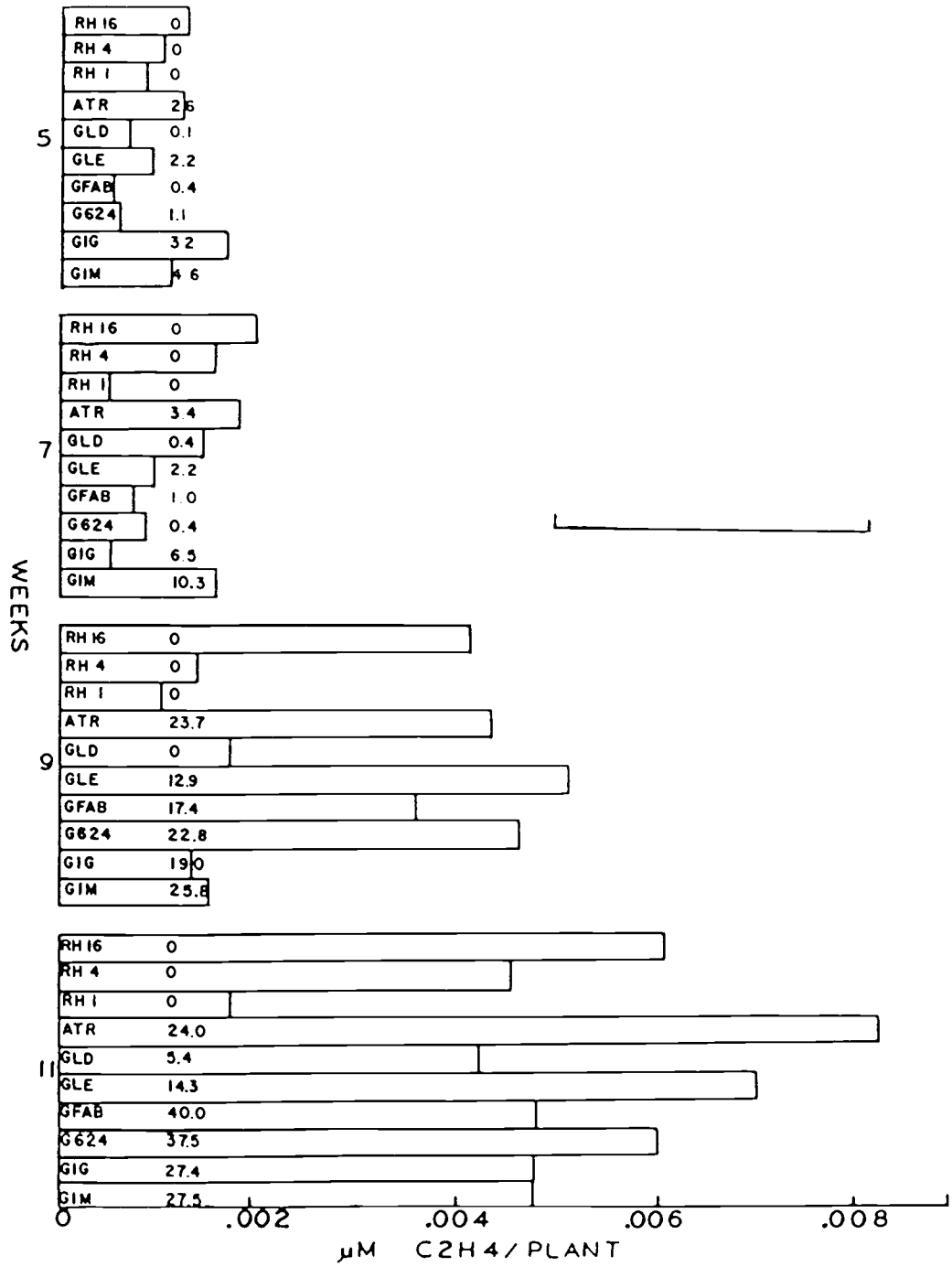
WEEKS
5
RH 16 0
RH 4 0
RH 1 0
ATR 2.6
GLD 0.1
GLE 2.2
GFAB 0.4
G624 1.1
GIG 3.2
GIM 4.6
7
RH 16 0
RH 4 0
RH 1 0
ATR 3.4
GLD 0.4
GLE 2.2
GFAB 1.0
G624 0.4
GIG 6.5
GIM 10.3
9
RH 16 0
RH 4 0
RH 1 0
ATR 23.7
GLD 0
GLE 12.9
GFAB 17.4
G624 22.8
GIG 19.0
GIM 25.8
11
RH 16 0
RH 4 0
RH 1 0
ATR 24.0
GLD 5.4
GLE 14.3
GFAB 40.0
G624 37.5
GIG 27.4
GIM 27.5
0
.002
.004
.006
.008
µM C2H4/PLANT

Figure 2-1.

Figure 2-2. Nitrogenase activity (μMoles • gm fresh weight nodule tissue) of subclover plants as affected by mycorrhizal colonization at four harvests.

(Rh16 - Rhizobium control with 4x full strength phosphate (172 ppm) LANS. Rh4 - Rhizobium control with full strength phosphate (43 ppm) LANS. Rh1 - Rhizobium controls with 1/4 strength phosphate (11 ppm) LANS. Atr - Acaulospora trappei. Gld - Glomus deserticola. Gle - G. epigaeum. Gfab - G. fasciculatum. G624 - G. fasciculatum isolate 624. Gig - Gigaspora gigantea. Gim - G. margarita. The numbers following each identifying code are the % root length colonization by VAM at each harvest time. The error bar is the least significant difference between adjacent means from the Student-Newman-Keul's test for $\alpha = 0.05$.

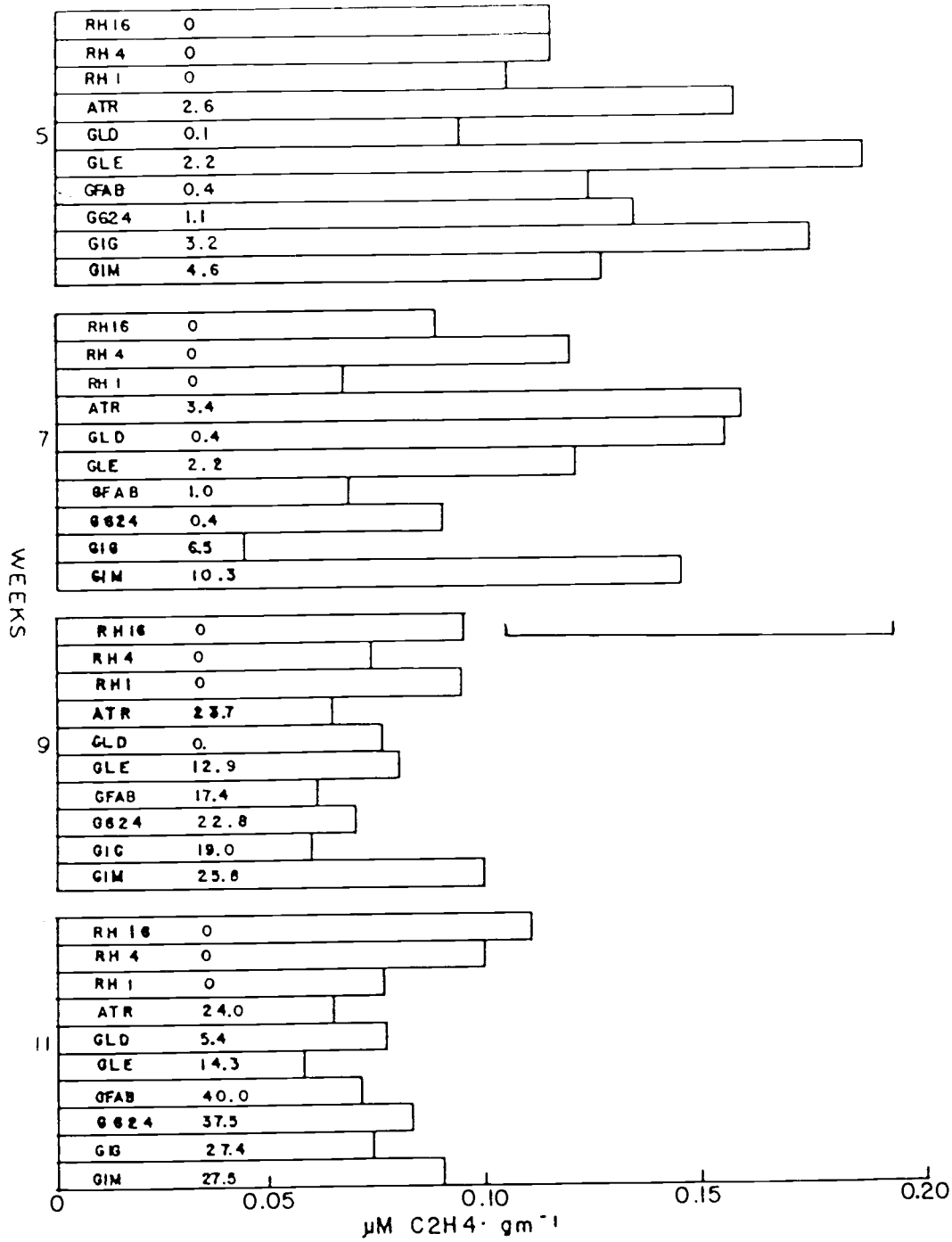
WEEKS
5
RH16 0
RH4 0
RH1 0
ATR 2.6
GLD 0.1
GLE 2.2
GFAB 0.4
GG24 1.1
GIG 3.2
GIM 4.6
7
RH16 0
RH4 0
RH1 0
ATR 3.4
GLD 0.4
GLE 2.2
GFAB 1.0
GG24 0.4
GIG 6.5
GIM 10.3
9
RH16 0
RH4 0
RH1 0
ATR 23.7
GLD 0.
GLE 12.9
GFAB 17.4
GG24 22.8
GIG 19.0
GIM 25.8
11
RH16 0
RH4 0
RH1 0
ATR 24.0
GLD 5.4
GLE 14.3
GFAB 40.0
GG24 37.5
GIG 27.4
GIM 27.5
0
0.05
0.10
0.15
0.20
µM C2H4 · gm⁻¹

Figure 2-2.

Figure 2-3. Dry weight of leaf tissue of subclover as affected by mycorrhizal colonization at four harvests. (Rh16 - *Rhizobium* control with 4x full strength phosphate (172 ppm) LANS. Rh4 - *Rhizobium* control with full strength phosphate (43 ppm) LANS. Rh1 - *Rhizobium* controls with 1/4 strength phosphate (11 ppm) LANS. Atr - *Acaulospora trappei*. Gld - *Glomus deserticola*. Gle - *G. epigaeum*. Gfab - *G. fasciculatum*. G624 - *G. fasciculatum* isolate 624. Gig - *Gigaspora gigantea*. Gim - *G. margarita*. The numbers following each identifying code are the % root length colonization by VAM at each harvest time. The error bar is the least significant difference between adjacent means from the Student-Newman-Keul's test for $\alpha = 0.05$.

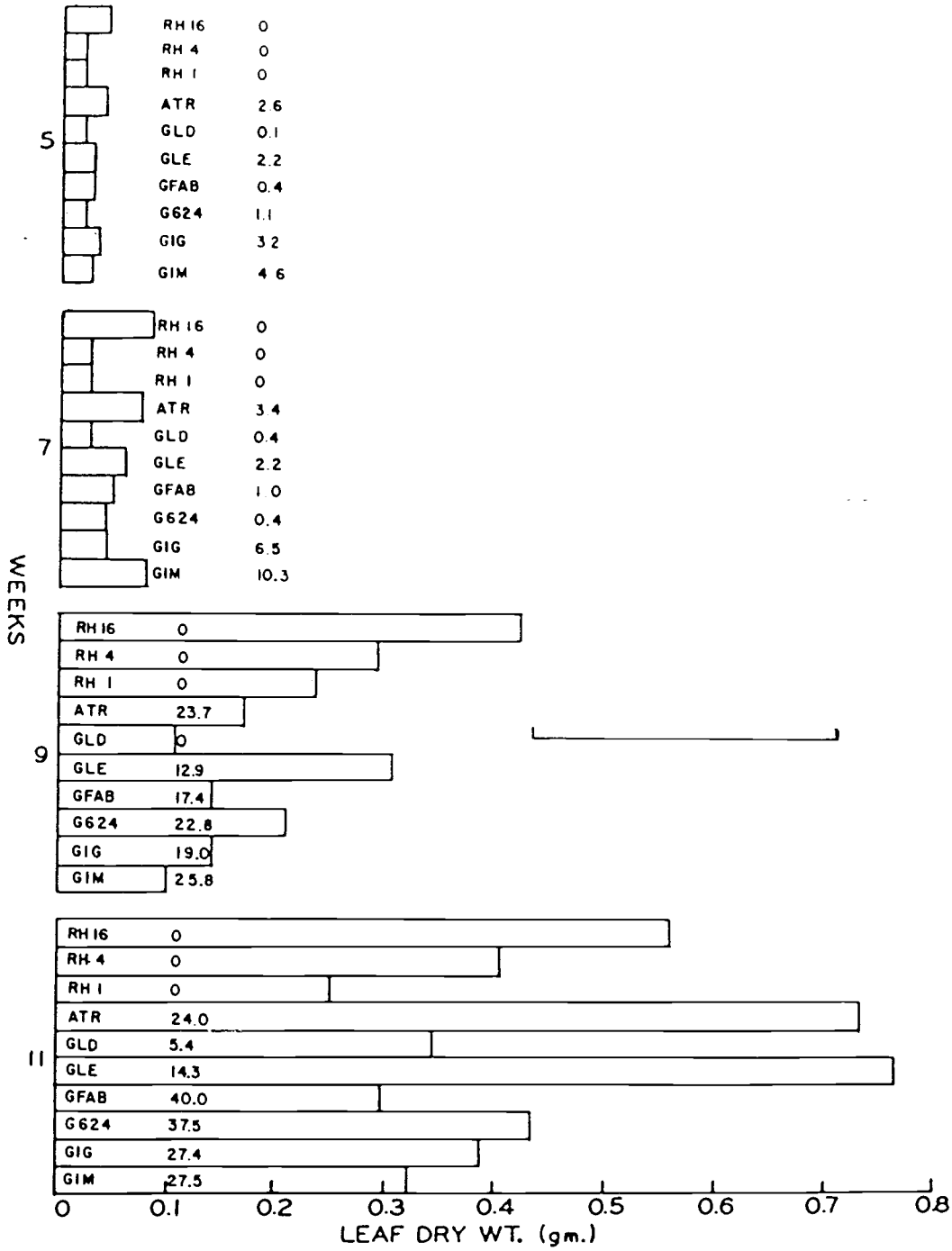
WEEKS
5
RH 16 0
RH 4 0
RH 1 0
ATR 2.6
GLD 0.1
GLE 2.2
GFAB 0.4
G624 1.1
GIG 3.2
GIM 4.6
7
RH 16 0
RH 4 0
RH 1 0
ATR 3.4
GLD 0.4
GLE 2.2
GFAB 1.0
G624 0.4
GIG 6.5
GIM 10.3
9
RH 16 0
RH 4 0
RH 1 0
ATR 23.7
GLD 0
GLE 12.9
GFAB 17.4
G624 22.8
GIG 19.0
GIM 25.8
11
RH 16 0
RH 4 0
RH 1 0
ATR 24.0
GLD 5.4
GLE 14.3
GFAB 40.0
G624 37.5
GIG 27.4
GIM 27.5
0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8
LEAF DRY WT. (gm.)

Figure 2-3.

Figure 2-4. Sand aggregation by subclover as affected by mycorrhizal colonization at four harvests.

(Rh16 - Rhizobium control with 4x full strength phosphate (172 ppm) LANS. Rh4 - Rhizobium control with full strength phosphate (43 ppm) LANS. Rh1 - Rhizobium controls with 1/4 strength phosphate (11 ppm) LANS. Atr - Acaulospora trappei. Gld - Glomus deserticola. Gle - G. epigaeum. Gfab - G. fasciculatum. G624 - G. fasciculatum isolate 624. Gig - Gigaspora gigantea. Gim - G. margarita. The numbers following each identifying code are the % root length colonization by VAM at each harvest time. The error bar is the least significant difference between adjacent means from the Student-Newman-Keul's test for $\alpha = 0.05$.

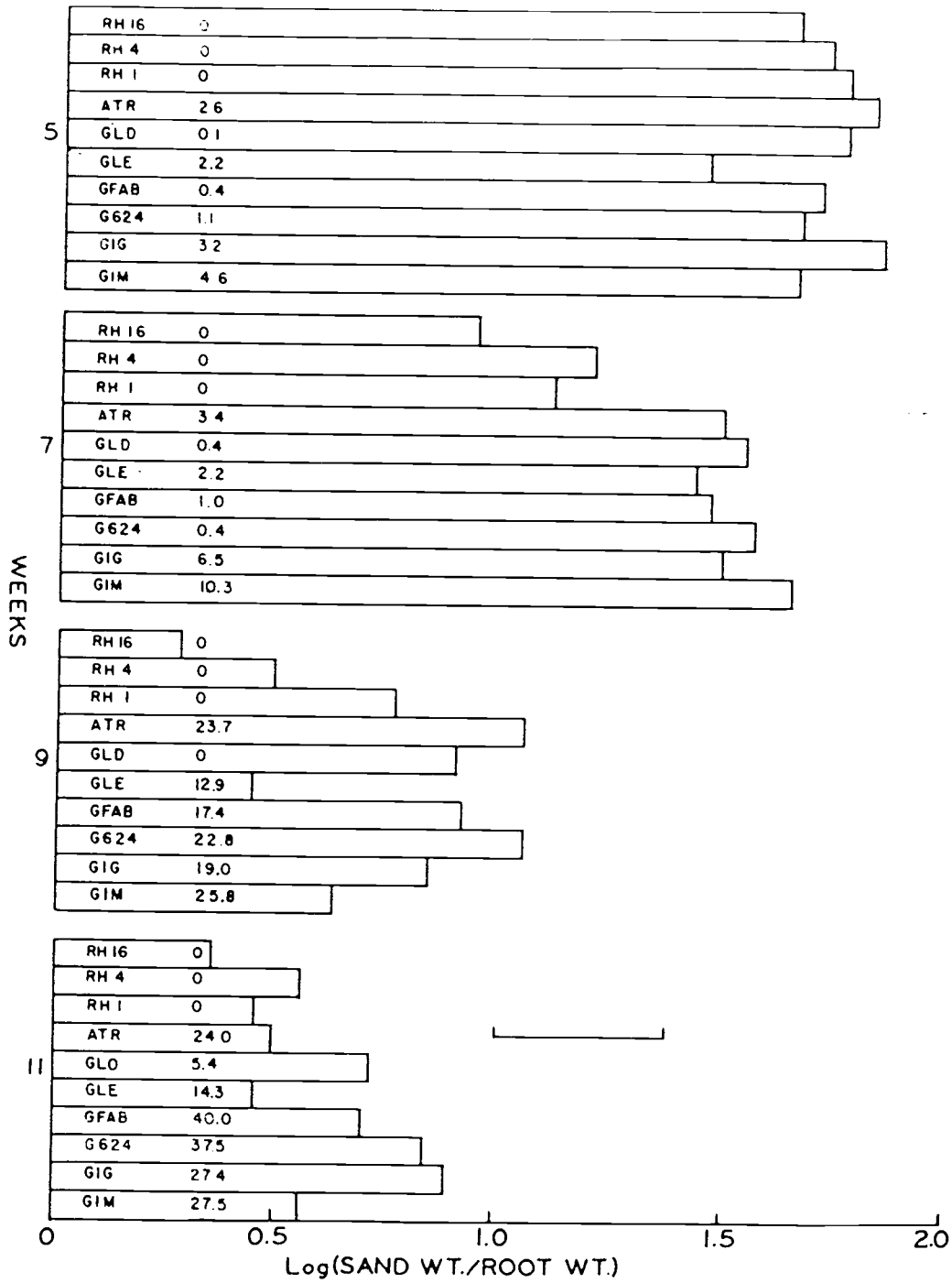
5
RH 16 0
RH 4 0
RH 1 0
ATR 2 6
GLD 0 1
GLE 2.2
GFAB 0.4
G624 1.1
GIG 3 2
GIM 4 6
7
RH 16 0
RH 4 0
RH 1 0
ATR 3 4
GLD 0.4
GLE 2.2
GFAB 1.0
G624 0.4
GIG 6.5
GIM 10.3
9
RH 16 0
RH 4 0
RH 1 0
ATR 23.7
GLD 0
GLE 12.9
GFAB 17.4
G624 22.8
GIG 19.0
GIM 25.8
11
RH 16 0
RH 4 0
RH 1 0
ATR 24 0
GLO 5.4
GLE 14.3
GFAB 40.0
G624 37.5
GIG 27 4
GIM 27.5
WEEKS
0
0.5
1.0
1.5
2.0
Log(SAND WT./ROOT WT.)

Figure 2-4.

REFERENCES

ABBOTT, L. K. & ROBSON, A. D. (1977). Growth stimulation of subterranean clover with vesicular arbuscular mycorrhizas. Australian Journal of Agricultural Research, **28**, 639-649.

ABBOTT, L. K. & ROBSON, A. D. (1982). The role of vesicular arbuscular mycorrhizal fungi in agriculture and the selection of fungi for inoculation. Australian Journal of Agricultural Research, **33**, 389-408.

ALLEN, M. F., MOORE, T. S., Jr. & CHRISTENSEN, M. (1980). Phytohormone changes in Bouteloua gracilis infected by vesicular-arbuscular mycorrhizae. I. Cytokinin increases in the host plant. Canadian Journal of Botany, **58**, 371-74.

AMES, R. N., REID, C. P. P., PORTER, L. K. & CAMBARDELLA, C. (1983). Hyphal uptake and transport of nitrogen from two ^{15}N-labelled sources by Glomus mosseae, a vesicular-arbuscular mycorrhizal fungus. New Phytologist, **95**, 381-396.

ASIMI, S., GIANINAZZI-PEARSON, V. & GIANINAZZI, S. (1980). Influence of increasing soil phosphorus levels on interactions between vesicular-arbuscular mycorrhizae and Rhizobium in soybeans. Canadian Journal of Botany, **58**, 2200-2205.

BETHLENFALVAY, G. J., BROWN, M. S. & PACOUSKY, R. S. (1982). Relationships between host and endophyte development in mycorrhizal soybeans. New Phytologist, **90**, 537-543.

BETHLENFALVAY, G. J. & YODER, J. F. (1981). The glycine-glomus-rhizobium symbiosis. I. Phosphorus effect on nitrogen fixation and mycorrhizal infection. Physiologia Plantarum, **52**, 141-145.

CRESS, W. A., THRONEBERRY, G. O. & LINDSEY, D. L. (1979). Kinetics of phosphorus absorption by mycorrhizal and non-mycorrhizal tomato roots. Plant Physiology, **64**, 191-206.

DAVIS, E. A., YOUNG, J. L. & LINDERMAN, R. G. (1983). Soil lime level (pH) and VA-mycorrhiza effects on growth responses of sweetgum seedlings. Soil Science Society of America Journal, **47**, 251-256.

GATES, C. T. & MÜLLER, W. J. (1979). Nodule and plant development in the soybean, Glycine max (L.) Merr.: Growth response to nitrogen, phosphorus and sulfur. Australian Journal of Botany, **27**, 203-215.

GERDEMANN, J. W. & NICOLSON, T. H. (1963). Spores of mycorrhizal Endogone species extracted from soil by wet seiving and decanting. Transactions of the British Mycological Society, **46**, 235-244.

GRAHAM, J. H., LINDERMAN, R. G. & MENGE, J. A. (1982). Development of external hyphae by different isolates of mycorrhizal Glomus spp. in relation to root colonization and growth of Troyer citrange. New Phytologist, **91**, 183-189.

HARLEY, J. L. (1978). Ectomycorrhizas as nutrient absorbing organs. Proceedings of the Royal Society of London, Series B, **203**, 1-21.

HEWITT, E. J. (1966). Sand and water culture methods used in the study of plant nutrition. Technical Communication No. 22 (2nd edition, revised). Commonwealth Agricultural Bureaux, London.

KORMANIK, P. P., BRYAN, W. C. & SCHULTZ, R. C. (1980). Procedures and equipment for staining large numbers of plant root samples for endomycorrhizal assay. Canadian Journal of Microbiology, **26**, 536-538.

KUCEY, R. M. N. & PAUL, E. A. (1982). Carbon flow, photosynthesis and N_2 fixation in mycorrhizal and nodulated faba beans (Vicia faba L.). Soil Biology and Biochemistry, **14**, 407-412.

MERTZ, S. M., Jr., HEITHAUS, J. J., III & BUSH, R. L. (1981). Mass production of axenic spores of the endomycorrhizal fungus Gigaspora margarita. Transactions of the British Mycological Society, **72**, 167-169.

MOSSE, B. (1959). Observations on the extra-matrical mycelium of a vesicular-arbuscular endophyte. Transactions of the British Mycological Society, **42**, 439-448.

MOSSE, B. (1972). Effect of different Endogone strains on the growth of Paspalum notatum. Nature, **239**, 221-223.

MOSSE, B., POWELL, C. L. & HAYMAN, D. S. (1976). Plant growth responses to vesicular-arbuscular mycorrhiza. IX. Interactions between VA mycorrhiza, rock phosphate, and symbiotic nitrogen fixation. New Phytologist, **76**, 331-342.

PHILLIPS, J. M. & HAYMAN, D. S. (1970). Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Transactions of the British Mycological Society, **55**, 158-161.

SANDERS, F. E., TINKER, P. B., BLACK, R. L. B. & PALMERLY, S. M. (1977). The development of endomycorrhizal root systems. I.

Spread of infection and growth-promoting effects with four species of vesicular-arbuscular endophyte. New Phytologist, **78**, 553-559.

SILBERBUSH, M. & BARBER, S. A. (1983). Sensitivity of simulated phosphorus uptake to parameters used by a mechanistic-mathematical model. Plant and Soil, **74**, 93-100.

SMITH, S. E. & DAFT, M. J. (1977). Interactions between growth, phosphate content and nitrogen fixation in mycorrhizal and nonmycorrhizal Medicago sativa. Australian Journal of Plant Physiology, **4**, 403-413.

SMITH, S. E. & WALKER, N. A. (1981). A quantitative study of mycorrhizal infection in Trifolium: separate determination of the rates of infection and of mycelial growth. New Phytologist, **89**, 225-240.

ST. JOHN, T. V. & COLEMAN, D. C. (1983). The role of mycorrhizae in plant ecology. Canadian Journal of Botany, **61**, 1005-1014.

SUTTON, J. C. (1973). Development of vesicular-arbuscular mycorrhizae in crop plants. Canadian Journal of Botany, **51**, 2487-2493.

SUTTON, J. C. & SHEPPARD, B. R. (1976). Aggregation of sand-dune soil by endomycorrhizal fungi. Canadian Journal of Botany, **54**, 326-333.

TINKER, P. B. (1975). Effect of vesicular arbuscular mycorrhizae on higher plants. Symposium of the Society for Experimental Biology, **29**, 325-350.

TISDALL, J. M. & OADES, J. M. (1979). Stabilization of soil aggregate by the root systems of ryegrass. Australian Journal of Soil Research, **17**, 429-441.

TOMMERUP, I. C. (1983). Spore dormancy in vesicular-arbuscular mycorrhizal fungi. Transactions of the British Mycological Society, **81**, 37-45.

VINCENT, J. M. (1970). A Manual for the Practical Study of Root-Nodule Bacteria. IBP Handbook No. 15, Blackwell Scientific, Oxford and Edinburgh.

WARNER, A. & MOSSE, B. (1982). Factors affecting the spread of vesicular-arbuscular mycorrhizal fungi in soil. I. Root density. New Phytologist, **90**, 529-536.

CHAPTER 3

Use of the Indirect Immunofluorescent Technique to Study the Vesicular-arbuscular Fungus *Glomus epigaeum* and Other *Glomus* Species

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SUMMARY

An antiserum for spores and hyphae of *Glomus epigaeum* was developed employing extracted chlamydospore walls as an antigen. The reaction with fungal material was visualized by an indirect immunofluorescent technique. The staining was most intense and uniform on hyphae from root preparations and on the inner spore walls and hyphal attachments from spore preparations. The staining reaction was strongest for *G. epigaeum*, but *G. mosseae* and *G. deserticola* also reacted. The

staining reaction appeared to be specific for the genus Glomus, as endomycorrhizal species of Gigaspora, Acaulospora and other soil and root-inhabiting fungi tested did not react.

INTRODUCTION

The study of the soil phase of vesicular-arbuscular mycorrhizae (VAM) has been hampered by the difficulty of tracing and distinguishing their hyphae from those of the other soil fungi. The fluorescent antibody (FA) technique has proved useful in examining other fungi including those inhabiting soil and roots (Burrell et al., 1966; Sen & Holland, 1970; Schmidt et al., 1974; Malajczuk, McComb & Parker, 1975; MacDonald & Duniway, 1979; Fitzell, Evans & Fahy, 1980; Fitzell, Fahy & Evans, 1980; Frankland et al., 1981). The use of the FA technique in VAM research has been impeded by the laborious methods involved in obtaining sufficient quantities of clean fungal material for use as an antigen. This difficulty is compounded by the present inability to culture VAM fungi in the absence of a living host plant.

The VAM fungus Glomus epigaeum produces large, relatively clean sporocarps on the soil surface of plant cultures (Daniels & Trappe, 1979). Surface contaminants can be removed by rinsing the spores, yielding a sufficient quantity of spore wall material to proceed with an immunological preparation. This study was designed to determine (1) if chlamydospores would serve as the antigen to produce serum that would react with spores and hyphae of the fungus and (2) if the

antiserum would be specific for G. epigaeum alone or if other VAM or non-VAM fungi would react.

MATERIALS AND METHODS

Preparation of fungal antigen

Mature sporocarps of G. epigaeum were collected from the soil surface of asparagus (Asparagus officinalis L.) pot cultures. The average weight of fresh sporocarps was 30 mg. This weight represents approximately 6000 spores. Twenty to 30 sporocarps were collected for each extraction. The chlamydospores were separated by hand dissection from the sporocarps in sterile distilled water (SDW) and sonicated until a suspension of individual chlamydospores was obtained. Care was taken to avoid heating during sonication. The spores were then washed in SDW, agitated, and centrifuged at 500 rev min^{-1} for 2 min. This procedure was repeated 10 times with SDW and twice with sterile phosphate buffered saline (PBS) (0.005 M $K_2HPO_4 \cdot 3H_2$), 0.005 M KH_2PO_4, 0.15% w/v NaCl). The spores were withdrawn in a minimum of PBS, and an aliquot plated on potato dextrose agar (PDA) to check for sterility. The remaining spores were frozen, lyophilized, and extracted by the method Ayers et al. (1976). Using this procedure, 0.1 g of fresh sporocarpic material yielded 35 to 50 mg of lyophilized, extracted material. The cell wall preparation was stored at -10 °C no more than 5 days before being suspended in Ringer's physiological saline for injection into rabbits.

Immunization and determination of titer

One milliliter of extracted wall suspension (50 mg ml^{-1}) was intravenously injected into the marginal vein of the ear in each of two rabbits. The injections were repeated on days 2 and 3, then the rabbits were rested for 11 days. On days 14 and 28 the injection sequence was repeated as before. On days 35 and 42 samples of approximately 5 ml were withdrawn from the marginal vein of the ear for determination of titer. The injection sequence was repeated on day 59 and the rabbits were bled on day 76. The blood was allowed to stand overnight to coagulate, after which the red blood cells were spun down. The serum was collected and subsequently used for the antibody titer assay.

The antibody titer was determined by a modification of the agglutination technique for mycelial wall preparations described by Malajczuk et al. (1975). Sonicated chlamydospores suspended in PBS were ground with a mortar and pestle, then left for 2 h to allow the larger fragments of cell walls to settle out. The remaining suspension was removed and used as an antigen in an agglutination test. The reaction with rabbit serum was carried out at 40 °C in a covered water bath for 12 h. The agglutination reaction was assessed by examination under a dissecting microscope. A positive reaction resulted in a diffuse cloud of agglutinated particles; a negative reaction was a clear suspension. Based on these results, all further serum collections were diluted with PBS (1:25) and stored in 10 ml aliquots at -10 °C until needed.

Specificity tests with spores, hyphae and roots

Chlamydospores of G. epigaeum were collected from sporocarps as described above, but were not extracted. Spores of other VAM species were obtained by sieving and decanting (Gerdemann and Nicolson, 1963) from pot cultures of asparagus (G. mosseae, G. deserticola, Gigaspora gigantea, Acaulospora trappei) or subclover (Gigaspora margarita and A. spinosa). The other fungi tested were from cultures growing on agar plates (Phytophthora cinnamomi, Verticillium dahliae, Fusarium oxysporum f. sp. pini) or in liquid culture (Pisolithus tinctorius and Cenococcum geophilum).

The fungal preparations to be examined were placed in a 25 mm membrane filter apparatus (Millipore Inc) equipped with a 0.45 μm nucleopore filter. They were washed with 500 ml sterile PBS after which 0.5 ml rabbit serum (diluted 1:25 with PBS) was introduced. This combination was allowed to react in a covered water bath at 40 °C for 1 h. Control fungal preparations with PBS but without rabbit serum or with non-immune rabbit serum were kept in the same chamber. The samples were then rinsed with 500 ml PBS, and 0.5 ml of 1:50 fluorescein-labeled goat anti-rabbit serum (Antibodies Inc, Davis, CA) was added to all samples including the controls. These were then rinsed with 500 ml of PBS, and stored in moist plastic seal bags in the dark at 4 °C for up to 2 days before examination.

Mycorrhizal root preparations of G. epigaeum, G. mosseae, G. deserticola, Gigaspora gigantea, and Acaulospora trappei from asparagus pot cultures were rinsed in 1.0 M NaOH for 5 h before staining to decrease non-specific staining of rhizoplane debris

(Mayersbach, 1969). After the NaOH rinse, the root preparations were rinsed with 500 ml of PBS, treated with immune rabbit serum or control solutions and incubated by the procedures described above. Root preparations were quenched for tissue autofluorescence with either 0.01% aqueous Evans blue or a 10% cold water extract of bark of Douglas-fir (B. B. Bohlool, pers. comm.). All preparations were examined under incandescent and u.v. illumination on a Zeiss standard microscope. The u.v. light source was a halogen lamp filtered to pass 450 to 490 nm wavelength light through the subject and allowing no wavelength shorter than 510 to 520 nm to pass through the eyepiece. Slides were taken with Kodak 160 ASA tungsten film processed to 320 ASA. Exposure times under u.v. illumination were from 30 to 60 sec.

RESULTS AND DISCUSSION

An antiserum to extracted chlamydospore walls of G. epigaeum reacted most intensely with crushed chlamydospores of G. epigaeum [Fig. 3-1(a)]. The fluorescein label was not uniformly distributed on the outer spore surface of G. epigaeum and was more intense on the inner walls and hyphae [Fig. 3-1(a), (e)-(h)]. Roots colonized by G. epigaeum alone and together with G. mosseae or G. deserticola also stained, but the label occurred on the spores and hyphae of all three fungi [Fig. 3-1(a)-(d)]. However, spore or root preparations of VAM species in other genera tested did not react. Hyphae of non-VAM aseptate fungi present on the root surface remained unlabeled [Fig. 1(3), (f)]. The mycelial preparations of ectomycorrhizal and

pathogenic fungi also did not label. There was autofluorescence present in the spore walls of some VAM species under u.v. illumination: Gigaspora margarita fluoresced a dull ochre color and some immature G. epigaeum spores an intense orange color. Both these reactions were easily distinguished from the apple-green color of the fluorescein label. The spotty nature of the stain on the outer spore wall of G. epigaeum could have resulted from the presence of pigmentation in the wall obscuring the antigenic sites (Sen & Holland, 1970; Fitzell et al., 1980). However, the spores and hyphae of G. deserticola are pigmented but reacted with the label [Fig. 1(c), (d)].

A problem existing in many FA studies of fungal material is the cross-reaction of the antiserum to different strains of the same or closely related species. Some researchers have been able to distinguish between species in the same genus using serum developed with sexual or asexual spores as antigens (Amos & Burrell, 1967). However, this was not the case in the present study as cross-reaction occurred between all of the Glomus species tested. Titers for cross-reaction were not determined because of the difficulty of obtaining sufficient quantities of the cross-reacting species. One means of eliminating cross-reaction is by the absorption of the cross-reacting globulins (Amos & Burrell, 1967; Sen & Holland, 1970; Fitzell et al., 1980; Frankland et al., 1981) but this procedure was not attempted due to the low antibody titer of the serum collected.

The titer of specific antibodies in the blood serum never exceeded 1:50 as determined by the agglutination tests. The serum

that was collected was therefore diluted 1:25 for use in these tests. Although this low titer was not an impediment in the present study, further globulin purification, removal of cross-reacting fractions, or direct labeling of the globulins with fluorescein isothiocyanate would require a higher titer. The extraction procedure is probably destructive to much of the antigenic material in spore walls. Ayers et al. (1976) found that their cell wall preparations of Phytophthora megasperma var. sojae contained only 10% protein. Since protein moieties are highly antigenic, a low protein content in extracted chlamydospores could result in reduced antigen activity in the preparations and hence a low titer. Since the chlamydospores were not contaminated after sonication and washing, a washed and crushed wall preparation could be employed as an antigen perhaps more successfully than the extracted preparations.

The FA technique should be useful in tracing the soil phase of VAM fungi and spores, especially when used in conjunction with a concentration technique based on soil sieving (Malajczuk, Bowen & Greenhalgh, 1978). The FA technique could also be used to study the development of hyphae along the root surface. Since the technique can distinguish between genera of VAM fungi, external colonization of roots by mixed inoculum, and differences in hyphal proliferation could be assessed. All this could be done with less disruption of the rhizosphere than presently occurs when using the clearing and staining technique of Phillips and Hayman (1970).

ACKNOWLEDGEMENTS

Nicholas Malajczuk was a recipient of a CSIRO postdoctoral fellowship.

Fig. 3-1. Photomicrographs of fluorescein-labeled VAM fungi viewed with u.v. illumination except (c), (e) and (g) which were viewed with incandescent illumination. (a) Crushed chlamydospore of G. epigaeum, arrow indicates location of occluded pore. Note intensity of staining on inner wall of spore. Bar = 10 µm. (b) Mature chlamydospore of G. mosseae with external vesicle (ves). Bar = 10 µm. (c), (d) Chlamydospore and pigmented hyphae of G. deserticola. Labeled hyphae presumably of G. epigaeum in background. Bar = 10 µm. (e), (f) G. epigaeum hyphae on asparagus root with unlabeled aseptate fungal hypha. (g), (h) G. mosseae hypha and vesicle (ves) above an asparagus root. Bar = 10 µm.

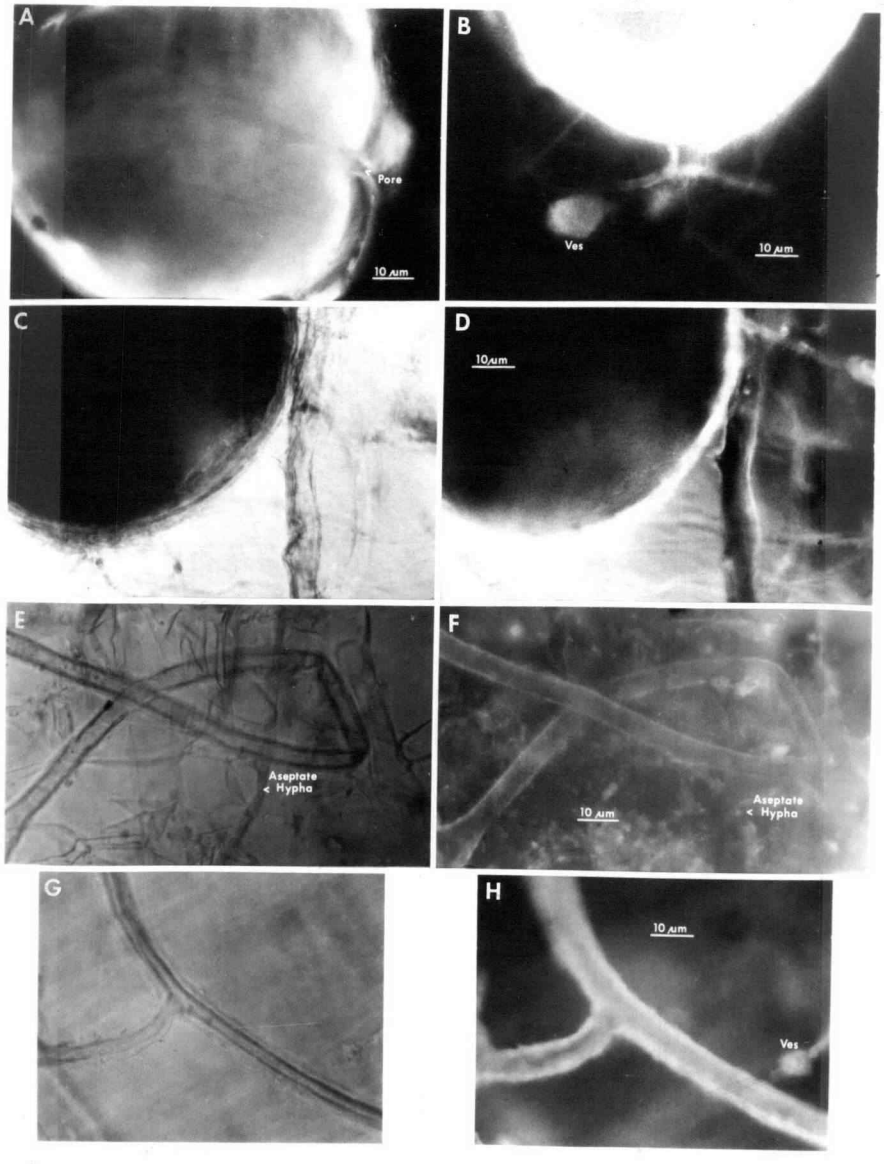
A
Pore
10 µm
B
Ves
10 µm
C
D
10µm
E
Aseptate
< Hypha
F
10 µm
Aseptate
< Hypha
G
H
10 µm
Ves

Figure 3-1.

REFERENCES

AMOS, R. E. & BURRELL, R. G. (1967). Serological differentiation in Ceratocystis. Phytopathology, **57**, 32-34.

AYERS, A. R., EBEL, J., VALENT, B. & ALBERSHEIM, P. (1976). Host-pathogen interactions X. Fractionation and biological activity of an elicitor isolated from the mycelial walls of Phytophthora megasperma var. sojae. Plant Physiology, **57**, 760-765.

BURRELL, R. G., CLAYTON, C. W., GALLEGLY, M. E & LILLY, V. G. (1966). Factors affecting the antigenicity of the mycelium of three species of Phytophthora. Phytopathology, **56**, 422-426.

DANIELS, B. A. & TRAPPE, J. M. (1979). Glomus epigaeus sp. nov., a useful fungus for vesicular-arbuscular mycorrhizal research. Canadian Journal of Botany, **57**, 539-542.

FITZELL, R., EVANS, G. & FAHY, P. C. (1980). Studies on the colonization of plant roots by Verticillium dahliae Kleb. with use of immunofluorescent staining. Australian Journal of Botany, **28**, 357-368.

FITZELL, R., FAHY, P. C., & EVANS, G. (1980). Serological studies on some Australian isolates of Verticillium spp. Australian Journal of Biological Science, **33**, 115-124.

FRANKLAND, J. C., BAILEY, A. D., GRAY, T. R. G. & HOLLAND, A. A. (1981). Development of an immunological technique for estimation of the mycelial biomass of Mycena galopus in leaf litter. Soil Biology and Biochemistry, **13**, 87-92.

GERDEMANN, J. W. & NICOLSON, T. H. (1963). Spores of mycorrhizal Endogone species extracted from soil by wet sieving and decanting. Transactions of the British Mycological Society, **46**, 235-244.

MacDONALD, J. D. & DUNIWAY, J. M. (1979). Use of fluorescent antibody to study the survival of Phytophthora megasperma and P. cinnamomi zoospores in soil. Phytopathology, **69**, 436-441.

MALAJCZUK, N., McCOMB, A. J. & PARKER, C. A. (1976). An immunofluorescent technique for detecting Phytophthora cinnamomi in soils. Australian Journal of Botany, **23**, 289-309.

MALAJCZUK, N., BOWEN, G. D. & GREENHALGH, H. G. (1978). A combined fluorescent antibody and soil sieving technique to count chlamydospores of Phytophthora cinnamomi in soil. Soil Biology and Biochemistry, **10**, 37-38.

MAYERSBACH. H. (1969). Unspecific interactions between serum and tissue in the fluorescent technique for tracing antigens in tissues. Journal of Histochemical Cytochemistry, **7**, 427.

PHILLIPS, J. M. & HAYMAN, D. S. (1970). Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Transactions of the British Mycological Society, **55**, 158-161.

SCHMIDT, E. L., BIESBROCK, J. A., BOHLOOL, B. B. & MARX, D. H. (1974). Study of mycorrhizae by means of fluorescent antibody. Canadian Journal of Microbiology, **20**, 137-139.

SEN, Y. C. & HOLLAND, A. A. (1970). Direct and indirect staining of Ophiobolus graminus Sacc. in culture and in the rhizosphere of cereal plants. Antonie van Leewenhoek, **36**, 549-554.

CHAPTER 4

Monitoring Extra-matrical Hyphae of a VA Mycorrhizal Fungus with the Immunofluorescence Assay and the Soil Aggregation Technique

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SUMMARY

An immunofluorescence assay (IFA) was used to compare the amount of extra-matrical hyphae of VAM fungi in 3 soil mixes with high or low root density. Differences in root density, accomplished by restricting root expansion into soil with nylon screens without affecting plant size. Hyphae were more frequent as root density increased. More external hyphae formed in river sand than in silt loam with similar percentages of intraradical colonization. Soil aggregation occurred in higher amounts in silt loam than in sand. A significant amount of aggregation occurred in soil without VAM

present perhaps due to non-VAM organisms, suggesting the aggregation method may be limited to use in sandy soil. The IFA detected G. epigaeum hyphae in mineral soil or sand, but autofluorescence of organic matter, especially in peat, was a major limitation of the technique.

Key words: Rhizosphere, Glomus epigaeum, external mycelium, soil aggregation.

INTRODUCTION

Vesicular-arbuscular mycorrhizae (VAM) benefit plant growth by increasing phosphorus uptake from soil. A possible mechanism for enhanced phosphorus uptake is exploration of greater soil volumes by external mycelium of the VAM fungus. This mycelium reaches sources of phosphate and other immobile nutrients otherwise unavailable to the plant root.

Activity of the VAM fungi is currently monitored by examining colonized root tissue in which these fungi form distinctive structures called arbuscules and vesicles within root cortical cells. However, intensity of root colonization does not always correspond to increased plant growth (Mosse, 1972; Graham et al, 1982) and soil factors have been shown to alter plant growth enhancement by VAM in spite of similar levels of root colonization (Davis et al., 1983; Skipper & Horace, 1979).

Radiotracers and heavy isotopes implicate VA mycorrhizal hyphae in nutrient uptake at considerable distances from colonized roots

(Rhodes & Hirrel, 1982). These isotope systems require physical separation of the roots and mycelium to insure only hyphal uptake of the isotopes. However, conclusions made about hyphal nutrient uptake and soil VAM proliferation from these studies may be inappropriate if proximity of root tissue affects the intensity of fungal soil colonization.

Most studies to date have been done with plants colonized by single isolates of VAM fungi. Such studies probably do not reflect the situation in natural soils where many species coexist in the root system (Abbott & Robson, 1977; Walker et al., 1982). Abbott and Robson (1979) have developed a method of classification to distinguish VAM fungus species in roots by certain peculiarities of the infective hyphae. This method identifies VAM endophytes but leaves unresolved their activities in the soil.

Adaptation of the immunofluorescent assay (IFA) to VA mycorrhizal fungi has recently been described (Aldwell et al., 1983; Kough et al., 1983, Wilson et al., 1983). This technique can be used to recognize endogonaceous fungal structures and distinguish them from other soil fungi. At present, antisera specificity is at the generic level only. However, this technique is one of the best means now available to study both the taxonomy and soil ecology of these ubiquitous root symbionts.

In the present study, the level of soil colonization by VAM fungi was determined using both IFA (Kough et al., 1983) and the sand aggregation method (Sutton & Sheppard, 1976; Graham et al., 1982). In addition, the effect of restricting root but not mycelial growth

on soil colonization by VAM fungi was examined. Plants were grown in three different soil types to test the possible effect of soil physical and/or chemical characters on hyphal proliferation.

MATERIALS AND METHODS

Preparation of specific antigens

Sporocarps of Glomus epigaeum Daniels & Trappe [= Glomus versiforme (Karst) Berch] were collected from the soil surface of host plant cultures of the fungus on Asparagus officinalis L., Thuja plicata J. Don ex D. Don and Sequoia sempervirens (D. Don) Endl. A suspension of single chlamydospores and hyphal debris was obtained by sieving and decanting sporocarps (Nicolson & Gerdemann, 1963). This spore suspension was filtered on a 10µM mesh nylon filter, washed with sterile deionized water (SDW), and placed in a solution of streptomycin (200 ppm) and gentiamycin (100 ppm) in sterile phosphate buffered saline (PBS) (0.15% w/v NaCl, 0.5 mM $K_2HPO_4 \cdot 3\ H_2O$, 0.5 mM KH_2PO_4, pH 7.2) to surface disinfest the spores (Mertz et al., 1981). After 2 wk at 6 °C in antibiotic suspension, the spores were removed, rinsed with SDW and placed on sterile filter paper in a petri dish to dry at 6 °C. At this time 150 spores were plated on potato dextrose agar or Kings medium B to check for sterility. Chlamydospores with no visible sign of contamination were weighed and mixed 1:1 (w/v) with PBS in a ground glass tissue macerator. Particulate and soluble fractions were separated by centrifugation (12,800 g for 3 min) after grinding. The soluble fraction without the lipid layer that had separated on top was retained and stored at

-20°C until needed. The particulate fraction was washed three times by suspension in PBS followed by centrifugation. The final pellet was suspended in PBS to give a final concentration of 150 mg fresh spore weight per ml and stored at -20°C until needed.

Immunization

The following immunization schedule was used for antibody production in rabbits:

Day 0. Thirty ml of blood were drawn from each of six male New Zealand white rabbits prior to injection to detect the presence of pre-existing antibodies to Glomus epigaeum. One-half ml of either soluble or particulate antigen preparation was injected intravenously (IV) into the marginal vein of the ear.

Day 14. A second IV injection of 1 ml of antigen preparation was administered. These IV injections were intended to induce a highly specific initial reaction.

Day 28. One-half ml of antigen preparation mixed 1:1 v/v with Freund's incomplete adjuvant (DIFCO, Detroit, MI) was administered as an intramuscular (IM) injection into a hind leg of each rabbit (Herbert, 1978). The use of IM injections and adjuvants was intended to increase antibody titer.

Day 42. Another 1 ml preparation of antigen and Freund's incomplete adjuvant was given as an IM injection in a hind leg.

Day 52. Fifty ml of blood were withdrawn from each rabbit to determine antibody titer. Surplus serum was stored in 5 ml aliquots

at -20 °C until needed. Since these initial antibody titers were low, another series of IM injections was initiated.

Day 82. Another 1 ml preparation of antigen and Freund's incomplete adjuvant was given as an IM injection in a hind leg.

Day 96. Another 1 ml preparation of antigen with Freund's adjuvant was administered IM in a rear leg.

Day 106. Blood samples were taken and antibody titers determined. As there was no change in antibody titer, the rabbits were bled by cardiac puncture and euthanized. All blood samples were allowed to clot overnight at 4 °C. The serum was removed, and stored in 5 ml aliquots at -20 °C until needed.

Titer determination and cross reaction tests

Titer was determined by agglutination in a series of wells as described in Malajczuk et al. (1976). Cross reactions were examined by indirect immunofluorescence on the fungal species Fusarium roseum, Fusarium oxysporum, Phytophthora cinnamomi, and species of Trichoderma and Penicillium isolated from soil dilutions as described in Kough et al. (1983).

Plant species and growth conditions

Natural seedlings of Chamaecyparis lawsoniana (A. Murr.) Parl. less than 2 cm tall were collected in the organic litter layer under a mature C. lawsoniana tree on the Oregon State University campus. Twenty-five seedlings were transplanted into 160 ml plastic tubes (Leach "Super Cells", Ray Leach Cone-Tainer Nursery, Canby, OR USA)

with pasteurized river sand (60°C/30 min) and inoculated with 20 ml of roots, soil, and spores of Glomus epigaeum pot culture as described in Chapter 1. The entire root systems of 30 C. lawsoniana seedlings from the same sward of organic duff were cleared and stained (Phillips & Hayman, 1970) and examined to determine if seedlings were colonized with VAM prior to transplanting.

The inoculated seedlings were maintained in a glasshouse (22/15°C average day/night temperature with supplemental lighting for 16 h photoperiod from high pressure sodium vapor lights with average irradiance of 200 μEinsteins$\cdot sec^{-1} \cdot M^{-1}$) for 6 mo with biweekly fertilization with 10 ml of 1/4 strength phosphate (11 ppm) Long Ashton's Nutrient Solution (LANS) (Hewitt, 1966). After 6 months 21 of the seedlings were measured for height and stem diameter and transplanted into 22 cm diameter plastic pots. The potting medium was one of the following: pasteurized river sand, pasteurized 1:1 river sand and silt loam, or pasteurized 1:1 river sand and sphagnum peat; all media were sieved to 2 mm prior to use. Roots of the four remaining seedings were cleared and stained and examined for VAM colonization (Biermann & Linderman, 1981).

Of the 7 seedlings transplanted into each soil, 3 seedling root systems were bound with 1 mm mesh nylon screening to restrict lateral growth of the root systems. The remaining 4 seedlings were transplanted without binding. All seedlings were planted near the center of the pots. The plants were maintained as described above except 50 ml of 1/4 strength phosphate (11 ppm) LANS fertilizer

solution was carefully poured around the edge of the pots at 2, 4, and 6 weeks after transplanting.

Harvest procedures

At 6 and 8 wk after transplanting, plants were measured for top height and stem diameter. A 1.8 cm diameter cork borer was used to remove 2 adjacent soil cores near the edge of each pot. The cores were measured and weighed. One of the cores was allowed to air dry for 48 h for determination of soil aggregation by VAM hyphae (Sutton & Shepard, 1976; Graham et al., 1982). The other core was used to determine the presence of VAM by the indirect IFA (Goldman, 1968; Johnson et al, 1978).

A modification of the technique of Malajczuk et al. (1978) was employed to determine presence of VAM in the soil fraction. Each weighed soil core was placed in 40 ml of 7.5 mM KOH and agitated to remove adherent debris. Visible root tissue was removed, cleared and stained to determine VAM presence (Phillips & Hayman, 1970). The agitated soil suspensions were allowed to settle for 5 h at 4 °C. The suspension above the settled soil fraction was withdrawn and passed through a 10 μM mesh nylon filter. Debris and hyphae remaining on the filter were rinsed twice with PBS and stained with serum for IFA and examined as described in Kough et al. (1983). Hyphal amounts in these IFA preparations were subjectively rated on a 0-5 scale (5 = maximum quantity of fluorescent fragments) based on total fluorescent hyphal fragments observed in the microscopic mounts of debris retained on the filter.

RESULTS

Titer determination and cross reaction tests

Using a shorter immunization schedule and antigenic preparations with Freund's incomplete adjuvant, a higher antibody titer was obtained than in previous studies (Kough et al., 1983). Titers for the sera ranged from 1:30 to 1:300 in agglutination tests. The second immunization series did not increase antibody titer, so the rabbits were bled and euthanized. Higher titers were generally obtained with the soluble antigen preparations (1:60 to 1:300) than the particulate fractions (1:30 to 1:60). However, in subsequent tests for cross reaction with common soil fungi, antisera produced to the soluble antigens showed cross reaction to Penicillium species making these sera unusable in a soil assay without cross absorption. Therefore, antisera to particulate antigen preparations with both highest titer and lowest level of cross reaction to soil fungi were used for subsequent assays.

Root density differences

Root development into soil occurred in both the presence or absence of nylon screening but they were generally fewer in number with nylon screening (Tables 4-1, 4-2). Roots were less frequently detected in the soil cores from plants with restricted roots especially at the 6 wk harvest. True differences in root density can therefore be assumed to have occurred. Seedling top height or stem

diameter did not differ significantly at either harvest due to these root differences.

VAM root colonization and adherent external hyphae

The roots of seedlings collected in the organic duff, cleared and stained at the time of VAM inoculation, were not colonized with VAM fungi. Considering the nonsterile conditions of germination and growth, the absence of adherent saprophytic fungi is notable. These could have been displaced during the process of clearing and staining, however. At the time of transplanting after 6 months growth, the roots of 4 random VAM-inoculated seedlings were colonized by VAM fungi over 46% of the root length with hyphal coils, intraradical hyphae and vesicles.

The root pieces in the soil cores used for IFA were examined for colonization by VAM fungi, presence of external hyphae and VAM entry points (Table 4-3). Roots from river sand had a larger amount of external mycelium still adherent to entry points and a greater number of entry points compared to roots from the other two soils. Percent root length colonization in the different soils was probably similar but the root sample size recovered from the cores was too small to analyze statistically. Root samples recovered from the cores with unrestricted root growth were only numerous enough in the 8 wk assay to justify a statistical analysis. A t-test showed no significant differences in root colonization among the three soils at that time.

The number of entry points and amount of adherent mycelium on roots was highest in the river sand and lowest in the peat:sand

mixture or roots that escaped the nylon mesh barrier. Again, these results can only suggest trends, since sample size was too small for statistical treatment.

Soil aggregate formation

Soil aggregation, presumably by VAM hyphae, differed between soil types used in this study. The total percentage of soil in aggregates was highest in the silt loam:river sand mixture and lowest in the river sand alone (Table 4-2). Within a soil type, no difference between the percentage of soil in aggregates from cores taken with or without root growth restriction occurred.

Values for aggregate formation, expressed as aggregate weight (gm) · root weight (gm^{-1}), could not be calculated for most of the cores with root growth restriction since root tissue was absent in most cores. However, values for aggregate formation decreased with increasing root length/volume values in river sand system with unrestricted root growth. From similarity in amount of soil aggregates, both over time and between root density treatments, both the silt loam and peat soils aggregate due to the presence of other microbes. VAM hypha-induced aggregation may be masked in these systems.

Immunofluorescence assay

The immunofluorescence assay (IFA) was not possible in peat soil due to high levels of background fluorescence in the organic fraction. Some fluorescence was nonspecific staining of the organic

particles, but the majority was from autofluorescence of organic matter.

In mineral soils VAM hyphae were easily detected since little debris collected on the filters besides soil fungi and tiny root fragments. There was a difference between the amount of VAM fragments present in cores with root tissue as opposed to those without (Table 4-1). Even after removal of root tissue, when it had been in the soil cores, more fragments of VAM hyphae were recovered than if roots were not present. This can be seen in the higher levels of IFA-detected fragments in cores with larger values of root length/cm^3.

More IFA detectable VAM hyphae were found in river sand than the silt loam. This may be an artifact of the system to recover the hyphae. More soil mycelium probably settled with the soil fraction and was not recovered. Another method to separate the mycelium might be employed such as the sucrose density recovery system described by Tisdale & Oades (1979).

DISCUSSION

Low root densities in soil affect the probability that extramatrical mycelium of VAM fungi will rapidly proliferate in the soil. High root densities have been previously shown to adversely affect the ability of VAM fungi to colonize root tissue (Warner & Mosse, 1982). The present study indicates low root density can adversely affect fungal colonization of soil even when the level of root

colonization is similar to the high root density situation. The presence of soil microbes can enhance proliferation of external mycelium of VA mycorrhizae (Sutton & Sheppard, 1976). If the responsible microbes are the rhizosphere microflora, a lower root density would reduce their numbers perhaps affecting hyphal proliferation. However, as indicated by Warner and Mosse (1982), host plant species is the dominant factor in root density VAM colonization effects. Therefore another plant species should be examined to confirm these results.

Ability of VAM fungus to proliferate in the soil between roots was affected by soil type. Many anomalous results with VAM symbiosis (Skipper & Smith, 1979; Davis et al., 1983) have implicated edaphic factors especially pH. While VAM fungi transport organic nitrogen to host plants from considerable distances (Ames et al., 1983) and grow into soil organic matter preferentially (St. John et al., 1983), the effect of organic matter on proliferation of soil hyphae is still unclear. Both the soil aggregate assay and IFA were not usable in soil with high organic matter content.

The IFA effectively detected VAM hyphae in soil. Careful removal of root pieces to confirm VAM root colonization also provided samples of roots with adherent VAM mycelium. While presence of mycelium correlated well with the subsequent results of IFA tests, considerable amounts of hyphae are apparently lost even with careful manipulation. Moreover, hyphal fragments that reacted on the IFA filter system were not always easy to confirm visually as VAM hyphae.

The immunofluorescence system as described is limited in usefulness to mineral soils until a method is found to eliminate background fluorescence of organic particles. Counterstaining or gelatin pretreatment effectively reduced a similar problem in other soil systems and may be effective here (Bohlool & Schmidt, 1968). Background fluorescence complications could be reduced if the soil organic fraction were absent. Sucrose density treatments may be applicable to remove unwanted debris (Tisdale & Oades, 1979). The affect of high osmotica on the antigenicity of these preparations, however, is unknown.

The use of the IFA technique will not eliminate the need to clear and stain roots to confirm VAM colonization. However, it provides a means to sample the soil nondestructively for the activity of VAM symbionts. At present the technique is limited in usefulness because the antisera developed are specific for VAM fungi only at the generic level (Aldwell et al., 1983; Kough et al., 1983; Wilson et al., 1983). Improved antisera specificity may permit one to trace development of external hyphae of a specific isolate in a soil system with a mixed population of VAM fungi.

Table 4-1. Presence of extra-matrical VAM hyphae measured by immunofluorescence in soil cores with differing root densities.

Soil type and root density*	6 wks after transplant		8 wks after transplant	
	Fluorescence rating†	Root length (cm)/cm^3 soil	Fluorescence rating†	Root length (cm)/cm^3 soil
Sand, roots free	2.5	.32	2.5	.65
Sand, roots restricted	0.5	--‡	1.5	.35
Silt loam, roots free	1.5	.38	1.5	.66
Silt loam, roots restricted	1.5	.27	0.5	.09

* Root density differences resulted from growth being restricted by the presence or absence of nylon screening.

† Fluorescence ratings were visually assessed on a 0-5 scale (5 = greatest abundance of fluorescent mycelia) for total mass of VAM hyphae retained on filter.

‡ No root tissue present in soil core.

Table 4-2. Aggregate formation by VAM hyphae in soil cores of river sand, silt loam and peat soil with different root densities.

Soil type and root density*	6 wks after transplant			8 wks after transplant		
	Aggregate formation			Aggregate formation		
	% total soil	Aggregate wt/ root tissue wt (gm)	Root length (cm)/cm^3 soil	% total soil	Aggregate wt/ root tissue wt (gm)	Root length (cm)/cm^3 soil
Sand, roots free	18	22.3	.32	25	8.9	.79
Sand, roots restricted	23	36.1	.08	36	36.1	.18
Silt loam, roots free	37	45.7	.13	46	19.4	.47
Silt loam, roots restricted	42	37.5	.04	37	61.0	.15
Peat, roots free	33	24.0	.45	24	21.4	.18
Peat, roots restricted	35	30.9	.18	31	34.9	.17

* Root density differences resulted from growth being restricted by the presence or absence of nylon screen.

Table 4-3. VAM root colonization in soil cores used for immunofluorescence assay

Soil type and root density*	6 wks after transplant			8 wks after transplant		
	Entry points	VAM % root length	Adherent external hyphae†	Entry points	VAM % root length	Adherent external hyphae†
Sand, roots free	79	80	3.0	70	60	4.0
Sand, roots restricted	--	--	--‡	14	62	3.0
Silt loam, roots free	46	54	2.0	43	57	2.0
Silt loam, roots restricted	22	35	1.0	31	42	1.5
Peat, roots free	7	43	1.0	24	30	1.5
Peat, roots restricted	--	--	--‡	--	--	--‡

* Root density differences resulted from growth being restricted by the presence or absence of nylon screening.

† Amount of external hyphae present and connected to infection points was estimated on a 0-5 scale, 5 = greatest amount of hyphae.

‡ No root tissue recovered from soil cores.

REFERENCES

ABBOTT, L. K. & ROBSON, A. D. (1977). The distribution and abundance of vesicular arbuscular endophytes in some western Australian soils. Australian Journal of Botany, 25, 515-22.

ABBOTT, L. K. & ROBSON, A. D. (1979). A quantitative study of the spores and anatomy of mycorrhizas formed by a species of Glomus, with reference to its taxonomy. Australian Journal of Botany, 27, 353-375.

ALDWELL, F. E. B., HALL, I. R. & SMITH, J. M. B. (1983). Enzyme linked immunosorbent assay (ELISA) to identify endomycorrhizal fungi. Soil Biology and Biochemistry, 15, 377-378.

AMES, R. N., REID, C. P. P., PORTER, L. K. & CAMBARDELLA, C. (1983). Hyphal uptake and transport of nitrogen from two ^{15}N-labelled sources by Glomus mosseae, a vesicular-arbuscular mycorrhizal fungus. New Phytologist, 95, 381-396.

BIERMANN, B. & LINDERMAN, R. G. (1981). Quantifying vesicular-arbuscular mycorrhizae: a proposed method towards standardization. New Phytologist, 87, 63-67.

BOHLOOL, B. B. & SCHMIDT, E. L. (1968). Nonspecific staining: Its control in immunofluorescence examination of soil. Science, 162, 1012-1014.

DAVIS, E. A., YOUNG, J. L. & LINDERMAN, R. G. (1983). Soil lime level (pH) and VA-mycorrhiza effects on growth responses of sweetgum seedlings. Soil Science Society of America Journal, 47, 251-256.

GERDEMANN, J. W. & NICOLSON, T. H. (1963). Spores of mycorrhizal Endogone species extracted from soil by wet sieving and decanting. Transactions of the British Mycological Society, **46**, 235-244.

GOLDMAN, M. (1968). Fluorescent Antibody Methods. Academic Press, New York.

GRAHAM, J. H., LINDERMAN, R. G. & MENGE, J. A. (1982). Development of external hyphae by different isolates of mycorrhizal Glomus spp. in relation to root colonization and growth of Troyer citrange. New Phytologist, **91**, 183-89.

HERBERT, W. J. (1978). Mineral oil adjuvants and the immunization of laboratory animals. In: Handbook of Experimental Immunology (ed. by D. M. Weir), Appendix 3. Blackwell Scientific, Oxford and Edinburgh.

HEWITT, E. J. (1966). Sand and water culture methods used in the study of plant nutrition. In: Technical Communication No. 22, 2nd Edn., revised. Commonwealth Agricultural Bureaux, London.

JOHNSON, G. D., HOLBOROW, E. J. & DORLING, J. (1978). Immunofluorescence and immunoenzyme techniques. In: Handbook of Experimental Immunology (ed. by D. M. Weir), chapter 15. Blackwell Scientific, Oxford and Edinburgh.

KOUGH, J. L., MALAJCZUK, N. & LINDERMAN, R. G. (1983). Use of the indirect immunofluorescent technique to study the vesicular-arbuscular fungus Glomus epigaeum and other Glomus species. New Phytologist, **94**, 57-62.

MALAJCZUK, N., McCOMB, A. J. & PARKER, C. A. (1976). An immunofluorescent technique for detection of Phytophthora cinnamomi in soils. Australian Journal of Botany, **23**, 289-309.

MALAJCZUK, N., BOWEN, G. D. & GREENHALGH, H. G. (1978). A combined fluorescent antibody and soil sieving technique to count chlamydospores of Phytophthora cinnamomi in soil. Soil Biology and Biochemistry, **10**, 37-38.

MERTZ, S. M., HEITHAUS, J. J. & BUSH, R. L. (1979). Mass production of axenic spores of the endomycorrhizal fungus Gigaspora margarita. Transactions of the British Mycological Society, **72**, 167-169.

MOSSE, B. (1972). Effect of different endogone strains on the growth of Paspalum notatum. Nature, **239**, 221-223.

PHILLIPS, J. M. & HAYMAN, D. S. (1970). Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Transactions of the British Mycological Society, **55**, 158-161.

RHODES, L. H. & HIRREL, M. C. (1982). Radiotracer methods for mycorrhizal research. In: Methods and Principles of Mycorrhizal Research (N. C. Schoenck, ed.), pp. 189-200. American Phytopathological Society, St. Paul, MN.

SKIPPER, H. D. & SMITH, G. W. (1979). Influence of soil pH on the soybean-endomycorrhiza symbiosis. Plant and Soil, **53**, 559-563.

ST. JOHN, T. V., COLEMAN, D. C. & REID, C. P. P. (1983). Association of vesicular-arbuscular mycorrhizal hyphae with soil organic particles. Ecology, **64**, 957-959.

SUTTON, J. C. & SHEPPARD, B. R. (1976). Aggregation of sand-dune soil by endomycorrhizal fungi. Canadian Journal of Botany, **54**, 326-333.

TISDALL, J. M. & OADES, J. M. (1979). Stabilization of soil aggregates by the root systems of ryegrass. Australian Journal of Soil Research, **17**, 429-441.

WALKER, C., MIZE, C. W. & McNABLE, H. S., Jr. (1982). Populations of endogonaceous fungi at two locations in Central Iowa. Canadian Journal of Botany, **60**, 2518-2529.

WARNER, A. & MOSSE, B. (1982). Factors affecting the spread of vesicular mycorrhizal fungi in soil. I. Root density. New Phytologist, **90**, 529-536.

WILSON, J. M., TRINICK, M. J. & PARKER, C. A. (1983). The identification of vesicular-arbuscular mycorrhizal fungi using immunofluorescence. Soil Biology and Biochemistry, **15**, 439-445.

DISCUSSION AND CONCLUSIONS

The extra-matrical hyphae of VAM are an integral part of the interaction of host plant, edaphic and fungal symbiont factors.

When the same host plant, VAM fungus species, and soil system were examined at different levels of soil phosphorus fertility, biomass differed significantly with the same percentage of root colonization. Factors other than root colonization appear to be responsible for the efficiency of the endomycorrhizal symbiosis. Similar results (Mosse, 1972; Graham et al., 1982) have suggested that the ability of the extra-matrical hyphae of VAM fungus to proliferate in the soil was altered and consequently, the beneficial effect of the VAM fungus on plant growth was reduced.

To determine if plant species has any effect on the growth promoting activity of the fungus, the same species of VAM fungus was examined on different host plants in the same soil. In both herbaceous and arboreal species, physiological requirements of the host plant were the major factors responsible for growth enhancement by the fungus. Since these fungi are obligate parasites, their growth will be affected by host factors nourishing the fungus. Host plant growth responsiveness to VAM is related to its phosphorus nutrition and often to root physical characteristics such as diameter and presence of root hairs (Baylis, 1975; Gerdemann, 1975; St. John, 1980). Current studies indicate similar root colonization on plants with different root morphologies such as trees with magnolioid roots and clover with fibrous roots resulted in very different levels of growth enhancement. Moreover, in plants such as clover and lotus

with similar root characteristics, a given VAM fungus with similar levels of root colonization gave significantly increased growth on clover and no effect on lotus.

The amount of extra-matrical hyphae clearly related to growth enhancement in some VAM isolates and not others. In the same soil system, some VAM species caused significantly greater aggregation of sand particles than others. This activity correlated with enhanced nitrogen fixation and greater plant growth in A. trappei and Glomus fasciculatum. However, G. epigaeum gave increased plant growth and nitrogenase activity with low levels of sand aggregation. Both Gigaspora species, caused significant aggregation of sand particles but did not increase plant growth and occasionally caused growth suppression.

Development of VAM external hyphae was apparently influenced by edaphic factors as well as root density. The amount of external hyphae of VAM was lower in silt loam than sandy soil. Lower root density produced a decrease in VAM external hypahe detected in either soil. The decrease in external mycelium may reflect a situation where the nutrient depletion zone around each individual root is smaller hence the VAM could obtain adequate phosphorus with less proliferation into the soil. Microbes enhance the proliferation of VAM external mycelium contrary to the situation in many soil fungi (Sutton & Sheppard, 1976). Decreased root tissue density would concurrently decrease rhizosphere microbes and thereby reduce the development of external VAM hyphae.

LITERATURE CITED

ABBOTT, L. K. & ROBSON, A. D. (1977). The distribution and abundance of vesicular arbuscular endophytes in some western Australian soils. Australian Journal of Botany, **25**, 515-22.

ABBOTT, L. K. & ROBSON, A. D. (1977). Growth stimulation of subterranean clover with vesicular arbuscular mycorrhizas. Australian Journal of Agricultural Research, **28**, 639-649.

ABBOTT, L. K. & ROBSON, A. D. (1979). A quantitative study of the spores and anatomy of mycorrhizas formed by a species of Glomus, with reference to its taxonomy. Australian Journal of Botany, **27**, 353-375.

ABBOTT, L. K. & ROBSON, A. D. (1982). The role of vesicular arbuscular mycorrhizal fungi in agriculture and the selection of fungi for inoculation. Australian Journal of Agricultural Research, **33**, 389-408.

ALDWELL, F. E. B., HALL, I. R. & SMITH, J. M. B. (1983). Enzyme linked immunosorbent assay (ELISA) to identify endomycorrhizal fungi. Soil Biology and Biochemistry, **15**, 377-378.

ALLEN, M. F., MOORE, T. S., Jr. & CHRISTENSEN, M. (1980). Phytohormone changes in Bouteloua gracilis infected by vesicular-arbuscular mycorrhizae. I. Cytokinin increases in the host plant. Canadian Journal of Botany, **58**, 371-74.

AMES, R. N., REID, C. P. P., PORTER, L. K. & CAMBARDELLA, C. (1983). Hyphal uptake and transport of nitrogen from two ^{15}N-

labelled sources by Glomus mosseae, a vesicular-arbuscular mycorrhizal fungus. New Phytologist, **95**, 381-396.

AMOS, R. E. & BURRELL, R. G. (1967). Serological differentiation in Ceratocystis. Phytopathology, **57**, 32-34.

ASIMI, S., GIANINAZZI-PEARSON, V. & GIANINAZZI, S. (1980). Influence of increasing soil phosphorus levels on interactions between vesicular-arbuscular mycorrhizae and Rhizobium in soybeans. Canadian Journal of Botany, **58**, 2200-2205.

AYERS, A. R., EBEL, J., VALENT, B. & ALBERSHEIM, P. (1976). Host-pathogen interactions X. Fractionation and biological activity of an elicitor isolated from the mycelial walls of Phytophthora megasperma var. sojae. Plant Physiology, **57**, 760-765.

BÄRTSCHI, H., GIANINAZZI-PEARSON, V. & VEGH, I. (1981). Vesicular-arbuscular mycorrhiza formation and root rot disease (Phytophthora cinnamomi) development in Chamaecyparis lawsoniana. Phytopathologische Zeitschrift, **102**, 213-218.

BAYLIS, G. T. S. (1962). Rhizophagus, the catholic symbiont. Australian Journal of Science, **25**, 195-200.

BAYLIS, G. T. S. (1975). The magnolioid mycorrhiza and mycotrophy in root systems derived from it. In: Endomycorrhizas (Ed. by F. E. Sanders, Barbara Mosse & P. B. Tinker), pp. 149-174. Academic Press, New York & London.

BETHLENFALVAY, G. J. & YODER, J. F. (1981). The Glycine-Glomus-Rhizobium symbiosis. I. Phosphorus effect on nitrogen fixation and mycorrhizal infection. Physiologia Plantarum, **52**, 141-145.

BETHLENFALVAY, G. J., BROWN, M. S. & PACOUSKY, R. S. (1982). Relationships between host and endophyte development in mycorrhizal soybeans. New Phytologist, **90**, 537-543.

BIERMANN, B. & LINDERMAN, R. G. (1981). Quantifying vesicular-arbuscular mycorrhizae: a proposed method towards standardization. New Phytologist, **87**, 63-67.

BIERMANN, B. & LINDERMAN, R. G. (1983). Increased geranium growth using pretransplant inoculation with a mycorrhizal fungus. Journal of the American Society of Horticulture, **108**, 972-976.

BOHLOOL, B. B. & SCHMIDT, E. L. (1968). Nonspecific staining: Its control in immunofluorescence examination of soil. Science, **162**, 1012-1014.

BURRELL, R. G., CLAYTON, C. W., GALLEGLY, M. E & LILLY, V. G. (1966). Factors affecting the antigenicity of the mycelium of three species of Phytophthora. Phytopathology, **56**, 422-426.

CHAPIN, F. S, III. (1983). Adaptation of selected trees and grasses to low availability of phosphorus. Plant and Soil, **72**, 283-287.

COOPER, K. M. (1981). The role of VA mycorrhizas in the development of a new commercial crop - tamarillo - in New Zealand. Proceedings of the 5th North American Conference on Mycorrhizae, Universite Laval, Quebec, p. 54 (Abstract).

CRESS, W. A., THRONEBERRY, G. O. & LINDSEY, D. L. (1979). Kinetics of phosphorus absorption by mycorrhizal and non-mycorrhizal tomato roots. Plant Physiology, **64**, 191-206.

DANIELS, B. A. & TRAPPE, J. M. (1979). Glomus epigaeus sp. nov., a useful fungus for vesicular-arbuscular mycorrhizal research. Canadian Journal of Botany, **57**, 539-542.

DAVIS, E. A., YOUNG, J. L. & LINDERMAN, R. G. (1983). Soil lime level (pH) and VA-mycorrhiza effects on growth responses of sweetgum seedlings. Soil Science Society of America Journal, **47**, 251-256.

FITZELL, R., EVANS, G. & FAHY, P. C. (1980). Studies on the colonization of plant roots by Verticillium dahliae Kleb. with use of immunofluorescent staining. Australian Journal of Botany, **28**, 357-368.

FITZELL, R., FAHY, P. C., & EVANS, G. (1980). Serological studies on some Australian isolates of Verticillium spp. Australian Journal of Biological Science, **33**, 115-124.

FRANKLAND, J. C., BAILEY, A. D., GRAY, T. R. G. & HOLLAND, A. A. (1981). Development of an immunological technique for estimation of the mycelial biomass of Mycena galopus in leaf litter. Soil Biology and Biochemistry, **13**, 87-92.

FURLAN, V., FORTIN, J. A. & PLENCHETTE, C. (1983). Effects of different vesicular-arbuscular fungi on growth of Fraxinus americana. Canadian Journal of Forest Research, **13**, 589-593.

GATES, C. T. & MÜLLER, W. J. (1979). Nodule and plant development in the soybean, Glycine max (L.) Merr.: Growth response to nitrogen, phosphorus and sulfur. Australian Journal of Botany, **27**, 203-215.

GERDEMANN, J. W. & NICOLSON, T. H. (1963). Spores of mycorrhizal Endogone species extracted from soil by wet seiving and decanting. Transactions of the British Mycological Society, **46**, 235-244.

GERDEMANN, J. W. & TRAPPE, J. M. (1974). The Endogonaceae in the Pacific Northwest. Mycologia Memoirs, **5**, 1-76.

GOLDMAN, M. (1968). Fluorescent Antibody Methods. Academic Press, New York.

GRAHAM, J. H., LINDERMAN, R. G. & MENGE, J. A. (1982). Development of external hyphae by different isolates of mycorrhizal Glomus spp. in relation to root colonization and growth of Troyer citrange. New Phytologist, **91**, 183-189.

GRIME, J. P. (1979). Plant Strategies and Vegetation Processes. John Wiley & Sons, Chichester and New York.

HARLEY, J. L. (1978). Ectomycorrhizas as nutrient absorbing organs. Proceedings of the Royal Society of London, Series B, **203**, 1-21.

HERBERT, W. J. (1978). Mineral oil adjuvants and the immunization of laboratory animals. In: Handbook of Experimental Immunology (ed. by D. M. Weir), Appendix 3. Blackwell Scientific, Oxford and Edinburgh.

HEWITT, E. J. (1966). Sand and water culture methods used in the study of plant nutrition. In: Technical Communication No. 22, 2nd Edn., revised. Commonwealth Agricultural Bureaux, London.

JANOS, D. P. (1981). VA mycorrhizae increase productivity and diversity of tropical tree communities. Proceedings of the 5th

North American Conference on Mycorrhizae, Universite Laval, Quebec, p. 18 (Abstract).

JOHNSON, G. D., HOLBOROW, E. J. & DORLING, J. (1978). Immunofluorescence and immunoenzyme techniques. In: Handbook of Experimental Immunology (ed. by D. M. Weir), chapter 15. Blackwell Scientific, Oxford and Edinburgh.

KORMANIK, P. P., BRYAN, W. C. & SCHULTZ, R. C. (1980). Procedures and equipment for staining large numbers of plant root samples for endomycorrhizal assay. Canadian Journal of Microbiology, **26**, 536-538.

KORMANIK, P. P., SCHULTZ, R. C. & BRYAN, W. C. (1982). The influence of vesicular-arbuscular mycorrhizae on the growth and development of eight hardwood tree species. Forest Science, **28**, 531-539.

KOUGH, J. L., MALAJCZUK, N. & LINDERMAN, R. G. (1983). Use of the indirect immunofluorescent technique to study the vesicular-arbuscular fungus Glomus epigaeum and other Glomus species. New Phytologist, **94**, 57-62.

KUCEY, R. M. N. & PAUL, E. A. (1982). Carbon flow, photosynthesis and N_2 fixation in mycorrhizal and nodulated faba beans (Vicia faba L.). Soil Biology and Biochemistry, **14**, 407-412.

MacDONALD, J. D. & DUNIWAY, J. M. (1979). Use of fluorescent antibody to study the survival of Phytophthora megasperma and P. cinnamomi zoospores in soil. Phytopathology, **69**, 436-441.

MALAJCZUK, N., BOWEN, G. D. & GREENHALGH, H. G. (1978). A combined fluorescent antibody and soil sieving technique to count

chlamydospores of Phytophthora cinnamomi in soil. Soil Biology and Biochemistry, **10**, 37-38.

MALAJCZUK, N., McCOMB, A. J. & PARKER, C. A. (1976). An immunofluorescent technique for detection of Phytophthora cinnamomi in soils. Australian Journal of Botany, **23**, 289-309.

MARCONI, D. J. & NELSON, P. V. (1984). Leaching of applied phosphorus in container media. Scientia Horticulturae, **22**, 275-285.

MAYERSBACH. H. (1969). Unspecific interactions between serum and tissue in the fluorescent technique for tracing antigens in tissues. Journal of Histochemical Cytochemistry, **7**, 427.

MEJSTRIK, V. & KELLEY, A. P. (1979). Mycorrhizae in Sequoia gigantea Lindl. et Gard. and Sequoia sempervirens Endl. Ceska Mykologie, **33**, 51-54.

MENGE, J. A. (1982). Effect of soil fumigants and fungicides on vesicular-arbuscular fungi. Phytopathology, **72**, 1125-1132.

MERTZ, S. M., Jr., HEITHAUS, J. J., III & BUSH, R. L. (1981). Mass production of axenic spores of the endomycorrhizal fungus Gigaspora margarita. Transactions of the British Mycological Society, **72**, 167-169.

MOSSE, B. (1959). Observations on the extra-matrical mycelium of a vesicular-arbuscular endophyte. Transactions of the British Mycological Society, **42**, 439-448.

MOSSE, B. (1972). Effect of different Endogone strains on the growth of Paspalum notatum. Nature, **239**, 221-223.

MOSSE, B., POWELL, C. L. & HAYMAN, D. S. (1976). Plant growth responses to vesicular-arbuscular mycorrhiza. IX. Interactions between VA mycorrhiza, rock phosphate, and symbiotic nitrogen fixation. New Phytologist, **76**, 331-342.

PARKE, JENNIFER LEE. (1982). Factors Affecting the Inoculum Potential of VA and Ectomycorrhizal Fungi in Forest Soils of Southwest Oregon and Northern California. Ph.D. Thesis, Oregon State University, Corvallis.

PHILLIPS, J. M. & HAYMAN, D. S. (1970). Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Transactions of the British Mycological Society, **55**, 158-161.

PLENCHETTE, C., FURLAN, V. & FORTIN, J. A. (1981). Growth stimulation of apple trees in unsterilized soil under field conditions with VA mycorrhiza inoculation. Canadian Journal of Botany, **59**, 2003-2008.

POPE, P. E., CHANEY, W. R., RHODES, J. D. & WOODHEAD, S. H. (1983). The mycorrhizal dependency of four hardwood tree species. Canadian Journal of Botany, **61**, 412-417.

RHODES, L. H. & HIRREL, M. C. (1982). Radiotracer methods for mycorrhizal research. In: Methods and Principles of Mycorrhizal Research (N. C. Schenck, ed.), pp. 189-200. American Phytopathological Society, St. Paul, MN.

SANDERS, F. E., TINKER, P. B., BLACK, R. L. B. & PALMERLY, S. M. (1977). The development of endomycorrhizal root systems. I. Spread of infection and growth-promoting effects with four

species of vesicular-arbuscular endophyte. New Phytologist, **78**, 553-559.

SCHMIDT, E. L., BIESBROCK, J. A., BOHLOOL, B. B. & MARX, D. H. (1974). Study of mycorrhizae by means of fluorescent antibody. Canadian Journal of Microbiology, **20**, 137-139.

SCHULTZ, R. C., KORMANIK, P. P., BRYAN, W. C. & BRISTER, G. H. (1979). Vesicular-arbuscular mycorrhiza influence growth but not mineral concentrations in seedlings of eight sweetgum families. Canadian Journal of Forest Research, **9**, 218-223

SEN, Y. C. & HOLLAND, A. A. (1970). Direct and indirect staining of Ophiobolus graminus Sacc. in culture and in the rhizosphere of cereal plants. Antonie van Leewenhoek, **36**, 549-554.

SILBERBUSH, M. & BARBER, S. A. (1983). Sensitivity of simulated phosphorus uptake to parameters used by a mechanistic-mathematical model. Plant and Soil, **74**, 93-100.

SKIPPER, H. D. & SMITH, G. W. (1979). Influence of soil pH on the soybean-endomycorrhiza symbiosis. Plant and Soil, **53**, 559-563.

SMITH, S. E. & DAFT, M. J. (1977). Interactions between growth, phosphate content and nitrogen fixation in mycorrhizal and nonmycorrhizal Medicago sativa. Australian Journal of Plant Physiology, **4**, 403-413.

SMITH, S. E. & WALKER, N. A. (1981). A quantitative study of mycorrhizal infection in Trifolium: separate determination of the rates of infection and of mycelial growth. New Phytologist, **89**, 225-240.

ST. JOHN, T. V. (1979). Root size, root hairs and mycorrhizal infection: a re-examination of Baylis's hypothesis with tropical trees. New Phytologist, **84**, 483-487.

ST. JOHN, T. V. & COLEMAN, D. C. (1983). The role of mycorrhizae in plant ecology. Canadian Journal of Botany, **61**, 1005-1014.

ST. JOHN, T. V., COLEMAN, D. C. & REID, C. P. P. (1983). Associations of vesicular-arbuscular mycorrhizal hyphae with soil organic particles. Ecology, **64**, 957-959.

STEEL, R. G. D. & TORRIE, J. H. (1960). Principles and Procedures of Statistics. McGraw-Hill, New York.

SUTTON, J. C. (1973). Development of vesicular-arbuscular mycorrhizae in crop plants. Canadian Journal of Botany, **51**, 2487-2493.

SUTTON, J. C. & SHEPPARD, B. R. (1976). Aggregation of sand-dune soil by endomycorrhizal fungi. Canadian Journal of Botany, **54**, 326-333.

TINKER, P. B. (1975). Effect of vesicular arbuscular mycorrhizae on higher plants. Symposium of the Society for Experimental Biology, **29**, 325-350.

TISDALL, J. M. & OADES, J. M. (1979). Stabilization of soil aggregates by the root systems of ryegrass. Australian Journal of Soil Research, **17**, 429-441.

TØMMERUP, I. C. (1983). Spore dormancy in vesicular-arbuscular mycorrhizal fungi. Transactions of the British Mycological Society, **81**, 37-45.

VINCENT, J. M. (1970). A Manual for the Practical Study of Root-Nodule Bacteria. IBP Handbook No. 15, Blackwell Scientific, Oxford and Edinburgh.

WALKER, C., MIZE, C. W. & McNABLE, H. S., Jr. (1982). Populations of endogonaceous fungi at two locations in Central Iowa. Canadian Journal of Botany, **60**, 2518-2529.

WARNER, A. & MOSSE, B. (1982). Factors affecting the spread of vesicular mycorrhizal fungi in soil. I. Root density. New Phytologist, **90**, 529-536.

WILSON, J. M., TRINICK, M. J. & PARKER, C. A. (1983). The identification of vesicular-arbuscular mycorrhizal fungi using immunofluorescence. Soil Biology and Biochemistry, **15**, 439-445.