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Title: Microbial Changes and Interactions in the Rhizosphere of Vesicular-arbuscular Mycorrhizal Plants

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Dr. Robert G. Linderman

The influence of vesicular-arbuscular mycorrhizae (VAM) on populations of general taxonomic and functional groups of naturally-occurring rhizosphere bacteria and actinomycetes associated with roots of sweet corn (Zea mays var. rugosa) and subterranean clover (Trifolium subterraneum L.) was assayed using selective media. Sporangial production by Phytophthora cinnamomi was also used as a bioassay to detect qualitative changes in rhizosphere soil microorganisms stimulatory or inhibitory to sporangium production.

Establishment of VAM increased total bacterial populations and numbers of fluorescent pseudomonads on the root surface (rhizoplane) of sweet corn and clover, but did not affect numbers of actinomycetes. Total numbers of bacteria and actinomycetes in the rhizosphere soil was not affected by VAM, but the presence of VAM did affect populations of specific groups of bacteria and actinomycetes in both the rhizosphere and rhizoplane. Facultative anaerobes increased, fluorescent pseudomonads, Streptomyces spp and chitinase-producing microorganisms all decreased in the VAM rhizosphere. Fewer

sporangia were formed by Phytophthora cinnamomi in leachates from VAM rhizosphere soil than in leachates from nonmycorrhizal root rhizosphere soil, and fewer zoospores were released. The data suggest that rhizosphere microbial populations associated with VAM are qualitatively different from those associated with nonmycorrhizal roots. The significance of changes in microbial populations or activities due to VAM are discussed.

In addition, plant growth and nodulation of subterranean clover were studied in nonsterile soil inoculated with a plant growth-promoting rhizobacterium (PGPR) isolate of Pseudomonas putida and indigenous VA mycorrhizal fungi. Although inoculation with either PGPR or VAM alone increased plant growth after 12 weeks, a significant increase in root dry weight, compared to uninoculated controls, was observed only when both PGPR and VAM were present. Shoot dry weight of plants inoculated with both PGPR and VAM was significantly greater than with PGPR alone, VAM alone or uninoculated controls. Nodulation was enhanced significantly by either PGPR or VAM alone (50% increase over controls at 12 weeks), but was significantly greater (103% increase over controls) when both PGPR and VAM were present. Inoculation with PGPR increased VAM infection from 7% to 23% of the root system infected at 6 weeks, but VAM infection levels were similar (ca 50%) at 12 weeks. Populations of PGPR increased similarly in the rhizosphere of both mycorrhizal and nonmycorrhizal plants. Concentrations (ppm) of Fe, Cu, Al, Zn, Co and Ni were considerably greater in the shoots of plants inoculated

with both PGPR and VAM than in plants inoculated with either PGPR or VAM alone. Possible mechanisms for these interactions are discussed.

MICROBIAL CHANGES AND INTERACTIONS IN THE RHIZOSPHERE OF VESICULAR-ARBUSCULAR MYCORRHIZAL PLANTS

by

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INTRODUCTION

The mycorrhizal association occurs in most woody and herbaceous plant species in nature. Vesicular-arbuscular mycorrhizae (VAM) are characterized by intracellular fungal colonization of the root cortical cells as contrasted to ectomycorrhizal (ECM) fungi which colonize intercellularly and surround the plant root with a fungal sheath. It is unlikely that the physiology of a root so densely colonized by a fungus would be the same as one which was not (Hayman, 1983). Both a change in root physiology and the presence of fungal mycelium in the rhizosphere can influence the microbial ecology of this environment (Foster and Bowen, 1982; Foster and Marks, 1967; Rovira and Davey, 1974). However, few studies have been reported on the role of either ECM or VAM in the microbial ecology of the rhizosphere.

The powerful influence of the plant root on populations of soil microorganisms is called the "rhizosphere effect", characterized by microbial populations which are quantitatively greater and qualitatively different compared to the soil population outside the rhizosphere. Several excellent reviews have been written on this important ecological phenomenon (Bowen and Rovira, 1976; Katznelson, 1970; Katznelson et al., 1948; Newman, 1978; Rovira, 1965; Rovira and Davey, 1974). The rhizosphere is a microbial continuum (Foster et al., 1983; Old and Nicholson, 1978) with no distinguishable borders

and thus sampling procedures are difficult to standardize. Two distinct regions are usually recognized: the rhizosphere, the soil directly adjacent to and influenced by the root; and the rhizoplane, the actual two-dimensional root surface (Rouatt and Katznelson, 1961; Rovira and Davey, 1974). The root surface is, however, also part of a microbial continuum because microorganisms are commonly embedded in epidermal and even cortical cells (Foster et al., 1983; Old and Nicholson, 1978). Heterogeneous populations of fungi, bacteria, actinomycetes, algae, protozoans and insects in the rhizosphere number in the tens of millions or more, but yet we know very little about what they do and how they influence the plant and each other (Katznelson, 1970; Rovira and Davey, 1974). The microbiological equilibrium in the rhizosphere is dynamic and determined not only by plant activity (root growth and exudation) but by microbial activity as well, with environmental factors influencing both (Katznelson, 1970).

A. G. Lochhead, H. Katznelson, M. I. Timonin and coworkers have made important contributions to the study of the rhizosphere effect using a qualitative approach in the isolation and classification of soil and rhizosphere microorganisms based on physiology and nutritional requirements (Katznelson, 1946; Katznelson, 1970; Katznelson and Rouatt, 1957; Lochhead, 1940; Lochhead, 1957; Lochhead and Thaxton, 1947; Timonin, 1940; Wallace and Lochhead, 1949). They obtained qualitative data on the metabolic activities on rhizosphere bacteria, and to a lesser extent actinomycetes and fungi, which has contributed much towards the elucidation of the microbial equilibrium

of the rhizosphere. Although the number of species of rhizosphere microorganisms is great and the taxonomy incomplete, much information can be obtained on the ecology of rhizosphere microorganisms and their effects on plants by asking questions about their physiology and function, e.g., what substrates they use, what metabolite they produce and how organisms affect and are affected by their environment. Microorganisms receiving particular attention have been those known to be directly involved in nutrient cycling, e.g., N_2-fixers, those involved in N cycling, solubilization of P and other minerals or the decomposition of organic matter. Plant pathologists have a particular interest in microorganisms which have the potential to antagonize plant pathogens. Many examples of microbial interactions, involving metabolite production or substrate competition which result in biological control of plant disease, are found in Baker and Cook (1974) and Cook and Baker (1983).

The study of interactions between ectomycorrhizae (ECM) and saprophytic rhizosphere microorganisms has focused mainly on qualitative differences in taxonomic and physiological groups of naturally-occurring microorganisms in the rhizosphere of ectomycorrhizal and nonmycorrhizal roots (Fontana and Luppi, 1966; Katznelson et al., 1962; Malajczuk and McComb, 1979; Neal et al., 1964; Oswald and Ferchau, 1968; Tribunskaya, 1958; Voznyakovskaya and Rhyzhkova, 1955). In most of these studies, both quantitative and qualitative differences in rhizosphere populations were observed when comparing mycorrhizal and nonmycorrhizal roots and even mycorrhizal roots of different ectomycorrhizal fungal species. Because of the

altered root morphology, the abundant fungal mycelium and establishment of a hyphasphere completely enclosing the plant root, these results are not surprising. Rambelli (1972) distinguished between the "mycorrhizosphere" and the rhizosphere of nonmycorrhizal roots. However, the implications of close associations and possibly concerted activities between ECM and saprophytic microorganisms for tree growth have not been pursued, in spite of such ecologically significant findings as N_2-fixing bacteria closely associated with ECM sporocarps by Li and Castellano (1984), which confirms earlier observations by Richards and Voigt (1964; see also Hassouma and Wareing, 1964).

Studies on the interactions between VAM and saprophytic nonpathogenic soil microorganisms are relatively recent and have focused mainly on associative N_2-fixing bacteria or bacteria which are able to solubilize inorganic phosphates. The associative N_2-fixing bacteria Azotobacter (Bagjaraj and Menge, 1978; Barea et al. 1973; Brown and Carr, 1979; Ho and Trappe, 1979), Azospirillum (Barea et al., 1983), and Beijerinckia moblis (Manjunath et al., 1981) have been studied in gnotobiotic conditions as well as in nonsterile soil. Strains of phosphate-solubilizing Pseudomonas spp. (Azcon et al., 1975; Azcon-Aquilar and Barea, 1978; Barea et al., 1975), Bacillus (Raj et al., 1981) and Aspergillus niger (Manjunath et al., 1981) have been tested for possible synergistic effects with VAM fungi on the P nutrition of plants. A study with Thiobacilli, a sulfur-oxidizing bacterium which can also influence the availability of phosphate ions and Glomus spp. is cited briefly by Gianinazzi-

Pearson and Diem (1982). Only one potential biocontrol agent, Streptomyces cinnamomeous, studied becaused of its ubiquity and plant growth-promoting effects, has been investigated in relation to VAM (Krishna et al., 1982). Some data on the relative numbers of bacteria (Ames et al., 1984; Bagjaraj and Menge, 1978; Brown and Carr, 1979), actinomycetes (Ames et al., 1984; Bagjaraj and Menge, 1978), spore-forming bacteria (Brown and Carr, 1979), and introduced mixtures of field-collected bacterial isolates (Ames et al., 1984) found in mycorrhizal compared to nonmycorrhizal root rhizosphere soil are available, but results are difficult to interpret because of differences in experimental procedures. Some generalizations from the studies with introduced saprophytic microorganisms are:

--VAM infection levels are often stimulated by the presence of the bacteria (Azcon-Aquilar and Barea, 1978; Barea et al., 1983; Brown and Carr; 1979),

--bacterial populations were sustained longer in the rhizosphere of mycorrhizal compared to nonmycorrhizal plants (Bagjaraj and Menge, 1978; Barea et al., 1975; Raj et al, 1981),

--dual inoculation with VAM fungi and associated N_2-fixing bacteria can substitute for N + P fertilizer under some conditions (Barea et al., 1983), and

--synergistic effects occur between introduced bacteria and VAM on plant growth (Azcon-Aquilar and Barea, 1978; Bagjaraj and Menge, 1978; Barea et al., 1975; Brown and Carr, 1979; Manjunath et al., 1981).

The importance of testing a potential beneficial bacterial inoculant on VAM development is illustrated by Krishna et al. (1982) who observed mutual antagonism between a Streptomyces cinnamomeous isolate and a VAM fungus which negated the plant growth-promoting potential of each organism introduced singly.

Plant growth-promoting rhizobacteria or PGPR (Suslow, 1982), isolated from the roots of various plant species, are currently receiving much interest for their plant-growth-promoting abilities. They produce strong siderophores (Kloepper et al., 1980), an Fe-chelating compound, and are thought to increase plant growth by altering the composition of the rhizosphere microflora favorably for plant growth through the inhibition of populations of deleterious microorganisms or subclinical pathogens, thereby allowing the plant to achieve more of its growth potential (Kloepper and Schroth, 1981a; Kloepper and Schroth, 1981b; Kloepper et al., 1980; Suslow, 1982; Suslow and Schroth, 1982). The importance of deleterious rhizosphere microorganisms in restricting plant growth is becoming more widely recognized (Suslow and Schroth, 1982; Woltz, 1978).

PGPR belong to the Pseudomonas fluorescens-putida group, a common and diverse group of soil bacteria whose effects on plants range from stimulatory to neutral to deleterious (Suslow, 1982). PGPR have been tested in both greenhouse and field trials on several agronomic plants and there are reports of increased plant growth and yield (Burr et al., 1978; Howie and Echandi, 1983; Kloepper et al., 1980; Suslow and Schroth, 1982b).

No study has considered the presence of VAM in evaluating growth promotion results from inoculation with PGPR, although VAM had probably developed in the nonsterile soil conditions used to test the efficacy of PGPR. Development of VAM can be stimulated by Pseudomonas spp. (Azcon-Aquilar and Barea, 1978; Mosse, 1962) and there is one report of severe depression of ectomycorrhizal formation in the presence of a pseudomonad (Bowen and Theodorou, 1979).

In this thesis, two questions have been addressed: Can the establishment of VAM have an influence on the composition of rhizosphere microbial populations? Secondly, do VAM and an introduced Pseudomonas strain with plant-growth-promoting capabilities interact to influence their mutal development or the growth of the plant?

CHAPTER 1

SELECTIVE INFLUENCE OF VESICULAR-ARBUSCULAR MYCORRHIZAE ON SOME TAXONOMIC AND FUNCTIONAL GROUPS OF RHIZOSPHERE BACTERIA AND ACTINOMYCETES

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SUMMARY

The influence of vesicular-arbuscular mycorrhizae (VAM) on populations of general taxonomic and functional groups of naturally-occurring rhizosphere bacteria and actinomycetes associated with roots of sweet corn (Zea mays var. rugosa) and subterranean clover (Trifolium subterraneum L.) was assayed on selective media. Total numbers of bacteria, but not actinomycetes, on the root surface (rhizoplane) increased on plants with VAM compared to plants without VAM. Neither bacteria nor actinomycetes were quantitatively affected in the rhizosphere of VAM plants. However, VAM affected specific groups of bacteria and actinomycetes in both the rhizosphere and rhizoplane. VAM rhizosphere soil contained more facultative

anaerobic bacteria isolated on low N medium, had fewer fluorescent pseudomonads, but the same number of gram negative bacteria compared to non-VAM rhizosphere soil. Of the actinomycetes assayed, populations of both Streptomyces spp and chitinase-producing actinomycetes decreased in the VAM rhizosphere. Leachates of VAM and non-VAM rhizosphere soil were also compared for the presence or activity of bacteria that could influence sporulation by the root pathogen Phytophthora cinnamomi.

Fewer sporangia and zoospores were produced by P. cinnamomi in leachates of rhizosphere soil compared to non-VAM plants, suggesting that either sporangium-inducing microorganisms had declined or sporangium-inhibitors had increased.

Assays for specific functional groups of microorganisms revealed changes even when total numbers seemed the same; the microbial equilibrium is evidently altered by establishment of VA mycorrhizae.

INTRODUCTION

The plant root primarily determines the nature and extent of the saprophytic rhizosphere microflora, so any condition affecting root growth or metabolism will be reflected in quantitative and qualitative changes in rhizosphere microbial populations (Katznelson, 1970). The dense colonization of plant roots by vesicular-arbuscular mycorrhizal (VAM) fungi and the resulting changes in root physiology and exudation (Graham, 1981; Hayman, 1983; Schwab, 1983) suggest that VAM fungi and establishment of the VA mycorrhizal association will influence the microbiology of the rhizosphere. Studies on VAM

microbial interactions in the rhizosphere have focused mainly on the introduction of organisms involved in nutrient cycling such as the associative N_2-fixing bacteria Azotobacter (Bagyaraj and Menge, 1978; Barea et al., 1979; Brown and Carr, 1979; Ho and Trappe, 1979), Azospirillum (Barea et al., 1983), and Beijerinckia moblis (Manjunath et al., 1981) or organisms which can solubilize inorganic phosphates (Azcon et al., 1975; Azcon-Aquilar and Barea, 1978; Barea et al., 1975; Raj et al., 1981). Stimulation of VAM infection by introduced bacteria (Azcon-Aquilar and Barea, 1978; Barea et al., 1983; Brown and Carr, 1979), sustained bacterial populations on mycorrhizal roots (Ames et al., 1984; Bagyaraj and Menge, 1978; Barea et al., 1975; Raj et al., 1981), synergistic effects between bacteria and VAM on plant growth (Azcon-Aquilar and Barea, 1978; Bagyaraj and Menge, 1978; Barea et al., 1975; Brown and Carr, 1979; Manjunath et al., 1981) as well as the mutual antagonism observed between a VAM fungus and a Streptomyces isolate (Krishna et al., 1982) are evidence that VA mycorrhizae and rhizosphere microorganisms can influence their mutual development and exert combined effects on plant growth. Because these studies were performed by introducing selected microorganisms, it is not yet known if VA-mycorrhizal and nonmycorrhizal roots support a qualitatively different rhizosphere microflora. However, qualitative differences in the microbial populations associated with ectomycorrhizae compared to non-ectomycorrhizal roots have been

studied and reported, (Katznelson et al., 1962; Neal et al., 1964; Oswald and Ferchau, 1968).

The aim of our study was to investigate the possible selective influence of VAM development on the composition of naturally-occurring, rather than introduced, rhizosphere bacterial and actinomycete populations. General taxonomic and functional groups of microorganisms were assayed in the rhizosphere and rhizoplane using selective media. Phytophthora cinnamomi, a root fungal pathogen whose reproduction is very sensitive to the microbial activity of the soil (Marx, 1970), was also used in a bioassay to detect microbial changes in extracts of rhizosphere soil from mycorrhizal compared to nonmycorrhizal plants.

MATERIALS AND METHODS

Preparation of plant material and treatments

Rhizosphere bacterial and actinomycete populations were compared on mycorrhizal and nonmycorrhizal sweet corn (Zea mays L. var. rugosa cv. Golden Cross Bantam) and subterranean clover (Trifolium subterraneum L. cv. Mt. Barker) grown in nonsterile soil in the greenhouse. Nonsterile soil free from indigenous VA mycorrhizal fungi was prepared by inoculating pasteurized soil (65°C/30 min) with a sieved (38 µm mesh) garden loam suspension (200 g/l H_2O) after which the soil was incubated moist for 1 to 2 weeks.

Sweet corn was inoculated with VAM by placing three 3-cm root pieces colonized by Glomus fasciculatum, from an 8 month-old

subterranean clover pot culture, beneath 2 surface-disinfested (3% H_2O_2 for 30 min) sweet corn seeds; controls received noninfected root pieces. The seedlings were thinned to one plant/15 cm pot after germination and harvested as the plants began to flower (8 to 9 weeks).

In subsequent experiments with subterranean clover, spores of Glomus fasciculatum, rather than root pieces, were used as VAM inoculum to reduce microbial contamination. The spores were obtained from a 9 month-old subterranean clover pot culture by wet-sieving and decanting (Gerdemann and Nicholson, 1963). They were surface-disinfested by stirring in an antibiotic solution (2% w/v chloramine T + 200 µg/ml streptomycin and 1 drop Tween 20) for 10 min, washing with 1/2 l 0.5% sodium hypochlorite solution in a sterile Büchner apparatus and rinsing with 2 l sterile distilled H_2O (Rose, 1981). The spores were mixed in soil which was used to fill hollow cylindrical space in the center of 15 cm pots created by filling the pots around an inverted 50 cm^3 tube, firming the soil and removing the tube. This was an effective method of concentrating the spores around the growing root and reducing the quantity of spores needed as inoculum. The inoculum density was approx. 100 spores/15 cm pot. Surface-disinfested (3% H_2O_2 for 30 min) clover seeds were sown and thinned to one plant/pot after germination. Plants were harvested at 6 and 12 weeks after sowing. All plants were grown in the greenhouse at 24°C day, 18°C night temperatures and a 16 h photoperiod of 240 µE $m^{-2}s^{-1}$ from high pressure sodium vapor lamps and were fertilized

every 14 days with 20 ml of Long Ashton nutrient solution (Hewitt, 1966) with 1/4 strength P (11 ppm).

Assay procedure

Plants were harvested by removing the tops with a sterile scalpel and shaking loosely adhering soil from the roots. Approximately one gram (fresh wt) of roots was removed with a sterile scalpel and forceps, tapped firmly against a hard surface to remove loosely adhering soil and placed in a sterile dilution bottle containing 100 ml 0.1% water agar dilutent. This dilutent was chosen because of its slight viscosity which resulted in more uniform dilutions. Corn roots develop yellow pigmentation when mycorrhizal, and young, yellow-pigmented lateral roots were chosen for sampling. Roots of similar size were chosen from nonmycorrhizal corn roots. Subterranean clover roots were sampled from the middle 1/3 of the root system. The roots were shaken by hand 25x in the dilution bottle, removed, and serial dilutions prepared from the soil remaining in the bottle. Total dry wt of rhizosphere soil sampled was determined by pipetting 25 ml of the original soil suspension into pretared weighing dishes, drying for 48 hr at 70°C and reweighing.

Rhizoplane microorganism counts were obtained by quickly weighing the fresh roots from the rhizosphere soil dilution bottle, and macerating with 100 ml sterile 0.1% water agar dilutent in a sterile Waring blender for 1 min at high speed. Serial dilutions were prepared in the same manner as for the rhizosphere soil and 2

dilutions were plated out onto five replicate plates/medium. The dry weight of the root sample was determined by calculating % moisture from the fresh and dry weights of the remaining roots. Shoot weight was determined after drying 48 h at 70°C. The number of Rhizobium nodules was counted on fresh roots of 6 week-old clover plants. VAM colonization was determined by clearing and staining an additional 0.5 g (fresh wt) root sample (Philips and Hayman, 1970) and determining the percentage of 1 mm^3 root sections containing arbuscules, vesicles, hyphae or spores of the VAM fungi.

General taxonomic and functional groups of bacteria and actinomycetes were assayed on the following media: total bacteria on 0.3% Tryptic Soy Agar (Difco), with 2 ppm crystal violet solution added to assay gram negative bacteria (Rovira et al., 1974); fluorescent Pseudomonas spp on Kings B + NPC (Sands and Rovira, 1970); total actinomycetes on 1.5% H_2O or chitin agar (Lingappa and Lockwood, 1962); chitinase-producing microorganisms on chitin agar (Lingappa and Lockwood, 1962); Streptomyces spp, identified by sporulation, on casein-glycerol medium (Kustar and Williams, 1964), and anaerobic bacteria on Rennie's combined carbon medium (Rennie, 1981) with 0.05% sodium thioglycollate added to the dilutent as a reducing agent. The plates were incubated at root temperature for 5 days (bacteria) to 14 days (actinomycetes). Anaerobic incubation was carried out for 10 days in a Gas Pak (BBL Microbiology Systems, Cockeysville, MD 21030) anaerobic chamber.

To compare microbial activity of rhizosphere soil affecting sporangia formation and zoospore release by Phytophthora cinnamomi,

the original rhizosphere soil suspensions were placed on a Lab-Line Orbit shaker overnight and vacuum filtered through Whatman #1 filters with a sterile Büchner apparatus. Ten ml of the suspensions were placed in each side of 100 mm 2-compartment petri plates. Three 5-mm plugs of P. cinnamomi from a 48 hr V-8 agar (Ribeiro, 1978) culture were placed in 3 replicate 2-compartment plates/plant. Sporangia were counted after 3 days at 50x. To determine viability of sporangia and numbers of zoospores released, the test solutions were chilled for 1 h at 5°C, warmed to room temperature, and two serial dilutions prepared in sterile distilled H_2O. One-half ml of the 2 dilutions were plated onto Phytophthora-selective PVRPH medium (Ribeiro, 1978). The plates were incubated in the dark for 2 days and number of colonies from germinated zoospores recorded. Except for a 12 week subterranean clover harvest, all experiments were repeated once, with at least 5 plant replicates/treatment. Tissue nutrient analysis was performed on 6 week-old subterranean clover plants with an ICB Spectrophotometer by the Plant Analysis Lab, Dept. of Horticulture, Oregon State University.

The data were analyzed by one-way analysis of variance.

RESULTS

Establishment of VAM increased total bacterial populations on the rhizoplane of sweet corn and clover, but did not affect numbers of actinomycetes (Fig. 1-1). Total numbers of bacteria and actinomycetes in rhizosphere soil were not affected by VAM.

However, the presence of VAM affected specific groups of bacteria and actinomycetes in both the rhizosphere and rhizoplane (Fig. 1-1 and 1-2). VAM increased the numbers of facultative anaerobic bacteria isolated from clover on low N medium, decreased the numbers of fluorescent Pseudomonas spp isolated from sweet corn, but had no effect on the total number of gram negative bacteria from the rhizosphere of either plant species. Populations of Streptomyces spp and chitinase-producing microorganisms, mainly actinomycetes, decreased in the VA mycorrhizosphere. Higher populations of fluorescent pseudomonads were isolated from the rhizoplane of mycorrhizal sweet corn (Fig. 1-1). Fewer sporangia were formed by Phytophthora cinnamomi in leachates of VAM rhizosphere soil compared to leachates from nonmycorrhizal root rhizosphere soil (Fig. 1-3) and fewer total zoospores were released.

Rhizobium nodulation was significantly enhanced on 6 week mycorrhizal clover roots, which supported 102 total nodules or 0.73 nodules/mg root compared to 7.5 total nodules or 0.13 nodules/mg nonmycorrhizal root. VA mycorrhizae were present in 90% of the sweet corn root system sampled at 9 weeks; VAM infection levels in clover roots were 20% at 6 weeks and 35% at 12 weeks. VAM significantly increased root (233% at 6 weeks; 158% at 12 weeks) and shoot wt (470% at 6 weeks; 360% at 12 weeks) of clover, but concentrations of N, P and K were comparable in both mycorrhizal and nonmycorrhizal plants at 6 weeks. Dry weights of mycorrhizal sweet corn did not differ from those of nonmycorrhizal plants at 9 weeks.

DISCUSSION

Stimulation or depression of root VAM or ectomycorrhizal infection by bacteria (Azcon-Aquilar and Barea, 1978; Barea et al., 1983; Bowen and Theodorou, 1979; Brown and Carr, 1979) is recognized as part of the ecology of these fungi. What is generally not considered, however, is that once mycorrhizae are established, the fungal symbiont is an integral part of the rhizosphere microflora and will thus contribute to the dynamic equilibrium of the rhizosphere. Our data suggest that the rhizosphere microbial populations associated with VAM are qualitatively different than those associated with nonmycorrhizal roots. This has been observed with ectomycorrhizae (Katznelson, 1962; Neal et al., 1964; Oswald and Ferchau, 1968) but had not been reported for VAM.

The ecological significance of changes in microbial populations or activities due to VAM are difficult to assess. However, the observed synergistic effects of VAM and introduced bacteria on plant growth in greenhouse trials indicate the possible contribution of VAM in microbiological processes affecting plant productivity and plant nutrition in nature. For example, beneficial interactive effects have been shown with VAM and a plant growth-promoting Pseudomonas putida isolate (see Chapter 2), associative N_2-fixing bacteria (Bagjaraj and Menge, 1978; Barea et al., 1983; Brown and Carr, 1979; Ho and Trappe, 1979; Manjunath et al., 1981), P-solubilizing bacteria (Azcon et al., 1975; Barea et al., 1975; Raj et al., 1981) and Frankia spp (Rose and Youngberg, 1981). The synergistic effects of

VAM on nodulation and fixation by Rhizobium spp in greenhouse and field trials are well-documented (Barea and Azcon-Aquilar, 1983).

Establishment of VAM has been shown to either reduce, have no effect or increase plant disease (Dehne, 1982; Schenck and Kellam, 1978). However, definitive field studies are lacking and most of the available data are from greenhouse experiments using treated soil (Schenck, 1981). Under these conditions, the results reflect mainly the direct influence of VAM fungi on plant physiology and susceptibility to disease. A further aspect of plant disease in the field is the microbial activity of the soil (Cook and Baker, 1983). Qualitative changes in microbial populations or activity in the rhizosphere by VAM fungi may have, therefore, an impact on plant disease. For example, sporangia formation by Phytophthora cinnamomi, used in this study in a bioassay of microbial activity, was significantly less in the rhizosphere soil extracts from mycorrhizal roots; fewer zoospores, the primary infective propagules of the pathogen, were released. Streptomyces spp and chitinase-producing microorganisms, both implicated in the biocontrol of some root-infecting fungi (Cook and Baker, 1983; Mitchell and Alexander, 1962), were found in lower populations in the rhizosphere of mycorrhizal clover and sweet corn, respectively. This indicates that biocontrol by these organisms would not be enhanced by VAM in the soil tested and secondly, that the most effective and realistic testing of potential biocontrol agents belonging to these groups would be in the presence of VA mycorrhizal fungi. Antagonism between VAM and Streptomyces cinnamomeous has been reported (Krishna et al., 1982).

The functional and taxonomic groups of bacteria and actinomycetes assayed in this study were chosen for their ease of isolation, potential biocontrol abilities, ubiquity and differing physiology. Other qualitative assays could be devised to determine which types or activities of microorganisms involved in nutrient transformations, nutrient availability to plant and fungus, hormone production or pathogen antagonism may change as VAM establish. An example of ecological significance is the isolation of a N_2-fixing bacterium from the sporocarps of ectomycorrhizal fungi by Li and Castellano (1984), confirming earlier studies by Richards and Voigt (1964), and from spores of Glomus fasiculatum (Thaxter sens. Gerd.), a VA mycorrhizal species (Ho and Trappe, 1979). In our study, more facultative anaerobes, possibly N_2-fixing, were isolated on low N medium (Rennie, 1981) from the rhizosphere of VA mycorrhizal roots than nonmycorrhizal roots. Qualitative assays of rhizosphere populations or activities associated with VAM can give some insight into how the rhizosphere environment is affected by mycorrhizal establishment and how the associated microflora contribute to the mycorrhizal effects on plants grown in various soils.

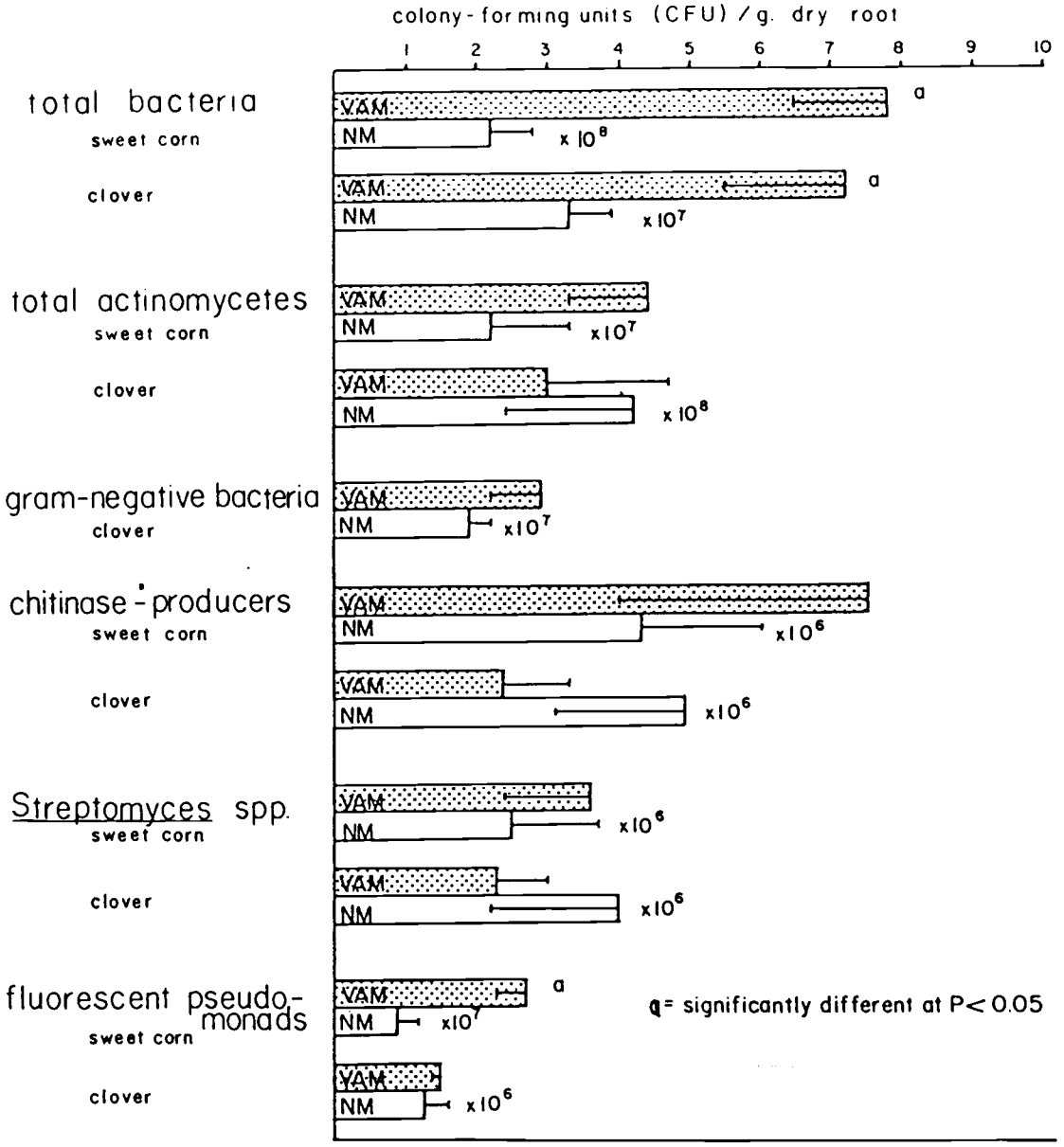

Fig. 1-1. Numbers of rhizoplane bacteria and actinomycetes isolated from vesicular-arbuscular mycorrhizae (VAM) or nonmycorrhizal (NM) roots.

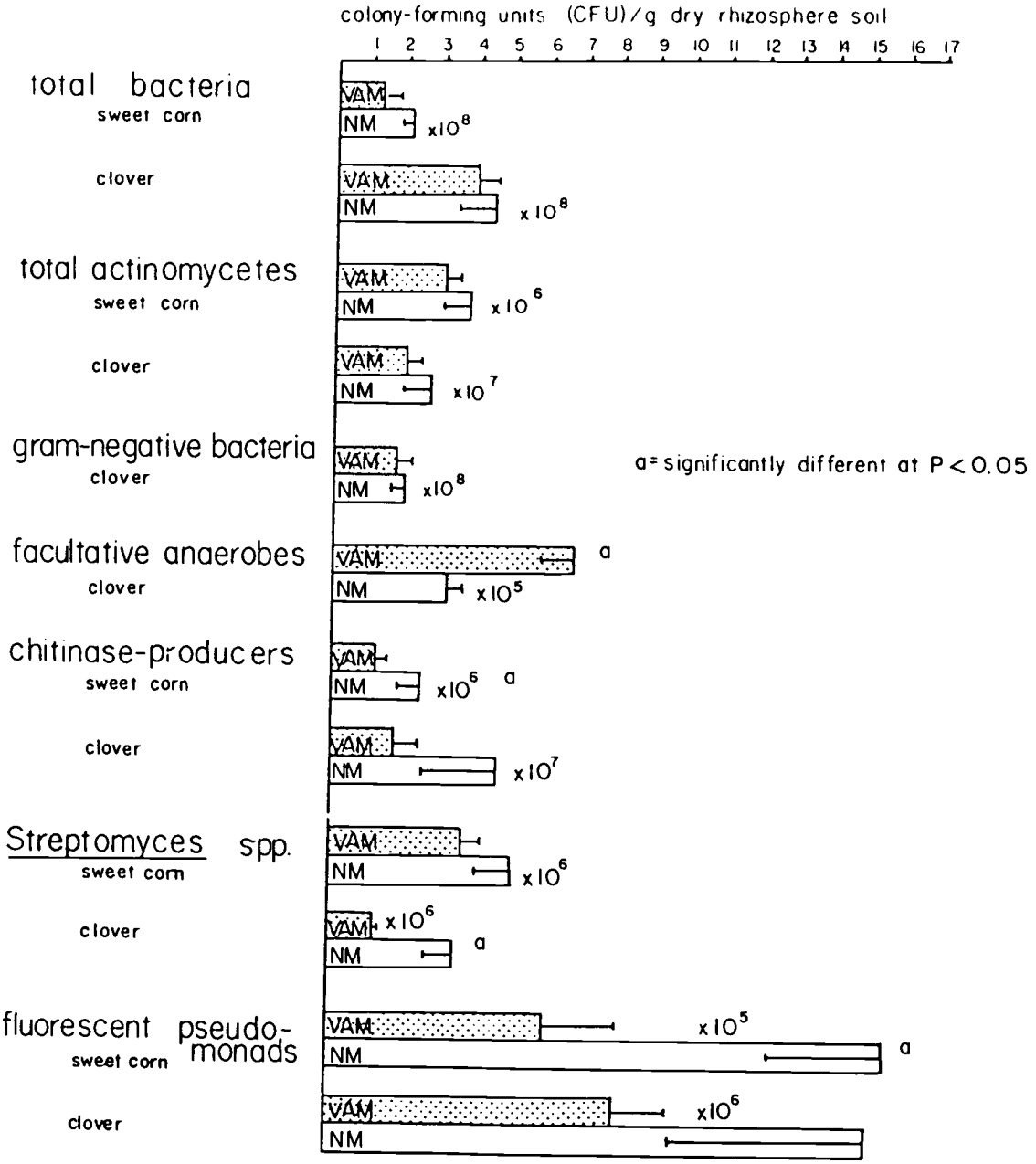

Fig. 1-2. Numbers of rhizosphere bacteria and actinomycetes isolated from vesicular-arbuscular mycorrhizae (VAM) or nonmycorrhizal (NM) roots.

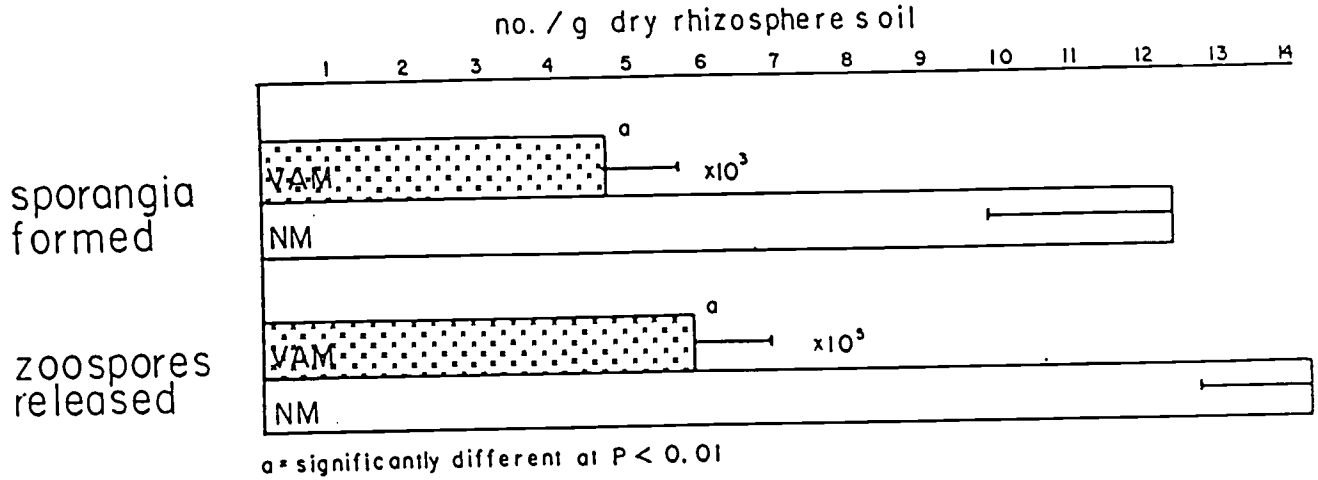

Fig. 1-3. Sporangia and zoospore production by Phytophthora cinnamomi in extracts of rhizosphere soil from vesicular-arbuscular mycorrhizae (VAM) and nonmycorrhizal (NM) roots.

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CHAPTER 2

RESPONSE OF SUBTERRANEAN CLOVER TO DUAL INOCULATION WITH VA MYCORRHIAL FUNGI AND A PLANT GROWTH-PROMOTING BACTERIUM, *PSEUDOMONAS PUTIDA*

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SUMMARY

Plant growth and nodulation of subterranean clover (*Trifolium subterraneum* L.) were studied in nonsterile soil inoculated with a plant growth-promoting rhizobacterium (PGPR) isolate of *Pseudomonas putida* and indigenous vesicular-arbuscular mycorrhizal fungi (VAM). Although inoculation with either PGPR or VAM alone increased plant growth after 12 weeks, a significant increase in root dry weight, compared to uninoculated controls, was observed only when both PGPR and VAM were present. Shoot dry weight of plants inoculated with PGPR and VAM was significantly greater than with PGPR alone, VAM alone or uninoculated controls. Nodulation was enhanced significantly by either PGPR or VAM alone (50% increase over controls at 12 weeks) but was significantly greater (103% increase over

controls) when both PGPR and VAM were present. Inoculation with PGPR increased VAM infection from 7% to 23% of the root system infected at 6 weeks, but VAM infection levels were similar (ca 50%) at 12 weeks. Populations of PGPR increased similarly in the rhizosphere of both mycorrhizal and nonmycorrhizal plants. Concentrations of Fe, Cu, Al, Zn, Co and Ni were considerably greater in the shoots of plants inoculated with PGPR and VAM than in plants inoculated with either PGPR or VAM alone. Possible mechanisms for these interactions are discussed.

INTRODUCTION

Specific strains of pseudomonads have been shown, when inoculated into soil, or on to roots or seed, to increase the growth and yield of some agricultural crops (Azcon-Aquilar and Barea, 1978; Burr et al., 1978; Howie and Echandi, 1983; Kloepper et al, 1980; Suslow and Schroth, 1982). Such plant growth-promoting rhizobacteria (PGPR) are believed to function mainly by inhibiting populations of deleterious microorganisms, called subclinical pathogens or exopathogens, in the rhizosphere (Kloepper and Schroth 1981a, 1981b; Suslow, 1982). When populations of deleterious microorganisms are reduced, the plant can achieve more of its growth potential (Kloepper 1981a, 1981b; Suslow, 1982; Woltz, 1978). Suslow (1982) discussed and reviewed the possible mechanisms involved.

Plants, including most agricultural plants, grown in nonsterile soil coexist with fungal root endophytes, forming mutualistic fungus-root associations called vesicular-arbuscular mycorrhizae (VAM). The

fungal partners of the association are ubiquitous in nature and the mycorrhizal condition is the normal condition for most plant species. Once the fungus-root association is formed, the plant can benefit from an increased supply of phosphorus and other ions which the fungus can acquire from the soil beyond the zone of depletion at the root surface and transport them back to the root (Rhodes and Gerdemann, 1980). Other effects of VAM on plant growth and physiology being studied are resistance to water stress and plant hormonal balance (Hayman, 1983) and resistance to plant pathogens (Dehne, 1983). The fungi are considered to be obligate symbionts, very well adapted to live with plant roots from which they obtain carbohydrates and a unique ecological niche protected from microbial antagonism in the soil (Hayman, 1978).

Because of the ubiquity of the VAM association in crop plants and their effects on plant growth, physiology and nutrition, their influence should be considered when testing the growth-promoting effects of beneficial bacterial inoculants such as PGPR. In order to achieve maximum benefit from beneficial microorganisms, the influence of bacterial inoculants on VAM development in nonsterile soil should also be studied for possible interactions, particularly antagonistic effects. Until now, no such studies on PGPR and VAM have been conducted. Therefore, we investigated the interactions between one PGPR and indigenous VA mycorrhizal fungi and their effects on the growth and nodulation of subterranean clover (Trifolium subterraneum L.) in nonsterile soil with natural soil Rhizobium inoculum.

MATERIALS AND METHODS

Soil and Inoculum Preparation

Nonsterile soil free from indigenous VA mycorrhizal fungi was prepared by inoculating pasteurized sand (65°C/30 min with aerated steam) with a sieved (38 m mesh) garden soil suspension (200 g soil/1 H_2O), containing indigenous Rhizobium bacteria, and allowing it to incubate moist for 2 weeks.

Spores of VA mycorrhizal fungi were isolated by wet-sieving and decanting (Gerdemann and Nicholson, 1963) soil from a 6-month-old sweet corn pot culture in garden soil containing an indigenous, probably mixed, population of VA mycorrhizal fungi. The spores were surface disinfested by suspending them in an antibiotic solution containing 2% w/v chloramine T, 200 g/ml streptomycin and a drop of Tween 20 for 10 min. They were then washed with 1/2 l 0.5% sodium hypochlorite solution through a sterile Büchner apparatus, rinsed with 2 l sterile distilled (SD) H_2O and resuspended in SD H_2O (Rose and Youngberg, 1981). One-half of the soil was inoculated with the VAM spore suspension by hand-mixing to a final concentration of 80 spores/soil volume. An aliquot of the final spore rinse water was mixed into the non-VAM soil to include any antibiotic or chlorox residues.

Pseudomonas putida isolate R20 marked with rifampicin antibiotic resistance (obtained form R. Osburn, University of California, Berkeley) can enhance growth of sugar beet (Osburn, unpublished) and decrease Pythium damping-off disease (Osburn, 1983). In preliminary tests, we reconfirmed the growth-promoting potential of this isolate

on clover and lettuce in mycorrhizae-free, nonsterile soil, prepared as above. In this study, the bacterium was grown for 48 h on Kings B (KB) medium amended with 100 µg/ml rifampicin. The colonies were scraped into a sterile 0.1 M $MgSO_4$ buffer solution to a final concentration of 10^8 CFU/ml. Aliquots of the bacterial suspension were mixed promptly by hand into one-half of the VAM spore-inoculated and noninoculated soil to a final concentration of 2×10^8 CFU/soil volume. Soil not inoculated with the PGPR suspension received an identical aliquot of sterile 0.1 M $MgSO_4$ buffer solution.

Surface-disinfested (3% H_2O_2 for 30 min) subterranean clover seeds (Trifolium subterraneum L. cv. Mt. Barker) were germinated on moist sterile filter paper in glass petri dishes. Fifty uniform 5-day-old seedlings were randomly assigned to one of the four treatments and transplanted into 50-cm^3 tubes (Ray Leach Cone-Tainer Nursery, Canby, OR). Plants were grown for 6 and 12 weeks in the greenhouse (24°C day 18°C night, 16 h photoperiod of 240 µE $m^{-2}s^{-1}$ from high pressure sodium vapor lamps) and fertilized every 14 days with 20 ml of Long-Ashton nutrition solution (Hewitt, 1966) with 1/4 strength phosphorus (11 ppm).

Assay procedure

Twenty replicate plants/treatment were harvested at 6 weeks and 28 replicates at 12 weeks. To estimate PGPR populations in the rhizosphere, the tops were removed and approx. 1 g (fresh wt) root samples were cut from the middle 1/3 of the root system with a sterile scalpel. The root samples were held with sterile forceps,

tapped firmly against a hard surface to remove loosely adhering soil and placed in a sterile dilution bottle containing 100 ml 0.1% water agar dilutent. The bottle was shaken 25x by hand, the roots removed and serial dilutions prepared. Five replicate KB agar plates amended with 100 g/ml rifampicin were inoculated with 0.5 ml of diluted rhizosphere soil suspension. The plates were incubated for 5 days at room temperature in the dark. Final dry wt of the rhizosphere soil samples was determined by pipetting 25 ml of the original suspension into pretared weighing dishes, drying in a 70°C oven for 48 hr and reweighing.

The population of PGPR in the rhizoplane was also assayed in the 12 wk harvest by quickly determining the fresh wt of the root sample, macerating roots in 100 ml of 0.1% water agar dilutent in a sterile Waring blender at high speed for 2 min and preparing serial dilutions in the same manner as for the rhizosphere soil sample.

Root colonization by VAM fungi was assessed by clearing and staining an additional root sample (approx. 0.5 g fresh wt) by a modification of the technique of Phillips and Hayman (1970) and determining the percentage of 1 mm^2 root sections containing mycorrhizal hyphae, vesicles, arbuscules or spores. Root nodules were counted on fresh roots and weights of tops and roots were taken after drying at 70°C for 48 h. Plant tissue analysis was performed with an ICB Spectrophotometer by the Plant Analysis Lab, Dept. of Horticulture, Oregon State University.

The data were analyzed with one-way analysis of variance, and the Least Significant Difference procedure was used to separate treatment means (Sokal and Rohlf, 1969).

RESULTS

Inoculation with PGPR, either with or without VAM, significantly stimulated plant growth after 6 weeks. Plants grown with VAM only had low VAM infection levels and were not significantly larger than control plants after 6 weeks (Table 2-1). Although the bacterium and VAM alone increased root and shoot weight after 12 weeks, a significant increase in root growth, as measured by tissue dry wt, compared to uninoculated controls, was observed only when both PGPR and VAM were present. Shoot growth of plants inoculated with both PGPR and VAM was significantly greater than from any other treatment (Table 2-1).

VAM infection in the roots was increased significantly from 7% to 23% by the presence of PGPR at 6 weeks (Table 2-1). At 12 weeks, VA mycorrhizae were present in about 50% of the root system of all plants grown in VAM-inoculated soil, both with and without PGPR. Arbuscules, many vesicles and abundant external hyphae were observed. Plants grown in soil not inoculated with VAM spores did not develop mycorrhizae.

Nodulation was significantly enhanced by PGPR at 6 weeks and 12 weeks and by VAM alone at 12 weeks. However, at both harvests, significantly more nodules had developed on the root systems of plants growth with both organisms compared to PGPR alone, VAM alone

or uninoculated controls (Table 2-1). The majority (> 60%) of the nodules of plants treated with PGPR, VAM or PGPR + VAM appeared firm and pigmented at 12 weeks. Nodules of control plants were noticeably smaller and less pigmented.

Populations of antibiotic-marked PGPR were easily monitored with rifampicin-amended medium. No colonies grew at all from soil not inoculated with PGPR, indicating that spontaneous antibiotic resistance had not occurred in natural soil microorganisms, and were therefore not being included in the PGPR population counts. Populations of PGPR in the rhizosphere soil of both mycorrhizal and nonmycorrhizal plants steadily increased over the 12 week experimental period from 2-3 x 10^6 CFU/g dry rhizosphere soil to 4-5 x 10^6 CFU. At 12 weeks, populations of PGPR on the rhizoplane of mycorrhizal and nonmycorrhizal PGPR-inoculated plants were comparable, averaging 6 x 10^5 CFU/g dry root.

Concentrations (ppm) of Fe, Al, Cu, Zn, Co, and Ni were considerably greater in the shoots of plants inoculated with both PGPR + VAM than in plants inoculated with either organism alone (Table 2-2). Concentration (% dry wt) of P was also higher in dual-inoculated plants. Concentration of N, K, Ca, Mg, Mn, S, and Mo in roots and shoots were similar in all treatments (Tables 2-2, 2-3).

DISCUSSION

The rapid and sustained establishment of the P. putida isolate in the rhizosphere of inoculated plants resulted in significantly increased plant growth, whether with or without VAM. Although the

indigenous VAM fungi used in this study did not significantly enhance growth when inoculated alone, it is noteworthy that the greatest growth and nodulation was achieved by plants grown with both organisms. Azcon-Aquilar and Barea (1978) noted similar results with whole-cell inocula of a Pseudomonas isolate, Rhizobium meliloti, and a selected VAM fungus on alfalfa. In this study, root weight was increased significantly over the uninoculated controls only when both organisms were present and shoot weight of dual-inoculated plants was significantly greater at 12 weeks than those grown with either PGPR or VAM alone.

Possible explanations for the increased benefit to the plant by dual-inoculation with PGPR + VAM might include 1) early VAM infection, and therefore early benefit by the VAM symbiosis, increased by the presence of PGPR; 2) VAM enhanced the uptake of elements solubilized by PGPR; and 3) both organisms contributed singly or in concert to nodule formation and/or activity. These mechanisms would not be necessarily mutually exclusive.

More rapid and extensive VAM formation could result in more rapid hyphal development and hence ion uptake and earlier induction of other physiological benefits from VAM. Early VAM infection has been associated with improved nodule formation and function (Smith et al., 1979). There is evidence that some pseudomonads can increase the plant susceptibility to VAM infection. Stimulation of VAM infection by a Pseudomonas sp was noted by Mosse (1962) when a Pseudomonas contaminant of otherwise sterile root + VAM spore culture induced changes necessary for fungal penetration of the roots. Low

concentrations of a sterile pectolytic + cellulolytic enzyme preparation or sterile bacterial filtrates were also effective, suggesting that changes in cell wall plasticity was involved. The presence of microorganisms on roots affects root exudation patterns and can thus affect root-infecting processes by fungi (Rovira, 1965). Azcon-Aquilar and Barea (1978) reported stimulation of VAM infection by a phosphate-solubilizing Pseudomonas strain and by a cell-free extract of the same. They suggested the involvement of increased concentrations of plant growth regulators in the rhizosphere and demonstrated stimulation of VAM infection by a mixture of IAA, gibberellins and cytokinins (Azcon et al., 1978). The P. putida isolate used in this study is an effective antagonist against Pythium spp (Osburn et al., 1983) and may have influenced the rhizosphere microflora favorably for VAM infection. Although the presence of Pseudomonas spp or other bacteria is not essential for VAM establishment, as demonstrated by successful VAM infection in axenic root organ cultures (Mosse and Hepper, 1975), infection by VAM fungi in nature may be aided by the better infection conditions created at or near the root surface by these common rhizosphere bacteria. Once infection is established, continued spread of infection may be less influenced by rhizosphere microorganisms and more sustained by the established nutrient source and induced changes in root physiology. This may explain why no further enhancement of VAM infection by PGPR was observed at the later harvest. It should be emphasized that pseudomonads in the rhizosphere are a diverse group of bacteria and their interactions with the plant and with

mycorrhizal fungi will differ between strains. For example, Bowen and Theodorou (1979) observed marked suppression in the development of several ectomycorrhizal species in Pinus roots by a fluorescent pseudomonad.

It is of interest that dual-inoculated plants had higher shoot tissue concentration of Fe, Al, Cu, Zn, Co and Ni compared to those inoculated with VAM or PGPR alone. As mycorrhizal and nonmycorrhizal PGPR-inoculated plants were the same size at 6 weeks, this indicates uptake of these elements by the VAM fungi. Fe, Al, Zn, Co, and Ni, among other elements, can be solubilized by 2-ketogluconic acid (Duff et al., 1963), a chelating agent produced through glucose metabolism by many pseudomonads (Richards, 1974) including P. putida (Grimes and Mount, 1984). A bacterial chelating action combined with fungal uptake may explain the higher concentration of these elements observed in PGPR + VAM plants. Phosphates are also solubilized by 2-ketogluconic acid and held in a form available to plants (Richard, 1974). P concentrations in dual-inoculated plants were slightly higher than those inoculated with PGPR or VAM alone but since the treatment replicates were combined, no statistical analysis could be performed on these data. PGPR have been shown to produce a strong siderophore (Kloepper et al., 1980) which is considered to play a role in the antagonistic abilities of these bacteria. To our knowledge, it is not known whether the Fe chelated on the siderophore, called ferric pseudobactin (Kloepper et al., 1980), is available to plants.

Our results confirm other studies in which Rhizobium nodulation

was enhanced by Pseudomonas spp (Krasilnikov and Korenyako, 1944; Azcon-Aquilar and Barea, 1978; Grimes and Mount, 1984). Nodulation of actinorrhizal plants by Frankia spp was greatly enhanced in gnotobiotic culture by bacterial isolates inoculated singly or together (Knowlton et al., 1980). The authors propose that the "helper" organisms including Pseudomonas cepacia strains facilitate a close association between the symbiont and host by causing massive root hair deformation which served to trap the Frankia filaments. They pointed out the close analogy of their study with the observations of Mosse (1962) in which infection by VAM was also greatly facilitated by a "helper" organism in gnotobiotic culture. The mechanisms by which inoculation with some Pseudomonas strains is thought to increase plant susceptibility to VAM infection, e.g. changes in cell wall plasticity and root exudation patterns, growth regulator concentration in the rhizosphere, nutrient availability and uptake, and perhaps alteration of the rhizosphere microflora, are also possible explanations for the enhanced Rhizobium nodulation by PGPR observed in this study, since these mechanisms have been implicated in the Rhizobium nodulation process (Gibson and Jordan, 1983; Nutman, 1970).

Enhanced Rhizobium nodulation and N_2-fixation in mycorrhizal plants is well recognized and is attributed to increased P nutrition, although other as yet undetermined secondary effects may also be involved (Asimi et al., 1980; Barea and Azcon-Aquilar, 1983; Crush, 1974; Daft, 1978; Smith et al., 1979). In our study, inoculation with PGPR or with VAM alone enhanced nodulation, but nodulation was

even more significantly increased when both organisms were present. This may be an additive effect of both microorganisms operating singly or in a concerted action, e.g. bacterial chelation of P, Fe, and Co, all essential nutrients for nodule formation and function (Gibson and Jordan, 1983), coupled with enhanced uptake by the fungal hyphae. The isolation of compounds from Phaseolus vulgaris roots which cause agglutination of P. putida cells (Anderson, 1983; Grimes and Mount, 1984) suggests a close binding of precipitated cells to the root surface, where bacterial metabolic activity would have the greatest potential to affect the plant.

The enhanced mineral uptake by mycorrhizal PGPR-inoculated plants, however, suggest a close associaton of the bacterium with the VAM hyphae. Abundant fungal mycelium was observed in the mycorrhizal plants and it is now recognized that VAM colonization spreads throughout the root by the growth of hyphae along the root surface from established infection points (Hayman, 1978). Because of the dynamic nature of microbial colonization and activity in the rhizosphere, the elucidation of cause and effect will have to take into account both indirect effects of each organism on the plant as well as direct microbial interactions. The ubiquity of VAM fungi, Pseudomonas spp and Rhizobium spp in the rhizosphere of legumes suggests consideration of an adaptation towards mutual enhancement, although not total dependency.

Our results indicate that some of the growth-promoting effects attributed to inoculation with PGPR may be due in part to combined effects with VAM fungi. Among some practical implications to be

explored in this context is the encouragement of effective VAM development by cultural practices, or the inoculation with efficient VAM strains, to ensure the efficacy of beneficial microorganisms. Although PGPR are known to be antagonistic towards some soil fungi and can affect species composition in the rhizosphere (Suslow, 1982; Osburn et al., 1983), our study indicates that this is not the case with VAM fungi.

Table 2-1. Dry weight, nodulation and mycorrhizal colonization of subterranean clover inoculated with a plant growth-promoting bacterium (PGPR), vesicular-arbuscular mycorrhizal fungi (VAM) or both.

Treatment	Shoot dry wt (g)		Root dry wt (g)		No. nodules		% VAM	
	6 wk	12 wk	6 wk	12 wk	6 wk	12 wk	6 wk	12 wk
Noninoculated control	0.039a[1]	0.24a	0.046a	0.20a	9.1a	32.6a	0	0
VAM	0.040ab	0.30ab	0.047a	0.23ab	12.7ab	49.3b	7a	50a
PGPR	0.062bc	0.36b	0.066a	0.25ab	20.5b	49.1b	0	0
PGPR + VAM	0.064c	0.46c	0.068a	0.028b	31.6c	66.2c	23b	44a
LSD	0.022	0.09	0.024	0.06	10.5	10.8		

[1] Column means followed by the same letter are not significantly different at P = 0.05.

Table 2-2. Shoot tissue nutrient content of subterranean clover (Trifolium subterraneum L.) inoculated with a plant growth-promoting bacterium (PGPR), vesicular-arbuscular mycorrhizae (VAM) or both[1]

	Nutrient[2]													
	Fe		Cu		Al		Zn		Co		Ni		P	
Treatment	6 wk	12 wk	6 wk	12 wk	6 wk	12 wk	6 wk	12 wk	6 wk	12 wk	6 wk	12 wk	6 wk	12 wk
Noninoculated	633	716	11	14	449	506	24	27	0.27	0.33	0.17	2.53	0.10	0.14
VAM	800	573	13	16	578	401	24	27	0.23	0.27	0.18	2.36	0.12	0.15
PGPR	841	578	19	15	540	375	24	26	0.35	0.30	0.24	2.57	0.10	0.10
PGPR + VAM	1638	528	28	14	942	374	31	26	0.48	0.28	0.39	2.31	0.14	0.13
	N		K		Ca		Mg		Mn		S		Mo	
Treatment	6 wk	12 wk	6 wk	12 wk	6 wk	12 wk	6 wk	12 wk	6 wk	12 wk	6 wk	12 wk	6 wk	12 wk
Noninoculated	2.61	3.18	2.16	2.58	2.06	2.11	.55	.55	161	120	.25	.26	2.2	1.9
VAM	2.42	2.99	2.22	2.40	2.05	2.14	.53	.50	160	115	.27	.25	1.8	1.5
PGPR	2.67	3.05	2.34	2.25	2.18	1.97	.53	.48	155	111	.23	.20	1.8	1.7
PGPR + VAM	2.46	3.30	2.29	2.16	1.39	2.09	.52	.46	170	113	.23	.23	1.9	1.4

[1] Analysis performed on combined treatment replicates.

[2] N, P, K, Ca, Mg and S values are expressed in % dry wt, all others are ppm.

Table 2-3. Root tissue nutrient content of subterranean clover (Trifolium subterraneum L.) inoculated with a plant growth-promoting bacterium (PGPR), vesicular-arbuscular mycorrhizae (VAM) or both[1]

	Nutrient[2]													
	Fe		Cu		Al		Zn		Co		Ni		P	
Treatment	6 wk	12 wk	6 wk	12 wk	6 wk	12 wk	6 wk	12 wk	6 wk	12 wk	6 wk	12 wk	6 wk	12 wk
Noninoculated	5284	6832	31	34	5705	5231	36	32	1.97	1.98	7.5	8.4	0.10	0.14
VAM	6591	7217	18	42	6262	5910	30	35	2.16	2.11	9.9	8.8	0.13	0.14
PGPR	8614	5236	33	23	6654	4249	34	25	2.78	1.87	11.2	10.0	0.12	0.11
PGPR + VAM	8004	6787	36	25	7689	5914	35	28	2.26	1.91	12.4	8.7	0.12	0.15

	N		K		Ca		Mg		Mn		S		Mo	
Treatment	6 wk	12 wk	6 wk	12 wk	6 wk	12 wk	6 wk	12 wk	6 wk	12 wk	6 wk	12 wk	6 wk	12 wk
Noninoculated	1.70	2.16	2.01	2.13	0.43	0.48	.96	1.3	170	178	.84	1.2	6	3.0
VAM	1.70	2.32	2.22	1.81	0.43	0.52	.99	1.1	177	186	.93	1.0	5.1	2.7
PGPR	1.84	2.30	1.89	1.68	0.42	0.47	.97	1.2	249	161	.83	0.9	6	2.7
PGPR + VAM	1.61	2.32	1.36	1.27	0.41	0.46	.68	1.0	240	169	.64	0.9	4.5	2.3

[1] Analysis performed on combined treatment replicates.

[2] N, P, K, Ca, Mg and S values are expressed in % dry wt, all others are ppm.

Fig. 2-1. Growth of subterranean clover (Trifolium subterraneum L.) inoculated with vesicular-arbuscular mycorrhizal fungi (VAM; upper right), a plant growth-promoting isolate of Pseudomonas putida (PGPR; upper middle), both VAM + PGPR (lower middle) or noninoculated (upper left).

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CONCLUSION

There is a tendency in plant-microbial experimentation to inoculate plants with selected microorganisms of interest and conclude that the plant growth results obtained are due to a direct cause-and-effect between the introduced microorganism and the plant. Although this is true, in part, the fact that the introduced microorganism is part of a dynamic microbiological community under both experimental and natural conditions is often overlooked. The experiments in this thesis provide evidence that interactions and possibly even concerted activities occur between microorganisms in the rhizosphere which will influence plant growth. Consideration of microbial interactions in experimental design to test microbial effects on plants can be important to the relevance, accuracy, and interpretation of the results.

Response of subterranean clover to PGPR inoculation, for example, was significantly greater in the presence of VAM fungi in this study. The greater concentrations of essential nutrients (Fe, Cu, Co, Zn and P) in the shoots of dual-inoculated plants indicate that bacterial chelation of complexed elements coupled with fungal uptake could explain the synergistic effects of dual-inoculation with PGPR and VAM on plant growth and nodulation. No previous study had considered the presence of VAM in evaluating the growth promotion results from inoculation with PGPR, although VAM had probably developed in the nonsterile soil conditions used to test the efficacy of PGPR isolates.

VA mycorrhizal fungi can also be influenced by the presence of PGPR. Colonization of subterranean clover roots by VAM fungi increased from 7% to 23% after 6 weeks in the presence of PGPR. In a second series of experiments, VAM formation exerted a selective influence on the composition of rhizosphere microbial populations, as indicated by qualitative assays on selective media. Evidently, saprophytic microorganisms can be involved in the establishment of the symbiosis and directly affected by it. It is hoped that these experiments will stimulate VAM research performed in a larger context of interacting microbial communities. This may lead to new interpretations on the effects of mycorrhizal fungi on plant growth and nutrition and on the role of mycorrhizal fungi in the ecosystem.

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