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Controls for rhizosphere microorganisms to study effects of vesicular-arbuscular mycorrhizae on *Artemisia tridentata*

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Abstract. Seven treatments were set up to test the effects of vesicular-arbuscular (VA) mycorrhizal fungi and other rhizosphere microorganisms on the growth of *Artemisia tridentata* ssp. *tridentata*. Soil sievings had no significant effect on root or shoot mass. Spores and surface-sterile spores were a poor inoculum source, but roots and fresh soil caused 45–75% mycorrhizal infection. Whereas root-inoculated plants still had low growth responses by the end of the experiment, fresh soil inoculum caused the greatest response, and partial fresh inoculum caused a lesser response. These results suggest that fresh soil is an appropriate inoculum for this plant-fungal-soil system, and that the major effect on plant growth of the fresh soil inoculum is from the mycorrhizal fungi and not from the other microorganisms, because the sievings had no effect on plant growth. In addition, soil dilution plating of saprophytic fungi showed 85% species similarity between sterile and fresh soil inoculum by the end of the experiment. Since the effects of non-VA microorganisms are complex and varied, we suggest that researchers work out the type of mycorrhizal controls that best suit their system.

Key words: *Artemisia tridentata* – Mycorrhizal controls – Saprophytic fungi – Soil sievings – Vesicular-arbuscular mycorrhizae

Introduction

In studying the effects of vesicular-arbuscular (VA) mycorrhizae on plant growth, a number of researchers have expressed concern that soil microorganisms other than mycorrhizal fungi may affect the growth of the plants, and that great care must be taken when setting up experimental controls and in interpreting the results (Ames et al. 1987; Hetrick et al. 1989, 1990; Wilson et al. 1988; Koide and Li 1989). Azcon-Aquilar and Barea (1992) summarized the effects of rhizosphere organisms on plant growth, and concluded that the effects of non-VA microorganisms may be to increase, decrease, or cause no change in plant growth, depending on which microbial species dominate. This may occur via different mechanisms, including alteration of plant nutrient availability by non-mycorrhizal microorganisms, production of growth-regulating factors, promotion or reduction of mycorrhizal infection, or plant pathogenesis.

The ideal way to study the effects of mycorrhizal fungi would be in an axenic culture containing a plant and mycorrhizal fungus only. Sterility is difficult to maintain, but has been achieved in agar cultures (Allen et al. 1979, 1981a, b), where the dry mass of *Bouteloua gracilis* was increased by about 30% with inoculation, comparable to results in soil. Sand culture has also been used in an attempt to create axenic cultures, although bacterial contamination was not eliminated (St. John et al. 1981). While axenic cultures have provided conclusive evidence that the VA mycorrhizal fungi, and not other microorganisms, are responsible for the observed growth responses, the cultures are difficult to maintain, and do not reflect the real world; thus mycorrhizal researchers must accept other microorganisms as a part of their experiments. The types of controls and interpretation of results are critical to an understanding of the potential effects of the various microbial species.

The most common method suggested to introduce non-VA mycorrhizal microorganisms in controlled studies is to use soil sievings. Ames et al. (1987) suggested that all experiments should include soil sievings, but pointed out that the sieve will only allow a select group of microorganisms to pass, depending on the sieve size. Soil sievings caused reduced plant growth when added to sterile, mycorrhizal-inoculated soil in some studies (Wilson et al. 1988; Hetrick et al. 1986, 1988, 1989, 1990; Koide and Li 1989), but caused no change in growth in others (Manjunath and Bagayaraj 1981; Meredith 1990), and increased plant growth in another study, possibly by promoting mycorrhizal infection (Azcon-Aquilar and Barea 1985). Fresh soil is often used as an inoculum source and, similar to sievings, has given

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conflicting results in different experiments. In some experiments, fresh soil was more effective as an inoculum than mycorrhizal spores (Hetrick et al. 1986, 1989, 1990; Wilson et al. 1988), but in others the soil was just as effective as spores (Allen and Allen 1984; Hetrick et al. 1989). Side effects of non-mycorrhizal organisms were cited to explain this difference.

We report here the results of controlled inoculum experiments to determine effects of VA mycorrhizae and rhizosphere microorganisms on the growth of *Artemisia tridentata* ssp. *tridentata*. We were concerned that conflicting results in the literature did not allow us to determine which method would be best for our plant-soil-fungal system, given some preliminary data that surface-sterilized spores collected from our field site germinated poorly (unpulished observations). In addition to examining plant growth responses, we also quantified saprophytic fungi to determine whether potential growth differences could be attributed to mycorrhizal or saprophytic fungi.

Materials and methods

The seeds and soil for this experiment were collected from a stand of *A. tridentata* ssp. *tridentata* at the Sky Oaks Biological Research Station, about 100 km NE of San Diego. The dominant associated species were the grasses *Stipa pulchra* and *Sporobolus cryptandrus*. The sandy loam soil was collected in May 1989 from underneath mature *A. tridentata* plants, and had 13.3 µg/g bicarbonate-extractable P and 0.88 mg/g total Kjeldahl N. The plants were subject to seven treatments to assess possible interacting effects of the mycorrhizal fungi with other species of microorganisms, at two levels of phosphorus. These were: (1) steam-sterilized soil, (2) sterile soil plus soil sievings, (3) sterile soil plus spores, (4) sterile soil plus surface-sterilized spores, (5) sterile soil plus roots, (6) sterile soil plus 20 g nonsterile soil, and (7) nonsterile soil only.

The treatments were prepared by filling tubes measuring 4×20 cm and holding 175 g with either steam-sterilized or (for treatment 7) fresh soil from the field. Soil sievings were made by collecting the filtrate that passed a 37-µm mesh screen from 175 g of fresh soil, pouring this filtrate onto sterile soil that partially filled the tubes, to 3–5 cm depth beneath the soil surface, and then filling the tube with the remainder of the 175 g sterile soil. Other treatments received an equal volume of tap water. Spores were separated by sucrose flotation (Allen et al. 1979) from 20-g soil samples, and buried at about 3–5 cm beneath the surface. Each sample contained a total of about 500 spores. Spores for the surface-sterilization treatment were immersed in 10% sodium hypochlorite for 1 min and then rinsed.

The root-inoculum treatment contained about 2 g fresh weight of roots which were also buried at a depth of 3–5 cm. The final two treatments contained either 20 g of fresh non-sterile soil added at a depth of 3–5 cm to 155 g sterile soil, or 175 g non-sterile soil only. Each of these treatments was made with soil with or without P fertilizer. Each fertilized tube received enough P in the form of KH_2PO_4 in aqueous solution poured onto the surface to raise the mean level of P in the tube to 50 µg/g. There were 10 replicates of each mycorrhizal/phosphorus treatment, giving a total of 140 tubes.

The seeds were planted in the greenhouse in early May and thinned to one seedling per tube as they emerged. The temperature was maintained at approximately 30° C daytime and 22° C nighttime with ambient day length and 85% of full sunlight. Plants were watered daily to avoid moisture stress in this sandy soil. The plants were harvested in early October, at which time the roots of the largest plants were pot-bound. Seedlings from several of the treatments without mycorrhizal inoculum survived poorly and had to be reseeded 2–3 times over a 1-month period during May. Even after reseeding, one treatment had only two surviving seedlings by the end of the experiment (the fertilized soil-sievings treatment), and two had six seedlings (the unfertilized spore and surface-sterile spore treatments). All other treatments had 7–10 seedlings.

The variables measured were shoot and root dry mass, shoot height, percent root infection, and shoot and root P and N concentrations. One-half of the root samples was assessed for mycorrhizal infection by staining with trypan blue and examining 50 1-mm root segments from each plant for the presence or absence of mycorrhizal fungi. The other half of the roots and all of the shoots were digested in acid for nutrient analysis, and N and P were detected colorimetrically. Some treatments produced too few samples or too little biomass for nutrient analysis and were omitted.

Just prior to harvesting, soil was sampled for dilution plating of saprophytic fungi using a 1 cm wide×6 cm deep core. Two treatments were selected for this analysis, the sterile soil and the sterile soil inoculated with 20 g of nonsterile soil, with five cores from each. The soil from within each sample was homogenized, and a 2-g aliquot was diluted in 10 l sterile water; a 2-ml sample of the diluent was then plated on soil extract agar with lactic acid to prevent bacterial growth. Three samples were plated from each of the five cores for fungal colony counts. Five random colonies were then isolated from each plate, or a total of 75 isolates from each of the two treatments. Dominant species were identified to genus, and all species were compared in the two treatments so that an index of similarity could be calculated. We used the equation $2w/(a+b)$, where w is the number of species in common to the two samples, a is the number of species in sample a, and b is the number of species in sample b.

Results

Only two of the treatments, 20 g nonsterile soil and nonsterile soil only, caused a significant growth response of *A. tridentata* compared to the sterile-soil controls, and these response patterns were similar for shoot dry mass, root dry mass, and shoot height (Fig. 1A–C). The soil sievings that should have added soil microorganisms other than mycorrhizal fungi to the soil had no effect on any of these measured growth parameters. Neither spores nor surface-sterilized spores had an effect on growth and, surprisingly, neither did the root inoculum.

The percentage of mycorrhizal infection was low (0–11%) for all treatments except the root, 20 g nonsterile soil, and nonsterile soil only treatments, which ranged from 43% to 75% (Fig. 1D). Mycorrhizal infection was statistically similar in these three treatments, even though dry mass production varied, with the root treatment having high infection but low biomass. The soil-sievings treatment caused a slight contamination by mycorrhizal fungi, although this was apparently not high enough to cause a corresponding increase in plant biomass (Fig. 1A, B).

The effects of P fertilization on dry mass, height and infection were variable. The P fertilization significantly increased shoot and root growth for one treatment, the nonsterile soil only inoculum, and did not affect plant height. Biomass may have been limited by P in the unfertilized tubes, as the nonsterile soil only treatment had

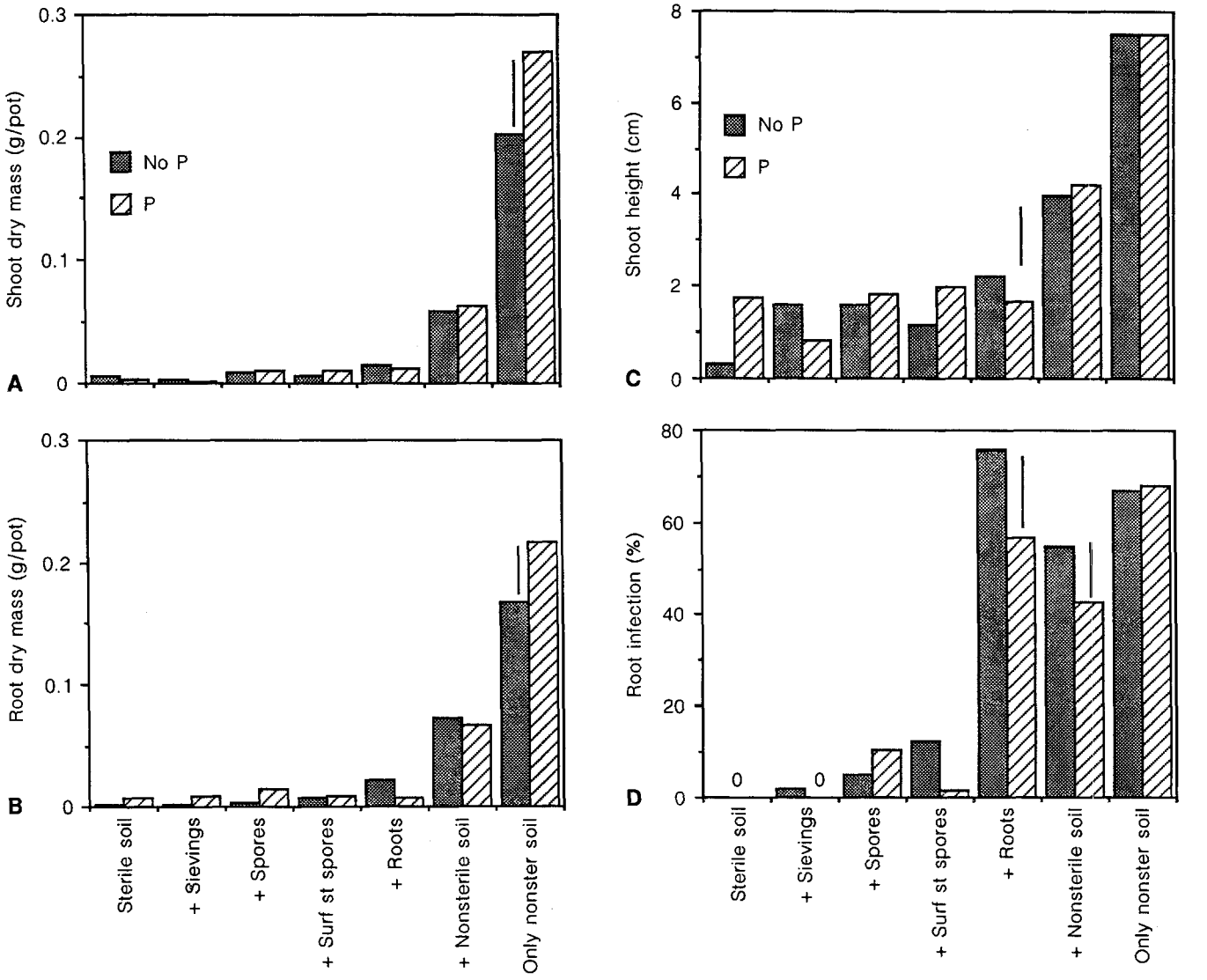

Fig. 1. Shoot dry mass (**A**), root dry mass (**B**), shoot height (**C**) and % root infection (**D**) of *Artemisia tridentata* subjected to seven inoculated or uninoculated treatments. Each treatment contained steam-sterilized soil alone or with different inoculum types, except one with only nonsterile soil. +*Surf st spores*, surface-sterilized spores; +*nonsterile soil*, sterile soil plus 20 g nonsterile soil. *Vertical bar*: $LSD_{0.05}$

by far the greatest plant growth and could have depleted the soil available P, but there was no change in root infection in this treatment (Fig. 1D). Two treatments, the root inoculum and 20 g nonsterile soil, showed decreased infection with added P.

Plant tissue nutrients had few significant responses to mycorrhizal infection (Fig. 2). The two nonsterile soil treatments with the greatest biomass response to mycorrhizae (20 g nonsterile soil, nonsterile soil only) did not have higher shoot P concentrations than the sterile-soil treatment or the other treatments that formed little or no infection, suggesting that the tissue P concentration even of plants in the unfertilized soil was sufficient (Fig. 2A). These treatments did have lower shoot N concentrations than some of the treatments that produced smaller plants, probably due to dilution of N in the larger plants (Fig. 2B). Only few roots were large enough to assess nutrient concentrations (Fig. 2C–D). There were no consistent effects of P fertilization on P- or N root uptake of the three treatments analyzed, again suggesting that P was not limiting in the unfertilized soils.

The dilution plating of soil fungi showed that the same species were present in the sterile control and the 20 g nonsterile soil treatment. A total of 20 species was found in the soil-inoculated treatment, while 19 were in the control that was initially steam sterilized, with an overlap of 17 species common to both. This gives a Simpson's index value of 0.87 similarity between the two treatments. The most abundant genera were *Penicillium*, *Aspergillus*, *Trichoderma*, and *Rhizopus*, which occurred in both treatments. Only the five least abundant (unidentified) species did not occur in both treatments, and these were detected only once or twice in the cultures. A species area curve (Fig. 3) shows that 75 isolates were sufficient to detect most of the culturable sa-

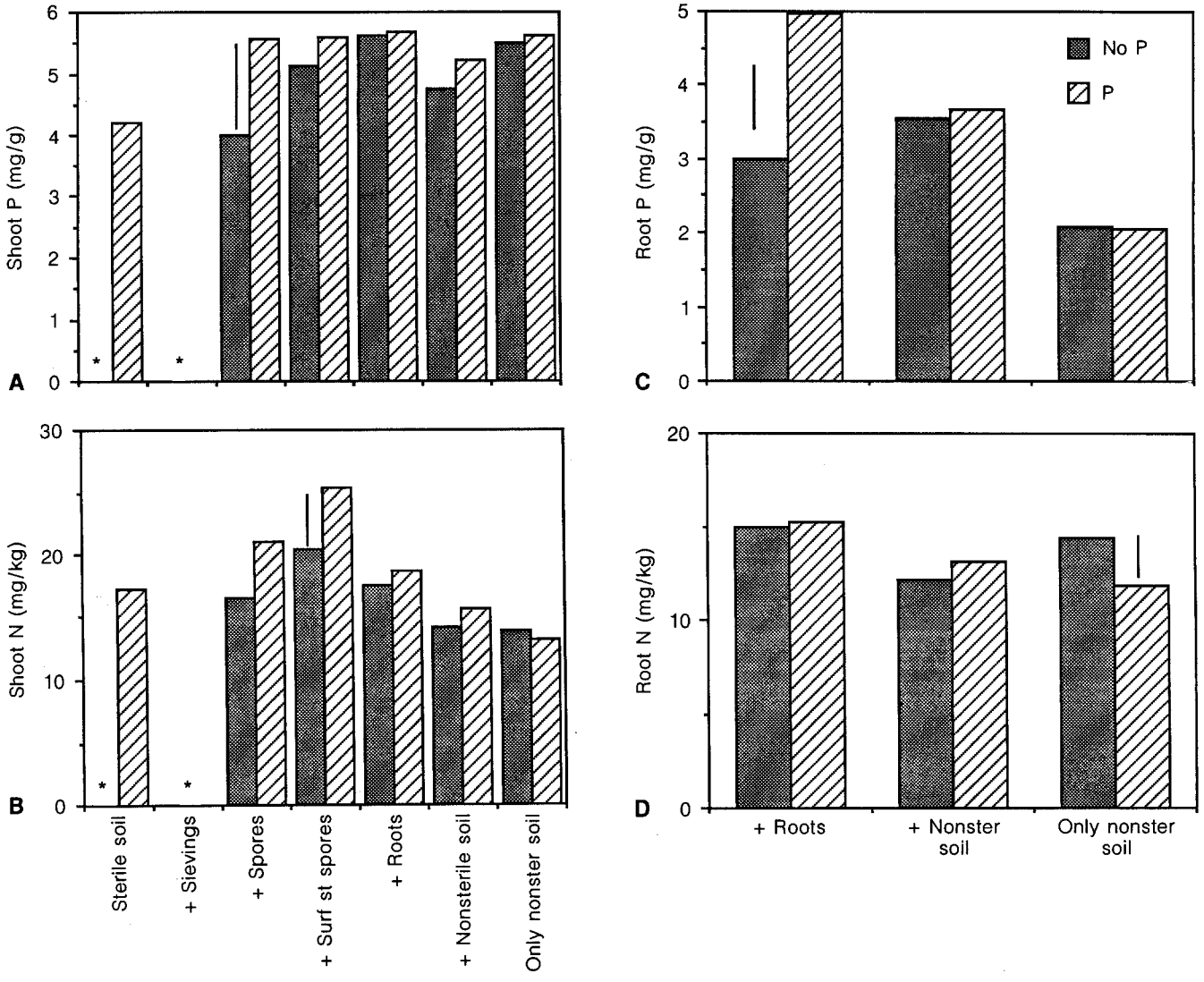

Fig. 2. Shoot P concentration (**A**), shoot N (**B**), root P (**C**), and root N (**D**) of *A. tridentata* subjected to seven inoculated or uninoculated treatments. Each treatment contained steam-sterilized soil alone or with different inoculum types, except one with only nonsterile soil. Only three treatments are shown for **C** and **D** because plant survival was low in the uninoculated treatments, and the root material was used to assess root infection. *Vertical bar*: $LSD_{0.05}$. *, Insufficient plant material

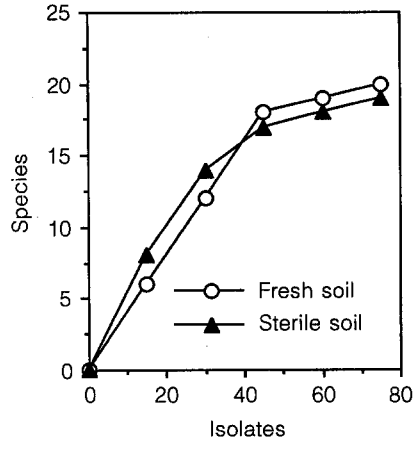

Fig. 3. Number of isolates of dilution-plated soil solution versus total number of saprophytic fungal species observed in samples from 20 g fresh soil inoculum and sterile soil

prophytic fungal species, as the curve begins to level at 17–18 species.

Discussion

Our results represent a significant departure from other controlled inoculum studies (e.g., Wilson et al. 1988; Hetrick et al. 1990) in that the plants growing in nonsterile soil had the highest growth response, even higher than those with only 20 g nonsterile soil. The root inoculum did not produce a large response even though the plants were infected. We suggest that washing the roots broke hyphal attachments and reduced their initial infectivity. Surprisingly, the root and the two nonsterile soil treatments had similar percent infection at the end of the experiment, even though they differed in dry mass. In other studies, analysis of the time course of in-

fection rate showed that denser inoculum types caused increased rates of infection and increased plant growth (e.g., Wilson et al. 1988). The three inoculum types most likely had different infectivities depending upon the initial hyphal mass, which is known to determine infectivity (Read 1992).

Soil sievings produced no plant growth response in our experiment, as shown by some studies (Manjunath and Bagyaraj 1981; Meredith 1990), while other studies showed a reduction in plant size with soil sievings (Wilson et al. 1988; Hetrick et al. 1989, 1990; Koide and Li 1989). The lack of plant response to sievings is not surprising in our study as the saprophytic fungal taxa were similar in the steam-sterilized soil and soil inoculated with 20 g nonsterile soil at the end of the experiment. In fact, the genera that did colonize the sterile soil are those known to be effective colonizers, while at the same time they are abundant soil fungi (Domsch et al. 1980). In addition, the genus *Fusarium* is a dominant in fresh soils from our field sites (S. Morris and M. Allen, unpublished observations), but was absent from our greenhouse isolates. Thus the soil microflora of greenhouse experiments cannot be compared to field experiments (Welvaert 1974).

Other researchers who assessed density of both fungi and bacteria in inoculated and uninoculated treatments at the end of their experiments (Koide and Li 1989; Meredith 1990) also found no differences in microbial counts, even though the sievings caused plant growth reductions in the former and no changes in the latter study. The microorganisms must of course be absent or very sparse in steam-sterilized soil at the beginning of the experiment, hence Koide and Li (1989) hypothesized that initial microbial effects on plant growth can still be observed several months later.

Without precise knowledge of what microbial species are affecting the growth of the plants, it is impossible to explain the discrepancies of plant response to the different inocula. Ames et al. (1987) suggested that the sievings do not simulate the non-VA mycorrhizal microorganisms because larger propagules will not pass through fine-meshed sieves. In addition, several of the studies were done with excessively large amounts of sievings, sometimes from three times the soil volume in which the plants were growing (Koide and Li 1989; Hetrick et al. 1990), and thus there may have been an inordinately high initial density of microorganisms that might affect plant growth.

Another problem in interpretation is the use of mycorrhizal spore inocula in several of the studies which are not the same composition as the species of spores found in nonsterile inoculum soil. Several of the studies used pot-cultured inoculum containing single spores of species known to be effective mycorrhizal fungi, and compared plant growth results to those from nonsterile soil inoculum with a mixture of mycorrhizal fungi (e.g., Wilson et al. 1988; Koide and Li 1989; Hetrick et al. 1989, 1990). Reduced growth in nonsterile soil in these studies does not necessarily argue for interference by non-VA mycorrhizal microorganisms. An alternative hypothesis is that the pot-cultured spores are more effective species in causing growth responses than those that infect from the inoculum soil. The most effective spore species in monoculture is not necessarily the most infective in mixture (Wilson and Tommerup 1992), and species with lesser effectiveness may infect the roots to a greater extent. In our study, we used spores extracted from 20 g soil to replicate the number of spores (about 500) in an equal mass of the inoculum soil. However, spores alone were not infective, and we have little recourse except to use roots or preferably whole soil as an inoculum source.

Our results also show that soil sievings are not needed as a control for our soil-plant-fungal system as they had no effect on plant growth. Since it is unclear which microbial species are added with soil sievings (Ames et al. 1987), an alternative control might be to compare the effects of surface-sterilized spores with nonsterile soil inoculum. This comparison cannot be made in our study because the spores were not infective, even though they were collected in the spring, a time of year when spore germination would be expected to take place naturally. However, in a previous study of grasses from sagebrush-steppe, spore and soil inocula produced no significantly different growth effects on two grass species (Allen and Allen 1984).

In determining the best set of controls for non-VA microorganisms, a researcher needs to consider both the degree of control and the degree of reality needed. For instance, to examine a specific biochemical or hormonal response of a plant, an axenic culture with a high degree of control but low reality is appropriate. Many studies on mycorrhizae involve growth and physiological responses of plants to mycorrhizae, and are performed in the greenhouse or growth chamber. Such studies have a lesser degree of control than axenic studies, but greater reality. As discussed above, the microflora that colonizes in these conditions may not simulate field conditions, and our study and others have shown that attempts to simulate the non-VA mycorrhizal microflora with soil sievings may eventually be masked by colonizing microflora. Field studies have the greatest degree of reality, but less control in eliminating mycorrhizal and other inocula, and therefore less assurance in interpretion of results. It was our intention to use this as a preliminary experiment before transplanting seedlings into the field, and we did not wish to loose reality by inoculating with an artificially cultured mycorrhizal fungus. Therefore, we concluded that fresh-soil inoculum was appropriate, given that the sievings had no effect on plant growth. Since the effects of non-VA mycorrhizal microorganisms are so complex and so varied, we suggest that the type of microbial controls to be used do not become a dogma, but that researchers work out controls best-suited to the question addressed and to their soil-plant-fungal system.

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References

Allen EB, Allen MF (1984) Competition between plants of different successional stages: mycorrhizae as regulators. Can J Bot 62:2625–2629

Allen MF, Moore TS, Christensen M, Stanton N (1979) Growth of vesicular-arbuscular mycorrhizal and nonmycorrhizal *Bouteloua gracilis* in a defined medium. Mycologia 71:666–669

Allen MF, Sexton JC, Moore TS Jr, Christensen M (1981a) Influence of phosphate source on vesicular-arbuscular mycorrhizae of *Bouteloua gracilis*. New Phytol 87:687–694

Allen MF, Smith WK, Moore TS Jr, Christensen M (1981b) Comparative water relations and photosynthesis of mycorrhizal and non-mycorrhizal *Bouteloua gracilis* (J. B. K.) Lag ex Steud. New Phytol 88:683–693

Ames RN, Mihara KL, Bethlenfalvay GJ (1987) The establishment of microorganisms in vesicular-arbuscular mycorrhizal and control treatments. Biol Fert Soil 3:217–223

Azcon-Aguilar C, Barea JM (1985) Effect of soil microorganisms on formation of vesicular-arbuscular mycorrhizas. Trans Br Mycol Soc 84:536–537

Azcon-Aguilar C, Barea JM (1992) Interactions between mycorrhizal fungi and other rhizosphere microorganisms. In: Allen MF (ed) Mycorrhizal functioning. Chapman and Hall, London, pp 163–198

Domsch KH, Gams W, Anderson T-H (1980) Compendium of soil fungi. Academic Press, London

Hetrick BAD, Kitt DG, Wilson GT (1986) The influence of phosphorus fertilizer, drought, fungal species, and nonsterile soil on the mycorrhizal growth response in tall grass prairie. Can J Bot 64:1199–1203

Hetrick BAD, Wilson GT, Kitt DG, Schwab AP (1988) Effects of soil microorganisms on mycorrhizal contribution to growth of big bluestem grass in non-sterile soil. Soil Biol Biochem 20:501–507

Hetrick BAD, Wilson GW, Hartnett DC (1989) Relationship between mycorrhizal dependence and competitive ability of two tallgrass prairie species. Can J Bot 67:2608–2615

Hetrick BAD, Wilson GWT, Todd TC (1990) Differential responses of C_3 and C_4 grasses to mycorrhizal symbiosis, phosphorus fertilization, and soil microorganisms. Can J Bot 68:461–467

Koide R, Li MG (1989) Appropriate controls for vesicular-arbuscular mycorrhiza research. New Phytol 111:35–44

Manjunath A, Bagyaraj DJ (1981) Components of growth of VA mycorrhizal inoculum and their effects on growth of onion. New Phytol 87:355–361

Meredith JA (1990) The influence of varied microbial substrate conditions on the growth and mycorrhizal colonization of little bluestem (*Schizachyrium scoparium* (Michx.) Nash). MS thesis, Illinois State University, Normal, Ill

Read DJ (1992) The mycorrhizal mycelium. In: Allen MF (ed) Mycorrhizal functioning. Chapman and Hall, London, pp 102–133

St John TV, Hayes RI, Reid CPP (1981) A new method for producing pure VAM-host cultures without specialized media. New Phytol 89:81–86

Welvaert W (1974) Evolution of the fungus flora following different soil treatments. Agro-Ecosystems 1:157–168

Wilson GT, Hetrick BAD, Kitt DG (1988) Suppression of mycorrhizal growth response of big bluestem by non-sterile soil. Mycologia 80:338–343

Wilson JM, Tommerup IC (1992) Interactions between fungal symbionts: VA mycorrhizae. In: Allen MF (ed) Mycorrhizal functioning. Chapman and Hall, London, pp 199–248