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MONITORING EXTRA-MATRICAL HYPHAE OF A VESICULAR–ARBUSCULAR MYCORRHIZAL FUNGUS WITH AN IMMUNOFLUORESCENCE ASSAY AND THE SOIL AGGREGATION TECHNIQUE

J. L. KOUGH
Department of Botany and Plant Pathology, Oregon State University, Corvallis, OR 97331, U.S.A.

and

R. G. LINDERMAN
U.S. Department of Agriculture, ARS, Horticultural Crops Research Laboratory, Corvallis, OR 97330, U.S.A.

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Summary—An immunofluorescence assay (IFA) was used to compare the amount of extra-matrical hyphae of the vesicular–arbuscular mycorrhizal (VAM) fungus *Glomus versiforme* (Karst) Berch colonizing seedlings of White Cedar, *Chamaecyparis lawsoniana* (A. Murr.) Parl., with different root densities in three soil mixes. Restriction (binding) of the root mass with nylon screening decreased root density and also decreased the density of VAM hyphae detected per root length or per soil volume. Top growth of transplanted seedlings was not affected by root restriction. More VAM fungal external hyphae, determined by IFA and the sand aggregation method, formed in river sand than in silt loam with similar percentages of intraradical VAM colonization. More soil aggregates formed in silt loam than in sand regardless of the presence of VAM fungal hyphae. Depending on experimental design, the aggregation method for estimating VAM external hyphae may be limited to use in sandy soil. IFA reacted specifically with *G. versiforme* hyphae in mineral soil or sand, but was ineffective in peat soil due to nonspecific staining and autofluorescence of organic matter.

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 VAM fungi is currently monitored by determining the extent of root cortex colonization and formation of distinctive structures such as arbuscules and vesicles. Abundant root colonization does not always correspond to increased plant growth (Graham *et al.*, 1982; Mosse, 1972) and soil factors alter plant growth enhancement by VAM, despite similar levels of root colonization (Davis *et al.*, 1983; Skipper and Smith, 1979).

Radiotracers and heavy isotopes demonstrate that nutrients can be taken up by VAM hyphae at considerable distances from colonized roots (Rhodes and Hirrel, 1982). To demonstrate these isotope systems, physical separation of roots and mycelium is required to insure that only hyphae absorb isotopes added to soil. However, conclusions made from such studies about nutrient uptake by VAM fungal hyphae and their growth into soil may be inappropriate if proximity of root tissue affects the intensity of fungal colonization of soil.

Immunofluorescence assay (IFA) (Aldwell *et al.*, 1983; Kough *et al.*, 1983; Wilson *et al.*, 1983) recognizes endogonaceous fungal structures and distinguishes them from structures produced by other soil fungi. At present, antisera used in those studies are specific only at the genus level. However, this technique is one of the best means available to study both taxonomy and soil ecology of VAM fungi.

We studied root colonization by VAM fungi on plants grown in three different soil types using both IFA (Kough *et al.*, 1983) and the sand aggregation method (Graham *et al.*, 1982; Sutton and Sheppard, 1976). The effect of root density on hyphal growth into soil by VAM fungi was also examined.

MATERIALS AND METHODS

Preparation of specific antigens

Sporocarps of *Glomus versiforme* (Karst) Berch were collected from the soil surface of asparagus (*Asparagus officinalis* L.), Western Red Cedar (*Thuja plicata* J. Don ex D. Don), and Coast Redwood [*Sequoia sempervirens* (D. Don) Endl.] pot cultures. A suspension of single chlamydospores and hyphal debris was obtained by sieving and decanting sporocarps (Gerdemann and Nicolson, 1963). This spore suspension was filtered onto 10 μm mesh nylon, and the retained spores were rinsed with sterile deionized water (SDW) and placed in a solution of streptomycin (200 μg ml^{-1}) and gentiamycin (100 μg ml^{-1}) 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 eliminate surface contamination (Mertz *et al.*, 1979). After 2 weeks at 6°C in antibiotic suspension, the spores were rinsed with SDW, placed on sterile filter paper and dried at 6°C for 3 days. An aliquot

of approximately 150 spores was then plated on potato dextrose agar or Kings medium B to determine sterility. If any spores were contaminated, that entire preparation was discarded. Chlamydospore preparations with no visible sign of contamination were weighed and mixed 1:1 (w/v) with PBS in a ground glass tissue macerator. Approximately 0.2–0.3 g of spores (from 5–10 sporocarps) or about 20,000 spores were thus used for each injection of 6 rabbits. Particulate and soluble fractions were separated by centrifugation (12,800 *g* for 3 min) after grinding. The soluble fraction (without the lipid layer that separated on top) was withdrawn and stored at −20°C. 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 ml^{-1} and stored at −20°C.

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 before injection to detect the presence of any antibodies to *G. versiforme*. One-half ml of either soluble or particulate antigen preparation was injected intravenously (i.v.) into the marginal vein of the ear of three rabbits.

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

Day 28. One-half ml of antigen preparation, mixed 1:1 v/v with Freund's incomplete adjuvant (DIFCO, Detroit, Michigan), was injected into the muscles of the hind leg of each rabbit (Herbert, 1978). The use of intramuscular (i.m.) injections and adjuvants was intended to increase antibody titer through prolonged antigen exposure.

Day 42. Another 1 ml preparation of antigen and Freund's incomplete adjuvant was given as i.m. injection into 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. These initial antibody titers were low, necessitating another series of 1-ml injections on days 82 and 96.

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 killed painlessly. All blood samples were allowed to clot overnight at 4°C. Serum was removed and stored in 5 ml aliquots at −20°C.

Titer determination and cross reaction tests

Titer was determined by agglutination of antigen suspensions (Malajczuk *et al.*, 1976). Cross reactions were examined by indirect immunofluorescence in preparations of *Fusarium roseum*, *F. oxysporum*, *Phytophthora cinnamomi*, and species of *Trichoderma* and *Penicillium*. These fungi were isolated from soil dilutions as described in Kough *et al.* (1983).

Plant species and growth conditions

Natural seedlings less than 2 cm tall of White Cedar (*Chamaecyparis lawsoniana* (A. Murr.) Parl.) were collected in the organic litter layer under a mature *C. lawsoniana* tree. Twenty-five seedlings were transplanted into 160 ml plastic tubes (Leach "Super Cells", Ray Leach Cone-Tainer Nursery, Canby, Oregon) containing pasteurized river sand (60°C for 30 min) amended with 20 ml of roots, soil, and spores of *G. versiforme* from an asparagus pot culture. The entire root systems of 30 *C. lawsoniana* seedlings from the same sward of organic duff were cleared and stained (Phillips and Hayman, 1970) to determine if seedlings had been colonized with VAM fungi before transplanting.

Inoculated seedlings were maintained in a glasshouse (22–15°C average day–night temperature with supplemental lighting for a 16 h photoperiod from high pressure sodium vapor lights with average irradiance of 200 $\mu E\ s^{-1}\ m^{-2}$) for 6 months with fertilization every 2 weeks with 10 ml of 1/4 strength phosphate (11 $\mu g\ ml^{-1}$) Long Ashton's Nutrient Solution (LANS) (Hewitt, 1966). Twenty-one of the seedlings then were measured for uniformity of height and stem diameter and transplanted into 22-cm dia plastic pots. The potting medium (sieved to less than 2 mm) was one of the following: pasteurized river sand, pasteurized 1:1 (v/v) river sand and silt loam, or pasteurized 1:1 (v/v) river sand and sphagnum peat. Roots of the four remaining seedlings were cut into 1 cm segments, cleared, stained and percent root length with VAM colonization determined (Biermann and Linderman, 1981).

Of 7 seedlings transplanted into each soil, 3 seedling root systems were placed within a cylinder of 1 mm nylon screen to restrict lateral growth of the root systems. The remaining 4 seedlings were transplanted without any treatment to restrict root growth. All seedlings were planted near the center of each pot. Plants were maintained as described above, except 50 ml of 1/4 strength phosphate (11 $\mu g\ ml^{-1}$) LANS fertilizer solution was carefully poured around the edge of the pots, 2, 4 and 6 weeks after transplanting.

Harvest procedures

At 6 and 8 weeks after transplanting, plants were measured for top height and stem diameter. A 1.8 cm dia cork borer was used to remove 2 adjacent soil cores near the edge of each pot. The cores were measured and weighed. One core was used to determine soil aggregation by VAM hyphae (Graham *et al.*, 1982; Sutton and Sheppard, 1976). The other core was used to determine VAM hyphae in soil by the indirect IFA (Goldman, 1968; Johnson *et al.*, 1978; Malajczuk *et al.*, 1978). Each soil core used for the IFA was placed in 40 ml of 7.5 mM KOH. Root pieces measured for length to the nearest mm under a stereomicroscope and then were cleared and stained to determine presence of mycorrhizas (Phillips and Hayman, 1970). Soil suspensions lacking root tissue were settled for 5 h at 4°C. The suspension 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 by fluorescent microscopy (Kough *et al.*, 1983). Hyphal amounts in IFA preparations were subjectively rated on a 0–5 scale based on total amount of fluorescent hyphal fragments observed in

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

Soil type and root density	6 weeks after transplant		8 weeks after transplant	
	Fluorescence rating[a]	Root length (cm cm ^{3} soil)	Fluorescence rating	Root length (cm cm ^{3} soil)
Sand, high root density	2.5	0.32	2.5	0.65
Sand, low root density	0.5	0[b]	1.5	0.35
Silt loam–sand high root density	1.5	0.38	1.5	0.66
Silt loam–sand low root density	1.5	0.27	0.5	0.09

[a]Fluorescence ratings were visually assessed on a 0–5 scale (5 = greatest abundance of fluorescent mycelia) for total mass of VAM fungal hyphae retained on filter, 0 = no hyphae; intermediate ratings reflected intermediate numbers of hyphae detected.
[b]No root tissue present in soil core.

each of 20–30 fields on each of two microscopic slide mounts of all debris retained on the filter. A rating of 0 = no fluorescing fragments; 1 = less than 10% of the fragments fluorescing; 2 = 10–30% fluorescing fragments; 3 = 30–50% fluorescing fragments; 4 = 50–70% fluorescing fragments; and 5 = 70–100% fluorescing fragments. Ratings of 4 were rarely given, and 5 was never given. Each field had 30–60 fragments per field, although generally all fragments in a field were not counted.

In the ratings estimating the amount of external hyphae adhering to roots, a 0–5 scale was also used except each division represented an estimation of hyphae per root length. In those estimations, ratings of 4 and 5 were common.

RESULTS

Titer determination and cross reaction tests

By using a short immunization schedule and antigenic preparations with Freund's incomplete adjuvant, higher antibody titer was obtained than by Kough *et al.* (1983). Serum antibody titers ranged from 1:30 to 1:300 in agglutination tests. As the second immunization series did not increase antibody titer, rabbits were bled and killed painlessly. Higher titers were generally obtained with soluble antigen preparations (1:60 to 1:300) than with particulate fractions (1:30 to 1:60). However, antisera produced to soluble antigens showed cross reaction to *Penicillium* species, making these sera unusable in a soil assay without prior cross absorption. Therefore, only antisera to particulate antigens with both highest titer and no detectable cross reaction to soil fungi were used for subsequent soil assays.

Root density differences

Nylon screening did not completely prevent root development into soil after transplanting (Tables 1 and 2), but roots were less frequently detected in soil cores from plants with restricted root systems, especially at the 6 week harvest. Although there were true differences in root density, seedling top height or stem diameter did not differ significantly at either harvest.

VAM fungal colonization of roots and adherent external hyphae

The roots of seedlings collected in the organic duff were not colonized with VAM fungi prior to inoculation. Roots of 6-month-old inoculated seedlings were colonized by VAM fungi (46% of the root length of examined segments) as indicated by hyphal coils, intraradical hyphae and vesicles.

Root pieces in soil cores later used for IFA were examined for colonization by VAM fungi, presence of external hyphae and VAM entry points after clearing and staining (Table 3). Roots recovered from river sand had the largest amount of external mycelium still adherent to entry points and the most entry points per root length. While percentage root length colonized by VAM fungi differed between soils, sample size of root pieces recovered from the cores was too small to analyze statistically except the root samples recovered from the cores with unrestricted root growth in the 8 week assay; in those samples, VAM colonization did not differ significantly among the three soils according to a t-test.

Number of entry points and amount of adherent mycelium was highest in river sand and lowest in peat:sand mixture on roots that escaped the nylon screen 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 the soil types used in this study.

Table 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 weeks after transplant			8 weeks after transplant		
	Aggregate formation			Aggregate formation		
	% Total soil	Aggregate wt/ root tissue wt (g)	Root length (cm cm ^{3} soil)	% soil	Aggregate wt/ root tissue wt (g)	Root length (cm cm ^{3} soil)
Sand, high root density	18	22.3	0.32	25	8.9	0.79
Sand, low root density	23	36.1	0.08	36	36.1	0.18
Silt loam–sand high root density	37	45.7	0.13	46	19.4	0.47
Silt loam–sand low root density	42	37.5	0.04	37	61.0	0.15
Peat–sand high root density	33	24.0	0.45	24	21.4	0.18
Peat–sand low root density	35	30.9	0.18	31	34.9	0.17

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

Soil type and root density	6 weeks after transplant: Entry points	VAM % root length	Adherent external hyphae[a]	8 weeks after transplant: Entry points	VAM % root length	Adherent external hyphae[a]
Sand, roots free	79	80	3.0	70	60	4.0
Sand, roots restricted	0	0	0[b]	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	0	0	0[b]	0	0	0[b]

[a]Amount of external hyphae present and connected to infection points was estimated on a 0–5 scale, 5 = greatest amount of hyphae, 0 = no hyphae; intermediate ratings reflected intermediate numbers of hyphae relative to the rating 5 level.

[b]No root tissue recovered from soil cores.

Total percentage of soil in aggregates was highest in the silt loam:river sand mixture and lowest in river sand alone (Table 2). Within a soil type, percentage of soil aggregates did not differ with root growth restriction treatment.

Values for aggregate formation, expressed as g aggregate weight g^{-1} root weight, could not be calculated for cores taken from root growth restriction treatments, since root tissue was absent in most cores. Aggregate formation decreased as cm root length cm^{-3} soil volume values increased in river sand. The silt loam–sand and peat–sand mixes had similar aggregate amounts regardless of root density or harvest times. Both these soil mixes formed aggregates even in the absence of VAM fungi. Therefore, VAM fungal hypha-induced aggregation may be masked in these soils.

Immunofluorescence assay

It was not possible to detect VAM fungal hyphae by immunofluorescence assay (IFA) in the peat–sand mix due to high amounts of background fluorescence in the organic fraction. Some fluorescence was associated with nonspecific staining of organic particles, but the majority was from autofluorescence of organic matter.

VAM fungal hyphae were easily detected in mineral soils since little debris collected on the filters other than soil fungi and tiny root fragments. Even after careful removal of colonized root pieces to retain all adherent external mycelium, more VAM fungus hyphal fragments were identified in the soil cores by IFA, and more of those were present in cores with root tissue than those without root tissue (Table 1). For a given soil type and harvest time, greater root density (cm root length cm^{-3} soil) gave greater detectable amounts of VAM fungus hyphal fragments.

More IFA-detectable VAM fungal hyphae were found in river sand than in silt loam–sand mix. This result may have been an artifact of the system used to retrieve VAM fungus hyphae from soil. Some mycelium could have settled with the soil fraction, and therefore was not recovered or detected.

DISCUSSION

Root density within the volume of soil around a plant affects the rate of proliferation of extra-matrical mycelium of VAM fungi into soil. Decreased intraradical VAM fungal colonization when root density was high was noted by Warner and Mosse (1982), but we observed that VAM fungal growth into the soil was less when root density was low compared to a higher root density even though the level of root colonization was similar. In light of the report that soil microbes can enhance proliferation of external mycelium of VA mycorrhizae (Sutton and Sheppard, 1976), rhizosphere microbes may be responsible for this effect. A lower root density would reduce rhizosphere populations and perhaps reduce hyphal proliferation between roots.

Ability of VAM fungus hyphae to proliferate in the soil between roots was affected by soil type. The VAM symbiosis is undoubtedly influenced by edaphic factors, such as pH (Skipper and Smith, 1979; Davis *et al.*, 1983). While VAM fungal hyphae transport organic nitrogen to host plants from considerable distances (Ames *et al.*, 1983) and may preferentially colonize soil organic particles (St. John *et al.*, 1983), the effect of organic matter on proliferation of hyphae in soil is unclear. Both soil aggregate and IFA techniques were not usable in soil with high organic matter content.

IFA effectively detected VAM fungal hyphae in soil. Careful removal of root pieces to confirm VAM fungal colonization also provided samples of roots with adherent VAM fungus mycelium. While presence of adherent mycelium correlated well with subsequent IFA tests, considerable amounts of hyphae are apparently lost even with careful manipulation. Moreover, hyphal fragments that reacted with the IFA filter system were not always easy to confirm visually as VAM hyphae, although previous results (Kough *et al.*, 1983) indicated there would be no cross reactions with other fungi.

The IFA system, as described, is limited in usefulness to mineral soils until a method can be found to eliminate background fluorescence of organic particles. Counter-staining or gelatin pretreatment effectively reduced a similar problem in other soil systems and may be effective here (Bohlool and Schmidt, 1968). Background fluorescence complications could be reduced if the soil organic fraction were absent. Sucrose density treatments may aid in removal of unwanted debris (Tisdall and Oades, 1979), but the effect of high osmotica on the antigenicity of these preparations is not known.

The IFA technique will not eliminate the need to clear and stain roots to confirm VAM colonization. However, it provides an additional means to sample the soil for activity of VAM symbionts. At present

the technique is limited in usefulness because 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.

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