# Population Dynamics of Two Vesicular-Arbuscular Endomycorrhizal Fungi and the Role of Hyperparasitic Fungi

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## ABSTRACT

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Populations of *Glomus macrocarpus* var. *geosporus* and *Gigaspora gigantea*, based on numbers of chlamydospores in roots and azygospores in soil, respectively, were followed through two growing seasons in small plots in which peanuts and soybeans were grown. During the second growing season, root infection data also were obtained. Populations of *G. macrocarpus* increased rapidly during the first season only in plots without *G. gigantea* which may indicate either that *G. macrocarpus* does not compete well with *G. gigantea*, or that other factors such as hyperparasites inhibit the former more than the latter. The inhibitory effect of high soil phosphorus on the percentage of root infection was less in doubly-infected roots than in roots infected with *G. macrocarpus* alone. Two hyperparasites, a species of *Phlyctochytrium* and a 'Pythium-like' fungus, were found attacking *G. macrocarpus* at the end of the second season. The hyperparasites were associated with a decline in chylamydospore production in soybean roots growing in soil from the plots. Hyperparasites of endotrophic mycorrhizal fungi are postulated to play an important role in limiting populations of certain of these beneficial fungi.

During the past decade, the effects of vesicular-arbuscular endomycorrhizal fungi on plant growth have received increasing consideration (5). Allegations of the importance of these fungi have been widely accepted, although their relevance under normal agricultural conditions in most cases is difficult to assess experimentally (2) because most soils contain indigenous endomycorrhizal fungi (EMF). To study and measure their effect, soils are commonly treated with heat, chemicals, or radiation to elminate or reduce indigenous EMF populations. Although researchers realize this "treated soil" is no longer natural, its use is necessary to conduct the experiments. The importance of the elimination of competing species of mycorrhizal fungi and other organisms usually is neglected.

Since 1968, our work has been conducted in 1 × 1.5-m plots (6, 7, 8) that were fumigated with methyl bromide, with or without chloropicrin, each season before adding mycorrhizal fungi and planting the soil. During 1974 and 1975, these plots were used to investigate the interrelationships of two species belonging to different genera of EMF on peanut and soybean. This report presents the results of this test, and also implicates the role of hyperparasites in the survival and/or the composition of EMF population densities.



## MATERIALS AND METHODS

The 12 plots used in this experiment were previously described (8). Six plots had dilute acid-extractable P levels of about 150 μg/g soil (ppm) and six had P levels of 60 μg/g soil (ppm). Soil was fumigated in 1974 with methyl bromide at the rate of 80 cc/m$^2$ approximately 6 wk prior to planting plots to peanuts (*Arachis hypogaea* L. 'Florigiant'). In 1975, each plot was divided in half by inserting a sheet of 0.152-mm (6-mil) plastic through the soil to a depth of 90 cm; peanuts were planted in one half and soybeans [*Glycine max* (L.) Merr. 'Davis'] in the other half. Plots were not fumigated in 1975.

Inoculum of *G. gigantea*, initiated from individually picked azygospores, was produced in the greenhouse for 4 mo on soybean roots grown in steamed soil. Soil with about 10,500 azygospores was distributed in a furrow in each of three plots of each P level, and peanuts were seeded above the inoculum.

Previous experiments had shown that soil fumigated with methyl bromide at 80 cc/m$^2$ usually does not totally eradicate *G. macrocarpus* if the fungus occurs at high population levels. Therefore, the initial *G. macrocarpus* population in 1974 was that remaining after fumigation.

Population densities of EMF were determined by spore counts and root infection. Roots were washed from a weighed amount of soil and blotted to a constant dry weight. Soil and water from the root extractions were passed through a 200-μm (80-mesh) screen that retained azygospores; these were counted in appropriate dilutions and expressed as the number per gram of root. Chlamydospores of *G. macrocarpus* were obtained by chopping the weighed roots in a blender for 30 sec and pouring the blend through a 200-μm screen to remove coarse debris; chlamydospores retained on a 53-μm (270-mesh) screen were counted and expressed in the same manner as the azygospores. Chlamydospores free in the soil were not extracted.

Root infection data were obtained by a modified method of Hayman (4). Roots washed from soil samples were cut into pieces approximately 1 cm long; 100 randomly-selected pieces were cleared in 10% KOH for 30 min at 90 C, rinsed with 10% acetic acid, and stained in 0.01% cotton blue in lactophenol for 45 min at 90 C. Stained root segments were placed on microscope slides and examined at ×100. The degree of arbuscule formation (light, medium, or heavy) and presence of vesicles or chlamydospores were recorded for each root piece. Because *G. gigantea* forms neither vesicles nor chlamydospores in roots and *G. macrocarpus* does so, these structures provided a measure of infection by *G. macrocarpus* in doubly-infected roots.

At the end of the 1975 growing season, 15-cm diameter pot cultures of soybean were established in the greenhouse in soil taken from each plot. These cultures provided a source of EMF for studies during the winter.

## RESULTS

**Interaction of mycorrhizal fungi.**—Data from the soil screenings and root assays in November 1974 indicated that, within each P level, *G. macrocarpus* chlamydospore formation was less in plots with *G. gigantea* than it was in plots without *G. gigantea* (Table 1). The number of chlamydospores in peanut roots taken from plots with both EMF was 1/20 that from peanut roots infected with *G. macrocarpus* alone. A similar pattern was found in 1975 with both peanut and soybean although the magnitude of difference was less in 1975 than in 1974. Chlamydospore population densities were less in 1975 than in 1974. Azygospore population densities in soil permeated by peanut roots increased roughly by a factor of five from 1974 to 1975, and were five times higher from soil planted to soybean than from soil planted to peanut. As previously reported (6), fewer spores were formed with high than with low soil P.

Root infection data from samples taken in August 1975 indicate that more roots became infected when both endophytes were present than when *G. macrocarpus* was

TABLE 1. Number of chlamydospores of *Glomus macrocarpus* var. *geosporus* recovered from soybean or peanut roots and azygospores of *Gigaspora gigantea* recovered from soil from plots infested with either *G. macrocarpus* var. *geosporus* or the latter plus *G. gigantea*[a]

| | | Chlamydospores per gram root | | | Azygospores per gram of root | | |
|---|---|---|---|---|---|---|---|
| | | Peanut | | Soybean | Peanut | | Soybean |
| Inoculum | Phosphorus level | 1974 | 1975 | 1975 | 1974 | 1975 | 1975 |
| *Glomus* | Low | 2,530 | 785 | 590 | ... | ... | ... |
| *Glomus + Gigaspora* | Low | 120 | 360 | 300 | 93 | 520 | 2,820 |
| | | *[b] | N.S. | N.S. | | | |
| *Glomus* | High | 1,350 | 210 | 270 | ... | ... | ... |
| *Glomus + Gigaspora* | High | 50 | 10 | 20 | 33 | 150 | 980 |
| | | ** | ** | ** | N.S. | N.S. | ** |

[a] Samples taken in November 1974 and September 1975.

[b] N. S., *, and ** refer to whether differences between treatments above are nonsignificant, or significant at $P = 0.05$ or 0.01, respectively.

TABLE 2. Effect of *Gigaspora gigantea* infection of peanut and soybean roots on the percentage of roots infected by *Glomus macrocarpus* var. *geosporus* at two soil phosphorus levels[a]

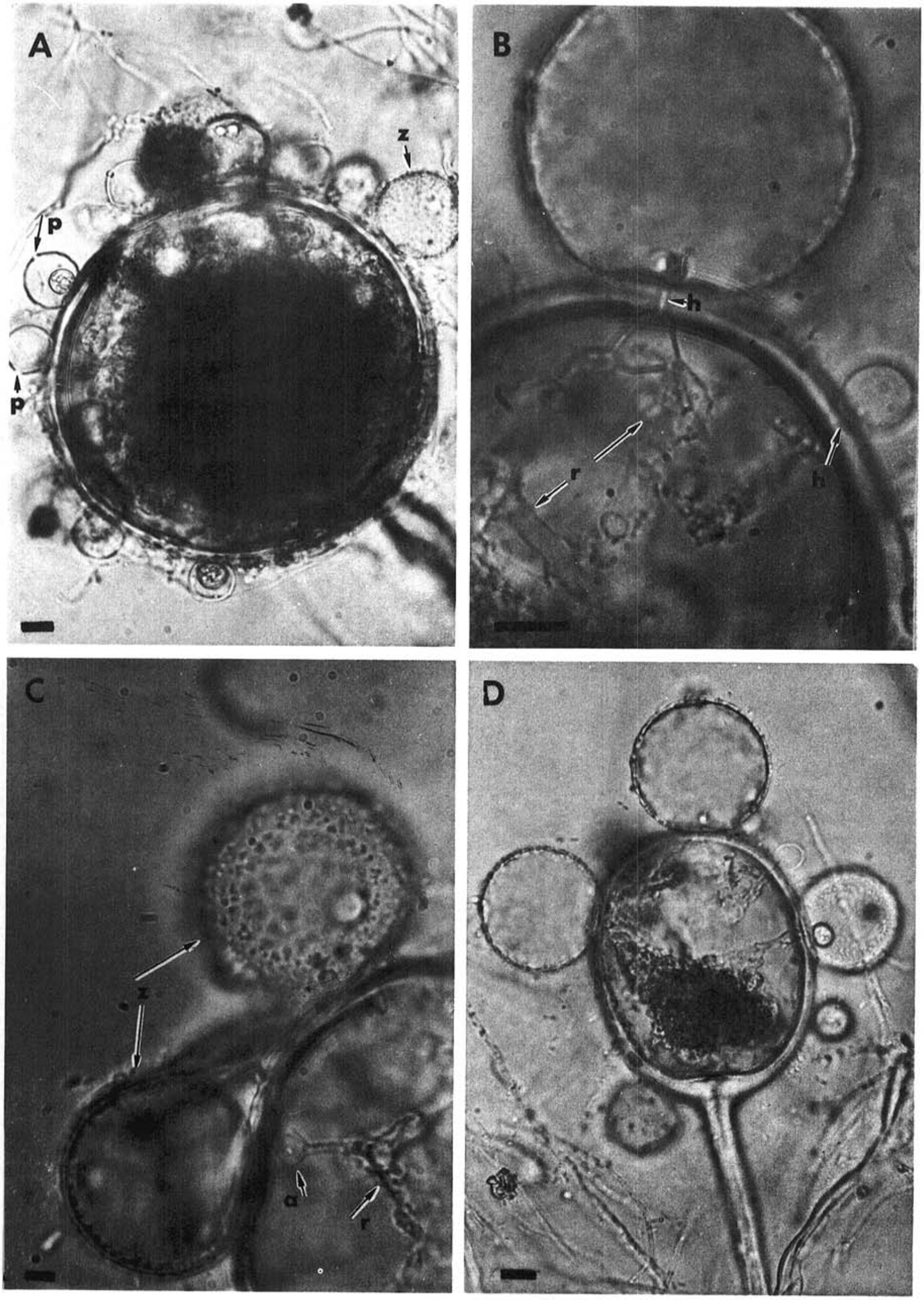
| | | Peanut | | Soybean | |
|---|---|---|---|---|---|
| Inoculum | Phosphorus level | Percentage of roots infected[b] | Percentage of roots with vesicles[c] | Percentage of roots infected | Percentage of roots with vesicles[c] |
| *Glomus* | Low | 79 | 36 | 80 | 34 |
| *Glomus + Gigaspora* | Low | 97 | 17 | 98 | 28 |
| | | *[d] | N. S. | ** | N. S. |
| *Glomus* | High | 58 | 31 | 64 | 34 |
| *Glomus + Gigaspora* | High | 92 | 18 | 94 | 19 |
| | | ** | N. S. | ** | * |

[a] Soil samples taken 8/22/75; data are avg. of three replications.

[b] Based on root pieces with arbuscules or hyphae.

[c] Indicates infection by *Glomus*.

[d] N. S., *, and ** refer to whether differences between treatments above are nonsignificant, or significant at $P = 0.05$ or 0.01, respectively.


A
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present alone, and that the percentage of root pieces with vesicles (indicating *G. macrocarpus* infection) was reduced when *G. gigantea* was present (Table 2). This was more noticeable with peanut (approximately 50%) than with soybean (30%). Higher soil P levels did not reduce vesicle formation (Table 2) as much as spore formation (Table 1). The effect of soil P levels on root infection was greater in soil infested with *G. macrocarpus* alone than in soil infested with both EMF.

**Hyperparasites of mycorrhizal fungi.**—Two fungi were found parasitizing chlamydospores of *G. macrocarpus* obtained from soybean roots grown in pots filled with plot soil at the end of the 1975 growing season. The development of one parasite, a species of *Phlyctochytrium*, was studied on chlamydospores extracted from roots. Chlamydospores were incubated in moist, washed quartz sand in size 00 Beem capsules, each with a 2-mm diameter opening made in the narrow end to allow drainage. After 7-14 days at approximately 24 C, chlamydospores were washed from the sand, decanted, and examined with a dissecting microscope at ×45. Infected chlamydospores were often less than 100 μm in diameter and frequently were covered with sporangia and resting spores of the chytrid (Fig. 1-A). Zoosporangia were spherical, 27-60 μm in diameter, and contained two-to-four inoperculate exit pores, their walls were minutely echinulate and 0.6-μm thick (Fig. 1-A). Formation of small, new sporangia seemed to occur by internal proliferation of sporangia. Resting spores were spherical, 14-23 μm in diameter, orange, and smooth-walled. Their germination was not observed. Zoospores released from sporangia were grooved, possessed one posterior whiplash-type flagellum, and rounded up (3, 6 μm) when coming to rest. Their germination was not observed. Some motile zoospores appeared to be fused in pairs and were misshapen. Occasionally spores believed to be encysted zoospores were found attached to chlamydospore walls.

A hypha penetrated through the chlamydospore wall and the apophysis usually was located just inside (Fig. 1-B). The apophyses varied in shape and size (approximately 7.5 μm in diameter for zoosporangia apophyses) and gave rise to rhizoidal hyphae within the chlamydospore (Fig. 1-C, D).

Infection experiments were performed with chlamydospores screened from chopped soybean roots obtained from the plots after the 1975 season. Spores were removed from debris by decanting and wet sieving, and were picked up with suction. Groups of 10-50 spores were washed into No. 00 Beem capsules about half full of moist, washed, quartz sand. The capsules then were filled with additional sand and incubated at 30 C. At 5- to 7-day intervals the sand and spores were washed out and spores decanted into a small dish and examined with a dissecting microscope for formation of sporangia of the chytrid. After 11 days, some of the chlamydospores were found to bear sporangia and resting spores. Presumably chlamydospores already infected when extracted provided the inoculum for new infections.

In another infection experiment, chlamydospores were recovered from a greenhouse culture of *G. macrocarpus* initiated from a single chlamydospore previously germinated aseptically on agar (to maximize the chances of being free from infection). These chlamydospores were picked out of the root debris and placed in sand in Beem capsules as described above. For inoculum, a chlamydospore bearing sporangi and resting spores of the chytrid were placed in the sand. Many chlamydospores became infected with the chytrid after 11 days.

An unidentified Phycomycete resembling a species of *Pythium* also was found in structures of *G. macrocarpus* within and outside of the soybean root. This "Pythium-like" fungus was isolated from a chlamydospore extracted from soybean roots (Fig. 2-A). Infected chlamydospores resembled healthy spores except that they tended to be smaller, and the contents consisted of rather uniform-sized spores of the parasite instead of the usual oil droplets characteristic of healthy chlamydospore cytoplasm (Fig. 2-A, B). When an infected chlamydospore was ruptured and incubated in a drop of water, spores of the parasite germinated en masse (Fig. 2-C). To isolate the parasite, an infected chlamydospore was rinsed in six changes of sterile distilled water and crushed with a sterile needle. Spherical spores (15μm in diameter) of the parasite were transferred onto the surface of 2% water agar in a petri plate where they readily germinated. To obtain pure cultures of the parasite, hyphal tips were transferred to corn meal agar containing 40 and 100 μg/ml of chlortetracycline and chloramphenicol, respectively, to produce a pure culture of the parasite. Growth of the parasite was inhibited on acidified media.

Neither zoosporangia nor sexual structures of this hyperparasite were ever seen, although it was grown at various temperatures on a variety of media (hempseed extract agar, hempseed halves in water, infested pieces of grass in pond water, snakeskin, and various agar media). The only spores observed were slightly pyriform to spherical structures 15 (12-20) μm in diameter, with 0.7-μm thick walls (Fig. 2-D, E, F). In certain instances the spores resembled oospores, but neither antheridia, oogonia, nor oospores were discerned. In older cultures, hyphae sometimes appeared vacuolate and spores turned light yellow; they were borne terminally or intercalarly and the proximal part of the stalk sometimes was walled off as part of the spore (Fig. 2-E). In certain media, hyphae were knobby or bulbous in places, and at times spores were formed on moniliform hyphae (Fig. 2-G, H, I). Spores were copiously produced on corn meal agar

**Fig. 1-(A to D).** Infection of chlamydospores of *Glomus macrocarpus* var. *geosporus* by *Phlyctochytrium* sp. Scale bar = 10 μm. **A)** Large empty zoosporangia (z) have discharged their zoospores; smaller structures are resting spores. Zoosporangium (z) shows finely echinulate wall; exit pores (p) are visible on some resting spores. Elements B, C, and D show thalli of *Phlyctochytrium* in *G. macrocarpus* chlamydospores with minimal contents showing epibiotic rudiments of sporangia, endobiotic apophyses (a), and a branched rhizoidal system (r). **B)** Penetration hyphae (h) of zoosporangium and resting spore through chlamydospore wall. **C)** Apophysis and reticulations of sporangium. **D)** Entire chlamydospore showing thalli of *Phlyctochytrium*.

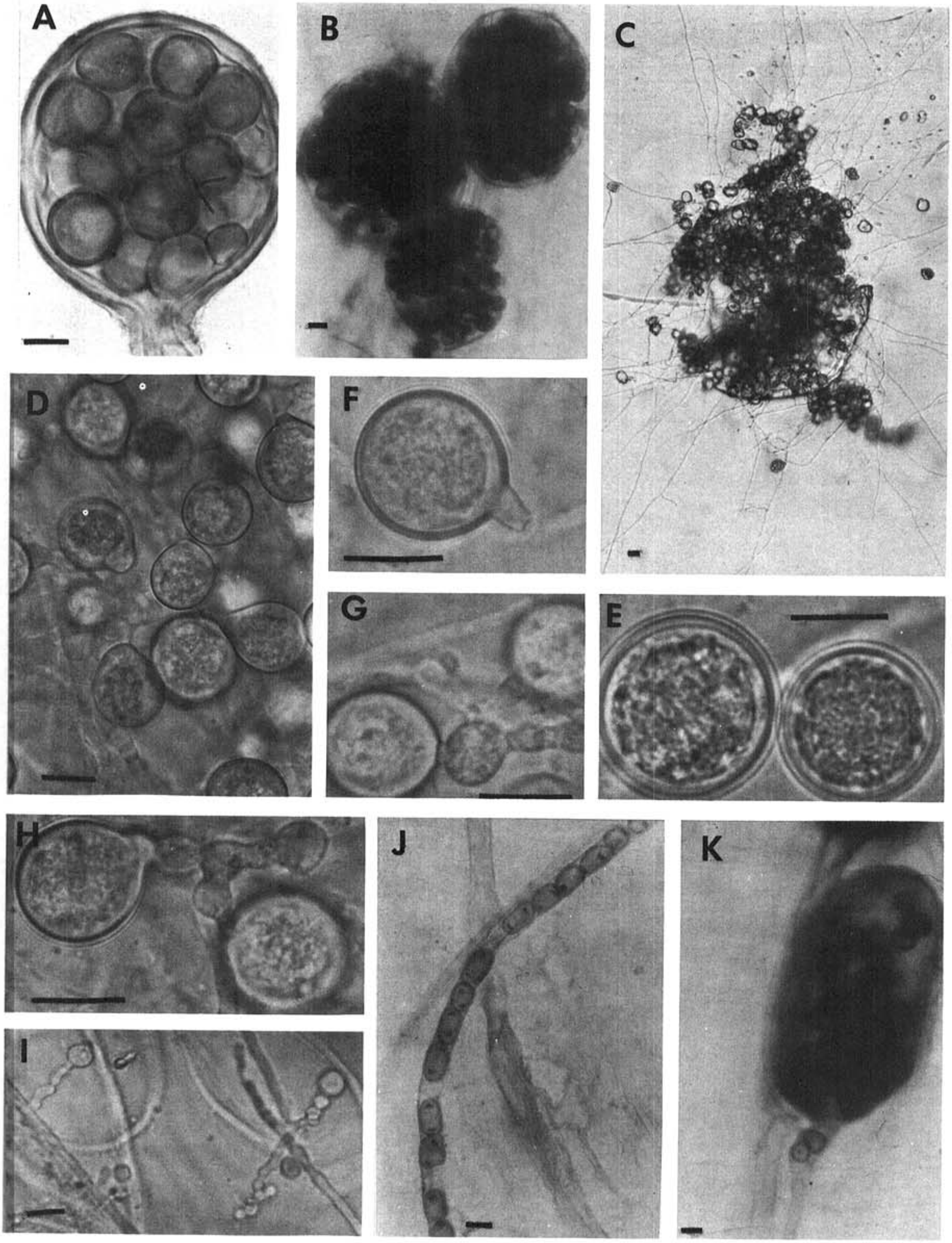


**Fig. 2-(A to K).** Structures of a "Pythium-like" fungus hyperparasitic on *Glomus macrocarpus* var. *geosporus*. Scale bar = 10 $\mu$m. **A, B)** Chlamydospores of *Glomus* filled with spores of the hyperparasite. **C)** Broken chlamydospore of *Glomus* and germination of the mass of the spores of the hyperparasite after 2 day's incubation in water. **D)** Spores of the hyperparasite growing on sterilized snakeskin showing various spore shapes. **E)** Spores of the hyperparasite with thickened walls after 2 wk of growth on hempseed extract agar. **F)** Spore with stalk from hyperparasite grown on corn meal agar. **G, H, I)** Production of spores on knobby or moniliform-like hyphae. Formation of spores within hyphae (**J**), and vesicle and stalk (**K**) of *G. macrocarpus* within soybean root tissue.

and optimum growth occurred at 28 C; on a variety of agar media, growth was most rapid on corn meal and slowest on potato dextrose. Growth on agar media tended to be submerged and at times growing hyphae coalesced in strands.

Inoculations with this hyperparasite were made in several ways. Small pieces of agar containing the fungus were placed near germinated, surface-disinfected chlamydospores of *G. macrocarpus* and azygospores of *G. gigantea* growing on water agar. After 3 days, the hyperparasite had not penetrated hyphae of the latter but had produced coils around them. Several weeks later, hyphae and spores of the hyperparasite were found in mycelium of both EMF stained with cotton blue.

In another inoculation test, 2-wk-old corn meal agar cultures of the parasite were chopped and added to methyl bromide-fumigated greenhouse soil with and without *G. macrocarpus*. Soybean seed were planted and after 1 mo root samples were taken and stained. The percentage of roots infected with *G. macrocarpus* was not reduced by the hyperparasite. Six wk later, hyphae, vesicles, and spores of *G. macrocarpus* in root tissue contained spores of the hyperparasite (Fig. 2-J, K; 3-A). Observations of stained roots from soybeans grown in soil infested with the hyperparasite alone revealed hyphae and spores of the latter in cortical cells near the primary xylem (Fig. 3-B, C). Hyperparasite spores that formed in *G. macrocarpus* hyphae or in soybean root tissue often were elongated and rounded on the ends rather than spherical as those formed in artificial culture. However, spores formed in larger structures (chlamydospores or vesicles) tended to be spherical and had dimensions similar to those formed on artificial media.

## DISCUSSION

At least three factors may have influenced the population of EMF in the plots: (i) soil P levels (1, 6); (ii) antagonism or competition between mycorrhizal fungi within root tissue; and (iii) hyperparasites of EMF. Comparisons involving the latter two factors should be limited to those plots of similar soil P levels because high P levels reduce mycorrhizal development.

Since *G. macrocarpus* and *G. gigantea* both parasitize similar host root tissue in a similar fashion (arbuscules are indistinguishable), competition between the two fungi may affect their population densities. The low *G. macrocarpus* chlamydospore population densities found in plots in 1974 infested with both EMF compared to those in plots with only *G. macrocarpus* may reflect an inability of the latter to compete with *G. gigantea*. The slow development of *G. gigantea* during 1974, as reflected by azygospore numbers, and its increase during 1975, coincided with an increase and decline in *G. macrocarpus* chlamydospore levels, respectively.

If *G. macrocarpus* is more susceptible to hyperparasitism than *G. gigantea*, and original hyperparasite populations were low (following fumigation in 1974), this could explain the high initial population buildup of *G. macrocarpus*. Similar high EMF buildup was observed in previous work when soybeans were grown in fumigated soil to which *G. macrocarpus* was added (6, 8). The resulting spore populations were much greater than those under field conditions and may reflect negligible hyperparasite activity. Increases in hyperparasitism of *G. macrocarpus* during 1975 could account for the decrease in its population. The increase in the *G. gigantea* population in 1975 suggests that it is not as susceptible as *G. macrocarpus* to these hyperparasites. Since both *Phlyctochytrium* and the "Pythium-like" fungus were found in spores of *G. macrocarpus*, but not in *G. gigantea* spores, the latter may not be affected as much as *G. macrocarpus* by the hyperparasites. The invasion of *G. gigantea* hyphae by the "Pythium-like" fungus on petri plate cultures, however, indicates that it is not immune.

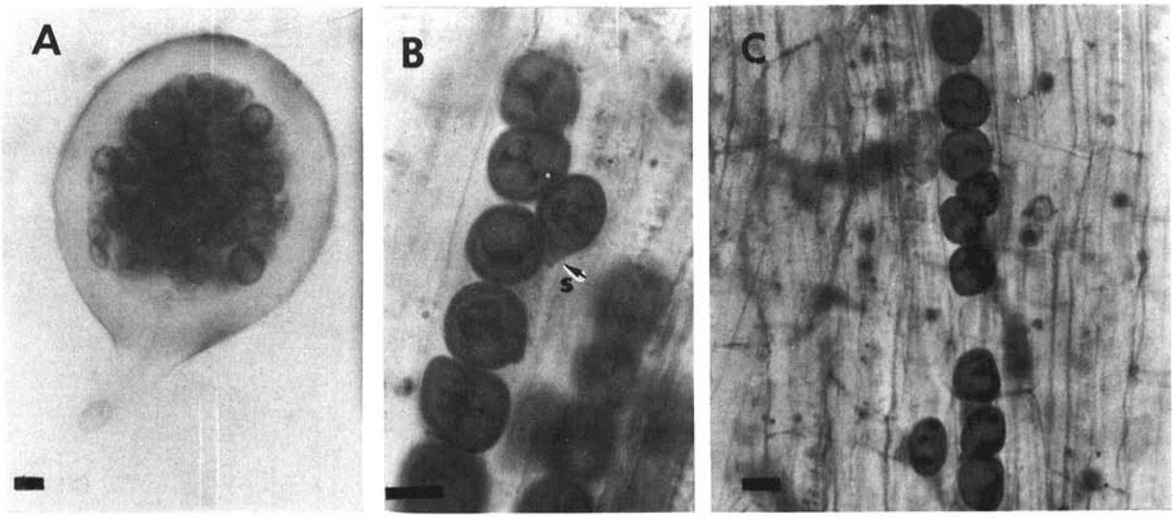


**Fig. 3-(A to C).** Spores of "Pythium-like" hyperparasite of *Glomus macrocarpus* var. *geosporus*. Scale bar = 10 μm. **A)** Chlamydospore of *G. macrocarpus* var. *geosporus* containing spores of hyperparasite in stalk and within spore cavity. **(B, C)** Formation of hyperparasite spores in soybean root tissue free of *G. macrocarpus* var. *geosporus*. **B)** A spore(s) of hyperparasite with stalk similar to those found in artificial culture. **C)** A cluster of variously-shaped spores.

Further evidence of the role of hyperparasites was obtained when soybeans were grown in the greenhouse in soil from the plots after the 1975 growing season. After 4 mo, the number of chlamydospores that developed in roots declined to near undetectable levels, although roots were infected by *G. macrocarpus*. *Phlyctochytrium* sporangia developed on some of the chlamydospores extracted from roots after incubation of the spores in vials of sand; hence, the parasite affects chlamydospore development. This could impair survival of *G. macrocarpus* in the absence of a host.

We have not yet determined when chlamydospores become infected with *Phlyctochytrium*. The chlamydospore infection experiments conducted in vials showed that free chlamydospores can be infected outside root tissue, presumably by zoospores. However, development of this hyperparasite on chlamydospores released from roots by the blending process may indicate that chlamydospores also become infected within or on root tissue.

The parasitism of soybean roots by the "Pythium-like" hyperparasite in the absence of EMF is similar to that of *Pythium acanthicum* reported to parasitize a wide range of fungi as well as higher plant roots (3). Whether the "Pythium-like" fungus exists merely as a resident in the root or has some pathogenic capabilities is not known since infected roots appeared to be normal. Its infection of *G. macrocarpus* hyphae and vesicles is detrimental to the mycorrhizal fungus, and chlamydospores are destroyed.

The two parasites described herein provide two examples of mechanisms of hyperparasitism of EMF: (i) attack within host tissue (the "Pythium-like" fungus); and (ii) parasitism of spores in the soil (*Phlyctochytrium*).

Hyperparasites of EMF may affect both the population density of a mycorrhizal fungus species and the physiological functions of the mycorrhiza. Recognition of such hyperparasites presents new information for an understanding of endomycorrhizal relationships. Soil treated to eliminate mycorrhizal fungi almost certainly is devoid of or has minimal hyperparasite populations. Interpretations of experiments dealing with EMF such as spore population densities, root infection, or physiological functions should be tempered by considerations that apparent treatment effects could be influenced by hyperparasite activity.

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