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HYPERPARASITES OF OOSPORES OF PHYTOPHTHORA MEGASPERMA VAR. SOJAE:
HOST RANGE, ENVIRONMENTAL PARAMETERS, AND BIOLOGICAL CONTROL

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HYPERPARASITES OF OOSPORES OF PHYTOPHTHORA MEGASPERMA VAR. SOJAE: HOST RANGE, ENVIRONMENTAL PARAMETERS, AND BIOLOGICAL CONTROL

by

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ABSTRACT

HYPERPARASITES OF OOSPORES OF *PHYTOPHTHORA MEGASPERMA* VAR. *SOJAE*: HOST RANGE, ENVIRONMENTAL PARAMETERS, AND BIOLOGICAL CONTROL

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Oospore hyperparasites were investigated as potential biocontrol agents of *Phytophthora* root rot of soybean. *Hyphochytrium catenoides*, *Pythium monospermum* and *Humicola fuscoatra* applied to soil 7 days before seeding reduced root rot severity by 38-66% in natural soil in various laboratory experiments.

Seed treatments with *Actinoplanes missouriensis* or *H. fuscoatra* increased plant stand 30-80% in soil artificially infested with *Phytophthora megasperma* var. *sojae* (*Pms*) in greenhouse tests.

Disease incidence in field plots was very low during three consecutive years, and biological control could not be adequately assessed. A soybean seedling assay of soil taken from treated rows, showed no reduction in disease incidence in soil to which *A. missouriensis* or *H. fuscoatra* were applied prior to planting in the spring, or to which *A. missouriensis*, *H. fuscoatra*, or *H. catenoides* were applied at seeding.

The host range of the *Pms* oospore parasites *A. missouriensis*, *Diheterospora chlamydosporia*, *H. catenoides*, and *P. monospermum* was extended to include *Aphanomyces cochlioides*, *A. euteiches*, *P. citrophthora*, *Pythium aphanidermatum*, and *P. ultimum*, except that *H. catenoides* did not infection *P. ultimum* oospores. *Pms* oospores were parasitized by two other *Actinoplanes* species, *A. philippinensis* and *A. utahensis*; *Amorphosporangium auranticolor*, *Ampullariella regularis*, and

Spirillospora albida, all members of the Actinoplanacae; *Micromonospora* sp. of the Micromonosporaceae. *Pms* oospores of races 1, 3, 4, and 7 were equally susceptible (>95%) to infection by *A. missouriensis* and *H. fuscoatra*. The age of host and parasite cultures had no effect on oospore infection by *A. missouriensis* and *H. fuscoatra*.

Using transmission electron microscopy, hyphae of *A. missouriensis* and *H. fuscoatra* were observed to directly penetrate the oogonial and oospore walls, and infect the oospore cytoplasm of *Pms* within 4 days. No specialized infection structures were formed by either hyperparasite, and no ultrastructural change in response to infection was observed in the host.

Conditions highly conducive to parasitism of *Pms* oospores in autoclaved soil by *A. missouriensis* were 0 bars matric potential, soil temperature 15-30 C, and soil pH at 5.5 or between 6.6 and 8.0. Optimum conditions for parasitism of *Pms* oospores by *H. fuscoatra* were -0.3 bars matric potential, soil temperatures of 20-30 C, and soil pH between 6.6 and 8.0.

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INTRODUCTION

Soybean root rot caused by Phytophthora megasperma Drechs. var. sojae Hildb., is the most important disease of soybeans in Michigan. Due to the ability of the pathogen to form races that will infect once resistant varieties, breeding programs may offer only a partial solution of control. Chemical control has not been practiced because it is expenseive, ineffective, and potentially hazardous. Biological control may offer a third alternative to the perplexing battle of controlling this pathogen.

Parasites of P. megasperma var. sojae oospores were discovered in studies of oospore germination in soil (187). In further studies, Humble and Lockwood (118) demonstrated the presence of oospore hyperparasites in each of 15 field soils tested. However, there was no relationship between the frequency of hyperparasitism and disease potential as determined by a seedling assay of soils from the various fields. When hyperparasites were applied to soil naturally infested with P. megasperma var. sojae, biocontrol was inconsistent, but Hyphochytrium catenoides caused significant disease reduction in two out of three experiments (117).

Evaluation of biocontrol potential of P. megasperma var. sojae by hyperparasites was the main objective of this study. This assessment was made in several ways. The efficacy of the oospore hyperparasites identified by Sneh et al. (187) was determined in laboratory experiments.

Host range studies using the parasites and plant pathogenic Oomycetes other than *P. megasperma* var. *sojae* were undertaken to determine the extent of hyperparasitic ability. Optimal environmental conditions for hyperparasitic activity were studied. Greenhouse and field tests to control *Phytophthora* root rot were conducted in artificially and naturally infested soils.

LITERATURE REVIEW

Parasitism of one microorganism by another has been documented as early as the late nineteenth century by workers such as deBary in Germany (67), Bainier in France (17) and Thaxter in America (194). Numerous reports of mycoparasitism have since been made, and several excellent reviews have collated most of this material (24, 25, 26, 28, 40, 71, 169). The parasitism by one microbe on another parasite is known as hyperparasitism, while the parasitism of one fungus by another is termed mycoparasitism (5). Lacking within the current usage of hyper- and mycoparasitism is a term describing parasitism of a saprophytic microorganism by another microorganism. Mycoparasitism and hyperparasitism have been used almost interchangeably to describe mycoparasitic interactions. In this review, the term hyperparasites will be frequently used since parasites of plant pathogens will be emphasized. Mycoparasitism is not entirely appropriate here because not all parasites of plant pathogens are fungi.

Hyperparasitism can be subdivided into two groups based on the mode of parasitism. If the parasite kills the host cells prior to utilization of host nutrients, the relationship is termed <u>necrotrophic</u> (24, 40). If the parasite obtains nutrients from the living host cell, the relationship is <u>biotrophic</u>. Further interpretation of these categories is dependent upon the researcher. Boosalis (40) subdivided the biotrophic group into those parasites whic are more destructive and kill the host, and those parasites which are balanced and can obtain nutrients from the living host causing little or no harm. Barnett (24), on the other hand, used the term biotrophic parasite interchangeably with balanced parasite, and necrotrophic interchangeably with destructive parasite. Barnett and Binder (28) subdivided biotrophic parasitism to include three types of parasitism: 1) internal, parasites developing within the host cell, 2) contact, parasitism occurring without invasion of host cells, and 3) haustorial, parasites forming haustoria within host cells. Because there are examples of internal, contact, and haustorial parasites that will kill the host cell [e.g., numerous internal oospore parasites (188), hyphal interference (120), and haustorial parasites such as Rhizidium richmondense (221) and Rhizidiomycopsis stomatosa (190) in which the rhizoidal system acts as the absorptive apparatus], it is deemed necessary to retain Boosalis' interpretation of biotropic parasitism to include both destructive and balanced parasites. Examples of internal, contact, and haustorial parasites may then be categorized for both destructive and balanced parasites. One might argue that classification of hyperparasitic relations is unwarranted until a nutritive exchange between host and parasite is proven experimentally. Terminology may need to be refined as information becomes available on host-parasite physiology.

Studies of hyperparasitism have had two major emphases: i) to understand basic anatomical and physiological processes of the parasitic relationship, and ii) to utilize the parasitic relationship to reduce natural populations of the host organism. Biological control has long been observed in nature by plant pathologists and has been held responsible for keeping powdery mildew and rust diseases in check during certain years, as will be discussed later. It is probable that biological control via hyperparasitic phenomena is already responsible for limiting populations of plant pathogens. Recently, there are increased efforts to introduce natural parasites of economically threatening plant pathogens into agronomic cropping systems. Accordingly, this literature review will emphasize the biological control potential of different hyperparasites.

Parasitism of microorganisms causing rots of fruits and vegetables

Members of the Mucorales are important economically because of their role in blossom blights and storage decay. These plant pathogens are susceptible to parasites that are members of the Mucorales. The literature dealing with hyperparasitic Mucorales is extensive, and much of it has been reviewed by Benjamin (33).

Choanephora cucurbitarum, the causal agent of blossom end rot of vegetables in the southern and eastern United States, is susceptible to attack by *Piptocephalis unispora* (125) and *P. virginiana* (34). Other hosts are *Rhizopus nigricans* which causes soft rot of vegetables, particularly sweet potatoes and fruits, and *Mucor* spp. which can cause decay of fruits. Most *Piptocephalis* spp. are limited in host range to the Mucorales except, however, *P. xenophila* is known to attack some species of *Penicillium*, *Aspergillus niger*, *A. repens*, *Cephalosporium* sp. and *Venturia inaequalis* (72).

On agar culture media, germ tubes of *P. virginiana* were attracted to *C. cucurbitarum* hyphae; upon contact, an appressorium developed on the host hypha (35). Penetration was accomplished by an infection peg and was thought to be largely due to mechanical forces (156). An haustorium then developed within the host hypha. A compatible interaction was established between *P. virginiana* and *C. cucurbitarum* only when the host hyphae were young (1-day-old) (155). The host cell wall was one layer in thickness after 24 h, but was composed of two layers by the time the hyphae were 7 days of age and resistant to attack. In the incompatible reaction, the hyperparasite dissolved the outer wall layer of the host. The inner layer became thickened and a papilla was developed by the host which limited the development of the hyperparasite (53). Haustoria of *P. virginiana* were excluded from host cytoplasm by a sheath in the early stages of penetration. Host origin of the sheath was demonstrated with autoradiography (157), and by the use of an enzyme inhibitor (158).

In the compatible interaction, no papilla formation was detected and sheath formation was delayed (153). Direct contact of *P. virginiana* was made with the protoplast of *C. cucurbitarum* (155). Cisternae of the endoplasmic reticulum surrounded the young parasitic haustorium, the disappearance of which appeared to coincide with sheath development. Lipid-containing sphaerosome-like bodies were found in the host and parasite (156).

A comparison was made between axenic cultures of *P. virginiana* and cultures of the parasite with its host (151). *Piptocephalis virginiana* developed to a very limited extent in sterile culture, and the few spores that were produced would not germinate in the absence of a host (143). Comparisons between spores produced with and without the host revealed the absence of lipid bodies in the axenically-cultured spores. Analyses using thin-layer and gas-liquid chromatography demonstrated the presence of the fatty acid, γ-linolenic acid, in spores produced in mixed culture but not in those produced in sterile culture (151). Further investigation by Manocha and Deven (154) revealed that environmental conditions including media composition, temperature and light conditions, and age of the culture affected the amount of γ-linolenic acid detected in C. cucurbitarum which in turn determined the degree and rate of parasitism by P. virginiana (152). Gamma linolenic acid is a major constituent of the phospholipid, diphosphatidylglycerol, which was also lacking in axenically cultured spores. Evidence was presented to suggest that diphosphatidylglycerol was closely associated with the mitochondrial system of yeasts (123); therefore, its absence may explain the poor growth of P. virginiana in the absence of a host (152).

Other workers also have shown that the medium composition (34, 38) and incubation temperature (34) will affect hyperparasitism. Jeffries and Young (124) investigated parasitism of the saprophyte Cokeromyces recurvatus by P. unispora. The same events occur in this relationship as just described for C. cucurbitarum and P. virginiana, although the role of γ-linolenic acid has not yet been established.

Piptocephalis virginiana is a biotrophic, balanced, haustorial parasite causing little if any damage to its hosts (35). Therefore, it does not appear to have biological control potential.

Dispira cornuta, a plant parasite itself (9), will infect C. cucurbitarum, Mucor spp., and Rhizopus nigricans (10). Unlike Pitocephalis, D. cornuta may be cultured axenically on an egg medium and still retain the ability to parasitize the Mucorales (19). Dispira cornuta has demonstrated a growth requirement for high levels of thiamine, biotin, and nitrogen (22). The parasitic ability of D. cornuta was altered when a host was grown on a culture medium with different carbon and nitrogen sources (10, 140). When near a suitable host, germinating spores of *D. cornuta* exhibited positive tropism (10). Penetration followed by haustorial development within the host occurred only if host hyphae were young. *Dispira cornuta* is a biotrophic, balanced, haustorial parasite, and probably has no value as a biological control agent.

Syncephalis spp. have been noted as parasites of *Choanephora*, *Mucor*, and *Rhizopus*. *Syncephalis* is a biotrophic, haustorial parasite as are *Piptocephalis* and *Dispira*. Some *Syncephalis* spp. may suppress host sporulation. Bainier (17) described the sporangial development of *S. curvata* on a hypha of *R. nigricans*. *Syncephalis sphaerica* was found to parasitize *M. mucedo* among other fungi, but did not appear to affect its host appreciably (20). Of the Mucorales tested, *Rhizopus* spp. were the most susceptible to infection by *S. californica* (119).

No evidence of tropism of germinating spores of *S. californica* has been observed (119). A single or multiple lobed appressorium, several times the diameter of vegetative hyphae, encircled the host hypha after chance contact, and an infection hyphae penetrated the hyphal wall. Localized swellings of the host near the appressorium were observed after infection. The size of the swellings was inversely proportional to their number. Once through the wall, the hyphae branched sparsely. Eventually the host cytoplasm disappeared to reveal the parasitic hyphae tightly packed in the swollen portions of the host. Some of these hyphae emerged from the host, and penetrated other portions of the host thallus. Although the host was not killed, infection by *S. californica* reduced sporulation of *R. oryzae* in agar culture. Ellis (85) was able to grow *Syncephalis* spp. on liver agar. In vitro, pH optima ranged from 7 to 8, and temperature optima from 18-22 C.

Biological control of *Rhizopus* decay may be possible using *S. californica*, but further work is needed to determine if the parasite is destructive enough to prevent mycelial spread of the pathogen. Cold storage is the major treatment presently used to prevent losses by *Rhizopus*.

Chaetocladium and *Parasitella* cause swellings or galls in their hosts much as *Syncephalis* does. Both parasites will infect *Choanephora*, *Mucor*, and *Rhizopus* (43). The similarity of the galls to the progametangia of the Zygomycetes caused Burgeff (44) to suggest that an imperfect sex reaction between host and parasite had initiated the development of the parasitic relationship. He presented evidence that the (+) strain of *Parasitella* would parasitize only a (-) strain of the host and vice versa. Satina and Blakeslee (180) tested a large number of host strains and concluded that the mating sex type was not entirely responsible for attraction between host and parasite, although there did seem to be a parasitic preference for opposite sex strains. The hyperparasite *Chaetocladium* is itself susceptible to attack by *Dispira simplex* (41) and *Piptocephalis* (209).

Other hyperparasites of the Mucorales include *Ampelomyces quisqualis* on *M. mucedo* (146) and *Fusarium lini* on *M. racemosus* (8). *Trichoderma* spp. have been reported to penetrate hyphae of *Rhizopus oryzae* (80, 81).

The chytrid, *Rhizophydium mycetophagum*, was described as a hyperparasite of the conidiophores and hyphae of an unidentified species of *Choanephora* (130). It produced numerous epibiotic sporangia on the host cell. The zoospores were released, then encysted on the host hyphae, penetrated, and formed a delicate but highly branched rhizoidal system within the host cell. The host protoplasm degenerated by the time the parasitic sporangia released their zoospores.

Another organism causing cucumber fruit decay, Rhizoctonia solani, and its hyperparasite, Laetisaria arvalis, will be discussed later with Rhizoctonia diseases.

Parasitism of organisms infecting aerial plant parts

Peronosporaceae. While the Oomycetes are generally known for causing root diseases, one family, the Peronosporaceae, contains a highly specialized group of obligate parasites causing downy mildews. Phlyctochytrium punctatum has been observed infecting the oospores of Peronospora tabacina, the cause of blue mold of tobacco (136). Sporangia formed epibiotically on the oospores. An apophysis developed within the oospore as the result of swelling of the base of the rhizoidal axis. The rhizoidal system was lobed or irregular. Oospores of Sclerospora sorghii (the causal agent of downy mildew of corn and sorghum) were attacked by an unidentified Phylyctochytrium sp. (133). Parasitism occurred as described for P. punctatum, although apophyses in the oospores were not consistently formed. Healthy and heat-killed oospores were attacked equally. The hyperparasite was cultured on agar and in liquid media, and survived storage on agar at 6 C for 28 months. Optimum temperature range for growth was 23-27 C. This hyperparasite was considered to have a high potential as a biological control agent.

Another chytrid, Rhizophidium pollinis, parasitized oospores of S. graminicola, which causes downy mildew of grasses and millet (159). Hyperparasite sporangia were sessile on the oospore, and a delicate rhizoidal system formed within. Zoospores encysted on contact with an oospore, germinated and penetrated the oospore, and eventually functioned as sporangia. Resting spores were produced inside old sporangia. *Sclerospora graminicola* is also susceptible to attack by the imperfect fungus, *Fusarium semitectum* (175), which invaded the oospores within the infected millet head and rendered them, for the most part, ungerminable. *Fusarium semitectum* was suggested as a potential biological control agent.

Ascomycetes and fungi imperfecti. *Claviceps purpurea* causes a disease of cereals known as ergot. This fungus replaces some of the cereal grain with its own sclerotia. An isolate of *Fusarium roseum* 'Sambucinum' was found to be an effective hyperparasite of *C. purpurea* in both laboratory and field tests (162). In addition to reducing the number of sclerotia formed by *C. purpurea*, *F. roseum* degraded ergotamine into inert compounds which did not cause deleterious effects on rats or rabbits. This *F. roseum* isolate did not cause disease on rye, and preliminary tests did not show that *F. roseum* was itself producing toxic substances. The biological control potential of *F. roseum* is readily apparent due to its ability to infect *C. purpurea*, and to inactivate the toxin produced by the plant pathogen.

Two plant pathogens that infect corn ears, *Fusarium moniliforme* and *Diplodia zeae*, were found to be hosts of the contact biotrophic hyperparasite *Acladium tenellum* (141). *Acladium tenellum* made contact with its host by the formation of specialized contact cells. Nutritional studies have demonstrated the requirement for thiamine and biotin in axenic culture. The destructiveness of *A. tenellum* was not alluded to, but if like other biotrophic contact parasites, it probably does not cause enough damage to be of potential value as a biological control agent.

Helminthosporium spp. cause leaf spots and blights of small grains and corn. *Sphaeronemella helvellae*, a perithicial ascomycete, was found to infect conidia and mycelia of *H. carbonum*, *H. maydis*, and *H. turcicum* (208). Infected conidia of *H. carbonum* were distorted, while mycelia were vacuolated and had a slower growth rate. Biological control experiments were not performed.

Trichoderma lignorum was isolated from soil collected from field plots of barley that had remained healthy despite heavy inoculation with pathogenic *H. sativum* (36). In biological control experiments, plants were less diseased in pots containing both *H. sativum* and *T. lignorum* then in pots containing only *H. sativum*. Parasitism was the mechanism implicated in disease control, but this was not established.

Alternaria diseases are common on many plants, usually occurring when plants are physiologically stressed. *Gonatobotrys simplex* is a biotrophic parasite of *A. tenuis* (217). In culture work on the host-parasite interaction, hyphae of *G. simplex* demonstrated tropic growth towards host spores. The parasite produced short, bulbous branches which contacted the host hyphae. Ultrastructural study of the host-parasite interface showed membrane-lined plasmodesmata connecting the fungi (106). The host wall was thickened. The contact cell of *G. simplex* was delimited from the main hypha by either an open or occluded septal pore. The parasite caused only a slight reduction in growth of its host (217). *Gonatobotrys simplex* would not grow in the absence of a host unless a water extract of the host or an extract of certain other fungi was added to the medium. A growth factor was partially purified from fungus extracts and named mycotrophein. This substance(s) has apparently never been characterized.

Nectria inventa and its *Verticillium* state have been cited as destructive parasites of *Alternaria brassicae* (198, 199, 201). In cultural studies of their interactions, usually the hyperparasite hyphae grew towards the host then grew along side or coiled around the host hyphae forming appressorium-like structures. Degeneration of contacted cells occurred with or without penetration; conidia were penetrated more often than hyphae. Penetration often occurred at septa of hyphae and mature conidia resulting in separation of cells. Because of the formation of large holes in the cell wall and the lack of indentation, invasion was thought to be enzymatic in nature. The host plasma membrane was invaginated beneath the point of contact in both hyphal and conidial cells. The area between the invaginated membrane and the host cell wall was electron transparent and contained electron-dense tubule structures that in later stages appeared as irregular deposits. Septal plugs were formed by the host hyphae delimiting the infected from the healthy area. When conidial cells were penetrated, an enlargement of the parasitic hyphae occurred at each septum. The hyphae became constricted with passage into the adjoining cell. Complete vacuolation of infected host cells eventually occurred. No biological control experiments have been performed to study *Nectria* as a hyperparasite of *Alternaria* in nature, but such attempts would appear promising since both hyphae and conidia are susceptible to hyperparasitic attack.

Hansfordia sp. has been discovered in nature as a hyperparasite of two foliar pathogens, *Cercosporidium personatum* which causes late leaf spot on peanuts and *Fulvia fulvum* (*Cladosporium fulvum*) which causes leaf mold of tomatoes (192, 193). Spores and hyphae of the hosts were penetrated by the parasite. *Cercosporidium personatum* was colonized by

Hansfordia in greenhouse tests. When the fungicides benomyl, maneb, or chlorothalonil were used in the greenhouse, *Hansfordia* sp. was not found on *Fulvia*-infected plants.

Several other examples of hyperparasites of fungi causing leaf spots are *A. tenellum*, a hyperparasite of *Diplocarpon rosae* (141), the causal agent of black spot of rose, and *Fusarium semitectum*, itself a secondary plant parasite, parasitizing three species of *Cercospora* (176). *Fusarium semitectum* coiled around host conidiophores and conidia sending out infection pegs at contact points. Hypertrophy of host cells was frequently observed.

Powdery mildews. Hyperparasitism of the powdery mildews by *Ampelomyces quisqualis* (*Cicinnobolus cesatii*) is perhaps one of the most frequently observed hyperparasitic relations in nature. DeBary in 1870 (87) gave a detailed account of the destructive internal parasitic action. *Ampelomyces quisqualis* invaded the host hyphae directly and quickly ramified through the hyphal cells passing through septa without constriction. Hyperparasite pycnidia commonly formed in conidiophores or ascocarps, replacing ascospores with pycnidiospores. Only mature conidia or ascocarps were not invaded by the parasite. The particular host and stage of development influenced the size and shape of the parasite pycnidium. Eventually the host protoplasm was degraded, and the parasite hyphae became shorter and thicker. *Ampelomyces quisqualis* then lived saprophytically on the dead tissue of the host plant.

Ampelomyces quisqualis has been reported on mildews of the genera *Erysiphe*, *Leveillula*, *Microsphaera*, *Oidium*, *Podosphaera*, *Phyllactinia*, and *Sphaerotheca* (7, 191). Biological control of all these genera except *Microsphaera*, which was not tested in the greenhouse, was obtained when repeated inoculations were made with the hyperparasite at 10-day intervals (191). It has been suggested that *A. quisqualis* has held in check powdery mildew of clover in Indiana (223) and of several herbaceous plants in Iowa (7) during certain years. One reason *A. quisqualis* is not used commercially for powdery mildew control may be because sulfur, an inexpensive chemical control, is readily available. In addition, powdery mildews will infect and cause disease at moisture conditions much lower than *A. quisqualis* may tolerate.

Biotrophic, contact hyperparasitism has been observed between *Tilletiopsis* sp. and the powdery mildew fungus, *Sphaerotheca fuliginea* (110). The hyperparasitic hyphae entwined the hyphae, conidiophores, and conidia of the host. Powdery mildew control was obtained in the greenhouse by spraying a spore suspension of *Tilletiopsis* onto detached cucumber leaves infected with *S. fuliginea*. Development of *S. fuligenea* was prevented when *Tilletiopsis* spores were applied up to 8 days prior to inoculation with *S. fuligenea*.

Rusts. The observations of naturally occurring biological control of the powdery mildews can only be matched in number by accounts of hyperparasitic associations with the rusts. The hyperparasite *Darluca filum* has been isolated from a large number of rust species belonging to the genera *Puccinia*, *Uromyces* (49, 131) and *Peridoermium* (1), and has been reported to attack the pycnial (=spermatial), aecial, uredial, and telial stages of the rust life cycle (1). Infection of the uredial stage has been reported most frequently. *D. filum* entered the host cell by direct penetration (52). A study on the ultrastructural relationship of *Puccinia graminis* and *D. filum* demonstrated the invasion of uredospores but not the sub-basal cells or mycelium of the host. After penetration of the spore walls, electron transparent channels were observed next to the walls of the hyperparasite. A haustorium was not formed. Complete destruction of the spores followed penetration. *Darluca filum* replaces the rust sporocarp with its own pycnidium, and can be recognized easily in nature by the abnormal black appearance of a normally orange-red rust lesions (166). The hyperparasite can be cultured on common laboratory media (32). Growth rates of *D. filum* conidial or mycelial isolates vary with different carbon and nitrogen sources (32, 163, 174). Due to the relatively common occurrence of conidia in nature, it was suggested that the conidial stage be used for nutritional and physiological studies. *Darluca filum* was thought to keep the spread of corn rust in check in Iowa (66).

Tuberculina maxima, like *D. filum*, is a fungus that has long been reported to be closely associated with the rusts on woody plants. Field surveys of several pine rusts have indicated the presence of *T. maxima*, but the hyperparasite's significance in natural rust control has been controversial (134, 160). *Cronartium* spp. and *Uredinopsis mirabilis* are hosts for *T. maxima* (116). The hyperparasite has been thought to directly attack the rust as a hyperparasite, but Wicker and Woo (220) could find no penetration of rust cells, nor could they find any evidence of direct attack upon *Cronartium ribicola*. However, the destruction of rust-infected white pine cells by *T. maxima* was apparent in histological sections. Evidence was presented to indicate that *T. maxima* was parasitic on the rust-parasitized plant cells and not directly on the rust. The implied role of the rust was to weaken the middle lamella which would allow secondary invasion of plant cells by *T. maxima*. *T. maxima* invaded healthy pine tissue in vitro but not in vivo (219), but rust-free pine tissues grown in vitro have poorly developed middle lamellae (220). Wicker and Woo (220) claimed that *D. filum* may act in a similar manner to *T. maxima*, but since that time, an ultrastructural study showed direct penetration of rust cells by *D. filum* (52). An attempt to inoculate naturally-occurring rust cankers with *T. maxima* met with little success (116).

Scytalidium uredinicola has been cited as a destructive hyperparasite of the rusts *Cronartium quercum* f. sp. *fusiforme* (139) and *Endocronartium harknessii* (220). The mode of action of *S. uredinicola* is similar to that of *T. maxima* because direct contact with the rust is not required for the rust to be harmed. Rust hyphae and spores were not penetrated, but rather disintegrated by the action of *S. uredinicola* much like those of *T. maxima*. It would be interesting to know whether the two hyperparasites operate by the same mode of action. *Scytalidium uredinicola* hyphae penetrated the rust-infected galls and were able to destroy rust hyphae within the gall, even below the invaded area, suggesting the involvement of a diffusible compound. Surveys in some areas of Alberta indicated that *S. uredinicola* was associated with greater than 80% of the rust galls (200). *Scytalidium uredinicola* may decrease aeciospore production by *C. quercum* f. sp. *fusiforme* by as much as 72% (138). The fact that the modes of action for *T. maxima* and *S. uredinicola* may involve the inadvertent destruction of the rusts does not diminish their value as biological control agents.

Monocillium nordinii was found as a parasite of *Cronartium coleosporioides* and *Endocronartium harknessii* on pine (197). *Monocillium nordinii* formed appressorium-like structures on the host spores, but penetration did not occur until after the host cells were devoid of most contents. Another hyperparasite, *Fusarium trichothecioides* (*F. bactridioides*), was found occurring on galls produced by *Cronartium* spp. (222). White pine blister rust cankers inoculated with *F. trichothecioides* were dead one year later. The hyperparasite was found sporulating on *C. harknessii* and *C. filamentosum* cankers several months after inoculation.

Rust diseases can be very devastating. Control is usually through resistant varieties, or removal of alternate hosts, although several systemic fungicides have been very effective. Biological control would be very useful if found effective.

Pathogens of woody plant parts. Hyperparasites of canker-producing organisms have been noted. *Gonatorrhodiella highlei*, a biotrophic, contact parasite has been found in close association with *Nectria coccinea*, causal agent of beech bark disease, and *N. cinnabarina*, a fungus responsible for dieback and canker of shade trees (11, 38). In agar culture, the germ tube upon contact with the host formed an appressorium which gave rise to hyphal hooks which wrapped around the host hyphae. A branched hyphal system developed from the appressorium and hook cells, giving rise to new appressoria and hook cells (37, 38). In several instances, Blyth (38) observed reversed parasitism, *Nectria* causing vacuolation of *G. highlei* hyphae. Gain and Barnett (90) isolated the hyperparasite by incubating cultures in the dark, and allowing the hyphae of the hyperparasite to grow from the colony edge of the host then transferring to an agar medium. To obtain an axenic culture of *G. highlei*, it was necessary to add mycotrophein, and a water soluble compound isolated from beech bark (90). Some nitrogen sources at concentrations greater than 42 mg/l caused inhibition of the hyperparasite due to the liberation of NH_3 by the host. Concentrations of thiamine had to be greater than the optimum for host development in order for parasitism to occur. Other plant parasitic hosts of the hyperparasite include N. galligena (11), Graphium sp., and Verticillium albo-atrum (90).

Calcarisporium parasiticum was isolated from a culture of Dothiorella quercina (Physalospora glandicola) which causes twig blight of oak (23). Several other Physalospora spp., Botryosphaeria ribis, Guignardia bidwelli, Diplodia pinea, Coniothyrium sp., and Dothiorella sp. were also hosts of C. parasiticum (29). Calcarisporium parasiticum is a biotrophic, contact mycoparasite which forms buffer or contact cells at the host-parasite interface. Unlike the positive tropic growth exhibited by some of the other contact hyperparasites, in this case the host hyphae were attracted to the spores of C. parasiticum. The host either produced lateral branches, or occasionally the main hypha would turn and grow to the parasite. After contact, a septum was formed in the germ tube of C. parasiticum (105). The septal pore was eventually plugged, leaving mitochondria, endoplasmic reticula, and vesicles contained in the contact cell. A pore was dissolved in the host wall and cytoplasmic exchange occurred between the two organisms. No movement of organelles was observed through the septum of the contact cell. Very little effect on the host was observed, although dry weights of the host cultures were lower when parasitized (29).

Gonatobotryum fuscum, another biotrophic, contact, balanced hyperparasite, was found to parasitize Ceratocystis spp. and related Deuteromycetes (186). Ceratocystis is important as the causal agent of several tree wilts and one species, *C. fimbriata*, causes sweet potato black rot. *Graphium* is one of the imperfect forms of *Ceratocystis* and was used frequently as a host for *G. fuscum* (27, 186). *Gonatobotryum fuscum* developed contact cells on host hyphae (186). Mycotrophein was necessary for axenic culture of the hyperparasite. A medium containing glucose, yeast extract, thiamine, and biotin plus mycotrophein favored good growth (48). Darkness enhanced development of the parasite on its hosts (186), the inhibitory effects of light being related to the destruction of vitamin B_6 (27). Vitamin B_6 production by the hyperparasite apparently stimulates the host to produce biotin needed for growth of *G. fuscum*.

The destruction of *Ceratocystis fagacearum* and *C. fimbriata* in agar culture by *Gliocladium roseum* has been studied (30, 185). Coiling around host cells was common and penetration, if it occurred, was after the death of host cells. Biological control of oak wilt using this hyperparasite was attempted by Shigo (30). Sixty-two infected red, black and scarlet oak trees were inoculated with *G. roseum*. The hyperparasite was reisolated from 38 of the trees 2 months later. When samples were taken further from the inoculation point, there was no indication that *G. roseum* had spread within the trees. *Ceratocystis fagacearum* was recovered from all of the hyperparasite-inoculated trees, demonstrating the failure of *G. roseum* to inhibit *C. fagacearum in vivo*.

Necrotrophic hyperparasitism of *C. fimbriata* and *C. fagacearum* by 15 wood-rotting basidiomycetes was reported to occur in culture (98). Contact by the parasites resulted in the death of spores or mycelium of the hosts. A role for hyperparasitism in microbial succession in stumps or logs was speculated. The necrotrophic hyperparasitism of *C. fimbriata* by *Hirschioporus pargamenus*, the causal agent of white rot of sapwood of several tree species was noted (195). Contact was not essential for this antagonistic interaction.

Parasitism of organisms causing root diseases

Chytrids. Black wart of potato, caused by *Synchytrium endobioticum*, is worldwide in distribution, but most severe in Europe. Kohler, in 1924, reported the hyperparasite, *Phlyctochytrium synchytrii*, as a parasite of the resting sporangia of *S. endobioticum* (189). *P. synchytrii* produced sporangia and resting spores epibiotically. The endobiotic portion was described as irregularly expanded or subspherical haustoria, flattened against the host wall. More research in this area is necessary before the prospects of *P. synchytrii* as a biological control agent can be assessed.

Oomycetes parasitized by slime molds. Two endoparasitic slime molds, *Sorodiscus cokeri* and *Woronina pythii*, have been shown to infect *Pythium* spp. Both are obligate parasites on species of *Pythium* (94, 95). *Sorodiscus cokeri* parasitized *P. irregulare* (94) which is sometimes associated with pea, lupine, or cucumber roots. *Woronina pythii* infected the plant pathogens *P. aphanidermatum*, *P. debaryanum*, *P. irregulare*, and *P. ultimum*, causing galls delimited by septa in the host cells (95). Both hyperparasites have similar life cycles. Zoospores encysted on host hyphae and then produced short penetration tubes through which the contents of the spores emptied into the host cell. Plasmodia eventually appeared, one or more per gall. After the plasmodia increased in size, zoospores were delimited and bulbous exit tubes released the zoospores to the outside. *Woronina pythii* is distinguished from *S. cokeri* by the formation of galls in basipetal succession and by the types of cystosorus.

Not enough is known about these slime molds to speculate on their use in biological control.

Oomycetes and their chytrid parasites. The aquatic nature of both the chytrids and the Oomycetes has undoubtedly led to the coexistence of the parasitic chytrids with the lower fungi. Many chytrids have been described as parasites of oomycetous hyphae. Reports have included parasites of saprophytic as well as plant parasitic oomycetes. It is not known if any of the mycoparasitic chytrids are pathogenic to the plant pathogenic oomycetes. The chytrids parasitic on Oomycete hyphae are listed here (189).

Host	*Parasite*
Aphanomyces spp.	*Chytriomyces parasiticus* *Olpidiopsis aphanomycis* *O. luxurians* *Phlyctidium mycetophagum*
Pythium spp.	*Lagenidium pythii* *Olpidiopsis brevispinosa* *O. curvispinosa* *O. gracile* *O. pythii* *Pythiella vernalis* *Rhizophydium pythii* *Rozella cuculus* *R. irregularis* *R. laevis* *Rozellopsis inflata* *Solutoparies pythii*
Phytophthora spp.	*Rozella barretti* (also sporangia) *Rozellopsis waterhouseii* (also sporangia)

Olpidiopsis aphanomycis was observed as a hyphal parasite of *Aphanomyces cladogamus* (218), a relatively unfamiliar plant pathogen cited as causing tomato rootlet injury. *Olpidiopsis aphanomycis*, an endobiotic hyperparasite, produced zoosporangia and resting spores in swellings of the host hyphae.

Pythium gracile, the cause of ginger rot, was a host for Rozella laevis (129). The hyperparasite caused marked hypertrophy of the host hyphae, and either filled or partially filled the swollen portion with its sporangium or resting spore. Pythium intermedium causes a damping-off of fern and was susceptible to attack by four chytrids: Olpidiopsis gracile, O. pythii, Rozella cuculus, and Rozellopsis inflata (47, 129, 189). Olpidiopsis gracile and O. pythii infected the host hyphae, and R. cuculus and R. inflata infected the sporangia. All were endobiotic parasites inducing hypertrophy. Hyphae of Pythium vexans, a weak parasite which will sometimes cause seed decay and seedling disease in corn may become infected by O. pythii and Rozella irregularis, causing local swellings. Rozella irregularis may induce septation and abnormal branching (47).

Rozella barretti was reported as a parasite of Phytophthora cactorum (129), the causal agent of collar rot of apples. The hyperparasite caused local hypertrophy of the hyphae and, in addition, infected the sporangia. Rozella barretti sporangia were not detectable in the host sporangia until zoospores formed. Rozellopsis waterhouseii invaded the sporangia of the root rotters P. cryptogea and P. megasperma, and occasionally caused hypertrophy (129).

Rhizophidium apophysatus and R. carpophilum were found to be epibiotic parasites of oogonia and oospores of nonpathogenic Oomycetes (130, 189). Sparrow (189) commented that species of Olpidiopsis and Rozella occur endobiotically in the hyphae of water molds, while Rhizidiomyces and Rhizophidium occur epibiotically on oogonia and oospores.

Sneh et al. (188) isolated of wide range of hyperparasites of oospores of Phytophthora megasperma var. sojae (=P. megasperma f. sp. glycinea) from soil. These included three different chytrids: Canteriomyces stigeoclonii, Hyphochytrium catenoides, and Rhizidiomycopsis japonicus. Both C. stigeoclonii and H. catenoides were found to be endobiotic hyperparasites. The thalli of C. stigeoclonii released zoospores through a discharge tube in the presence of free water. The thalli of H. catenoides developed into sporangia, and released zoospores through a discharge tube that terminated in a vesicle. R. japonicus, an epibiotic parasite, was observed previously parasitizing oogonia of Aplanes sp. (189). Sporangia of the hyperparasite developed on the oospore while a rhizoidal system developed within. These three hyperparasitic chytrids were able to infect oospores of Aphanomyces euteiches, Pythium sp., and Phytophthora cactorum, in addition to P. megasperma var. sojae. In a survey of soil samples from 33 soybean fields, H. catenoides was identified most frequently, followed by C. stigeoclonii and R. japonicus, when soils were flooded (118).

Soil moisture influenced the kinds of oospore hyperparasites obtained (188). Chytrids were found only in flooded soils, while hyphomycetes dominated when the soils were drier. To determine if a relationship existed between hyperparasitism and disease potential, soil was collected from a site in an apple orchard where trees were infected with P. cactorum, and also from a site with healthy trees. Soil samples were baited with oospores of A. euteiches, Pythium sp., P. cactorum, and P. megasperma var. sojae. After 1 or 3 weeks, significantly more oospores were parasitized by chytrids in the soil collected from the healthy site. In a separate study by Humble and Lockwood (118), chytrid parasitism of oospores incubated in different soils could not be correlated with *P. megasperma* var. *sojae* root rot potentials.

The oospore hyperparasite, *H. catenoides*, has received more attention than any other chytrid as a potential biological control agent. It was identified as a parasite of the oospores of *Aphanomyces*, *Pythium*, and *Phytophthora* species (15, 188). The infection process began when zoospores of *H. catenoides* encysted on the host cell. An infection peg penetrated the oogonial and oospore walls and entered the oospore cytoplasm. Sporangia of *H. catenoides* eventually developed within the oospore (15, 188). Greatest zoospore production and motility occurred in a pH range of 6.0 to 7.0, and at temperatures from 20-30 C (15). Growth rate of *H. catenoides* in agar culture increased with increasing temperatures, 16 C being the minimum and 28 C the maximum (118). Invasion of *P. myriotylum* oospores was optimum between 20 and 30 C after six days (15). Parasitism of *P. megasperma* var. *sojae* oospores by *H. catenoides* in flooded steamed soil increased with temperature to a maximum of 85% at 28 C after 10 days (Humble & Lockwood, in press). When tested in distilled water, oospores of *P. aphanidermatum*. *P. ultimum*, and *A. euteiches*, and *P. myriotylum* were infected (Ayers & Lumsden, 1977). Infection percentages of *P. aphanidermatum* and *P. ultimum* oospores were 21-26%, and for *A. euteiches* and *P. myriotylum* 65%, after 2 weeks. Oospore infection in soil by *H. catenoides* was slower, but after 60 days, 77% of the oospores of *P. myriotylum* were infected compared to 13% for *P. aphanidermatum* and 11% for *P. ultimum*. An isolate of *P. megasperma* var. *sojae* race 7 was more resistant than isolates of races 1 and 3 to attack by *H. catenoides* (118). *Hyphochytrium catenoides* was detected in five of 15 tested agricultural soils. This chytrid is being evaluated as a biological control agent for *Phytophthora megasperma* var. *sojae* (Hsu &

Lockwood, unpublished results).

Oomycetous hyperparasites of other Oomycetes. Members of the Oomycetes may, on occasion, act as mycoparasites. *Aphanomyces exoparasiticus* parasitized many Phycomycetes tested, including members of the *Mucoraceae*, *Peronosporaceae* and *Saprolegniaceae*, but did not infect selected members of the Ascomycetes and Basidiomycetes (60). The parasite coiled around the host hyphae and developed superficial rhizoidal attachments. Cross-walls were developed by the host where infection occurred. Another Oomycete, *Pythiella vernalis*, developed galls containing 1-4 sporangia in the hyphae of *Pythium gracile* and *P. dictyosporum* (61).

Pythium spp. may be hyperparasites of other *Pythium* spp. Hyphae of *Pythium debaryanum*, *P. irregulare*, *P. myriotylum*, and *P. ultimum* were susceptible to attack by *P. acanthicum*, *P. oligandrum*, *P. periplocum* (75). Invasion of *P. myriotylum* hyphae was initiated by appressoria. The parasite either penetrated and formed haustorial hyphae which were delimited by host septa, or the host responded by increasing the thickness of the outer and inner walls. In the latter case, the hyphae either remained healthy or the cytoplasm degenerated.

Biological control of damping-off of sugar beet was achieved by one of the aforementioned *Pythium* antagonists, *P. oligandrum* (210). Beet seed emergence and plant weights were increased in pot trials by treatment of beet seed with a suspension of 4 x 10^6 oospores of *P. oligandrum*/ml. The biocontrol treatment was superior to a chemical seed-dressing with Hermal (70% thiram).

An unidentified *Pythium* sp. was observed parasitizing oospores of *P. megasperma* var. *sojae* in studies performed by Sneh et al. (188). This organism was subsequently identified as *P. monospermum* (118). In steamed soil, *P. monospermum* parasitized more than 70% of *P. megasperma* var. *sojae* oospores. *P. monospermum* was an effective parasite over the range of 16-28 C, but maximum parasitism occurred at 24 C. In agar culture, linear growth of *P. monospermum* increased as temperature increased up to 28 C. Oospores of races 1 and 3 of *P. megasperma* var. *sojae* were more susceptible to attack by *P. monospermum* than those of race 7. The basis for the difference in susceptibility was not determined.

Leptolegnia sp. is another oomycete that was found infecting oospores of *P. megasperma* var. *sojae* (188). Hyphae of this hyperparasite emerged from the infected oospore to form sporangia and zoospores.

Pythium acanthicum has a microbial host range that is too numerous to list (102, 109). The hyperparasite hyphae were attracted to the host. When *P. aphanidermatum* was parasitized, the hyperparasite hyphae coiled around the host causing cytoplasmic changes, but no penetration occurred. The hyphae at the contact zones appeared dead.

Phycomyces blakesleeanus, a saprophyte, reacted to *P. acanthicum* contact by producing very large papillae with a fibrillo-granular matrix, which coalesced in response to multiple attacks (109). The host plasma membrane separated the papillae from the cytoplasm. The papillae increased in size until the hyperparasite penetrated the papillae and the plasma membrane, at which time the rate of development of the papillae decreased. By the time penetration of cytoplasm occurred, the host cells appeared dead. In coenocytic hosts, septa-like structures slowed hyperparasitic advancement. In Basidiomycete hosts, the cytoplasm appeared dead a few minutes after papilla formation. *Sclerotinia sclerotiorum* and *Sclerotium rolfsii* caused *P. acanthicum* hyphae to lyse before contact was made. Deacon (64) also observed lysis of *P. oligandrum* by some basidiomycetes.

Preliminary evidence indicates that hyperparasitic *Pythium* spp. merit further investigation as biological control agents. Caution will be needed to find genetically stable isolates that will not pose the threat of eventual development of phytopathogenicity.

Fungi Imperfecti as hyperparasites of Oomycetes

Other hyperparasites of *Pythium* oospores include several Deuteromycetes. Drechsler reported six species of *Dactylella* as parasites of *Pythium* oospores: *D. anisomeres*, *D. helminthodes*, *D. passalopaga*, *D. stenocrepis*, *D. stenomeces*, and *D. spermatophaga* (73, 76, 77, 78). Parasitism by all species was accomplished in essentially the same manner. Appressoria of the hyperparasite developed when contact was made with oogonia or oospores. Penetration pegs then penetrated the oogonial wall. Occasionally, a second appressorium formed on the oospore wall preceded penetration of this structure. Eventually, after the oospore cytoplasm was absorbed, the assimilatory hyphae became visible. *Dactylella helminthodes* infected *P. debaryanum* oospores (76). *D. stenocrepis* parasitized *P. butleri*. *Dactylella anisomeres* was found to parasitize *P. butleri* and *P. debaryanum* (77). *Pythium debaryanum*, *P. irregulare*, and *P. ultimum* were hosts for *D. stenomeces* (78) and *D. passalopaga* (76). *Dactylella spermatophaga* was destructive to *P. acanthicum*, *P. arrhenomanes*, *P. butleri*, *P. debaryanum*, *P. graminicola*, *P. myriotylum*, *P. oligandrum*, *P. paroecandrum*, *P. periplocum*, *P. ultimum*, *P. vexans*, and many other saprophytic *Pythium* spp. (73). In addition, *D. spermatophaga* parasitized oospores of *Aphanomyces euteiches*, *Phytophthora* cactorum and P. megasperma, and chlamydospores of P. cinnamomi. Drechsler suggested that the role of D. spermatophaga would be minimal in controlling the pythiaceous fungi in nature because of the slow growth rate of this hyperparasite (73). However, in the same paper he also suggested that by manipulation, D. spermatophaga had great potential for attainment of "soil sanitation". Drechsler also described two species of Trichothecium as oospore parasites (75, 76). T. arrhenopum invaded P. graminicola oospores (74), and T. polyctonum was destructive to oospores of the saprophyte P. spinosum (76). In addition, P. butleri oospores were parasitized by Trinacrium subtile (73). Parasitism occurred essentially as described for Dactylella. Hoch and Abawi (107) isolated Fusarium merismoides from oospores of P. ultimum. Thirty to 74% of the oospores of P. ultimum were parasitized by this hyperparasite over an 8 week period in sterile or natural soils.

Sneh et al. (188) identified Alternaria alternata, Cephalosporium sp., Diheterospora chlamydosporia, Fusarium oxysporum, and Humicola fuscoatra as parasites of P. megasperma var. sojae oospores. These Hyphomycetes were found to be the dominant hyperparasites of oospores of P. cactorum (188) and P. megasperma var. sojae (118, 188) when soil moistures were held below saturation.

Biological control of P. ultimum was obtained when cotton seed was treated in the furrow with Gliocladium virens (113). Seedling emergence and survival increased significantly with the treatment. In addition to acting as a hyperparasite, one of two antibiotics isolated from culture filtrates of G. virens was inhibitory to P. ultimum. Mutants deficient for antibiotic production did not control damping-off, while those with enhanced antibiotic production were superior to the parent strain. The

The mode of parasitism was not elucidated. *Gliocladium* has been associated with a complex of fungi causing seedling blights; therefore, it would be advisable to test a wide range of higher plants as potential hosts prior to field application.

Trichoderma spp. may be promising as biological control agents for *Pythium* diseases. A wide range of species and isolates of *Trichoderma* were assessed for their coiling response to potential host fungi (70). In each species the majority of isolates would coil around hyphae of *P. ultimum* inducing vacuolation and coagulation of cytoplasm. Occasionally, hyphae of *P. ultimum* were penetrated by isolates of *T. harzianum* and *T. viride*. Two isolates of *T. viride* also on occasion penetrated hyphae of *Phytophthora cactorum* and *P. erythroseptica*. Growth of *Fomes annosus*, *Fusarium oxysporum*, and *Rhizoctonia solani* halted after contact with the hyperparasite, regardless of whether antibiotics were being produced. Reisolation of host fungi was usually not possible after contact with the hyperparasites.

A non-antibiotic producting isolate of *T. hamatum* in a methyl cellulose slurry was effective in controlling root rot of peas planted in a soil naturally-infested with *P. aphanidermatum*, *P. oligandrum*, and *P. ultimum* (100). A similar finding was reported by Chet and Baker (55). Better protection was obtained when pea seed was treated with a concentration of *Trichoderma* conidia greater than 10^6/ml than at lower concentrations, and when soil temperatures were between 17 and 34 C.

Trichoderma hamatum seed treatment amended with powdered, unbleached chitin decreased disease incidence (101). Other amendments of peat or *Rhizoctonia solani* cell walls increased the population of *T. hamatum*, but did not reduce *Pythium* seed rot. *Rhizobium* inoculum added to the *T.*

hamatum pea seed treatment did not interfere with control. *Trichoderma hamatum* controlled pre-emergence damping off as well as chemical seed treatments, but did not protect pea as well against post-emergence damping off (100). In a replanting with untreated pea seed into the same pots, disease control by *T. hamatum* was comparable to that by chemicals. In a second replanting with untreated pea seed, plants in soils originally treated with *T. hamatum* or fenaminosulf (Dexon) showed less disease than captan-treated or untreated soils. *Chaetomium globosum* has also been applied to seed as a potential biocontrol agent both alone and in combination with *T. hamatum* (100). Although some control was achieved, *C. globosum* was less effective than *T. hamatum*, and it did not augment control by *T. hamatum*.

Another *Trichoderma* sp., *T. harzianum*, did not control pea seed rot as a seed treatment (100), or pineapple root rot caused by *Phytophthora cinnamoni* when applied to soil as a granule preparation (132).

Hyperparasitism of some of the lower fungi by Rhizoctonia.

Rhizoctonia solani and other *Rhizoctonia* spp. pathogenic to strawberry roots were also hyperparasites of the *Mucorales*, *Peronosporales* and *Ambylosporium botrytis*, a Deuteromycete (45). Included as hosts were *Mucor mucedo*, *Pythium butleri*, *P. debaryanum*, *P. irregular*, *P. vexans*, *Phytophthora cinnamomi* and (+) and (-) strains of *Rhizopus nigricans* (45, 46). On the basis of pathogenicity to *P. debaryanum* and *R. nigricans*, Butler (45) recognized four physiological races of *R. solani*.

Penetration of the host occurred with or without coiling (45). When parasitism occurred without coiling, a few infection hyphae penetrated the host cell and developed a hyphal system within the host cell. Penetration with coiling took place at frequent intervals with many infection pegs. An extensive mycelium ramified throughout the infected area. Although the parasitic hyphae replaced the host protoplasm in infected cells, noninfected portions survived. When *R. solani* parasitized *Syncephalastrum* sp., young hyphae were the most susceptible (45).

Resistance of the host was expressed by walling off infection hyphae or by inducing lysis of the *R. solani* hyphae (45). Barrel- shaped cells and sclerotia were produced in high numbers in parasitized cultures (46). There was no relationship between mycoparasitic abilities and plant pathogenicity (45).

Rhizoctonia solani will probably never intentionally be used as a biological control agent because it is a plant parasite. The study of the host-hyperparasite relationship of the lower fungi with *R. solani* is interesting because this ability may be of survival value for *Rhizoctonia* spp.

Ascomycetes and Fungi Imperfecti. *Gliocladium roseum*, already discussed as a parasite of *Ceratocystis*, was also a hyperparasite of hyphae and sclerotia of *Botrytis allii*, an organism commonly associated with neck rot of onion (211). *Gliocladium roseum* exhibited tropic growth towards *B. allii* hyphae, appressoria were formed on host hyphae, and haustoria-like structures appeared within the *B. allii* hyphae. Further development of the absorptive hyphae took place intracellularly. Of 40 naturally occurring *B. allii* sclerotia plated on agar, 8 were infected with *G. roseum*. In a controlled environment of 21 C and 100% relative humidity, the rate of *B. allii* infection of both wounded and healthy onion bulbs decreased when *G. roseum* was placed in close contact with *B. allii* inoculum.

A wide range of economically important diseases are caused by

Fusarium spp. including wilts, and root and stem rots. *Gliocladium catenulatum* was tested as a hyperparasite of *F. equiseti*, *F. oxysporum*, *F. poae*, and *F. sporotrichioides* (114). Macroconidia and hyphae of all species were susceptible to *G. catenulatum*, but chlamydospores did not show signs of infection. Host cells were killed on contact. *G. catenulatum* formed pseudoappressoria which produced hyphae that did not penetrate host cells. *Sclerotinia sclerotiorum* was also susceptible to this hyperparasite.

Stephanoma phaeospora was found associated with a *Fusarium* sp. in nature (173). Other hosts were *Ceratocystis fimbriata*, responsible for sweet potato black rot, *Septoria apii*, causal agent of late blight of celery, *Thielaviopsis basicola*, cause of black root rot, and the smuts, *Ustilago avena* and *U. maydis*. *Stephanoma phaeospora*, a contact, biotrophic hyperparasite, caused no visible damage to its hosts. *Axenic* culture of *S. phaeospora* has been possible with the addition of a growth factor from specific fungi, a condition much like that previously mentioned for the hyperparasite *Gonatobotrys simplex*. The contact cells of *S. phaeospora* are of two shapes - hooked, or elongated and flattened. No attraction of the parasite hyphae towards the host was observed.

Trichoderma spp. have been reported to be antagonistic to *Fusarium*. Chi (57) noted hyphal coiling of *Trichoderma* and its hosts around each other, and cytoplasmic changes in the hyphae of *F. oxysporum*, *F. roseum*, and *F. solani* infected by two species of *Trichoderma*. Pots of sterilized soil infested with *F. culmorum* and *T. lignorum* contained fewer diseased barley plants than pots with *F. culmorum* alone (36).

Pythium acanthicum, *P. oligandrum* and other mycoparasites were easily isolated by placing small pieces of soil organic matter on agar plates precolonized by *Phialophora radicicola* var. *radicicola* (65, 66). *Pythium acanthicum* and *P. oligandrum* were recognized by spiny oogonia produced on these plates. The interaction of *P. oligandrum* and susceptible host fungi was evaluated *in vitro* using amount of suppression of filter paper cellulolysis as a measurement of parasitism, *P. radicicola* var. *radicicola* was classified as highly susceptible, *Fusarium roseum* f. sp. *cerealis*, *Gaeumannomyces graminis* var. *tritici*, and *P. radicicola* var. *graminicola* were moderately susceptible, *G. graminis* var. *graminis* was resistant, and several basidiomycetes were antagonistic to *P. oligandrum* (64). Susceptibility of host hyphae was greatest when young, declined as hyphae matured, and then increased as hyphae aged. The narrow, hyaline hyphae of *Phialophora* and *Gaeumannomyces* were more susceptible to lysis by the parasite than the broad, darkly pigmented hyphae. Deacon (64) suggested that melanization might be related to resistance. Hyphal coiling by the parasite was observed only on resistant hyphae. *P. acanthicum*, like *P. oligandrum*, parasitized *G. graminis* var. *tritici*, *P. radicicola* var. *graminicola*, and *P. radicola* var. *radicola*. All *P. acanthicum* isolates but one, a plant pathogen, caused a reduction in filter paper cellulolysis by host fungi (66). It was suggested that *Pythium* isolates that are highly phytopathogenic may be only slightly mycoparasitic and vice versa. This relationship was not shown for mycoparasitic *Rhizoctonia* isolates (45).

Another hyperparasite of *G. graminis* was identified as the Ascomycete, *Didymella exitialis* (187). Disease was reduced by 50% in the greenhouse under sterile conditions when wheat seedlings were co-inoculated with both host and hyperparasite. The hyphae of *G. graminis* were penetrated and destroyed by *D. exitialis* in the rhizophere.

Combinations of free amino acids from culture filtrates of *D. exitialis* reduced pathogenicity of *G. graminis* by 40%, while single amino acids caused a 20-30% reduction.

An isolate of *Macrophomina phaseolina*, causal agent of damping-off and charcoal rot of slash pine, was found to be parasitized by an unidentified Basidiomycete (69). The hyperparasite either directly penetrated the host cell and colonized it, or first formed an appressorium from which a penetration peg developed. In the laboratory, aseptically grown slash pine seedlings inoculated with agar disks of *M. phaseolina* plus the hyperparasite showed no disease symptoms, whereas *M. phaseolina* alone caused disease. In a field experiment, equal amounts of inoculum of the pathogen or parasite produced on vermiculite supplemented with potato-dextrose broth, were spread over nursery plots. The treatments included *M. phaseolina*, *M. phaseolina* + hyperparasite, and the hyperparasite. Pine seeds germinated equally well in all treatments, but seedling survival was increased 27% with the addition of the hyperparasite. Hyperparasitism by this organism also occurred on *Cylindrocladium floridanum*, *P. ultimum*, *P. cinnamomi*, and *R. solani*.

Sclerotia. Fungi of the genus *Sclerotinia* cause root and stem diseases, and are destructive to many crops. Mycelium infects susceptible tissue, and as it spreads, sclerotia are formed in cavities or on the surface of infected plant parts. Sclerotia (and/or mycelium on plant tissue) overwinter to produce apothecia in the spring. Many studies have been done on the survival of sclerotia in soil (58). Sclerotia of *S. sclerotiorum* were buried in soil or left on the soil surface and retrieved at different times of the year to study the sclerotial microflora (172). Organisms associated with the sclerotia varied with the time of year. Buried sclerotia were colonized less than those at the soil surface. Of those organisms isolated, Cunninghamella echinulata, Fusarium oxysporum, Rhizopus orrhizus, and Trichoderma lignorum caused a 20-35% decrease in sclerotia germination in vivo. The parasitic nature of these organisms was not established, but antibiosis was not detected in culture. Penicillium citrinum, P. funiculosum, P. pallidum, and P. steckii were aggressive sclerotia parasites. Aspergillus niger and A. ustus were also parasitic, but less so than the Penicillium spp. Both Aspergillus- and Penicillium-colonized sclerotia did not produce apothecia in the field.

Species of Chaetomium, Codinaea, Fusarium, Gliocladium, Paecilomyces, Talaromyces, and Trichoderma isolated from surface-sterilized sclerotia were associated with a decline in populations of sclerotia of S. minor in four agricultural soils (89). In preliminary tests, Chaetomium fusiforme and P. elegans decreased disease of lettuce caused by S. minor in the greenhouse.

Gliocladium virens parasitized sclerotia and mycelium of S. sclerotiorum (202). Appressorium-like structures were formed on the host. By the use of transmission electron microscopy, the presence of G. virens inside the sclerotial cells was demonstrated. The hyperparasite prevented the formation of sclerotia in culture, and those sclerotia that were parasitized did not germinate. G. virens also parasitized Rhizoctonia solani.

Trichoderma viride was isolated from sclerotia of S. sclerotiorum (127). Four single spore isolations were made, of which one decayed the majority of sclerotia on moist sand or in buried soil, while the other three decayed only a few sclerotia on sand and none on soil. In a culture medium, however, all four isolates parasitized *S. sclerotiorum* hyphae and sclerotia. These results indicate the importance of isolate selection.

Coniothyrium minitans, another hyperparasite of *Sclerotinia* spp., directly penetrated the host cell wall of *S. sclerotiorum* and caused cytoplasmic disintegration of hyphae and sclerotia (115). In hyphal infections, cells adjoining the infected area appeared vacuolated and unhealthy. In response to infection, new hyphae of *S. sclerotiorum* were produced within the infected areas but showed only limited growth. *Coniothyrium minitans* produced pycnidia on infected sclerotia (204). Sclerotia of *S. cepivorum*, *S. minor*, *S. trifoliorum* and some isolates of *Botrytis cinerea* were also susceptible to *C. minitans* (204). Apothecia were also parasitized. The first signs of infection occurred as brown spots which expanded and turned black. The apothecium remained functional for some time before being destroyed (196). The optimum temperature for sclerotial infection was 20 C, but parasitism occurred as low as 10 C. Isolates of *S. sclerotiorum* demonstrated great variability in susceptibility (204). Sclerotia were more susceptible to infection by *C. minitans* when produced in agar culture than on autoclaved carrots (204), or on brussel sprouts than on sterile oat seeds (50).

In an attempt to reduce numbers of sclerotia of *S. sclerotiorum*, pycnidiospore preparations of *C. minitans* were placed in direct contact with sclerotia in sterile sand or were applied to sterile sand containing sclerotia. Apothecial formation was inhibited by both treatments (50). In other work, a cornmeal-sand culture of *C. minitans* was mixed into a loamy sand or a heavy clay field soil in which sclerotia of *S. trifoliorum* were buried (196). Within 11 weeks, 85-99% of the sclerotia were killed. Coniothyrium minitans was not pathogenic to several species of plants (204). C. minitans appears to be very promising as a biological control agent.

Sporidesmium sclerotivorum was recently described as a sclerotial parasite of S. sclerotiorum (205). Glucose or mannitol as a carbon source, casamino acids or glutamine as a nitrogen source, thiamine, and an initial pH of 5.0-5.5 provided a suitable nutritional environment for axenic culture of the hyperparasite (31). Sporidesmium sclerotivorum infected S. cepivorum (12), S. minor (13) and Sclerotium sclerotivorum, in addition to S. sclerotiorum (12). The hyperparasite grew through moist sand from one S. sclerotivorum sclerotium substrate to another (12). Conidia as far as 9 mm from a sclerotium of S. minor in moist natural soil were stimulated to germinate (13). In 10 weeks or less, 95% of the sclerotia were destroyed by the hyperparasite in sand or soil (steamed or natural) infested with sclerotia at a rate of 1% (w/w) (12). Maximum infection occurred at 20-25 C, pH 5.5-7.5, and a soil moisture of -8 bars matric potential or higher (12). Concentrations as low as 2 X 10^3 S. sclerotivorum spores per 100 g of soil caused infection and decay of host sclerotia.

Naturally occurring S. sclerotivorum caused a decline of S. minor populations in a field soil (3). Populations of sclerotia were 90% in June and 30% in September, of that in March. The hyperparasite was observed on 13-22% of the sclerotia recovered for each assay from May to September. In a field test where the hyperparasite was introduced at a rate of 100 spores/g soil, a similar decline was observed. Selected naturally infested onion fields in New Jersey demonstrated an association between naturally occurring S. sclerotivorum and decreased populations of

S. cepivorum. In five potato fields in Oregon, infection of introduced sclerotia of *S. sclerotivorum* by *S. sclerotiovorum* was low in four fields which had a moderate to high incidence of stem rot, while it was low in the fifth field where little stem rot had occurred. Commercial production of the hyperparasite may be the next step for use of *S. sclerotivorum* as a biological control agent.

Sclerotium spp. infects a wide range of crops as seedlings or mature plants, and can also cause severe losses during the transport of fruits and vegetables. It is a problem primarily in warm climates, and in the United States diseases caused by *Sclerotium* are prominent in the South. Control of *Sclerotium* is difficult, and an effective biological control agent would help in reducing losses caused by this pathogen. Two genera of hyperparasites that have been studied in terms of biological control are *Coniothyrium* and *Trichoderma*.

Coniothyrium minitans forms its own pycnidia within the infected sclerotia of *S. cepivorum* (92). Control of white rot of onion by *C. minitans* was studied in the greenhouse (4). The hyperparasite was prepared as a pycnidial dust and used in soil treatments at rates of 50 or 100 times that of *S. cepivorum* sclerotia, or as a seed dressing at one-third the rate of sclerotial inoculum. *C. minitans* gave disease control equal to that of the fungicide Calomel, and the biological control was recommended for further evaluation.

Trichoderma spp. have been effective both in field and greenhouse in reducing diseases caused by *Sclerotium* spp. Biological control of *S. rolfsii* on beans was achieved in the greenhouse using a wheat bran culture of *T. hazianum* (56, 82). *T. harzianum* decreased disease approximately 40-60% over a temperature range of 22-34 C (82). Control in the greenhouse was enhanced when *T. harzianum* was applied in combination with PCNB (56), or when soil was pretreated with solar heat at 47 or 50 C (83). A wheat bran preparation of *T. harzianum* also reduced bean disease caused by *S. rolfsii* about 15% and increased green pod yield by 20%, 48 days after planting in a field test (82).

Trichoderma hamatum also has been evaluated for control of *S. rolfsii* on beans (55). Although no antagonism was observed between *T. hamatum* and *S. rolfsii* in agar culture, disease incidence in the greenhouse was decreased by about 40% after 14 days when *T. hamatum* was added to the soil at 10^6 propagules per g. A second planting into the treated soil 14 days after the first provided 15% greater disease control than was achieved in the first planting, after 14 days.

A *T. harzianum* isolate capable of parasitizing *S. rolfsii*, *Sclerotinia trifoliorum* and *Botrytis cinerea*, but not *Rhizoctonia solani*, was tested as a biological control agent in greenhouse and field tests (216). *Trichoderma harzianum* grown in a rye seed-soil mixture gave 100% control of *S. rolfsii* on tomato or peanuts and 91% control on lupine, when equal weights of *S. rolfsii* on the same material was used in a greenhouse experiment (216). *Trichoderma harzianum* gave 70% or more disease control in field tests in soil naturally infested with *S. rolfsii* when applied when plants were 4 cm high, again 9 days later, and finally 7 days later. In another field experiment, plants from *T. harzianum* plots were at least 88% disease-free, almost twice that of untreated plots, when the hyperparasite was applied at the time tomatoes were cut back to approximately 2 cm. Elad et al. (82) also achieved a 20% reduction in *S. rolfsii* disease of tomatoes in the field using a different *T. harzianum* isolate.

A hyperparasitic isolate of *T. harzianum* gave significant control of *S. rolfsii* in naturally-infested peanut fields using wheat bran inoculum (56) or molassses-impregnated diatomaceous earth granules (18) on which the fungus was cultured. *T. harzianum* applied as granules increased peanut yield equal to that of PCNB treatment.

Two species of *Trichoderma* were selected for field trials on the basis of their *in vitro* abilities to degrade the sclerotia of an *S. rolfsii* isolate responsible for *Sclerotium* blight of turf (171). *Trichoderma* grown on molasses-impregnated diatomaceous earth was sprayed at rates of 110 kg/ha and 140 kg/ha onto three diseased golf greens. Populations of *Trichoderma* increased over time, but biological control was inconsistent.

Other organisms also have been reported to degrade sclerotia. *Teratosperma oligocladum* parasitized sclerotia of *Sclerotinia minor*, *S. sclerotiorum*, *S. trifoliorum*, *Sclerotium cepivorum* and *Botrytis cinerea* (14). In soil infested with *T. oligocladium*, sclerotia of *Sclerotinia* spp. were infected within 2 weeks, and sclerotia of *S. minor* were destroyed by 10 weeks. *Acrostalagmus roseus*, *Gliocladium roseum*, *Gliocladium* sp., *Fusarium* sp., *T. viride*, and *Verticillium* sp. were able to infect the sclerotia of *Botrytis cinerea*, *Claviceps purpurea*, *Sclerotinia borealis*, *S. sclerotiorum*, and *S. trifoliorum* *in vitro* (150). Resistance of the *Sclerotinia* spp. to the parasites was related to the rate of regeneration of the sclerotial cortex.

Rhizoctonia. *Rhizoctonia solani* is world-wide in distribution and causes seedling rot and root rot on a wide range of crop plants. Many hyperparasites have been observed on *R. solani* and attempts have been made to utilize them in biological control of this pathogen.

Gliocladium roseum caused the hyphae of *R. solani* to collapse in dual culture, presumably without penetration (170). Another isolate of the hyperparasite did not control *R. solani* in field trials with cucumbers (144). Another species of *Gliocladium*, *G. virens*, parasitized *R. solani* hyphae (203), in much the same manner as this hyperparasite infected *Sclerotinia sclerotiorum* (203) (see previous section). Hyphae of *G. virens* were found within sclerotial cells of *R. solani* in thin sections (203). In culture, sclerotial formation was deterred by *G. virens*. Disease of white beans caused by *R. solani* was decreased proportionately to the amount of hyperparasite inoculum added to artificially infested soil in greenhouse tests.

Penicillium vermiculatum was investigated as a potential biological control agent of *R. solani* by Boosalis (39). *P. vermiculatum* parasitized *R. solani* hyphae by the development of penetration pegs at contact sites. The hyphae of the hyperparasite either ramified throughout the host or was limited within an area. Of 30 isolates of *R. solani* tested for their susceptibility to *P. vermiculatum*, all were susceptible, though in different degrees. The amount of dextrose in the agar medium influenced the severity of infection in mixed cultures. An insignificant amount of parasitism occurred when 10 g dextrose was used, while 20 g resulted in severe infection.

Biological control experiments were performed by simultaneous artificial infestation of sterile or non-sterile soil with *R. solani* and *P. vermiculatum*. After 10 days of incubation, pea seeds were planted. Control of *R. solani* was obtained in sterilized soil system but not in unsterile soil. Use of *P. vermiculatum* as a biological control agent does not appear promising.

Papulospora stoveri was isolated from R. solani-infected sugar beet seedlings (212). P. stoveri parasitized R. solani hyphae in vitro by rapidly coiling around the host hyphae causing the host cytoplasm to retract and eventually disintegrate at contact points. Hyphae of the hyperparasite were often observed growing within the host hyphae. The same mode of parasitism was seen when the host-parasite interaction was studied in soil using buried slides. In biological control experiments, corn meal-sand cultures of the host and hyperparasite were mixed with soil. After 4, 24, and 55 days of incubation, sugar beet seed pieces were planted. Control efficiency increased with incubation time, and was dependent on the strain of R. solani.

Laetisaria arvalis [Corticium sp. (42)] was investigated as a control for R. solani (144, 164, 165). Plantings of L. arvalis-treated seed into nonsterilized soil infested with R. solani resulted in increased stands of dry beans, soybeans, and sugar beets in greenhouse and field tests (164, 165). A second planting gave continued protection, although results varied depending on the R. solani isolate and soil type (164). Population density of R. solani in soils with L. arvalis was decreased by 28%, while that in soil treated with PCNB was decreased by 35%. The addition of sugar beet pulp to L. arvalis inoculum gave better protection of sugar beets than either treatment alone (165). The mechanism of control of R. solani was not determined, but other L. arvalis isolates were hyperparasites of R. solani (165).

Field tests using L. arvalis as a control for Rhizoctonia fruit rot of cucumber gave variable results (144). Control appeared to be dependent on the method of infestation. When L. arvalis was disked into the soil, treatments were as effective as the fungicide captafol, but about 20% less effective than the fungicide chlorothalonil 6F. If the hyperparasite inoculum was plowed into the soil, control was approximately 20% less than in the case of disking in the inoculum. There may be better biological control agents than *L. arvalis* for *R. solani*.

Recently, much information has been published on the use of *Trichoderma* spp. for the biological control of plant diseases. There have been more experiments done with *Trichoderma* than with any other hyperparasite. There are several reasons for this. First, *Trichoderma* is commonly found in nature. Second, unlike many of the hyperparasites discussed, it is easy to grow axenically. Third and most important, it is an effective hyperparasite. Weindling, in the 1930's, was the first to describe the parasitic nature of *Trichoderma* and to test this hyperparasite as a biological control agent for control of *R. solani*. Antibiotic production by *Trichoderma* strains will not be discussed here. There are hyperparasitic *Trichoderma* strains that control plant pathogens that do not produce antibiotics effective against the test organism (54, 55).

The mode of parasitism appears to be the same for different species of *Trichoderma* (147, 213). *Trichoderma* responded to the host by coiling around *R. solani* hyphae. The host protoplasm degenerated and hyphal cells separated at the septa. *T. lignorum* hyphae were observed to flourish at points where host cell contents were released into the environment (213). Penetration of *R. solani* by *T. harzianum* was not observed (147). However, Weindling (213) observed internal parasitism by *T. lignorum* of a strain of *R. solani* parasitic in potatoes. Differences in host susceptibility to parasitism by *T. lignorum* have been found among isolates of *R. solani*, *Phytophthora parasitica*, *Pythium* spp., *Rhizopus* spp., and *Sclerotium rolfsii*. The extent of the host range of *Trichoderma* has been dependent on the isolates of both hyperparasite and host (55, 82, 99).

Soil conditions play a large role in determining the effectiveness of biological control by *Trichoderma*, the fungus being most favored by moist soils and high acidity (147). Hyperparasitism of *R. solani* by *T. harzianum* was significantly greater at -0.3 or -0.1 bars matric potential than at -0.5 bars (97). *Trichoderma harzianum* was more effective in suppressing disease caused by *R. solani* and over a longer time period at high than low soil moisture levels (147).

Biological control experiments with sour orange seedlings indicated that acidification of soil with aluminum sulfate, in conjunction with the natural presence of *Trichoderma* spp., provided better control of damping-off caused by *R. solani* than either aluminum sulfate or *Trichoderma* alone (215). As pH decreased, growth of *T. harzianum* increased, and disease incidence decreased (54, 147). It has been suggested that pH affects antibiotic production by *Trichoderma* strains which in turn affects their parasitic ability (214). The influence of lower pH on parasitic ability may, however, be due to growth stimulation of *Trichoderma* and not to increased antibiotic production. Biological control of *R. solani* by an isolate of *T. harzianum* that did not produce antibiotics in vitro was increased by acidic conditions (54, 147).

Trichoderma harzianum has been used in greenhouse and/or field tests to control *R. solani* diseases of alfalfa (54), bean (56, 83, 99), carnation (84), cotton (62, 82), cucumber (147), eggplant (56, 99), potatoes (83), radishes (54, 55, 103, 147), sugarbeets (54), and tomatoes (99). *Trichoderma hamatum* has been used to control *R. solani* as a pathogen of radishes (55, 100, 101).

In most of the biological control experiments cited, Trichoderma was cultured on sterilized wheat bran or wheat bran-sawdust mixtures. An increase in concentration of Trichoderma inoculum resulted in decreased disease. For example, Elad et al. (84) investigated rate and placement of T. harzianum for control of Rhizoctonia root rot of carnation. Significant disease reduction was achieved when T. harzianum was broadcast in the field at 100 g/m^2 or added to the rooting medium at 13.5 g/m^2. The rooting medium treatment gave the best disease control, and may be appealing to commercial carnation growers because of the ease with which this method may be used.

Rhizoctonia solani on beans (99) and on eggplant (56, 99) grown in the greenhouse was controlled by T. harzianum inoculum. Disease incidence of beans after a second planting in artificially infested soil was greatly decreased when soil was infested with 1.7, 2.3, and 2.9 x 10^{10} T. harzianum spores/kg soil prior to planting (99). Bean disease in naturally infested soil was significantly reduced by 21% using T. harzianum at a rate of 3 g preparation/kg soil (56), or in other work by 41% at 6 g/kg, but not at 3 g/kg (99). Disease on beans caused by R. solani was very low (only about 13% after 48 days) in a field test, but disease onset was delayed and disease incidence was reduced by about 50% in the T. harzianum treatment. Three different concentrations of T. harzianum delayed development of R. solani on eggplant and significantly reduced disease by approximately 15% after 3 days (99).

In a field experiment, Rhizoctonia damping-off of cotton was reduced approximately 50% by the use of the hyperparasite, T. harzianum (82). PCNB at 2 and 10 g/g soil, when combined with T. harzianum, provided an additive control effect, but the cotton herbicides, prometryne, fluometuran, and trifluralin interfered with control by *Trichoderma* (62).

Trichoderma harzianum was investigated in field trials as a biological control for potato diseases caused by *R. solani* and in addition by *Sclerotium rolfsii* or *Verticillium dahliae* (83). In 1977, *T. harzianum* was used alone at 400 kg inoculum/ha, or was applied in conjunction with solar heating. *Trichoderma harzianum* caused a significant decrease in disease symptoms in potato shoots at 86 days, but no significant symptom decrease in shoots after 54 days, or in potato tubers after 133 days. Solar heating in combination with *T. harzianum* gave a significant decrease in disease, but the decrease cannot be attributed to *T. harzianum* since solar heating alone gave equal control. *Trichoderma harzianum* did not decrease *Verticillium* wilt at 86 days, but solar heating alone or with *T. harzianum* caused a decrease in this disease. There were no differences in potato yields between any of the treatments.

In 1978, *T. harzianum* was used alone, or following solar heating or methyl bromide treatment. There was no disease reduction on potato tubers or beans planted in the greenhouse in soil from the field, using *T. harzianum* alone. Methyl bromide or solar heating followed by *T. harzianum* did not cause a decrease in *R. solani* in tubers or in beans. However, the combination of methyl bromide with *T. harzianum* resulted in a significant increase in bean yields . There were no differences in incidence of *S. rolfsii* or *V. dahliae* diseases on potatoes that could be related to control by *T. harzianum*. Yields of potatoes were not different among the treatments. Results from these field experiments do not indicate that *T. harzianum* is an adequate control agent for *R. solani*, *S. rolfsii*, or *V. dahliae* on potatoes. Possibly, *R. solani* isolates from potatoes are more resistant to T. harzianum than other isolates.

Soils taken from the potato plots in a field experiment at 0, 90, and 135 days after potato planting, were planted with beans three consecutive times at 28 day intervals (83). Disease in soil taken from T. harzianum, solar heating, or solar heating plus T. harzianum treatments, was approximately 10-60% less than that in the control, except in the sample collected at 135 days, in which disease incidence was the same in the T. harzianum treatment and the control in all three consecutive plantings.

Hyperparasitic Trichoderma spp. may induce soil suppressiveness. Suppressive soils can be defined as soils that resist the introduction a plant pathogen; or in which establishment of a plant pathogen is successful but no disease occurs; or in which the pathogen is established, disease occurs, but decreases in intensity with continuous cropping (19). This characteristic may be either expressed as soil conduciveness or soil suppressiveness (103). Soil conduciveness is expressed by the equation $CI = 1 - \frac{X}{A}$, where CI = conducive index, A = number of healthy plants in the noninoculated control, and X = number of healthy plants in the inoculated control. The formula used to express the suppressiveness index (SI) is $SI = 1 - \frac{X}{A}$ where X = number of diseased plants in the inoculated treatment and A = number of healthy plants in the uninoculated treatment. The SI value will range from 0 to 1. The lower the SI, the greater number of diseased plants. Henis et al. (103) found that suppressiveness of a soil to R. solani could be increased by successive monoculture of radish plants. In studying control of radish damping-off caused by R. solani, 8.2 X 10^9 T. harzianum conidia/kg soil, pentachloronitrobenzene (PCNB) at 4 g/kg soil, or T. harzianum plus

PCNB at those concentrations were added to a loamy sand soil and incubated for 1 week. Soils were seeded with radish five consecutive times at 1 week intervals. All of the treatments resulted in a decrease in disease with time, but the combination of *T. harzianum* and PCNB gave the most effective control of radish damping-off. Populations of *R. solani* followed the same trends as disease incidence.

In further work, soils suppressive to *R. solani* were developed by successive replantings of radish and cucumber (147). Mycelial mats of *R. solani* were buried in untreated soil, soil made suppressive by successive weekly replanting of radishes, or soil planted with radishes in the same manner but without *R. solani*. *Trichoderma* spp. were retrieved from almost all of the mycelial mats incubated in the suppressive soil, but only from a small percentage from those incubated in the other two treatments. *Aspergillus* sp., *T. aureoviride*, *T. koningii*, and two isolates of *T. harzianum* were compared for their ability to induce suppressiveness in a conducive soil. The Fort Collins isolate of *T. harzianum* was most effective in inducing suppressiveness. Suppressiveness has also been developed in low pH soils consecutively replanted to alfalfa or sugarbeet (54). Natural populations of *T. harzianum* increased as suppressiveness increased.

Trichoderma hamatum also has been under investigation as a biological control agent (55, 100, 101). In agar culture, the hyperparasite coiled around the hyphae of *R. solani*, and eventually caused the lysis and separation of the host cells (55).

A pH preference for slightly acid soils was also indicated for *T. hamatum* (55), as for *T. harzianum* (147). In a clay loam soil that was artificially infested with *T. hamatum* and *R. solani*, damping-off of radishes in the first three plantings was significantly lower in soil acidified to pH 6.0 than in soil at pH 8.1 (55). By the fourth planting, disease was very low in both treatments. Possibly, the increase in *Trichoderma* populations induced by consecutive plantings overcame the pH effect.

Soil temperature influenced the efficacy of *T. hamatum* as a biological control agent (101). Radish seeds treated with *T. hamatum* were planted into *R. solani*-infested soil and damping-off was assessed at temperatures from 20 C to 35 C. Disease was controlled at all temperatures tested except 35 C, which also is not conducive to growth of *T. hamatum* in culture. Concentration of *T. hamatum* inoculum affected the degree of biological control obtained (55, 101).

Trichoderma hamatum as a seed treatment was superior to two fungicides, Captan and PCNB, for control of *R. solani* damping-off of radishes (100). PCNB gave as much control as *T. hamatum* 3 days after planting, but was not as effective by 5 or 7 days. In a replanting with untreated seeds, the *T. hamatum* treatment still was effective in reducing disease. By 7 days, almost all of the plants were diseased in the chemical treatments.

Trichoderma hamatum was compared with *Chaetomium globosum* for controlling damping-off of radish (101). Radish seeds treated with *T. hamatum* gave the least disease. The combination of *T. hamatum* and *C. globosum* was not significantly different from *T. hamatum* alone, and *C. globosum* by itself provided no protection. Results were similar when the same soil was replanted with untreated seeds. Soil seeded with *T. hamatum*-treated seed contained the fewest *R. solani* propagules, and the most propagules of *Trichoderma* spp. Soil sown with seeds treated with *T.*

hamatum and C. globosum contained about half as many Trichoderma spp. propagules and almost three times the number of R. solani propagules as soil sown with seeds treated with only T. hamatum.

The percentage of diseased radish seedlings decreased when seed were treated with T. hamatum plus chitin, R. solani cell walls, or nonpathogenic R. solani (101). Trichoderma hamatum alone did not significantly reduce disease in this experiment. Cellulose or peat did not augment the effect of T. hamatum, yet the population density of Trichoderma was very high in these treatments. The authors suggest that T. hamatum may have utilized organic compounds in peat, and that peat should also decrease the pH of the soil environment. Trichoderma hamatum produces cellulase, therefore, the ability to utilize cellulose as a carbon source was reflected in the increased in T. hamatum population (100). It may be possible that cellulose or peat enhanced the saprophytic but not the hyperparasitic qualities of T. hamatum during this experiment. In other work, T. hamatum added to R. solani-infested soil decreased incidence of stem rot of beans (55).

Studies have indicated that T. hamatum was as effective as T. harzianum as a biological control agent against Rhizoctonia diseases (55, 100, 101).

Basidiomycetes. There are very few examples of hyperparasites of the root-infecting basidiomycetes. Armillaria mellea was parasitized by Trichoderma via coiling followed by lysis in agar culture at pH's of 3.4 -5.1, but not at pH 7.0 (16). When studying the interaction of Peniophora gigantea and Heterobasidium annosum (Fomes annosus), no evidence was found that the inhibition caused by P. gigantea was due to antibiotics (121, 122). However, H. annosum hyphae in direct contact with P. gigantea became granulated and vacuolated. This interaction was termed hyphal interference, and may be likened to destructive contact hyperparasitism. Peniophora gigantea is used in Europe as a biological stump treatment for control of H. annosum.

Parasitism of beneficial root-infecting fungi, the endomycorrhizae

When spores were first observed within the chlamydospores of Endogonaceae spp., researchers weren't sure if these spores were part of the chlamydospores or were those of a hyperparasite (91, 161). Godfrey (93) isolated a slow-growing, non-sporulating fungus with septate mycelium from infected spores of a mycorrhizal fungus, then identified as Endogone. Sparrow (189) described the chytrid, Rhizidiomycopsis stomatosa as an epibiotic parasite of Gigaspora margarita, the rhizoidal system of which arose from an apophysis inside the parasitized chlamydospore. This parasite was first isolated by Schenck & Nicolson (181), who found that the chytrid was prevalent in chlamydospores after very heavy rains. The hyperparasitic host range included other Gigaspora spp., Glomus spp., and Acaulospora laevis.

Another chytrid, Phlyctochytrium sp., has been observed as a parasite of Glomus macrocarpus var. geosporus (179), G. epigaeces, G. fasiculatus, and Gigaspora margarita (63). Spores of G. epigaeus were more susceptible to parasitism by Phlyctochytrium sp. after soaking in H_2O_2, NaOCl, or KOH, but less susceptible if soaked in HCl (63). It was suggested that melanin content of spore walls may be related to susceptibility of chlamydospores to hyperparasites.

Two members of the Fungi Imperfecti, Anguillospora pseudolongissisma and Humicola fuscoatra, were identified as parasites of Glomus spp.

chlamydospores (63). A. pseudolongissima infected 69.3% of the G. epigaeus spores or 94.6% of the G. fasciculatus spores in autoclaved soil while H. fuscoatra infected 70.9% of G. epigaeus spores, or 91.2% of G. fasciculatus spores in autoclaved soil. Parasitism was less in water agar. A. pseudolongissima-infected spores contained sausage-shape hyphae while H. fuscoatra-infected spores contained slender hyphae and/or aleurospores. H. fuscoatra has also been noted as a parasite Phytophthora megasperma var. sojae oospores (188).

The parasitism of beneficial mycorrhizae may be a negative side aspect to the use of hyperparasites as biological control agents. An attempt to control hyperparasites of G. egigaeus in sterile sand with fungicides interfered with germination of mycorrhizal spores (63). It is suggested that mycorrhizae be included in host range tests of potential hyperparasitic biological control agents.

Potential Biological Control Agents

Not one of the promising hyperparsitic agents discussed is used in commercial agricultural production to date. This is not to say that biological control will not be effective on a commercial scale, or is not taking place in certain microenvironments. The advancement of biological control in plant pathology has only recently attracted the renewed interest of researchers.

A wide range of organisms have been cited as hyperparasites. The biotrophic, contact or haustorial hyperparasites do not appear to cause enough damage to their hosts to be of value as biological control agents. Other hyperparasites are themselves plant pathogens, which may preclude their use. The destructive hyperparasites, however, can actively suppress and in some cases destroy their hosts. This is the group that plant pathologists will need to exploit as hyperparasitic biological control agents.

Once the efficacy of a potential biological control agent is established, other considerations become important. The variability of isolates of parasites and the host must be considered. For example, some strains of *T. harzianum* infect only *R. solani*, whereas others will infect other plant pathogens as well (55, 82, 99). Strains of *R. solani* may differ in susceptibility to *Trichoderma* (215). Strain selection could be important to the success of biological control.

A few researchers are investigating the inclusion of biological control agents in integrated pest management systems. Already, the additive effect of *T. harzianum* combined with PCNB in control of *S. rolfsii* (56) or *R. solani* (83, 103), and of *T. harzianum* following methyl bromide for *R. solani* control (83) has been demonstrated.

Biological control efficacy may be adversely affected by the use of pesticides. The detrimental effects of cotton herbicides on the effectiveness of *Trichoderma* in controlling *R. solani* was revealed by Curl et al. (62). Fungicides prevented the establishment of *Hansfordia* as a biological control of leaf spot in the greenhouse (193). Presently, *T. harzianum* mutants are being screened for tolerance to fungicides (167). Information obtained on the interaction of biological and chemical controls may aid in establishing a successful disease control program.

Although there is not yet a hyperparasitic biological control agent in commercial use, it may not be long before *Trichoderma* will be the first.

MATERIALS AND METHODS

Host fungi

The Oomycetes selected for study were *Aphanomyces cochlioides* Drechs. obtained from C. L. Schneider, Michigan State University, East Lansing; *Aphanomyces euteiches* Drechs. isolate L30 from W. F. Pfender, University of Wisconsin, Madison, Wisconsin; *Phytophthora cactorum* Leb. and Cohn (Schroet.) from J. E. Mitchell, University of Wisconsin, Madison, Wisconsin; *Phytophthora megasperma* (Drechs.) var. *sojae* Hildb. [=*P. megasperma* (Drechs.) f. sp. *glycinea* (Kuan and Erwin) (137)] races 1, 3, and 4 from A. F. Schmitthenner, Ohio Agricultural Research and Development Center, Wooster, Ohio; *P. megasperma* var. *sojae* race 7 from F. A. Laviolette, Purdue University, Lafayette, Indiana; *Pythium aphanidermatum* (Edson) Fitzpatrick from R. D. Lumsden, USDA, Soilborne Diseases Laboratory, Beltsville, Maryland. *Pythium ultimum* Trow was isolated from diseased soybeans (182), and was maintained in our laboratory. The following were obtained from the University of California, Riverside, California: *Phytophthora capsici* Leonian, A1 isolate P504 and A2 isolate P505; *Phytophthora citrophthora* (Smith and Smith) Leonian, isolate P405; *Phytophthora megasperma* Drechs. [=*P. megasperma* (Drechs.) f. sp. *medicaginis* (Kuan and Erwin) (137)] isolate P1057; *Phytophthora parasitica* A1 isolate P991 and A2 isolate P731.

Aphanomyces spp. and *Pythium* spp. were maintained on water agar. *P. megaspmera* var. *sojae* (*Pms*) and *P. cactorum* were normally maintained on

Schmitthenner's *Phytophthora* selective medium (83). The other *Phytophthora* spp. were maintained on modified V-8 juice agar (178). To obtain oospores, two agar disks from a culture plate of the colonized maintenance medium were transferred to opposite sides of petri dishes containing the appropriate medium. *A. cochlioides* and *A. euteiches* were grown for 40-45 days in oatmeal broth (184). All *Phytophthora* spp. were grown for 30-34 days in clarified V-8 juice supplemented with 30 mg/l of cholesterol (15). *P. aphanidermatum* and *P. ultimum* were grown in clarified V-8 juice for three weeks. All cultures were incubated in the dark at 23±2 C.

Cultures of *A. cochlioides*, *A. euteiches*, *P. cactorum*, and *P. megasperma* var. *sojae* were frozen at -12 C to -20 C for 1 hour to kill mycelium and to aid in oospore separation. Mycelial mats of *P. aphanidermatum* were dried on filter paper in sterile petri dishes prior to oospore extraction. Cultures of *A. cochlioides*, *A. euteiches*, *P. aphanidermatum*, and *P. ultimum* were ground in an Omni-mixer (Ivan Sorvall, Norwalk, Connecticut) at a rheostat setting of 70 using 10, 5-second pulses. Those of all *Phytophthora* spp. were similarly ground using a rheostat setting of 40 for 10 min. The oospores were passed through an 80 µm nylon mesh screen which retained mycelial fragments. The fragments were reground using the same procedure, to release more oospores. The oospore suspension was centrifuged for 15 seconds at 3100 *g*. The supernatant was removed by suction, leaving the oospores in the pellet. The oospores were resuspended in sterile deionized distilled water (SDDW) and centrifuged again. The oospores were stored in SDDW at 4±2 C until use.

Chlamydospores of the mycorrhizal fungus *Glomus etunicatus* Becker

Gerdeman, as identified by B. A. Daniels, Kansas State University, Manhattan, were isolated from soil by N. Bolgiano and C. E. Nelsen, Michigan State University, East Lansing, using methods described by Caveness and Jensen (53). Chlamydospores were collected on an 8 μm membrane filter (Millipore Filter Corporation, Bedford, Massachusetts) in a Millipore Swinnex-13 holder. The spores were surface-sterilized in the filter holder with 5.25% NaOCl for three minutes, and then rinsed three to five times with SDDW. The membrane filter was placed in SDDW in a sterile petri dish to release the spores. Chlamydospores were used immediately.

Maintenance of potential hyperparasites

The parasites previously isolated from Pms oospores (118, 188) that were used in laboratory, greenhouse and/or field experiments, included *Diheterospora chlamydosporia* (Komishko) Barron and Onions, *Humicola fuscoatra* Traaen., and *Hyphochytrium catenoides* Karling which were maintained on potato-dextrose agar (PDA), and *Pythium monospermum* Pringsh. which was maintained on water agar. F4 was maintained on PDA. F4 was a fungus isolated from an infected oospore by the author. It had septate hyphae, but failed to sporulate on various culture media. Other test organisms that had no past history as hyperparasites were several members of the *Actinoplanaceae*: *Amorphosporangium auranticolor* Couch isolate 255, *Ampullariella regularis* Couch isolate 31, and *Spirillospora albida* Couch isolate 163; a member of the *Micromonosporaceae*, *Micromonospora* sp. isolate 44, all provided by J. N. Couch, University of North Carolina, Chapel Hill, North Carolina. Another oospore hyperparasite (88) used was *Actinoplanes missouriensis* Couch isolate 431. Two other species of Actinoplanes tested for their ability to parasitize Pms oospores were A. philippinensis Couch isolate 455, and A. utahensis Couch isolate 37. The cultures of the Actinoplanes spp. were obtained from M. M. Hoehn, Lilly Research Laboratories, Indianapolis, Indiana. All actinomycete isolates had been lypholized and in storage since 1957 or 1958. They were easily recovered by transfer to chitin agar (145). All isolates were maintained on Czapek's sucrose-nitrate agar (59) made with soil water (J. N. Couch, personal communication). Soil water was prepared by heating, but not boiling, 100 g of field soil in 1000 ml of deionized distilled water for 1.5 h. The soil water was filtered through Whatman #1 filter paper.

Production of potential hyperparasites for laboratory use

H. catenoides was grown in potato-dextrose broth for 30 days. The culture was ground in an Omni-mixer at a rheostat setting of 40 for 10 min, then centrifuged for 15 seconds at 3100 g. The supernatant broth was removed by suction, and the sporangia were resuspended in SDDW. P. monospermum was grown in clarified V-8 juice broth for 4 weeks. The mycelial mats of P. monospermum were harvested and ground in an Omni-mixer at a rheostat setting of 70 using 10, 5-second pulses. D. chlamydosporia and H. fuscoatra were cultured on PDA for 30-32 days. Suspensions were prepared by scraping hyphae and spores from agar plates, and grinding with SDDW in an Omni-mixer at a rheostat setting of 40 for 10 minutes. All the actinomycetes were grown on Czapek's sucrose nitrate agar for 30-32 days. Sporangia and mycelia were harvested by scraping the agar plates and grinding the scrapings in an Omni-mixer at a rheostat setting of 30 for 10 min. All test organisms were stored at 4±2 C as aqueous suspensions until use.

Evaluation of hyperparasitism *in vitro*

The membrane filter-water agar technique used by Jimenez-A (126) to assess oospore germination, was adopted here for host-parasite studies. Approximately 10^3 spores of the host fungi were applied using mild suction to 0.4 µm polycarbonate membrane filters (Nuclepore, Pleasanton, California), 1 X 1 cm square. Membranes bearing the oospores were floated on SDDW until used. Two percent water agar was poured into 5-cm-diameter plastic petri dishes. Just before the agar solidified, membrane filters were placed, spores down, on the agar surface. After the agar had solidified, the outline of the membrane was traced with a marking pen on the bottom of each plate to allow for easy location of the spores when viewing microscopically. The membranes were removed from the agar, which was inoculated with a suspension of the test hyperparasite, at a concentration of 10^4 propagules per plate. The propagule types were sporangia for *H. catenoides*, oospores for *P. monospermum*, chlamydospores for *D. chlamydosporia*, and aleuriospores for *H. fuscoatra*. Sporangial counts were used to determine actinomycete concentrations for *Actinoplanes* species, while concentrations of the other actinomycetes were determined by plating on Czapek's sucrose-nitrate agar and counting colony forming units.

In accordance with moisture conditions that were thought to favor each hyperparasite (118, 188), the agar was either flooded with 2 ml of SDDW or a thin water film was obtained by using 0.5 ml of SDDW. Plates were incubated for two weeks at 25±1 C in the dark. All experiments were arranged in a completely randomized design. There were 2-4 replications per test organism per experiment, and each experiment was repeated 1-6 times. At the end of the incubation period plates were viewed immediately, or were held at 4±2 C for no longer than a week and then viewed. On each plate, 100 spores were evaluated for infection by the test hyperparasite. Oospores were considered infected when mycelia or spores of the hyperparasite had replaced the oospore cytoplasm, or if the cytoplasm was granulated and disorganized. A vital stain was sometimes used to aid in assessment of hyperparasitism.

Evaluation of host spore viability using tetrazolium bromide

Tetrazolium bromide [3-(4,5-dimethyl-thiazol-2-yl)-2*H*-tetrazolium-bromide] (MTT) was used by Zimmerman and Young (224) to assess oospore viability from crossed pairings of *Phytophthora palmivora* and *P. capsici*. Ribeiro (177) used the stain for detection of viable oospores of *Phytophthora infestans*, and also recommended MTT for other *Phytophthora* species. MTT blocks the electron transport chain, and results in a rose-colored formazan product (6). It was tested as a stain for oospores of *A. cochlioides*, *A. euteiches*, *P. cactorum*, *Pms*, *P. aphanidermatum*, and *P. ultimum*. A 1:1 mixture of a 0.1% aqueous solution of MTT (Aldrich Chemical Company, Inc., Milwaukee, Wisconsin) and an oospore suspension (usually 0.5 ml of each) was prepared in a sterile test tube. The oospores were incubated at 23±2 C or 35±1 C for 2, 24, 47, and 72 hr to determine the optimum time period and temperature for staining. Two replications were prepared per treatment. Each replication was sampled three times, with 100 oospores counted in each sample. Rose-colored oospores were scored as healthy.

Environmental factors affecting hyperparasitism of *P. megasperma* var. *sojae* oospores by *A. missouriensis* and *H. fuscoatra*

The soil used was Capac loam from a family of fine-loamy, mixed, mesic Aeric Ochraqualfs (206, 207). The moist bulk density ranged from 1.43-1.73 g/cm^3 and organic matter, 1-3%. The soil pH was 5.4 as measured in water (1:1, v/v). The soil was collected from a *Phytophthora*-infested field in Shiawassee County in 1980. It was necessary to sterilize the soil prior to use because of interference by natural hyperparasites. Nine cm-diameter glass petri dishes were used as experimental units.

MES, MOPS, and HEPPS buffers (0.05M) in SDDW were used to adjust soil pH (96). The buffers were adjusted with NaOH to a pH dependent on the buffering capacity of the soil such that when added to soil the desired soil pH would be obtained. A range of pH from 4.7 to 8.0 was obtained at approximately 0.5 unit intervals. MES was used to obtain pH 4.7-4.8, 5.4-5.5 and 6.0. MOPS was used to obtain pH 6.5-6.6 and 6.7-7.2. HEPPS was used to obtain pH 7.3-7.7 and 7.9-8.0. Twenty grams of air-dried soil were weighed into each of 21 petri dishes which were autoclaved for 1 h. Thirty ml of the buffer solutions were added to each dish. Equilibration for at least one week was required for pH to stabilize.

Approximately 10^3 *Pms* oospores were applied with suction to 1 X 1 cm squares of 25 μm pore size monofilament nylon fabric (Tetko, Elmsford, New York) (149). A suspension of *A. missouriensis* or *H. fuscoatra* was applied directly on top of the oospores at a concentration of 10^4 propagules per square. Mesh squares with only oospores served as controls. For each pH treatment, six mesh squares were placed on the soil in each petri dish. After one week of incubation in the dark at 25±1 C, the membranes were retrieved and 100 oospores on each membrane were assessed for parasitism. In some experiments, oospores were stained with MTT.

Soil that had not been air-dried to avoid error caused by hysteresis (104) was used in experiments to study the effect of soil moisture on hyperparasitism. The soil was autoclaved, and 700 g was placed on a ceramic pressure plate (Soil Moisture Equipment Company, Santa Barbara, California) and adjusted to -0.3, -1, -5, -10, and -15 bars matric potential. The soil was then removed from the plate and quadruple-bagged in plastic until used. Soil weights showed no significant loss of water over time. Two soil plates were prepared for each moisture treatment per parasite. For the 0 bar treatment, 40 g of soil were weighed into each petri dish and flooded with 40 ml deionized distilled water. For treatments less than 0 bars, 20 g of the adjusted soil was placed in a petri dish, on top of which were placed a 9 cm diameter circle of 0.05 mm mesh nylon fabric. Twenty grams of soil was placed on top of the mesh. Three mesh squares with oospores and sporangia of _A. missouriensis_, or spores of _H. fuscoatra_, or with only oospores were placed under the 9 cm-diameter mesh fabric for water potentials less than 0 bars or were immersed under the water for 0 bars.

To study the effect of temperature on parasitism of oospores by _A. missouriensis_, soil was flooded and adjusted to a pH range of 6.6-6.8 with MOPS buffer, as described previously. For _H. fuscoatra_, the soil was adjusted to -0.3 bars, but the pH was left at 5.4. The petri dishes were placed in incubators at 5±1, 10±1, 15±1, 20±1, 25±1, or 30±1 C. One dish was prepared per temperature per hyperparasite or control. Six mesh squares bearing oospores plus parasite were placed in each dish.

After one week of incubation, parasitism was assessed by counting 100 oospores per square. Control oospores were stained with MTT for viability determination.

Electron microscope studies of oospore penetration

Transmission electron microscopy was used to investigate the parasitism of *Pms* oospores by *A. missouriensis* and *H. fuscoatra*.

Membrane filter-water agar plates were prepared with *Pms* oospores and suspensions of *A. missouriensis* or *H. fuscoatra* as described in the section Evaluation of Hyperparasitism *in vitro*. After 4 days of incubation in the dark at 25 C, a square of agar including the interaction zone was removed and prepared for transmission electron microscopy by K. K. Baker (Director, Electron Optics Laboratory, Michigan State University, East Lansing) using methods of Hooper et al. (111). Specimens were examined with a Phillips 300 transmission electron microscope operated at 60 kV.

Biological control experiments *in vitro*

A variety of soils was collected from soybean fields with plants infected with *Pms*. Each of these samples was tested in the laboratory for the presence of *Pms* using two-day-old Hark soybean seedlings as baits (89). The Hark variety is susceptible to all races of *Pms* known to be present in Michigan (148). Twenty grams of coarsely-sieved (5 mm) soil was placed in a glass petri dish and flooded with 30 ml distilled water for five days to stimulate oospore germination. Five seedlings approximately 36 h old, germinated in vermiculite, were placed in each plate, 5 seedlings/plate. Ten soils were evaluated and five replications were used per sample. Disease was rated after 3-4 days on a scale from 0 to 3 with 0 = no disease, 1 = mild disease identified by slight browning of the roots, 2 = moderate disease identified by root browning and the absence of secondary roots, and 3 = severe disease identified by stunting, browning and absence of secondary roots (118). Positive identification of Phytophthora infection was obtained by observing the sporangia of Pms on the root surface with the aid of a dissecting microscope. The natural soils causing severe disease were used in later experiments.

The same petri dish bioassay was used to assess biological control by H. fuscoatra, H. catenoides, and P. monospermum. Aqueous suspensions of each of the hyperparasites were added to 20 g of naturally infested Pms soil in petri dishes at a concentration of 10^4 propagules/dish. The volume was brought up to 30 ml of liquid. Plates were incubated 7 days before seedlings were added. Disease was assessed as described above after 3-4 days.

Production of inoculum for greenhouse and field experiments

Pms inoculum was produced in clarified V-8 juice broth (15), on millet seed (126), or in soybean seedlings (118). Inoculum was usually grown for approximately 30 days, and frozen for 1-4 h before use to kill the mycelium. In an attempt to identify suitable media for production of hyperparasite inoculum, different substrates were inoculated with H. fuscoatra, H. catenoides, or P. monospermum. These included seeds of barley, millet, soybean, wheat, and rye; ground preparations of wheat bran, alfalfa hay, linseed meal, beet pulp, sawdust, and crab shell; ground stems and leaves of wheat, corn, and barley; and onion leaves. Onion leaves had been found to be a suitable substrate for H. catenoides

(221). Fifty ml of each substrate was placed in 125 ml Erlenmeyer flasks. Fifteen ml distilled water was added to flasks inoculated with *H. fuscoatra* and 30 ml to flasks inoculated with *H. catenoides* or *P. monospermum*. The flasks were autoclaved for 1 h each on successive days. After 3-4 weeks, the cultures were assessed for growth of the respective parasite.

Difficulties in growing *H. catenoides* stimulated an attempt to grow this chytrid on an Oomycete that had already colonized a substrate. *Pythium aphanidermatum*, a known host of *H. catenoides* (15) was selected. Barley seed, prepared as above except with 40 ml of distilled water, was inoculated with *P. aphanidermatum*. After 8 weeks when the barley was completely colonized, the cultures were inoculated with *H. catenoides*.

Growth of *H. catenoides* was also tested on the following liquid media: Chytrid Medium [(=Emerson's YpSs (86)] prepared without agar; corn meal extract prepared by steaming 20 g corn meal in 400 ml of distilled water for 30 min, straining, and autoclaving; diluted V-8 juice broth made with 50 ml of V-8 juice and 950 ml distilled water; potato-dextrose broth; and *Pms* extract prepared by grinding six 3-week-old mycelial mats of *Pms* in 200 ml distilled water in an Omni-mixer at a rheostat setting of 40 for 10 min, autoclaving, centrifuging and using the supernatant liquid. Fifty ml of each medium was dispensed into 250 ml Erlenmeyer flasks. Each medium was inoculated with a colonized agar disk of *H. catenoides*.

A coarse grade of perlite (Krum Horticultural Perlite, Silbrico Corporation, Chicago, Illinois) was used as a substratum (J. A. Lewis, personal communication) for *A. missouriensis* and *H. fuscoatra*. A given amount of perlite in flasks or autoclavable plastic bags was saturated with either Czapek's broth made with soil water for A. missouriensis or potato-dextrose broth for H. fuscoatra. The flooded perlite was autoclaved, the excess solution decanted, and the media were inoculated with sporangial or spore suspensions, respectively.

Carriers for biological control agents

Carriers used for A. missouriensis, H. fuscoatra, H. catenoides, P. monospermum, or F4, an unidentified parasite, included soybean seed (as a seed treatment), vermiculite (Grace Horticultural Products, Cambridge, Massachusetts), Turface (=Montmorillonite clay, International Minerals and Chemical Corporation, Des Plaines, Illinois), attapulgus clay 18/35 AA RVM (The Upjohn Company, Kalamazoo, Michigan), and Readi-earth mix (Grace Horticultural Products, Cambridge, Massachusetts). Spores and mycelial suspensions prepared as described under the section on "Production of Potential Hyperparasites for Laboratory Use" were applied to each carrier by mixing in a plastic bag. The treated material was spread in a thin layer and allowed to air-dry. Normally when seed were treated with a given hyperparasite, no sticking agents were used. Soybean seeds absorbed water very rapidly, and the seeds were dried as quickly as possible by rolling the treated seeds on filter paper. Colonized barley seed and perlite also were used as carriers for H. fuscoatra. A given volume (800-1000 ml) of barley was autoclaved for 1 h in an autoclavable bag, fitted with a 0.05 cm diameter glass tube plugged with cotton for air exchange. The barley was inoculated with a spore suspension, and the bags were occasionally shaken to provide for inoculum dispersal. The method for growing inoculum on impregnated perlite was described under Production of Inoculum for Greenhouse and Field Experiments.

To determine concentrations of propagules on the seeds, 10 seeds were soaked in 10 ml water for 15-30 min. Seed coats were removed from the seed and added to the liquid which was brought up to 50 ml, then ground in an Omni-mixer for 10 min at a rheostat setting of 30. The suspension was poured through an 80 m sieve and enumerated by the microsyringe method (135), or by counting colonies of *A. missouriensis* on Czapek's agar and *H. fuscoatra* on potato-carrot agar (1:1, potato-dextrose agar:carrot agar). Concentrations of organisms on vermiculite or Readi-earth mix were determined by microsyringe counts, whereas those on clays or perlite were determined by counting colony forming units.

Greenhouse experiments

The soybean cultivar Hark was used in all experiments. Conover loam from a family of fine-loamy, mixed, mesic Udollic Ochraqualfs (206), collected from a severely diseased soybean field in 1978, was used initially in experiments. Soils were diluted with coarse sand (1:1 and 1:3, v/v). Other potting media were coarse sand, Michigan State University greenhouse mix (1:1:1, sand:peat:soil, v/v), and Readi-earth potting mix. Pots were either 8 cm-diameter styrofoam cups, 20 x 14 x 5 cm diameter styrofoam containers, 20 x 10 x 5.5 cm plastic containers, or 11 cm diameter clay pots.

Pms oospores were added to the potting media with continuous mixing to ensure uniform distribution of the plant pathogen. Clay or barley seed bearing the hyperparasites was mixed into the potting media following infestation with *Pms* inoculum. *A. missouriensis*, *H. fuscoatra*, *H. catenoides*, and *P. monospermum* were also tested without carriers. The containers were incubated on greenhouse benches for 3-7 days, and kept flooded or just below saturation (preferable for *H. fuscoatra* treatments) with tap water. The excess water in the flooded treatments was allowed to evaporate for approximately 24 h before planting to improve emergence of the soybeans. Treated or untreated seeds were directly seeded into the infested potting media, or when carriers other than seed were used, untreated seeds were germinated in vermiculite for about 36 h before transplanting. Controls in biological control experiments included soil with or without *Pms*. *Pms* inoculum was always frozen for 1-4 h at -12 C to -20 C to kill the mycelium prior to use. The effects of culture medium, oospore concentration, and watering regimes on disease production by *Pms* oospores were investigated independently of biological control tests.

Field experiments: Biological control with hyperparasites

During the summer of 1978, soybean fields were observed for *Phytophthora* root rot in Ingham, Genesee, and Shiawassee Counties, Michigan. Two severely diseased fields were observed. One field was located on the east side of Reed Road, almost midway between Cronk and Juddville Roads in Section 32 of Shiawassee County. This property belongs to Mr. John Majzel. The other field was located on the east side of Byron Road, between Lansing and Newburg Roads, north of the railroad tracks, near the intersection of Lansing and Byron Roads in Section 14 of Shiawassee County. This area was being farmed by the Leo Shuman family. Soil samples were collected from both farms, and disease potential was assessed using the petri dish assay. S. D. Cohen, Senior Research Assistant, Michigan State University, determined that only race 1 of *Pms* was present in each field using the petri dish assay (148) and differential cultivars (142). Arrangements were made to place a field plot in each of these areas in 1979.

1979. In Mr. Majzel's field, a plot 6.4 m X 18.3 m (21' X 60') was laid out in a split-plot design consisting of four whole plots, each including four hyperparasites. The whole plots were replicated three times, and two carrier systems comprised subplots. The rows were 3 m (10') in length with 0.4 m (15") row spacing. Approximately 16 seeds were planted per meter using a belt seeder. In Mr. Shuman's field the plot was 4.6 m X 54.8 m (15' X 180'). The rows were 3 m (10') in length with 0.6 m (24") row spacing. Seeds were sown at a rate of 25 seeds per meter using a belt seeder. The experiment was designed as a split-plot with five whole plots, each including five hyperparasites. The whole plots were replicated three times, and two carrier systems comprised subplots.

A. missouriensis, H. fuscoatra, H. catenoides, P. monospermum, and an unidentified oospore parasite (F4) were tested as biological control agents. F4 was used only at the Shuman Farm. The other hyperparasites were cultured as described in Production of Potential Hyperparasites for Laboratory Use. Cultures of all organisms were ground for about 10 min with sterile distilled water using an Omni-mixer. The suspensions were then washed with repeated centrifugation and reground for 1 min. All parasites were used to coat soybean seeds, cv. Hark, by applying 45 ml of a hyperparasite suspension plus 0.45 g of Cellulose Gum (Hercules Powder Company, Wilmington, Delaware) to 450 seeds. Excess liquid was removed rapidly from the seeds by drying them on filter paper. Controls included seed treated with cellulose gum and untreated seed.

Vermiculite was used as a carrier for all five hyperparasites. Czapek's broth was added to the vermiculite for A. missouriensis and PDB for H. catenoides and P. monospernum, but not for H. fuscoatra and F4. Aqueous suspensions of the hyperparasites were mixed with vermiculite in a plastic bag. The ratio of liquid to vermiculite was 1:2 (v/v) in all treatments. The treated vermiculite was air-dried at 23±2 C. For each row, 50 ml vermiculite inoculum of A. missouriensis, H. fuscoatra, and F4 or 40 ml for H. catenoides or P. monospermum was spread on top of 75 untreated Hark seed on the belt of the seeder, and incorporated in the row with the seed. Untreated vermiculite was included as a control.

Inoculum also consisted of H. fuscoatra and F4 grown on barley seed. After the seed was thoroughly colonized (1.5 months) the seed was dried at 23±2 C. Sixty ml of the F4-colonized barley seed or 35 ml of H. fuscoatra-colonized barley was mixed with 75 seed and applied to each row with the belt seeder.

Soybean emergence was counted and post-emergence disease was also evaluated in each plot.

1980. Four separate biological control experiments were done. In one experiment, field soils was naturally infested with Pms and in another, Pms-colonized millet seed was used to artificially infest the soil. The third and fourth experiments were designed to test the efficacy of two potential biological control agents when applied in the fall or spring prior to planting.

An experimental plot, 4.9 m X 53.3 m (16' X 175'), was put in Mr. Shuman's field, naturally infested with Pms, and adjacent to last year's plot. A similar experiment was set up at the Botany and Plant Pathology Farm, Michigan State University, in a 9.1 m X 24 m (30' X 80') area, in which the soil was artificially infested with Pms. In both experiments a completely randomized design was utilized. The rows were

6.1 m (20') in length with a 0.9 m (3') spacing between rows. Seeding was done with a belt seeder at a rate of 33 seeds/m.

A. missouriensis, *H. fuscoatra*, and *H. catenoides* were applied as seed treaments using methods previously described. The average concentration of inoculum per seed was 2.5 x 10^5 sporangia for *A. missouriensis*, 1 x 10^5 spores for *H. fuscoatra*, and 2.5 x 10^4 sporangia for *H. catenoides*. Other carriers used were Turface for *A. missouriensis*, colonized barley seed for *H. fuscoatra*, and vermiculite for *H. catenoides*. Mycelium and sporangia of *A. missouriensis* grown in nutrient broth were used to treat the Turface. Two hundred ml of Turface inoculum was applied per row. One hundred ninety-five ml of barley seed colonized by *H. fuscoatra* (30 days old) was applied per row. A control of barley seed colonized by *Trichoderma harzianum* Rifai was applied at the rate of 195 ml/row. Inoculum of *H. catenoides* (prepared by Su-chan Hsu, Michigan State University, East Lansing, Michigan) was grown in suspension cultures of 0.2 to 0.5% PDA which were later added to vermiculite. Approximately 1 l of the inoculum was applied per row. Untreated controls were included. Inoculum of *Pms*, for the plot at the Botany and Plant Pathology Farm, was produced on millet seed and was applied to each row with a belt seeder at seeding. The final concentration of oospores (mycelium killed by freezing) was 10^6/row or 30/g of treated area 5 cm wide X 7 cm deep X 3 m long (2" X 3" X 120").

In the other two field experiments, additional time between application of the hyperparasites and seeding was allowed. Inoculum of *A. missouriensis* and *H. fuscoatra* was incorporated into two plots in the *Phytophthora*-infested field of Mr. Shuman. Untreated rows served as controls. One plot was treated November 19, 1979 and the other May 28, 1980, prior to planting in June 1980. The size of each plot was 9 m X 9 m (30' X 30'). Rows were 2.4 m (8') in length with a row spacing 0.8 m (36"). Both plots were arranged in a randomized complete block design with 9 replications.

An aqueous suspension of A. missouriensis was obtained by grinding scrapings from 85 agar plates for 5 min in 60 ml sterile distilled water in an Omni-mixer at a rheostat setting of 40. H. fuscoatra inoculum was prepared from 60 PDA plates in a similar manner. For each treatment, 60 ml of the hyperparasite suspension was sprayed onto 500 ml Readi-earth mix after it was finely ground dry in a Waring Blendor. Sterile distilled water was sprayed on the mix to serve as the control. All preparations were air-dried at 23±2 C. Five hundred ml of inoculum was spread on the soil surface by hand in a band about 0.15 m (6") in width, then was roto-tilled into the rows of both plots to a depth of 0.15 m (6"). Based on microscopic counts made in the laboratory before application to the soil, final concentrations were 84 sporangia/g soil for A. missouriensis and 2.6 X 10^3 spores/g soil for H. fuscoatra. Weed control was provided by periodic rototilling, but prior to planting, Round-up herbicide (Monsanto Agricultural Products Company, St. Louis, Missouri) was used at a rate of 0.9 kg/ha (2 lbs/A) in the fall-treated plot. Both plots were seeded at the same time with a belt seeder at a rate of 33 untreated seeds cv. Hark/m.

Soybean emergence was counted and post emergence disease was evaluated in each plot.

The relative concentration of Pms was roughly estimated using the soybean seedling assay (89), and using soybean stem segments as a substitute for leaf disks as baits (51). Usually, three soil subsamples were assayed per row. For the seedling assay, 10 seedlings cv. Hark were tested with 20 g soil samples flooded with 30 ml distilled water. Soybean stem segments, 3-4 mm in length, were cut from 2-week-old Hark soybean plants between the cotyledons and primary leaves. Ten segments were placed on 20 g soil in each plate and incubated for 18-22 h. The segments were then transferred to a selective medium (183). After three days, the agar plates were flooded with DW, rinsed after 3-4 h, and then reflooded. Plates were viewed under a dissecting microscope at 75X. Each stem segment was rated for presence or absence of Pms sporangia.

1981. In an effort to establish a higher concentration of Pms oospores in soil, microplots (21), employing clay tiles 0.2 m X 0.3 m (8" X 12") were used. The tiles were buried vertically 0.9 m (3') apart in the field at the Botany and Plant Pathology Farm, with about 2 cm extending above the soil surface. Treatments were arranged in a completely randomized design with 5 replicates per treatment.

The hyperparasites A. missouriensis and H. fuscoatra were applied as seed treatments at concentrations of 4.5 X 10^3 and 3 X 10^4 sporangia or spores/seed, respectively. Inoculum of hyperparasites on carriers and of Pms inoculum were thoroughly mixed into the top 10 cm of field soil in each tile by hand using a trowel. A. missouriensis on attapulgus clay was added at a final concentration of 4 X 10^4 CFU/g soil. Thirty-day-old H. fuscoatra-colonized perlite was added to the soil to give a final concentration of 2 X 10^5 CFU/g. Inoculum of H. catenoides was produced by Su-chan Hsu in suspension cultures in 0.2-0.5% PDA for approximately 40 days. Five hundred ml of cultures of H. catenoides were mixed into the soil in each tile.

P. megasperma var. sojae was grown in clarified V-8 juice broth with

30 mg/l of cholesterol for 2-4 weeks. The mycelial mats were harvested, frozen for two hours at -20 C, and ground in a Waring Blendor for ten minutes. The final concentration of oospores was 53/g soil. The control tiles were untreated. Twenty-five soybean seeds (cv. Hark) were planted per tile. The relative concentration of Pms was roughly estimated using the soybean seedling assay (89).

During the growing season of 1981, soybean emergence was counted and post emergence disease was also evaluated.

Statistical Analyses

Where appropriate, tests for statistical significance were performed using analysis of variance. Differences between means were detected with Duncan's Multiple Range Test, or in some cases by Student's t-test.

RESULTS

Tetrazolium bromide as an oospore stain

Staining efficacy varied with the Oomycete (Table 1). Low percentages of Aphanomyces cochlioides, A. euteiches, and Pythium ultimum oospores stained 2-4 days, while 65% or more of the oospores of Phytophthora cactorum, P. megasperma var. sojae (Pms) race 1, and Pythium aphanidermatum stained after 1-2 days. The color reaction of oospores was categorized using the Horticultural Colour Chart (112). Color intensity varied slightly with different oospore lots of each Oomycete, and was always within the limits of the specific colors listed in Table 1. The difference in color may have been due to contamination of MTT with other tetrazolium salts. P. aphanidermatum oospores varied in the shade of Spirea Red manifested within a given batch.

In a time course study done at 23±2 C, none of the test organisms showed significant staining up to the 72 h sampling time. At 35±1 C, not more than 9-10% of the oospores of A. euteiches and A. cochlioides, or 30% of the oospores of P. ultimum were stained after 72 h. In one experiment, sample tubes of these three organisms were left to incubate at 35±1 C for 20 days. After this time period, still only very few oospores of A. euteiches were stained, but 99% of the oospores of A. cochlioides stained Rose Red. Many of the P. ultimum oospores were stained black.

An average of 87% and 79% of the oospores of Pms and P. aphanidermatum, respectively, were stained after 8 h, and more than 85%

Table 1. Stain reactions of several Oomycetous fungi with tetrazolium bromide (MTT)

Fungus	Stained oospores, %[a]	Color[b]	Time
Aphanomyces cochlioides	4	Rose Red	3 days
A. euteiches	4	Spirea Red	3 days
Phytophthora cactorum	89	China Rose	1-2 days
P. megasperma var. *sojae*	85	China Rose	1-2 days
Pythium aphanidermatum	85	Spirea Red	2 days
P. ultimum	30	Spirea Red Rose Red Magenta Rose	2 days 3 days 4 days

[a] Average % of oospores stained from subsamples of two replicate tubes.

[b] Color reaction of the oospores were identified using Horticultural Colour Chart.

of both species were stained after 24 h (Figure 1). The remainder were either not stained or were stained black. The oospores of *Pms* were stained more intensively nearer the oospore wall than in the center, whereas those of *P. aphanidermatum* were evenly stained. At 24 h, the China Red oospores of *Pms* changed to Spirea Red and the cytoplasm became evenly stained in most of oospores viewed. After 24 h, the population of *P. aphanidermatum* oospores began to display different intensities of Spirea Red, and uneven color within a given oospore, even though total numbers of stained oospores increased from that at 8 h. Eighty-nine percent of the *P. cactorum* oospores were stained China Rose by 24 h. The cytoplasm near the oospore wall was more intensely stained in several *P. cactorum* oospores, but most oospores were evenly stained. After 24 h, the numbers of black oospores of *Pms* and *P. cactorum* increased causing a decrease in numbers of rose colored oospores. There was little change in the number of unstained oospores. Staining of *P. aphanidermatum* oospores remained essentially the same. In preliminary experiments, MTT staining of *Phytophthora* oospores after 48 h at 35 C averaged 71, 88, and 77% for *P. capsici*, *P. citrophthora* and *P. parasitica*, respectively. Sixty two % of the oospores of the hyperparasite, *Pythium monospermum*, stained Spirea Red in a 0.1% solution of MTT at 23±2 C.

To test whether MTT was acting as a vital stain, dead oospores killed by autoclaving were compared with living oospores. Three hundred autoclaved or untreated oospores were stained in each of six tubes. Eighty-one % of untreated oospores were stained, whereas only 5% of autoclaved oospore were stained. Oospores not stained rose color were unstained and were not black. Similar results were obtained in another experiment. Staining of viable oospores with MTT also has been

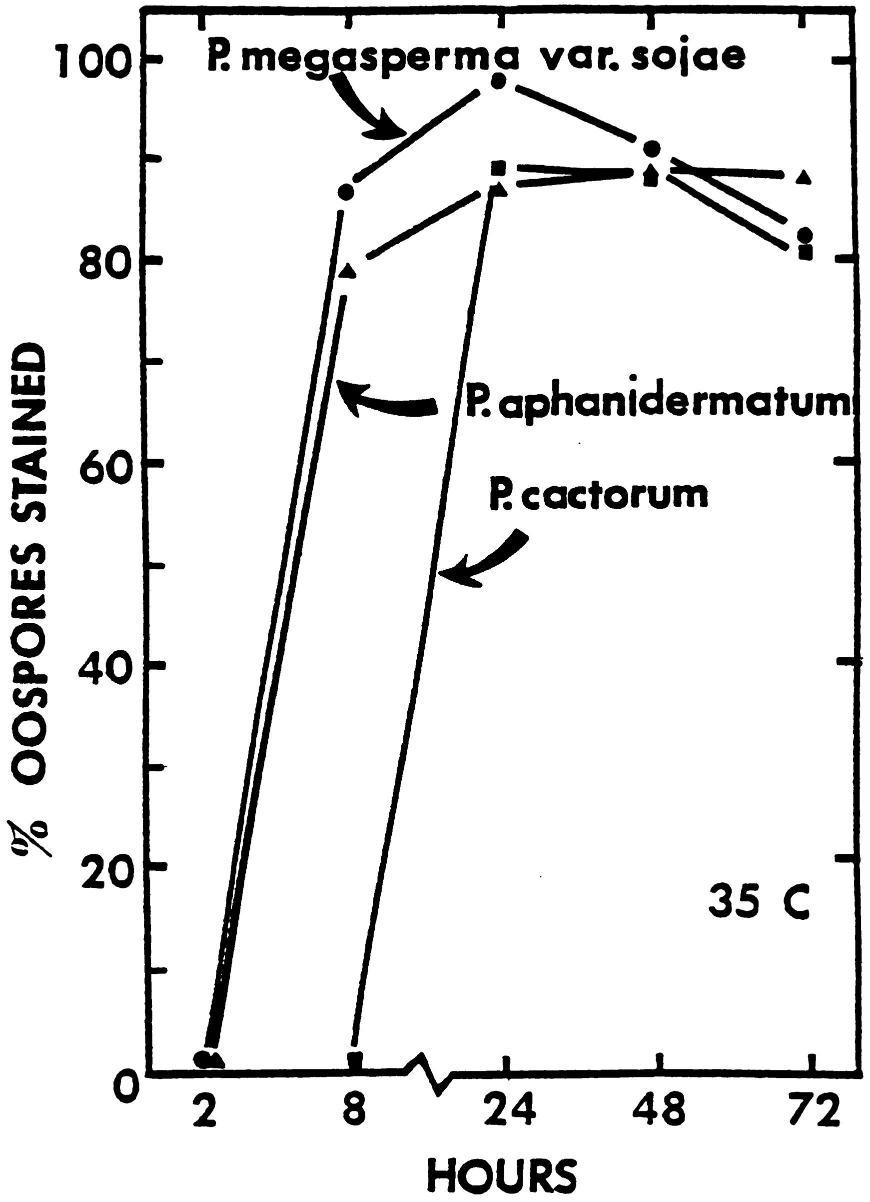


Figure 1. Staining of oospores of three Oomycetes during 2-72 h incubation in a 0.1% suspension of tetrazolium bromide (MTT) at 35 C.

correlated with oospore germinability (Sutherland and Cohen, unpublished results).

It was discovered in host range experiments that MTT also stained the mycelia and sporangia of *Actinoplanes missouriensis* within infected oospores stained purple, after approximately 15 min. at 23±2 C. Since healthy oospores never stain purple this permitted selective staining of the parasite and facilitated microscopic evaluation of parasitism, especially in the presence of soil particles. The actinomycetes, *Actinoplanes philippinensis*, *Actinoplanes utahensis*, *Ampullaria regularis*, *Amorphosporangium auranticolor*, and *Spirillospora albida*, within infected oospores, also were stained purple in MTT at 23±2 C after about 15 min.

Host range experiments

All possible combinations of the following potential hosts and parasites were tested: *Aphanomyces cochlioides*, *A. euteiches*, *Phytophthora citrophthora*, *P. megasperma* var. *sojae* race 1, *Pythium aphanidermatum*, and *P. ultimum* as potential hosts, and *Actinoplanes missouriensis*, *Diheterospora chlamydosporia*, *Humicola fuscoatra*, *Hyphochytrium catenoides*, and *Pythium monospermum* as potential oospore parasites. All host species were parasitized by all parasites except *P. ultimum* by *H. catenoides* (Table 2). Invasion of *A. cochlioides* and *A. euteiches* by *H. catenoides* and *P. monospermum* after two weeks was less than 50%, while parasitism by the other organisms was greater than 66%. *P. citrophthora* and *Pms* appeared to be equally susceptible to *A. missouriensis*, *D. chlamydosporia*, and *H. fuscoatra*, parasitism being greater than 90% in all cases. Susceptibility of *Pms* oospores to *H.*

Table 2. Parasitism of oospores of six plant pathogenic fungi by five hyperparasites after 14 days of incubation on water agar.

	Oospores parasitized, %[a]				
Host	*Actinoplanes missouriensis*	*Diheterospora chlamydosporia*	*Humicola fuscoatra*	*Hyphochytrium catenoides*	*Pythium monospermum*
Aphanomyces cochlioides	66 ± 5	82 ± 10	88 ± 3	24 ± 6	45 ± 14
A. euteiches	74 ± 6	71 ± 6	81 ± 4	17 ± 6	24 ± 6
Phytophthora citrophthora	96 ± 2	91 ± 2	92 ± 3	71 ± 4	79 ± 3
P. megasperma var. *sojae*	96 ± 2	93 ± 1	96 ± 2	54 ± 4	55 ± 13
Pythium aphanidermatum	45 ± 10	34 ± 10	79 ± 4	20 ± 7	14 ± 4
P. ultimum	33 ± 6	42 ± 10	34 ± 7	0	77 ± 4

[a] Average % of oospores parasitized ± standard error on 3-5 replicate plates in each of 2-5 experiments; 100 oospores were counted per plate. Parasitism was determined by microscopic observation of the hyperparasite within the oospore, or by degradation of oospore wall and cytoplasm.

catenoides and *P. monospermum* was about 20% less than the susceptibility of *P. citrophthora* to the same parasites. *P. aphanidermatum* and *P. ultimum* were not parasitized as frequently as the other hosts (45% or less), except in the interactions between *P. aphanidermatum* and *H. fuscoatra* or *P. ultimum* and *P. monospermum* where oospore infection averaged 79% and 77%, respectively.

Glomus etunicatus was also tested as a host for *A. missouriensis* and *H. fuscoatra* to determine the susceptibility of a mycorrhizal fungus to oospore hyperparasites. The deep pigmentation of chlamydospores made assessment of parasitism by *H. fuscoatra* difficult. Empty chlamydospores were rated as infected by *H. fuscoatra*. After 34 days of incubation, an average of 47% of the chlamydospores were parasitized by *H. fuscoatra* in one experiment and 38% in a second experiment. After 2 weeks 39% of the chlamydospores were infected in a third experiment. Chlamydospores incubated with *A. missouriensis* remained healthy for at least 4 weeks.

Phytophthora cactorum, *P. capsici*, *P. megasperma*, and *P. parasitica* also were tested as hosts for *A. missouriensis* and *H. fuscoatra* (Table 3). Oospore infection of each species by both parasites was approximately the same, average percentages ranging from 89-97% for parasitism of *P. parasitica* to 95-99% for *P. capsici*. Races 1, 3, 4, and 7 of *P. megasperma* var. *sojae* were also tested to determine their susceptibility to *A. missouriensis* and *H. fuscoatra*. Parasitism of each *Pms* race averaged 95% or higher (Table 4). Similar results were obtained in a second experiment.

Two other *Actinoplanes* species, *A. philippinensis* and *A. utahensis*, and *Amorphosporangium auranticolor*, *Ampullariella regularis*, and *Spirillospora albida*, all members of the Actinoplanaceae, and

Table 3. Parasitism of oospores of four *Phytophthora* species by *Actinoplanes missouriensis* or *Humicola fuscoatra* after 14 days of incubation on water agar.

Host	Oospores parasitized, %[a]	
	A. missouriensis	*H. fuscoatra*
Phytophthora cactorum	97 ± 1	98 ± 1
P. capsici	95 ± 1	99 ± 0
P. megasperma	93 ± 2	96 ± 3
P. parasitica	97 ± 1	89 ± 2

[a] Mean % of oospores parasitized ± standard error on 3-4 replicate plates; 100 oospores were counted per plate. Parasitism was determined as described in Table 2.

Table 4. Comparative susceptibilities of oospores of four races of *Phytophthora megasperma* var. *sojae* to the hyperparasites *Actinoplanes missouriensis* and *Humicola fuscoatra* after 14 days of incubation on water agar.

Hyperparasite	Oospore race	Oospores parasitized, %[a]
Actinoplanes missouriensis	1	99 ± 1
	3	99 ± 1
	4	95 ± 3
	7	96 ± 2
Humicola fuscoatra	1	96 ± 4
	3	95 ± 2
	4	98 ± 1
	7	95 ± 1

[a] Mean % of parasitized oospores ± standard error on three replicate plates; 100 oospores were counted on each plate. Parasitism was determined as described in Table 2.

Micromonospora sp. of the Micromonosporaceae, were not known to be oospore parasites. These organisms were tested as potential hyperparasites by incubating each actinomycete with Pms oospores for two weeks. Colony forming units per petri dish used in testing hyperparasitism were 5 X 10^5 for A. auranticolor, 2 X 10^5 for A. regularis, 7 X 10^4 for Micromonospora sp., and 9 X 10^5 for S. albida. Concentrations of 10^4 sporangia per petri dish were used for A. philippinensis and A. utahensis. All of the test organisms were successful oospore parasites with percentages of parasitism ranging from 94-99% (Table 5).

Pms oospores were inoculated with four different single spore isolates obtained from a culture of H. fuscoatra to test the hyperparasitic stability of this organism. There were no gross morphological differences among the isolates. No significant differences were detected in parasitism of Pms oospores by the isolates in either of two experiments.

Pigmentation ranging from white to gray to black was observed in culture of H. fuscoatra. Since the occurrence of cultures bearing albino spores was rather high (roughly 1 in 10), the parasitic capability of albino and dark spores was compared. Hyperparasitism of Pms oospores was compared using aleuriospores derived from albino and black (wild type) cultures of H. fuscoatra. Parasitism by albino spores was 80% and 97% in two experiments whereas that by pigmented spores was 78% and 93%.

Invasion of living vs. killed oospores

To determine whether killed Pms oospores could be used to stimulate an increase in the populations of indigenous oospore parasites, the extent to which autoclaved oospores were invaded was tested. Oospores of

Table 5. Parasitism of *Phytophthora megasperma* var. *sojae* oospores by six actinomycetes species after 14 days of incubation on water agar.

Hyperparasite	% Oospores infected[a]
Actinoplanes philippinensis	95 ± 2
A. utahensis	99 ± 1
Amorphosporangium auranticolor	95 ± 2
Ampullariella regularis	99 ± 1
Micromonospora sp.	95 ± 3
Spirillospora albida	94 ± 1

[a] Average % of parasitized oospores ± standard error, on three replicate plates; 100 oospores were counted per plate. Parasitism was determined by microscopic observation of the hyperparasite within the oospore.

Pms were autoclaved for 15 min in a 1 ml volume of water. Identical batches of oospores were not autoclaved. Using the membrane filter-water agar technique, plates were inoculated with suspensions of A. missouriensis or H. fuscoatra. Invasion of killed oospores by H. fuscoatra was nearly equal to that of healthy oospores, but A. missouriensis invaded only about one-third the number of killed oospores (Table 6). Vital staining of oospores initially, as indicated by staining in MTT, was 88% for the untreated oospores, but after killing was 6%. The experiment was repeated with similar results. The results suggested that killed oospores of Pms might be useful to stimulate natural parasites, especially H. fuscoatra.

Effect of Oomycete culture age on susceptibility of oospores to hyperparasites

Pms oospores were harvested from cultures 16, 22, or 30 days of age. There was no significant difference in hyperparasitism of the oospores by A. missouriensis or H. fuscoatra (Table 7). As an indication of viability prior to the addition of hyperparasites, oospore staining with 0.1% MTT was 89% or greater for each age. A second experiment gave similar results.

Effect of hyperparasite culture age on hyperparasitism

To determine if culture age of the hyperparasites would have any bearing on biological control potential, A. missouriensis sporangia were collected from cultures 13, 29, or 45 days of age, and H. fuscoatra aleuriospores were collected from cultures 12, 28, or 78 days of age and used as inoculum. Oospore parasitism by both organisms was greater than 95% at all ages tested (Table 8). Results were similar in a second experiment.

Table 6. Invasion of living vs. killed oospores of *Phytophthora megasperma* var. *sojae* by *Actinoplanes missouriensis* or *Humicola fuscoatra* after 14 days of incubation on water agar.

Treatment	Oospores invaded, %[a]		MTT staining of control oospores[b]
	Actinoplanes missouriensis	*Humicola fuscoatra*	
Untreated oospores	94 ± 2	97 ± 1	88 ± 3
Autoclaved oospores	31** ± 6	99 ± 1	6 ± 2

[a] Mean % of oospores invaded ± standard error on three replicate plates; 100 oospores were counted per plate. The value with double asterisks (**) is significantly lower (*P*=0.01) than that for untreated oospores. Parasitism was determined as described in Table 2.

[b] Mean % of oospores stained as an estimate of oospore viability; 100 oospores were counted in each of three replicate plates.

Table 7. Susceptibility of Phytophthora megasperma var. sojae (Pms) oospores of three ages to parasitism by Actinoplanes missouriensis or Humicola fuscoatra after 7 days of incubation on water agar.

Pms culture age, days	Oospores parasitized, %[a] Actinoplanes missouriensis	Humicola fuscoatra	MTT staining of of oospores[b]
16	91 ± 4	94 ± 2	89 ± 3
22	86 ± 4	93 ± 4	90 ± 2
30	95 ± 2	90 ± 4	94 ± 2

[a] Average % of oospores parasitized ± standard error on three replicate plates; 100 oospores were counted per plate. Parasitism was determined as described in Table 2.

[b] Mean % of oospores stained as an estimate of viability on three replicate plates; 100 oospores were counted per plate.

Table 8. Parasitism of oospores of Phytophthora megasperma var. sojae by Actinoplanes missouriensis and Humicola fuscoatra of different ages after 7 days of incubation on water agar.

Hyperparasite	Culture age, days	Oospores parasitized, %[a]
Actinoplanes missouriensis	13	96 ± 1
	29	98 ± 1
	45	99 ± 1
Humicola fuscoatra	12	98 ± 1
	28	97 ± 2
	78	97 ± 1

[a] Average % of oospores parasitized ± standard error on three replicate plates; 100 oospores were counted per plate. Parasitism was determined as described in Table 2.

The influence of soil pH, moisture, and temperature on hyperparasitism of P. megasperma var. sojae oospores by A. missouriensis and H. fuscoatra

Environmental parameters were tested in order to assess the range of conditions that *A. missouriensis* and *H. fuscoatra* would tolerate as hyperparasites. As soil pH increased from 4.7 to 8.0, parasitism of *Pms* oospores by *A. missouriensis*, in Experiment 1, increased from 4 to 100% (Table 9). At the lower pH's, 4.7 to 6.0, parasitism was not greater than 16%. Rasing the pH to 6.6, greatly increased oospore infection to 91%. Infection remained high (98-100%) at pH's 7.0 to 8.0. In a second experiment, oospore parasitism by *A. missouriensis* increased with increasing pH up to 98% oospore infection at pH 8.0. However, in this experiment parasitism greatly increased at pH 5.5 to 83% and steadily increased thereafter. Parasitism of *Pms* oospores by *H. fuscoatra* increased from 5 to 50% as soil pH increased from 4.7 to 7.9 (Figure 2). Parasitism was 6% or less at pH's 4.7 to 6.0 then increased to 27% at pH 6.6, and 50 to 53% at pH's 7.2 to 7.9. Results were similar in a second experiment. Parasitism by *A. missouriensis* or *H. fuscoatra* in soils adjusted with MES, MOPS, or HEPPS buffers and NaOH to a common pH (6.7 to 7.0) did not differ. It is not known why oospore parasitism by *A. missouriensis* differed between experiments at the low pH's, 4.7 to 6.0.

Parasitism of *Pms* oospores by *A. missouriensis* and *H. fuscoatra* was studied at matric potentials from 0 to -15 bars (Figure 3). Mean values for *A. missouriensis* in six experiments ranged from 59-85%, and for *H. fuscoatra* from 67 to 91% in five experiments. Oospore infection by *A. missouriensis* was 85% at 0 bars and gradually decreased with decreasing matric potential to 59% at -15 bars. Parasitism of *Pms* by *H. fuscoatra* was 67% at 0 bars, increased to 91% at -0.3 bars, then decreased to 67% at -15.0 bars.

Table 9. Effect of pH on parasitism of Phytophthora megasperma var. sojae oospores by Actinoplanes missouriensis after 7 days of incubation in autoclaved soil.

pH[a]	Oospores parasitized, %[b]	
	Experiment 1	Experiment 2
4.7	4 ± 1	36 ± 1
5.5	11 ± 1	83 ± 1
6.0	16 ± 1	89 ± 1
6.6	91 ± 1	93 ± 1
7.0	98 ± 1	94 ± 1
7.7	98 ± 1	96 ± 0
8.0	100 ± 0	98 ± 1

[a] Soil pH was adjusted using 0.05 M concentrations of the following buffers: MES (pH 4.7, 5.5, and 6.0), MOPS (pH 6.6 and 7.0), and HEPPS (pH 7.7 and 8.0).

[b] Average % per plate of soil for six replicate membranes ± standard error; 100 oospores were counted per membrane. Parasitism was determined by microscopic observation of the hyperparasite within the oospore.

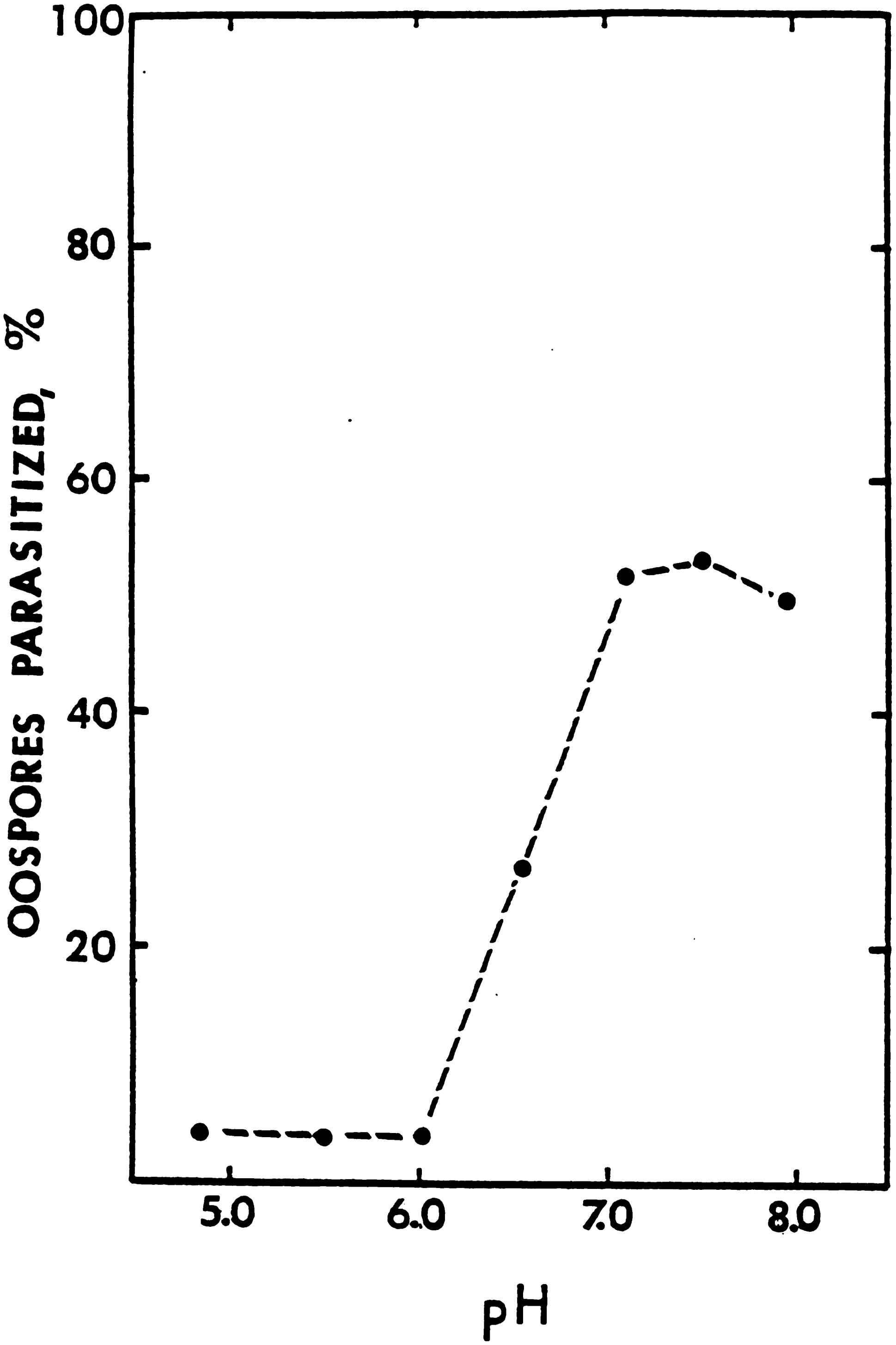


Figure 2. The effect of pH on parasitism of Phytophthora megasperma var. sojae oospores by Humicola fuscoatra after 7 days of incubation in autoclaved soil. Standard errors ranged from 1-3%. Soil pH was adjusted as described in Table 9.

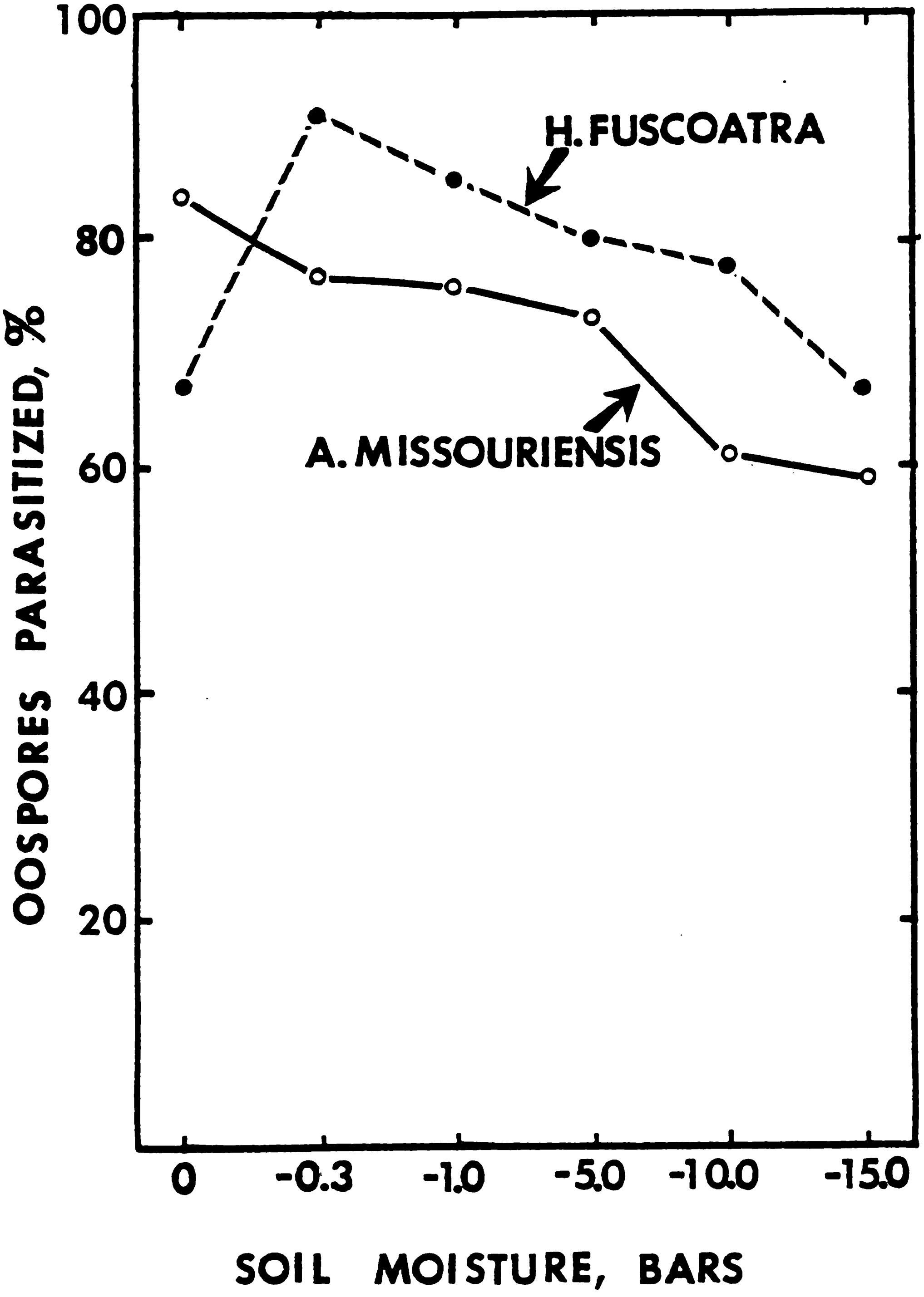


Figure 3. Hyperparasitism of Phytophthora megasperma var. sojae oospores by Actinoplanes missouriensis and Humicola fuscoatra in autoclaved soil adjusted to different matric potentials, after 7 days of incubation. The values represent averages (± standard errors of 2-6%) of six experiments with A. missouriensis and averages (± standard errors of 2-5%) of five experiments with H. fuscoatra.

As soil temperature increased from 5 to 30 C, parasitism of Pms oospores by A. missouriensis and H. fuscoatra increased from a minimum of 4% at 5 C to a maximum of 99% for A. missouriensis or 92% for H. fuscoatra at 30 C (Figure 4). Very little hyperparasitism (4 to 6%) occurred at 5 and 10 C by either oospore parasite. At 15 C, oospore infection increased to 63% for A. missouriensis but remained low (9%) for H. fuscoatra. From 20 to 30 C, both hyperparasities parasitized greater numbers of oospores as the temperature increased. The same trends were shown in two additional experiments for each hyperparasite. The effect of temperature on hyperparasitism of Pms oospores by A. missouriensis also was tested on water agar over a two-week period. After 7 days results were similar to those in autoclaved soil, but by 14 days hyperparasitism at 10 and 15 C had approached that at 20 to 25 C. The hyperparasite did not appear to be active at 5 C. Therefore, parasitism in autoclaved soil at 10 and 15 C may also have increased with a longer inoculation time.

Electron microscopic studies of the host-parasite interaction

A. missouriensis hyphae were found throughout the infected Pms oospore four days after incubation of oospores with the hyperparasite (Figure 5). A. missouriensis hyphae were observed to directly penetrate the oogonial wall (Figure 6) and the oospore wall (Figure 8), with little change in the diameter of the penetration hyphae. High concentrations of hyperparasite hyphae commonly occurred in the periplasmic space between the oogonial and oospore walls (Figure 7). H. fuscoatra also penetrated directly through the oogonia wall, but the penetration hyphae appeared narrower than other hyphae (Figure 10). A hypha grew through the periplasmic space (Figure 9), penetrated the oospore wall then immediately

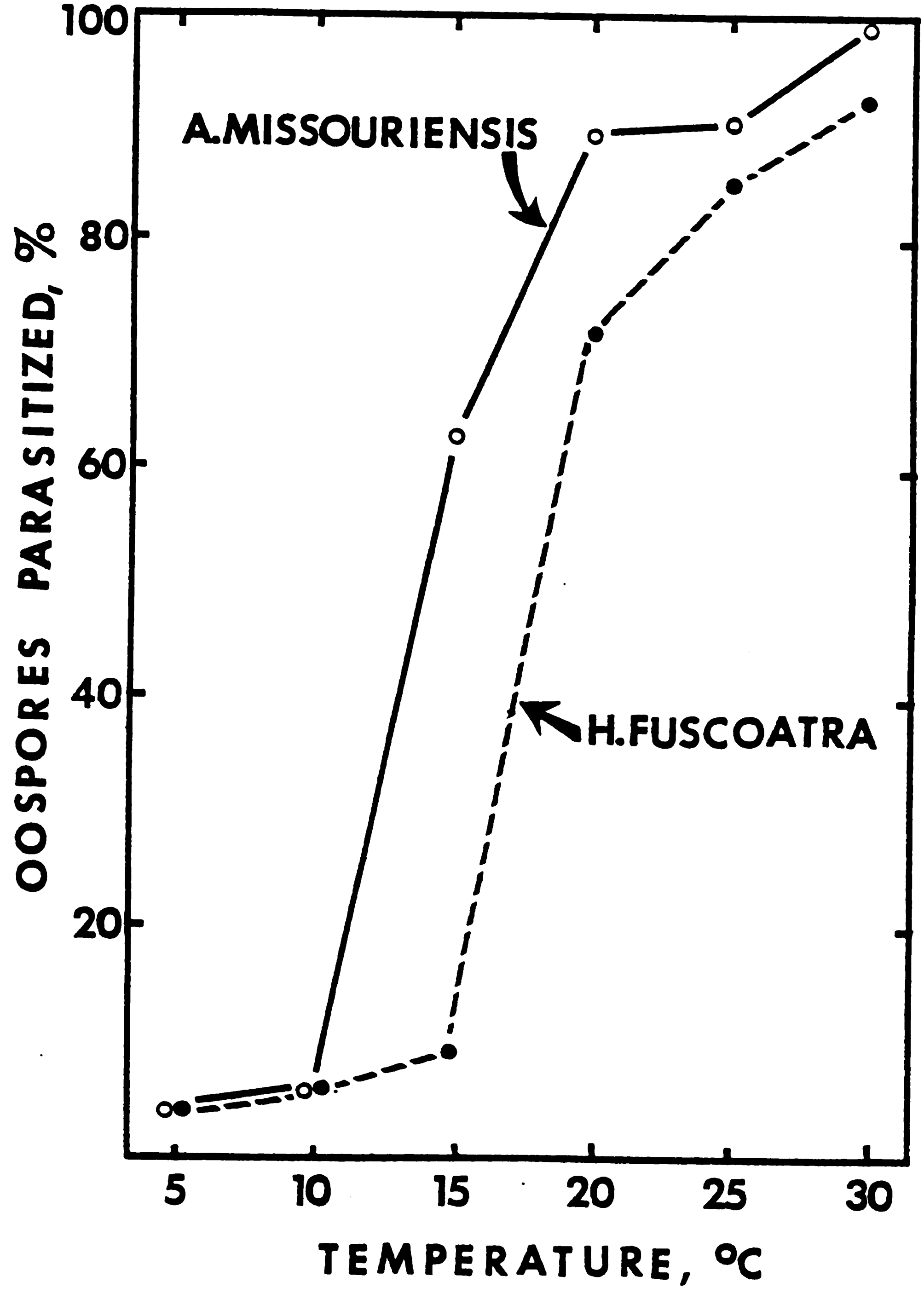


Figure 4. The effect of temperature on parasitism of Phytophthora megasperma var. sojae oospores by Actinoplanes missouriensis or Humicola fuscoatra after 7 days of incubation in autoclaved soil. Standard errors ranged from 0-4% in the A. missouriensis experiments and 1-4% in the H. fuscoatra experiments.

Figures 5-8. Invasion of oospores of Phytophthora megasperma var. sojae by Actinoplanes missouriensis.

Figure 5. Overview showing hyphae of the hyperparasite on the oogonial wall, in the periplasmic space between the oogonial and oospore wall, the oospore wall, and the oospore cytoplasm. X 2,592.

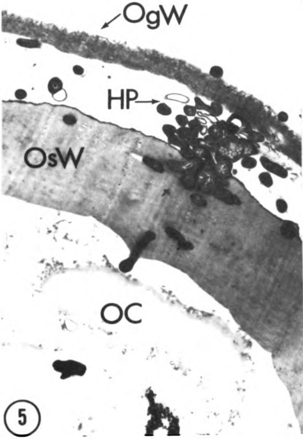
Figure 6. Penetration of the oogonial wall. X 17,280.

Figure 7. Concentration of A. missouriensis hyphae in the periplasmic space. X 4,320.

Figure 8. Penetration of the oospore wall. X 17,280.

KEY TO LETTERING ON FIGURES 5-8.

HP = hyperparasite, OC = oospore cytoplasm, OgW = oogonial wall, OsW = oospore wall, PS = periplasmic space.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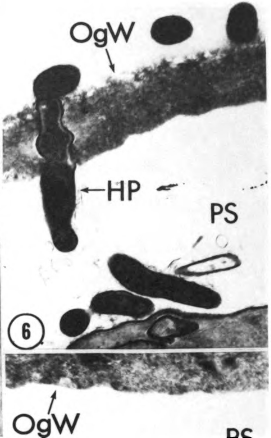
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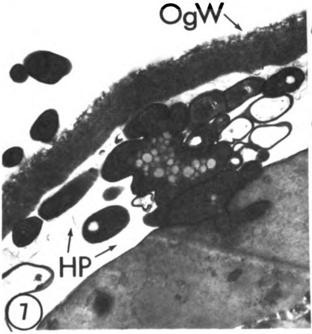
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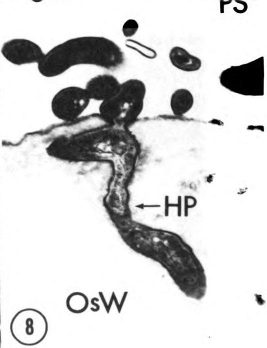
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Figures 9-11. Invasion of oospores of *Phytophthora megasperma* var. *sojae* by *Humicola fuscoatra*.

Figure 9. Overview showing hyphae of the parasite penetrating the oogonial wall, within the periplasmic space, and in the oospore wall. X 8,250.

Figure 10. Penetration of the oogonial wall. X 20,000.

Figure 11. Penetration of the oospore wall. X 15,000.

KEY TO LETTERING ON FIGURES 9-11.

HP = hyperparasite, OC = oospore cytoplasm, OgW = oogonial wall, OsW = oospore wall, PS = periplasmic space.

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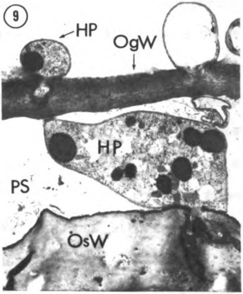

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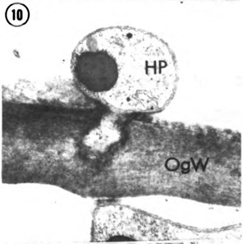

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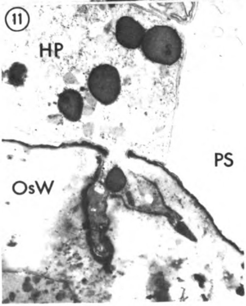

branched (Figure 11). Parasitism by both hyperparasites was similar in that no specialized cells, e.g., appressoria or haustoria, were formed and penetration occurred directly. Indentations of the host walls were not observed at penetration sites of either hyperparasites. There was no clearing of the host wall adjacent to the parasite suggesting that the hyperparasite may penetrate mechanically without enzymatic degradation. The Pms oospores appeared to lack a physical defense system as no change in oospore morphology or internal structure was detected in response to hyperparasite penetration.

Biological control experiments using the soybean seedling assay

Soil naturally infested with Pms was used to evaluate the biological control potential of four hyperparasites in the laboratory. *H. fuscoatra*, *H. catenoides*, *P. monospermum*, *H. catenoides* in combination with *P. monospermum*, or an unidentified hyperparasite, F4, were tested at concentrations of 10^4 propagules/petri dish (20 g soil). Partial control of *Phytophthora* root rot was obtained in two of three experiments by all hyperparasites tested except *H. fuscoatra* which reduced disease in one experiment (Table 10). A lower disease index was obtained with the combination of *H. catenoides* and *P. monospermum* in the first, but not in the second or third experiments. Disease incidence and severity were decreased 38-66% in Experiment 1, and 38-56% in Experiment 2.

Attempts to enhance indigenous hyperparasites through soil treatment with oospores

An alternative approach to the addition of hyperparasites in biological control is to enhance indigenous hyperparasitic organisms. To test whether such an approach might be feasible, oospores of *Pythium acanthicum*, itself a hyperparastie, and autoclaved oospores of Pms were

Table 10. Biological control of *Phytophthora* root rot of soybean in the laboratory using hyperparasites in naturally infested soil.

Treatment	Disease index[a]		
	Experiment 1[b]	Experiment 2	Experiment 3
Control	2.9 A	2.7 A	2.9 A
H. fuscoatra	-	1.5 B	2.9 A
Pythium monospermum	1.8 B	1.6 B	2.7 A
Hyphochytrium catenoides	1.5 B	1.2 B	2.9 A
P. monospermum + *H. catenoides*	1.0 B	1.7 B	2.9 A
F4	1.3 B	1.8 B	2.5 A

[a] Mean values for three replicate plates each containing five seedlings. Seedlings were rated on a scale from 0 = no disease to 3 = severe root rot. Numbers followed by the same letter are not significantly different (*P*=0.05, Experiment 1; *P*=0.10, Experiment 2).

[b] *H. fuscoatra* was tested in unsaturated soil and was compared with an unsaturated control. The disease index was 1.7 for both treatments.

added to soils naturally infested with *Pms*.

Oospores of *P. acanthicum* (2 X 10^5) were added to petri dishes containing 10 g of soil naturally infested with *Pms*. The soil was flooded to bring the final volume of water to 20 ml, and petri dishes were incubated 10 days. Four replications of treated and untreated soil were prepared. The disease indices after 5 days were identical in the control and *P. acanthicum*-amended soils.

Autoclaved *Pms* cultures were added to two soils naturally infested with *Pms* at the rate of 3 X 10^3 oospores/g soil in five replicate petri dishes per soil. All soils including control soils without added autoclaved *Pms* were flooded and incubated 10 days. No differences in disease were detected in the soils (Table 11).

Inoculum substrates for hyperparasites

Humicola fuscoatra grew successfully on barley, millet, soybean, wheat, or rye seed; wheat bran, linseed meal, beet pulp; ground wheat, corn, or barley stems and leaves; or onion leaves. Barley seed was selected as the food base for *H. fuscoatra* in greenhouse and field tests. The same substrates were not colonized by *H. catenoides* or *P. monospermum* under the conditions used.

Because of the difficulty in growing *H. catenoides*, attempts were made to culture this hyperparasite on oospores of *Pythium aphanidermatum*, a previously identified host (15). *P. aphanidermatum* which had been cultured on sterilized barley seed was inoculated with *H. catenoides*. However, *H. catenoides* failed to colonize the culture. *H. catenoides* was grown successfully in several liquid media, including Emerson's Chytrid medium, corn meal extract, diluted V-8 juice broth, PDB, and *Pms* extract.

Table 11. The effect of *Pythium acanthicum* or killed *Phytophthora megasperma* var. *sojae* (*Pms*) oospores on *Phytophthora* root rot in soils naturally infested with *Pms*.

Treatment	Disease index for indicated soil[a] Brookston-Conover loam association	Parkhill loam	Conover loam
Control	2.8	2.4	2.5
Pythium acanthicum	2.8	-	-
Autoclaved *Pms*	-	2.3	2.4

[a] Average numbers of five replicate plates each containing 10 seedlings. Plants were rated on a scale from 0 = no disease to 3 = severe root rot.

Although colony counts were not made, it was obvious that the greatest number of new *H. catenoides* colonies formed in the shortest period of time in the *Pms* extract. Fewer new colonies formed in the Chytrid medium, but they were much larger. Corn meal extract and diluted V-8 broth supported approximately equal growth of *H. catenoides*, but were inferior to *Pms* extract and Chytrid medium. No new *H. catenoides* colonies formed in PDB, but there appeared to be more growth than in the other media.

Perlite saturated with Czapek's broth made with soil water or with PDB were tested as substrates for *A. missouriensis* and *H. fuscoatra*, respectively. *A. missouriensis* did not colonize the perlite very rapidly. *H. fuscoatra* grew well on perlite with PDB, and this medium was used in further work.

Greenhouse experiments

Effect of potting medium on disease. Several different potting media were used to compare disease using culturally grown oospores at a concentration of 114 oospores/g soil. Two hundred ml of Michigan State University greenhouse mix (1:1:1, sand:peat:soil), Readi-earth potting mix, Oshtemo-Boyer loamy sand, or riverbed coarse sand were measured into pots. The potting medium in each of five pots was infested with *Pms*, while five other pots of the potting media were not infested. The potting media were kept flooded for 4 days after which time five, 2-day-old seedlings were transplanted to each pot. An additional treatment was included to test the effect of direct seeding on disease incidence in riverbed coarse sand. Emergence was recorded as an indication of disease. Plants grown in non-infested potting media were healthy (Table 12). Pre-emergence disease was significantly higher (P=0.05) and only 1-2 seedlings/5 survived when greenhouse mix, Readi-earth mix, riverbed coarse sand, or Oshtemo-Boyer loamy sand were used as the potting media. Emergence was less when seed were directly planted in riverbed coarse sand.

In a similar experiment, six pots of sterilized Capac loam (a soil naturally infested with Pms) each were infested with 100 oospores/g soil. The oospores had been produced in V-8 juice broth. Details were as described above. No disease was obtained in the artificially infested soil.

Effect of culture medium on infectivity of P. megasperma var. sojae oospores. Pms oospores produced on millet seed or in clarified V-8 juice broth were harvested when cultures were 35 days old. Eight pots of Readi-earth mix were infested (50 oospores/g) with each oospore type. Eight pots of mix were left untreated. All pots were flooded for five days. On the seventh day, two-day-old soybean seedlings were transplanted into all pots. The experiment was repeated using an oospore concentration of 100/g soil, and four replications per treatment. Oospores produced on V-8 juice broth caused the same amount of disease as those produced on millet seed in both experiments (P=0.01, Experiment 1; P=0.05, Experiment 2) (Table 13).

Effect of inoculum density on disease using two different potting media. Two hundred ml of riverbed coarse sand or Readi-earth potting mix were infested with Pms oospores produced in V-8 juice broth at concentrations of 1, 10, 30 or 50/g soil. Each treatment was replicated seven times. Potting media were kept flooded for five days to stimulate oospore germination. Two days later, four 2-day-old seedlings were

Table 12. Effect of potting medium on Phytophthora root rot of soybean in a greenhouse experiment using oospores produced in V-8 juice broth at a concentration of approximately 114 oospores/g potting medium.

Potting medium	Mean emergence/five seedlings/pot[a]
Transplanted seedlings[b]	
Controls[c]	5 A
Greenhouse mix	2 B
Readi-earth mix	2 B
Riverbed coarse sand	1 B
Oshtemo-Boyer loamy sand	3 B
Direct seeding	
Control	4 A
Riverbed coarse sand Directly seeded	4 A

[a] Mean values for five replicate pots, each with five seedlings. Numbers followed by the same letter are not significantly different (P=0.05).

[b] Transplanted seedlings were two days old with the radicle approximately 1 cm in length.

[c] Controls included five pots of each potting medium without Phytophthora.

Table 13. Comparison of virulence of Phytophthora megasperma var. sojae oospores produced on millet seed or in clarified V-8 juice broth.

	Mean emergence/five seedlings/pot[a]	
Inoculum source	Experiment 1	Experiment 2
Control	5 A	5 A
Millet seed	3 B	4 AB
V-8 juice broth	3 B	3 B

[a] Seedlings two days old with the radicle approximately 1 cm in length were transplanted. Means for eight replicate pots in Experiment 1 and four replicate pots in Experiment 2, each with five seedlings. Values followed by the same letter were not significantly different (*P*=0.01, Experiment 1; *P*=0.05, Experiment 2).

transplanted into the pots. Plant stand was significantly reduced 25-50% ($\underline{P}$=0.05) at oospore concentrations of 10, 30 and 50/g soil in Readi-earth potting mix, but not in riverbed coarse sand.

Effect of length of time of flooding on disease. Oospore germination by Pms requires free water. To determine the optimum time of flooding soil, 20 pots each containing 200 ml of Readi-earth mix were infested with oospores (100/g) produced in V-8 juice broth, then were flooded. Twenty additional pots of flooded soil without Pms were left to serve as controls. Pots of mix were planted 1, 2, 3 and 4 days after flooding. Five pots containing either infested or non-infested soil were directly seeded with soybeans, five seeds per pot; after seeding, soils were watered as needed. No disease occurred in any treatment. After the experiment was concluded, 10 g mix was taken from one pot each of one of the Pms-infested treatments. Disease potential, assessed using soybean seedlings (89), showed severe root rot of seedlings. It is not known why disease was not obtained in the greenhouse.

Biological control in the greenhouse by A. missouriensis applied as an aqueous suspension. Mycelial/spore suspensions of A. missouriensis were tested for biological control under different conditions. In one experiment, ground Pms-colonized millet seed was used as inoculum. Fifty ml of the colonized seed was ground in 50 ml DW in an Omni-mixer at a rheostat setting of 30 for 1 min. The Pms-seed suspension was incorporated into Readi-earth mix by thorough mixing in a plastic bag. This infestation procedure was repeated for 11 bags. The average oospore concentration was 7 X 10^3 oospores/g soil. Millet seed controls were prepared in the same manner except that Pms-colonized millet seed was autoclaved for 20 min. at 121 C, before grinding. A. missouriensis was added to 6 pots of mix infested with *Pms* or mix without *Pms* by adding the *A. missouriensis* while mixing the potting medium. The final concentration of *A. missouriensis* was approximately 10^3 sporangia/g soil. Soils were flooded for 4 days. On the fifth day, seedlings germinated in vermiculite for 36 h were transplanted to the soils at 5/pot.

In a second experiment, *Pms* oospore inoculum was produced in V-8 juice broth. Washed and steamed riverbed coarse sand was infested with *Pms* oospores at a concentration of 450/g. An aqueous suspension of *A. missouriensis* was applied at a concentration of 38 sporangia/g. After 4 days of flooding, water was withheld and five seedlings were transplanted to each pot.

Both kinds of *Pms* inocula caused severe seedling rot (Table 14). *A. missouriensis* applied as a mycelial/spore suspension did not control pre-emergence seedling rot in either experiment (*P*=0.01). In experiment 1 where *Pms*-colonized millet seed was used, emergence in the millet seed control and *A. missouriensis* treatments was less than that in the control, but this was due to toxicity from the millet seed. Soybean emergence in the *A. missouriensis* control was not different from emergence in the untreated control.

Biological control with A. missouriensis and H. fuscoatra as seed treatments. In three experiments, *A. missouriensis* and *H. fuscoatra* were evaluated as seed treatments for soybeans grown in Michigan State University greenhouse mix. After *Pms* inoculum was mixed into the soil and prior to planting, soils were flooded 4-5 days. Watering was then withheld until needed. Five replications were prepared per treatment. *Pms* oospores from cultures grown 11 and 14 days in V-8 juice broth, were used to infest soil in two experiments at a concentration of

Table 14. Lack of biological control of <u>Phytophthora megasperma</u> var. <u>sojae</u> (<u>Pms</u>) in the greenhouse by <u>Actinoplanes missouriensis</u> applied as a mycelial/spore suspension under different conditions.

	Mean emergence/five seedlings/pot[a]	
Inoculum source	Experiment 1[b]	Experiment 2[c]
Control (no treatment)	5 A	5 A
Millet seed control[d]	4 A	-
<u>Pms</u> on millet seed	1 B	-
<u>Pms</u> from V-8 juice broth	-	1 B
<u>Actinoplanes missouriensis</u>	4 A	5 A
<u>A</u>. <u>missouriensis</u> + <u>Pms</u>	1 B	2 B

[a] Seedlings two days old with the radicle approximately 1 cm in length were transplanted. Means of six replicate pots in Experiment 1 or five replicate pots in Experiment 2, each with five seedlings. Values followed by the same letter were not significantly different (<u>P</u>=0.01).

[b] <u>Pms</u> was grown on millet seed for 30 days then ground with water. Fifty ml of <u>Pms</u> suspension was used to infest Readi-earth potting mix at a concentration of 7 X 10^3 oospores/g mix. <u>A</u>. <u>missouriensis</u> was incorporated at a concentration of 10^3 sporangia/g mix.

[c] <u>Pms</u> oospores grown in V-8 juice broth for 30 days were used to infest steamed riverbed coarse sand at a concentration of 450 oospores/g sand. <u>A</u>. <u>missouriensis</u> was applied as a drench at a concentration of 38 sporangia/g sand.

[d] Autoclaved <u>Pms</u>-colonized millet seed.

approximately 3 X 10^3/g. Populations of *A. missouriensis* or *H. fuscoatra* on the seed averaged 10^5 CFU or 5 X 10^4 CFU, respectively. In a third experiment, *Pms* oospores produced in soybean tissue (117) were used at a concentration of 200 oospores/g soil. The cultures were 6-8 months old, and unlike other *Pms* cultures used were frozen for 3 days instead of 1-4 h prior to use. Concentrations on the seed were approximately 3 X 10^4 CFU's of *A. missouriensis* or *H. fuscoatra*.

In all three experiments plant stand was increased significantly (*P*=0.05) with the hyperparasites as seed treatments (Table 15). The reason for the poor emergence of controls in Experiments 1 and 2 is unexplained, but may have been due to poor seed quality or seedling decay pathogens present in the mix. Twice the number of plants emerged when *A. missouriensis*-treated seed were planted in soil artificially infested with *Pms* in Experiment 1. In Experiment 2 when seeds were treated with *H. fuscoatra*, almost one-third more plants emerged. In Experiment 3, *A. missouriensis* increased emergence by 5 times, while *H. fuscoatra* increased plant stand 3 times, as compared to stand in soil artificially infested with *Pms*.

Additional biological control experiments. Many experiments that were done to test hyperparasites failed because *Pms* inoculum did not cause disease. Briefly, the agents, method of delivery, and numbers of experiments done were as follows:

A. missouriensis:	aqueous suspension,	5
	attapulgus clay,	1
	seed,	4
H. fuscoatra:	aqueous suspension,	5
	attapulgus clay,	2
	barley seed,	1
	seed,	2

Table 15. Biological control of _Phytophthora_ _megasperma_ var. _sojae_ (_Pms_) in the greenhouse by _Actinoplanes_ _missouriensis_ and _Humicola_ _fuscoatra_ applied as seed treatments.

	Emergence/12 seeds planted/pot[a]		
Treatment	Experiment 1	Experiment 2	Experiment 3
Control	6 A	10 A	4 A
Pms	3 B	7 B	1 B
Actinoplanes missouriensis + _Pms_	6 A	-	5 A
Humicola fuscoatra + _Pms_	-	10 A	3 A

[a] Means of five replicate pots, each with 12 seeds. Values followed by the same letter are not significantly different (P=0.05, Duncan's multiple range test). _Pms_ inoculum for Experiments 1 and 2 was produced in V-8 juice broth; that for Experiment 3 was produced in soybean tissue.

H. catenoides: aqueous suspension, 2

P. monospermum: aqueous suspension, 1

Failure was independent of Pms inoculum age, culture medium, or concentration; potting medium; or time period of flooding prior to planting.

Effect of viable Pms mycelium on disease incidence and biological control

In three different experiments in which Pms inoculum was not frozen to kill the mycelium, significant disease was obtained. A. missouriensis as a seed treatment was tested once, and A. missouriensis and H. fuscoatra on clay were tested twice in attempts to obtain biological control of both mycelium and oospores. No disease control was obtained, probably because the mycelium is not susceptible to parasitism.

Field experiments

In the summer of 1979, biological control was tested in fields naturally infested with Pms on John Majzel's farm. Phytophthora root rot incidence was very low (0-3 infected plants/50 seeds planted) despite severe disease being present the preceding year. No significant differences were found among the treatments. Woodchuck damage to soybean plants was considered too extensive for the data collected at the Shuman farm to be meaningful.

Biological control was evaluated in 1980 in an artifically infested plot at the Michigan State University Botany and Plant Pathology Farm, and in a naturally infested plot at the Leo Shuman Farm. Disease incidence was low in both areas, only 0-3 infected plants/200 seeds planted at the Botany Farm and 4-12 infected plants/200 seeds planted at the Shuman Farm. The addition of hyperparasites caused no statistically detectable beneficial or detrimental effects. Using the soybean seedling assay, Pms was detected in the soil samples collected from the plot at the Shuman Farm. There were no differences in the disease indices among soil samples from rows treated with *A. missouriensis*-treated Turface (2.0), *H. fuscoatra*-colonized barley seed (1.6), and *H. catenoides*-treated vermiculite (2.2) and controls (1.9). Therefore, no disease control was indicated using these hyperparasites. The soybean stem segment assay proved to be an unreliable indicator of *Pms* occurrence.

Disease incidence was very low in naturally infested field plots in which fall or spring hyperparasite application prior to seeding was evaluated in 1980. No significant differences in numbers of infected plants were found among the control or *A. missouriensis*- and *H. fuscoatra*-infested soils at either time of hyperparasite application. Disease incidence in the Fall plot ranged 3-4 plants/80 seeds planted and in the Spring plot ranged from 4-6 plants/80 seeds planted. Soil samples were collected from the rows of the spring plot, and baited with soybean seedlings or stems to estimate the occurrence of *Pms*. Results showed no differences in the presence of *Pms* among treatments (range = 1.5-1.7), and therefore indicate no biological control had taken place with hyperparasite application in the spring prior to planting. The stem baiting method gave results too inconsistent to be interpreted.

Biological control was assessed in 1981 in microplots at the Michigan State University Botany and Plant Pathology Farm. Microplots were artificially infested with *Pms*. Of 25 seeds planted per microplot, 0-3 plants emerged even in uninfested controls. Stems and epicotyls of some of the seedlings that emerged appeared red and shriveled, and subsequently died. Soil samples collected from each microplot were indirectly rated for disease potential using the soybean seedling assay. All seedlings in the plates were rapidly destroyed, but microscopic examination revealed *Fusarium* macroconidia, and two oospore types, neither similar to *Pms* oospores. Plants in a soybean plot adjoining the hyperparasite trial were diseased due to *Fusarium*. It was therefore concluded that soybean losses in the microplots were not due to *Pms*.

DISCUSSION

The most significant new findings of this research are: i) the extension of the host range of *Pms* oospore parasites to include other Oomycetes, ii) the discovery of several new oospore hyperparasites, iii) the apparent direct penetration and rapid colonization of oospores by *Actinoplanes missouriensis* and *Humicola fuscoatra* as seen with electron microscopy, iv) the definition of the range of soil temperature, water potential, and pH that influence oospore parasitism by *A. missouriensis* and *H. fuscoatra*, and v) the demonstration of partial biological control of *Phytophthora* root rot of soybean in the laboratory and greenhouse.

The wide host ranges of the hyperparasites suggest that in nature many Oomycetes are susceptible to hyperparasitic attack. The plant pathogenic Oomycetes are, in general, difficult to control because host resistance and chemical methods are not always available. The diversity of the hyperparasites and the wide host range of many of them should lend encouragement to their further study as potential biological control agents.

Ayers and Lumsden (15) found in tests using agar that *Hyphochytrium catenoides* infected 26% of *Pythium aphanidermatum* oospores, 21% of *P. ultimum* oospores, and 64% of *A. euteiches* oospores. My work showed that *H. catenoides* parasitized 20±7% of the *P. aphanidermatum* oospore population, but only 17±6% of *A. euteiches* oospores and no *P. ultimum* oospores. Humble and Lockwood (118) reported that approximately 64% of *Pms* oospores were infected by *H. catenoides* in steamed soil after 14 days. *H. catenoides* parasitized 54±4% of the oospores of *Pms* in experiments on water agar after 14 days in this study. The same isolates of host and parasite as used by Humble and Lockwood (118) were also used in my work. Differences between my results and those of Ayers and Lumsden (15) may have been due to somewhat different methods employed, or to the use of different isolates of hyperparasites and hosts.

Humble and Lockwood (118) determined that nearly 90% of *Pms* oospores were infected in steamed soil by *Humicola fuscoatra* after three weeks. In my work, using different test media, but the same isolates of host and parasite as Humble and Lockwood (118), parasitism of *Pms* oospores by *H. fuscoatra* was greater than 90% on water agar after 14 days, and in autoclaved soil after only 7 days. Persistent microorganisms in the steamed soil may have delayed parasitic action by *H. fuscoatra* in the studies of Humble and Lockwood (118). Sterile conditions and a higher hyperparasite concentration may account for the higher rate of parasitism of *Pms* oospores by *H. fuscoatra* in my work. Daniels and Menge (63) detected more infection of *Glomus* spores by *Anquillospora pseudolongissima* and *H. fuscoatra* in autoclaved soil than on water agar. In the present work, agar was used because of its convenience. It is not known if parasitism was underestimated using water agar, but in any case, host range results are useful for comparative purposes.

Several new hyperparasites, all members of the actinomycetes, were discovered. Two *Actinoplanes* species, *A. philippinensis* and *A. utahensis*, and *Amorphosporangium auranticolor*, *Ampullariella regularis*, and *Spirillospora albida*, members of the Actinoplanacae, and *Micromonospora* sp. of the Micromonosporaceae, were found for the first time to infect *Phytophthora* oospores. Hyperparasitism by *Actinoplanes missouriensis* was commonly observed in flooded natural soil (188). The role of these organisms, other than *A. missouriensis*, in nature has not been established, but it may be possible that they play a role in the population dynamics of Oomycetes in soil. The aquatic nature of the *Actinoplanes* spp., *Spirillospora albida*, *Hyphochytrium catenoides*, and *Pythium monospermum* may have led to the co-evolution of these organisms with the plant pathogenic Phycomycetes. The production of zoospores by these hyperparasites may be expected to increase the chances of contact with oospores in soil.

Tetrazolium bromide (MTT) stain aided the assessment of parasitism, and was frequently used when *Pms* was the test host. High percentages of oospores of *Pms* and the other *Phytophthora* spp. were stained pink or red in a 0.1% MTT solution at 35±1 C after 24 h. In contrast, only low numbers of oospores of *Aphanomyces* spp. and *Pythium ultimum* stained with MTT, presumably due to the lack of stain penetration. This was suggested by the observation that oospores of these species reacted to MTT when in the initial stages of invasion by a hyperparasite. A higher temperature, higher MTT concentration, a longer incubation time, or a combination of these conditions may allow for increased staining of *A. euteiches*, *A. cochlioides*, or *P. ultimum*. The majority of *A. cochlioides* oospores were stained rose-colored after an extended period of time, 20 days, in 0.1% MTT.

In addition to the value of MTT as an oospore stain, hyphae, sporangia, and spores of the hyperparasites stained blue-black or black and were thus highlighted by the stain. Sporangia of the Oomycetes and

H. catenoides stained pink after approximately 15 min at 23±2 C and turned blue-black within 1 h or even sooner at 35±1 C. Mycelia and sporangia of the hyperparasitic actinomycetes turned a deep purple after 15 min incubation in MTT. The differential staining response of host and parasite greatly facilitated the determination of oospore parasitism, and MTT was used frequently in parasitic assessment of oospores by *A. missouriensis* and *H. fuscoatra*.

Little is known about the process of infection by oospore hyperparasites. All descriptions to date have been based upon observations made with the light microscope. Parasitism of oospores of *Pythium myriotylum* by *H. catenoides* was described briefly by Ayers and Lumsden (15). Zoospores of the hyperparasite encysted on the host wall. An infection peg entered the cytoplasm. Sporangia developed within the host oospore and released zoospores via short exit tubes into the environment. In the case of the Imperfect Fungi, *Dactylella anisomeres*, *D. helminthodes*, *D. stenocrepis*, *D. stenomeces*, *Tricothecium arrhenopum*, and *T. polyctonum* (74, 76, 77, 78), appressoria of various shapes were usually formed, except occasionally with *D. helminthodes*, *D. stenomeces* or *T. polyctonum*, where no differentiation occurred (76). An infection peg penetrated the oogonial wall. Sometimes, a second enlargement or appressorium occurred prior to penetration of the oospore wall by *D. helminthodes*, *D. stenocrepis*, *D. stenomeces*, and *T. polyctonum*. Assimilative branches of the parasite became visible as the oospore cytoplasm was absorbed.

In the present work, no evidence was found to indicate that appressoria were formed by *A. missouriensis* or *H. fuscoatra* by light or electron microscopy. Indentations of the host walls were not observed at penetration sites, possibly due to the inherent rigidity of the walls.

The hyphae of A. missouriensis directly penetrated the oogonial and oospore walls, and entered the cytoplasm without any noticeable change in the diameter of the hyphae. However, penetrating hyphae of H. fuscoatra appeared narrower than the vegetative hyphae (Figure 10). There was no indication of enzymatic degradation of the oogonial or oospore wall adjacent to penetrating hyphae of either hyperparasite, but without biochemical tests, the role of enzymes in oospore parasitism cannot be ruled out. The host did not respond to invasion by papillae formation, which may possibly be explained by the dormancy of the oospores. Once the hyperparasites infected the oospore cytoplasm, it rapidly disappeared, and was replaced with the thallus of the hyperparasite. The mechanism(s) of host-parasite recognition by A. missouriensis and H. fuscoatra is still completely unknown. It is interesting to note, however, that infection by these two organisms, a prokaryote and a eukaryote, appears to be similar.

Experiments to determine the effect of environmental parameters on parasitism by A. missouriensis and H. fuscoatra were done in sterile soil. The exclusion of indigenous parasites by autoclaving the soil was necessary to clarify the hyperparasitic relationship. Conditions highly conducive to parasitism of Pms oospores by A. missouriensis after 7 days were 0 bars matric potential, soil temperatures of 15-30 C, and soil pH's 5.5 or 6.6-8.0. Optimum conditions for parasitism by H. fuscoatra were -0.3 bars matric potential, soil temperature of 20-30 C, and soil pH's of 6.6-8.0. A parasitic preference for flooded soil by A. missouriensis and unsaturated soil for H. fuscoatra had been alluded to by other workers (118, 188). Soil pH in my experiments could not be maintained with Good's buffers at -0.3 bars matric potential, the optimum soil moisture for oospore parasitism by _H_. _fuscoatra_. To resolve this difficulty, pH was tested at 0 bars where it could be stabilized. Parasitism of _Pms_ oospores by _H_. _fuscoatra_ was less than would be expected had soils been at -0.3 bars.

It is difficult to eliminate variables that could interfere with assessment of pH effects on hyperparasitism. The addition to soil of buffers may bring about changes due to microbial utilization, degradation, or specific toxicity. The collection of various soils with a wide pH span, such as 5-8, would likely introduce variability due to other soil properties (e.g., structure, texture). Good's buffers were selected for use because of their relative stability. The buffers MES, MOPS, and HEPPS, when adjusted to a common pH, did not appear to interfere with hyperparasitism of _Pms_ oospores by _A_. _missouriensis_ or _H_. _fuscoatra_. Thus, it appears that the buffers used were not differentially inhibitory to the hyperparasites. The results, however, should be verified using different buffer systems.

It was hoped that results from the experiments on environmental parameters might be used to define the limits of biological control with _Pms_ hyperparasites. Since there was such difficulty in obtaining _Phytophthora_ root rot, this could not be accomplished.

There was never any difficulty obtaining disease using the soybean seedling-petri dish assay in early biological control work. On the contrary it was thought disease might be too severe to detect biological control. Therefore, greenhouse experiments were initiated using naturally infested _Pms_ soil. Although a reduction of plant height and root length was demonstrated in one experiment, there was not enough disease to evaluate biological control. The problem was never solved throughout the course of this work in spite of much effort directed toward this goal.

Biological control of Pms in the greenhouse was demonstrated using A. missouriensis- and H. fuscoatra-treated seed. These results suggest that under some conditions biological control of Pms may be feasible. Difficulties obtaining disease in the greenhouse may reflect the inconsistent nature of disease in the field. Most often soybean plants in the field are infected in a linear pattern. Plants adjacent to each other, in groups of three to five, frequently show cankers. Mr. Shuman's soybean field in 1978, however, was evenly infected with Pms and it was difficult to find a healthy plant in approximately 50 acres. The soil in this area was assayed in June of 1979, and at that time the disease index averaged 2.7 on a scale from 0 to 3. In the summer of 1980, soil samples from selected areas caused root rot in the seedling assay indexed at 1.7 to 1.9. If the assay is reliable, indigenous Pms populations would appear to have declined from 1979 to 1980. Credibility of the assay would have been strengthened had there been more disease in the field plots.

Very little disease was obtained during the three years of field experiments using natural or artificially grown Pms inoculum. It is possible that the environment was not conducive to severe infection of soybeans, or to symptom expression. It may also be possible that Pms populations were reduced by indigenous parasites, and that biological control was taking place without the intervention of man. These alternative hypotheses need to be evaluated in future work.

The difficulty in obtaining Phytophthora root rot in the greenhouse and field experiments does not preclude biological control as a viable means to control disease and diseases caused by related pathogens. Several findings of this research lend support to the feasibility of using hyperparasites to reduce disease caused by *Phytophthora*. These entail the wide range of oospore hyperparasites now identified (15, 74, 76, 77, 78, 118, 188), including several actinomycetes found for the first time to be oospore hyperparasites. The broad host range of these parasites also offers many options for their applied usage. Many of these hyperparasites grow well on common laboratory media and, therefore, the mass production of inoculum in fermentation vats designed for commercial production of microorganisms may be feasible. Large scale fermentation may be especially adaptable to the culture of the hyperparasitic actinomycetes due to their bacteria-like metabolism. Culture age of the hyperparasites *A. missouriensis* and *H. fuscoatra* did not affect their hyperparasitic abilities, which allows flexibility in the duration of inoculum production. The parasitic ability of *H. fuscoatra* may be a genetically stable trait, since each of four single spore isolates, and an albino isolate, were highly virulent. The population densities of *A. missouriensis* and *H. fuscoatra* on treated seed and other carriers remained stable for 2-12 months after drying. This stability suggests that the shelf-life of biocontrol preparations of these organisms may be adequate. Finally, the reduction in *Phytophthora* root rot obtained by hyperparasites introduced into naturally infested soil in the laboratory, and by hyperparasite-treated seeds in the greenhouse suggests that this approach to control *Phytophthora* diseases merits continued research effort.

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