Approaches to Biological Control of Soybean Root Diseases

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INTRODUCTION

Soilborne root-infecting fungi are among the most destructive pathogens of soybean. A diversity of such pathogens exists in Michigan, including *Pythium ultimum* (seed decay and seedling blight), *Phytophthora megasperma* f. sp. *glycinea* (root and stem rot), *Thielaviopsis basicola* (black root rot), *Rhizoctonia solani*, *Fusarium oxysporum* and *Fusarium solani* (seed, seedling and root rots), *Macrophomina phaseolina* (charcoal root and stem rot), and *Phialophora gregatum* (brown stem rot, a vascular disease).

For many of these diseases, chemical control is not feasible for reasons of cost and insufficient efficacy. Moreover, varieties resistant to most of these diseases are not available and cultural practices are only partially effective. Therefore, it was considered worthwhile to investigate the possibilities of biological control as alternate means of dealing with some of these pathogens.

We have investigated the use of dried, green plant material as soil amendments for *T. basicola*; oilseed meals and urea as soil amendments for *T. basicola*, *P. ultimum* and *M. phaseolina*; and hyperparasites of the oospores of *P. megasperma* f. sp. *glycinea* for control of this pathogen, as a third approach. Some encouraging results have been obtained with all three approaches in laboratory and greenhouse experiments, but only those with urea have been verified in field experiments.

Control of *Thielaviopsis basicola* by germination-lysis.

Black root rot is a common disease of soybean in Michigan, especially during cool, spring periods when the root cortex can become badly decayed and the taproot frequently girdled. Papavizas[26] and Papavizas and Adams[27] studied the effects of organic amendments on black root rot of bean, and found that alfalfa hay stimulated germination of the chlamydospores of the pathogen in soil and that the germ tubes lysed without formation of new endoconidia or chlamydospores. We investigated this method for black

root rot of soybean and examined the mechanism of biocontrol in detail[31].

Alfalfa, red clover, rye and triticale were collected during flowering and were dried and ground (0.25 mm). All four plant residues, when mixed with soil at 1% (w/w), reduced chlamydospore populations of *T. basicola* to about 0.005 of the original population after 28 days. Disease was correspondingly reduced in soybean seedlings planted in treated soil 3-4 weeks after amendment. By contrast, chitin applied at 0.2% (w/w) reduced chlamydospore populations by only about 0.25, and was much less effective than the other amendments in reducing disease. In further studies with alfalfa, concentrations of 1% or 0.7% were more effective in reducing chlamydospore populations than 0.5%, which was more effective than a 0.1% concentration.

Higher alfalfa concentrations also stimulated greater germination of chlamydospores in soil than lower concentrations. Their germination on membrane filters in soil was stimulated during the first 1-2 days following incorporation of alfalfa. This was followed by lysis of the germ tubes, which was manifested by a decline in the number of chlamydospores with germ tubes, until at the 6th day no chlamydospores with germ tubes were seen. The stimulatory effect of the alfalfa amendment on germination was short-lived. Maximum germination took place when chlamydospores were applied to soil at the same time as the amendment. When chlamydospores were applied 1-3 days later, germination declined markedly and after 4 days no germination was seen. Chlamydospore populations showed a similar trend, i. e., the greatest decline occurred when chlamydospores and the amendment were applied simultaneously, whereas the application of chlamydospores 3 days after amendment resulted in only a slight decline in population.

The germination-lysis mechanism was confirmed by scanning electron microscopy. In alfalfa-amended soil, germ tubes emerged after 1-2 days. A few newly formed endoconidia, but no new chlamydospores, were seen. From the 3rd day on, hyphae collapsed and gradually lysed apparently by autolysis, since colonization of the hyphae by other microorganisms was not seen. Chlamydospores recovered after long periods were empty and badly eroded. In nonamended soil, the chlamydospores remained ungerminated and intact.

Studies were done to determine which components of the alfalfa hay were responsible for germination stimulation. Unsaturated fatty acids had previously been implicated as the stimulatory substances[1,28], based on germination stimulation in soil by individual fatty acids, triglycerides and vitamin E acetate, but not by single simple sugars, amino acids and sterols[28]. However, in our work[31], aqueous extracts of alfalfa were more stimulatory in soil than extracts made with the fat solvents diethyl ether or chloroform-methanol. Neutral, cationic and anionic fractions of the aqueous extracts all were stimulatory, but less so than the combined fractions or the original aqueous extract. Chlamydospore populations in soil were reduced much more by aqueous extracts than by organic solvent extracts of alfalfa.

It seems clear that biological control was achieved through stimulation of

germination of chlamydospores, mainly by a mixture of water-soluble components of alfalfa hay, and that this was followed by autolysis of hyphae and degradation of the chlamydospores, resulting in a precipitous population decline. A secondary mechanism might also be involved: the fungistatic capacity of soil was elevated following application of alfalfa hay. Alfalfa (1%) was applied to soil, then 9 days later chlamydospores were applied simultaneously with a second application of alfalfa (1%) to stimulate germination. Germination in soil which had received the first amendment was 55%, whereas that in soil which received only the second amendment was 79%. Adams and Papavizas[1] also showed that treatment of soil with alfalfa hay increased fungistasis against *T. basicola*. Thus, germination in rhizospheres (and hence numbers of root infections) would be expected to be less in soil treated with green plant residues, and in fact chlamydospores and endoconidia of *T. basicola* adjacent to germinating bean seeds failed to germinate in soil treated 7 days earlier with alfalfa hay, whereas an appreciable number germinated in soil not so treated[1].

The destruction of inoculum by the germination-lysis method of biological control is conceptually an attractive one, and should be persistent since inoculum density is reduced. Populations of a number of other pathogens have been reduced by this same mechanism, but the method is not generally applicable to all [30]. However, the method is difficult to apply in practice due to a requirement for a large amount of organic material (ca. 1% by weight), the probable necessity for a homogeneous mixture of the residue with the soil, and a waiting period of 3-4 weeks for phytotoxicity to disappear. It was suggested that a winter cover crop of rye might be plowed down in the spring several weeks before seeding soybeans[31]. This was attempted in small field plots, but dry, hardened soil prevented deep and thorough mixing of the plant material with the soil. Even so, populations of *T. basicola* was reduced by about 50% (J. L. Lockwood and J. E. Bailey, unpublished results).

There may be alternate means of stimulating germination of propagules in soil, possibly by specific chemicals, particularly volatile compounds which can permeate the soil. Brief exposure of soil to distillates of alfalfa hay containing methanol, isobutyraldehyde and isovaleraldehyde stimulated germination of microconidia of *Verticillium albo-atrum*, followed by a precipitous decline in population[18]. Such materials might be effective in concentrations too low to support growth, and hence might have broader applicability among fungi. For example, certain fatty acids and their volatile peroxidation products stimulated germination of conidia of *Alternaria alternata* and *Fusarium solani* f. sp. *pisi* in soil at extremely low concentrations [19]. The most stimulatory compound, 2,4-hexadienal, stimulated germination at 4 nL/petri dish. The stimulation of germination of propagules with low concentrations of volatile compounds which cannot act as substrates for colonization would appear to be an attractive area for further study.

Control of several pathogens by oilseed meals and urea.

Experiment with oilseed meals. *Fusarium oxysporum* and *F. solani* are involved in soybean seedling and root rots, and their response to several orga nic amendments in soil was investigated. Later, *P. ultimum* also was included.

Ten different kinds of residues were evaluated: ground mature stems of barley, wheat, corn and soybean; commercial sugarbeet pulp; alfalfa meal; linseed, cottonseed and soybean meals; and ground crabshell[36]. When applied at 1% (w/w) to a sandy loam soil in closed containers, only the oilseed meals and chitin reduced *Fusarium* populations, after 4 weeks. The oilseed meals were most effective, reducing populations to 0.001 to 0.0001 of the original population. Therefore, these products were studied further.

The three oilseed meals were effective at concentrations as low as 0.25%[36]. Composting the oilseed meals and alfalfa meal neither increased nor decreased their effectiveness. Populations of actinomycetes, bacteria and fungi were reduced in soil treated with oilseed meals, but fungi were reduced the most. Soybean meal reduced populations of *Fusarium* to a similar extent in soils adjusted to -3.0, -0.2, -0.03 and 0 bars matric potential, but cottonseed and linseed meals were most effective at -0.2 and -0.03 bars.

The oilseed meals were less effective in fine-textured than in coarse-textured soils[36]. For example, *Fusarium* populations in Capac loam (2.96% organic matter) were reduced less than those in Oshtemo-Boyer sandy loam (2.02% organic matter), and populations in Brookston loam (6.46% organic matter) were not reduced at all, even after 10 weeks.

In view of the encouraging results in the laboratory, two field experiments were done in a loamy sand soil using linseed and cottonseed meals at 1% concentration in uncovered plots[36]. However, after 4-5 weeks *Fusarium* populations were equivalent to or higher than those in the untreated plots; after 9-12 weeks, *Fusarium* populations in amended plots were higher than those in control plots.

The discrepancy between laboratory and field results led to studies of the mechanism of action of the oilseed meal amendments in the hope of improving field performance[37]. The greater reduction of *Fusarium* populations in oilseed meal-supplemented soil in closed than in open containers, and the failure of oilseed meals to reduce *Fusarium* populations in field experiments done in uncovered plots, suggested the involvement of volatile degradation products of the meals. This was confirmed by the near extinction of *Fusarium* in small soil samples contained in planchets incubated on the surface of, but separated from contact with, a larger mass of soil to which 1% oilseed meals had been added. Further, alkaline volatiles were trapped in small beakers of distilled water or 2% boric acid solution placed in the containers. Soil treated with soybean meal yielded the greatest amount of titratable alkalinity of the three oilseed meals, in boric acid traps. The presence of trap solutions nullified the toxic effect of soybean meal, but not that of linseed and cottonseed meals, suggesting a more complex array of volatile toxic substances in soil containing these amendments.

The trapping of alkaline volatiles in boric acid suggested the presence of ammonia and this was confirmed by gas chromatography of the boric acid trap solutions[37]. Ammonia was first detected in 2-3 days, then increased rapidly in concentration until about the fifth day when the rate of production declined. Ammonia production was in the order: soybean meal linseed meal cottonseed meal. Peak ammonia concentrations were 3747, 2187 and 1394 μg/ml of trapping solution, respectively, for the three amendments. By contrast, the ammonia concentration in nonamended soil or alfalfa-amended soil was 10 μg/ml. Amines were not detected. The C/N ratios of the oilseed meals were 6.7-7.4 as compared with 17.1 for alfalfa meal, apparently accounting for the production of ammonia by the oilseed meals.

The behavior of *Fusarium* chlamydospores in oilseed meal-amended soils was studied microscopically. When chlamydospores were introduced into soil simultaneously with the amendments, 95% or more germinated after 24 hours, whereas none germinated in nonamended soil. After 2-3 days, hyphae lysed and new chlamydospores were produced. In subsequent observations, these new chlamydospores failed to germinate in soil and all hyphae disappeared. The chlamydospores progressively lost viability as shown by tests on agar, until at 21 days none remained germinable, whereas in nonamended or alfalfa-amended soil, 65% were still germinable after 21 days. When chlamydospores were added to soil 7 days after amendment, their ability to germinate when transferred to agar 7 days later was near zero, whereas those incubated for 7 days in nonamended or alfalfa-amended soil germinated 80% or more.

The lethal effect of volatiles from oilseed meals was not restricted to *F. oxysporum* and *F. solani*. Chlamydospores of *F. lateritium*, *F. moniliforme*, *F. rigidisculum*, *F. roseum*, and *F. tricinctum* also where decimated when incubated in soil planchets placed on soil amended with oilseed meals. Incubation in the presence of nonamended or alfalfa-amended soils had no effect.

It is interesting that the oilseed meals initially nullified soil fungistasis, promoting germination of *Fusarium* chlamydospores. This was followed by growth of hyphae and production of new chlamydospores, which were killed, apparently by the ammonia and possibly other toxic volatiles generated during decomposition of the meals. Thus. the mechanism of biological control of *Fusarium* by the oilseed meals differs from that for *T. basicola* by dried, green plant material. In the latter case, the fungus was eradicated after germination because of autolysis, presumably from nutrient stress[24], and because this fungus, unlike *Fusarium* spp., was unable to produce new chlamydospores.

Subsequent to the above work, soybean meal was shown to reduce populations of *P. ultimum* to undetectable levels in covered containers of soil[5,14]. In open containers, the population first increased, then declined to a lower level over time. In field experiments with 1% soybean meal in covered and uncovered plots, populations of *P. ultimum* increased, sometimes many-fold, then declined, but frequently to levels no less than those

of the original population[14]. The ineffectiveness of the oilseed meals against *Fusarium*[35] and *P. ultimum*[14] in field conditions was due apparently to their being colonized by the pathogens before lethal concentrations of ammonia were generated. For reasons as yet unknown, the ammonia subsequently formed did not bring about control.

Experiments with urea. To circumvent the problem of colonization of the amendment by the pathogen, urea was evaluated as an alternative source of ammonia, since it does not serve as an energy substrate. In laboratory experiments, urea at 1% concentration in soil reduced the population of *P. ultimum* without an initial increase[5]. Therefore, urea was tested for its effect on populations of *P. ultimum*, *M. phaseolina* and *T. basicola* in a sandy loam soil in the field[7]. The soil was artificially infested with the three pathogens and packed into clay tiles sunk into the surrounding field soil. After an equilibration period, this soil was watered with an aqueous solution of urea to give 0.1 and 1% (w/w) urea in trials 1 and 2 and 0.25, 0.5 and 1% urea in trial 3.

Populations in untreated soil remained stable throughout the 30-40 days of each experiment. In soil treated with 1% urea, populations of all three pathogens were either reduced to extinction or to very low levels. Their relative sensitivity was in the order: *P. ultimum T. basicola M. phaseolina*. Urea at 0.25 and 0.5% also drastically reduced populations, but the rate of reduction was slower at these concentrations. 0.1% urea reduced populations in trial 1 but not in trial 2. Trial 2 was done in the autumn when soil temperatures were frequently 10 C, possibly retarding the rate of formation of ammonia or reducing its toxicity. These results are consistent with those of Papavizas *et al.*[29] that bean root rot caused by *T. basicola* was reduced in soil amended with soybean meal at 35, 28 and 22°C, but not at 16°C. Urea treatments of soil were accompanied by an increase in soil pH, depending on the concentrations applied; with 1% urea, pH rose to 8.7-9.0 after 4 days, then declined to about pH 8.0 at the end of the experiment. With 0.25% urea, pH rose to 8.0, then declined to the original value of 5.7 before the end of the experiment.

Ammonia concentrations in soil with 1% urea were about 500 mg/kg soil throughout the experiments; with 0.25% urea, ammonia reached a peak of about 300 mg/kg at 4 days, then declined to about 100 mg/kg.

Thus, what began as a biological control approach to root disease control through the use of plant materials as soil amendments ended as a chemical control approach. The practical utility of the application of a high concentration of nitrogenous material to agricultural soils is questionable. Even at 0.25%, the equivalent application of about 5000 kg urea/hectare is 6-8 times the normal high rates for fertilizing wheat and corn. Excess nitrogen could be detrimental to crops and would be expensive. However, further research might focus on urea applications to localized foci of infection, or the use of lower concentrations, which might bring about a gradual population decline.

Effect of ammonia on exudation from fungal propagules. Since the

decline in pathogen populations in soil proceeded much more slowly at low than at high concentrations of urea, it seemed possible that this slow decline might be related to gradual attrition of the propagules than to a direct lethal effect. Exudation of ^{14}C -labelled compounds from fungal propagules is increased in soils and has been associated with the imposition of soil fungistasis[15,16,24]. Prolonged incubation of fungal propagules in soil can debilitate them, resulting in elevation of their nutrient requirements for germination[4,16], in progressive loss of viability[4,16], and in decreased virulence[2,13]. These aspects of fungal behavior in soil can be mimicked by a model apparatus in which fungal propagules are incubated on a bed of sand through which a dilute salts solution is percolated, thereby steepening the diffusion gradient away from the propagules in a manner analogous to that brought about by microbial competition for substrate in soil. Therefore, studies were done to determine whether ammonia might increase exudation from fungal propagules *in vitro* and in soil, and to determine the effects of the increased exudation on propagule viability.

Exposure of conidia of *Drechslera victoriae* and sclerotia of *M. phaseolina* for a few hours to high free ammonia concentrations (up to 10,000 mg/L) in the model system stimulated a short burst of exudation, which was associated with death of the propagules[6]. Lower concentrations increased exudation less, but the increased exudation tended to be maintained for a longer period. Viability of the propagules was reduced when they were continually exposed to doses that were nonlethal for shorter durations. High pH by itself also increased exudation somewhat, but did not account for the greater exudation effected by ammonia, nor for its lethal effects. In another model system imposing a lesser diffusion stress, ammonia at low concentrations (50 mg/L) actually decreased exudation and viability was not affected. However, as ammonia concentration was increased (50 mg/L), exudation was stimulated and viability declined[6]. The decreased exudation and lack of toxicity of low concentrations of ammonia also were observed by Loffler and Schippers[25] for conidia of *F. solani* f. sp. *phaseoli*, *D. victoriae* and *Botrytis cinerea*.

Since concentrations of ammonia from treatments of soil with 0.25% urea exceeded 50 mg/L, it is possible that low concentrations of ammonia can accelerate the natural attrition of propagules occurring under nutrient stress in soil, through increased exudation depleting endogenous reserves.

Biologica control of Phytophthora megasperma f. sp. glycinea by hyperparasites of the oospores

Laboratory experiments. During observation of the behavior of *P. megasperma* f. sp. *sojae* (*Pmg*) in soil, numerous oospores of the pathogen were found to contain fungal, actinomycete, and less frequently bacterial parasites[32]. Only a few hyperparasites of oospores in soil had been described[9–12,20], and therefore it was surprising to find a wide range of microorganisms parasitizing oospores of *Pmg*. The hyperparasites found initially included the Oomycetes *Pythium monospermum* and *Leptolegnia*

sp., the Hyphomycetes *Dactylella spermatophaga*, *Diheterospora chlamydosporia*, *Humicola fuscoatra*, *Fusarium oxysporum*, *Cephalosporium* sp. and *Alternaria alternata*; the Actinomycete, *Actinoplanes missouriensis*; three Hyphochytridiales, *Rhizodiomycopsis japonicus*, *Canteriomyces stigeoclonii*, and *Hyphochytrium catenoides*; and the bacterium *Pseudomonas* sp. Subsequently[35], six additional Actinomycetes isolated from sources other than fungal oospores were found to be efficient parasites of oospores *in vitro*. These included *Actinoplanes philippinensis*, *A. utahensis*, *Amorphosporangium auranticolor*, *Ampullariella regularis*, *Spirillospora albida* and *Micromonospora* sp.

Oospores were parasitized most frequently by Oomycetes, Hyphochytridiomycetes and *A. missouriensis* in wet soils and by Hyphomycetes in drier soils, suggesting that different groups are adapted for hyperparasitic activity under different soil moisture conditions[32].

The ability of several hyperparasites to parasitize oospores was compared by incubating oospores of *Pmg* on membrane filters on sterile soil that had been infested individually with several hyperparasites[22]. *H. fuscoatra*, *H. catenoides* and *P. monospermum* were the most efficient parasites, infecting at least 76% of the oospores within three weeks. *H. catenoides* and *P. monospermum* reached high levels of parasitism within 1 week; *H. fuscoatra* showed no parasitism during the first 2 weeks, but by the third week nearly 90% of the oospores were parastized. Parasitism by *Cephalosporium* sp., *D. spermatophaga*, *D. chlamydosporia* and *F. oxysporum* was 25% or less. These same seven fungi did not parasitize mycelium of *Pmg* in paired cultures on cornmeal agar or PDA, nor were there inhibition zones or any other evidence of interaction[22].

Some environmental parameters for parasitism by specific hyperparasites were studied in steamed soil. Parasitism by *H. catenoides* in flooded soil during 10 days did not occur at 16°C, but was substantial (60%) at 20°C and maximum (85%) at 28°C[22]. *P. monospermum* parasitized 32% of the oospores at 16°C, reached a maximum of 67% at 24°C, then sharply declined to parasitize 20% at 28°C. Parasitism by *H. fuscoatra* after 7 days at -0.3 bar matric potential was very low at 5-15°C, but was progressively greater at 20, 25, and 30°C, with 92% of oospores parasitized at 30°C[35]. Results for *A. missouriensis* at 0 bars were similar, except that parasitism at 15°C was quite high (63%). Parasitism by *A. missouriensis* at constant 25°C was maximum (85%) at 0 bars matric potential and decreased with decreasing matric potential to 59% at -15 bars[35]. The frequency of oospore parasitism by *H. fuscoatra* was 62% at 0 bars; it increased to 91% at -0.3 bars, then decreased to 67% at -15 bars. Thus, those parasites so far tested were active over a fairly broad range of moisture and temperature conditions.

The oospore hyperparasites appear to be quite nonspecific with respect to their oomycete host range. Oospores of *Pmg*, *Phytophthora cactorum*, *Pythium* sp. and *Aphanomyces euteiches*, all were parasitized by Hyphochytridiomycetes in flooded soils[32], but in such a test infection could have been by different organisms. In tests on water agar,

isolates of *A. missouriensis*, *H. fuscoatra*, *H. catenoides*, *P. monospermum* and *V. chlamydoporia* were tested in all possible combinations against oospores of *Pmg*, *P. aphanidermatum*, *P. ultimum*, *A. cochlioides*, *A. euteiches* and *Phytophthora citrophthora*[35]. After two weeks all host species were parasitized by all parasites except *P. ultimum* by *H. catenoides*. Parasitism ranged from a low of 14% to a high of 96% (3 instances), and in 18 of the 30 combinations, in excess of 50% of oospores were parasitized. *A. missouriensis*, *H. fuscoatra* and *V. chlamydosporia* were the most efficient of the five parasites overall. Although *H. catenoides* did not parasitize *P. ultimum* in our experiments, it did so in those of Ayers and Lumsden[3].

The actinomycetes *A. philippiensis*, *A. utahensis*, *A. auranticolor*, *A. regularis*, *S. albida* and *Micromonospora* sp., each parasitized 94-99% of *Pmg* oospores *in vitro*[35]. These organisms were not known previously to be oospore parasites.

Oospores from cultures 16, 22, or 30 days old were infected equally by *A. missouriensis* and *H. fuscoatra*. Sporangia of *A. missouriensis* from cultures 13, 29 or 45 days old and aleurospores of *H. fuscoatra* from cultures 12, 28 or 78 days old, all parasitized 95% of *Pmg* oospores[35]. Thus, within these limits, oospore susceptibility and hyperparasitic capacity were unaffected by age.

A question can be raised whether oospores produced in culture were similar in susceptibility to parasitism to those produced in natural substrates. Thus, oospores produced during 5 weeks in V-8 juice broth were compared with those produced during 5 weeks in aseptically grown soybean seedlings[22]. Oospores from the two sources did not differ greatly in susceptibility to parasitism when incubated in six different soils for 1 or 2 weeks. In flooded soils there was no difference between the two types of oospores; in drier soils there was more infection of culture-grown than of seedling-grown oospores in four of 12 comparisons, but no difference in the other eight comparisons.

There is also a question whether the oospores were viable. With respect to *Pmg*, other work in our laboratory frequently showed germinability as great as 50%[23]. Thus, viability was at least this great and probably greater, since virtually all of the oospores contained apparently living protoplasm, and as many as 65-70% assumed the "activated" stage. Since the incidence of parasitism often exceeded 90%, living oospores must have been parasitized. Another test of oospore viability is staining with a vital stain. Over 85% of *Pmg* oospores from various sources were stained with tetrazolium bromide (MTT)[34]. Hence, it appears that a high proportion of the oospores used in experiments with hyperparasites were viable.

There appears to be little specificity of parasitism with respect to race of *Pmg*. Humble and Lockwood[22] found that 60-65% as many oospores of race 7, as compared with races 1 and 3, were parasitized by *H. catenoides* and *P. monospermum*, but Sutherland and Lockwood[35] found more than 95% of the oospores of races 1, 3, 4 and 7 parasitized by *A. missouriensis* or *H. fuscoatra*.

The invasion of oospores of *Pmg* by

A. missouriensis and *H. fuscoatra* was compared by transmission electron microscopy. Both organisms rapidly penetrated the oogonial and oospore walls without production of specialized structures and with no evidence of enzymatic degradation of the walls. No apparent morphological or internal structural changes occurred in response to hyperparasitic invasion. Thus, infection by a prokaryote and eukaryote appeared to be similar.

Biological control experiments. Although hyperparasitism of oospores introduced into natural soils occurs frequently[22,23], whether it is of biological significance in reducing populations of oospores is unknown. In one approach to this question, two soil samples from an apple orchard, one where trees were infected with *P. cactorum* and the other where trees were not infected, were incubated with oospores of *P. cactorum*, *Pmg*, *Pythium* sp. and *A. euteiches*. Oospore infection of all four species was significantly greater in soil collected near healthy trees than in soil near trees infected with *P. cactorum* (e. g., 58% vs. 24%, respectively, for *P. cactorum*), suggesting a possible relationship of a high incidence of parasitism to pathogen suppression.

An attempt was made to determine if such a relationship existed in soils of different potentials for Phytophthora root rot of soybean[22]. Soils from 33 soybean fields were assayed for Phytophthora root rot potential by incubating soybean seedlings in flooded soil samples. Fifteen soils were selected, five giving severe root rot, five moderate root rot and five no disease. Oospores of *Pmg* were incubated for 1, 2, and 3 weeks in soils kept either flooded or at a lower water potential. However, there was no significant relationship between the percentages of oospores parasitized and the disease potentials of the soil at either moisture condition. In flooded soils, in which parasites could be identified, parasitism was mainly by Actinoplanaceae or Hyphochytridiales, but the root rot potentials of soils could not be correlated with particular parasite distribution patterns. It is possible that our methods were not sufficiently precise to demonstrate a correlation between hyperparasite activity and disease potential. Possibly, an incubation period longer than 3 weeks may be necessary to allow full expression of hyperparasitic activity.

Despite our finding no evidence for a role for hyperparasitism in suppressing Phytophthora root rot of soybean in field soils, the possibility that an augmented population might effect biological control was considered worthy of testing.

An essential character of biological control agents is that they not be pathogenic themselves to the plant species they are being used to protect. Soybeans were planted in steamed soil that had been infested with *D. spermatophaga*, *D. chlamydosporia*, *H. catenoides*, *H. fuscoatra* and *Cephalosporium* sp[22]. After 5 weeks, the emergence, survival, and dry weights of plants grown in the infested soils did not differ significantly from control plants. In addition, roots and foliage of treated and control plants were comparable in color, length and general vigor. Thus, there was no evidence for pathogenicity of the hyperparasits to soybeans.

In preliminary biocontrol experiments

in the laboratory, *H. fuscoatra*, *H. catenoides*, *P. monospermum* and a combination of *H. catenoides* and *P. monospermum* were incubated for 7 days in soil naturally infested with *Pmg*, then soybeans seedlings were applied[35]. *H. catenoides* and *P. monosporum* or the combination of the two reduced severity of disease in two of three experiments; *H. fuscoatra* reduced disease in one experiment. This led to a number of biocontrol experiments in the greenhouse.

Mycelia of *H. catenoids* applied on a vermiculite carrier to soil artificially or naturally infested with *Pmg*, afforded a high degree of protection against Phythophthora root rot[21]. Seedling emergence, plant height and root health frequently approached those of plants grown without the pathogen, but disease was never completely eliminated. Control was similar whether soil was treated with *H. catenoides*, then flooded for 7 days before seeding, or if the flooding and prior incubation periods were eliminated.

In other experiments, hyperparasites were applied as coatings on soybean seeds. In experiments done in a greenhouse mix artifically infested with *Pmg* oospores, seeds coated with aqueous suspensions of *A. missouriensis* or *H. fuscoatra* gave plant stands 1.4- to 4-fold higher than those from noncoated seeds [35]. Roots of plants from coated seeds appeared to be healthy. Biocontrol was achieved whether oospores were produced in V-8 juice broth or in soybean tissue.

In further work, soybean seeds were coated with the actinomycetes *A. missouriensis*, *A. utahensis*, *Micromonospora* sp. or *A. auranticolor*, or the fungus *H. catenoides*[17]. The organisms were grown in yeast extract-starch broth for 4-6 weeks, then comminuted, washed and applied to soybean seeds in a carrier of 1% carboxymethyl cellulose + 1% soluble starch in water. Seeds were sown in a loam soil either naturally infested with *Pmg* or supplemented with 400 oospores of *Pmg*/*g*.

Stands of soybeans from seeds coated with *A. missouriensis*, *A. utahensis* or *Micromonospora* sp. were increased over those from noncoated seed, in three of four experiments; *A. auranticolor* was effective in one experiment. In the experiment giving maximum control, stands were increased 2- to 2.6-fold and in some instances to levels not significantly different from healthy control plants in steamed soil. Treasments giving increased stands also tended to have reduced root rot severity and the surviving plants frequently had increased root and shoot weights, as compared with plants from nontreated seeds. *H. catenoides* was not effecive as a seed treatment, which was surprising since it gave excellent control when appled to soil in other experiments[21].

The disease control achieved by seed treatments[17] and by application of *H. catenoides* inoculum to soil at the same time as oospore inoculum[21] raises a question about the mode of action of the hyperparasites. In such experiments, can they parasitize oospores rapidly enough to account for disease control, or are they acting by some other means. The question cannot now be answered with certainty, since there is no precise means of quantifying changes in the inoculum density of *Pmg*. An approach to this

question was made using soybean seedlings as baits for *Pmg* in hyperparasite-treated soil. Soil was incubated for 1 and 3 weeks after which 10- fold dilutions were made and soybean seedlings applied. Soil treated with *A. utahensis* or *A. missouriensis*, in one experiment, gave less seedling disease than control soil at the greater soil dilutions. In a second experiment, all five hyperparasites gave less disease than control seedlings at the greater soil dilutions. Though this approach provides evidence for reduction in oospore populations, it still does not say how rapidly they can act. Electron microscopic studies of parasitized oospores *in vitro* showed extensive invasion of the oospores by *A. missouriensis* and *H. fuscoatra* after only 4 days of incubation [33], but earlier examinations were not made.

Relatively little has been done to evaluate different media for the production of inoculum of hyperparasites or the longevity of harvested inoculum in storage. Selection of appropriate growth media is especially important for the Hyphochytridiales and Actinoplanaceae, since these organisms grow slowly. Of several different media evaluated for prodution of *H. catenoides*, tryptone-glucose-yeast extract agar (TGYE) [21] supported the most growth, and growth was further enhanced when the medium was prepared with 0.2% agar to make semi-solid medium in which the fungus grew suspended. Mycelia of *H. catenoides* from TGYE cultures mixed with sterile vermiculite and stored at 25°C in plastic bags yielded about 60% of the original number of colonies after 6 months. Filonow and Lockwood[17] obtained satisfactory growth of several Actinoplanaceae and *H. catenoides* in Emerson's yeast extract-starch broth.

Hyperparasites have demonstrated potential for biological control of Phytophthora root rot of soybean in laboratory and greenhouse experiments. These results, the great diversity and broad host ranges of the hyperparasites, and the fairly wide range of soil temperatures and moistures over which parasitism occurs lend encouragement to further work on this group of biocontrol agents, not only for control of Phytophthora root rot of soybean, but for other oomycetous root diseases as well. Seed coating would seem to be the method of choice in further studies. It has given satisfactory results in greenhouse tests and would likely be a more economical and acceptable practice than broadcast application or incorporation with the seed. Further research should focus on such factors as strain selection, concentration and form of inoculum, carriers of inoculum, culture media for inoculum production, and inoculum storage potential. Field evaluations of efficacy also need to be made. Attempts so far have been frustrated because of inability to obtain sufficient disease[35].

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