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PROGRESS REPORT
PROPOSAL NUMBER 6.250
"MICROBIAL ANTAGONISMS: THE POTENTIAL FOR SELECTED STRAINS OF RHIZOBIUM TO INHIBIT LEGUME ROOT PATHOGENS"

Submitted by:
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GENERAL PROGRESS

During the past six months we have accomplished several specific tasks necessary for the successful completion of this project. The results of these experiments are discussed below. In addition, we have established communication with Dr. Eduardo C. Schroder at the University of Puerto Rico. Dr. Schroder has proposed work similar to the goals of our project, but with Macrophomina phaseolina, the causal agent of ashy blight of bean, and antagonistic Rhizobium to AID. Exchange of information, in order to coordinate research activities, is already occurring between the investigators and we are hopeful that we will be able to exchange Rhizobium cultures (or other rhizosphere bacteria) that are inhibitory to root pathogens. The interaction between Dr. Schroder and us will provide us with increased opportunity to examine the spectrum of antagonistic activity that individual Rhizobium strains exhibit.

Serita Frey, a second semester senior in the Department of Environmental Sciences at the University of Virginia, will be completing an undergraduate research project this semester that developed as a result of her work on this project. Serita is an outstanding student who will begin her graduate work this spring. Her graduate research topic will consider microbial antagonisms in the rhizosphere and will make a significant contribution to this project.

RESULTS

One of the objectives of our work has been to develop a reliable, relatively quick method of screening for inhibitory interactions between large numbers of Rhizobium and fungi. In the last progress report, three methods of screening (streak plates, double-layer plates, and spent culture media) were compared. We suggested that, in general, the methods gave consistent results although the techniques might detect different types of antagonisms. There are several advantages to the spent culture media (SCM) technique (described in previous progress reports). The technique allows for quantitative estimates (as reduction in biomass) of fungal inhibition. Using biomass

measurements also allows for direct comparison between antagonists so that they can be ranked in inhibitory activity. It is clear that Rhizoctonia solani growth in spent culture media differs greatly as a function of the Rhizobium strain from which the media were derived (Table 1). In addition, replicate tests of inhibition using SCM are consistent, although the relative ability of the Rhizobium strains may be somewhat different between tests (Table 1).

Tests using SCM are most likely to detect antagonisms that result from the production of inhibitory metabolic products. Frequently, these inhibitory products are inactivated by heating. The procedure that has been used in our experiments involve autoclaving, so that it is possible that antagonistic activity has been substantially altered or lost. Thus, spent culture media were filter sterilized and inhibition of R. solani compared to autoclaved SCM. The results are presented in Table 1. In all but 4 cases, fungal biomass production was less in the filter sterilized SCM than in the autoclaved SCM. In the three of the instances in which biomass production was not less in the filter sterilized SCM, the inhibition observed did not change the ranking of the R. phaseoli strains substantially (Table 2). It is important to note that in two cases, Bacillus pumulis and R. phaseoli 526 203 CR, filter sterilization caused a significant decrease in the ability of R. solani to grow in the SCM (Table 1) and an increase in the relative (change in rank in comparison with the other organisms tested) inhibitory ability of the bacteria (Table 2). It is likely that the difference between autoclaving and filter sterilization is a result of heat inactivation of an inhibitory compound excreted into the growth medium by B. pumulis and R. phaseoli 526 203 CR.

When fungal growth is expressed as the % of growth observed in untreated culture medium and the results from the autoclaved and filter sterilized SCM experiments are plotted against one another (Figure 1), it is clear that both methods yield similar results for 85% (14 of 16) of the strains tested. In the case of B. pumulis and 526 203 CR, the error in prediction of the effect of the two strains was conservative. That is, the strains were classified as unable to inhibit the fungus. Since the autoclaved SCM technique yields results similar to the filter sterilized SCM technique, although more conservative, and because the autoclaved SCM technique is much less labor intensive, the autoclaved SCM technique was chosen for all further laboratory screening work.

The approach that we have used to screen the Costa Rican R. phaseoli and R. solani is to test the rhizobia against fungi isolated from the same fields. This has yielded some very intriguing results. In all of the cases tested to date (some of the data are not included here), the Costa Rican rhizobia were not inhibitory to R. solani from the same plots and in several cases actually stimulated the growth of the pathogen (Table 3). When R. solani isolate 12.1 was tested with R. phaseoli 576 2535 and 574 127k17 (rhizobia which were classed as inhibitory in previous tests), fungal biomass production was drastically

reduced. We are presently pursuing the idea that the best antagonists may be organisms that have been isolated from areas that widely separated geographically. This line of investigation involves predicting the relative ability of R. phaseoli strains to inhibit R. solani isolates based on where the organisms were isolated and comparing the results to fungal biomass production obtained using the SCM technique. Exchange of strains with Dr. Schroder will enhance this aspect of the investigation. As was suggested in the last progress report, comparison of the R. phaseoli strains antibiotic resistance patterns and presence of plasmids might also help to elucidate the possible mechanisms responsible for fungal inhibition. It would certainly be exciting if antibiotic resistance and plasmid composition are have a biogeographic basis.

We have begun the first set of greenhouse trials to test the ability of those R. phaseoli strains that caused greater than 50% reduction in biomass production using the autoclaved SCM technique. B. pumulis and R. phaseoli 526 203CR will also be tested in order to further evaluate the differences between autoclaving and filtering SCM. Preliminary experiments were done to determine the protocol that results in consistent Phaseolus vulgaris response to the presence of R. solani in soil. A description of the method that will be used for the greenhouse experiments follows. Oven dry commercial potting soil for 12 hours at 80°C. Place 450 g of dry soil and 55 g of chopped raw potato in a 1000 ml beaker and moisten. Autoclave the chopped potato-soil mixture for 3 hours. Next, add a standard R. solani inoculum (described in earlier progress reports) to the potato-soil mixture and incubate for 7 days at 30°C. This mixture is then used to inoculate soil that will be planted with bean seeds. For the R. solani treatment, one beaker of fungal inoculum is mixed with 450 g of moist potting soil and placed in autoclaved clay pots. A similar mixture of uninoculated chopped potato soil and potting soil is prepared for the uninoculated treatments. The pots are watered and allowed to incubate for 2 days prior to planting. Treated seeds (gum arabic or gum arabic and rhizobia) are planted in the pots at one inch square spacing. After three weeks, the plants are clipped at the soil surface, dried, and weighed. Plants in uninoculated pots were vigorous and showed no sign of damping-off. Seeds in the R. solani pots germinated equally as well as those in the uninoculated pots however the seedlings were significantly smaller than the uninoculated based on dry weight measurements of the plant foliage. All plants in the inoculated pots had large lesions at the soil surface. If the plants were allowed to grow for longer than three weeks, the tops began to fall over. We have had very good success at replication within an experiment and between experiments using the methods described above. Tests with plant growth pouches were more difficult to set-up and gave inconsistent fungal infection of the plants. We believe that the soil pot method will provide a better measure of the type of plant response that will be observed in the field than plant growth pouches.

REMAINING ACTIVITIES

In the next six months, we expect to complete screening of all of the Costa Rican R. phaseoli strains against the R. solani strains isolated from the same location. Most, if not all, strains exhibiting antagonistic activity will have been tested in the greenhouse, depending on the number of strains that are classified as antagonistic as a result of screening in SCM. After a meeting between the PI's to outline the field testing protocol, field trials with some of the antagonistic strains will begin in Costa Rica this spring and continue the following fall and spring. In addition, we will continue to investigate the mechanism of the antagonistic activity for those strains that give the greatest degree of root-rot control as determined in the greenhouse tests. Another question that we hope to pursue is the stimulation of the pathogen by organisms isolated from the same location.

Table 1. Growth of *R. solani* 18619 in media prepared from spent culture media prepared by autoclaving or filtration.

Source of Spent Medium	% of Uninhibited in autoclaved medium	% of Uninhibited in filtered medium
NONE	100.00	100.00
Rs 18619	103.00	97.06
Pseudomonas fluorescens	61.42	74.76
Bacillus pumulis	86.04	37.36
526 203CR	69.76	23.82
576 2535	36.88* (44.04)[1]	20.37
573 127K14	41.92 (36.30)	21.51
574 127K17	37.75	20.74
523 127K17	52.09	37.36
127K12b	59.86	48.95
575 127K21	84.85	90.35
668 6-3SE1N	76.80	ND[2]
127K105	57.45	ND
127K80e	74.14	78.50
127K47	52.63	48.85
693 523 SESN	41.75	29.49
127K44	68.24*	54.57
127K86	37.64	ND
127K102	51.36*	69.84

* Mean of 3 replicates, all others N=4.

(1) Numbers in parentheses are the results from an independant replicate experiment.

(2) ND = not done.

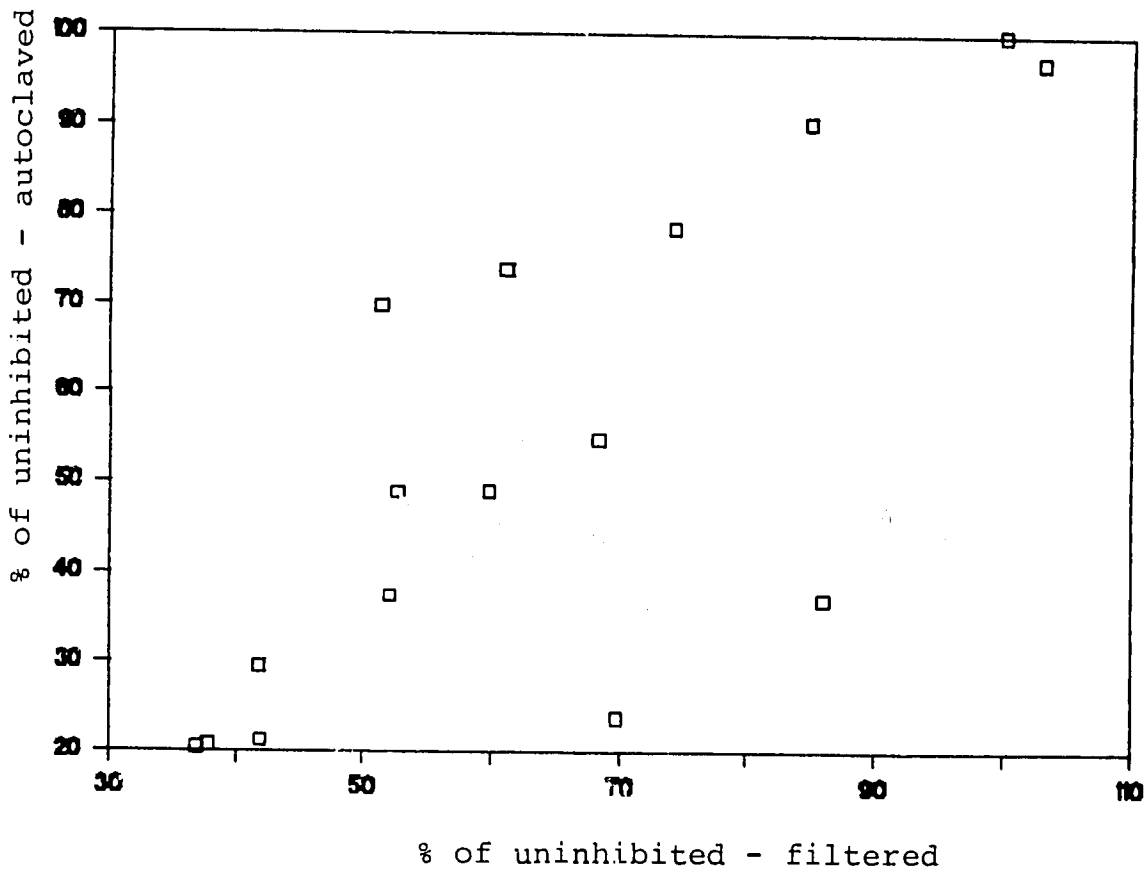
TABLE 2. Relative ablility of bacteria to inhibit the growth of *R. solani* 18619 (Rs 18619). Organisms with number designations are *R. phaseoli* strains. Least inhibition = 1, greatest inhibition = 16.

Source of Spent Medium	Autoclaved Medium Rank	Filtered Medium Rank
NONE	2	1
Rs 18619	1	2
Pseudomonas fluorescens	8	5
Bacillus pumulis	3	10.5
526 203CR	6	13
576 2535	16	16
573 127K14	13	14
574 127K17	15	15
523 127K17	11	10.5
127K12b	9	8
575 127K21	4	3
127K80e	5	4
127K47	10	9
693 523 SESN	14	12
127K44	7	7
127K102	12	6

TABLE 3. Growth of Costa Rican R. solani in autoclaved spent media. Costa Rican R. phaseoli (stains preceded by "CR") were isolated from the same location as the R. solani isolate. Percent uninihibited are calculated from the mean of 4 replicates.

Source of Spent Medium	Rhizoctonia Inoculated	% of Uninhibited
CR4020	RH 9.1	85.14
CR4022	RH 12.1	535.00
CR4001	RH 5.1	126.50
CR4003	RH 6.1	156.80
CR4004	RH 7.1	71.86
CR4011	RH 7.1	95.07
576 2535	RH 12.1	24.02
574 127K17	RH 12.1	22.25

FIGURE 1. Comparison of SCM prepared by autoclaving and filter-sterilization.



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PROGRESS

We have completed a number of tasks that were listed in Table 2, Time Frame and Responsibilities, of the original proposal which is attached to this progress report as Table 1. Tasks that have been completed include selection of locations in Costa Rica for the field trials, collection and analysis of soil samples from the Costa Rican study sites, and isolation and preservation (by lyophilization) of Rhizobium phaseoli and Rhizoctonia solani strains. We have also made significant progress in screening rhizobia for antagonistic activity to the pathogen.

A large number of Rhizobium phaseoli and Rhizoctonia solani strains from the United States and Costa Rica have been collected. At this time we have obtained 15 R. phaseoli strains from the U.S., have purchased two R. solani strains from the American Type Culture Collection, and isolated one strain from soil collected at the University of Virginia's Research Farm, Blandy Experiment Station. During the last six months we have also isolated several hundred strains of R. phaseoli from Phaseolus vulgaris nodules and over 100 strains of R. solani from infected bean plants growing in actively farmed P. vulgaris fields in Costa Rica. Eighty (80) R. phaseoli and 18 R. solani strains from the Costa Rican isolates have been randomly selected for use in screening tests of antagonistic activity. The Costa Rican strains were shipped to the U.S. in July, 1987.

It is important that we have a reliable, relatively quick method of screening for inhibitory interactions between the rhizobia and fungi in order to test large numbers of bacterial and fungal strains. Thus, we chose to begin working out methods of testing for antagonism with U.S. strains while the Costa Rican microorganisms were being isolated. Tests with three different screening techniques, streak plates, double-layer plates, and spent culture media, have been completed. The methods are described below, and the results of these tests summarized in Tables 2 and 3. In addition to these 3 techniques, we are presently testing two additional screening procedures, seed germination/seedling survival in soil and cellophane plant

pouches. The results of all 5 screening techniques will be compared for consistency between the techniques and one or more techniques selected for screening the Costa Rican strains.

METHODS

STREAK PLATE: Petri dishes containing yeast extract mannitol (YEM), a medium that will support the growth of both rhizobia and fungus, were prepared. One half of a plate was streaked with *Rhizobium* and the other half inoculated with a *R. solani* sclerotium. The plates were incubated at 30 °C for 5 days and fungal growth in the presence of the rhizobium compared to that of the fungus grown on YEM in the absence of the bacterium.

DOUBLE-LAYER PLATE: *R. phaseoli* strains were grown in YEM broth until a heavy suspension of cells developed (3-4 d). Bacterial cultures were centrifuged and the cells suspended in 10 ml of YEM broth. One milliliter of the suspension was added to each petri dish and tempered YEM agar was added to the cells. Rhizobia were distributed throughout the agar by gently swirling the plates. After the agar solidified, another layer of sterile, uninoculated YEM was poured over the first layer. Plates were incubated at 30 °C for 24 h. One *R. solani* sclerotium was placed on each plate and the plates incubated at 30 °C for 2-5d. Fungal growth in the plates containing rhizobia was compared to growth on YEM without rhizobia.

SPENT CULTURE MEDIA: *R. phaseoli* strains were grown in YEM broth until a heavy suspension of cells developed. Cultures were centrifuged and the supernate divided into 20 ml portions. Each aliquot was amended with mannitol and salts to insure no carbon and mineral limitations. The supplemented, spent media were autoclaved, cooled, and inoculated with standard sized cubes of agar containing even, 3 day old growth of *R. solani*. Earlier experiments showed that this method of inoculating with the fungus gives consistent results between replicates. The inoculated flasks were incubated at room temperature for 6 d and the fungal biomass measured after filtering and drying. Fungal biomass production in fresh YEM broth was used as a standard for comparison. Growth in the spent medium was expressed as the percent reduction in fungal biomass (Table 3). 50% or greater reduction in growth was arbitrarily considered to be evidence for an inhibitory effect of the rhizobia on fungal growth (Table 2).

DISCUSSION

Comparison of the methods for evaluating the antagonistic affect of bacteria on fungi shows that the results tend to be consistent between techniques for some strains but not for other strains (Table 3). Six of 15 tested strains of rhizobia showed some degree of inhibition to *R. solani* 18619. In the case of

strains 574, 576, and 573 the results of all three screening procedures indicated some degree of inhibition. Strain 526 inhibited the fungus when tested using the streak plate and double-layer plate methods. Although fungal growth was reduced by 36% in the spent medium assay with rhizobium 526, no inhibition was recorded in Table 2 for this strain since we arbitrarily set the cutoff for inhibition at 50%. Strains 693 and 127K86 reduced fungal growth by over 60% in spent media trials, but did not prove to be inhibitory in the streak plate and double-layer plate tests.

Differences in the inhibitory activity of the rhizobia between techniques may be related to the mechanism of antagonism. For example, antagonisms that result from competition for carbon would not be evident in the spent medium assay since the bacterium and fungus are not grown together and the medium is supplemented with carbon prior to inoculation with the fungus. If the inhibition is a result of a bacterially produced extracellular toxin, the spent medium assay may be more sensitive as the toxin would be well distributed throughout the medium during shaking and would not have to diffuse through solid medium used in the streak plate and double-layer plate tests.

It is interesting to note that all of the strains exhibiting antagonism (Table 3) are also resistant to at least one antibiotic with the exception of strain 693 which has not been tested for sensitivity to antibiotics. The correlation between antibiotic resistance and antagonistic activity is an area that merits further exploration.

REMAINING ACTIVITIES

During the next six months we will plant the preliminary bean plots (September) at both field sites, complete assessment of the screening techniques with the U.S. strains of R. phaseoli and R. solani, and begin screening of the Costa Rican strains. We plan to begin field tests with the antagonistic rhizobia during the spring 1988 planting season and continue with field trials the following fall and possibly spring.

TABLE 1: Progress Report
Table 2. Time frame and responsibilities

TIME FRAME AND RESPONSIBILITIES OF INVESTIGATORS

TASK	YEAR ONE				YEAR TWO				YEAR THREE			
	W	S	S	F	W	S	S	F	W	S	S	F
Select field locations	R&L*											
Establish plots	R&L											
Plant preliminary bean plots		R	R									
Isolate R. solani			R	R								
Isolate R. phaseoli			R	R								
Sample soil and characterize					R	R						
Screen R. phaseoli isolates for antagonistic activity					L	L	L	L				
Field trials with antagonistic rhizobia and data collection								R&L	R	R	R	
Data analysis and manuscript preparation											R&L	R&L

* R = Ramirez's responsibility

L = Lennox's responsibility

TABLE 2. Comparison of the streak plate, double-layer, and spent culture media techniques for measuring inhibition (no inhibition, -; possible inhibition, +/-; inhibition, +) of R. solani 18619 by R. phaseoli strains.

STRAIN	STREAK PLATE	DOUBLE-LAYER	SPENT MEDIA
None	-	-	-
574	+/-	+/-	+
575	-	-	-
127K105	-	-	-
576	+	+/-	+
127K86	ND*	-	+
127K12b	-	-	-
127K47	-	-	-
127K102	-	-	-
523	-	-	-
127K44	-	-	-
668	-	-	-
526	+	+/-	-
573	+	+	+
693	-	-	+
127K80e	ND	ND	-

*ND = NOT DONE

TABLE 3. Growth of *R. solani* 18619 in media prepared from *R. phaseoli* spent culture media.

Source of Spent Medium	Fungal Biomass*(g)(SD)	% of Uninhibited
no rhizobia	0.01618 (0.00103)	100
575	0.01373 (0.00807)	84.9
668	0.01243 (0.00080)	76.8
127K80e	0.01200 (0.00196)	74.2
127K44	0.01104 (0.00052)	68.2
526	0.01030 (0.00098)	63.7
127K12b	0.00969 (0.00028)	59.9
127K105	0.00930 (0.00129)	57.5
127K47	0.00852 (0.00097)	52.7
523	0.00843 (0.00111)	52.1
127K102	0.00831 (0.00035)	51.4
693	0.00691 (0.00054)	42.7
573	0.00611 (0.00074)	40.0
574	0.00609 (0.00043)	37.8
127K86	0.00609 (0.00114)	37.6
576	0.00597 (0.00096)	36.9

*Mean of 4 replicates except strains 127K44, 127K102, and 576 where N = 3.