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

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GENERAL PROGRESS

The past six months have proven to quite productive in several aspects including experimental results, papers and manuscripts, interaction between Costa Rican and U.S. investigators, and continued communication and cooperation with Puerto Rican investigators. Specific experimental results are discussed below. We have submitted an abstract for an oral presentation to the American Phytopathological Society to be presented at the November meeting in San Diego. In addition, the first draft of a manuscript describing the screening methods that were developed as part of this project is complete and I expect that the final draft will be finished within the month and submitted to Applied and Environmental Microbiology. We have exchanged antagonistic Rhizobium phaseoli cultures with Dr. Eduardo Schroder at the Univ. of Puerto Rico and are making plans for Eduardo to present a seminar at the Univ. of Virginia this fall. We are hopeful that Eduardo's stay in Beltsville during the fall will facilitate discussion and cooperation between the Univ. of Virginia and Univ. of Puerto Rico research groups.

In addition to Serita Frey beginning graduate school this fall, a Costa Rican woman, Gabriela Soto M., will be arriving in Charlottesville to begin her Master's program. Both of these students have been awarded graduate research assistantships and will be supported by funds from this AID grant. I am very pleased to have two such enthusiastic students working on biological nitrogen fixation and biological control of plant diseases. We are expecting Gabriela to arrive in Charlottesville any day.

Serita Frey and I traveled to Costa Rica at the beginning of July. During our trip we were able to see first hand the significant impact that Rhizoctonia solani seed rot and damping-off has on bean crops in Costa Rica. With the advise of Dr. Carlos Ramirez and Gabriela, we selected two field sites, one at San Antonio on the western coastal plain and the other at Esparza in the Central Valley, for field testing our best Rhizobium antagonists. Neither site has been planted to Phaseolus vulgaris within the past 5 years.

In May, both fields were planted with uninoculated beans to determine the incidence of indigenous R. solani and infective Rhizobium phaseoli. Mature bean plants at San Antonio showed no evidence of web blight or seed rot, but infrequently lesions typical of damping-off were found. No R. solani root lesions were detected at a minimum dilution of 1×10^{-4} using an MPN technique with bean plants. Thus, we believe that R. solani populations in this field are much lower than those found at Esparza. However, populations of indigenous rhizobia are quite high (1×10^{5} cells/gram of soil based on MPN tests with beans in greenhouse tests). The nodules formed by the indigenous rhizobia on plants examined in the field appear to be effectively fixing nitrogen since no signs of nitrogen deficiency were observed and the nodules were quite pink when cut open. No tests have been done to determine rates of fixation at this time.

Beans at the Esparza site were highly impacted by web blight. The causal agent of web blight is R. solani anasotmosis groups 1 and 2. Seed rot and damping-off (anasotmosis group 4) were also observed. MPN tests for R. solani damping-off lesions were positive at dilutions of 1×10^{-5} of the soil. This is at least a 2-fold difference in the pathogens population between the two sites. No Rhizobium nodules were found on roots from field-grown plants. No nodules were detected by the plant MPN technique at a dilution of 1×10^{-3}, thus the population of indigenous rhizobia capable of infecting beans is much lower than observed at San Antonio.

Using two sites with differing fungal and rhizobial populations will allow us to test a variety of interactions including competition between inoculated and indigenous rhizobia, and antagonism between the rhizobia and the fungal pathogen. Plans were finalized for the field tests during our visit to Costa Rica. A minimum of three separate tests of three of the most antagonistic rhizobia (as determined in greenhouse tests) at each location will be done. Ms. Floribeth Mora Umana, a student at the Univ. of Costa Rica, will be doing this portion of the work and it will serve as the basis for her thesis research. Parameters that will be used to evaluated the effectiveness of biocontrol of the fungus include number of seeds germinated after 2 weeks, number of plants surviving to maturity, dry weight of surviving plants, carbon/nitrogen ratio of surviving plants, number and dry weight of root nodules, disease incidence and disease severity (see later in this report), and in one set of experiments serotyping of rhizobia in the nodules for strain identification. Identical experiments will also be conducted in the greenhouse. It will be necessary for us to receive an extension of the granting period to be able to complete 3 sets of field trials.

RESULTS

In the January, 1988 progress report, I indicated that several of the most antagonistic rhizobia were also strains that we knew were resistant to a number of antibiotics. I speculated that antibiotic resistance might be correlated with antagonism. We have followed up on this line of investigation. Petri dishes containing yeast extract mannitol (YEM) agar were spread with broth cultures of individual Rhizobium strains and filter paper discs impregnated with antibiotics placed on the surface of the agar. The plates were incubated at 27 ^{o}C and examined after 2 and 4 days of incubation. Growth in the presence of six common antibiotics, tetracycline, erythromycin, kanamycin, streptomycin, penicillin, and chloramphenicol, was noted for 4 of the most antagonistic strains and 5 strains that showed either no inhibition or were stimulatory to R. solani. The results are shown in Table 1. It is clear that there is no correlation between antibiotic resistance and degree of antagonism. It is interesting to note that, in general, the pattern of antibiotic resistance is very similar between R. phaseoli strains. One additional experiment to examine several of the Costa Rican rhizobia will be done for completeness and unless the results differ significantly from previous experiments, this line of research will not be continued.

We are pursuing the idea that the best antagonists may be organisms that have been isolated from areas that are widely separated geographically. Combinations of bacteria and fungi isolated from different regions are being tested in the laboratory. Preliminary results with two Costa Rican rhizobia that are stimulatory to fungal isolates from the same geographic area and inhibitory to one U.S. R. solani suggest that the C.R. fungi and the U.S. fungi respond differently to the rhizobia. These combinations of organisms are presently being tested in the greenhouse. This line of research is quite intriguing and will be continued during the next six months.

In spite of the extremely hot weather this summer and almost continual problems with the cooling system at our greenhouses, we have completed several sets of experiments with one of our most antagonistic Rhizobium strains, 127K17. The results of these tests are presented in Tables 2 and 3. The methods used for the greenhouse experiments were described in the last progress report. In our first greenhouse experiment, R. phaseoli 127k17 was tested with red kidney bean and R. solani 18619. The plants were examined weekly for 4 weeks. Although 127k17 is normally infective on red kidney bean, none of the plants were nodulated. Protection with fungicide was much more effective (90% germinating, 93% of those germinating surviving for 4 weeks) than protection with rhizobia (32% germinating, 72% surviving). No difference was observed in the number of seeds that germinated in the R. solani treated seeds (66%) compared to the R. solani and 127k17 treated seeds (64%). However, of the seeds that did germinate and emerge from the soil, the number of plants surviving for 4 weeks was much greater when the seeds were

inoculated with both R. solani and R. phaseoli (72%) than with only the fungus (52%). These results indicate that 127k17 has little effect on seed rot, but is most effective at controlling the incidence of damping-off.

An index of disease severity and disease incidence was included as a measure of the effectiveness of control of damping-off in the second greenhouse trial. Disease incidence is expressed as the percentage of the surviving seedlings that have signs and/or symptoms of damping-off. For disease severity, each plant is examined for lesions and given a score from 0 - 5, where 0 = no lesions, 1 = lesions less than 2.5 mm long, 2 = lesions between 2.5 and 5.0 mm long, 3 = lesions greater than 5.0 mm long, 4 = lesions girdling plants and visibly wilting leaves, and 5 = seedlings damped-off or dead. The individual plant scores are summed for each treatment and divided by the number of seedlings observed. Thus, a low score indicates less disease than a high score.

In the second greenhouse experiment, white kidney beans were grown for 6 weeks and all inoculated white kidney beans were very heavily nodulated. The results of this experiment have not been tested yet for statistical significance, but in cases where appropriate, the standard error of the mean is indicated. Approximately 98% of the seeds germinated when inoculated with 127k17 and R. solani, while 90% of the seeds germinated when inoculated with the fungus only (Table 3). Thus, 127k17 may be providing some measure of protection from seed rot, while measures of damping-off (i.e., the number of survivors, disease indices, and dry weights) are quite similar although the foliage dry weights may be reduced in the presence of the pathogen without fungicide or rhizobia.

The results of the greenhouse tests are not conclusive and the results with white kidney beans are different than those observed for red kidney bean where the effect of the antagonistic rhizobia was to protect the plants from damping-off. These differences are most likely related to the plant types used for the experiments. Measures of foliage dry weights and disease indices would have provided important additional information for comparison between experiments. Subsequent greenhouse tests are in progress and will include measures similar to those used for white kidney bean. The question of the effect of plant type on expression of antagonism will also be addressed.

REMAINING ACTIVITIES

During the next six months, greenhouse tests with our best antagonists will continue. In addition, in some very preliminary tests with no replication, we have had some success with a Bacillus species that Carlos Ramirez has used for control of Monilinia brown rot of coffee beans. This organism will be added to our list of potential biocontrol agents. We will continue to

screen various combinations of rhizobia and fungi in the laboratory. Field trials are ready to start at the beginning of September and should yield information on biocontrol as well as competition between inoculated vs. indigenous rhizobia. The field tests will be repeated several times throughout the next year. We also plan to examine the relationship between stimulatory rhizobia and fungi in the soil-plant system.

Table 1. Antibiotic resistance: Comparison of strains with varying antagonistic activity. + = antibiotic resistance, - = antibiotic susceptible, 0 = no lawn. The antibiotics used are Te = tetracycline, E = erythromycin K = kanamycin, S = stsreptomycin, P = penicillin, C = chloramphenicol.

Strain	Te	E	K	S	P	C
	Strains inhibitory to R. solani					
526203CR	-	+	-	+	+	+
57317K14	-	+	-	+	+	+
5762535	-	+	-	+	+	+
574127K17	-	+	-	+	+	+
	Strains not inhibitory to R. solani					
575127K21	-	±	+	+	+	-
127K44	-	-	-	-	-	-
127K80e	-	+	-	±	-	-
6686-3SE2N	+	+	-	+	+	+
127k12b	0	+	+	+	0	0

Table 2. Test of antagonistic activity in a soil-plant-microbe system with red kidney bean (P. vulgaris). No nodules were observed in any treatment

	# Germinated	% Germinated	# Survivors	% Survivors
None (5)	50	100	50	100
Captan (5)	45	90	42	93.3
Ph[1] (4)	39	97.5	39	100
Rs[2] (5)	33	66	17	51.5
RR[3] (5)	32	64	23	71.8

[1] Rhizobium phaseoli
[2] Rhizoctonia solani
[3] R. phaseoli + R. solani

Table 3. Test of antagonistic activity in a soil-plant-microbe system with white kidney bean (*P. vulgaris*). Numbers in parentheses represent the mean for n pots.

Treatment (n)	# Germinated	% Germinated	# Survivors	% Survivors	Disease Severity	Disease Incidence	Top Dryweight
None (3)	30 (10 ± 0)	100	30 (10 ± 0)	100	0	0	10.3 ± 1.78
Captan (4)	39 (9.8 ± .4)	97.5	35 (8.8 ± 1.3)	89.7	2.84 ± 0.80	87	8.85 ± 2.69
Ph[1] (3)	30 (10 ±0)	100	30 (10 ± 0)	100	0	0	10.54 ± 0.82
Rs[2] (3)	27 (9 ± 0.8)	90	26 (8.7 ± 0.5)	96.2	2.88 ± 0.10	100	7.34 ± 1.15
RR[3] (4)	39 (9.8 ± .4)	97.5	33 (8.3 ± .4)	84.6	3.44 ± 0.13	100	8.98 ± 1.87

[1] *Rhizobium phaseoli*
[2] *Rhizoctonia solani*
[3] *R. phaseoli* + *R. solani*