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Final Progress Report
(THIRD SUBMISSION)
January 31, 1995
PSTC Project No. 6.250
"Microbial Antagonisms: Potential for Selected Strains of Rhizobium to Inhibit Legume Root Pathogens"

SUMMARY OF PROJECT FINDINGS

1. The degree of virulence of 12 isolates of *Rhizoctonia solani* obtained from bean stems from different regions of Costa Rica was determined. Autoclaved pots and soil were inoculated with agar cultures of the fungus and *Phaseolus vulgaris* var. Negro Huasteco were planted two days after soil inoculation with the fungus. Significant differences were observed in stem length and infection index. Isolate R4, from Rosario de San Carlos, Alajuela Province was the most virulent strain while R10 from Canas Gordas, Coto Brus, was the least virulent. The most virulent strains were used in experiments described below.

2. Strains of *Rhizobium leguminosarum* biovar. *phaseoli* (Rlp) were screened for antagonistic activity toward the root-rot pathogen *Rhizoctonia solani*. Four screening techniques were compared: streak plates, double-layer plates, autoclaved culture filtrates, and filter-sterilized culture filtrates. Bacterial strains identified as antagonistic with a given screening method were consistently identified as antagonistic when tested with the other screening techniques, although the type of antagonism detected may have varied among techniques. The autoclaved spent-medium technique was determined to be the most rapid and reliable method. This method is particularly useful since it allows for quantification of the degree of inhibition based on fungal biomass production. Nine Rlp strains were found to inhibit *R. solani* biomass production by at least 50%. In greenhouse trials, inoculation of *Phaseolus vulgaris* seeds with Rlp 574-127K17, the best antagonist, increased seedling survival by 20.3%. Selection for antagonistic *Rhizobium* species and seed inoculation with these nitrogen-fixing bacteria may provide biological control of seed and root diseases of leguminous plants.

3. Common beans, *Phaseolus vulgaris*, are often inoculated with *Rhizobium leguminosarum* biovar. *phaseoli* (Rlp) to alleviate the need for extensive nitrogen fertilization. Because rhizobia are competent rhizosphere colonizers, are frequently capable of survival in the soil, and are known to produce bacteriocins and iron chelating compounds (siderophores), rhizobia have the potential to reduce the severity of seed and root decay diseases. Little is known about the interaction between rhizobia and root pathogens in the rhizosphere and the effect on plant health. The objective of this set of experiments was to compare the ability of Rlp strains to reduce the severity of seed decay and damping-off of *P. vulgaris*.

The approach used was to screen rhizobia for their ability to inhibit biomass production in laboratory culture by *Rhizoctonia solani*, a root pathogen of common beans. The fungus was inoculated into media in which the rhizobia had been previously grown and removed. Prior to fungal inoculation, supplemental sugars were added to the spent media to eliminate carbon and

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energy limitations. Those *Rhizobium* strains which reduced the fungal biomass by at least 50% were considered to be inhibitory, while those strains that increased growth by greater than 115% were considered to be stimulatory. Forty-nine *Rhizobium* strains were tested with six different fungal isolates. Six of the stains were inhibitory, while 6 were stimulatory.

After the initial screening, several inhibitory and stimulatory rhizobia were examined for the production of fluorescent pigments on King's medium B (KMB), used to detect the production of water-soluble, yellow-green pigments that are characteristic of siderophores. The KMB was amended with mannitol and vitamins to allow for *Rhizobium* growth. Only *Rhizobium* strains capable of fungal inhibition produced a fluorescent pigment. However, not all (2 of 6) inhibitory strains produced pigment. The addition of 1.5 μM $FeCl_3$ to KMB allowed for typical fungal colony formation, while growth on KMB was limited by the pigment-producing rhizobia; siderophore production by the pigment-producing rhizobia may be responsible for the inhibition observed in spent culture media.

Several strains that exhibited the greatest degree of antagonism and fluorescent pigment production and one strain that enhanced fungal biomass production were used to study the effect of rhizobia on disease severity in greenhouse trials with *P. vulgaris*. Five treatments were used in each experiment: (1) no *Rhizobium* or fungus, (2) fungus plus the fungicide, captan, (3) fungus only, (4) *Rhizobium* only, and (5) fungus plus *Rhizobium*. The number of seeds which germinated, number of seedlings surviving to 28 days, disease severity, and foliage dry weight per pot were determined.

Seed inoculation with 574-127K17 did not protect the seed from decay, however, for those seeds that germinated, seedling survival was significantly enhanced. For strains 573-127K14 and 576-2535, inoculation with these rhizobia decreased both the incidence of damping-off (increased seedling survival) and the index of disease severity. With strain 4022, a stimulatory strain, disease severity was significantly greater in the presence of the *Rhizobium*.

The results of these experiments suggest that screening of *Rhizobium* inoculants for their effect on seed and root disease may be as critical as screening for effectiveness (N_2-fixation) and competitive ability (nodule occupancy).

4. Because a primary goal of *Rhizobium* ecology is to understand what factors determine strain survival in the rhizosphere and success in nodule formation, the competitive interaction between strains of *Rhizobium leguminosarum* biovar phaseoli in the rhizosphere of *Phaseolus vulgaris* was examined. We attempted to examine the effect that genetic relatedness of the Rlp strains has on the severity of competition and on the pattern of nodulation at harvest. Six isolates of Rlp of known genetic relatedness were obtained. Genetic relatedness was based on electrophoretically detectable allelic variation at 15 enzyme loci and ranged from 20% to 87% similar. All six strains were found to be infective and effective on *P. vulgaris* L. var. Duchess. Although the strains selected were in general genetically heterogeneous, the polyclonal fluorescent antibodies produced for this study lacked the specificity necessary for differentiation of the test strains. It

is recommended that genetic probe technology be employed for future studies which require enumeration of related *Rhizobium* strains. This technique is more specific and more sensitive than immunofluoresence techniques.

5. The effect of pH on competition for nodule occupancy by Rlp type I and type II strains was also examined. A nonsterile soil system was used to examine the competitive abilities of type I and type II strains under moderately acidic and circumneutral conditions. Nodule occupancy, nodule number, and plant yield were determined. Additionally, the rhizosphere populations of competing type I and type II strains were monitored during the first ten days following inoculation. The results indicate that soil pH can influence which symbiotype of Rlp will competitively nodulate beans and that the observed patterns of nodule occupancy were not related to the relative numbers or specific growth rates of competing strains in the host rhizosphere prior to nodulation.

6. To determine the combinations of environmental variables that influence the geographic distribution of indigenous populations of *Rhizobium leguminosarum* biovar phaseoli (Rlp) in Costa Rica, soil samples from 47 sites representing a wide range of soil types and climate conditions were collected. At each sampling location, two soil samples were collected; one from a field planted with beans and the other from a field that had not been planted with beans for at least the past three years. For each field, the agricultural history was obtained from the farmer and climate data were obtained form the Instituto Climatologico Nacional including rainfall distribution, mean annual temperature, mean maximum and mean minimum temperature, and mean daily hours of light. A complete soil analysis, including texture, organic matter, pH, P, Ca, Mg, K, interchangeable acidity, Fe, Mn, Zn, and Cu was done for each sample. The numbers of Rlp were determined by a most probable number technique using the bean variety Negro Huasteco. A separate principal component analysis was done to relate the abundance of Rlp to soil, climate, agricultural practices and biological factors.

The major correlations observed between Rlp abundance and environmental conditions were: 1.) a negative correlation with organic matter, Fe, and Mn concentrations; 2.) a negative correlation with K and pH; 3.) at bean sites only, a positive correlation with the use of fertilizer and a negative correlations with slope; 4.) at non-bean sites only, a positive correlation with herbicide and flooding; and 5.) a positive correlation with the prior presence of beans, the presence of nodules at sampling time, and the total bacterial counts. Factor 5, was expected because a preliminary examination of the data in which a t-test was used, revealed a significant difference ($P<0.05$) in Rlp abundance between bean and non-bean sites. Mean values of $5.6 \times 10^4 +/- 1.1 \times 10^3$ cells g of $soil^{-1}$ at bean sites, and $1.8 \times 10^3 +/- 4.5 \times 10^3$ cell g $soil^{-1}$ at non-bean sites. A strong correlation with climate was not observed, however, some of the Rlp variation at bean sites was positively correlated with temperature and daily hours of light, while at non-bean sites the opposite was observed. The geographic distributions of each of these correlations was prepared: a more regional distribution of Rlp was observed for soil and climate variables, while a patchy Rlp distribution was observed for most agricultural practices. This work is the first study to attempt to examine the geographic distribution of a non-spore-forming soil bacterium, and to determine the environmental conditions that affect that distribution. The

correlations mentioned above provide insight into the combinations of variables that may be critical to understanding the mechanisms that determine indigenous Rlp distribution.

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