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Impact of Pseudomonas putida on nodulation in the common bean, Phaseolus vulgaris.

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IMPACT OF *PSEUDOMONAS PUTIDA* ON NODULATION IN THE COMMON BEAN, *PHASEOLUS VULGARIS*

A Thesis Presented

By

HOWARD DEAN GRIMES

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For My Parents

James and Marcia

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ABSTRACT

Pseudomonas putida, a potential biocontrol agent, is being investigated for its effects on the beneficial soil bacterium *Rhizobium phaseoli*. A random block field plot design was used with treatments consisting of no bacteria, *P. putida*, *R. phaseoli*, and combinations of these. *R. phaseoli* was applied directly to the soil. *P. putida* inoculation was performed by soaking seeds for 15 minutes in a dH_2O-suspension. Statistical analysis of the first year's field data indicates that *P. putida* is able to stimulate *R. phaseoli* nodulation approximately 150% over the *Rhizobium* controls. *P. putida* seems to have no effect on yield in the common bean. In conclusion, *P. putida* is not detrimental to *Rhizobium phaseoli* nodulation of the common bean, and may be very stimulatory and beneficial.

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INTRODUCTION

Pseudomonas putida (Trevisan) Migula has been known to inhibit other microorganisms under *in vitro* conditions and thereby has potential as a biocontrol agent. Although *in vitro* antibiosis often yields variable success under field conditions, *P. putida* shows promise in a number of crop plants including wheat, potatoes, squash, and cucumbers.

P. flourescens and *P. putida* also have been investigated for their growth promoting effects on potatoes, sugar beets, and radishes. In most cases, they seem to both increase yield and control some disease problems.

If *P. putida* is to be utilized as a biocontrol agent in the field, it is also necessary to obtain basic information on the effects of *P. putida* against beneficial soil microorganisms. This study was undertaken to provide such information with three major objectives in mind: 1) to determine the impact of *P. putida* on field and greenhouse nodulation in the common bean, *Phaseolus vulgaris* L. Burk.; 2) to determine the yield potential of beans utilizing various *P. putida* treatments; and 3) to obtain insight into the mechanism of the impact of *P. putida* on nodulation.

C H A P T E R II

LITERATURE REVIEW

The nodulation of leguminous plants by species of Rhizobium is an extremely important biological phenomenon. It is important not only from a biological point of view, but also because of economic and agronomic interests. Current estimates of the amount of dinitrogen fixation place it between 100 and 200 million metric tons of nitrogen fixed each year by nitrogen fixing organisms (2).

In many of the developed countries of the world, agriculture is becoming increasingly dependent on energy. This energy is invested not only in highly mechanized farm operations, but also in the production of fertilizers and other agricultural chemicals. These energy costs constitute an ever-increasing burden for the farmer and undoubtedly will be augmented as fossil fuel supplies are depleted. In this larger context, the importance of understanding nitrogen fixation, and discovering ways to improve upon it, becomes clear.

The nodule is the center of all interactions between the rhizobia and the plant. Hence, it is only natural that the nodule has received intensive study. Certain well defined developmental stages are recognized. During infection and nodule genesis, rhizobia are the aggressors, as shown by the mode of their entry, dissemination,

multiplication, and incitation of tissue proliferation (33,51).

The first step towards establishing a nodule is for the plant host to recognize the symbiont cell (12,33,62). Subsequent to this initial recognition, the host plant responds by synthesizing several nodule-specific peptides, termed nodulins, that are thought to be the first lines of communication between the rhizobia and the legume host (63).

Entry of rhizobia into leguminous roots occurs through infection of root hairs, broken epidermal and cortical cells, and ruptured tissue at the site of lateral root emergence (2,9,13,20,40,75). Although the exact mechanism of entry remains a mystery, the aforementioned nodulins are thought to play a role (63). The number of rhizobia functioning in the infection process is not proportional to the size of the initial inoculum. Only about four to five percent of the root hairs are normally infected (2, 10,17,51).

Infection of the root hair is followed immediately by an alignment of the rhizobia within a thread which proceeds to grow directly to the basal cell. Migration of the rhizobia from the site of the infection to the inner wall of the epidermal cell requires between 18 and 48 hours (2).

In order for rhizobia to induce proliferation of the

root cortical cells, they must be liberated from the infection threads. Release of the rhizobia is generally thought to occur through breakage of the threads during cell enlargement and division. After the rhizobia are released, they begin to multiply and secrete hormones, primarily β-indole acetic acid, which stimulate the host cells to enlarge and divide, thus forming the nodule (44, 47,64).

Many methods have been employed to study nodulation including sterile and unsterile soil, sand, and isolated root segments on agar (8,17,18,20,21,25,26,39,54,73,79). All of these techniques have inadequacies and consequently there is no entirely adequate method to study the interaction of soil microorganisms in relation to nodulation (25,56,58). In spite of these barriers, many workers have utilized greenhouse and field methods to illuminate various aspects of the interaction between rhizobia and other microorganisms (1,3,4,5,7,16,20,23,27,28,29,30,37,39,60, 61,68,76).

An extremely interesting interaction being studied is between rhizobia and vesicular-arbuscular (VA) mycorrhizae fungi. These fungi have been known to stimulate plant growth. The mechanism for this stimulation appears to lie in the ability of these fungi to solubilize soil phosphate and transport it directly into the plant (68,

71,76).

Several workers have reported that plants inoculated with both VA mycorrhizae and Rhizobium will stimulate nodulation over Rhizobium and VA mycorrhizae controls (5, 27,28,68). This stimulation of nodulation also is believed to be related to the ability of VA mycorrhizae fungi to solubilize soil phosphate. Proper nutrition of the legume is very important for nodulation and an ample supply of phosphate is a prerequisite for adequate nodulation (28, 40,43,81). Consequently, the VA mycorrhizae stimulate nodulation by increasing the level of phosphate in the plant (28,40).

Some bacteria, especially Bacillus and Pseudomonas species, have the ability to solubilize soil phosphate which can alter the nutritional status of the plant and the chemistry of the rhizosphere (3,6,16,22,31,34,36,61), although this phenomenon has not been studied as extensively as the VA mycorrhizae effect. Furthermore, several workers have reported a stimulation of nodulation by various soil bacteria, usually pseudomonads (1,3,60,74). This stimulation of nodulation is attributed to hormones produced by the added bacteria although no data is presented to support this conclusion (5,16,18,24,46,53,57, 64,65).

Pseudomonads, besides their ability to solubilize soil phosphates, also are being investigated for use as bio-control agents. Many of the pseudomonad species produce antibiotics which may play a role in their ability to antagonize other microorganisms (7,13,15,50,83). Pseudomonas putida, in particular, has been under investigation in many laboratories due to its ability to both stimulate plant growth and inhibit various plant pathogenic microorganisms (7,22,49,55,56,57,58,59,60,83). The in vitro production of antibiotics by pseudomonads and the antibiosis directed toward other organisms is well documented (7,13,45,49,56,59,83). It is important to realize that in vitro production of an antibiotic does not mean that the antibiotic will be synthesized under field conditions. Furthermore, assuming the antibiotic is synthesized and released, there is a large body of literature which suggests that it will form a molecular complex with various soil components, thus rendering the antibiotic ineffective (14,15,69,70,72,75,77,78). Kloepper and Schroth, however, recently have published a report in which they claim that "the ability to produce antibiotics in vitro is directly related to the capacity of rhizobacteria to significantly increase plant growth" (59). This implies that the antibiotics produced in vitro by their bacteria, many of them pseudomonads, are synthesized

in the soil and are functionally active in the soil environment. Kloepper and Schroth arrived at these conclusions by using several *in vitro* antibiotic producing bacterial strains and 16 mutants which lacked *in vitro* antibiotic production, and compared the ability of these two groups to displace other rhizosphere organisms (59). The importance and extent of antibiotic involvement in the soil ecosystem needs further study before any definitive conclusions can be made.

With the role of the antibiotic undetermined, there is a gap in the understanding of the mechanism of antibiosis. Some authors feel that the control of diseases observed is because of competition between the introduced agent and existing microflora (7,13,66,67), while others suspect that the mechanism may be a more sophisticated chemical mechanism such as siderophore activity (57,58). Although these are both plausible explanations, no conclusive work has been completed.

C H A P T E R III

MATERIALS AND METHODS

Bacteria and Culture Conditions

Bacterial Strains. Rhizobium phaseoli Dangeaard (ATCC 14482) was obtained initially from the American Type Culture Collection and was generously provided by W. J. Manning, University of Massachusetts Department of Plant Pathology. A second R. phaseoli culture (K80) was obtained from Winston Brill, University of Wisconsin Department of Bacteriology, for use during the second season of field work.

Pseudomonas putida (Trevisan) Migula M17 was isolated from over-ripe tomato fruit by Richard Mytkowicz in the laboratory of M. S. Mount, University of Massachusetts Department of Plant Pathology. This strain was selected for its ability to inhibit Erwinia carotovora subsp. carotovora Dye (isolate EC14) in vitro. A mutant of M17, M174, was obtained from P. D. Colyer, University of Massachusetts Department of Plant Pathology. P. putida M174 was isolated from a nitrosoguanidine mutagenized culture of M17.

Maintenance of Bacteria. Unless otherwise specified, P. putida isolates M17 and M174 were stored on nutrient agar

(NA, Difco) slants (1.5%) at 30 C.

R. phaseoli was grown and stored on yeast mannitol agar (YEM: 0.05% K_2HPO_4; 0.02% $MgSO_4 \cdot 7H_2O$; 0.01% NaCl; 1.0% mannitol; 0.04% Difco yeast extract; 1.5% agar) slants at 30 C.

Overlay Procedure

The ability of P. putida M17 to inhibit R. phaseoli was screened using an overlay technique (82). A bottom layer of 25 ml of solid medium in a 100 x 15 mm petri plate was spotted in the center with a drop from an overnight culture of M17 or M174. The plates were incubated for 48 h at 24 C after which time they were exposed to chloroform vapor for 1 h to kill the test bacteria. Chloroforming the plates consisted of inverting the bottom of each plate over a glass petri plate containing a chloroform saturated 5.5 cm round Whatman #1 filter paper. Plates were then left standing for 30 min under a sterile hood to allow excess chloroform vapors to dissapate. One-tenth of 1 ml of an overnight culture of R. phaseoli was added to tubes of 3.0 ml soft-agar (0.5% water agar) after the agar had been molten and then cooled to approximately 43 to 50 C. The inoculated soft agar then was poured over the chloroformed M17 and the plates were incubated for 24 h at 30 C. Inhibition by

M17 or M174 was determined by measuring the clear zone in the R. phaseoli lawn.

Field and Greenhouse Experiments

Greenhouse Experiments. Greenhouse experiments were performed to facilitate the understanding of field results. One litre each of R. phaseoli, P. putida M17 and M174 were grown in YEM and NB, respectfully, for 48 h. Cells were pelleted by centrifugation at 8,000 x g for 20 min. The pellets were washed in sterile dH_2O and recentrifuged. The resulting pellets were combined and diluted to 800 ml with sterile dH_2O, such that there were three cultures of 800 ml each.

Seeds were sterilized by rinsing 3x in dH_2O, 1x in 98% ethanol, and finally soaked for 10 min in 20% chlorox. Seeds with intact seed coats were plated onto water agar (3%). The petri plates were stored in a styrofoam cooler (for darkness) at room temperature for 72-96 h. Only seeds which had no brown discoleration of the roots were selected for further experimentation.

Two types of soil were used for these experiments; a greenhouse mix (soil:sand:peat in a ratio of 2:1:1) which had been electrically sterilized and non-sterilized field soil taken from the same field as the field experiments. Each soil type received the following six

treatments:

1) Ø Control - germinated seeds planted after soaking for 30 min an sterile dH_2O
2) Rhizobium Control - germinated seeds planted and 8 ml of R. phaseoli placed into the hole with the seed and root
3) P. putida M17 - seeds planted after soaking for 30 min in a suspension of M17
4) P. putida M174 - same as #3 but with M174
5) Rhizobium + M17 - seeds soaked in M17 for 30 min and then planted. R. phaseoli (8 ml) placed into hole with the seed and root
6) Rhizobium + M174 - same as #5 but with M174.

Plants were placed into two growth chambers (Percival) with a 12 h photoperiod and a 12 h day/night cycle with 80 F day and 70 F night temperature cycles. The plants were watered everyday for one month. After this time, the plants were removed from their pots and the root nodules counted.

Field Experiments. The field was previously cropped to corn and the soil texture was very sandy (Montague farm; pH 4.5).

1980. A random block design was used with four blocks, each containing 13 plots. Six treatments were employed, with a disproportionate number of each treat-

ment in the blocks. Phaseolus vulgaris (var. Provider) seeds were obtained from Agway, Inc. in 8 oz. bags.

Bacteria were grown for 72 h (c.a. 10^{10} cells/ml). A total of 12 L of Rhizobium phaseoli (YEM) and P. putida (M17 and M174) were grown, 4 L for each bacterial type. Cells were pelleted by centrifugation at 8,000 x g for 20 min. Cells were resuspended to 3 L in sterile dH_2O.

Individual treatments were performed in the following manner:

1) Ø Control - seeds soaked in sterile dH_2O for 60 min and then planted directly in the soil.
2) Rhizobium Control - 50 lb. of peat humus (Agway, Inc.) was mixed with 3 L of suspended R. phaseoli. Peat was applied to the furrows of each Rhizobium-receiving plot (40 altogether; Rhizobium control, R. phaseoli + M17, and R. phaseoli + M174). Seeds were planted after they had been soaked in sterile dH_2O for 60 min.
3) M17 - seeds were soaked for 4 h in a 3 L suspension of M17 and planted
4) M174 - same as #3 but with M174
5) Rhizobium + M17 - seeds soaked for 4 h in M17 and planted on top of the R. phaseoli inoculated peat.
6) Rhizobium + M174 - same as #5 but with M174.

After all seeds were planted, the rows were covered with approximately ½ inch of soil. The entire field was

immediately watered to insure survival of the bacteria. Watering was continued, virtually every day, throughout the summer due to drought conditions.

Harvesting of the plants was performed by loosening the soil with a spade about one foot on each side of the beans. Plants were removed carefully and loose soil shaken from the roots. Ten plants from each block were harvested and a fresh weight obtained. Each individual plant was carefully washed and the root nodules counted and scored. A total of 540 bean plants were analyzed.

1981. The same field was used as in 1980 except that an extra portion was added. This added portion previously was uncropped and only turf was grown. The entire field received 5-10-10 fertilizer at a rate of 1 ton/acre. A random block design was used with three blocks consisting of 18 plots per block. Three replicates of each of six treatments were performed in each block.

The same six treatments used in 1980 again were utilized with the only differences in protocol being the Rhizobium inoculation of the soil and seed soaking (for M17 and M174) time reduced to 30 min. Dry peat humus was applied to all rows of the field. R. phaseoli was applied to the appropiate plots by means of a backpack sprayer (Solo). Bacteria were grown and diluted in the same manner as those experiments during 1980. The entire field was

watered immediately after planting and thereafter as needed.

Harvesting was performed by loosening the soil around the beans and carefully lifting plants out. The plants from each row (30/plot) were placed into large paper bags and returned to the laboratory. All bags were weighed and fresh weight recorded. The number of nodules on each plant was subsequently counted and recorded. A total of 1620 plants were analyzed.

Data analysis for both years was performed within the Statistical Package for the Social Sciences (SPSS) and included Anova, Breakdown, Condescriptive, Crosstabs, and other statistical tests. Duncan's Multiple Range test was used to delimit treatment differences.

Agglutinin Isolation and Bioassay

Agglutinin Isolation. Eight 15 cm diameter plastic pots were filled with sterilized greenhouse soil max and four Phaseolus vulgaris (var. Provider; Agway, Inc.) seeds were planted in each pot. Plants were grown for three weeks in the greenhouse. The roots were collected and washed carefully. The agglutinating compound was isolated via the protocol outlined in Figure 1.

Agglutinin Bioassay. Bacteria (R. phaseoli, M17, and M174) to be assayed were harvested from 100 ml cultures grown to

Figure 1. Purification scheme for isolation of agglutinin from the roots of P. vulgaris.

100 gm root plus 100 ml sterile dH_2O blended for 1 min in Waring blender at 4 °C

↓

filter homogenate through cheesecloth

↓

centrifuge homogenate at 12,000 g for 20 min

↓ supernatent (crude extract) | ↓ discard pellet

↓

mix supernatent with DEAE-Sephadex A-50-120 and shake for 20 min (30 ml hydrated resin/100 ml extract)

↓

filter slurry through Buchner funnel (save filtrate)

↓

add DEAE filtrate to CM-Sephadex C-50-120 (30ml hydrated resin/ 100 ml extract) and shake for 20 min

↓

filter slurry and save filtrate

↓

mix filtrate with 3 volumes cold 95% EtOH and incubate at 4 °C for 18-24 h

↓

collect precipitate by centrifuging at 12,000 g for 10 min

↓

resuspend pellet in 100 ml sterile dH_2O

↓

homogenize in blender for 30 sec

↓

add 150 µl octanol

↓

recentrifuge at 12,000 g for 10 min

↓

collect supernatent

↓

dialyze against dH_2O for 6 h

late log phase in nutrient broth (NB, Difco) on a gyratory shaker at room temperature. Cells were harvested by centrifugation at 12,000 x g for 10 min at 4 C. The pelleted cells were washed twice by suspension in sterile distilled water (SDW) followed by recentrifugation. The washed bacterial cells were resuspended in SDW to yield a suspension of about 10^{10} cells/ml.

The agglutination of bacterial cells in suspension was used to determine agglutinin activity. The assay consisted of adding 50 µl of various dilutions of test solution containing 150 µl of 1mM $MgCl_2$. Control was 50 l H_2O rather than agglutinin. The assay tubes were placed on a wrist-arm shaker for 15 min and then read visually for the presence of precipitated bacterial cells. The degree of precipitation in the tubes was scored from ++++ for complete precipitation of all cells to a single +, which indicated a fine granular appearence.

Paper Chromatography of 2-Ketogluconic Acid

Five milliliters of an overnight NB culture of P. putida M17 was inoculated into a 1 L culture of glucose-yeast extract medium (4% glucose; 0.002% yeast extract) and allowed to grow for 10 days on a reciprocating water bath shaker at 30 C (34,35). Cells were pelleted by centrifugation at 12,000 x g for 20 min. The supernatent

was millipore-filtered through a 0.45 micron filter. 250 µl drops of sterile supernatent and a control of 2-ketogluconic acid (barium salt; Sigma Co., St. Louis, MO) were spotted onto the paper (Whatman #1) and allowed to dry. The paper was subjected to chromatography in a 1-butanol: acetic acid:distilled wated (4:1:5) solvent system. Approximately five hours were required to properly develop the chromatogram. After the run, the chromatogram was air-dried for 30 min. It then was sprayed with aniline hydrogen phthalate dissolved in dH_2O-saturated butanol to yield a 0.1 M solution. Aniline hydrogen phthalate will react colorimetrically with only ketogluconic acids. After spraying, the chromatogram was dried in a 105 C oven for 10 min to allow visualization of the spots (45). Reddish spots indicated 2-ketogluconic acid while brown spots represented 5-ketogluconic acid.

C H A P T E R IV

RESULTS

Data obtained from the P. putida M17 versus R. phaseoli overlays indicated that M17 was inhibitory to R. phaseoli. Figure 2 shows that M17 demonstrated an extremely strong antibiosis towards R. phaseoli as indicated by the clear zone surrounding the M17 colony. These results were first observed by M. Hinteregger while elucidating the biospectrum of M17 (49), and later confirmed against different strains of R. phaseoli.

The in vitro observations seem to indicate that M17, via direc t. antibiosis, would suppress nodulation simply by decreasing the population levels of R. phaseoli in the soil. Upon testing this hypothesis in the field, it was demonstrated that M17 stimulated nodulation. Figure 3 and Table 1 show the increase in nodulation caused by M17 and its antibiotic deficient mutant, M174, over the residual Rhizobium populations. Inoculation of seeds with M17 and M174 stimulated nodulation to approximately the same level as artificial Rhizobium nodulation. Furthermore, combination treatments of R. phaseoli + M17 and R. phaseoli + M174 stimulated nodulation over the Rhizobium control. Table 2 represents a summary of 1980 and 1981 field data.

Figure 2. Antibiosis of R. phaseoli by P. putida M17.


RHIZOBIUM
PHASEOLI

Figure 3. Graphical representation of field nodulation results.

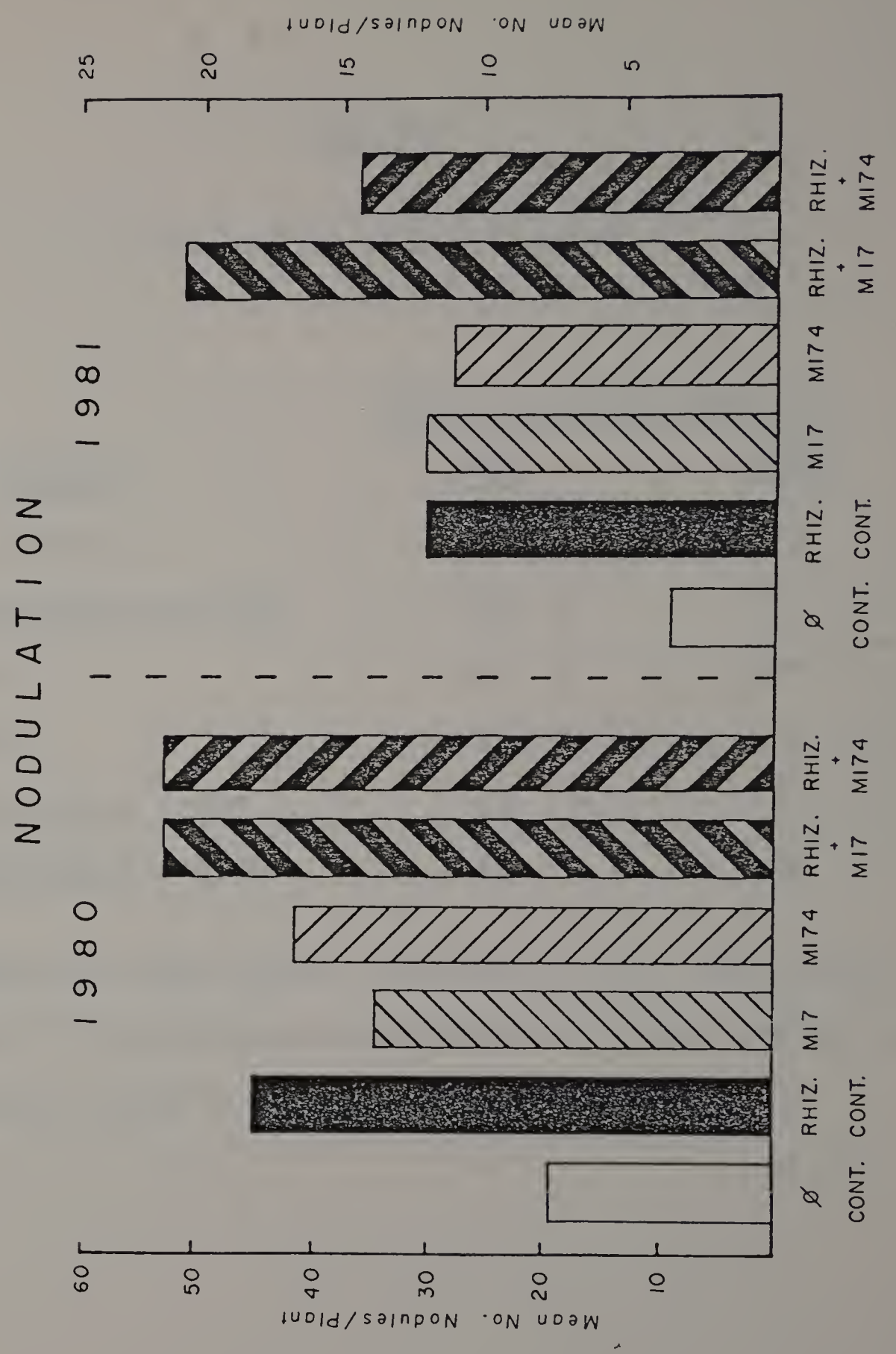

NODULATION
1981
Mean No. Nodules/Plant
25
20
15
10
5
Ø RHIZ.
M17
M174
RHIZ. + M17
RHIZ. + M174
CONT. CONT.
TREATMENT
1980
Mean No. Nodules/Plant
60
50
40
30
20
10
Ø RHIZ.
M17
M174
RHIZ. + M17
RHIZ. + M174
CONT. CONT.
TREATMENT

TABLE 1

Nodulation Field Results

Treatment	Mean Number of Nodules 1980		1981	
ø Control	19	a	3	a
Rhizobium Control	46	b	12	b
M17	34	b	12	b
M174	41	b	11	b
Rhizobium + M17	53	c	23	c
Rhizobium + M174	53	c	14	c

Means in each column followed by the same letter are not significantly different at the p=0.05 level using Duncan's multiple range test.

TABLE 2

Summary of Nodulation Field Results

1980

Treatment	Treatment	% Increase
ø Control	M17	100
Rhizobium Control	*Rhizobium* + M*	16
ø Control	*Rhizobium* Control	137

1981

Treatment	Treatment	% Increase
ø Control	M17	229
Rhizobium Control	*Rhizobium* + M	54
ø Control	*Rhizobium* Control	270

*The "M" represents an average of the *P. putida* M17 and M174 treatment means.

A statistical analysis of the field data indicated high significance with F values for 1980 treatment equal to 22.7 and 1981 treatment equal to 34.6. Duncan's multiple range test at p=0.05 was performed on the field data and yielded three internal homogenous subsets; subset A = ∅ Control, subset B = R. phaseoli, M17, and M174 treatments, and subset C = R. phaseoli + M17 and R. phaseoli + M174. These three homogenous subsets were identical in both 1980 and 1981.

Several other statistical tests and analyses were performed looking for more subtle effects from the various treatments. An interesting effect was observed for the three treatments R. phaseoli control, M17, and M174. Although these three treatments constitute the same Duncan's homogenous subset, the distribution of nodulation was different between the R. phaseoli control and the M17 and M174 treatments. Table 3 represents an interval grouping histogram of nodulation by treatment. This table shows that the R. phaseoli control has a greater number of plants with a low number of nodules as compared to the P. putida treatments which have more plants with a high number of nodules (c.a. 4.5% of the plants having 60 nodules per plant). P. putida appeared to be shifting the profile of nodulation upwards.

TABLE 3

Interval grouping histogram for number of nodules by treatment

Interval Group for Number of Nodules	Treatments (with % of plants within the interval group)					
	Ø Cont.	Rhiz. Cont.	M17	M174	Rhiz + M17	Rhiz + M174
0.0 x 12.4	90	44	33	46	21	27
12.4 x 24.8	10	45	44	27	42	39
24.8 x 37.2	0	8	14	18	30	26
37.2 x 49.6	0	4	4.5	7	5	5.5
49.6 x 62.0	0	0	4.5	3	2.5	3.3

The same six bacterial treatments were performed in growth chambers using two soil types, a sterile greenhouse mix and unsterilized field soil. Results from these experiments are shown in Table 4. The results are comparable to the field results with the basic trends manifesting themselves again. One exception was in the sterile soil where M17 and M174 failed to increase nodulation. This was interpreted to be due to the sterilization process which eliminated the residual population of Rhizobium.

Shoot and bean fresh weight was determined in 1980 and 1981 and 1981, respectfully (Figures 4 and 5). For both of these yield parameters, there were no significant differences between treatments, with one exception. In 1980, M17 increased shoot fresh weight significantly. This increase is an isolated event, since M17 failed to increase shoot or bean fresh weight in 1981.

In an attempt to elucidate the mechanism for the observed stimulation of nodulation, two experiments were performed. First, an extracellular preparation was obtained from M17 and subjected to paper chromatography. After the chromatogram had been removed from the solvent system, it was sprayed with aniline hydrogen pthalate which detects colorimetrically the presence of 2-ketogluconic acid. A sketch of the resulting chromatogram appears in Figure 6. Upon analysis of the chromatogram it was evident that

TABLE 4

Greenhouse Nodulation Results

Treatment	Mean Number of Nodules			
	Sterile Soil		Field Soil	
ø Control	0.125	a	19.125	a
Rhizobium Control	10.625	b	23.5	ab
M17	1.0	a	26.125	ab
M174	1.125	a	32.375	b
Rhizobium + M17	19.625	c	46.0	c
Rhizobium + M174	20.375	c	45.375	c

Means in each column followed by the same letter are not significantly different at the p=0.05 level using Duncan's multiple range test.

Figure 4. Graphical representation of shoot fresh weight.

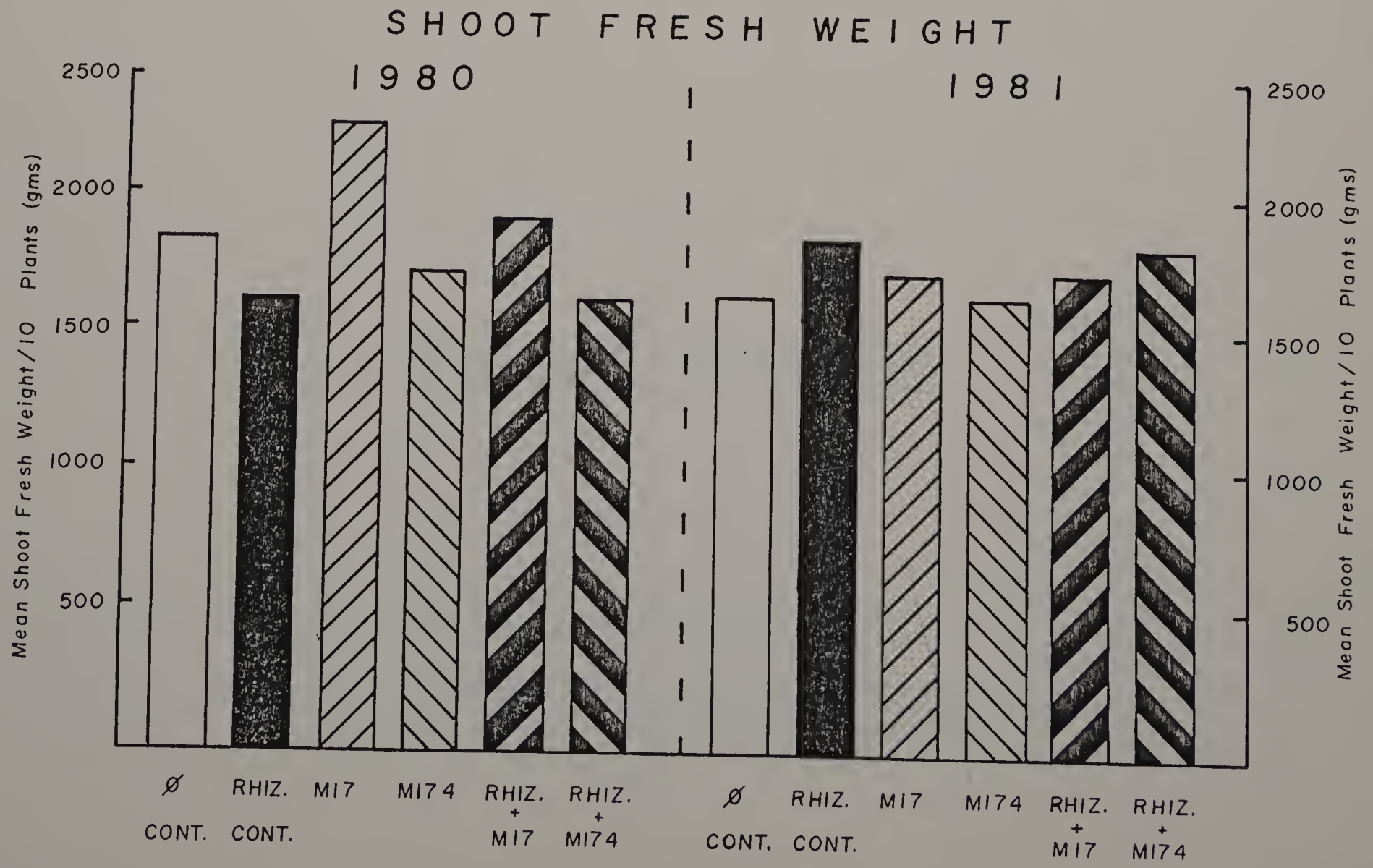
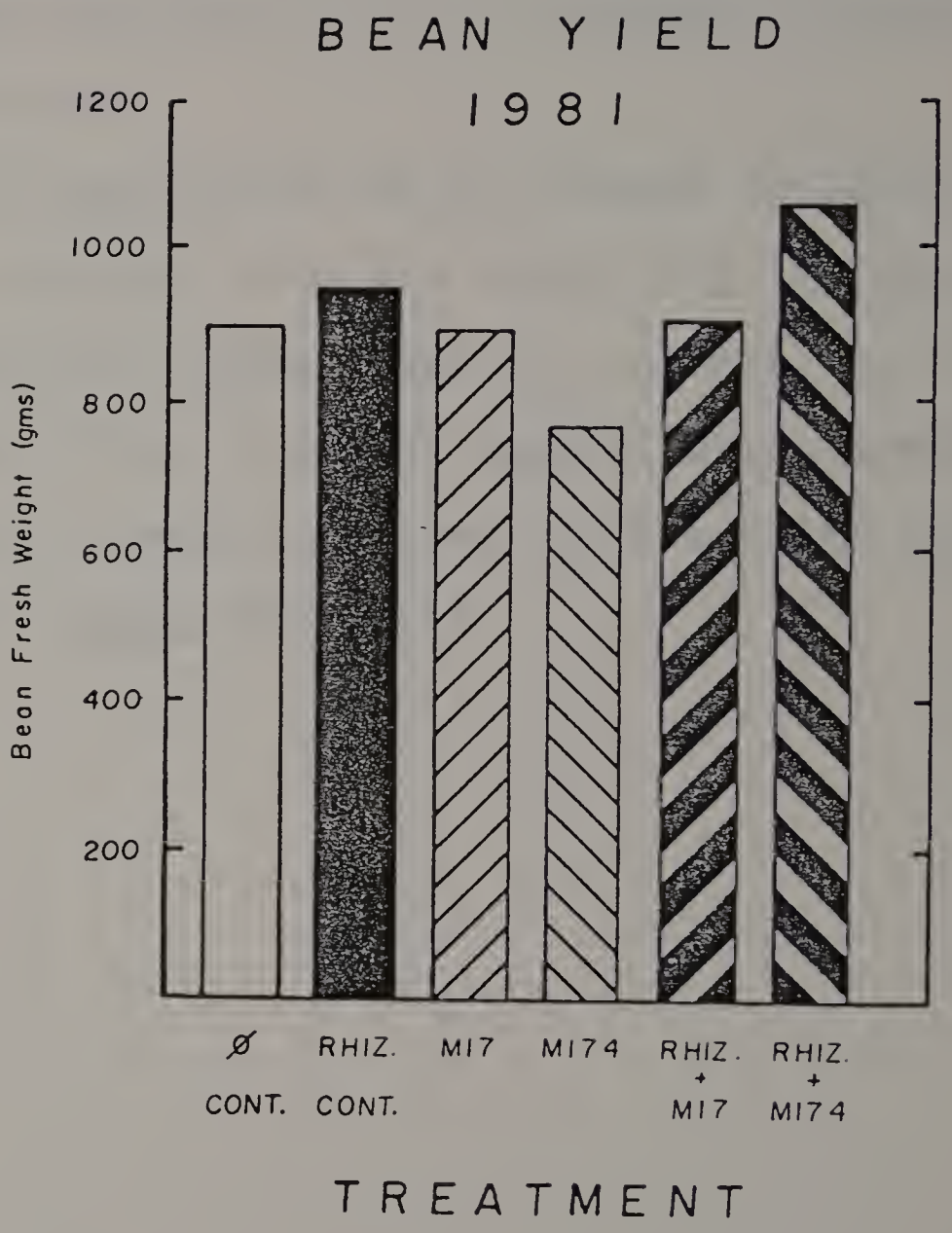

SHOOT FRESH WEIGHT
1980
1981
Mean Shoot Fresh Weight/10 Plants (gms)
2500
2000
1500
1000
500
Ø CONT.
RHIZ. CONT.
M17
M174
RHIZ. + M17
RHIZ. + M174
TREATMENT

Figure 5. Graphical representation of bean fresh weight.

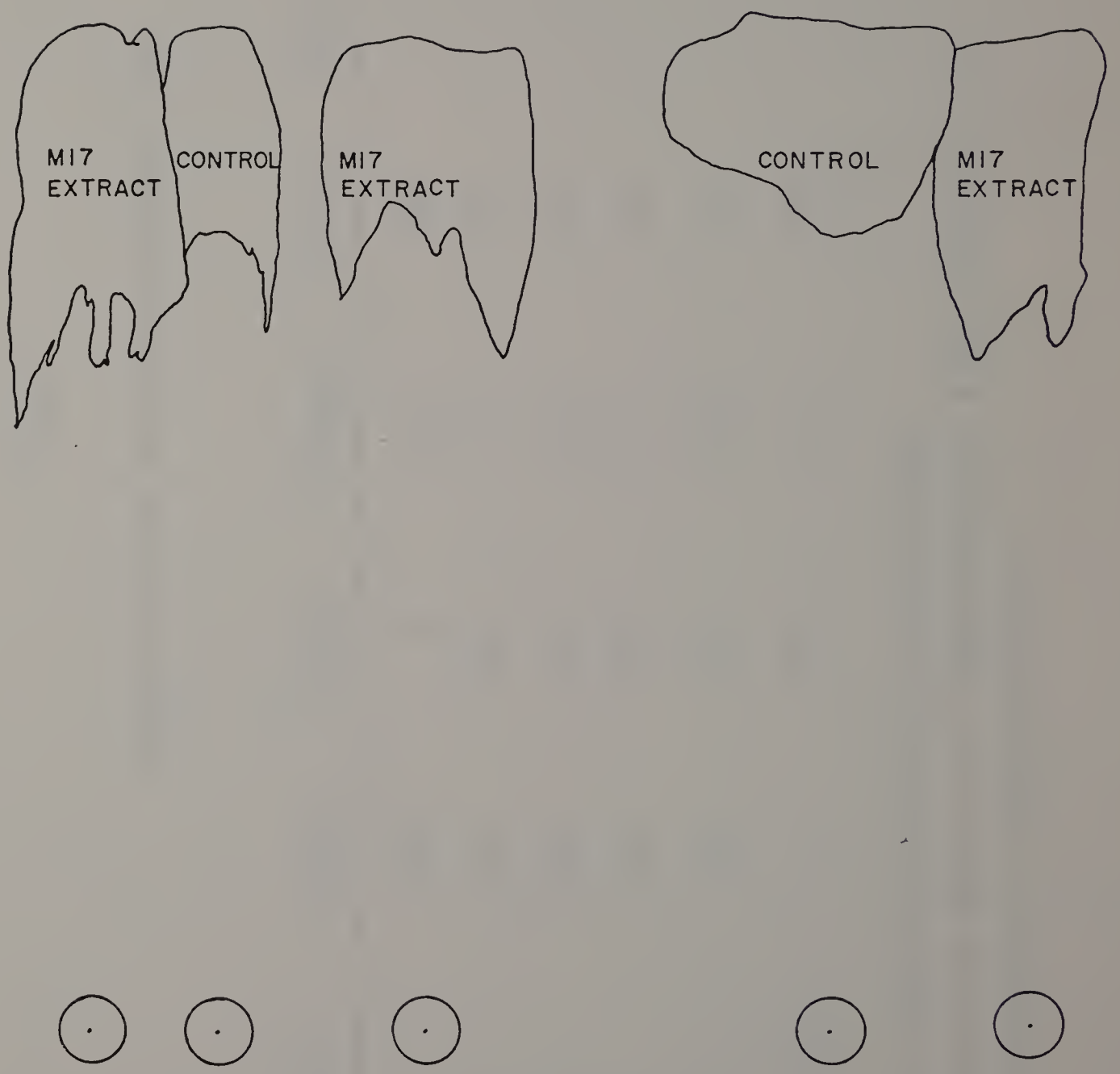

BEAN YIELD
1981
Bean Fresh Weight (gms)
1200
1000
800
600
400
200
Ø CONT.
RHIZ. CONT.
MI7
MI74
RHIZ. + MI7
RHIZ. + MI74
TREATMENT

2-ketogluconic was produced by M17 as evidenced by the color of the spot and an identical Rf value of 0.707 for both the 2-ketogluconic acid control and the 2-ketogluconic acid found in M17. Furthermore, based on the size of the spots, M17 was considered to be an abundant producer of 2-ketogluconic acid.

The second experiment was an attempt to isolate an agglutination compound from the roots of P. vulgaris, with activity towards P. putida. Table 5 reports the results of the bioassay of the isolated agglutination compound. P. vulgaris had a compound present in its roots that agglutinated P. putida M17 cells,

Figure 6. Sketch of the chromatogram showing the presence of 2-ketogluconic acid in the supernatent of P. putida M17. Control is 2-ketogluconic acid (barium salt; Sigma, Co.). Rf value for both the control and M17 supernatent was 0.707.

SOLVENT FRONT



ORIGIN

TABLE 5

Agglutination Bioassay Against M17

Tube #	Vol_{agg}	Vol_{H_2O}	Vol_{MgCl_2}	Vol_{bact}	Agglutination
1	500*	0	150	50	+ + + +
2	250	250	150	50	+ + +
3	200	300	150	50	+ +
4	100	400	150	50	+
5	50	450	150	50	+
Control	0	500	150	50	-

*all quantities are in microlitres
+indicates degree of agglutination (see Chapter III)
- indicates no agglutination

C H A P T E R V

DISCUSSION

P. putida M17 was strongly antagonistic towards *R. phaseoli* as indicated by *in vitro* antagonism studies. The observed antagonism resulted from an antibiotic produced by M17 (49). This finding has practical agricultural ramifications since M17 has been examined for its biocontrol potential (58,59,83). For example, Vrany (83) has examined a strain of *P. putida* for its biocontrol potential on wheat and *Gaeumannomyces* and has found promising results. Since wheat, and perhaps other crops which may be protected by *P. putida*, is often rotated with leguminous crops there is the possibility of a detrimental effect on nodulation of the legume. This would prove disadvantageous to the farmer who rotates with legumes in order to raise the nitrogen content of the soil.

Under field conditions, however, it was observed that M17 stimulated nodulation rather than suppressed it. M17 by itself, with only residual *Rhizobium* populations present, significantly increased nodulation to approximately the same level as the *Rhizobium* control. Combination treatments of *Rhizobium* and *P. putida* significantly increased nodulation over the *Rhizobium* control level. These results forced a dramatic modification of the

original hypothesis. Furthermore, it was necessary to develop mechanistic possibilities to explain the results obtained.

Five possibilities to explain the increase in nodulation were developed from both the existing literature and further research. The first explanation suggests a displacement of rhizosphere organisms by the introduced bacteria, thus altering the rhizosphere constituents. This possibility has been discussed in the literature by several workers (7,13,37,50,54,58,83). In particular, Vrany (83) has reported that inoculation of wheat seeds by P. putida results in an increase in pseudomonads in the rhizosphere and a decrease in other bacteria, namely Agrobacterium, and various soil fungi. Similiarly, M17 may have been able to effectively colonize the rhizosphere in beans; thus displacing some microorganisms that are effective competitors with Rhizobium. Having displaced the Rhizobium competitors, perhaps M17 has facilitated the colonization of roots by Rhizobium spp., thus increasing nodulation.

Direct bacterial stimulation of R. phaseoli by M17 constitutes the second possibility. Malcolm (66) has established several types of possible interactions between organisms, and he differentiated between when the interaction was "on" or "off." The interactions termed

"commensalism" and "unnamed #2" best apply to the *Rhizobium* - M17 interaction. M17 could be producing a nutrient or vitamin which stimulates rhizobial growth, thus producing a positive effect in *Rhizobium* with M17 receiving neither a beneficial nor detrimental effect. With the interaction off, commensalism would require M17 to exert a negative effect on *Rhizobium*. Since this is not likely, unnamed #2 offers the best possibility for categorizing the interaction between rhizobia and M17. In unnamed #2 , there are no effects between the interacting organisms when the interaction is off. Krasil' Nikov (60) initially projected this concept towards rhizobia nodulation. He also observed an increase in nodulation after inoculating with "soil bacteria" which in all liklihood were largely pseudomonads. In his report, he suggested that the soil bacteria can alter the virulence and activity of rhizobia by producing "stimulatory compounds."

The third possibility concerns the production of hormones by pseudomonads. Many workers have reported that various soil bacteria produce hormones which stimulate plant growth (3,19,47,53,64). Azcon-Aguilar (3) has demonstrated that certain strains of pseudomonads produce hormones of the same type and amount as *Rhizobium* spp. He also has investigated the relationship between

Rhizobium nodulation and various soil bacteria as well as VA mycorrhizal fungi (3,4,5). From his results, he has proposed that soil bacteria, largely pseudomonads, stimulate nodulation by supplying hormones in addition to those already produced by rhizobia (3). To confirm his hypothesis, he obtained an extracellular preparation of soil "phosphobacteria," distributed it onto pot-grown Medicago, and observed an increase in nodulation which he attributed to hormones present in the extracellular preparation. Unfortunately, no attempt was made to use isolated hormones for this experiment, thus placing his conclusions in doubt.

The fourth proposed mechanism is that M17 may be able to solubilize insoluble soil phosphate and make it available to the plant. This increase in phosphate level would cause an increase in nodulation. The importance of adequate phosphate supply to nodulation is well documented (5,27,28,29,30,68,81). Van Schreven (81) has reported that incremental increases in soil phosphate levels resulted in concurrent increases in nodulation. In addition, inoculation of plants with both VA mycorrhizal fungi and Rhizobium has yielded more nodules than inoculation with Rhizobium alone. This increase has been shown to occur because of the increased phosphorous made available by the VA mycorrhizal fungi (5,27,28,29,30,68).

Several aspects of this fourth possibility need to be discussed. Duff and Webley (35) have reported that various soil bacteria, including some pseudomonads, produce 2-ketogluconic acid which served as a natural chelating agent of soil phosphorous. The presence of 2-ketogluconic acid in M17 has been confirmed.

Azcon-Aguilar (3), however, has investigated the effect of inoculation with "phosphobacteria" on nodulation. Although he has observed an increase in nodulation, he dismissed the phosphorous hypothesis primarily for one reason. Upon performing an assay of the soil phosphate levels before and after inoculation with phosphobacteria, he found no significant changes. From this he concluded that even though the bacteria have the ability to chelate soil phosphorous, they are nevertheless unable to solubilize enough phosphorous to modify the nodulation process. Therefore he attributed the increase in nodulation to hormones produced by these phosphobacteria (3). A subtle, yet crucial, criticism of his phosphorous-assaying methodology needs to be made. He performed the assay from a soil sample taken from the soil surface. The plant, however, does not obtain its phosphorous from this region of the soil; rather, the phosphorous is extracted from the rhizosphere and immediately surrounding areas. Small changes in the phosphate level of the rhizosphere may

have been undetected, but still could be sufficient to stimulate nodulation. In contrast to Azcon-Aguilar's findings, Raj et. al. (74) used Bacillus as a soil phosphate chelator and observed that the phosphorous levels do increase from 12 to 65 days after soil inoculation with this bacterium. These data suggest that the possibility of M17 stimulating nodulation via chelating soil phosphorous remains a viable hypothesis.

The fifth mechanistic possibility can be viewed as an extension of the fourth. Simply stated, M17 may be agglutinated onto the roots of P. vulgaris thus placing 2-ketogluconic acid in precisely the place where it would be most effective. It has been demonstrated that P. vulgaris roots contain a lectin-like compound that will agglutinate P. putida cells. If M17 is agglutinated, and thereby localized in the rhizosphere, the role of 2-ketogluconic acid takes on added importance. At the very least, it becomes apparent that rhizosphere phosphorous levels need to be carefully analyzed before the chelating agent hypothesis is rejected.

It is clear that no thorough understanding of P. putida induced stimulation of nodulation is available. Besides the five mechanistic possibilities discussed, it is entirely possible that more than a single mechanism may be at work. Perhaps the mechanism involves a syn-

ergistic combination of the identified possibilities. On the other hand, there may be yet a sixth, unidentified, explanation. More work is necessary before any conclusive statements can be made.

It was evident, though, that the antibiotic produced by M17 has little or no effect on nodulation. Two observations supported this statement. First, there were no significant differences in the number of nodules obtained from M17 versus its antibiotic-deficient mutant, M174. Secondly, if the antibiotic was active in the soil, then nodulation would be expected to decrease due to fewer numbers of rhizobia.

There are many possibilities to explain why the antibiotic had no effect on field nodulation. For instance, it is possible that the antibiotic is not synthesized under field conditions. It may be synthesized and never released or it may be released and then rendered inactive by complexing with various soil components. There is a large body of literature which suggests that most antibiotics aren't effective in the soil because of soil component-antibiotic complexes (69,70,75,77,78). A recent article by Kloepper and Schroth, however, suggests that <u>in vitro</u> antibiotic production is related to rhizosphere colonization ability (59). This implies that the antibiotic is present and

active in the soil. More work needs to be performed before the question of antibiotic effectiveness in the soil is answered.

The impact of P. putida M17 on bean yield also was studied, but found to have no significant effect at p = 0.05. Only one exception was observed; M17 in the 1980 field trials significantly increased shoot fresh weight approximately 400 grams over other treatments. The 1981 field data is very difficult to interpret due to significant amounts of virus in the field, infecting 25% of all plants. No attempt was made to identify or further quantitate the virus. Tu, et. al. (80) has demonstrated that virus infection will decrease soybean yield and nodulation. Since the virus was distributed randomly throughout the field with no significant differences between plots or treatments, it seems likely that yield was uniformly depressed. Computer analyses of the data failed to show any correlation between yield loss and virus percent by treatment. It appeared, therefore, that P. putida M17 had no effect on yield in P. vulgaris.

It would be expected that the increase in nodulation would result in a concurrent yield increase, as has been reported in other systems (2,30). Why didn't the dramatic increase in nodulation observed in this study result in a yield increase? Although there are many

possibilities, perhaps the most likely answer revolves around the crop plant itself. P. vulgaris has an extremely short growing season, with plants reaching the harvest stage in 58 days. The nodules are not present long enough, or efficient enough, to result in appreciable yield differences. On another crop, soybeans for instance, the probability of observing a yield increase because of a nodulation increase would be somewhat higher.

In summary, this work has demonstrated that P. putida M17 stimulated Rhizobium nodulation of P. vulgaris, even though M17 exhibited strong antibiosis against R. phaseoli in vitro. Although much work remains before field use becomes practical, one important consideration emerges with regard to the literature. Many laboratories across the world are interested in stimulating nodulation and have worked with VA mycorrhizal fungi inoculations to this end. VA mycorrhizal fungi do stimulate nodulation, due to an increase of phosphate availability (4,5,23,27, 28,29,30,68,71,74). However, VA mycorrhizal fungi do not seem to show as much biocontrol potential as P. putida. Since inoculation with P. putida results in a comparable increase in nodulation to VA mycorrhizal fungi, it would seem that P. putida may be a more cost-efficient agent to use for stimulating nodulation because of its capability of serving as both a biocontrol agent and a

nodulation stimulator. Furthermore, it may prove especially useful in crop systems that are rotated, corn and soybeans for instance, where it would serve a dual purpose. This clearly distinguishes P. putida from VA mycorrhizal fungi apart from their similiar effects on nodulation.

LITERATURE CITED

1. Abdel-Ghaffar, A. S. and O. N. Allen. 1950. The effects of certain microorganisms on the growth and function of rhizobia. Internat. Congr. Soil Soc. 4 Amsterdam, Trans. 3: 93-96.

2. Allen, E. K. and O. N. Allen. 1950. Biochemical and symbiotic properties of the rhizobia. Bact. Rev. 14: 273-330.

3. Azcon-Aguilar, C. and J. M. Barea. 1978. Effects of interactions between different culture fractions of "phosphobacteria" and Rhizobium on mycorrhizal infection, growth, and nodulation of Medicago sativa. Can. J. Microbiol. 24: 520-524.

4. Azcon-Aguilar, C., R. Azcon, and J. M. Barea. 1979. Endomycorrhizal fungi and Rhizobium as biological fertilizers for Medicago sativa in normal cultivation. Nature 279: 325-327.

5. Azcon-Aguilar, C. and J. M. Barea. 1981. Field inoculation of Medicago with VA mycorrhiza and Rhizobium in phosphate-fixing agricultural soil. Soil Biol. Biochem. 13: 19-22.

6. Bacon, J. D. 1968. The chemical environment of bacteria in soil. In "The ecology of soil bacteria." (eds. Gray and Parkinson). Univ. Toronto Press. pp. 25-43.

7. Baker, R. 1968. Mechanisms of biological control of soil-borne pathogens. Ann. Rev. Phytopathol. 6: 263-294.

8. Burrios, S., N. Raggio, and M. Raggio. 1963. Effect of temperature on infection of isolated bean roots by rhizobia. Plant Physiol. 38: 171-174.

9. Bergerson, F. J. and G. L. Turner. 1967. Nitrogen fixation by the bacteroid fraction of breis of soybean root nodules. Arch. Biochem. and Biophys. 141: 507-515.

10. Bergerson, F. J. and G. L. Turner. 1968. Comparitive studies of nitrogen-fixation by soybean root nodules, bacteroid suspensions and cell-free extracts. J. Gen. Microbiol. 53: 205-220.

11. Berenue, A. and K. T. Williams. 1958. A colour test for 5-ketoaldonic acids on paper chromatograms. Arch. Biochem. Biophys. 71: 477-478.

12. Bhuvaneswari, T. V. 1981. Recognition mechanisms and infection process in legumes. Econ. Bot. 35: 204-223.

13. Bowen, G. D. and A. D. Rovira. 1976. Microbial colonization of plant roots. Ann. Rev. Phytopathol. 14: 121-144.

14. Brian, P. W. 1949. The production of antibiotics by microorganisms in relation to biological equilibria in soil. Symposia Soc. Exptl. Biol. 3: 357-372.

15. Brian, P. W. 1957. The ecological significance of antibiotic production. In "Microbial ecology." (eds. Williams and Spicer.). Cambridge Univ. Press, London, pp. 189-217.

16. Broadbent, P., K. F. Baker, N. Franks, and J. Holland. 1977. Effects of Bacillus spp. on increased growth of seedlings in steamed and in non-treated soil. Phytopathology 67: 1027-1034.

17. Broughton, W. J., A. M. van Egeraat, and T. A. Lie. 1980. Dynamics of Rhizobium competition for nodulation of Pisum sativum cv. Afghanistan. Can. J. Microbiol. 26: 562-565.

18. Brown, M. E., R. M. Jackson, and S. K. Burlington. 1968. Growth and effects of bacteria introduced into soil. In "The ecology of soil bacteria." (eds. Gray and Parkinson.) Univ. Toronto Press. pp. 531-551.

19. Brown, M. E. 1972. Plant growth substances produced by microorganisms of soil and rhizosphere. J. Appl. Bacteriol. 35: 443-451.

20. Brown, M. E. 1974. Seed and root bacterization. Ann. Rev. Phytopathol. 12: 181-197.

21. Bunting, A. H. and J. Horrocks. 1964. An improvement in the Raggio technique for obtaining nodules on excised roots of Phaseolus vulgaris L. in culture. Ann. Bot. 28: 229-237.

22. Burr, J. J., M. N. Schroth, and I. Suslow. 1978. Increased potato yields by treatment of seed pieces with specific strains of Pseudomonas flourescens and P. putida. Phytopathology 68: 1377-1383.

23. Carling, D. E., W. G. Riehle, M. R. Brown, and D. R. Johnson. 1978. Effects of a vesicular-arbuscular mycorrhizal fungus on nitrate reductase and nitrogenase activities in nodulation and non-nodulating soybeans. Phytopathology 68: 1590-1596.

24. Chan, E. S., H. Katznelson, and J. W. Rouatt. 1963. The influence of soil and root extracts on the associative growth of selected soil bacteria. Can. J. Microbiol. 9: 187-197.

25. Clark, F. E. 1965. The concept of competition in microbial ecology. In "Ecology of soil-borne pathogens." (eds. Baker and Snyder.) Univ. Calif. Press, Berkely, Calif. pp. 339-345.

26. Conn, H. J. 1948. The most abundant groups of bacteria in soil. Bact. Rev. 12: 257-273.

27. Crush, J. R. 1974. Plant growth responses to vesicular-arbuscular mycorrhize VII. Growth and nodulation of some herbage legumes. New Phytol. 73: 743-752.

28. Daft, M. J. and A. A. El-Giahmi. 1974. Effect of Endogone mycorrhiza on plant growth VII. Influence

of infection on the growth and nodulation in french bean (Phaseolus vulgaris). New Phytol. 73: 1139-1147.

29. Daft, M. J. and A. A. El-Giahmi. 1976. Studies on nodulated and mycorrhizal peanuts. Ann. Appl. Biol. 83: 273-276.

30. Daft, M. J. 1978. Nitrogen fixation in nodulated and mycorrhizal crop plants. Ann. Appl. Biol. 88: 461-462.

31. Dart, P. J. and F. V. Mercer. 1964. The legume rhizosphere. Archiv. fur Mikrobiologie 46: 344-378.

32. Davis, R. J. 1962. Resistance of rhizobia to antimicrobial agents. J. Bacteriol. 84: 187-188.

33. Dazzo, F. B. and W. J. Brill. 1977. Receptor site on clover and alfalfa roots for Rhizobium. Appl. Env. Microbiol. 33: 132-136.

34. Duff, R. B., D. M. Webley, and R. O. Scott. 1963. Solubilization of minerals and related materials by 2-ketogluconic acid-producing bacteria. Soil Sci. 95: 105-114.

35. Duff, R. B. and D. M. Webley. 1959. 2-ketogluconic acid as a natural chelator produced by soil bacteria. Chem. Ind. (London): 1376-1377.

36. Eklund, E. 1970. Secondary effects of some pseudomonads in the rhizosphere of peat grown cucumber plants. Acta Agric. Scand., Suppl. 17:1-57.

37. Estermann, E. F. and A. D. McLaren. 1961. Contribution

of rhizoplane organisms to total capicity of plants to utilize organic nutrients. Plant Soil 15: 243-260.

38. Felsted, R. L., J. Li, G. Pikrywda, M. T. Egorin, J. Spiegal, and R. K. Dale. 1981. Comparison of Phaseolus vulgaris cultivars on the basis of isolectin differences. Int. J. Biochem. 13: 549-557.

39. Fogle, C. E. and O. N. Allen. 1948. Associative growth of actinomycetes and rhizobia. Vol. 1. pp. 53.

40. Gates, C. T. 1974. Nodule and plant development in Stylosanthes humulis H. B. K. symbiotic responses to phosphorous and sulpher. Aust. J. Bot. 22: 45-55.

41. Gilboa-Garber, N. and L. Mizrahi. 1981. Binding of Pseudomonas aeruginosa lectin to Rhizobium sp. J. Appl. Bacteriol. 50: 21-28.

42. Graham, P. H. 1963. Antibiotic sensitivities of the root-nodule bacteria. Aust. J. Biol. Sci. 16: 557-559.

43. Graham, P. H. 1963. Vitamin requirements of root-nodule bacteria. J. Gen. Microbiol. 30: 245-258.

44. Graham, P. H. and C. A. Parker. 1964. Diagnostic features in the characterisation of the root-nodule bacteria of legumes. Plant Soil 20: 383-396.

45. Hais, I. M. and K. Macek. 1963. Paper chromatography a comprehensive treatise. Academic Press, New York and London. 955 pp.

46. Hanus, F. J., J. G. Sands, and E. O. Bennett. 1967.

Antibiotic activity in the presence of agar. Appl. Microbiol. 15: 31-34.

47. Hata, K. 1962. Studies on plant growth accelerating substances. Part I. the isolation method of soil microbes which produce plant growth accelerating substances. Agric. Biol. Chem. 26: 278-287.

48. Heenan, D. P. and L. C. Campbell. 1980. Soybean nitrate reductase activity influenced by magnisium nutrition. Plant Cell Physiol. 21: 731-736.

49. Hinteregger, M. 1980. Isolation and characterization of an antibiotic produced by Pseudomonas putida. M. S. Thesis. Univ. of Massachusetts, Amherst, MA. 91 pp.

50. Howell, C. R. and R. D. Stipanovic. 1980. Suppression of Pythium ultimum induced damping off of cotton seedlings by Pseudomonas flourescens and its antibiotic, pyoluteorin. Phytopathology 70: 712-715.

51. Hunter, W. J. and C. J. Fahring. 1980. Movement by Rhizobium and nodulation of legumes. Soil Biol. Biochem. 12: 537-542.

52. Jasalavich, C. A. and A. J. Anderson. 1981. Isolation from legume tissues of an agglutinin of saprophytic pseudomonads. Can. J. Bot. 59: 264-271.

53. Katznelson, H. and S. E. Cole. 1965. Production of gibberellinlike substances by bacteria and

actinomycetes. Can. J. Microbiol. 11: 733-741.

54. Katznelson, H., A. G. Lochhead, and M. I. Timonin. 1948. Soil microorganisms and the rhizosphere. Bot. Rev. 14: 543-587.

55. Kloepper, J. W. and M. N. Schroth. 1981. Development of a powder formulation of rhizobacteria for inoculation of potato seed pieces. Phytopathology 71: 590-592.

56. Kloepper, J. W. and M. N. Schroth. 1981. Plant growth promoting rhizobacteria and plant growth under gnotobiotic conditions. Phytopathology 71: 642-644.

57. Kloepper, J. W., M. N. Schroth, and T. D. Miller. 1980. Enhanced plant growth by siderophores produced by plant growth-promoting rhizobacteria. Nature 286: 885-886.

58. Kloepper, J. W., M. N. Schroth, and T. D. Miller. 1980. Effects of rhizosphere colonization by plant growth-promoting rhizobacteria on potato plant development and yield. Phytopathology 70: 1078-1082.

59. Kloepper, J. W. and M. N. Schroth. 1981. Relationship of *in vitro* antibiosis of plant growth promoting rhizobacteria to plant growth and the displacement of root microflora. Phytopathology 71: 1020-1024.

60. Krasil'Nikov, N. A. and A. I. Korenyako. 1944. Influence of soil bacteria on the virulence and

activity of nodule bacteria. Mikrobiologiya 13: 39-44.

61. Kundu, B. S. and A. C. Gaur. 1980. Establishment of N_2-fixing and phosphate solubilising bacteria in rhizosphere and their effect on yield and nutrient uptake of wheat crop. Plant Soil 57: 223-230.

62. Law, I. J. and B. W. Strigdom. 1977. Some observations on plant lectins and Rhizobium specificity. Soil Biol. Biochem. 9: 79-84.

63. Legocki, R. P. and D. S. Verma. 1980. Identification of "nodule-specific" host proteins (nodulins) involved in the development of Rhizobium-legume symbiosis. Cell 20: 153-163.

64. Lilly, D. M. and R. H. Stillwell. 1965. Probiotics: growth-promoting factors produced by microorganisms. Science, N. Y. 147: 747-748.

65. Lochhead, A. G. 1958. Soil bacteria and growth promoting substances. Bact. Rev. 22: 145-153.

66. Malcolm, W. M. 1966. Biological interactions. Bot. Rev. 32: 243-254.

67. Meers, J. L. 1978. Growth of bacteria in mixed cultures. Ann. Appl. Biol. 89: 143-171.

68. Mosse, B., C. L. Powell, and D. S. Hayman. 1976. Plant growth responses to vesicular-arbuscular mycorrhiza IX. interactions between VA mycorrhiza, rock phosphate, and symbiotic nitrogen fixation.

New Phytol. 76: 331-342.

69. Pinck, L. A., W. F. Holton, and F. E. Allison, 1961. Antibiotics in soils 1. Physico-chemical studies of antibiotic-clay complexes. Soil Sci. 91: 22-28.

70. Pinck, L. A., D. A. Soulides, and F. E. Allison. 1961. Antibiotics II. Extent and mechanism of release. Soil Sci. 91: 94-99.

71. Powell, C. L. and J. Daniel. 1978. Mycorrhiza fungi stimulate uptake of soluble and insoluble phosphate fertilizer from a phosphate-deficient soil. New Phytol. 80: 351-358.

72. Pramer, D. 1958. The persistence and biological effects of antibiotics in soil. Appl. Microbiol. 6: 221-224.

73. Raggio, M., N. Raggio, and J. G. Torrey. 1957. The nodulation of isolated leguminous roots. Amer, J. Bot. 44: 325-334.

74. Raj, J., D. J. Bagyaraj, and A. Manjunath. 1981. Influence of soil inoculation with vesicular-arbuscular mycorrhiza and a phosphate-dissolving bacterium on plant growth and ^{32}P-uptake. Soil Biol. Biochem. 13: 105-108.

75. Siminoff, P. and D. Gottlieb. 1951. The production and role of antibiotics in the soil I. The fate of streptomycin. Phytopathology 41: 420-430.

76. Smith, S. E., D. J. Nicholas, and F. A. Smith. 1979. Effect of early mycorrhizal infection on nodulation and nitrogen fixation in Trifolium subterraneum L. Aust. J. Plant Physiol. 6: 305-316.

77. Soulides, D. A., L. A. Pinck, and F. E. Allison. 1962. Antibiotics in soils. V. Stability and release of soil adsorbed antibiotics. Soil Sci. 94: 239-244.

78. Soulides, D. A., L. A. Pinck, and F. E. Allison. 1961. Antibiotics in soils III. Further studies on the release of antibiotics from clays. Soil Sci. 92: 90-93.

79. Tribe, H. T. and P. A. Williams. 1967. Investigations into the basis of microbial ecology in soil, illustrated with reference to growth of soil diphtheroids and Azotobacter in a model system. Can. J. Microbiol. 13: 467-480.

80. Tu, J. C., R. E. Ford, and S. S. Quiniones. 1970. Effects of soybean mosaic virus and/or bean pod mottle virus infection on soybean nodulation. Phytopathology 60: 518-523.

81. Van Schreven, D. A. 1958. Some factors affecting the uptake of nitrogen by legumes. In "Nutrition of the legume." (ed. E. G. Hallsworth.). Butterworth Press, London.

82. Vidaver, A. K., M. L. Mathys, M. E. Thomas, and M. L. Schuster. 1972. Bacteriocins of the phytopathogens

Pseudomonas syringae, *P. glycinia*, and *P. phaseolicola*. Can. J. Microbiol. *18*: 705-713.

83. Vrany, J., V. Vancura, and M. Stanek. 1981. Control of microorganisms in the rhizosphere of wheat by inoculation of seeds with *Pseudomonas putida* and by foliar application of urea. Folia Microbiol. *26*: 45-51.