FACTORS LIMITING SYMBIOTIC DINITROGEN FIXATION IN PHASEOLUS VULGARIS

A final report proposed for

AID PASA AC/TAB 610-9-76 (USDA-SEA-CR 616-15-189)

By

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Field, greenhouse and laboratory studies were conducted to better define factors that limit N fixation in Phaseolus vulgaris. All studies were based on selected isolates from an initial screening of 150 isolates of Rhizobium phaseoli for effectiveness of N fixation in beans. Antisera were prepared to selected isolates and conjugated with fluorescein isothiocyanate (FITC). In field studies, the recovery of and competition between isolates of R. phaseoli applied to soil was studied on beans. Of three isolates applied, 4, 20 and 36% of nodules were derived from inoculant strains CIAT 255, IP347B4 and Kimberly-5, respectively. Kimberly-5 occupied 100 and 92 % of bean nodules when added equally in the inoculum with IP347B4 and CIAT 255, respectively. Thus, Kimberly-5 was highly competitive with both native and applied strains. Neither inoculation nor N fertilizer increased bean seed yield or nodulation in spite of the absence of a crop, nitrogen fertilizer, or applied inoculum in the past 20 years in the field. Soil R. phaseoli population was $<10$ most probable number (MPN) g$^{-1}$. It is difficult to determine why a nodulation response was not obtained with such a low population of native soil rhizobia.

To determine which soil factors were related to nodulation, 26 commerical bean fields were evaluated for selected soil parameters and nodulation of P. vulgaris. Significant and negative correlations were obtained between nodulation and soil organic matter, and between nodulation and total inorganic N. No significant correlations were obtained between bean variety, pH, P and K and nodulation of dry beans. Above 200 kg ha$^{-1}$ of inorganic soil N, nodulation was drastically reduced. As in the field-plot study, many fields were low in R. phaseoli population, with half being less than 10 MPN rhizobia g$^{-1}$. These values are low in spite of the fact that beans have been grown at various times in the rotation. Significant correlations were not obtained between serogroup distribution and any of the soil parameters studied. These studies indicate that excessive levels of soil N were a major limitation to nodulation in the Columbia Basin.

The effect of bean root rot caused by Fusarium solani f. sp. phaseoli on nodulation and N fixation of P. vulgaris (cv. Viva Pink) was evaluated in greenhouse studies. Fusarium inhibited nodulation and plant growth, but it was not determined whether the reduction in nodulation was caused from a direct effect of the Fusarium on Rhizobium or indirectly from the reduction in plant vigor.

Various flocculating agents were evaluated for recovery of fast- and slow-growing Rhizobium in course- and fine-textured soils. Cells were enumerated by the FA technique. Recoveries of R. japonicum and R. phaseoli from two Idaho soils averaged 63, 40, 24 and 18%, respectively, for CaCl$_2$, AlCl$_3$, FeCl$_3$ and Ca(OH)$_2$: MgCO$_3$ at 60 min of flocculation. In general, a greater recovery (44%) was obtained for R. japonicum as compared to fast-growing R. phaseoli (33%). Interactions were noted between flocculant and Rhizobium spp.; 10% R. phaseoli and 61% R. japonicum cells were recovered with FeCl$_3$ from a Michigan soil.

When suspensions containing added cells and soil were flocculated in 24% sucrose containing 0.2% CaCl$_2$ and later added on top of a gradient containing 50% sucrose, recoveries markedly increased after centrifugation. Cells remained in the 24% phase. Greater than 91% of added R. phaseoli cells were recovered
when added in the range of $10^4$ to $10^7$ cells g\(^{-1}\) soil. With conventional CaCl\(_2\) flocculation, no cells were recovered when added at $10^4$; only 33\% were recovered at $10^5$ cells/g soil. In incubation studies at 300 days, enumeration by FA using sucrose density centrifugation was up to 300\% greater than when cells were extracted by conventional flocculation.

Current studies have shown quantitative recoveries of Rhizobium added at $5 \times 10^3$ cells g\(^{-1}\) of soil. This technique will be very useful, especially in studying the ecology of *R. phaseoli* where populations are often low.

The relatively low populations of *R. phaseoli* in the Columbia Basin soils prompted the study of the survival characteristics of these rhizobia. There is evidence to suggest that the slow-growing rhizobia survive better than the fast-growing types and that survival is better in fine- than coarse-textured soil. Coarse-textured soils are common in central Washington and periods of soil disiccation are common.

In the laboratory, sterile and non-sterile soils were maintained at -0.3 bar or -30 bars and inoculated with fast-growing (*R. leguminosarum* or *R. phaseoli*) or slow-growing (*R. japonicum*) cells. Survival was monitored for 90 days by the FA technique in two fine-textured and two coarse-textured soils at 25°C. Numbers of both fast- and slow-growing Rhizobium increased in sterile soils held at -0.3 bars and were above the value added at the end of 90 days. Survival of all Rhizobium cells decreased under non-sterile conditions at -0.3 bars and at -30 bars. Survival of fast-growing rhizobia was considerably greater in fine- than coarse-textured soils. It has been postulated that the protection afforded the fast-growers in fine-textured soils is a function of cell charge density and soil clay.

By comparing FA counts with plate counts on sterile samples and FA with MPN on non-sterile samples, differences in cell numbers were noted at the different bars. Plate counts were comparable to FA and MPN at high bars. However, at -30 bars, FA estimates were considerably higher than estimates from MPN or plate counts, presumably because a relatively large proportion of non-viable cells were counted with the FA technique. These data indicate limitations to the FA technique in quantifying Rhizobium populations under dry soil conditions. Survival of *R. phaseoli* was less at -30 bars than at -0.3 bar when enumerated by MPN. About equal survival would be predicted if cells were to be enumerated by FA.
In the Columbia Basin of Washington, approximately 10,000 hectares are planted to dry beans annually. With the necessity for irrigation, average bean yields at 2,000 kg ha\(^{-1}\) are among the highest reported in the US. However, these yields are obtained only under irrigation and with supplemental N at rates to 112 kg ha\(^{-1}\) of N. Although the climate in the Columbia Basin may not be typical of that in many developing countries, some soil conditions may typify those found in some foreign locations. In spite of irrigation, periods of hot and dry conditions are common, which could cause stress conditions for survival and persistence of native and applied Rhizobium.

Bean seed yield in Washington is limited by a number of soil-borne and foliar diseases. Notably, root rot (Fusarium solani f. sp. Phaseoli) seriously limits bean production. In fumigation studies, yields were nearly doubled by the application of chloropicrin, a response attributed largely to the reduction of root rot organisms. Reinoculation with R. phaseoli after fumigation did not increase bean yield, which suggests that the root rot did not directly influence nodulation. In other studies, response to inoculation has been minimal.

With the financial assistance of a grant from SEA/AR-AID, studies were conducted to evaluate factors that limit nodulation and N fixation in Phaseolus vulgaris. Objectives were to:

1. Determine the competitive ability of selected serologically-known isolates of R. phaseoli with each other and with the native population.
2. Determine the effect of measurable soil parameters on nodulation of P. vulgaris by R. phaseoli.
3. Evaluate the effect of Fusarium solani f. sp. Phaseoli on nodulation of R. phaseoli.
4. Determine the effect of soil texture and soil water potential \(\psi\) on survival of fast- and slow-growing rhizobia.
5. To evaluate methods for recovery of Rhizobium from soil for enumeration by the fluorescent antibody technique.
Field, greenhouse and laboratory studies were conducted to determine what factors influence nodulation and N fixation of *P. vulgaris*.

Major conclusions are:

1. The non-yield response from added inoculant strains of *R. phaseoli* and from combined N was surprising in view of the fact that a crop was not grown in this field for about 20 years. Fertilizer N was not applied during this time. The relatively good recovery (4 to 36%) of applied strains of *R. phaseoli* in the inoculum may be a reflection of the low native population of *R. phaseoli* (<10 MPN g⁻¹ of soil). However, it is difficult to reconcile the relatively good nodulation (greater than 1 g of nodules per plant) from the control soil containing less than 10 MPN *R. phaseoli* g⁻¹ of soil. It is suggested that the most probable technique should be reevaluated as a reliable index for levels of soil rhizobia.

2. The negative correlation of total inorganic soil N with nodulation in beans from 26 commercial fields suggests that added fertilizer N and residual soil N are limiting nodulation of beans in Washington. However, the native population of *R. phaseoli* in half of the fields was less than 10 MPN g⁻¹. These results suggest that there may be factors limiting the persistence of *R. phaseoli* in these soils. Whether these factors are biotic, abiotic, or both is not immediately known. From other studies on inoculation of *R. japonicum* into rhizobia-free soils, excellent nodulation and N fixation was obtained for similar soils in the first year of study. These results place some doubt as to the ability of *R. phaseoli* to persist in these soils. Since soybeans are not generally grown in Washington, data on the persistence of *R. japonicum* are not available.

3. From results of laboratory studies on the survival of fast (*R. phaseoli*) and slow (*R. japonicum*)-growing rhizobia in soil, better survival was obtained for *R. japonicum* than for *R. phaseoli* in both coarse- and fine-textured soils at -0.3 bars for 50 days. Survival was considerably greater for both fast and slow growers in fine- than coarse-textured soils. Assuming the availability of water was equal to both *R. japonicum* and *R. phaseoli* in coarse- and fine-textured soils held at -0.3 bars, factors such as lower nutrients or reduced rate of nutrient diffusion to cells may better explain the reduced survival in coarse-textured soils. Basic differences in cellular membrane constituents, nutrient requirements, and growth rate between the fast- and slow-growing rhizobia may explain the better survival of the slow-growing rhizobia in all soils.

4. Survival of *R. phaseoli* at -30 bars was also less than for *R. japonicum* when populations were determined by the FA technique. However, survival was about equal in sterile and non-sterile soils held at -30 bars. It was determined later that rhizobial populations in soils held at -30 bars were overestimated by two to four log numbers with the FA technique as compared to MPN. Probably at -30 bars, a large number of non-viable cells were stained with the FA conjugate and counted as viable. Therefore caution must be exercised in using the FA technique for enumeration of *Rhizobium* at low soil pH. Population estimates between MPN and FA were comparable in soils held at -0.3 bars. When populations of *R. phaseoli* were compared using MPN, survival was greater at -0.3 than at -30 bars.

CONCLUSIONS AND RECOMMENDATIONS
ACKNOWLEDGEMENTS

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INTRODUCTION

Recovery of serologically known isolates of rhizobia by legumes has been shown by agglutination reactions (Dudman and Brockwell, 1968; Gibson et al., 1966; Johnson et al., 1965; and Roughley et al., 1976) and gel immune diffusion (Dudman and Brockwell, 1968). Gibson et al., (1976) obtained recovery rates of greater than 90% for four of five strains of R. trifolii, indicating that these isolates were able to compete successfully with the native rhizobial population for nodule sites. Using four serologically distinct strains of R. phaseoli and five varieties of P. vulgaris, Das and Bhaduri (1974) demonstrated host specificity towards different strains of R. phaseoli. They obtained from 17 to 36% recovery of applied isolates in the inoculum by the varieties studied under field conditions. It is apparent that lower recovery of applied isolates by legumes was obtained for R. phaseoli as compared with R. trifolii. However, even lower recoveries (5%) of applied isolates specific for R. japonicum were reported in soybeans (Johnson, et al., 1965).

The use of fluorescent antibody (FA) technique to determine serological recovery of rhizobial strains was first studied by Schmidt et al., (1968) with R. japonicum bacteroids and later by Trinick (1969) and Jones and Russell (1972) with R. trifolii bacteroids.

The objectives of this study were to determine the competitive ability of selected isolates of R. phaseoli with each other and with the native rhizobial population, in the Columbia Basin of Washington.

MATERIALS AND METHODS

About 100 isolates of R. phaseoli were screened for their effectiveness on dry beans (Phaseolus vulgaris L. cv. Viva Pink). The majority of the R. phaseoli isolates were obtained from the nodules of numerous P. vulgaris genotypes grown in pots containing native and cultivated Washington soils. Genotypes were obtained from the Centro Internacional de Agricultura Tropical (CIAT), at Cali Columbia, South America. In addition, isolates were included from CIAT and from Dr. D. Westermann in southern Idaho. Evaluation of isolates for effectiveness were carried out in the greenhouse using modified Leonard jars (Leonard, 1943) and sterile nitrogen-free nutrient solution. The plants were evaluated for top weight and for N₂ (C₂H₂) fixation rate. Based upon the effectiveness of the isolates on P. vulgaris, selected isolates were chosen for antiserum production.

Field Plots

Field plots were initiated in May, 1978, at the Washington State University Irrigated Agricultural Research and Extension Center at Othello. Viva Pink dry beans were inoculated with eight isolates of R. phaseoli either singularly or in combination with another isolate. Four of the isolates tested were obtained from Dr. Joe Burton (The Nitragin Company, Milwaukee, Wisconsin). The
four remaining isolates were obtained from a culture collection of *R. phaseoli* at Washington State University. All isolates were sent to The Nitragin Company, where they were formulated with a granular-type inoculant (Soil Implant\(^1\)). Selection of isolates was based upon results of preliminary experiments in the greenhouse.

Using a randomized complete block design, 25 treatments were studied with six replications. Treatments included eight single isolates of *R. phaseoli*, combinations of two isolates, a N treatment of 112 kg/ha N as \(\text{NH}_4\text{NO}_3\) and a noninoculated control. Four row plots, 7.6 m in length with a row spacing of 55 cm were used. Seed and inoculum were mixed together in the planter box and planted with a cone planter at a rate of 120 seeds per row. The inoculum was added at twice the recommended rate of 5.6 kg/ha.

During mid-July, when the plants were in the early stages of podfill, roots were collected from six randomly selected plants from each of the sites. The nodules were then carefully removed from the roots, pooled, washed, determined for fresh weight and frozen.

The plots were harvested on August 14, 1978, 14 weeks after planting, wind rowed and allowed to dry before thrashing. The seed was cleaned and weighed.

**Production and Conjugation of Antisera**

Antisera were produced from either whole cells injected into the ear vein of a rabbit or from intramuscular injection using Freund's incomplete adjuvant (Vincent, 1970). When a titer of 1:1000 was obtained, approximately 100 ml of blood was obtained by cardiac puncture. Serum was separated by centrifugation and divided into 2-ml aliquots and frozen. Fine-ml fractions of serum were treated with 5-ml of 80\% saturated ammonium sulfate to precipitate the gammaglobulin fraction. After sequential precipitation and dialysis, the globulin fraction was conjugated with fluorescein isothiocyanate (FITC) as described by Schmidt (1974). The conjugated gamma globulin was purified on Sephadex G-50 by eluting with phosphate buffered saline (PBS) (pH 7.2). The faster moving band was collected and allowed to stand overnight at 4\(^\circ\)C. The resulting protein preparation was filtered through a 0.45 \(\mu\) Millipore filter, divided into 2-ml aliquots and frozen.

**Determination of Nodule Occupancy**

Thirty-six nodules from each of eight treatments from three blocks were sampled during early pod-fill, crushed in 2.5 ml of saline and mixed vigorously to release bacteroids. The nodule macerate was placed on to microscopic slides, air-dried and fixed in 95\% ethanol for 5 minutes. Fluorescein-labeled antisera were added to the slides which were incubated in a moist chamber for 30 minutes. Excess unreacted antisera were washed from the slides with PBS (pH 7) and the slides were allowed to stand in the buffer for 20 minutes. After drying, glass cover slips were mounted on the slides with buffered glycerol mounting medium (Baltimore Biological Laboratories, Cockeysville, Maryland). The smears were examined for the fluorescence using a Zeiss model 14 microscope equipped with a 100-
watt quartz-halogen light source in the epiilluminator mode. A primary interference filter emitting from 450 to 490 nm was used in conjunction with a dichroic reflector emitting less than 510 nm and a secondary filter emitting greater than 520 nm. A fluorescence reaction of 2+ or greater on the scale presented by Trinick (1969) was considered as a positive reaction to the antiserum. Fluorescence of FITC antibody to homologous bacteriods was 2+ or greater, whereas the nonhomologous combinations were 2 or less.

Using all combinations of antisera and antigens, the cross reactivity of the four antisera used in this study were evaluated. All antisera and antigens were tested by both Ouchterlony and tube agglutination analysis following the procedure of Vincent (1970).

RESULTS AND DISCUSSIONS

Tube agglutination analysis of the four isolates of R. phaseoli used for recovery and competition study showed that two of the four chosen isolates were serologically distinct (Table 1). Analysis of the similarity between isolates IP566A5 and Ciat 255 showed at least one common antigen by Ouchterlony. As a result, isolate IP566A5 was dropped from the study. No cross reactivity of the remaining isolates was shown by fluorescent antibody (FA) analysis.

Serogroup Distribution and Recovery of Added Inoculum

Inoculation of the soil with all of the test isolates of R. phaseoli produced significant deviations in the serogroup distribution from the normal distribution of the uninoculated control (Table 2). However, from 47 to 70% of the nodules tested did not react with any of the test antisera. The change in serogroup distribution for Kimberly-5 and Ciat 255 was significantly different from the control at $p < 0.005$ whereas, IP347B4 was significantly different at $p < 0.025$.

When the frequency of a given isolate in the control nodules was subtracted from the frequency of the isolate applied in the inoculum, the recovery of the applied isolate was computed. The recoveries ranged from 4 to 36% (Table 2) which agree favorably with the values of from 17 to 36% reported by Das and Bhaduri (1974). Recovery of Kimberly-5 was the highest at 36%, which indicates that this isolate was able to compete favorably with the native rhizobial population. A 20% recovery rate obtained for IP347B7 indicates a moderate competitive ability with the native population. The recovery rate for Ciat 255 of 4% was considerably lower than that obtained for the other isolates, which indicates that this isolate was a poor competitor with the native population.

The relatively high recovery of Kimberly-5 and IP347B4 as compared with Ciat 255, could, in part, be explained by the origin of each of the isolates. Ciat 255 was obtained from Dr. P. H. Graham at Centro Internacional de Agricultura Tropical in Cali, Colombia. Since the climatic conditions are considerably different between Columbia and Washington, Ciat 255 may not have adapted well to soil conditions in Washington. Kimberly-5, which was isolated from southern Idaho and IP347B4, which was isolated from soil in the Columbia Basin of Washington, would be expected to survive better under soil conditions in the Northwest.
Table 1.--Cross reactions between antisera used at Othello in 1978.†

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Kimberly-5</th>
<th>IP566A5</th>
<th>IP347B4</th>
<th>Ciat 255</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kimberly-5</td>
<td>1:200</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>IP566A5</td>
<td>NR</td>
<td>1:1600</td>
<td>NR</td>
<td>1:3200</td>
</tr>
<tr>
<td>IP347B4</td>
<td>NR</td>
<td>NR</td>
<td>1:6400</td>
<td>NR</td>
</tr>
<tr>
<td>Ciat 255</td>
<td>NR</td>
<td>1:1600</td>
<td>NR</td>
<td>1:3200</td>
</tr>
</tbody>
</table>

†Values represent the highest dilution for a positive reaction by tube agglutination. NR: no reaction at a dilution of 1:50.
Table 2.--Effects of isolates of *Rhizobium phaseoli* applied on serogroup distribution and recovery of applied isolates by *Phaseolus vulgaris*.

<table>
<thead>
<tr>
<th>Isolate Applied</th>
<th>Kim-5</th>
<th>IP347B4</th>
<th>Ciat 255</th>
<th>Other</th>
<th>$\chi^2$</th>
<th>$p^+$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kim-5</td>
<td>45</td>
<td>8</td>
<td>0</td>
<td>47</td>
<td>42.44</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>IP347B4</td>
<td>3</td>
<td>56</td>
<td>1</td>
<td>40</td>
<td>10.72</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>Ciat 255</td>
<td>12</td>
<td>14</td>
<td>4</td>
<td>70</td>
<td>15.87</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>16</td>
<td>13</td>
<td>3</td>
<td>68</td>
<td>17.13</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Control</td>
<td>9</td>
<td>36</td>
<td>0</td>
<td>55</td>
<td>--------</td>
<td>------</td>
</tr>
</tbody>
</table>

Recovery of Applied Isolate $^\dagger$

| Recovery of Applied Isolate $^\dagger$ | 36 | 20 | 4 | -- | ----- | ----- |

$^+\text{Probability of a difference in Chi square distribution from the control.}$

$^\dagger\text{Contribution of control has been subtracted.}$
It is apparent that the applied isolates varied in their competitive ability with the native rhizobial population. However, differences were noted in the competitiveness between applied isolates when added together in the inoculum. When added equally in the inoculum with IP347B4, Kimberly-5 occupied 100% of the nodules reactive to the two homologous antisera, and accounted for 51% of the total nodules produced (Table 3). When added equally with Ciat 255, Kimberly-5 produced 92% of the nodules which were reactive to the two homologous antisera. Together these isolates accounted for 31% of the nodules produced in this treatment. These results also show that Ciat 255 was slightly more competitive with Kimberly-5 for nodules sites than was PI347B4. When added equally in the inoculum with IP347B4, Ciat 255 showed a reduced level of competitiveness by occupying only 38% of the node sites produced by the two isolates as compared with 62% which was occupied by IP347B4. Together, the two isolates produced 43% of the nodules in this treatment.

**Yield and Nodulation**

No significant differences were obtained for seed yield in response to added N or to inoculation with different isolates of *R. phaseoli* at Othello in 1978. Seed yield varied from 1666 kg/ha in the control to a high of 1879 kg/ha for Kimberly-5. The lack of yield response to inoculation and N fertilization may be explained by the relatively high level of inorganic N (69 kg/ha to a depth of 30 cm in the soil at this site). The high level of inorganic N was surprising since the soil had not been cropped or fertilized in at least 20 years. Perhaps, the best explanation for the high values was from the mineralization of organic N present in the native vegetation.

Nodulation (from 1.5 to 2.0 g nodules/plant) was also not significantly influenced by either inoculation strain or N treatment. The relatively high levels of inorganic N probably reduced the impact of the applied inoculum. Although beans were not grown on this site for 20 years, the low level of nodulation obtained was undoubtedly a result of a low background level of *Rhizobium* that persisted over the years in the absence of a bean crop. A most probable number (MPN) assay, according to the procedure of Vincent (1970), showed less than 10 MPN rhizobia per g of soil. Under conditions of less soil N, response to inoculation would be more likely.
Table 3.--Competitiveness of applied isolates of *R. phaseoli* applied to the inoculum.

<table>
<thead>
<tr>
<th>Applied Isolates</th>
<th>Proportion of nodules reacting to:‡</th>
<th>Nodules Nonreactive†</th>
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<tbody>
<tr>
<td></td>
<td>Kim-5</td>
<td>IP347B4</td>
</tr>
<tr>
<td>Kim-5 and IP347B4</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Kim-5 and Ciat 255</td>
<td>92</td>
<td>--</td>
</tr>
<tr>
<td>IP347B4 and Ciat 255</td>
<td>---</td>
<td>62</td>
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‡Total nodules reacting to the single test antisera as a percent of nodules reacting to both test antisera.

†Percent of total nodules nonreactive to all test antisera.
PART 2

FACTORs RELATED TO NODULATION OF

PHASEOLUS VULGARIS

INTRODUCTION

The relatively poor nodulation in *Phaseolus vulgaris* has not only been observed in the US, but in many developing countries. Low soil pH, high available soil N, poor survival of Rhizobium and genotype are some of the reasons often given for poor nodulation. In the Columbia Basin of Washington, it is a common practice to apply from 40 to 100 kg of N/ha for maximum bean yield. Residual N from previously-grown crops requiring high fertilization rates has also added to the level of available N for a bean crop. Nodulation under these conditions is generally poor. However, poor nodulation with beans has also been observed in beans where no added N was applied (D. F. Bezdicek, unpublished data). Excellent nodulation has been reported for soybeans grown in these same type of soils (Bezdicek, et al., 1978).

Dramatic differences in the serogroup distribution of Rhizobium are often noted with variations in soil type, pH and the level of available N. Johnson and Means (1963) demonstrated marked variations in serogroup distribution of *R. japonicum* in soybean nodules from soils in Iowa, Maryland, Mississippi and North Carolina. In Iowa, Damirgi et al., (1967) found that serogroup 123 dominated in soils with pH values ranging from 6.2 to 7.5. In the same study, serogroup 135 dominated in soils with a pH of 8.0 to 8.3. Further, Damirgi et al., (1967) showed that isolates of serogroup 135 died off when inoculated into sterilized soils with a pH less than 7.5. Ham et al., (1971) showed a similar distribution of *R. japonicum* serogroups with respect to pH. Bezdicek (1972) showed a negative correlation between organic N and serogroup 123 and a positive correlation between available soil N and organic N with serogroup 110.

Limited research has been reported on the effect of soil factors on nodulation of *P. vulgaris*. In this study, the relation of soil parameters was correlated with nodulation in dry beans for the Columbia Basin of Washington.

MATERIALS AND METHODS

Site Selection and Sample Preparation

Twenty-six sites of various soil types and management practices were chosen for use in this study with the aid of bean dealers in Adams and Grant counties of Washington. Each site was planted to dry beans by the grower with one of three varieties: Rufus Red Mexican, Pinto UI-114 and Viva Pink. Differences in management practices included previouscroppings of wheat, alfalfa hay and seed, corn, sugar beets and turnips. In most cases, beans were previously grown in the rotation.

In May 1978, 12 soil cores were randomly sampled from a representative area in the 26 fields from 0 to 23 and from 23 to 55 cm in depth. All samples were refrigerated until air-dried, within seven days of collection. Following 96 hours of drying, the samples were crushed, passed through a 2-mm screen and thoroughly mixed. Samples were maintained at room temperature until they were analyzed.
Soil pH was determined on a saturation paste using a Beckman Zeromatic pH meter. Soluble salts were determined on the saturation extracts with a model RD-26 "Solu-bridge." Organic matter was determined by the Walkley-Black method as modified by Greweling and Pech (1960).

Soil P and K were extracted with 0.5N NaHCO₃ for 30 minutes and filtered through Whatman No. 1 filter paper (Olsen, et al., 1954). Phosphorous was determined in the extract by the method of Olsen et al., (1954), whereas K was determined using a Perkin-Elmer 52C flame photometer. Ammonium and NO₃-N were analyzed from 2N KCl extracts by steam distillation according to the method of Bremner (1965).

During mid-July, when the bean plants were in the early stages of podfill, roots from 21 randomly selected plants were collected from each of the sites. The nodules were carefully removed from the roots, pooled, washed, weighed and frozen. Data were compiled and statistically analyzed for a correlation matrix.

RESULTS AND DISCUSSION

Soil and plant characteristics determined for the survey are shown in Table 4. Considerable variation was noted for many of the soil parameters. Nodulation at many of the sites was very low, with fewer than 0.5 g of nodules per plant observed at many locations. From 1 to 2 g of nodules were observed for the recovery study (Part I). It is difficult to reconcile the relatively good nodulation at some sites where MPN of R. phaseoli was so low. The same observation can be made for the recovery study, where relatively good nodulation was obtained in a soil less than 10 MPN R. phaseoli/g.

Table 5 shows the range and means of values obtained from the soil parameters selected in the 1978 survey. The range of pH values from 5.7 to 8.1 represent values for noncalcareous and calcareous soils found in this area. Levels of organic matter in these sites varied from 0.1% in a coarse-textured soil to 1.7% in a finer-textured soil. Phosphorous varied from a deficient level of 3.4 ppm to 57.4 ppm which is excessive for beans (Dow, 1976). With P, K varied from a deficient level of 65 ppm to 675 ppm which is in excess of the level required by dry beans (Dow, 1976). The electrical conductivity varied from 0.25 mmhos/cm, below a level effecting the yield of beans, to 2.10 mmhos/cm, which has been reported to cause a 25% reduction in the yield of beans (Berstein, 1964).

The levels of inorganic N in these samples were determined to two depths (0 to 23 and 23 to 55 cm) because NO₃ leaches readily under irrigation and because bean roots are commonly found below 23 cm. The wide range of NH₄⁺-N from 10 to 37 kg/ha in the 0 to 23-cm depth and from 7 to 265 kg/ha in the 23 to 55-cm depth probably represent differences in the time and rate of fertilizer application. Differences in soil NO₃-N were about tenfold for both depths, with a high value of 202 kg/ha being noted in the 23 to 55 cm depth.

Since NH₄⁺ will readily nitrify to NO₃⁻, the total inorganic N was also considered. The high value of 441 kg/ha of N is excessive and would be expected to seriously limit nodulation. A recommended rate of N fertilization for the Columbia Basin is 80 kg/ha of N on soils which exhibit low levels of soil N. Based on the values reported in Table 4, most of the fields would not have required any additional applied N in the 1978 growing season.
Table 4.--Soil and plant characteristics for survey locations in 1978.

<table>
<thead>
<tr>
<th>Site No.</th>
<th>pH</th>
<th>O.M.</th>
<th>P</th>
<th>K</th>
<th>E.C.</th>
<th>( \text{NH}_4^+ )</th>
<th>( \text{NO}_3^- )</th>
<th>( \text{NH}_4^+ + \text{NO}_3^- )</th>
<th>Nodulation</th>
<th>Rhizobia</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.5</td>
<td>0.4</td>
<td>8.7</td>
<td>135</td>
<td>0.40</td>
<td>16</td>
<td>10</td>
<td>55</td>
<td>67</td>
<td>148</td>
</tr>
<tr>
<td>2</td>
<td>6.3</td>
<td>0.5</td>
<td>25.6</td>
<td>190</td>
<td>0.35</td>
<td>27</td>
<td>17</td>
<td>68</td>
<td>26</td>
<td>138</td>
</tr>
<tr>
<td>3</td>
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<td>0.8</td>
<td>57.4</td>
<td>675</td>
<td>0.32</td>
<td>18</td>
<td>19</td>
<td>161</td>
<td>41</td>
<td>239</td>
</tr>
<tr>
<td>4</td>
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<td>0.5</td>
<td>16.5</td>
<td>270</td>
<td>0.50</td>
<td>35</td>
<td>18</td>
<td>82</td>
<td>55</td>
<td>190</td>
</tr>
<tr>
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<td>0.6</td>
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<td>650</td>
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<td>9</td>
<td>9</td>
<td>177</td>
<td>87</td>
<td>282</td>
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<tr>
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<td>0.8</td>
<td>19.4</td>
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<td>192</td>
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<tr>
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<td>0.4</td>
<td>10.6</td>
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<td>0.58</td>
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<td>19</td>
<td>96</td>
<td>56</td>
<td>186</td>
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<tr>
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<td>0.8</td>
<td>17.4</td>
<td>362</td>
<td>0.55</td>
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<td>0.7</td>
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<td>25</td>
<td>19</td>
<td>109</td>
<td>58</td>
<td>211</td>
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<tr>
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<td>1.4</td>
<td>40.0</td>
<td>335</td>
<td>1.50</td>
<td>18</td>
<td>43</td>
<td>177</td>
<td>203</td>
<td>441</td>
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<tr>
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<td>1.7</td>
<td>38.0</td>
<td>19</td>
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<td>118</td>
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<td>166</td>
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<tr>
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<td>0.5</td>
<td>27.3</td>
<td>110</td>
<td>0.50</td>
<td>14</td>
<td>21</td>
<td>102</td>
<td>71</td>
<td>203</td>
</tr>
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<td>0.7</td>
<td>3.4</td>
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<td>1.60</td>
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<td>172</td>
<td>18</td>
<td>112</td>
<td>315</td>
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<td>0.4</td>
<td>2.3</td>
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<td>30</td>
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<td>90</td>
<td>69</td>
<td>200</td>
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<tr>
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<td>0.5</td>
<td>22.8</td>
<td>185</td>
<td>0.36</td>
<td>14</td>
<td>8</td>
<td>35</td>
<td>54</td>
<td>111</td>
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<tr>
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<td>0.6</td>
<td>13.1</td>
<td>170</td>
<td>0.50</td>
<td>23</td>
<td>21</td>
<td>94</td>
<td>63</td>
<td>201</td>
</tr>
<tr>
<td>20</td>
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<td>0.5</td>
<td>34.7</td>
<td>242</td>
<td>0.55</td>
<td>31</td>
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<td>19</td>
<td>54</td>
<td>123</td>
</tr>
<tr>
<td>21</td>
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<td>0.8</td>
<td>20.2</td>
<td>102</td>
<td>1.78</td>
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<td>125</td>
<td>13</td>
<td>164</td>
</tr>
<tr>
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<td>24.2</td>
<td>65</td>
<td>0.65</td>
<td>21</td>
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<td>97</td>
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<td>197</td>
</tr>
<tr>
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<td>0.4</td>
<td>25.7</td>
<td>135</td>
<td>0.45</td>
<td>1.1</td>
<td>11</td>
<td>98</td>
<td>21</td>
<td>201</td>
</tr>
<tr>
<td>24</td>
<td>7.7</td>
<td>0.6</td>
<td>20.6</td>
<td>21</td>
<td>0.80</td>
<td>2</td>
<td>1</td>
<td>0.3</td>
<td>71</td>
<td>123</td>
</tr>
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<td>0.5</td>
<td>32.8</td>
<td>315</td>
<td>0.65</td>
<td>13</td>
<td>18</td>
<td>81</td>
<td>43</td>
<td>155</td>
</tr>
<tr>
<td>26</td>
<td>8.1</td>
<td>0.1</td>
<td>13.9</td>
<td>160</td>
<td>0.90</td>
<td>13</td>
<td>14</td>
<td>35</td>
<td>27</td>
<td>99</td>
</tr>
</tbody>
</table>
Table 5.--Range and means of soil and plant parameters for the survey in 1978.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Range</th>
<th>Mean</th>
<th>Parameter</th>
<th>Range</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nodule Wt.</td>
<td>0.06-1.50</td>
<td>0.49</td>
<td>NH₄⁺ (0-23 cm)</td>
<td>10-37</td>
<td>20</td>
</tr>
<tr>
<td>pH</td>
<td>5.70-8.10</td>
<td>6.60</td>
<td>NH₄⁺ (23-55 cm)</td>
<td>7-265</td>
<td>27</td>
</tr>
<tr>
<td>O.M.-%</td>
<td>0.10-1.70</td>
<td>0.68</td>
<td>NO₃⁻ (0-23 cm)</td>
<td>10-178</td>
<td>94</td>
</tr>
<tr>
<td>P-ppm</td>
<td>3.40-57.40</td>
<td>25.20</td>
<td>NO₃⁻ (23-55 cm)</td>
<td>27-202</td>
<td>64</td>
</tr>
<tr>
<td>K-ppm</td>
<td>65.00-675.00</td>
<td>242.80</td>
<td>Total (0-23 cm)</td>
<td>27-195</td>
<td>114</td>
</tr>
<tr>
<td>EC-mmhos/cm</td>
<td>0.25-2.10</td>
<td>0.74</td>
<td>Total (23-55 cm)</td>
<td>16-26</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total (0-55 cm)</td>
<td>99-4</td>
<td>205</td>
</tr>
</tbody>
</table>

†C.M. (organic matter); E.C. (electrical conductivity of saturation extract).
Since the objectives of this study were to determine which soil parameters were related to nodulation and N fixation potential of *P. vulgaris*, significant correlation coefficients are listed in Table 6. Only three parameters were statistically related to nodulation. A significant negative correlation was obtained between total inorganic N and nodulation. This correlation was best expressed by a curvilinear relationship, where above 200 kg/ha of total inorganic N, nodulation was drastically reduced. Nodulation was not appreciably influenced by inorganic N up to 150 kg of N/ha.

A significant and negative correlation was also obtained between nodulation and electrical conductivity. Since a positive correlation was observed between total soil inorganic N and EC (0.46*), the higher levels of inorganic N may have contributed to an increase in the level of soluble salts. Thus, the relation of nodulation with EC may be more of an indirect effect on inhibition of nodulation by soil N. It should be mentioned that soluble salts are known to reduce the yield of field beans. Bernstein (1964) showed that field bean yields were reduced 10 to 50% at EC values of 1.5 and 3.0, respectively.

Organic matter was negatively correlated at the 5% level of probability with nodulation. This may be explained by the greater potential for mineralization of organic N in soils high in organic matter. There was not a significant relationship between serogroup distribution and other parameters studied. However, several apparent double infections were noted with the fluorescent antibody technique. The double infections were observed when cells of the same nodule macerate were stained with two different FITC conjugates.
Table 6.--Significant correlation coefficients for nodulation and soil parameters from the survey in 1978.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NW</th>
<th>pH</th>
<th>OM</th>
<th>P</th>
<th>K</th>
<th>E.C.</th>
<th>NH$_4^+$-N (s)†</th>
<th>NH$_3^+$-N (s)</th>
<th>NO$_3^-$-N (ss)†</th>
<th>Total N (s)†</th>
<th>Total N (ss)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nodule wt. (NW)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>Variety (VA)</td>
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<td></td>
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<td>pH</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Organic Matter (O.M.)</td>
<td>-0.49*</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Phosphorous (P)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Potassium (K)</td>
<td>0.42*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Electrical Conductivity (E.C.)</td>
<td>-0.46*</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NH$_4^+$-N (s)†</td>
<td></td>
<td>-0.48*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NH$_4^+$-N (ss)†</td>
<td></td>
<td></td>
<td>0.63**</td>
<td>0.61**</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>NO$_3^-$-N (s)†</td>
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<td></td>
<td></td>
<td></td>
<td>0.42*</td>
<td></td>
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</tr>
<tr>
<td>NO$_3^-$-N (ss)†</td>
<td></td>
<td></td>
<td>0.47*</td>
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<td>Total N (s)†</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.57**</td>
<td>0.91**</td>
</tr>
<tr>
<td>Total N (ss)†</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.45*</td>
<td></td>
<td></td>
<td></td>
<td>0.73**</td>
<td>0.48**</td>
</tr>
<tr>
<td>Total N</td>
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<td>0.53**</td>
<td></td>
<td></td>
<td></td>
<td>0.46*</td>
<td></td>
<td></td>
<td></td>
<td>0.63**</td>
<td>0.72**</td>
</tr>
</tbody>
</table>

*, ** significant at the 5 and 1% level of probability, respectively,

†Symbols: NH$_4^+$-N (s), 0-23 cm; NH$_4^+$-N (ss), 23-55 cm.
INTRODUCTION

Soil microorganisms live in a complex environment, with soil clays and organic macromolecules playing a role in the attraction of bacteria to solid surfaces. Production of enzymes by bacteria, extracellular polysaccharides and polymer fibrils, metal oxides, and the ionic strength of the soil solution all play a role in the attraction of bacterial to solid surfaces. These interacting factors are not only recognized in the attraction of bacteria to solid surfaces, but are involved when one wishes to separate bacteria from soil colloids for enumeration and characterization.

The study of soil microbial ecology has been limited by the lack of techniques which permit quantitative enumeration and direct observation of bacterial cells in soil. This has been particularly true for Rhizobium, since a specific culture medium is not available for its enumeration by dilution plating from soil. Direct observation of Rhizobium cells has not been possible until recently because these cells cannot be distinguished from other soil bacteria. As a result, considerable research has been conducted using sterile soils, where the applicability to natural environments is questionable. The fluorescent antibody (FA) technique, as a quantitative means of studying soil microbial ecology (Schmidt, 1974), has been applied to a wide range of soil environments (Boholand Brock, 1974; Fliermans and Schmidt, 1975; Strayer and Tiedje, 1978).

With the FA approach, soils containing bacterial cells are dispersed with a surfactant and flocculated with an inorganic salt. The supernatant containing the bacterial cells is passed through an appropriate filter (0.45 μ dia.), followed by adding a specific antiserum conjugated with a fluorochrome. Observation and enumeration of the specific fluorescent cells on the filter surface can be made with a microscope equipped with proper filters and optics. However, a major limitation of this technique is the difficulty in separating bacterial cells from soil colloids. Certain flocculating agents, which provide a clarified supernatant for filtration, often co-flocculate cells and colloids resulting in low bacterial recovery. Less efficient flocculation of colloids and cells often provides a turbid suspension which limits the quantity of supernatant that can be passed through the filter and hence prevents enumeration of soil bacteria at low populations. Recovery of added Rhizobium cells varies with soil type and flocculating agents. For example, recoveries of 20 to 80% (Vidor and Miller, Soil Biol. Biochem., in press), 33 to 44% (Donaldson et al., 1979) and less than 1 to 100% (Kingsley and Bohlool, 1979) have been reported.

The primary object of this study was to evaluate various flocculants for recovery of added Rhizobium from soil and to develop new approaches for recovery of bacteria from soil.

MATERIALS AND METHODS

Cultures Used

The slow-growing R. japonicum strain 110, came from the USDA-Beltsville collection and was supplied by Dr. D. F. Weber. Two fast-growing strains were studied. R. leguminosarum strain C1-204 isolated from Washington and R. phaseoli strain Kim-5 isolated from Idaho. Antisera against these isolates were prepared according to the procedure of Vincent (1970). Conjugation of antisera with fluorescein isothiocyanate (FITC) was conducted as described by Schmidt, 1974. For enumeration of cells, a Zeiss model 14 microscope equipped with a 100-watt, quartz-halogen light source was used in the epi-illumination mode. A primary interference filter emitting from 450 to 490 nm was used in conjunction with a dichroic reflector at 510 nm and with secondary filter emitting greater than 510 nm. A X 100 Neofluar objective was employed.

For recovery and survival studies, Rhizobium cells were grown in yeast extract mannitol broth (Vincent, 1970) on a rotary shaker at 24°C to mid-log phase (about 10⁸ cells/ml) and then pelleted by centrifugation at 7000 × g for 15 min. Cells were resuspended in a minimum volume of water. This process was repeated twice. Final cell concentration varied with the experiment. Cells enumerated directly by hemacytometer and by FA (Schmidt, 1974) were in close agreement.

FLOCCULATION STUDIES

Studies were conducted to determine the efficiency of various chemicals to flocculate coarse- and fine-textured soils for recovery of added Rhizobium cells. Turbidity was used as a first estimate of flocculation efficiency. To 1 g of both fine-textured (Parkhill sandy clay loam and Portneuf silt loam) and coarse-textured soils (Odell loamy sand and Winchester fine sandy loam) placed in 10-ml tubes containing glass beads, 8.5 ml water and 0.5 ml of a Rhizobium-cell suspension were added to provide about 10⁷ cells per g of soil. One drop each of a surfactant Tween 80 and Antifoam B Emulsion (Sigma Chemical Co.) were added. After mixing with a Vortex mixer for 1 min, 0.5 ml of the following flocculants were added to these final concentrations: 0.01, 0.05, 0.1 or 1% FeCl₃; 0.1 or 1% AlCl₃; 1% CaCl₂; 1% MgCO₃; 2% Ca(OH)₂: MgCO₃ (2:5) was used (Schmidt, 1974). After mixing for 1 min, tube contents were allowed to settle for either 10, 20, 40, 60 or 120 min at which time 3 ml were removed from the top and read for optical density (OD) at 660 nm. Aliquots to 2 ml were then passed through a Millipore filter (0.45 μm dia.) for enumeration by FA. Control soils were not added Rhizobium FA conjugates.

Properties of soils studied are summarized in Table 7. From various concentrations of FeCl₃ and AlCl₃ added to soil, 0.1% AlCl₃ and 0.5% FeCl₃ were selected to be optimal; all other concentrations tested either lacked an adequately clear solution for filtration or cleared within 10 min.

Of the flocculants, sample turbidity (OD) for Ca(OH)₂:MgCO₃ decreased rapidly within the first 10 min (Fig. 1). The use of this flocculant in FA studies is questionable in these soils because microbial recovery would
Table 7. Properties of soils used in flocculation and recovery studies.

<table>
<thead>
<tr>
<th>Soil Type</th>
<th>Soil Order</th>
<th>Origin</th>
<th>Soil pH</th>
<th>Soil Organic Matter %</th>
<th>Soil Water Content at -30 bars ( \psi )</th>
<th>(-0.3 \text{ bar } \psi )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Odell loamy sand</td>
<td>Mollisol</td>
<td>Michigan</td>
<td>6.2</td>
<td>2.3</td>
<td>2.3</td>
<td>8.8</td>
</tr>
<tr>
<td>Parkhill sandy clay loam</td>
<td>Inceptisol</td>
<td>Michigan</td>
<td>6.6</td>
<td>3.2</td>
<td>5.6</td>
<td>16.3</td>
</tr>
<tr>
<td>Portneuf silt loam</td>
<td>Aridisol</td>
<td>Idaho</td>
<td>7.8</td>
<td>1.3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Winchester fine sandy loam</td>
<td>Entisol</td>
<td>Idaho</td>
<td>8.0</td>
<td>0.8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Burke loam</td>
<td>Aridisol</td>
<td>Washington</td>
<td>7.7</td>
<td>1.1</td>
<td>4.1</td>
<td>14.5</td>
</tr>
<tr>
<td>Royal sandy loam</td>
<td>Aridisol</td>
<td>Washington</td>
<td>7.9</td>
<td>0.3</td>
<td>2.8</td>
<td>7.8</td>
</tr>
</tbody>
</table>
FLOCCULATION OF COMBINED SOILS

OD_{660nm} vs TIME (min)

- FeCl₃
- CaCl₂
- AlCl₃
- Ca(OH)₂/MgCO₃

Fig. 1.
likely charge considerably with time. The OD values for the other flocculants decreased at a similar rate. However, at 120 min, little difference was observed in OD for all the flocculants tested.

When recoveries of added *R. japonicum* cells were determined from the supernatant phase, Ca(OH)$_2$:MgCO$_3$ was the least effective of flocculants studied (Fig. 2). Relatively poor recovery was also obtained from FeCl$_3$, even though a substantial colloidal fraction remained in suspension as determined by OD (Fig. 1). Of the flocculants studied, CaCl$_2$ provided the greatest recovery, even though its OD values were lower than for FeCl$_3$. This suggests that CaCl$_2$ was preferentially flocculating soil colloids as opposed to *Rhizobium* cells. The increase in *Rhizobium* recovery from 40 to 60 min was frequently observed, especially for CaCl$_2$. This may represent a release of cells from colloids in the sedimented soil phase to the upper solution since bacterial cells are less dense than most clays. Similar results were obtained for the fast-growing *R. phaseoli* and *R. leguminosarum* in Idaho soils.

It is recognized that the fast- and slow-growing *Rhizobium* have different charges on their cell surfaces (Marshall, 1968) which may affect their flocculating efficiencies. Table 8 shows that the recovery of *R. japonicum* cells with FeCl$_3$ was about as good as obtained with CaCl$_2$ in Michigan soils. However, 10% or less of added *R. phaseoli* cells were recovered with FeCl$_3$. These trends for FeCl$_3$ were not observed in the Idaho soils. Flocculating efficiency from CaCl$_2$, AlCl$_3$ and Ca(OH)$_2$:MgCO$_3$ was about the same for both fast- and slow-growing *Rhizobium* regardless of soil type studied.

When turbidity (OD) of soil-flocculant suspensions was related to recovery of applied *Rhizobium* cells, recovery with FeCl$_3$ at a given OD was considerably lower than for either CaCl$_2$ or for AlCl$_3$ (Fig. 3). This suggests that even though two of these flocculants were trivalent cations, Al$^{3+}$ (0.05% AlCl$_3$) was more efficient in separating soil colloids from *Rhizobium* cells than was Fe$^{3+}$ (0.1% FeCl$_3$). Values of OD for soil suspensions treated with Ca(OH)$_2$:MgCO$_3$ were less than 0.3 (Fig. 1). Therefore these data are not presented. In a study of saturating cations with sorption of Azotobacter *chroococcum* by Volusia silt loam, Peele (1935) indicated that the cell sorption capacity by trivalent cations (Al$^{3+}$, Fe$^{3+}$) on the soil was much greater than for divalent cations. However, flocculation of soil colloids alone with tri or divalent cations would be expected to follow roughly the same order. Therefore, co-flocculation of soil colloids and bacterial cells would be expected from the addition of these cations and hence recovery of cells with a flocculating agent should be influenced by surface properties of both the cell and soil colloid. Of the flocculants studied, 1% CaCl$_2$ provided the greatest consistency of recovery with changes in soil texture and with type of *Rhizobium*.

**Sucrose Density Centrifugation**

Low recovery of *Rhizobium* cells from soils, relatively low detection levels in soil and the interaction of flocculant type with *Rhizobium* species led us to investigate other methods of recovery bacteria from soil. Initial attempts at bacterial separation from soil with 1,1,2,2-tetramethylethane (used in density separation of minerals) were unsuccessful. The use of sucrose in separation of bacterial cell components led us to investigate sucrose density centrifugation for recovery of *Rhizobium* from soil. Initially, several dispersing agents and various concentrations of CaCl$_2$ were studied by adding soil and *Rhizobium* cells
Table 8. Comparative recoveries of *R. phaseoli* and *R. japonicum* as determined by FA with various flocculants.

<table>
<thead>
<tr>
<th>Flocculant</th>
<th><em>R. phaseoli</em></th>
<th><em>R. japonicum</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>40 min.</td>
<td>120 min.</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>83</td>
<td>55</td>
</tr>
<tr>
<td>AlCl₃</td>
<td>28</td>
<td>26</td>
</tr>
<tr>
<td>Ca(OH)₂-MgCO₃</td>
<td>31</td>
<td>18</td>
</tr>
<tr>
<td>FeCl₃</td>
<td>10</td>
<td>3</td>
</tr>
</tbody>
</table>

Michigan Soils
RECOVERY VS TURBIDITY - SLOW GROWER

% RECOVERY

OD660 nm

CaCl2
AlCl3
FeCl3

F9.31
to a 24% sucrose solution (sp. gr. 1.0990). This suspension was layered on a 50% sucrose solution (sp. gr. 1.2296) in a 50-ml centrifuge tube. After centrifugation at various gravitational forces, aliquots from the 24% phase were removed and determined for cell recovery by FA. Difficulty was encountered in quantitatively removing cells from the 24% sucrose phase without disturbing the 50% sucrose phase which contained soil. This difficulty was most evident with $10^5$ or fewer Rhizobium cells/g of soil. Later a device described below was designed which permitted most of the soil to be removed during centrifugation.

For enumeration at greater than $10^4$ Rhizobium cells/g of soil, 1 g of soil was transferred to a 15-ml centrifuge tube followed by adding 2 drops (Pasteur pipette) of Tween 80, 1 drop of Antifoam B, 4 ml 24% sucrose (w/v) and 0.05% ml of 20% CaCl$_2$ to provide a final concentration of 0.2% CaCl$_2$. Either 0.5 ml of a Rhizobium cell suspension or 0.5 ml H$_2$O were added to provide a final soil:solution ratio of 1:5. The contents were shaken with a Vortex mixer for 1 min and centrifuged at 100 x g for 15 min. A 3-ml aliquot of the supernatant was removed and diluted if necessary. Three ml of the suspension were layered in a 15-ml centrifuge tube placed inside a 50-ml polypropylene centrifuge tube containing 17 ml of 50% sucrose (Fig. 4). A hole at the tip of the 15-ml centrifuge tube permitted soil to pass through during centrifugation. The entire apparatus was centrifuged at 718 x g for 15 min in a centrifuge equipped with a swinging bucket. Contents within the inner centrifuge tube were removed by placing a rubber stopper (equipped with a glass rod) on the inner tube and lifting the entire tube upward after placing a finger on the glass rod. The contents were released onto a 0.45 µm Millipore filter. When the maximum quantity (3 ml) of 24% sucrose was added to the gradient, approximately 0.5 ml of 50% sucrose remained at the bottom of the 15-ml centrifuge tube which was sufficient to stop the downward movement of cells, yet permit most of the soil to pass to the outer 50-ml centrifuge tube. Contents within the 15-ml tube were clear after centrifugation. Unless otherwise specified, Rhizobium cells were added to air-dry soil and extracted within an hour. A time study conducted at 4°C of R. phaseoli cells added to Parkhill sandy clay loam showed essentially 100% recovery for 5 days. Similar results were reported by Wollum and Miller (1979). Thus, it was deemed not necessary to incubate the soil-bacterial mixtures for recovery of cells.

As described above, density gradient centrifugation capitalizes on the lower density of bacterial cells relative to that of clays and non-silicate minerals. The use of a flocculating agent (CaCl$_2$) was necessary in the 24% sucrose phase. The Tween 80-Dow Antifoam combination used as described was a more useful dispersing agent than either NaOH, NaCl or sodium pyrophosphate.

A comparison of Rhizobium recovery by CaCl$_2$ flocculation with sucrose density centrifugation is shown in Table 9. The lower limit of detection by CaCl$_2$ flocculation is about $10^5$ organisms g$^{-1}$ soil. At this level, only 33% of applied Rhizobium cells were recovered. A maximum of about 2 ml of supernatant could be passed through the filter without causing plugging or covering of cells by colloids. A higher recovery obtained at higher cell densities was because the supernatant was diluted of interfering colloids prior to addition to the Millipore filter.

Recovery of added Rhizobium by sucrose density centrifugation was greater than by flocculation at all levels of added cells. The lower limit of recovery was $10^4$ cells g$^{-1}$ (one log number below that obtained by CaCl$_2$ flocculation). Recovery by sucrose density centrifugation was greater than 90% for all
15 ml Centrifuge Tube

50 ml Centrifuge Tube

24% Sucrose

50% Sucrose

Glass Rod

Rubber Stopper
Table 9. Comparative recovery of *R. phaseoli* as determined by FA with CaCl$_2$ flocculation and density centrifugation.

<table>
<thead>
<tr>
<th>Cells Added $g^{-1}$ soil</th>
<th>%Recovery$^1/$ flocculation</th>
<th>%Recovery$^2/$ density centrifugation</th>
</tr>
</thead>
<tbody>
<tr>
<td>$1 \times 10^7$</td>
<td>76 (28)</td>
<td>135 (14)</td>
</tr>
<tr>
<td>$1 \times 10^6$</td>
<td>62 (36)</td>
<td>110 (18)</td>
</tr>
<tr>
<td>$1 \times 10^5$</td>
<td>33 (61)</td>
<td>97 (31)</td>
</tr>
<tr>
<td>$1 \times 10^4$</td>
<td>ND</td>
<td>91 (109)</td>
</tr>
</tbody>
</table>

$^1$/ND non detectable; values in parenthesis represent the coefficient of variation for 30 microscopic fields.

$^2$/Average of 60 microscopic fields.
population levels. Few differences in recovery were noted between soil type and between fast- and slow-growing Rhizobium (data not shown). Thus, this method should be useful for recovering other bacteria from a range of different soils.

The recoveries in excess of 100% are due to a concentrating of cells in the 24% sucrose layer after centrifugation (Table 9). Concentration of Rhizobium cells within the 24% sucrose was less at the top and greater near the 24-50% sucrose interface (M. D. Donaldson and D. F. Bezdieck, manuscript in preparation). Although cells tended to move downward during centrifugation, there was not a significant movement into the denser, 50% sucrose. A similar approach using sucrose density centrifugation was reported by Wollum and Miller (1979), who obtained about 100% recovery of added Rhizobium at 10^6 cells/g of soils representing various taxonomic orders.
PART 4

SURVIVAL OF RHIZOBIUM IN SOIL

INTRODUCTION

The large increases in soybean yield in the Columbia Basin of Washington from inoculation with R. japonicum (slow growing) (Bezdicek, et al., 1978) is in sharp contrast to the lack of yield response from inoculation of beans with R. phaseoli (fast growing). This effect may have resulted from a different capacity of these two Rhizobium species to persist and multiply in these soils. Low populations of R. phaseoli ranging from 10 to 10^3 g^-1 have been reported in these soils (W. R. Ellis, M. S. Thesis, Washington State University, Pullman, 1978). Soils in this region are generally sandy in texture and despite irrigation are subject to periods of hot and dry conditions. Marshall (1964) suggested that the majority of legumes associated with the slow-growing rhizobia are found in the more arid regions of Australia; legumes associated with fast-growing rhizobia are well-established in semi-arid areas, but are restricted to heavier-textured soils. Evidence suggests that the slow-growing rhizobia are better able to survive periods of desiccation than the fast-growing rhizobia (Marshall, 1964; Marshall, 1968; Bushby and Marshall, 1977).

Bushby and Marshall (1977), showed that the addition of montmorillonite offered protection to fast-growing, but not slow-growing rhizobia in sterile soils. Marshall (1968) suggested that clays offer protection to the fast-growing rhizobia because of the attraction of positively charged edge-to-edge surfaces of clay with the negatively charged (carboxyl) bacterial cells. It was postulated that the montmorillonite envelope offered protection to cells by modifying the rate of water loss from the cell.

MATERIALS AND METHODS

Since many of the above studies were conducted under sterile soil conditions, an investigation of the survival of Rhizobium in non-sterile soils was initiated using the FA technique. Survival was monitored at two water potentials (Ψ); -0.3 bar, which represents a moisture level conducive to microbial activity and -30 bars, which represents a moisture level permitting only minimal bacterial activity (Griffin, 1972). Specific Ψ was determined since little attention has been given to the energy status of soil water on survival of Rhizobium.

To 25 g of both fine-textured (Parkhill sandy clay loam and Burke loam) and course-textured soils (Odel loamy sand and Royal sandy loam), calibrated concentrations of either fast- or slow-growing Rhizobium strains (about 10^6 cells g^-1 soil) were added to both sterile or non-sterile soil. Cultures and soils used are described in Part 3. The soils were sterilized in 50-ml Erlenmeyer flasks by autoclaving four 4 hours on two consecutive days. Non-sterile soils were incubated in 100-ml polypropylene bottles. Sufficient solution (cell suspensions and water) was added to bring the final Ψ to either -0.3 or -30 bars after thorough mixing. Flasks were placed in a humid chamber at 25°C. Water potential from -1 to -30 bars was determined by thermocouple psychrometry (Campbell, et al., 1966), whereas gravimetric water (θ) was obtained at -0.3 and -1 bar by the pressure membrane (Richards, 1965). A linear relation of log θ with log Ψ was obtained after adding various quantities of water to each air-dry soil. Periodically, 1-g samples were removed where Rhizobium numbers were enumerated by FA. At 100 days, soils
initially at -0.3 bar \(Y\) remained at that value. However, soils initially at
-30 bars increased in \(Y\) (between -15 to -10 bars). Direct counts of Rhizobium
from sterile soils were made on yeast extract mannitol agar (Vincent, 1970).
When appropriate, Rhizobium cells were also enumerated in non-sterile soils
by most probable number, MPN (Weaver and Frederick, 1972).

RESULTS AND DISCUSSION

Survival of \(R.\) phaseoli and \(R.\) japonicum from the coarse- and fine-textured
Washington and Michigan soils is shown in Fig. 5. At -0.3 bar \(Y\), an increase
in growth from one-half to two log numbers was observed at 40 days; the greatest
growth observed for \(R.\) japonicum in the fine-textured soils. Growth for \(R.
phaseoli\) in the coarse-textured soils reached a peak early and declined slowly.
In general, survival was greatest in the fine-textured soils which probably re­
resents a greater level of available substrate in these higher-organic matter
soils (Table 7).

Under non-sterile conditions at -0.3 bar, a gradual decrease in survival
was obtained for both fast- and slow-growing Rhizobium in both fine- and coarse-
textured soils (Fig. 6). However, slight increases in numbers were noted at
9 days for \(R.\) phaseoli and at 32 days for \(R.\) japonicum. Throughout the study,
survival was greater in fine-textured soils and was greater for the slow-growing
Rhizobium strain. These results are in agreement with those of Bushby and
Marshall (1977) under sterile soil conditions. However, since our studies
were conducted at near ideal soil moisture (-0.3 bar), the lower survival of
the fast-growing Rhizobium strain cannot be attributed to conditions of des­
ication. Rather, lower levels of substrate, presumed for soils of coarser
texture and lower organic matter, are probably more significant.

Survival data for rhizobia in sterile and non-sterile soils held at -30
bars \(Y\) (shown in Fig. 7) were combined since they were similar. Survival
under these relatively dry conditions was much better than expected. Again
as shown previously, survival was greater for the slow-growing Rhizobium.
No apparent difference in survival was observed between the fine- and coarse-
textured soils. Although these results agree with those of Marshall (1964)
and Bushby and Marshall (1974) with regard to the better survival of the
slow-growing Rhizobium, the degree of survival was much greater in our study.
The \(Y\) used in their studies was not reported and therefore a direct comparison
cannot be made on the relative potential for desiccation of cells.

Because of the relatively high survival at -30 bars \(Y\) compared with -0.3
bar \(Y\), Rhizobium from soil was enumerated by plate counts at 109 days in
sterile soils and by MPN in non-sterile soils at 143 days (Table 10). The
relatively large survival observed in sterile soils held at -0.3 bar represents
the increased growth shown in Fig. 4. Enumeration by FA tended to over estimate
counts as compared with plate counts. While this difference is not great, it
may result from some cells that are antigenically active to conjugated anti-sera,
but are not viable. However, at -30 bars \(Y\), counts by FA were from two to
nearly four log numbers higher than by plate count. These results suggest
that antigenic determinants on the cell surface are not destroyed by disiccation
at -30 bars \(Y\) and are subject to reaction with the conjugated antisera when
the cell itself is not viable.

Recovery of added \(R.\) phaseoli by FA and by MPN from non-sterile soils is
shown in Table 11. Recoveries by FA and MPN were comparable in non-sterile
soils held at -0.3 bar \(Y\). However, the FA technique used with soils held at
-30 bars \(Y\) again over estimated bacterial populations from \(10^2\) to nearly \(10^3\)
cells g\(^{-1}\). Little difference in survival was observed when comparing sterile
Fig. 5. B. Sterile soils.
-0.3 BAR NS

\[ \text{LOG}_{10} \]
\[ \% \text{ SURVIVAL} \]

\[ \text{TIME (days)} \]

\[ \text{SLOW GROWER} \]
\[ \text{FAST GROWER} \]

\[ \circ \text{ FINE TEXTURE} \]
\[ \circ \text{ COARSE TEXTURE} \]
\[
\text{LOG}_{10} \quad \% \text{ SURVIVAL}
\]

-30 BAR

TIME (days)

- FAST GROWER
- SLOW GROWER

FINE TEXTURE
COARSE TEXTURE
Table 10. Comparative survival of added *R. phaseoli* as determined by FA and plate counts from sterile soils.

<table>
<thead>
<tr>
<th>Soil Texture</th>
<th>-0.3 bars $\psi$ FA</th>
<th>Plate Count</th>
<th>-30 bars $\psi$ FA</th>
<th>Plate Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fine textured</td>
<td>1913</td>
<td>494</td>
<td>22</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>Coarse textured</td>
<td>178</td>
<td>34</td>
<td>20</td>
<td>&lt;0.003</td>
</tr>
</tbody>
</table>

Michigan Soils
Table 11. Comparative survival of added *R. phaseoli* as determined by FA and most probable number (MPN) from non-sterile soils.

<table>
<thead>
<tr>
<th>Soil Texture</th>
<th>Survival - % at 143 days; non-sterile soils</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-0.3 bars $\Psi$</td>
</tr>
<tr>
<td></td>
<td>FA</td>
</tr>
<tr>
<td>Fine textured</td>
<td>135</td>
</tr>
<tr>
<td>Coarse textured</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Michigan and Washington Soils Combined
and non-sterile soils at -30 bars ψ by plate count and MPN. However, _R. phaseoli_ survived better in the coarse-textured soil under non-sterile as compared to sterile conditions. Plate counts and MPN were not determined for _R. japonicum_. At -30 bars ψ, the differences in survival between coarse- and fine-textured sterile soils determined by plate count was much greater than those determined for non-sterile soils by MPN.

Since the energy at which water is held in both fine-textured and coarse-textured soils was presumed to be the same at -30 bars ψ, the decreased Rhizobium survival in coarse-textured soils is partially attributed to lower levels of substrate, reduced rate of diffusion of substrate to the cell, or both. Thus, careful control of the soil ψ is important when determining the effect of desiccation on bacterial survival.

These results also suggest that under periods of soil desiccation, enumeration of Rhizobium cells by the FA procedure will lead to an overestimation of a population relative to the viable soil population. At this low ψ (-30 bars), the potential for competition and antagonism by other soil organisms is minimized. Thus, survival in sterile and non-sterile soils held at -30 bars ψ was similar as determined by FA. The appearance of FA conjugated Rhizobium cells under the microscope from soils held at -30 bars ψ were different from cells held at -0.3 bar ψ. Cells held at -30 bars ψ had a "ghost-like" appearance, with reduced fluorescence at the outer cell wall.

Rewetting of soils held at -30 bars ψ was not attempted, but should bring about a rapid degradation of non-viable Rhizobium cells in non-sterile soil. The period of time required to establish an equilibrium between the production of new cells and degradation of old cells is not known.

Under most field conditions, periods of desiccation and wetting frequently occur. Therefore, the limitations of the FA technique for enumeration of Rhizobium at low ψ will not be as severe. Since the number of Rhizobium cells added to soils in our studies were relatively large, the difference of cell survival determined by FA as compared to other methods would be less in more "normal" cell densities (approximately $10^4$ g⁻¹). However, enumeration of bacteria by FA from soils at low ψ must be viewed with caution. Surface soils are often at a low ψ when plants are not under any apparent moisture stress.
LITERATURE CITED - PART 1

   Sci. 40B:554-561.

   bacteria introduced into field environments. I. A survey of field 
   performances of clover inoculants by gel immune diffusion serology. 

   parison of competitiveness and persistence amongst five strains of 

   sites between strains of R. japonicum applied as inoculum and strains 

   techniques to host plant/nodule bacteria selectivity experiments using 


   Trifolium subberranean by introduced rhizobia in competition with 


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LITERATURE CITED - PART 2


