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THE ECOLOGICAL SIGNIFICANCE OF pH AND MOISTURE ON THE SURVIVAL OF SELECTED RHIZOBIUM PHASEOLI STRAINS

The Ohio State University

Ph.D. 1981

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THE ECOLOGICAL SIGNIFICANCE OF pH AND MOISTURE ON THE SURVIVAL OF SELECTED RHIZOBIUM PHASEOLI STRAINS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Denis Sewa Amara, B.Sc. (Agric.); M.S. (Agron.)

* * * *

The Ohio State University
1981

Reading Committee:
Professor R. H. Miller
Professor E. O. McLean
Professor T. J. Logan

Approved by:

Adviser

Department of Agronomy
To my beloved parents,
Pa Amara Golia and Madame Beika Amara
ACKNOWLEDGEMENTS

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VITA

August 28, 1947.............................Born in Kpetewoma, Southern Province, Sierra Leone

1954-1961...................................St. Francis Primary School, Bo, Sierra Leone

1961-1967...................................Christ the King College, Bo, Sierra Leone

1967-1971...................................B.Sc. (Agric.), Njala University College, Njala, Sierra Leone

1971-1972...................................Teaching and Research Assistant, Department of Agronomy, Njala University College, Njala, Sierra Leone

1972-1974...................................M.S. (Agronomy), University of Illinois at Urbana-Champaign

1974-1977...................................Lecturer, Department of Agronomy, Njala University College, Njala, Sierra Leone

1977-1981...................................Graduate Research Associate, Department of Agronomy, The Ohio State University

PUBLICATIONS


Major field: Soil Microbiology

Soil Microbiology and Biochemistry: Dr. R. H. Miller

General Microbiology: Drs. C. I. Randles, J. I. Frea, and J. P. Kreier

Food Microbiology: Dr. G. J. Banwart

Water Microbiology: Dr. O. Tuovinen

Virology: Dr. P. Kimball

Biochemistry and Molecular Biology: Drs. J. F. Snell and R. Ross

Soil Fertility and Tropical Soils: Dr. T. Arscott

Soil Chemistry: Dr. E. O. McLean

Plant Physiology: Dr. M. Evans
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INTRODUCTION

The survival of Rhizobium in soil is affected by biotic and abiotic factors. Some biotic factors that have received research attention include, among others, predation by protozoa or myxomycetes, lytic activity of bacteriophage and Bdellovibrios, and toxin or antibiotic production by soil or rhizosphere microorganisms. Some important abiotic factors include soil pH, soil temperature, salt tolerance, resistance to desiccation, sensitivity to flooding, and Al and Mn toxicity.

In Latin America, it has been found that nodulation of Phaseolus vulgaris is generally poor, resulting in substantial yield reductions. Research has also shown (Graham, 1973) that P. vulgaris cultivars inoculated with selected strains of R. phaseoli were well nodulated in greenhouse flats or sand-nutrient solutions. However, these same cultivars were poorly nodulated, if at all, under field conditions, even though inoculated with the same strains. These nodulation difficulties occur severely in highly weathered oxisols and ultisols which are the major agricultural soils in the region.

Nodulation problems arise from a number of different interrelated factors involving the host, the rhizobium, and the soil. With the knowledge that oxisols and ultisols are usually acidic, poorly buffered, low in organic matter, low in nutrients especially P, and are high in Al and Mn, it seems appropriate to suggest that one major reason for
nodulation failures in these soils is associated with poor survival of \textit{R. phaseoli}. Any one of the stress factors mentioned could influence survival of the bacteria. Research is needed to delineate and provide a better understanding of which factors are most important for the survival of the microsymbiont.

A possible reason for some unsuccessful legume inoculation trials is that the criterion on which the strains are selected, i.e. effectiveness in the greenhouse or sand-nutrient solution, is inadequate. \textit{Rhizobium} strain selections should be designed to include conditions that reproduce important stress factors that both the \textit{Rhizobium} and its host are likely to encounter under field situations.

With these in mind, this research was initiated to fulfill the following objectives:

1. To select dinitrogen fixing strains of \textit{R. phaseoli}.
2. To select strains that are acid and/or aluminum and manganese tolerant, and test their survival in acid soils with moderate to high aluminum contents.
3. To identify strains that can survive soil moisture stress conditions, i.e. excess moisture and desiccation, and to test, where possible, whether these stress conditions would affect the dinitrogen fixing capacity of the strains.
4. To provide some insight as to the salt tolerance of some \textit{R. phaseoli} strains.

Information gathered from such studies would help guide the selection of edaphically competent \textit{R. phaseoli} strains for legume inoculation trials.
1. LITERATURE REVIEW

A. Effect of abiotic factors on growth and survival of Rhizobium.

1. pH

1.1. Direct effects of pH on growth and survival of Rhizobium.

As a general rule, the optimum pH range for growth and survival of Rhizobium is pH 6-8. There is both intra and inter species variation when growth occurs below pH 6.0 or above pH 8.0.

Vincent (1965) noted that the major limiting effect of excess H-ions is on growth and survival of Rhizobium. The effects of excess H-ions can be demonstrated by determining the critical pH for growth of various Rhizobium species and strains. Bryan (1922) defined critical pH as the reaction at which the bacteria do not show or produce any visible growth during a certain period of time. As early as 1918, Fred and Davenport determined the critical pH of the common rhizobial groups and arranged them in the order R. meliloti > R. leguminosarum > R. trifolii > R. japonicum > R. lupini. Bryan (1923) supported Fred and Davenport's results when he reported that R. meliloti was killed at pH 5.0, R. trifolii at pH 4.5, and R. japonicum at pH 3.5 to 3.9.

Bryan (1922) also observed small differences in critical H-ion concentration of different strains of R. japonicum. Differences in the growth or behavior of Rhizobium under different acidity conditions were attributed by Stevens (1925) to inherent differences in the nature of the organisms. Wright (1925) suggested that the ability to produce
gun appeared to be type characteristic and may be related to the
critical H-ion concentration of the various species. The results of
these earlier researchers have been corroborated by several other
investigators, including Graham and Parker (1964), who surveyed abilities
of 79 rhizobial strains to grow in yeast mannitol liquid media of
different initial pH. Their results established diversity of tolerance
within major groups of rhizobia. They divided rhizobia into fast-growers
and slow-growers and noted that acid tolerance was infrequent in the
former and common in the latter. This characteristic was observed by
Stevens (1925) and Norris (1965).

In culture studies, pH affects not only the growth of rhizobia
but also their morphology. The effect was probably first studied by
Bewley and Hutchinson (1920) who showed that the rod shaped organism
changed into the preswarmer form in calcareous soils; acid soils caused
the production of heavily vacuolated cells and eventually killed the
organism, while a slightly alkaline soil was found to be capable of
supporting vigorous growth without altering the form of the cells.
Cabezas de Herrera (1956) obtained similar results when he reported
that many of the strains he tested at pH 4.0 were modified into
bacteroid, coccolid or L-shaped forms. These abnormal forms were unable
to produce nodulation of lucerne but returned to the normal infective
form when supplied with a nutrient solution at pH 5 to 6.

According to comparative studies done in soil (Bryan, 1922, 1923;
Vincent and Walters, 1954; Mulder et al, 1966; Damirgi et al, 1967;
Uhliar and Mucuhova, 1968; Jensen, 1969; Ham et al, 1971; Rice et al,
1977) the critical H-ion concentration for growth and survival of
rhizobia is approximately the same as in pure culture. Vincent and Crofts quoted by Vincent (1965) reported the limiting role of H-ion concentration on the occurrence of rhizobia in soil. Acid soils adjacent to neutral to alkaline soil did not contain any *R. meliloti*. Heavy inoculation of acid soils with *Rhizobium* may lead to beneficial effects due to the provision of sufficient numbers of survivors to cause nodulation (Spencer, 1950; Mulder and van Veen, 1960; Robson and Loneragan, 1970).

For most useful rhizobial strains (Munns, 1978) pH requirements for growth approximately resemble those for nodulation of the appropriate host. Some studies indicate that nodulation failure under acid conditions is due to poor survival of rhizobia (Albrecht, 1933; Loneragan and Dowling, 1958; Mulder and van Veen, 1960; Spencer, 1950; Lie, 1971). In this respect, the critical pH for nodule formation corresponds with that for rhizobial growth. Some critical values reported for soybean (pH 3.2), clover (pH 4.7-4.8), and *Medicago* (pH 5.8-5.9) correspond with those that limited the growth of the respective bacteria (Bryan, 1922, 1923; Vincent, 1965; Mulder and van Veen, 1960). Canizo et al (1978) studied the infectivity of crushed nodule suspension of *Coriaria myrtifolia* towards its host and the development of nodules formed was followed in culture. Nodule formation occurred most freely over the pH range 6-9. Also, the capacity of the host plant to tolerate relatively low pH levels exceeded that of the nodule organism.

Evidence of direct inhibitory effect of excess H-ions on nodule formation was provided by Munns (1968). His experiments showed that the only acid-sensitive step was the curling of the root hairs. The
acid-sensitive step for *Medicago sativa* occurred at pH 4.4. At this pH, no root hair curling occurred even though rhizosphere populations were present. Raising the pH from 4.4 to 5.4 allowed curling and subsequent steps to proceed. Lie (1969) reported results which clearly showed that the sensitivity of inoculated pea plants to the inhibitory effect of an acid medium on nodulation existed a short period of time following inoculation. The acid-sensitive step was identified during the first four days after inoculation. When the plants during the period were exposed to neutral pH conditions, nodule initiation started and the development of nodules proceeded when the plants were subsequently transferred to acid media of pH 4.5. Failure of nodulation at low pH was probably not due to elimination of rhizobia, but an alteration of the root surfaces as a result of which infections were prevented. The following reasons were advanced for reaching this conclusion: 1) Addition of much larger numbers of nodule bacteria did not improve nodulation; 2) Root exudates from pea roots growing in acid solution supported the growth of nodule bacteria; 3) Acid nutrient solution inoculated with bacteria and in which pea roots failed to form nodules still contained active nodule bacteria capable of inducing nodules; 4) Pea plants exposed to a low pH for 4 days immediately after inoculation before being transferred to a neutral solution did not resume nodulation in the latter even though effective rhizobia were present. Fahraeus and Ljunggren quoted by Munns (1969) suggested that enzymatic breakdown of pectin in the wall of the root hair initiates infection by nodule bacteria. This hypothesis was tested by Munns (1969) in *Medicago sativa*. He reported that acidity in the range of pH 4.5-5.5 which inhibited nodule formation, also inhibited the action of pectinase
on pectin. Acidity inhibited enzyme production negligibly, compared to its effect on enzyme action or nodulation. In similar studies conducted by Dart (1977) the involvement of pectinase enzymes was not confirmed.

pH affects not only the growth and survival of Rhizobium but also its symbiotic effectiveness. Mulder et al (1966) found that in acid soils, red clover plants bore relatively large numbers of small yellow-white nodules which were ineffective as indicated by the color of the leaves. The effectiveness of *R. trifolii* strains was found by Holding and King (1963) to be positively correlated with soil base status. Similar results have been reported by Jones and Burrows (1969), van Schreven (1972), Hagedorn (1978), and Barber (1980). Some rhizobial species e.g., *R. trifolii* (Masterson, 1968; Nutman and Ross, 1969), and the cowpea miscellany (Keyser et al, 1979; Munns et al, 1979) have been shown to combine acid tolerance with symbiotic effectiveness.

Calcium and magnesium, both as liming materials and as nutrient elements, play a significant role in alleviating the harmful effects of pH on the growth, survival and symbiotic activity of Rhizobium. As early as 1924, Swanson studied the calcium content of soil in relation to absolute reaction. There was a close correlation between the calcium content and pH value of soils of similar physical texture collected under similar climatic conditions. Several investigators (Scalan, 1928; Albrecht and Davis, 1929; Albrecht and Poirot, 1930; Albrecht, 1932, 1933; Spencer, 1950; Mulder and van Veen, 1960; Jones et al, 1964; Jones, 1966; Vincent, 1965; Munns, 1965, 1970; Parker et al, 1976; Mengel, 1978; Barber, 1980) have reported a similar
correlation between the calcium content of soil or culture medium and
the growth, survival, and symbiotic activity of Rhizobium, since
calcium raises pH to a favorable range for bacterial growth and
survival. The amount of calcium needed by Rhizobium is low compared
with that of the host. Calcium maintains normal growth and morphology
of Rhizobium, and can be replaced by strontium but not by barium or
magnesium (Vincent and Colburn, 1961). Although magnesium cannot meet
the specific needs of calcium, it has been reported (Norris, 1958b,
1959a; Vincent, 1962b) that it is required in greater concentration
than calcium and its deficiency is more readily demonstrated.

Loneragan, quoted by Vincent (1965) considered it unlikely that
magnesium would itself limit the growth of rhizobia in soils, except
at concentrations well below the plant's needs.

Practical ways of improving Rhizobium survival and growth in acid
soils have been used. They include banding with lime and dolomite
(Albrecht, 1941) and using lime-pelleted seeds, a method first employed
by Australian researchers (Burton, 1976). Another recently tested method
(Research and Development Report, 1980) involves mixing inoculated seed
with fertilizer. Results indicate that mixing seed with fertilizer may
not cause inoculation problems as long as the seed is well coated with
rhizobia (at least $10^6$ per seed); as long as the seed-fertilizer mix is
prepared just prior to application, and as long as the pH of the
fertilizer is above 4.0. It was also reported that lime coated seed
protects the rhizobia longer than the seed-fertilizer mix.
1.2. Indirect effects of pH on growth and survival of *Rhizobium*.

The effects of acidity on growth, survival and symbiotic activity or *Rhizobium* are not simply an effect of H-ion concentration; toxicity resulting from iron, aluminum and manganese, and deficiency of calcium, magnesium or molybdenum are most pronounced at low pH, and one or all of these factors may be the cause of poor survival, growth or symbiotic activity of *Rhizobium* (Dobereiner, 1966; Alexander, 1977; Sanchez, 1976). The effects of aluminum and manganese on *Rhizobium* and the *Rhizobium*-legume symbiosis will be briefly reviewed.

Few studies have been done on the effects of aluminum on rhizobial growth and survival. Munns (1976) suggested that in order to conduct such studies, a combination of low phosphate and adequate pH-buffering at low pH would be required so that an effective concentration of free aluminum can be maintained. He also noted that for acid-tolerant legumes, such studies would require a pH too low to permit nodulation, but in legumes capable of nodulating at low pH, effects of aluminum on rhizobial growth might have important influence. Keyser and Munns (1979a) assessed tolerance of 65 strains of rhizobia, 52 from the cowpea miscellany and 13 from *R. japonicum*, to acidity, high aluminum (50 μM) and low phosphate (5 μM), in defined liquid media. Low pH and low phosphate were tested alone and in combination with high aluminum. Their data showed that all the strains varied in their response. Aluminum was identified as the most potent stress to growth of rhizobia. The cowpea rhizobia tended to have more tolerance to aluminum than *R. japonicum*. In another related study by Keyser and Munns (1979b), in which other
acidity stresses including low calcium (50 μM) and high manganese (200 μM) were studied together with high aluminum, aluminum was again identified as the most potent stress factor. It was established by these studies that acid-tolerance and aluminum tolerance are separate because strains that showed tolerance at low pH were not necessarily tolerant to aluminum. Rerkasen (1977) and de Carvalho (1978), both quoted by Keyser and Munns (1979a) have reported that some slow-growing rhizobia can survive high aluminum concentrations at low pH in both solution media and in soil. Some fast-growing rhizobia might also have useful acid and aluminum tolerance. In acid soils containing moderate amounts of aluminum, studies by Munns (1965a) and Jensen quoted by Keyser and Munns (1979a) suggested that R. trifolii might contain strains tolerant to aluminum. Miller (Interim report, 1979) reported that some fast-growing R. phaseoli strains tested in his laboratory for tolerance to aluminum between 0 and 100 μM differed in their response. The majority of the strains evaluated were rather sensitive to aluminum, although some resistant strains were identified.

Even for tolerant strains, aluminum reduces the growth rate and lengthens the lag phase (Vincent and Walters, 1954; Keyser and Munns, 1979a). Ability of strains to recover after a large initial decline in viability may imply physiological adaptation or selection of genetically tolerant variants (Keyser and Munns, 1979b). However, such effects could be critical for colonization of soil and rhizosphere, and for induction of nodulation (Munns, 1968; Jones, 1963; Vincent, 1974). de Carvalho quoted by Keyser and Munns (1979a) showed that aluminum inhibited nodulation of Stylosanthes spp. Keyser et al (1979c) implied from
their studies in acid aluminum containing soils that both acidity and aluminum were potent inhibitors of nodulation through their effects on rhizobial growth. In an earlier study, Keyser and Munns (1979b) it was showed that calcium had a statistically significant protective effect against aluminum but the effects were small and probably of no biological or practical significance. Rerkasen quoted by Keyser and Munns (1979c) also reported that calcium prevented the decline in viability of a fast-growing *Rhizobium* at pH 4.3, but did not overcome the negative effects of aluminum addition. A slow-grower that was not affected by acidity or aluminum did not respond to calcium.

Some studies have investigated the effects of manganese on growth and survival of *Rhizobium*. Wilson and Reisenauer (1970a, 1970b) reported that Mn-ion, even at a high concentration of 100 μM did not inhibit the growth of *R. meliloti*, cowpea rhizobia, *R. japonicum*, and *R. phaseoli*. Similar results have been reported by other workers (Holding and Lowe, 1971; Masterson, 1968). In some studies (Wilson and Reisenauer, 1970b) attempts to include a level of manganese that was toxic to rhizobia were unsuccessful because of the partial precipitation of the manganese by phosphate. Under these conditions, tolerance of the rhizobia studied was so large that inhibition to growth or survival in acid soils seemed unlikely. However, Dobereiner (1966) reported that fast-growing *R. phaseoli* strains differed in their tolerance to acid soils with manganese toxicity. Miller (Interim report, 1979) also reported that the response of *R. phaseoli* strains to concentrations of manganese between 0 and 320 μg/ml showed that there were differences in sensitivity to manganese. These strains showed sufficient diversity to
manganese tolerance to provide the potential for survival in soils containing high manganese content. Slow-growing rhizobia of the cowpea and *R. japonicum* groups showed little, if any, stress to 200 μM manganese in acid media containing 50 μM calcium (Keyser and Munns, 1979b).

It has been shown (Dobereiner, 1966; Masterson, 1968; Jones, 1963; Holding and Lowe, 1971) that high concentrations of manganese in the culture or soil medium containing rhizobia leads to loss of effectivity in the bacteria. However, the ability to fix nitrogen could be regained by subculturing the bacteria on media containing no manganese.

Calcium moderates the adverse effects of manganese on rhizobial growth and nodulation (Munns, 1977a, 1977b; Keyser and Munns, 1979b). The beneficial effects of liming acid soils containing toxic levels of manganese have also been demonstrated by other researchers (Morris, 1948; Lohnis, 1951, 1960; Holding and King, 1963; Mulder and van Veen, 1960).

1.3. Selection of *Rhizobium* for acid tolerance.

Mulder et al. (1966) suggested that breeding or selection of adapted plant varieties and selection of acid-resistant *Rhizobium* strains will enable the cultivation of nodulated legumes on acid soils. Date (1976) supported the idea by stating that one of the criteria for the selection of *Rhizobium* strains is tolerance to pH.

Selection of rhizobia for acid tolerance is conventionally done in yeast-mannitol-mineral salts broth or agar cultures adjusted to various pH's. Bacteria which demonstrate consistent growth at a low pH are then considered tolerant or resistant at that pH. However, this
method of selecting acid-tolerant rhizobia has been fraught with
difficulties since the report (Graham and Parker, 1964; Norris, 1965)
that slow-growing rhizobia produced alkali in their growth medium. A
change in the pH of the media which are initially acid could indicate
either genuine ability to multiply at low pH or capacity to progressively
increase the pH of the medium to an adequate level for growth. *Rhizobium*
strains which grow as a result of the latter characteristic during the
test are unlikely to be successful in more highly buffered acid soils.
Graham (1964) attempted to overcome this problem by comparing strains
in the early phase of their growth before a change in pH of the medium
could be detected. Date (1979) indicated that this method had the
disadvantages that strain differences have not become fully pronounced
in the early phases of growth, and that the growth must be estimated by
extremely tedious viable count method.

Availability of an acid medium in which the pH does not rise permits
a simple test in which the attainment of visual turbidity indicates
ability to grow at low pH. Keyser and Munns (1979a) used turbidity as
a measure of growth but they used low inoculum levels (10^3 or 10^5/ml)
in liquid glutamate medium maintained at pH 4.6. It was reported that
the population required to significantly raise the pH of the medium was
about 10^7 cells/ml. Although most of the strains evaluated produced an
alkaline reaction, measurements taken throughout the growth period
showed that the rhizobia had to make several-fold growth before changing
the pH by 0.1 unit. The pH rose only when densities greater than or
equal to 10^7/ml were reached. Thus growth reflected real tolerance to
stress because rhizobia did not first raise the pH and then grow.
Date (1979) developed a defined acid medium in which visual turbidity could be estimated as a reliable measure of tolerance to acidity. He demonstrated that *Rhizobium* from acid soil origin (pH 4.5) grew best in medium of pH 4.5 while *Rhizobium* from an alkaline soil (pH 8.5) did not grow at all. Of great significance and interest was his observation that growth of *Rhizobium* of acid soil origin in acidified media incorporating mannitol, the standard carbon source recommended for culturing rhizobia, raised the pH from 4.5 to approximately 7.5. However, in acidified media in which arabinose, or alternatively galactose, was substituted for mannitol, the pH decreased slightly to 3.8, yet growth, as measured by viable counts, was essentially the same as in media incorporating mannitol. In both media, the acid soil origin strain showed exponential growth, increasing from an inoculum of $7 \times 10^3$ to $1 \times 10^9$ viable cells per ml, whereas the number of viable cell per ml of alkaline soil origin strain diminished from $4 \times 10^6$ to $2 \times 10^2$.

Norris' (1965) suggestion that alkali production by slow-growing rhizobia was an ecological attribute has been questioned by the finding (Parker, 1971; Date, 1979) that alkali production by these bacteria is dependent on the organic or carbohydrate component of the culture medium.

Munns (1977) suggested that rhizobia be selected routinely under conditions that reproduce the important stresses the host legume is likely to encounter, so as to reduce the number of candidate strains quickly and avoid wasteful inclusion of edaphically incompetent strains in field trials. Stress factors including low phosphate, low calcium, high manganese and high aluminum have been tested alone or in combination with each other in acid media (Dart, 1979; Keyser and Munns, 1979a, 1979b;
Keyser et al, 1979c). Identification of strains of *Rhizobium* having superior tolerance to mineral stresses in addition to low pH may be a step towards improving chances of selecting successful inoculants for acid infertile soils.

2. Moisture

2.1. Effects of excess moisture on survival and symbiotic nitrogen fixation.

The diversity among *Rhizobium* spp in ability to survive at different soil moisture levels has been documented. Vandecaveye (1927) reported that populations of *R. leguminosarum* in pots of sterile soil were greatly reduced after two week's flooding. The viability of streptomycin resistant strains of *R. trifolii* and *R. meliloti* (Danso and Alexander, 1974) was not markedly reduced when these bacteria were introduced into moist soils. Parker et al (1977) commented on the work of Schroder and Comensero who attributed the reduction of nodule numbers on *Centrosema* in a previously flooded soil to the poor survival of the rhizobia. It was noted that the inoculant strains were more sensitive to excess water than the native strains. On the other hand, waterlogging has had little or no effect on survival of *R. trifolii* in waterlogged soils in Western Australia (Parker et al, 1977). Osa-Afiana and Alexander (1979) studied the survival of two streptomycin-resistant strains of *R. trifolii* and *R. japonicum* in sterile and non-sterile soil adjusted to 10, 22, 35 and 45% moisture. Survival of *R. trifolii* was not significantly different in sterile and non-sterile soil but *R. japonicum* maintained greater numbers in the non-sterile soil at 10%
moisture. However, survival of both species was greater in the sterile than in the non-sterile conditions. Flooding studies conducted in soil with and without sucrose indicated a greater suppression of growth of both species of *Rhizobium* in the sucrose-amended soil. These data suggested a role for protozoa at high moisture levels and of organic acids and low pH in the sugar-amended soils in the decline of the bacterial population. Similar results were obtained by Kremer and Peterson (1980) who related the quality of inoculant carriers of two strains of peanut rhizobia to survival at 24, 12, and 1.5% soil moisture. The data indicated that an improved inoculant carrier (an oil-base carrier) enhanced the survival of rhizobia when subjected to moisture stress. Boonkerd and Weaver (1980) reported that two *Rhizobium* spp survived well under air-dry, moist and saturated soils stored at 25 and 35°C. However, the 35°C temperature in conjunction with air-dry or saturated soil was the most detrimental to survival. The population of rhizobia decreased from $10^4$ to $10^3$ cells/g soil during 45 days of incubation.

Fred et al (1932) reviewed the work of several researchers and concluded that a relatively high water content of the soil but not waterlogging was desirable for maximum nodulation and nitrogen fixation. The optimum amount of soil moisture required for nodulation and nitrogen fixation differs for the various *Rhizobium*-legume symbiosis. The optimum for the symbiosis in lucerne and soybean (Fred et al, 1932; Tu and Hietkamp, 1977), and acacia (Habish, 1970) was found to be 60-80% of water holding capacity. In general, the number of nodules formed and the longevity of nodules are adversely affected by soil moisture
deviating from the optimum (Fred et al., 1932; Wilson, 1931). Recent studies indicate that nitrogen-fixing activity of the nodules is affected by water condition around the nodules. A thin layer of water around the nodules (Schwinghamer et al., 1970; Hume et al., 1976; Varma and Subba Rao, 1975) suppressed nitrogen fixation due to reduced oxygen supply of the nodules. Sprent and Gallacher (1976) provided evidence that excess water conditions also lead to stimulation of fermentative pathways of carbohydrate metabolism, thereby leading to production of inhibitory concentrations of ethanol. Both bacteroids and plant tissues were found to produce ethanol when rendered anoxic. Van Straten and Schmidt (1975) reported that a layer of water had no effect on soybean nodules so long as the nodule surface was undamaged. However, flooded or waterlogged soil conditions have consistently (Minchin and Pate, 1975; Minchin and Summerfield, 1976; Parker et al., 1977; Huang et al., 1975; Hong et al., 1977; Sprent, 1971b) caused detrimental effects on nodulation and symbiotic nitrogen fixation. Cowpea (*Vigna unguiculata* (L) walp) nodules formed under waterlogged conditions had increased nodule cortication and conspicuous lenticels (Minchin and Summerfield, 1976). Both conditions were regarded as adaptive anatomical responses which facilitated continued symbiotic nitrogen fixation. Hong et al. (1977) observed that screening for symbionts producing "lenticel-type" nodules might reduce the effect of waterlogging.

Few studies have been done on the effect of preplant flooding on nodulation and nitrogen fixation. Wu et al. (1968) reported that previously submerged paddy rice soils reduced nodulation of soybeans only 15% as compared to plants developing in soil not previously submerged. DePolli (1973) flooded soils for 0, 25, 50 and 100 days
before sowing Centrosema. Nodule numbers were reduced by previous flooding, indicating an adverse effect on survival of Rhizobium. Flooding durations of 0, 1, and 3 months were used by Carroll and Dunigan (1977) to study the effect of preplant flooding on survival of R. japonicum and yields. It was found that nodule numbers and nodule weight were affected significantly by duration of flooding. There were also significant differences in soybean yields as a result of duration of flooding.

2.2. Effects of desiccation on survival and nitrogen fixation.

Since Rhizobium does not produce endospores (Graham et al, 1963), desiccation is one of the most important abiotic factors affecting its survival in soil. Desiccation causes death of rhizobia in soils. Vincent et al (1962) found that die-back of R. trifolii associated with desiccation occurred in two phases. First, a death phase over the first few hours when the main loss of water occurred. Second, a slow gradual decline in numbers over a more prolonged time period. Danso and Alexander (1974) found that the maximum decrease of R. meliloti in two dry soils occurred during the first week, with the population of R. trifolii remaining constant thereafter until the completion of the study at 8 weeks. Almost no R. meliloti could be detected after four weeks in dry sterile sand. Pena-Cabriales and Alexander (1979) showed that the decline in numbers of the Rhizobium spp they studied was biphasic. The initial rapid phase coincided with the time of major water loss, and the subsequent slow rate of decline of viability occurred as the soil lost little water. The finding by Bushby (1964) and Bushby and Marshall (1977a, b, c) that fast-growing rhizobia are more susceptible
to desiccation than slow-growing rhizobia was not confirmed. Other workers (Sen and Sen, 1956; Foulds, 1971; Chatel and Parker, 1973b) have shown differences among fast-growing and slow-growing species of *Rhizobium* in regard to their susceptibility to desiccation. The fast-growing species have, in general, shown greater susceptibility to desiccation than the slow-growing forms.

Bushby and Marshall (1977a) have investigated the reasons for greater desiccation susceptibility of the fast-growing rhizobia compared to the slow-growers. This phenomenon was related to larger amounts of water retained by the desiccation-sensitive fast-growing strains at any relative vapour pressure. The greater moisture retaining ability of the fast-growing strains correlated with higher surface energies and greater availability of adsorptive surface area. Van Rensburg and Strijdom (1980) reported that under conditions of mild desiccation, fast-growing rhizobia survive better than slow-growing ones. It was purported that the low internal water-retaining ability of the slow-growing rhizobia at any relative vapour pressure is disadvantageous under mild desiccation conditions because insufficient moisture is available to allow the functioning of vital enzymes. A decline in the respiratory activity of bacteria at low water potentials (Wilson and Griffin, 1975) is a factor in the decline of rhizobial populations in soil. Mahler and Wollum (1980) showed that soil-water potential between 5 and 15 bars had a detrimental effect on the survival of *Rhizobium japonicum*. Krautmann and Weaver (1979) reported the detrimental effects of high temperature and low relative humidity on survival of *Rhizobium trifolii*. Bushby and Marshall (1977c) suggested that death of root-nodule bacteria on desiccation and rehydration was associated with changes in membrane permeability. The fluorescent
probe l-anilino-8-naphthalene sulphonate (ANS), which reacted with materials leaking from the cell walls of the bacteria, was used to establish differences between fast and slow-growing rhizobia.

The soil colloids (montmorillonite, illite and kaolinite) have been reported to afford protection to *Rhizobium* against the detrimental effects of desiccation (Marshall, 1964; Danso and Alexander, 1974; Danso, 1975; Bushby and Marshall, 1977b). The fast-growing rhizobia are better protected than the slow-growers. Other compounds like maltose, sucrose, polyvinylpyrrolidone and polyethylene glycol 600, also offer varying degrees of protection (Vincent et al, 1962; Bushby and Marshall, 1977b). The ability of sugars to provide protection against desiccation depends on the degree to which they penetrate the cell walls of bacteria under test (Zimmerman, 1962; Cox, 1967; Hambleton, 1970). The capacity to develop at low water activities or high internal osmotic tension (Chen and Alexander, 1973), and the production of extracellular polysaccharides (Kilbertus et al, 1979; Pena-Cabriales and Alexander, 1979) have been related to ability to resist desiccation, but neither of these phenomena could be confirmed by Bushby and Marshall (1979b).

Desiccation or droughty soil conditions have been shown to have deleterious effects on nodulation and the legume-*Rhizobium* symbiosis. Wilson (1931) reported that shedding of nodules occurred when droughty conditions followed nodulation. Hamdi (1971) showed that the movement of *Rhizobium* through soil was slowed down with increasing water tension and ceased when water-filled pores became discontinuous. Under such conditions, restricted migration of rhizobia could result in nodulation.
failures, even at water tensions permitting germination of legume seeds. Mohan Rao et al (1977) showed that nodules were reduced in number and size, with an increase in the number of ineffective nodules, under water stress. Reduction in soil moisture from 0.36 to 3.6 bars (Worrall and Roughley, 1976) decreased the number of infection threads and completely inhibited nodulation of *Trifolium subterraneum*, although the number of rhizobia was not affected. Several investigators (Sprent, 1969, 1971a, 1971b, 1972a, 1972b; Engin and Sprent, 1973; Mederski and Jeffers, 1973; Doss, 1974; Pankhurst and Sprent, 1975; Momen et al, 1979; Mahler et al, 1979; Peterson et al, 1980; Haque et al, 1980a, 1980b) have reported significant yield reductions in cowpeas and soybeans due to reduction in soil moisture content. These reductions have been attributed to serious decline in the respiratory and nitrogen fixing activity of the nodules. Sprent (1971a; 1972a) found that desiccation of nodules resulted in the destruction of the plasmodesmata connecting the plant cells in the nodules, and shrinkage and breakdown of the cytoplasm from the cortical cells surrounding the bacteroid tissue. Damage to the cortical cells or plasmodesmata results in reduced protection of the bacteroids against oxygen and a limited supply of substrates from the nitrogen fixing tissue (Sprent, 1972a; Boyer and McPherson, 1975). Recovery of stressed plants is possible if water is supplied before critical destruction is done to plant and nodule cells.
3. Effects of salts on growth, survival and dinitrogen fixing activity of *Rhizobium*.

Little research information exists on the salt tolerance of rhizobia. This is probably because much of the research on legumes grown in saline soils has focused on the macro rather than on the microsymbiont.

According to Graham and Parker (1964), 2% sodium chloride is lethal for most *Rhizobium* spp. However, some workers (Pillai and Sen, 1966; Yadav and Vyas, 1970, 1971a, 1973; Subba Rao et al, 1972; Okafor and Alexander, 1975) have demonstrated species and strain differences in salt tolerance. These, and other researchers (Steinborn and Roughley, 1975; Moffett and Colwell, 1968) showed that salt concentrations as low as 0.2% inhibited the growth of *Rhizobium*. Yadav and Vyas (1973) reported that the salt effect appeared to be ion specific, with chlorides being more toxic than sulphates of sodium, potassium, and magnesium. Similar observations were made by Steinborn and Roughley (1975) when they reported that the toxicity of sodium chloride was due to chloride, and that calcium chloride was more toxic than sodium chloride in both broth and peat culture studies of *R. trifolii* and *R. meliloti*. Pillai and Sen (1969) studied the effect of salinity on the formation of polysaccharide gum by some strains of *Rhizobium*. The results showed that gum formation by the organisms increased with increasing salinity and that the strains varied in their capacity to form gum in the presence of equal amounts of salt.
The effects of salinity on the population of rhizobia in soil have not been fully investigated. Steinborn and Roughley (1974) reported that peat containing > 0.2 sodium chloride was likely to cause considerable loss of viability of Rhizobium. In pot experiments using a saline alkali soil, it was shown that pelleting of seeds with either lime or gypsum gave some protection against salinity (Chhonkar et al., 1971).

Subba Rao et al (1972) indicated that salinity tolerances for the host plant, for nodulation, and for the symbiosis were different. Lucerne seeds did not germinate at 1.5% sodium chloride, initial nodulation was delayed at 0.4%, and at 0.7% the plants failed to nodulate. Bernstein and Ogata (1966) reported results which showed that nodulation of alfalfa was relatively resistant to salinity, and the relative yields of plus and minus nitrate cultures were similarly affected by salinity. By contrast, nodulation of soybeans was strongly affected by salinity, and the relative yields declined more sharply when plants were dependent on dinitrogen fixation than when nitrate was supplied. Glycine wightii behaves similarly, and there is evidence of the adaptability of the Rhizobium-legume symbiosis to increased substrate salinity (Wilson, 1970).

B. Effect of biotic factors on growth of Rhizobium.
1. Effect of root exudates on Rhizobium.

Several investigators (Rovira, 1956a, 1956b, 1959, 1965, 1969; Hale et al, 1971; Peters and Alexander, 1966; Vincent, 1974; Dart, 1974; Tuzimura and Wantanabe, 1962a; Vidor, 1977) have studied the effect of
root exudates on *Rhizobium*. In general, the presence of root exudates results in a positive rhizosphere effect under natural soil conditions or increase in turbidity in pure culture studies. Vidor (1977) reported only small positive differences in cell turbidity were observed when *R. japonicum* strains grew in the presence of soybean root exudate. He also noted that there was no relationship between cell turbidity and total number of cells estimated by plate counting.

Some investigators (Nutman, 1962, 1965; Brown et al, 1968; Egeraat, 1972) have discussed the concept of specific stimulation. This concept describes the situation whereby the appropriate *Rhizobium* symbiont is selectively favored by its host root and responds with overwhelming growth in the host rhizosphere as a preliminary to successful nodulation. Evidence, however, exists that legumes other than the host legume enhance the growth of heterologous *Rhizobium* spp (Tuzimura and Wantanabe, 1962b; Peters and Alexander, 1966; Reyes and Schmidt, 1980). Similar evidence for grass species has been reported (Robinson, 1967; Rovira, 1961; Tuzimura and Wantanabe, 1962b, Reyes and Schmidt, 1980).

The chemical nature of root exudates has been extensively reviewed by Rovira (1969). The principal compounds isolated from root exudates are sugars (glucose and fructose being the most abundant), amino acids, vitamins, organic acids, and growth hormones (Rovira, 1969). These compounds are stimulatory to microorganisms but others including hydrocyanic acids, glycosides, and saponins have been found to be toxic (Egeraat, 1972). The amino acid homoserine is stimulatory to *R. leguminosarum* but inhibitory to *R. phaseoli* and *R. trifolii* (Egeraat, 1972). Elkan (1961) has also reported that a non-nodulating soybean
line released compounds which altered the morphology of *R. japonicum* and prevented nodulation of the host legume.

Rovira (1969) listed nine factors which affect root exudation, two of them being plant species and microorganisms. The composition of root exudates varies both within and between plant species, as well as between seed and seedlings of the same species (Vancura, 1964; Vancura and Hanzlikova, 1972). Microorganisms may affect exudation in several ways, the main ones being: a) an effect upon the permeability of the root cells, b) an effect upon the metabolism of roots, and c) absorption of certain compounds in root exudates by microorganisms and excretion of others (Rovira, 1969). Rao (1976) showed that upon inoculation of *Medicago sativa* with *R. meliloti*, there was an increase in the exudation of non-reducing sugars, orthodihydroxy phenols, amino nitrogen, polygalactouronase, and pectin methylesterase, and a decrease in reducing sugars and total phenols. The root exudate of plants inoculated with homologous rhizobia differed quantitatively from those of plants inoculated with heterologous rhizobia and non-inoculated plants.

The root zone immediately behind the tip has been considered to be the major source of root exudates (Pearson and Parkinson, 1961). Exudation of ninhydrin positive compounds from bean roots was confined to the zone behind the root tip. Carbon-14 studies (Rovira, 1973; McDougall, 1968; McDougall and Rovira, 1965, 1970) have shown that the tips of the main and lateral roots of wheat plants are the sites of ninhydrin positive exudates. Egeraat (1975a,b) obtained similar results for pea seedlings.
The mechanisms responsible for the release of root exudates are still not thoroughly investigated. Rovira (1969) suggested that exudation could be controlled by metabolic processes or that it is simply a leakage of compounds, i.e., a function over which the plant has no control.


Antibiotics are organic substances of microbial origin which are either toxic or growth-inhibitory to other microorganisms. These substances have selective toxicity towards their target, and their modes of action include inhibition of cell wall and protein synthesis, and disruption of membrane functions (Stanier et al, 1976).

The selective action of antibiotics has been used in ecological studies to isolate *Rhizobium* from contaminated sources. Graham (1969) devised a selective medium from which he isolated *Rhizobium* from other contaminants. This medium contained actidione, sodium benzyl penicillin, chloramphenicol, neomycin, sulphafurazole and pentachloronitrobenzene (PCNB). Pattison and Skinner (1974) also formulated a medium which contained sodium azide, brilliant green, and PCNB. This medium facilitated the isolation of *Rhizobium* from heavily contaminated sources, but was very inhibitory to several *Rhizobium* spp when supplemented with 1 i.u./ml of penicillin. However, of the six antibiotics (chloramphenicol, erythromycin, penicillin, streptomycin, sulphafurazole and tetracycline) tested against 47 strains of *Rhizobium*, penicillin was the least inhibitory. Recently, tetracycline has been used as a selective agent (Olivares et al, 1980) in infectivity studies involving *R. meliloti*. The antibiotic was put into the root medium of the inoculated plants and a coefficient
of infectivity was calculated according to the number of nodules formed with and without the addition of the antibiotic. It was claimed that this method was simpler and easier to use than the test of competence between strains.

*Rhizobium* strains vary in their susceptibility to antibiotics. Landerkin and Lochhead (1948) noted that strains of *Rhizobium japonicum* were less susceptible to actinomycete antibiotics than other soil organisms tested. Graham (1963) commented on the work of Fogle and Allen where lupin-rhizobia and *R. japonicum* were found to be more susceptible to streptomycete antibiotics than other species of rhizobia. Slow-growing rhizobia have been found to be more susceptible to antibiotics than fast-growing rhizobia (Graham, 1963).

Bacterial resistance to antibiotics is acquired either by mutation of a chromosomal gene or by a plasmid belonging to the class of resistant factors (Stanier et al, 1976). These plasmids often confer simultaneous resistance to several antibiotics, as they carry genes which encode enzymes that catalyze chemical modifications of antibiotics, converting them to derivatives without antibiotic action.

resistant strains of *Rhizobium* were obtained with little difficulty and the antibiotic of choice to date has been streptomycin.

There are indications in the literature that antibiotic resistance is associated with loss of the dinitrogen fixing ability of *Rhizobium*. Schwinghamer (1964, 1967) reported loss of effectiveness in fast-growing strains selected for resistance to viomycin and neomycin. In contrast, loss of effectiveness occurred infrequently in clones resistant to kanamycin or polymyxin, and was not observed in streptomycin-resistant mutants. Ability to form nodules on the homologous host was retained in all mutant strains, although in other cases some streptomycin-resistant clones of *R. trifolii* (Zelazna-Kowalska, 1971) and *R. phaseoli* (Pena-Cabriales and Alexander, 1979) had become non-infective. Retention of effectiveness has been reported in some slow-growing rhizobia including the cowpea group (Konde, 1975), *R. japonicum* (Levin and Montgomery, 1974), and lotus *Rhizobium* (Pankhurst, 1977).

Two-step mutation to high level resistance to viomycin in *R. meliloti* could involve selective reduction in the permeability of cells towards viomycin (Hendry and Jordan, 1969; Schwinghamer, 1967) due to accumulation in the cell envelope of phospholipids able to complex with this antibiotic. No significant physiological difference was found to account for ineffective kanamycin-resistant mutants of *R. trifolii* and *R. meliloti* (Damery and Alexander, 1969). Naturally occurring resistant strains of *Rhizobium* are not necessarily ineffective.
3. Parasitism, predation and antagonism.

Parasitism, predation and antagonism are important microbial interactions which affect the survival of Rhizobium in natural soils. Stolp and Starr (1963) isolated *Bdellovibrio bacteriovorus*, a predatory and ectoparasitic microorganism with lytic activity, which has been implicated in parasitizing Rhizobium (Parker and Grove, 1970; Keya and Alexander, 1975). However, large numbers of Rhizobium survive in the presence of the parasite. This has been attributed in part to the high density of rhizobia needed to initiate feeding and replication by the parasite.

Keya and Alexander (1975) studied factors which affect growth of *Bdellovibrio* on Rhizobium. Their results showed that decline in the population density of Rhizobium exposed to *Bdellovibrio* was markedly reduced in the presence of clay minerals and colloidal organic matter. In a two-membered system containing clay minerals, *Bdellovibrio* and not Rhizobium was retained by the clay minerals. It was suggested that retention of the parasite might preclude or retard its attachment to or penetration of the rhizobia. On the other hand, a clay layer surrounding the rhizobia might protect them from invasion. Roper and Marshall (1978) have reported similar results for *E. coli* in aquatic systems.

Stolp and Starr (1963) reported that the physiological state of the bacteria was a factor in their susceptibility to *Bdellovibrio*. They found that cells derived from young cultures were often more susceptible to lysis by the parasite than old cells, a fact confirmed by Keya and Alexander (1975).
Bacteriophages have been shown to have some effect on the survival of *Rhizobium* in soil. However, Marshall and Vincent (1954), Bruch and Allen (1957), and Parker and Allen (1957) have shown that bacteriophages have limited host ranges. Hitchner (1930) and Marshall (1956) reported that some strains of *Rhizobium* become lysogenic when grown together with parasitic phages. Vidor and Miller (1980) have also reported the existence of lysogenic forms of indigenous *R. japonicum* strain 123. Because of these factors, i.e., limited-host range and lysogeny in the host bacterium, bacteriophage is not considered to have a significant effect on the survival of *Rhizobium* under natural conditions.

Golebiowska et al (1971) reported the occurrence of strain specificity in phage-*Rhizobium* association, as did Vidor and Miller (1980) who provided evidence that a bacteriophage specific for *R. japonicum* strain 123 might be responsible for the decline in population of this strain.

Antagonism by soil actinomycetes, bacteria and fungi also affect *Rhizobium* survival. Damirgi and Johnson (1966) showed that some actinomycete isolates were antagonistic to *R. japonicum*. Patel (1974) demonstrated some antagonistic effects of actinomycetes on *R. trifolii* and considered microbial antagonism as the reason for the unsuccessful establishment of clover pastures in New Zealand. Smith and Miller (1974) showed that eight out of nine rhizosphere bacteria isolate inhibited *R. japonicum* on agar cultures. They found that nodulation of soybean in vermiculite or in soil was not affected, but one isolate caused severe tap root damage. In the rhizoplane and rhizosphere from a *Cajanus cajan* field, there was a close correlation between fungistasis
and microbial populations (Mishra and Pandey, 1977).

Chatel and Parker (1972) showed that water extracts from soils in which clovers nodulated poorly were inhibitory to *R. trifolii*. Prushothaman and Balaraman (1973) provided evidence of the inhibitory effects of soil phenolics on the growth of *Rhizobium spp.* p-Amino benzoic acid and hydroquinone were the most inhibitory.

Protozoa play an important role in the soil ecological niche because they feed selectively on bacteria (Singh, 1946). Predatory protozoa have been shown to be responsible, either wholly or partially, for the decline in *Rhizobium* population in non-sterile soil (Danso et al, 1975; Osa-Afiana and Alexander, 1979; Sardesh Pande et al, 1977; Nikolyuk, 1980; Ramirez and Alexander, 1980). These studies indicate, in general, that the *Rhizobium* population is not completely decimated by the predator. A relatively high density of rhizobia persists in the soil despite the enormous numbers of rhizobia needed by the predator. The reason that has been advanced for this is that the energy gained by the predator in feeding at low population densities of the rhizobia is less than the energy expended in searching for the host. Another reason that has been purported is that the host multiplies faster than it is consumed. Nikolyuk (1980) interestingly pointed out that the physiological activity of the bacteria increased during the feeding of the predator. This was due to the fact that the protozoa excreted indole compounds and gibberellin-like substances.
2. MATERIALS AND METHODS

A. Strain evaluation for dinitrogen fixation.

Nineteen *Rhizobium phaseoli* strains were evaluated in the growth chamber for dinitrogen fixing efficiency. These strains, along with their source and origin, are given in Appendix A. The experiment was conducted in Leonard jars with sand or exfoliated vermiculite as the growth medium. The Leonard jar assemblies containing approximately 250 ml nutrient solution (Appendix B) were sterilized for 3 hours at 121°C and 1 atm pressure.

*Phaseolus vulgaris* seeds, cultivar Turrialba 4 (a black bean), were sterilized by soaking in ethanol for 5 minutes, followed by 6 to 7 consecutive washings in sterile water. Four seeds were planted per jar, and thinned to two plants after germination.

The *R. phaseoli* cultures were grown on yeast-mannitol slants, washed off with 0.85% saline when 3-4 days old, and dispersed with a vortex mixer. Turbidity was adjusted using Klett-Summerson photoelectric colorimeter (Model 800-3, Klett MFO Co. Inc., NY) to give approximately $1 \times 10^8$ cells/ml. One ml of each cell suspension was used to inoculate the seeds.

The plants were exposed to a 14-hour day-length, day/night temperatures of 30/26°C, 70-80% relative humidity, and light intensity of approximately 3900-7990 foot candles. The experimental design was a randomized complete
block (RCB) with 3 replications. The plants were harvested after 4 weeks, and the dinitrogen fixing ability of each strain was estimated by the amount of dry matter produced.

B. Biochemical and physiological characterization of strains.

A number of studies were conducted in liquid culture on *R. phaseoli* strains selected on the basis of their ability to fix dinitrogen. The media used in these studies was soil extract-yeast extract-mannitol (SYM) (ATCC medium 111, ATCC catalogue of strains, 13th edition, 1978) except where noted. The composition of this medium is given in Appendix C.

1. Biochemical tests.

The composition of the biochemical media used is given in Appendix D. The tests performed include the following:

a) Nitrate reductase.

Tubes containing the medium were inoculated with each organism and incubated at 30°C. An uninoculated control was included. Tests for the presence or absence of NO\textsubscript{3} were done after 4 days, using Greiss' A and B solutions. After adding a few drops of the solutions to the broth, the presence of a red color indicated the reduction of NO\textsubscript{3} to NO\textsubscript{2}. If no red color developed, a few grains of zinc dust were added and the appearance of a red color indicated that NO\textsubscript{3} was not reduced by the bacteria. If no color developed after the addition of zinc, NO\textsubscript{3} was reduced to ammonia or nitrogen gas.
b) Hydrolysis of gelatin.

Frazier's gelatin agar plates were prepared and the organisms were spotted on the surface. Incubation of the plates at 30°C lasted for 4 days, after which they were flooded with mercuric chloride solution (15 grams HgCl\(_2\) and 20 ml concentrated HCl in 100 ml of water).

If a clear zone developed in the medium surrounding the area of growth, the organism had produced an exoenzyme which had broken down the gelatin. If the media turned cloudy around the growth, gelatin was not broken down but was denatured (precipitated) in the HgCl\(_2\) solution.

c) Hydrolysis of citrate.

Simmon's citrate slants were prepared and inoculated with *Rhizobium* spp. The slants were streaked and the butt stabbed; an uninoculated control was retained. The tubes were incubated at 30°C for 3 to 4 days. The slants were observed for growth which indicates a positive result. Growth indicates that the organism is able to use citrate as a sole carbon source and usually accompanied by the medium turning to a deep prussian blue color.

d) Hydrolysis of starch.

Starch agar plates were prepared and spot inoculated on the surface with 3 to 4 organisms. The plates were incubated at 30°C for 3 to 4 days, after which they were flooded with Gram's iodine solution.

The presence of a clear zone surrounding the area of growth indicates the production by the organism of an exoenzyme which has broken down the starch. If the area around the growth turns blue-violet, the organism did not hydrolyze the starch.
e) The action of *Rhizobium* upon milk.

Dehydrated litmus milk (Difco) was rehydrated by dissolving 105 grams in 1 liter distilled water. Ten ml aliquots were dispensed into 18 x 150 mm test tubes, covered with plastic caps, and sterilized in the autoclave for 15 minutes at 121°C and 1 atm.

The tubes were inoculated separately with each organism, retaining an uninoculated control. They were incubated at 30°C and observations were made on each tube for color, coagulation, peptonization and reduction of litmus over a period of 2 months.

f) Catalase and oxidase.

A loopful of each organism to be tested was placed on a glass slide and a few drops of H$_2$O$_2$ added using a Pasteur pipette. The presence of catalase is indicated by the appearance of bubbles of oxygen. If no bubbles appear, then the organism is catalase negative.

After performing the catalase test, a few drops of oxidase reagent were added directly to the growth on the slant. If oxidase is present, a positive result is indicated by a color change to pink, red, magenta, and black successively. No color change gives a negative oxidase reaction.

2. Antibiotic resistance/sensitivity testing.

Resistance or sensitivity of ten *R. phaseoli* strains was determined using Bacto sensitivity discs on spread plates. The following antibiotics were used: Kanamycin, Viomycin, Penicillin, Novobiocin, Streptomycin, Doxycycline, Chloromycetin, Sulphathiazole, Neomycin, Polymyxin B, and Bacitracin.
The plates were incubated at 30°C and checked after 2 to 3 days. Zones of inhibition, where found, were noted and measured.

3. Carbohydrate utilization.

A wide range of carbon compounds were tested as sources of carbon for a number of *R. phaseoli* strains. The compounds included carbohydrates and organic acids as follows:

<table>
<thead>
<tr>
<th>Carbohydrates</th>
<th>Organic Acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabinose</td>
<td>Fumaric acid</td>
</tr>
<tr>
<td>Maltose</td>
<td>Gluconic acid</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>Glutaric acid</td>
</tr>
<tr>
<td>Mannitol</td>
<td>Malic acid</td>
</tr>
<tr>
<td>Dextrin</td>
<td>Pyruvic acid</td>
</tr>
<tr>
<td>Mannose</td>
<td></td>
</tr>
<tr>
<td>Galactose</td>
<td></td>
</tr>
<tr>
<td>Raffinose</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td></td>
</tr>
<tr>
<td>Rhamnose</td>
<td></td>
</tr>
<tr>
<td>Inulin</td>
<td></td>
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<tr>
<td>Sucrose</td>
<td></td>
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<tr>
<td>Lactose</td>
<td></td>
</tr>
<tr>
<td>Xylose</td>
<td></td>
</tr>
<tr>
<td>Levulose</td>
<td></td>
</tr>
</tbody>
</table>

Media containing phenol red and the appropriate carbon source were dispensed in 10 ml aliquots into 18 x 150 mm test tubes containing inverted Durham tubes. The tubes were stoppered with plastic caps and autoclaved at 121°C and 1 atm for 20 minutes. They were inoculated and incubated at 30°C for 3 to 4 days after which carbohydrate utilization was visually determined by growth and color change from red to yellow. The color change indicates acid production in the growth medium.
4. Response of *Rhizobium phaseoli* to root exudates.

Studies were undertaken to determine the response of *R. phaseoli* to root exudates of its homologous legume, black bean (*Phaseolus vulgaris*) var Turrialba, a heterologous legume, soybean (*Glycine max*), and a heterologous non-legume, corn (*Zea mays*).

a) Preparation of root exudates.

Seeds of the various plant species were sterilized by soaking in alcohol for 5 minutes and subsequently washing in 6 to 7 changes of sterile water. The seeds were spread on a wire mesh which was placed in a tray containing approximately 1 liter of sterile water, with the water about 3 cm from the top of the wire mesh. All steps were done under aseptic conditions.

After germination, the roots of the plants were allowed to grow in the water for 2 weeks during which time root exudates were released into the water. The root exudates were concentrated by flash evaporation, filter-sterilized, and stored at 4°C.

b) Organic matter content of root exudates.

The organic matter content of the root exudates was determined by the potassium bichromate-sulfuric acid method. This reagent (0.15 N) was prepared by dissolving 1.27 g of potassium bichromate in 200 ml of 96% (no dilution because $\text{H}_2\text{SO}_4$ has an acidimetric assay of 96%) $\text{H}_2\text{SO}_4$.

Two ml aliquots of root exudate were pipetted into 18 x 150 mm test tubes, and 4 ml of potassium bichromate-sulfuric acid solution added. The tubes were shaken and allowed to stand for 30 minutes. The samples were read at 645 nm against a blank of 2.0 ml of distilled water and 4 ml of bichromate solution. The amount of organic matter was determined
by reference to a sucrose standard curve. The sucrose equivalent organic matter content of black bean, soybean, and corn was 0.17, 0.11, and 0.96 mg/ml, respectively.

c) Preparation of media containing root exudates.

Twenty-five ml of SYM medium was dispensed into side-arm flasks which were plugged with cotton wool and sterilized at 121°C and 1 atm for 20 minutes. The flasks were inoculated with 1 ml of young cultures of R. phaseoli strains (-10⁶ to 10⁷/ml) and, using the sucrose content of black bean as standard, aliquots of each root exudate were added to give 0.11 mg sucrose per flask. Controls without root exudate were included.

The flasks were shaken on a mechanical shaker at 200 rpm, and growth curves were determined over a period of 96 hours.

5. Effect of salts on the growth of R. phaseoli.

Five R. phaseoli strains, P-442, S-442, QA 1062, CIAT 255, and C-05 were used in this study. The chlorides and sulfates of sodium, calcium, potassium and magnesium were used and salt treatments included 0, 0.1, 0.3, 0.5, and 1.0%.

Twenty-five ml of SYM broth containing the various levels of salt were transferred quantitatively to side-arm flasks and sterilized at 121°C and 1 atm for 20 minutes. The flasks were inoculated with about 10⁶ to 10⁷ cells/ml and shaken mechanically at about 200 rpm. Growth as measured by cell turbidity was determined over a 96 hour period.
6. pH studies.
   a) Selection of strains for acid tolerance on acid agar.

   Ten *R. phaseoli* strains were streaked on SYM agar plated adjusted
to pH 4.0, 4.5, and 5.0. Colonies which developed were transferred
several times until they were established. Strains which demonstrated
ability to grow at low pH were considered as acid-tolerant selections
and were prefixed S, while the parent strains were prefixed P, for
identification purposes.

   b) Stability testing in acid broth.

   Parent and acid-tolerant strains were grown in soil extract,
   yeast extract, galactose (SYG) broth adjusted to pH 4.0, 4.5, and 5.0,
in order to test their ability to demonstrate consistent growth at low
   pH. Galactose was substituted for mannitol because of the finding by
   Date (1979) that media containing galactose as the carbon source had
   little or no change in pH.

   Side-arm flasks containing 25 ml of the adjusted broth media were
sterilized and inoculated with low populations (~10^5/ml) of the test
organisms in order to further minimize changes in the pH of the medium
due to drastic increase in rhizobial population (Keyser and Munns, 1979a).
Media adjusted to pH 7.2 were included as controls.

   The flasks were shaken mechanically at 200 rpm and turbidity
measurements made over a 96 hour period. The pH of medium was measured
at the end of the growth period.

   c) Determination of the pH range for growth.

   Soil extract-yeast extract-galactose medium was used to determine
the pH range for growth of 10 strains of *R. phaseoli*. The medium was
adjusted to pHs of 4.0, 4.5, 5.0, and 7.2.
Twenty-five ml of the medium was dispensed into side-arm flasks and sterilized at 121°C and 1 atm for 20 minutes. The flasks were inoculated with approximately $10^5$ cells/ml and shaken mechanically for 96 hours during which time growth was measured turbidometrically. The final pH of the medium was measured at the end of the growth period.

d) Effect of aluminum and manganese on growth of *R. phaseoli*.

The acid-tolerant strain S-442 was evaluated for its ability to grow in SYG broth (pH 4.0) containing various concentrations of aluminum and manganese. The aluminum concentrations ranged from 0 to 50 μM as \( \text{AlCl}_3 \cdot 6\text{H}_2\text{O} \) and Mn concentration ranged from 22 to 352 μg/ml as \( \text{MnCl}_2 \cdot 4\text{H}_2\text{O} \).

Side-arm flasks were prepared and inoculated as described in 6a and 6b. The incubation period lasted 96 hours and 120 hours for aluminum and manganese studies, respectively. Growth was measured turbidometrically. The studies were repeated to include both parent and acid-tolerant strains of *R. phaseoli* strain 442, with the pH of the medium adjusted to 5.0.

e) Evaluation of parent and acid-tolerant strains for dinitrogen fixation.

The parent and acid-tolerant strains of *R. phaseoli* strain 442 were evaluated in the growth chamber for their ability to fix dinitrogen. Leonard jars containing exfoliated vermiculite were used. The procedures followed and environmental conditions were as given in section A. The total nitrogen content and dry weight of shoots were used to compare the relative dinitrogen fixing ability of the strains.
C. Preparation and conjugation of antisera.

1.1. Preparation of antisera for studies in non-sterile soil.

Antisera were prepared against 4 strains of *R. phaseoli* P-442, QA 1062, CIAT 255, and C-05. These strains were selected on the basis of their excellent ability to fix dinitrogen. Cell antigens were prepared and adjusted to about 200 Klett units. Flagellar antigens were inactivated by heating in boiling water for 30 minutes. The antigens were stored in serum bottles at 4°C until ready for use.

The cell antigens were injected intravenously into the large marginal vein of the ear of a New Zealand white rabbit according to a schedule described by Bohlool and Schmidt (1970): day 1, 0.5 ml; day 2, 1.0 ml; day 3, 1.5 ml; day 4 to 6, rest; day 7, 1.5 ml; day 8, 2.0 ml; and day 9, 2.0 ml. Prior to injection, a 10 ml blood sample was obtained by heart puncture to check for background antibodies against the *Rhizobium* antigens.

1.2. Evaluation of serum titer and collection of blood.

Three weeks after the completion of the injection schedule, 10 ml blood samples were collected to determine antibody titers. The blood samples were stored overnight at 4°C to extrude the serum. The clot was cut and the serum centrifuged for 5 minutes in a serofuge (Clay-Adams, Inc., New York 10, NY). Merthiolate was added to the supernatant at a final concentration of 1:10,000 and the serum was stored in serum vials at -20°C.

The titer of the antisera was checked against the homologous antigens according to a procedure described by Vincent (1970). Antiserum-antigen
dilutions of 1/50, 1/100, 1/200, 1/400, 1/800, 1/1600, 1/3200, and 1/6400 were prepared. The greatest dilution which agglutinated with the antigen was taken as the titer of the antiserum.

When a satisfactory titer was obtained, about 30 ml of blood was obtained by heart puncture and the antiserum prepared as before. All sera were preserved in merthiolate, dispensed in 5 ml serum vials and stored at -20°C. Final titers of each antiserum were determined and recorded.

1.3. Conjugation of antisera with fluorescein isothiocyanate (FITC).

a) Fractionation.

Ten ml of each antiserum was fractionated at 4°C by dropwise addition of 10 ml of cold saturated ammonium sulfate (760 g/l), with continuous stirring. The mixture was held overnight at 4°C to complete precipitation, and then centrifuged at 3000 rpm (13820 x g) for 15 minutes. The white precipitate of Immuno-g-globulin (IgG) was dissolved in 10 ml of cold distilled water and reprecipitated with 10 ml of cold ammonium sulfate. This step was repeated until a clean white precipitate was obtained. The precipitate was resuspended in cold distilled water and transferred to a dialysis bag and dialyzed against 0.85% NaCl in the cold until free of NH₄⁺. The presence of NH₄⁺ was determined by use of Nesler's reagent.

b) Conjugation with FITC.

Prior to conjugation, the amount of protein in each antiserum was determined according to a procedure described by Levin and Braver (1951), and given in Appendix E. Ten ml of the dialyzed antiserum with its protein
content adjusted to 1% were mixed with 4 ml of 0.15 M phosphate buffer (pH 9.0), followed by addition of 4 ml of 0.1 M phosphate buffer (pH 8.0) containing 1.4 mg FITC/ml to give 50 µg FITC/mg protein. The pH was adjusted to about 9.0 with 0.1 M NaOH, followed by adjustment of the volume to 20 ml with 0.15 M NaCl. The mixture was stirred overnight at 4°C in order to improve conjugation. Cold solutions were used throughout the preparation.

c) Purification of antiserum-FITC.

Antiserum conjugated with FITC was purified by gel-filtration chromatography as described by Curtain (1961). Twenty ml of crude conjugate were filtered through a Sephadex column (25 x 400 mm packed with Sephadex G-25 fine) connected to a reservoir containing 0.01 M phosphate buffer (pH 7.0). The weak fluorescent band containing pure conjugated FITC-antiserum travelled with the solvent front and was collected in the first 20 ml of effluent. Merthiolate was added to the purified FITC-antiserum at a final concentration of 1:10,000 to prevent microbial growth. The antisera were dispensed in 5 ml serum vials and stored at -20°C until ready for use. Agglutination tests were performed as described previously and titers of the conjugated antisera were reported.

d) Preparation of conjugated gelatin-rhodamine isothiocyanate (RhITC).

The procedure followed was that described by Bohlool and Schmidt (1968). One gram of gelatin was dissolved in 50 ml of distilled water with gentle heating. When the gelatin was completely dissolved, the temperature was brought to near room temperature and the pH adjusted to 11.0 with 1 N NaOH. The suspension was autoclaved for 10 minutes at 121°C and 1 atm
pressure, and the pH readjusted to 11.0. Ten mg of rhodamine isothiocyanate were dissolved in 2 ml of acetone (RhITC does not dissolve completely) to provide a minimum amount of 8 mg RhITC/g gelatin. This solution was added to the gelatin suspension.

The mixture was held overnight at room temperature with gentle stirring to improve conjugation. The RhITC was purified, collected, preserved, and stored in a manner similar to that previously described for antiserum-FITC.

2.1. Soil studies.
2.1.1. Soils used in the studies.

a) Rossmoyne soil (Aquic fragiudalf). This is a silt loam soil that was used in the moisture and dessication studies. The surface 15 cm (litter removed) was collected from the Southern branch, Ohio Agricultural Research and Development Center (OARDC). It was passed through a 2 mm sieve and stored at 4°C.

b) Santo Angelo (Haplorthox). This soil was used in the pH studies. It was obtained from Dr. Caio Vidor, Instuta de Pesquisus Agronomicas, Porto Alegre, Brazil. Surface soil samples were passed through a 2 mm sieve and stored at 4°C.

c) Bom Jesus (Haplumbrept). This soil was also used in the pH studies. Surface soil samples were also obtained from Dr. Caio Vidor. They were sieved and stored at 4°C.

Some physical and chemical properties of the above soils are given in Table 1; microbiological data are given in Table 2.
Table 1. Chemical and physical properties of Rossmoyne, Santo Angelo, and Bom Jesus soils.

<table>
<thead>
<tr>
<th>Soils</th>
<th>Particle Size Analyses</th>
<th>pH (1:1)</th>
<th>CEC Al</th>
<th>Organic C</th>
<th>Total N</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sand</td>
<td>Silt</td>
<td>Clay</td>
<td></td>
<td>me/100 g</td>
</tr>
<tr>
<td>Rossmoyne (Aquic Fragiudalf)</td>
<td>11.8</td>
<td>70.8</td>
<td>17.4</td>
<td>6.8</td>
<td>13.1</td>
</tr>
<tr>
<td>Santo Angelo (Haplorthox)</td>
<td>18</td>
<td>25</td>
<td>62</td>
<td>4.2</td>
<td>8.9</td>
</tr>
<tr>
<td>Bom Jesus (Haplumbrept)</td>
<td>16</td>
<td>31</td>
<td>52</td>
<td>4.6</td>
<td>20</td>
</tr>
</tbody>
</table>
Table 2. Microbial population of Rossmoyne, Santo Angelo, and Bom Jesus soils as determined by different methods.

<table>
<thead>
<tr>
<th>Soil Type</th>
<th>Bacteria and actinomycetes&lt;sup&gt;a&lt;/sup&gt; (No. of cells or propagules/g dry soil)</th>
<th>Fungi&lt;sup&gt;b&lt;/sup&gt; (No. of cells or propagules/g dry soil)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rossmoyne</td>
<td>$3.3 \times 10^7$</td>
<td>$3.2 \times 10^4$</td>
</tr>
<tr>
<td>Santo Angelo</td>
<td>$1.1 \times 10^6$</td>
<td>$4.5 \times 10^4$</td>
</tr>
<tr>
<td>Bom Jesus</td>
<td>$8.5 \times 10^5$</td>
<td>$1.1 \times 10^5$</td>
</tr>
</tbody>
</table>

<sup>a</sup>Plate counting on soil extract agar (Appendix F).

<sup>b</sup>Plate counting on Rose bengal-Streptomycin agar (Appendix G).
2.1.2. Survival of *R. phaseoli* in soil adjusted to different moisture tensions.

2.1.3. Survival studies in sterile soil.

Ten grams (dry weight basis) of sieved Rossmoyne soil were weighed out into 50 ml Erlenmeyer flasks and sterilized by autoclaving at 121°C and 1 atm for 1 hour. Prior to sterilization, the moisture content of the soil was determined. The moisture treatments used were 17% (1.2 bar), 23% (0.33 bar), 40% (0.1 bar) and flooding. Soil moisture tension curve for the Rossmoyne soil is shown in Appendix H.

*Rhizobium phaseoli* strains C-05, QA 1062, CIAT 255, P-442, and S-442 were used. Three to four day old cultures were washed off yeast-mannitol slants with 0.1% peptone, mixed with a vortex mixer, and adjusted to 50 Klett units. Plate count data, on SYM agar showed a range in population of $10^7$ to $10^8$/ml. One ml of each culture and enough sterile water were added to the soil to get the appropriate soil moisture treatment. For the flooding treatment, 1 ml of culture and enough water were added so that approximately 2 cm of water remained above the soil surface.

Twenty samples were prepared per treatment and incubated in a constant temperature room maintained at 28°C. Duplicate samples of each treatment were sacrificed at 0, 3, 5, 10, 15, 20, 25, and 30 days to make population counts. Each soil sample was transferred into a 250 ml Erlenmeyer flask containing 95 ml sterile water and 10 g glass beads (6 mm diameter). The flasks were shaken for 5 minutes on a rotary shaker at approximately 200 revolutions per min (rpm). Decimal dilutions were aseptically prepared in sterile 0.1% peptone, and 0.1 ml was plated in triplicate at the selected dilutions. The plates were incubated at
30°C until they were counted after 3 to 4 days.

2.1.4. Survival studies in non-sterile soil.

Two strains QA 1062 and C-05, and two soil moisture treatments, 0.33 bars and flooding, were used in this study. These strains were chosen because it was expected that their survival patterns would be similar to the other strains, since all strains studied under the sterile situation behaved similarly at all moisture levels. Ten grams of soil were dispensed into 50 ml Erlenmeyer flasks which were inoculated, adjusted to the desired moisture contents, and incubated similar to procedures described earlier for the sterile situation.

Survival counts were done after 0, 3, 7, 14, and 21 days by the Fluorescent Antibody (FA) technique described by Schmidt (1974) with slight modifications. Recovery of cells for FA counting was achieved by a density gradient centrifugation method recently developed by Wollum and Miller (1980), also with slight modifications.

For cell recovery, ten grams of soil were quantitatively transferred to a stainless steel blender cup with 95 ml of a 1% CaCl₂ solution. One drop (Pasteur pipette) of Tween 80 (Difco Laboratories) and three drops (Pasteur pipette) of antifoam agent (Dow-B, Fisher Scientific Co.) were added. The suspension was blended for 5 minutes on a multimixer (Prince Castle Manufacturing Div., Inc., Sterling, IL) after which a 20 ml portion was withdrawn and transferred into a 100 ml clear polypropylene centrifuge tube containing 10 ml of a 1.33 g/cm³ (855 g sucrose in 450 ml distilled water) sucrose solution. This soil-sucrose ratio differed from that used in the original procedure where a ratio
of 10:20 was used. The former ratio gave a better clarification of the sucrose layer after centrifugation, and a better recovery of cells (100-104% vs. 65-75%). The soil suspension-sucrose solution was thoroughly mixed, and 30 ml of a 1.33 g/cm$^3$ sucrose solution was layered beneath the soil suspension.

Samples were centrifuged at 1600 rpm (715 x g) for 15 minutes, using an International centrifuge with a swinging bucket rotor. After centrifugation, 10 ml aliquots were withdrawn from the topmost 2 cm layer and mixed with 90 ml of distilled water.

Five to 10 ml aliquots were withdrawn for immunofluorescent analysis. This volume was transferred to a filter assembly fitted with a polycarbonate membrane Nucleopore filter (25 mm, 0.4 μm; Nucleopore Corp.) which had been soaked overnight in a dilute solution of surfactant to overcome the hydrophobic properties of the filter. The cells were concentrated on the filter by suction with a vacuum pump, after which the tip of the filter holder was sealed with parafilm and just enough gelatin-rhodamine added to cover the surface of the filter. Without adding water to the filter as described by Wollum and Hiller (1980), the entire filter holder was removed from the filter flask and gently shaken to uniformly moisten the entire filter surface with the gelatin rhodamine. Another deviation from the original method was that the filter assembly was not returned to the suction flask to remove excess rhodamine. Instead, it was placed in an oven kept at about 50°C to allow near-dryness of the surface of the filter. Drying was achieved in approximately 7 to 10 minutes, after which the filter was left to cool.
The filter was stained with the appropriate antiserum-FITC and the entire filter assembly was stored in the dark for 30 minutes. Subsequently, the filter was washed with at least 250 ml of filtered physiological saline.

To assume a dark background, each Nucleopore filter was placed on a microscope slide directly over an india-ink stained membrane filter (25 mm diameter, 0.45 μm; HABG, Millipore Corp.) and treated with mounting fluid (Difco). The fluid was allowed to soak and penetrate the filter for at least 30 minutes. Each filter was covered with a glass cover slip and the cells counted by using a Leitz Ortholux microscope equipped for epifluorescent microscopy.

2.1.5. Effect of moisture on dinitrogen fixation by R. phaseoli.

Five R. phaseoli strains, S-442, P-442, C-05, QA 1062, and CIAT 255, were tested in the growth chamber for their ability to fix dinitrogen at various soil moisture contents. The Rossmoyne soil was used as the growth medium.

Triplicate 1.5 kg soil samples were weighed out into plastic pots and inoculated with young cultures of R. phaseoli. The growth on the surface of SYM agar slants was washed with 0.85% saline and mixed with a vortex mixer. Turbidity was adjusted using Klett-Summerson photoelectric colorimeter to give approximately \(1 \times 10^8\) cells/ml. Liquid inoculum of each strain was added to the soil at a population of about \(1 \times 10^7\) cells/kg soil and left to equilibrate for 30 minutes. Enough distilled water was added to the soil to give the following
moisture treatments:

1. Field capacity;
2. Pre-plant flooding for 2 weeks;
3. Pre-plant flooding for 4 weeks;
4. Continuous saturation;
5. Uninoculated control with nitrogen (100 kg N/ha);
6. Uninoculated control without nitrogen

Treatments 1 and 4 were maintained at the indicated moisture levels for 4 weeks. After 4 weeks, all but the continuous saturation treatments were kept at or near field capacity. Four sterilized seeds of black bean var Pijao, were then planted per pot and thinned to 2 after germination. Plant growth was maintained at field capacity and saturation until harvest after 4 weeks.

The environmental conditions were the same as described in section A. The experimental design was the randomized complete block. The position of the pots inside each block was randomly changed every other week to control variability due to changes in temperature, light intensity and humidity in the growth chamber. The parameters used to measure dinitrogen fixing ability were dry shoot weight, nodule numbers and dry weight, and total nitrogen.


3.1.1. Survival studies in sterile soil.

Studies were done in Rossmoyne soil to determine survival of *R. phaseoli* strains P-442, S-442, QA 1062, C-05, and CIAT 255 in soils undergoing drying. The strains used were chosen on the basis of their
excellent ability to fix dinitrogen. The preparation, sterilization, and inoculation of the samples were as described in section 2.1.1. The soils were adjusted to 1/3 bar moisture and left to dry slowly in a desiccator using CaSO$_4$ as desiccant.

Decimal dilutions were aseptically prepared from duplicate samples as given in section 2.1.1. Population counts were made after 0, 10, 20, 30, 60, and 90 days.

3.1.2. Survival studies in non-sterile soil.

Two strains, P-442 and CIAT 255, were used in this study. These strains were selected on the basis of the resistance of the former, and the susceptibility of the latter, to desiccation. These characteristics were demonstrated during studies in sterile soil.

Changes in cell numbers were estimated by the FA technique using procedures described in section 2.1.3. Rhizobium numbers were determined after 0, 5, 10, 20, 30, and 60 days on duplicate samples.

4.1. Effect of pH on survival of *R. phaseoli*.

4.1.1. Survival studies in non-sterile soils.

*Rhizobium phaseoli* strains P-442 and S-442 were studied in two Brazilian soils, Santo Angelo (Haplorthox) and Bom Jesus (Haplumbrept). Natural soil pH for Santo Angelo was 4.2 while that for Bom Jesus 4.6. These soils were each amended with 20% CaCO$_3$ and left to equilibrate for 2 weeks. The pH achieved was 6.6 for Santo Angelo and 6.8 for Bom Jesus.

Ten grams of soil were quantitatively transferred to 50 ml Erlenmeyer flasks, which were inoculated with 1 ml of young cultures of the test
organisms. Enough water was added so that the moisture content of the soils was approximately 1/3 bar. The flasks were incubated at 28°C.

Survival counts were determined at 0, 3, 7, 14, 21, and 30 days. Cell enumeration was by the FA technique as described previously in section 2.1.3.
3. RESULTS AND DISCUSSION

A. Strain evaluation for dinitrogen fixation.

Results of two growth chamber studies are given in Tables 3a and 3b. Both N positive controls (54 μg/ml N) and N negative controls were employed.

Of the 12 strains evaluated in the first study (Table 3), only five strains, QA 1062, 442, C-05, 400 and 127K17, resulted in dry matter yields greater than those of the minus N controls. Nodulation of these strains was excellent, while the other strains formed no nodules. Strain 127K21 formed nodules but was ineffective. None of the effective strains was capable of fixing nitrogen equivalent to 54 μg/ml N in the nutrient solution.

In the second study, seven CIAT strains and four strains selected from the previous study were included. Dry matter yields and relative degree of nodulation are given in Table 4. All but one strain (CIAT 114) produced dry matter yields greater than the minus N control, indicating that these strains have the capacity to fix dinitrogen. Only one strain (442) fixed dinitrogen to produce dry matter yields equivalent to the N positive control (Table 4), a situation contrary to the previous results (Table 3a). This discrepancy could be attributed to fluctuation in environmental conditions in the growth chamber, or to differences in plant vigor after germination.
Table 3. Shoot dry weight (g) and relative degree of nodulation of *P. vulgaris* inoculated with 12 strains of *R. phaseoli*.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mean dry weight</th>
<th>Relative degree of nodulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>QA 1062</td>
<td>2.76</td>
<td>+++</td>
</tr>
<tr>
<td>442</td>
<td>2.29</td>
<td>+++</td>
</tr>
<tr>
<td>C-05</td>
<td>1.40</td>
<td>++ +</td>
</tr>
<tr>
<td>400</td>
<td>1.28</td>
<td>++ +</td>
</tr>
<tr>
<td>127K17</td>
<td>0.77</td>
<td>+++</td>
</tr>
<tr>
<td>445</td>
<td>0.26</td>
<td>---</td>
</tr>
<tr>
<td>439</td>
<td>0.26</td>
<td>---</td>
</tr>
<tr>
<td>C-09</td>
<td>0.25</td>
<td>---</td>
</tr>
<tr>
<td>C-01</td>
<td>0.24</td>
<td>---</td>
</tr>
<tr>
<td>C-14</td>
<td>0.23</td>
<td>---</td>
</tr>
<tr>
<td>127K21</td>
<td>0.21</td>
<td>++</td>
</tr>
<tr>
<td>427</td>
<td>0.20</td>
<td>---</td>
</tr>
<tr>
<td>Control (+N)</td>
<td>4.37</td>
<td>---</td>
</tr>
<tr>
<td>Control (-N)</td>
<td>0.26</td>
<td>---</td>
</tr>
</tbody>
</table>

14 hour day length, day temperature 30°C, night temperature 26°C.

Mean of 3 replications.

+++ excellent nodulation; ++ fair nodulation; --- no nodulation.
Table 4. Shoot dry weight (g) and relative degree of nodulation of *P. vulgaris* inoculated with 11 strains of *R. phaseoli*.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mean dry weight</th>
<th>Relative degree of nodulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIAT 255</td>
<td>2.27</td>
<td>+++</td>
</tr>
<tr>
<td>CIAT 57</td>
<td>2.06</td>
<td>+++</td>
</tr>
<tr>
<td>CIAT 632</td>
<td>2.02</td>
<td>+++</td>
</tr>
<tr>
<td>CIAT 137</td>
<td>1.67</td>
<td>+++</td>
</tr>
<tr>
<td>CIAT 75</td>
<td>1.60</td>
<td>+++</td>
</tr>
<tr>
<td>CIAT 161</td>
<td>1.45</td>
<td>+++</td>
</tr>
<tr>
<td>CIAT 114</td>
<td>0.34</td>
<td>---</td>
</tr>
<tr>
<td>442</td>
<td>2.82</td>
<td>+++</td>
</tr>
<tr>
<td>C-05</td>
<td>1.36</td>
<td>+++</td>
</tr>
<tr>
<td>QA 1062</td>
<td>1.02</td>
<td>+++</td>
</tr>
<tr>
<td>400</td>
<td>0.74</td>
<td>+++</td>
</tr>
<tr>
<td>Control (+N)</td>
<td>2.53</td>
<td>---</td>
</tr>
<tr>
<td>Control (-N)</td>
<td>0.43</td>
<td>---</td>
</tr>
</tbody>
</table>

1, 2, and 3 See Table 3a for explanations.
It is evident from the results obtained that a broad range of dinitrogen fixing efficiency exists among strains of *R. phaseoli*. Detection of these differences from the conduct of strain evaluation studies is significant if the best and most efficient strains are to be used for commercial inoculant preparation.

Since most of the strains selected produced dry matter yields less than that of the +N control, it was hypothesized that their dinitrogen fixing efficiency can be improved by supplementing inoculated plants with inorganic nitrogen. An experiment was therefore designed to compare dry weight yields of inoculated plants with and without supplemental nitrogen (15 µg/ml) in the nutrient solution.

Four selected strains (442, QA 1062, 127K17, and CIAT 632) were used in this study. The experiment was conducted in Leonard jars containing vermiculite. Growth chamber conditions were as described in section A. Plants were harvested four weeks after germination. The data which are expressed as means of triplicate treatments and yield indexes are presented in Table 5.

The results indicate that all combinations of inoculated plants supplemented with nitrogen in the nutrient solution exceeded the N+ control but only the most efficient strain 442 resulted in a statistically significant positive yield response. Of significance was the fact that all plants, regardless of strain, were nodulated in the presence of nitrogen in the nutrient solution.

Allos and Bartholomew (1959), working with several legume species, reported that nitrogen application tended to increase dinitrogen fixation
Table 5. Dry shoot weight of inoculated *P. vulgaris* with and without supplemental nitrogen.

<table>
<thead>
<tr>
<th>Treatment (strain)</th>
<th>Weight (g)</th>
<th>Yield index*</th>
</tr>
</thead>
<tbody>
<tr>
<td>N (-), Control</td>
<td>0.59</td>
<td>0.14</td>
</tr>
<tr>
<td>N (+), Control</td>
<td>4.35</td>
<td>1.00</td>
</tr>
<tr>
<td>442</td>
<td>2.62</td>
<td>0.60</td>
</tr>
<tr>
<td>442, with N</td>
<td>5.22</td>
<td>1.20</td>
</tr>
<tr>
<td>QA 1062</td>
<td>1.62</td>
<td>0.37</td>
</tr>
<tr>
<td>QA 1062, with N</td>
<td>4.50</td>
<td>1.03</td>
</tr>
<tr>
<td>127K17</td>
<td>1.58</td>
<td>0.36</td>
</tr>
<tr>
<td>127K17, with N</td>
<td>4.47</td>
<td>1.03</td>
</tr>
<tr>
<td>CIAT 632</td>
<td>2.11</td>
<td>0.49</td>
</tr>
<tr>
<td>CIAT 632, with N</td>
<td>4.60</td>
<td>1.06</td>
</tr>
</tbody>
</table>

*Comparison with the uninoculated N (+) control as 1.00.*
which did not supply sufficient nitrogen for maximum growth. The experiments were conducted in gravel culture in the greenhouse.

Yoshihara and Kawanshee quoted by Ohlrogge (1960) concluded from sand and soil culture studies that combined nitrogen was needed for the first five weeks in order to increase the yields of the forages they used. In western Nigeria, Kang (1975), working with soybean variety TK-5, observed that inoculation alone was inadequate to supply the nitrogen needs of the crop. Thirty kg N/ha was needed with inoculation, and 60 kg N/ha without inoculation for maximum yield.

Some researchers, however, have reported data which indicate that combined nitrogen is not needed to improve yields of inoculated legumes. Weber (1966) showed that the amount of symbiotic nitrogen fixation in soybean decreased rapidly with increases in fertilizer nitrogen. He explained that nitrogen fixed symbiotically was more desirable for plant growth than non-symbiotic nitrogen because the energy required for seed production, respiration of nodule bacteria, and reduction of N₂ to NH₂ was less than that required for absorption of NO₃⁻ and its subsequent reduction to NH₃. Similar results have been reported by Beard and Hoover (1971), Pal and Saxena (1975), and Olsen et al (1975).

It appears that the requirement or non-requirement of fertilizer nitrogen in combination with inoculation depends on the inherent ability of the microsymbiont to fix dinitrogen, nitrogen status of the growth medium, and the legume host. In either case, it may be necessary to supplement the legume-Rhizobium symbiosis with a booster dose of
fertilizer nitrogen during the initial phases of plant growth when
dinitrogen fixation is inoperative. The results obtained for
Phaseolus vulgaris in this research need to be investigated further to
include more strains, different cultivars, and different growth media.

B. Biochemical and physiological characterization of strains.

Some biochemical and physiological characteristics of Rhizobium
have been used as diagnostic features in their classification and
identification (Graham and Parker, 1964; Moffet and Colwell, 1968;
Fred et al, 1932). Some tests that have been reported in the literature
and also used in this research include the following:

1. Biochemical tests.

   a) Nitrate reductase activity.

   This enzyme was absent in five but present in six of the strains
tested (Table 6). Nitrate reducing capacity is an integral process in
N assimilation by microorganisms. Nitrogen is released to the cell
by reduction of the \( \text{NO}_3^- \) to \( \text{NH}_3 \), and \( \text{NH}_3 \) is then built up into cellular
nitrogen (assimilatory nitrate reduction). Graham and Parker (1964),
showed that nitrate reduction was of significant diagnostic value in
the characterization of Rhizobium spp. Fred et al (1932) summarized
the work of several researchers who reported wide variation in nitrate
reductase activity among Rhizobium spp. In general, however, the
fast-growing rhizobia have been shown to possess more nitrate reductase
activity than the slow-growing ones.

   b) Litmus milk reaction.

   Table 6 shows results of the reaction of Rhizobium upon litmus milk.
Some strains produced acid, some produced alkali, all without coagulation.
Table 6. Some biochemical reactions of *R. phaseoli*.

<table>
<thead>
<tr>
<th>Strains</th>
<th>NO₂⁻-reduction</th>
<th>Litmus milk reaction</th>
<th>Acid/pink color</th>
<th>Alkaline/blue color</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No reaction</td>
<td>NO₂⁻ → NO₃⁻</td>
<td>No coagulation</td>
<td>No coagulation</td>
</tr>
<tr>
<td>422</td>
<td>+ a*</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>400</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>127K17</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>C-05</td>
<td></td>
<td></td>
<td>+</td>
<td>+ a</td>
</tr>
<tr>
<td>QA 1062</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>CIAT 161</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CIAT 632</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>CIAT 75</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CIAT 57</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>CIAT 137</td>
<td>+</td>
<td>+</td>
<td></td>
<td>++</td>
</tr>
<tr>
<td>CIAT 255</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a* Gelatin, citrate, and starch hydrolysates were negative for all strains; catalase and oxidase were positive for all strains.

**a** Positive reaction.

"Observation made after two months."
The changes in litmus milk are of value in the separation of the different species of \textit{Rhizobium}. The pH of the medium turns acid when glucose or a small amount of lactose is utilized, and alkaline when the proteins lactalbumin and casein are attacked. Fred et al (1932), Graham and Parker (1964), and Moffet and Colwell (1968), noted that fast-growing rhizobia produce serum zones in litmus milk, whereas slower-growing ones do not.

c) Gelatin, starch and citrate hydrolysates were negative. The \textit{R. phaseoli} strains do not have the exoenzymes gelatinase, amylase, or permease to effect the various hydrolysates.

d) Catalase and oxidase were positive for all the strains. Catalase breaks down $\text{H}_2\text{O}_2$ into water and oxygen. Hydrogen peroxide is frequently the end product of biological oxidation carried out by chemoorganotrophs in the presence of oxygen. Oxidase is a cytochrome oxidase similar to cytochrome C found in the electron transport system. Aerobic and facultatively anaerobic bacteria produce this enzyme.


The resistance/sensitivity of 10 \textit{R. phaseoli} strains is given in Table 7. There was differential sensitivity of the strains to the antibiotics tested. The degree of sensitivity was of the order 422 = CIAT 75 > CIAT 255 > QA 1062 = CIAT 57 > CIAT 161 > 127K17 > 400 = C-05 > CIAT 137.

Of the antibiotics tested, streptomycin, doxycycline, neomycin, kanamycin, viomycin, and chloromycetin were the most frequent inhibitors, while penicillin, sulfathiozole, bacitracin, polymyxin B, and novobiocin were the least effective. These results agree, in general, with those
Table 7. Antibiotic resistance/sensitivity of *R. phaseoli* strains.

<table>
<thead>
<tr>
<th>Strains*</th>
<th>Antibiotics</th>
<th>442</th>
<th>400</th>
<th>127K17</th>
<th>C-05</th>
<th>QA 1062</th>
<th>CIAT</th>
<th>161</th>
<th>75</th>
<th>137</th>
<th>255</th>
<th>57</th>
<th>% of strains sensitive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kanamycin (10 µg)</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
<td></td>
<td>86</td>
<td></td>
</tr>
<tr>
<td>Viomycin (5 µg)</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>80</td>
</tr>
<tr>
<td>Penicillin (3 µg)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td></td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Novobiocin (10 µg)</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Streptomycin (5 µg)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Doxycycline (10 µg)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>Chloromycetin (10 µg)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>Polymyxin B (166 µg)</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>Neomycin (10 µg)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>Sulfathiazole (150 µg)</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Bacitracin (125 µg)</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>30</td>
<td></td>
</tr>
</tbody>
</table>

% antibiotics sensitive

- Sensitive strains
+ Resistant strains
of other investigators including Davis (1962) and Pattison and Skinner (1974).

Interest in determining antibiotic resistance of *Rhizobium* has increased because of the need to develop methods for ecological studies. The information provided would aid in the selection of strains for such studies.

3. Carbohydrate utilization.

Table 8 shows that the *R. phaseoli* strains can metabolize a wide range of carbohydrates. Utilization of the various carbohydrates resulted in the production of moderate to strong acidity in most cases, as indicated by a change in the color of the phenol red medium to yellow. Changes in the H-ion concentration of culture media are frequently used as an index of carbohydrate utilization.

None of the organic acids were utilized by the rhizobia, except for gluconate, where only moderate growth was demonstrated.

Similar studies and results have been reported by Fred et al (1932), Graham (1964), Graham and Parker (1964). Based on such studies, *Rhizobium spp* have been separated into two groups: fast-growing strains and slow-growing strains. The former are acid-producing and the latter alkali-producing. Martinez de Drets and Arias (1972) reported an enzymatic basis for the separation of *Rhizobium* into fast-growing and slow-growing species. They grew the organisms in yeast-extract glucose medium and followed the enzymes involved in the degradation of the carbohydrate. On the basis of their G-phosphogluconate dehydrogenase (GPGD) activity, the fast-growers were found to possess NADP-GPGD activity, whereas the slow-growers did not. Most of the carbohydrates tested
Table 8. Carbohydrate utilization by some strains of *R. phaseoli*.

<table>
<thead>
<tr>
<th>Carbon Source</th>
<th>400</th>
<th>442</th>
<th>127K17</th>
<th>C-05</th>
<th>QA 1062</th>
<th>CIAT 161</th>
<th>CIAT 612</th>
<th>CIAT 75</th>
<th>CIAT 57</th>
<th>CIAT 137</th>
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<td>Lactose</td>
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<td>Raffinose</td>
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<td>++</td>
<td>++</td>
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<td>++</td>
<td>++</td>
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<td>++</td>
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<tr>
<td><strong>Polysaccharides</strong></td>
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<td>Dextrin</td>
<td>+ 3</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<td>Inulin</td>
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<td>+++</td>
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<td>+++</td>
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<td>+++</td>
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<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
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<tr>
<td><strong>Polyhydric alcohol</strong></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>Mannitol</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

1+++ = strongly acidic  
2++ = moderately acidic  
3+ = very slightly acidic  
40 = no change
occur in soil. The ability of the rhizobia to utilize a wide range of them may be a factor in improving their "saprophytic competence" under natural soil conditions.

4. Response of *R. phaseoli* to root exudates.

Results in Table 9 show that *R. phaseoli* strains can grow in the presence of root exudates of plants other than their homologous species. In some cases, growth stimulation by exudates of the heterologous species was slightly better than the homologous legume.

Mean Relative Growth Ratios (RGR) (Table 9) indicate that growth of the bacteria was stimulated to the same degree regardless of the source of the root exudate. This shows that the organisms responded to them to the same degree. These results support those of other workers (Robinson, 1967; Rovira, 1961; Tuzimura and Wantanabe, 1962b; Peters and Alexander, 1966; Reyes and Schmidt, 1980) who have reported that the concept of specific stimulation is not valid. This concept describes the situation whereby the appropriate *Rhizobium* symbiont is selectively stimulated by root exudates from its host's root as a prelude to successful nodulation.

Although non-specific stimulation occurs in the rhizosphere of plants, nodulation of legumes is mediated by specific recognition mechanisms between the host and its homologous *Rhizobium* strain. In order to effect nodulation, the bacteria recognize specific plant lectins, called glycoproteins, on the surface of the root. Extensive research information (Bohlool and Schmidt, 1974; Dazzo et al 1976) exists on the mechanism of nodulation. Non-specific stimulation is probably important in agricultural systems where crop rotation is practised.
Table 9. Relative growth ratios* of *R. phaseoli* strains grown in SYM broth supplemented with various root exudates.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Black bean</th>
<th>Corn</th>
<th>Soybean</th>
</tr>
</thead>
<tbody>
<tr>
<td>422</td>
<td>0.7</td>
<td>0.7</td>
<td>0.5</td>
</tr>
<tr>
<td>CIAT 255</td>
<td>1.9</td>
<td>1.4</td>
<td>1.4</td>
</tr>
<tr>
<td>CIAT 137</td>
<td>1.4</td>
<td>1.1</td>
<td>1.1</td>
</tr>
<tr>
<td>QA 1062</td>
<td>1.0</td>
<td>1.0</td>
<td>1.1</td>
</tr>
<tr>
<td>CIAT 632</td>
<td>1.0</td>
<td>1.1</td>
<td>0.8</td>
</tr>
<tr>
<td>C-05</td>
<td>0.9</td>
<td>1.0</td>
<td>0.9</td>
</tr>
<tr>
<td>127K17</td>
<td>1.0</td>
<td>1.0</td>
<td>0.7</td>
</tr>
<tr>
<td>CIAT 161</td>
<td>0.9</td>
<td>1.0</td>
<td>0.9</td>
</tr>
<tr>
<td>CIAT 75</td>
<td>0.7</td>
<td>1.0</td>
<td>0.6</td>
</tr>
<tr>
<td>Mean</td>
<td>1.1</td>
<td>1.0</td>
<td>0.9</td>
</tr>
</tbody>
</table>

*Relative growth ratio (RGR) = Growth (Klett units) with root exudate/Growth without root exudate.
In a black bean-corn-black bean rotation, for instance, a high population of *Rhizobium phaseoli* would be maintained to effect nodulation of the legume without frequent inoculation.

5. Effect of salts on the growth of *R. phaseoli*.

Table 10 shows concentrations of the chlorides and sulfates of Na, K, Ca, and Mg which affect the growth of five strains of *R. phaseoli*. Sodium and potassium chloride were more inhibitory (0.3%) than Ca and Mg chloride (1.0%). The effects of Na, K, and Ca sulfate were similar to Na and K chlorides at 0.3%, except for C-05 which was inhibited at 0.1%. Magnesium sulfate was similar to MgCl at 1.0%.

The report by Yadav and Vyas (1973) that the salt effect was ion specific with the chlorides being more toxic than sulfates was not confirmed. In fact CaSO$_4$ was more toxic than CaCl$_2$.

Comparative growth of the bacteria at 0.1% salts shows, in general, that growth of all strains was stimulated to the same degree by both chlorides and sulfates (Table 11). However, growth of P-442 was inhibited by both ionic species, but inhibited less than S-442. Mean RGR values show that there was a differential effect of salts on the rhizobia with Mg showing the greatest and Na the least positive effects, respectively. This seems to support the report by Norris (1958b) that Mg is required in greater concentration than Ca for the growth of *Rhizobium*.

There is evidence in the literature that *Rhizobium spp* differ in their tolerance to salts. Alfalfa rhizobia can grow at very high salt concentrations, e.g. 2% NaCl (Graham and Parker, 1964; Steinborn and Roughley, 1975). By contrast, soybean rhizobia and other slow-growers
Table 10. Chloride and sulfate concentrations of Na, Ca, K, and Mg lethal to the growth of some strains of *R. phaseoli*.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Chlorides %</th>
<th>Sulfates %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na</td>
<td>K</td>
<td>Ca</td>
</tr>
<tr>
<td>QA 1062</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>P-442</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>S-442</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>C-05</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>CIAT 255</td>
<td>0.3</td>
<td>0.3</td>
</tr>
</tbody>
</table>
Table 11. Relative growth ratios (RGR) of *R. phaseoli* strains grown in SYM broth containing 0.1% chlorides and sulfates of Na, K, Ca, and Mg.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Chlorides</th>
<th>Sulfates</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Na</td>
<td>K</td>
<td>Ca</td>
</tr>
<tr>
<td>CIAT 255</td>
<td>0.89</td>
<td>0.76</td>
<td>0.89</td>
</tr>
<tr>
<td>QA 1062</td>
<td>0.84</td>
<td>0.85</td>
<td>0.90</td>
</tr>
<tr>
<td>S-442</td>
<td>0.51</td>
<td>0.65</td>
<td>0.67</td>
</tr>
<tr>
<td>P-442</td>
<td>0.38</td>
<td>0.42</td>
<td>0.28</td>
</tr>
<tr>
<td>C-05</td>
<td>0.27</td>
<td>0.85</td>
<td>0.85</td>
</tr>
<tr>
<td>Mean</td>
<td>0.58</td>
<td>0.71</td>
<td>0.72</td>
</tr>
</tbody>
</table>
are less salt tolerant (Graham and Parker, 1964). Soybeans when symbiotic are more sensitive to salts than when N-fertilized (Bernstein and Ogata, 1966). In detailed studies with Glycine wightii, Wilson (1970) showed that addition of NaCl at 75 or 150 mM to nodulated plants without N caused almost immediate cessation of dinitrogen fixation and plant growth, with a drop in leaf percent N. When salt was removed, nodules recovered effectiveness.

Salt effects on Rhizobium are a significant constraint to legume production in arid areas. The results obtained here might serve as a basis for further research on salt stress factors on R. phaseoli. Future research should include an assessment of the effect of salts on dinitrogen fixation of strains used in this research.

C. Moisture studies.
1. Survival of R. phaseoli in soil adjusted to different moisture tensions.
1.1. Survival studies in sterile and non-sterile soil.

Studies in sterile soil indicate, in general, that there was an increase in Rhizobium population as percent moisture increased, and an excellent survival of the strains at all moisture levels (Figures 1 through 4). No significant negative effects of moisture was observed except at the 17% (1.2 bar) moisture level (Figure 1) where strains C-05 and S-442 declined in populations from an initial level of $10^7$ to less than $10^5$ for the former and from about $10^7$ to $10^6$ for the latter. This die-back may be due, in part, to the effects of high concentration of soluble salts and ammonia (Rovira and Bowen, 1966) and increased Mn (Messing, 1965) which accompanied steam sterilization of soil. It may also be an indication of the sensitivity of these strains to low
Figure 1. Survival of *R. phaseoli* strains in sterile Rossmoyne soil adjusted to 17% (1.2 bar) moisture.
Figure 2. Survival of *B. subtilis* strains in sterile Rossmoynes soil adjusted to 23% (0.33 bar) moisture.
Figure 3. Survival of *R. phaseoli* strains in sterile Rossowyn soil adjusted to 40% (0.1 bar) moisture.
Figure 4. Survival of *R. phaseoli* strains in sterile Rosswoyne soil subjected to flooding.
soil moisture. All strains increased in numbers between 3 and 5 days after incubation, probably in response to high soil nutrient levels, but the population stabilized at approximately $10^8$ as nutrients were depleted.

Studies were conducted to compare relative survival of *R. phaseoli* strains C-05 and QA 1062 in non-sterile soil with that in sterile soil. These strains were chosen on the basis of their excellent survival in sterile soil at all moisture levels studied.

Survival curves established over a three week period using the FA technique are shown in Figure 5. Curves obtained from studies in sterile soil are superimposed for comparison.

Survival of both strains in non-sterile soil was similar at the two moisture treatments. However, the cell population at 0.33 bar moisture was at least 0.4 log higher than at flooding. A decrease of about 1 log below the population in sterile soil occurred for both strains at the two moisture levels. Higher survival in sterile than non-sterile soil (Figure 5) implied that biological agents may be responsible for an initial decrease of the rhizobia. However, the populations stabilized after 7 days. Since there was no significant differential effect of moisture on survival of the bacteria, the influence of moisture in regulating microbial antagonism was not significantly demonstrated in this experiment. These results contradict those of Osa-Afiana and Alexander (1979) who reported differential effects of soil moisture on survival of *R. trifolii* and *R. japonicum* in non-sterile soil. These workers implicated predatory protozoa at moderate moisture levels in the decline of the *Rhizobium* populations.
Figure 5. Survival of R. phaseoli strains C-05 and QA 1062 in sterile (S) and non-sterile (NS) Rossmeine soil at 23% (0.33 bar) moisture and flooding.
Because there was no evidence of a precipitous decline in *Rhizobium* numbers in the present study, no investigation was initiated to identify the biological agents that may be responsible for the early and moderate population decline observed.

1.2. Effect of moisture on dinitrogen fixation by *R. phaseoli*.

Based on the relatively good survival of the strains studied in non-sterile soil, an experiment was designed in the growth chamber to test whether flooding of soil inoculated with *R. phaseoli* would affect survival of the rhizobia and thus reduce dinitrogen fixation. The parameters used to evaluate the dinitrogen fixing ability of the strains were dry matter production (Table 12), nodule numbers (Table 13), and total nitrogen (Table 14).

The results demonstrate that flooding did not have any significant effect on the survival (as shown by the excellent nodulation at these treatments) and dinitrogen fixing ability of the *R. phaseoli* strains. In fact, short term flooding appeared to have stimulated the activity of the *Rhizobium* strains. Continuously saturated soil conditions significantly reduced the dinitrogen fixing efficiency of the rhizobia, as indicated by all the parameters measured. This was because saturated soils reduced the $O_2$ supply of the nodules and roots, thereby reducing the amount of energy produced for utilization in the dinitrogen fixing process.

Reasons for the stimulatory effect of flooding are as yet unknown. It is speculated that the flooding, draining, and partial drying of the soils before planting may have resulted in the accumulation of organic
Table 12. Shoot dry weight (g) of *P. vulgaris* grown in Rossmoyne soil inoculated with *R. phaseoli* strains subjected to various moisture treatments.

<table>
<thead>
<tr>
<th>Moisture treatments</th>
<th>P-442</th>
<th>S-442</th>
<th>C-05</th>
<th>QA 1062</th>
<th>CIAT 255</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-plant flooding for 2 weeks</td>
<td>4.03a</td>
<td>4.03a</td>
<td>4.56a</td>
<td>4.23a</td>
<td>4.36a</td>
</tr>
<tr>
<td>Pre-plant flooding for 4 weeks</td>
<td>3.86a</td>
<td>4.33a</td>
<td>3.93a</td>
<td>4.73a</td>
<td>4.17a</td>
</tr>
<tr>
<td>Field capacity</td>
<td>3.71a</td>
<td>3.90a</td>
<td>4.06a</td>
<td>3.57a</td>
<td>4.04a</td>
</tr>
<tr>
<td>Continuous saturation</td>
<td>2.02b</td>
<td>2.42b</td>
<td>1.78b</td>
<td>1.89b</td>
<td>1.11b</td>
</tr>
</tbody>
</table>

*R. phaseoli Strains* - Mean of three replicates. Means followed by the same letter are not significantly different at \( P = .05 \).
Table 13. Mean nodule numbers of *P. vulgaris* grown in Rossmoyne soil inoculated with *R. phaseoli* strains subjected to various moisture treatments.

<table>
<thead>
<tr>
<th>Moisture treatments</th>
<th>P-442</th>
<th>S-442</th>
<th>C-05</th>
<th>QA 1062</th>
<th>CIAT 255</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-plant flooding for 2 weeks</td>
<td>180a**</td>
<td>182a</td>
<td>169a</td>
<td>183a</td>
<td>190a</td>
</tr>
<tr>
<td></td>
<td>(0.27)</td>
<td>(0.27)</td>
<td>(0.25)</td>
<td>(0.26)</td>
<td>(0.27)</td>
</tr>
<tr>
<td>Pre-plant flooding for 4 weeks</td>
<td>170a</td>
<td>165a</td>
<td>173a</td>
<td>178a</td>
<td>186a</td>
</tr>
<tr>
<td></td>
<td>(0.25)</td>
<td>(0.24)</td>
<td>(0.24)</td>
<td>(0.25)</td>
<td>(0.25)</td>
</tr>
<tr>
<td>Field capacity</td>
<td>113b</td>
<td>112b</td>
<td>121b</td>
<td>130b</td>
<td>134b</td>
</tr>
<tr>
<td></td>
<td>(0.18)</td>
<td>(0.18)</td>
<td>(0.19)</td>
<td>(0.19)</td>
<td>(0.19)</td>
</tr>
<tr>
<td>Continuous saturation</td>
<td>82c</td>
<td>81c</td>
<td>37c</td>
<td>41c</td>
<td>38c</td>
</tr>
<tr>
<td></td>
<td>(0.08)</td>
<td>(0.08)</td>
<td>(0.05)</td>
<td>(0.03)</td>
<td>(0.04)</td>
</tr>
</tbody>
</table>

*Mean of three replicates. Means followed by the same letter are not significantly different at P = .05.

**Values in parenthesis represent weight (g) of dry nodules.
Table 14. Total N (%) of *P. vulgaris* grown in Rossmoyne soil inoculated with *R. phaseoli* strains subjected to various moisture treatments.

<table>
<thead>
<tr>
<th>R. phaseoli Strains*</th>
<th>P-442</th>
<th>S-442</th>
<th>C-05</th>
<th>QA 1062</th>
<th>CIAT 255</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-plant flooding for 2 weeks</td>
<td>1.6a</td>
<td>1.6a</td>
<td>1.9a</td>
<td>1.8a</td>
<td>2.1a</td>
</tr>
<tr>
<td>Pre-plant flooding for 4 weeks</td>
<td>1.4a</td>
<td>1.7a</td>
<td>1.9a</td>
<td>1.8a</td>
<td>2.1a</td>
</tr>
<tr>
<td>Field capacity</td>
<td>1.4a</td>
<td>1.4a</td>
<td>1.8a</td>
<td>1.6a</td>
<td>1.5a</td>
</tr>
<tr>
<td>Continuous saturation</td>
<td>1.1a</td>
<td>1.1a</td>
<td>0.8b</td>
<td>0.9b</td>
<td>0.8b</td>
</tr>
</tbody>
</table>

*Mean of three replicates. Means followed by the same letter are not significantly different at P = .05
and inorganic excretion products by the activity of the soil microflora; these products, which probably include growth factors and nutrients, might have stimulated the legume-Rhizobium symbiosis during the initial phases of the growth cycle. The results obtained from this study do not agree with published reports by De Polli et al (1973) and Carrol and Dunigan (1977) where pre-plant flooding negatively affected nodulation and yield of the legumes. However, the finding that saturated soil conditions caused detrimental effects on nodulation and symbiotic nitrogen fixation confirms similar reports published in the literature. The contradictions noted earlier are expected since inherent differences between species and strains, in their ability to withstand certain stress factors, are certain to exist.

2. Survival of *R. phaseoli* in sterile and non-sterile soil undergoing desiccation.

Differences exist between *R. phaseoli* strains in their ability to withstand desiccation (Figure 6). In sterile soil, the order of resistance was as follows: P-442 = S-442 > C-05 > QA 1062 > CIAT 255. The population of QA 1062 and CIAT 255 fell markedly (to < 10^2/g) 20 and 60 days after incubation, respectively. In non-sterile soil, CIAT 255 was again more susceptible to desiccation than P-422 (Figure 6). However, CIAT 255 survived better in the non-sterile than in the sterile situation. These differences may again reflect response of the bacteria to physical and chemical changes which occur in steam-sterilized soils. However, since a similar pattern of desiccation resistance or susceptibility was demonstrated both under sterile and non-sterile conditions, it is more likely that the differences observed are due to the effects of desiccation
Figure 6. Survival of R. phaseoli strains in sterile (S) and non-sterile (NS) Rossmoyne soil undergoing desiccation, using CaSO$_4$ as desiccant.
than to other soil factors.

For all strains, the decline in population coincided with increase in soil moisture tension or decrease in percent soil moisture. Desiccation-induced death occurred primarily at moisture tensions > 15 bars. Similar observations were reported by Pena-Cabralles and Alexander (1979), and Mahler and Wollum (1980). A decline in respiratory activity of bacteria at low water potentials, a plausible explanation advanced by Wilson and Griffin (1975) may be responsible for the decline in rhizobial populations. Another reason for the decline in population is that as the water films in which the bacteria exist become smaller as desiccation progresses, osmotic pressure increases and nutrient availability decreases.

Reasons for the differential resistance or susceptibility to desiccation may be any or all of the following which have been reported in the literature: 1) differences in the water content of the cells at any relative vapor pressure; 2) ability to resist desiccation-induced cell damage; 3) amount of polysaccharides produced during desiccation; and 4) ability to develop at low water activities or low internal osmotic tension. It should be noted that for the strains studied, there was no relationship between capacity to grow at low water activity and ability to resist desiccation. This is because relative growth of these strains in media containing 0.1% salts (Table 11) showed that strains which gave the best growth were the most susceptible to desiccation, and vice versa.
Perhaps the significance of these studies lies in the fact that the Rhizobium strains have been studied under conditions that reproduce the important stresses the host legume is likely to encounter in field trials. In general, Rhizobium strains have been found which survive reasonably well under these stress conditions. With reservations about extrapolating from the results of these studies to situations in the field, it seems probable that some of the Rhizobium strains studied can survive stress conditions of excess moisture or desiccation long enough to initiate nodule formation.
D. pH studies.

1. Selection of strains for acid tolerance and determination of the pH range for growth.

Some colonies of strains QA 1062, 400, 127K17, C-05, CIAT 161, CIAT 75, CIAT 57, CIAT 632, and CIAT 255 were isolated and found to be acid-tolerant but they did not demonstrate a stable and consistent growth in acid media. Only one strain was identified as an acid-tolerant selection from the parent strain of 442. The selected strain S-442 demonstrated consistent growth on acid agar plates at pH 4.0 while the parent (P-442) did not grow below pH 5.0.

Growth studies conducted in SYG broth at pH 4.0, 4.5, 5.0, and 7.2 revealed striking differences between the parent and the acid-tolerant strain (S-442) in their ability to grow at low pH (Figures 7 through 9). These studies also showed that strain S-442 was indeed acid-adapted because it demonstrated differential ability to grow in acid media. Growth in acid SYG broth was not affected by pH and the organism grew as well at pH 4.0 as it did at pH 4.5 or 5.0 (Figure 10).

The pH range for growth was determined for nine parent *R. phaseoli* strains in SYG broth adjusted to pH 4.0, 4.5, 5.0, and 7.2. Growth, as determined by turbidity measurements over a 96-hour period, was expressed as Relative Growth Ratio (RGR) values as shown in Table 15. These values were calculated at the end of the exponential phase of growth which was usually after 72 hours.

Relative Growth Ratio (RGR) is defined by the following relationship:

\[
\text{RGR} = \frac{\text{Growth (Klett units) at acid pH}}{\text{Growth (Klett units) at pH 7.2 (control)}}
\]
Figure 7. Comparative growth rate of the parent culture of *R. phaseoli* strain 442 (P-442) and an acid-tolerant selection (S-442) at pH 7.2 and 4.0.
Figure 8. Comparative growth rate of the parent culture of P. phascoli strain 442 (P-442) and an acid-tolerant selection (S-442) at pH 7.2 and 4.0 (Repeated).
Figure 9. Comparative growth rate of the parent culture of *R. phaseoli* strain 442 (P-442) and an acid-tolerant selection (S-442) at pH 4.5 and 5.0.
Figure 10. Comparative growth of the acid-tolerant strain S-442 in solution culture at pH 4.0, 4.5 and 5.0.
Table 15. Relative growth ratios* of *R. phaseoli* strains grown in SYG broth adjusted to three acid pHs.

<table>
<thead>
<tr>
<th>Strains</th>
<th>pH 4.0</th>
<th>pH 4.5</th>
<th>pH 5.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-442</td>
<td>1.07</td>
<td>1.03</td>
<td>1.00</td>
</tr>
<tr>
<td>P-442</td>
<td>0.13</td>
<td>0.09</td>
<td>1.00</td>
</tr>
<tr>
<td>C-05</td>
<td>1.04</td>
<td>0.96</td>
<td>---</td>
</tr>
<tr>
<td>400</td>
<td>0.09</td>
<td>0.18</td>
<td>1.06</td>
</tr>
<tr>
<td>QA 1062</td>
<td>0.06</td>
<td>0.10</td>
<td>0.92</td>
</tr>
<tr>
<td>127K17</td>
<td>0.06</td>
<td>1.00</td>
<td>---</td>
</tr>
<tr>
<td>CIAT 161</td>
<td>0.12</td>
<td>0.92</td>
<td>---</td>
</tr>
<tr>
<td>CIAT 137</td>
<td>0.07</td>
<td>0.12</td>
<td>0.44</td>
</tr>
<tr>
<td>CIAT 57</td>
<td>0.05</td>
<td>0.88</td>
<td>0.91</td>
</tr>
<tr>
<td>CIAT 75</td>
<td>0.09</td>
<td>0.10</td>
<td>0.91</td>
</tr>
<tr>
<td>CIAT 632</td>
<td>0.05</td>
<td>0.20</td>
<td>0.89</td>
</tr>
</tbody>
</table>

*Relative Growth Ratio (RGR) = \( \frac{\text{Growth (Klett units) at acid pH}}{\text{Growth (Klett units) at pH 7.2}} \)
From the RGR values given in Table 15, the Rhizobium strains were grouped into three categories with respect to their pH range for growth.

<table>
<thead>
<tr>
<th>pH 4.0-7.2</th>
<th>pH 4.5-7.2</th>
<th>pH 5.0-7.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-442</td>
<td>S-442</td>
<td>S-442</td>
</tr>
<tr>
<td>C-05</td>
<td>127K17</td>
<td>P-442</td>
</tr>
<tr>
<td></td>
<td>CIAT 161</td>
<td>400</td>
</tr>
<tr>
<td></td>
<td>CIAT 57</td>
<td>QA 1062</td>
</tr>
<tr>
<td></td>
<td>C-05</td>
<td>CIAT 75</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CIAT 632</td>
</tr>
</tbody>
</table>

The above results indicate that the Rhizobium strains used have differential sensitivity to pH, and that some of them have inherent ability to grow at low pH in broth culture. Some strains, S-442, C-05, and 400 (Table 15) grew better at slightly acidic pH than at pH 7.2. This finding was also observed by Dart (1979), and contradicts the commonly accepted view that Rhizobium has a pH growth optimum of 6 to 8.

Acid production by the fast-growing R. phaseoli strains in SYG medium containing galactose as a carbon source was followed by measuring the initial and final pH of the growth medium. Data in Table 16 shows that, in general, when the initial pH was in the acid range (pH 4.0-5.0), there was a pH drop of one unit or less. However, there was a pH drop of about one to three units when the initial pH was near neutral (pH 7.2). The former observation supports the finding by Dart (1979) that acid media containing galactose as the carbon source did not change in pH appreciably as a result of growth of the organism. In contrast, growth of Rhizobium in acid media containing mannitol, the standard carbon source recommended for culturing rhizobia, raised the pH from 4.5 to 7.5. Norris' (1965)
Table 16. Initial and final pH of SYG broth used to grow *R. phaseoli* strains over a 96-hr period.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Initial pH</th>
<th>Final pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-442</td>
<td>4.0</td>
<td>4.3</td>
</tr>
<tr>
<td></td>
<td>4.5</td>
<td>4.6</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>4.8</td>
</tr>
<tr>
<td></td>
<td>7.2</td>
<td>6.3</td>
</tr>
<tr>
<td>P-442</td>
<td>5.0</td>
<td>4.1</td>
</tr>
<tr>
<td></td>
<td>7.2</td>
<td>4.1</td>
</tr>
<tr>
<td>C-05</td>
<td>4.0</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td>7.2</td>
<td>4.4</td>
</tr>
<tr>
<td>400</td>
<td>5.0</td>
<td>4.3</td>
</tr>
<tr>
<td></td>
<td>7.2</td>
<td>4.6</td>
</tr>
<tr>
<td>QA 1062</td>
<td>5.0</td>
<td>4.6</td>
</tr>
<tr>
<td></td>
<td>7.2</td>
<td>4.3</td>
</tr>
<tr>
<td>127K17</td>
<td>4.5</td>
<td>4.6</td>
</tr>
<tr>
<td></td>
<td>7.2</td>
<td>5.1</td>
</tr>
<tr>
<td>CIAT 161</td>
<td>4.5</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td>7.2</td>
<td>4.6</td>
</tr>
<tr>
<td>CIAT 137</td>
<td>5.0</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>7.2</td>
<td>5.1</td>
</tr>
<tr>
<td>CIAT 57</td>
<td>5.0</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td>7.2</td>
<td>4.0</td>
</tr>
<tr>
<td>CIAT 75</td>
<td>5.0</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>7.2</td>
<td>4.0</td>
</tr>
</tbody>
</table>
report that fast-growing rhizobia were acid producers was confirmed by the large drop in pH mentioned above. He noted that under soil conditions, an organism growing on the roots of a legume in an already acid rhizosphere would be at a disadvantage if it produced more acid in its environment. With the evidence produced by this research that some *Rhizobium* strains can grow well at pH 4.0-5.0, it can be argued that in acid soil, factors other than acidity per se could affect the growth and survival of the organism.

2. **Effect of Al and Mn on *R. phaseoli* strains P-442 and S-442 in solution culture.**

   a) **Effects of Aluminum.**

   At pH 4.0, S-442 was tolerant to 10 µM Al but was partially and strongly inhibited at 25 and 100 µM Al respectively (Table 17). At pH 5.0, a high degree of tolerance was exhibited at 25 µM where growth was almost equal to the control. Partial inhibition occurred at 50 and 100 µM. P-442 was partially inhibited at 25 µM and strongly inhibited at 100 µM.

   As expected, tolerance to Al at pH 5.0 was greater than at pH 4.0 due to the presence of different Al-ion species at these pHs. At the higher pH, more hydroxy-aluminum ions than Al\(^{3+}\) exist, so the effects of the metal on the bacteria were minimized. The opposite effect was expressed at the lower pH where more Al\(^{3+}\) than hydroxy-aluminum ions occurs.

   There were striking differences between the two strains in their tolerance to aluminum. Even at pH 5.0, the S-442 strain demonstrated superior ability to grow at relatively high concentrations of aluminum. It is perhaps logical to predict that in soils where acidity and aluminum are potent stress factors, S-442 would survive better than
Table 17. Effect of aluminum on growth of *R. phaseoli* strains P-442 and S-442 in solution culture at pH 4.0 and 5.0.

<table>
<thead>
<tr>
<th>Relative Growth Ratios* (RGR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aluminum Concentration (µM)</td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>10</td>
</tr>
<tr>
<td>25</td>
</tr>
<tr>
<td>50</td>
</tr>
<tr>
<td>100</td>
</tr>
</tbody>
</table>

In Klett units at aluminum concentration
*RGR = Growth (Klett units) at aluminum concentration/Growth (Klett units) of control
b) Effects of manganese.

Table 18 shows that S-442 could tolerate between 22 and 176 µg/ml at pH 4.0, but was strongly inhibited at 352 µg/ml. At pH 5.0, S-442 showed a similar degree of tolerance and inhibition as at pH 4.0 (Table 18). Also at pH 5.0, Figure 11 shows that the lag phase of growth which occurred between 0 and 48 hours, coincided with the inhibition of P-442 at concentrations ranging from 44 to 176 µg/ml. Exponential growth occurred thereafter between 48 and 72 hours.

From the results presented in Table 18, it can be concluded that S-442 is more tolerant to manganese than P-442.

c) Tolerance to acidity vs. tolerance to aluminum and manganese.

From the pH, aluminum, and manganese studies, it can be concluded that S-442 combines tolerance to acidity with improved tolerance to aluminum and manganese. In another study, Mohdjadi (1981) showed that 127K17 was tolerant to manganese and CIAT 161 was tolerant to both aluminum and manganese. These results provide evidence of the potential for survival of these strains in acid soils containing high aluminum and/or manganese content.

Some mechanisms by which microorganisms protect themselves against heavy metal toxicities have been reported. Bitton and Freihofer (1978) studied the effect of capsular polysaccharides on copper and cadmium toxicity toward *Klebsiella aerogenes*. Their data showed that the capsulated strain survived better than the non-capsulated strain after exposure to toxic levels of the metals. This is because the polysaccharide complexed the metals, thereby protecting the organism against the
Table 18. Effect of manganese on growth of *R. phaseoli* strains P-442 and S-442 in solution culture at pH 4.0 and 5.0.

<table>
<thead>
<tr>
<th>Manganese Concentration (µg/ml)</th>
<th>pH 4.0</th>
<th>pH 5.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>22</td>
<td>1.00</td>
<td>0</td>
</tr>
<tr>
<td>44</td>
<td>1.00</td>
<td>0.76</td>
</tr>
<tr>
<td>88</td>
<td>0.90</td>
<td>0.82</td>
</tr>
<tr>
<td>176</td>
<td>0.49</td>
<td>0.44</td>
</tr>
<tr>
<td>352</td>
<td>0.09</td>
<td>0.04</td>
</tr>
</tbody>
</table>

* RGR = Growth (Klett units) at manganese concentration
  Growth (Klett units) at control
bactericidal effects of the metals. Mohdjadi (1981) has also suggested a similar protection mechanism for the *R. phaseoli* strains he found tolerant to aluminum and manganese.

Another protective mechanism involves accommodation of the organism to stresses in its environment. This is usually demonstrated by a long lag phase of growth, after which normal exponential growth is resumed. Mitra et al (1975) observed this phenomenon in *E. coli*. They reported that accommodation of *E. coli* to the presence of cadmium involved exclusion of the ion from the cell and reversal of damage caused by prior exposure to the ion. It was noted that this reversible phenomenon of accommodation to growth-inhibiting concentrations of cadmium did not appear to result from selection of mutant cells. Although this phenomenon was not investigated in this research, it perhaps characterizes the effect of manganese on growth of P-442 (Figure 11).

Zwarum (1973) related tolerance of *E. coli* to toxic levels of cadmium to the ability of the cell wall to protect the organism. He explained that the mechanism involved was that the cell wall acted as a passive ion exchanger similar in action to an inert cation exchanger such as a clay particle.

In light of the above mechanisms, it is suspected that tolerance of the mutant strain S-442 to aluminum and manganese is related to protection afforded by the polysaccharide elaborated by the organism. However, the protective roles of the other mechanisms cannot be entirely precluded.
Figure 11. Effect of manganese (μg/ml) on growth of strain P-442 in solution culture at pH 5.0.
3. Effects of pH on survival of *R. phaseoli* strains P-442 and S-442 in soil.

This experiment was conducted to test whether: 1) the acid and aluminum tolerance demonstrated by S-442 in culture solution would be duplicated in soil, and 2) survival of the strains would be affected by factors other than pH.

The Santo Angelo and Bom Jesus soils were used in this study. The chemical and physical characteristics of these soils have been given in Table 1. Santo Angelo was chosen because of its low natural pH (pH 4.2) and low aluminum content (0.8 me), and Bom Jesus was chosen also because of its low pH (pH 4.6) and high aluminum content (5.0-6.0 me).

a) Santo Angelo - pH 4.2 and 6.6.

Survival of the two strains was similar at both pHs, with only about 1 log drop in population by the end of the incubation period (Table 19). At pH 6.6, the initial decline coincided with a slight increase in pH of the soil from 6.6 to 7.1. This pH increase, though moderate, may have enhanced the growth of antagonistic or predatory organisms to populations high enough to effect the decline observed in the Rhizobium population (Damirgi and Johnson, 1966; Keya and Alexander, 1975).

Surprisingly, at pH 4.2, the comparative survival of S-442 and P-442 differed only slightly. There is no explanation concerning why only a small difference occurred in survival of the two strains differing greatly in acid-tolerance.

b) Bom Jesus - pH 4.6 and 6.8.

Except for P-442, survival at pH 4.6 was similar to that at pH 6.8 (Table 20). In the acid soil condition, the population of S-442 remained
Table 19. Survival of P-442 and S-442 in Santo Angelo soil at pH 4.2 and 6.6.

<table>
<thead>
<tr>
<th>Time (Days)</th>
<th>pH 4.2</th>
<th>pH 6.6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S-442</td>
<td>P-442</td>
</tr>
<tr>
<td>0</td>
<td>6.7</td>
<td>6.5</td>
</tr>
<tr>
<td>3</td>
<td>6.6</td>
<td>6.0</td>
</tr>
<tr>
<td>7</td>
<td>5.8</td>
<td>5.6</td>
</tr>
<tr>
<td>14</td>
<td>5.6</td>
<td>5.4</td>
</tr>
<tr>
<td>21</td>
<td>5.7</td>
<td>5.4</td>
</tr>
<tr>
<td>30</td>
<td>5.5</td>
<td>5.3</td>
</tr>
</tbody>
</table>
Table 20. Survival of P-442 and S-442 in Bom Jesus soil at pH 4.6 and 6.8.

<table>
<thead>
<tr>
<th>Time (Days)</th>
<th>pH 4.6</th>
<th></th>
<th>pH 6.8</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S-442</td>
<td>P-442</td>
<td>S-442</td>
<td>P-442</td>
</tr>
<tr>
<td>0</td>
<td>6.5</td>
<td>6.5</td>
<td>6.5</td>
<td>6.5</td>
</tr>
<tr>
<td>3</td>
<td>6.3</td>
<td>6.3</td>
<td>6.4</td>
<td>6.3</td>
</tr>
<tr>
<td>7</td>
<td>6.1</td>
<td>5.5</td>
<td>6.1</td>
<td>6.1</td>
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<tr>
<td>14</td>
<td>6.2</td>
<td>5.1</td>
<td>6.0</td>
<td>6.0</td>
</tr>
<tr>
<td>21</td>
<td>6.1</td>
<td>5.1</td>
<td>6.1</td>
<td>6.1</td>
</tr>
<tr>
<td>30</td>
<td>6.1</td>
<td>5.3</td>
<td>6.2</td>
<td>6.1</td>
</tr>
</tbody>
</table>
relatively stable throughout the incubation period, and was at least 1 log higher than P-442 from the seventh through the 30th day of incubation. This pattern of survival is suggestive of the tolerance of S-442 and the susceptibility of P-442 to acid soil conditions. Although biotic factors may be implicated in the population decline in the acid soil, it is more likely that a pH effect was demonstrated since a relatively stable population was maintained at the higher pH.

It is noteworthy that the pH of the soil dropped during the study from 4.6 to 4.2. Since the Bom Jesus soil contains a relatively high amount of aluminum (5.0-6.0 me), a pH drop accompanied by aluminum toxicity may also have had a detrimental effect on the Rhizobium population.

A comparison of survival in the two soils suggests that pH alone may have little effect on survival. The combination of low pH and high aluminum produces a greater stress.

4. Evaluation of P-442 and S-442 for dinitrogen fixation.

Based on the striking differences observed between P-442 and S-442, as shown by the pH, aluminum and manganese studies, a growth chamber study was conducted (Section B, No. 6e) to compare the dinitrogen fixing ability of these strains, without inclusion of such environmental variables. For P-442, the amount of dry matter produced, nodule dry weight, and percent N in the tops were 3.54 g, 0.26 g, and 3.3%, in the order given. Similar values for S-442 were 3.40 g, 0.29 g, and 3.3%. There was no significant difference in the amount of dinitrogen fixed by each strain.
In summary, the following points can be emphasized from the results of the overall study:

1) An acid-adapted strain S-442 was identified by strain selection in acid media.

2) The acid-tolerant selection combines acid-tolerance with aluminum (25 µM) and manganese (176 µg/ml) tolerance.

3) Acid-tolerance was demonstrated in the Bom Jesus soil but not in the Santo Angelo soil, probably due to the high aluminum content (5.0-6.0 me) of the former and low aluminum content (0.8 me) of the latter.

4) Even in the Bom Jesus soil, survival of both strains at a population greater than $10^5$ cells/g of soil after 30 days suggests that survival of *R. phaseoli* would not limit nodule initiation or dinitrogen fixation. Similar results were reported by Vidor in Brazil (per. comm.) using antibiotic resistant mutants.
4. SUMMARY

The dinitrogen fixing efficiency of nineteen *R. phaseoli* strains was tested in the growth chamber. These experiments were conducted in Leonard jars with exfoliated vermiculite or sand N-free nutrient solution as the growth medium. Ten strains were efficient dinitrogen fixers as measured by the amount of dry matter produced. In most cases, the dinitrogen fixing efficiency was below the +N control. This was improved by supplementing inoculated plants with 15 μg/ml nitrogen in the nutrient solution.

Some biochemical and physiological studies on the strains revealed variation in nitrate reductase activity, litmus milk reaction, carbohydrate utilization, antibiotic resistance, response to root exudates, and salt tolerance. Strain 442, CIAT 75, and CIAT 255 were the most sensitive, while CIAT 137 was the most resistant to the antibiotics tested. The inhibitory concentration of salts ranged from 0.1 to 1.0%. All the strains lacked the exogenous enzymes gelatinase, amylase, and permease, but all gave positive catalase and oxidase reactions.

Studies in acid broth adjusted to various pHs showed that the *R. phaseoli* strains have differential sensitivity to pH. Some strains have inherent ability to grow at pH < 5 but the majority of them grew at pH 5.0 to 7.2. An acid-tolerant selection (S-442) was identified from the parent strain 442 (P-442) during strain selection studies in acid media. This acid-tolerant selection combines acid-tolerance
with aluminum (25 \mu M) and manganese (176 \mu g/ml) tolerance.

Survival studies of P-442 and S-442 were done in Santo Angelo (Haplorthox) and Bom Jesus (Haplumbrept) using the FA technique. Acid tolerance was demonstrated by S-442 in the Bom Jesus but not in Santo Angelo soil, probably due to the high (5.0-6.0 me) and low (0.8 me) aluminum contents of the soils, respectively. Even in the Bom Jesus soil, survival of both strains at a population greater than $10^5$ cells/g of soil after 30 days suggests that survival of \textit{R. phaseoli} would not limit nodule initiation or dinitrogen fixation.

Plate count and FA studies showed that there was no significant differential effect of moisture on the strains studied. Survival was excellent under both sterile and non-sterile conditions. Short-term flooding of soils did not affect the survival and symbiotic dinitrogen-fixing capacity of the bacteria. In fact, this treatment appeared to have stimulated the activity of the \textit{Rhizobium} strains, as indicated by increase in nodulation and yield. However, excess moisture stress severely reduced dinitrogen fixation.

Differences exist between \textit{R. phaseoli} strains in their ability to withstand dessication. In sterile soil, the order of resistance was: P-442 > S-442 > C-05 > QA 1062 > CIAT 255. A similar order was obtained in non-sterile soil, with P-442 > CIAT 255. The decline in population occurred during periods of severe dessication, i.e. at moisture tensions greater than 15 bars.

Data obtained from these studies lead to the conclusion that nodulation failures associated with stress conditions like pH, high aluminum, and moisture stress in the tropics may be more an effect on
nodule initiation and the symbiosis, or on the host plant, than on the survival of the specific Rhizobium.
Appendix A

Sources and origin of *R. phaseoli* strains used in this research.

<table>
<thead>
<tr>
<th>Culture Number</th>
<th>Source of Culture</th>
<th>Original Number and Source (if known)</th>
</tr>
</thead>
<tbody>
<tr>
<td>QA-1062</td>
<td>Dr. E. L. Schmidt, University of Minnesota</td>
<td></td>
</tr>
<tr>
<td>127K17</td>
<td>Dr. J. Burton, Nitragin Co., Milwaukee</td>
<td>127K17 Nitragin Co., U.S.A.</td>
</tr>
<tr>
<td>127K21</td>
<td>Dr. J. Burton, Nitragin Co., Milwaukee</td>
<td>127K21 Nitragin Co., U.S.A.</td>
</tr>
<tr>
<td>400</td>
<td>Dr. Calo Vidor, Instituta de Pesquisus Agronomicas, Porto Alegre, Brazil</td>
<td></td>
</tr>
<tr>
<td>427</td>
<td>Dr. Calo Vidor, Instituta de Pesquisus Agronomicas, Porto Alegre, Brazil</td>
<td></td>
</tr>
<tr>
<td>439</td>
<td>Dr. Calo Vidor, Instituta de Pesquisus Agronomicas, Porto Alegre, Brazil</td>
<td></td>
</tr>
<tr>
<td>442</td>
<td>Dr. Calo Vidor, Instituta de Pesquisus Agronomicas, Porto Alegre, Brazil</td>
<td></td>
</tr>
<tr>
<td>445</td>
<td>Dr. Calo Vidor, Instituta de Pesquisus Agronomicas, Porto Alegre, Brazil</td>
<td></td>
</tr>
<tr>
<td>C-01</td>
<td>Dr. Elke Cardoso, Dept. of Plant Pathology</td>
<td></td>
</tr>
<tr>
<td>C-05</td>
<td>Escola Superior of Agricultura</td>
<td></td>
</tr>
<tr>
<td>C-09</td>
<td>Luiz de Queiroz, Univ. de Sao Paulo</td>
<td></td>
</tr>
<tr>
<td>C-14</td>
<td>Piracicaba, Brazil</td>
<td></td>
</tr>
<tr>
<td>CIAT 57</td>
<td>CIAT, Cali, Columbia</td>
<td>CC511; CSIRO Canberra, Australia</td>
</tr>
<tr>
<td>CIAT 75</td>
<td>CIAT, Cali, Columbia</td>
<td></td>
</tr>
<tr>
<td>CIAT 114</td>
<td>CIAT, Cali, Columbia</td>
<td></td>
</tr>
<tr>
<td>CIAT 137</td>
<td>CIAT, Cali, Columbia</td>
<td></td>
</tr>
<tr>
<td>CIAT 161</td>
<td>CIAT, Cali, Columbia</td>
<td></td>
</tr>
<tr>
<td>CIAT 255</td>
<td>CIAT, Cali, Columbia</td>
<td>Z272, Aislado por PROMYP, Honduras</td>
</tr>
<tr>
<td>CIAT 632</td>
<td>CIAT, Cali, Columbia</td>
<td>21, ICTA, Guatemala</td>
</tr>
</tbody>
</table>
Appendix B

Nutrient solution used in *R. phaseoli* strain selection.

### N-Free Solution

<table>
<thead>
<tr>
<th>Stock Solution (SS)</th>
<th>Grams/l</th>
<th>Full Strength Nutrient Solution (ml SS/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 M K$_2$SO$_4$</td>
<td>87.00</td>
<td>5.0</td>
</tr>
<tr>
<td>1.0 M MgSO$_4$ · 7 H$_2$O</td>
<td>246.00</td>
<td>2.0</td>
</tr>
<tr>
<td>0.05 M Ca(H$_2$PO$_4$) · H$_2$O</td>
<td>12.60</td>
<td>10.0</td>
</tr>
<tr>
<td>0.01 M CaSO$_4$</td>
<td>1.72</td>
<td>200.0</td>
</tr>
</tbody>
</table>

### Complete Nutrient Solution

<table>
<thead>
<tr>
<th>Stock Solution</th>
<th>Grams/l</th>
<th>Full Strength Nutrient Solution (ml SS/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0 M KH$_2$PO$_4$</td>
<td>136.1</td>
<td>1.0</td>
</tr>
<tr>
<td>1.0 M KNO$_3$</td>
<td>101.1</td>
<td>5.0</td>
</tr>
<tr>
<td>1.0 M Ca(NO$_3$)$_2$ · 4 H$_2$O</td>
<td>236.2</td>
<td>5.0</td>
</tr>
<tr>
<td>1.0 M MgSO$_4$ · 7 H$_2$O</td>
<td>246.5</td>
<td>2.0</td>
</tr>
</tbody>
</table>

### Micronutrient Solution

<table>
<thead>
<tr>
<th>Solution</th>
<th>Grams/l</th>
<th>Use 1 ml of SS per liter of nutrient solution.</th>
</tr>
</thead>
<tbody>
<tr>
<td>H$_3$BO$_3$</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>MnCl$_2$ · 4 H$_2$O</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>ZnCl$_2$</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>CuCl$_2$ · 2 H$_2$O</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>MoO$_3$</td>
<td>0.05</td>
<td></td>
</tr>
</tbody>
</table>

### Iron Solution

<table>
<thead>
<tr>
<th>Solution</th>
<th>Grams/l</th>
<th>Use 1 ml of iron solution per liter of nutrient solution.</th>
</tr>
</thead>
<tbody>
<tr>
<td>FeCl$_3$</td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td>Tartaric acid</td>
<td>5.0</td>
<td></td>
</tr>
</tbody>
</table>
**Appendix C**

**Soil extract-yeast extract-mannitol or galactose medium.**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast extract</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Mannitol/Galactose</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Soil extract*</td>
<td>200 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>800 ml</td>
</tr>
<tr>
<td>Agar</td>
<td>15 g</td>
</tr>
</tbody>
</table>

Adjust pH to 7.2 or as required.

*To 1000 g fertile soil add 1 liter of tap water and autoclave at 121°C and 1 atm for 20 minutes. Add 0.5 g CaCO$_3$ to flocculate colloidal material and filter to clarify.*
Appendix D

Biochemical Media

1. Nitrate Broth: Reduction of nitrates.

Formula: Grams/liter of distilled water.

Beef extract 3.0
Peptone 5.0
KNO₃ 1.0


Formula: Grams/liter of distilled water.

Gelatin 20.0
Glucose 0.05
Nutrient broth 1.0
KH₂PO₄ 0.5
K₂HPO₄ 0.5
Agar 15.0

pH 7.0 ± 0.2

3. Simmons' Citrate Slants.

Formula: Grams/liter of distilled water.

MgSO₄ 0.2
KH₂PO₄ 1.0
K₂HPO₄ 1.0
NaCl 5.0
Sodium Citrate 2.0
Agar 15.0
Bromthymol blue 0.08

pH 7.0 ± 0.2

Formula: Grams/liter of distilled water.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Grams/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef extract</td>
<td>3.0</td>
</tr>
<tr>
<td>Peptone</td>
<td>5.0</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0</td>
</tr>
<tr>
<td>Starch</td>
<td>2.0</td>
</tr>
</tbody>
</table>

pH 7.0 ± 0.2

5. Catalase and Oxidase.

3% H₂O₂

1% para-aminodimethylaniline oxalate (oxidase reagent)
-- must be prepared fresh.
Appendix E

Determination of proteins by the biuret method.

Biuret reagent: 0.175 g of CuSO₄ · 5H₂O was placed in a 100 ml volumetric flask and dissolved in a few milliliters of distilled water. Thirty ml of concentrated NH₄OH, 50 ml of cold distilled water, and 20 ml of 75% NaOH solution were added. The temperature of the mixture was brought to room temperature and the volume adjusted to 100 ml with distilled water. The reagent was stored in a dark container at a temperature of 4°C.

Standard curve: 0.1 g of bovine serum albumin (BSA) crystals was diluted in 50 ml of 0.85% NaCl solution to give a concentration of 2 g BSA/l. From this stock solution, the following concentrations were prepared: 0, 0.1, 0.3, 0.5, 0.7, 0.9, and 1.1 g BSA/l by taking 0, 1, 3, 5, 7, 9, and 11 ml from the stock solution and diluting it to 20 ml with physiological saline.

Procedure: 0.5 ml of antiserum of unknown concentration was diluted with 9.5 ml of physiological saline to give a 1/20 dilution. Three ml of the antiserum samples and of each standard were added to different test tubes and mixed with 2 ml of biuret reagent. The optical density of the mixtures was determined at 540 nm immediately after mixing. Protein concentration in the sample was obtained from the standard curve and multiplied by the dilution factor to obtain the total protein concentration in the sample.
Appendix F

Soil extract agar.

- Glucose: 1.0 g
- $\text{K}_2\text{HPO}_4$: 0.5 g
- KNO$_3$: 0.1 g
- Agar: 15.0 g
- Soil extract*: 100 ml
- Tap water: 900 ml

Adjust to pH 6.5-7.0.

*To 1000 g of a fertile soil add 1000 ml of tap water and autoclave at 121°C and 1 atm for 20 minutes. Add 0.5 CaCO$_3$ to flocculate colloidal material and filter to clarify.
Appendix G

Rose-bengal-streptomycin agar.

Glucose 10.0 g
Peptone 5.0 g
KH₂PO₄ 1.0 g
MgSO₄ · 7H₂O 0.5 g
Agar 15.0 g
Tap water 1000 ml
Rose-bengal 0.033 g

Streptomycin: Sterilize the medium by autoclaving at 121°C and 1 atm for 20 minutes. When ready to pour agar into plates add streptomycin. The antibiotic solution must be sterilized by filtration and added aseptically to the medium at a final concentration of 30 µg/ml.
Appendix H

Moisture tension curve for Ross moyne soil.

17% = 1.2 bar
23% = 0.33 bar
40% = 0.1 bar
Bibliography


212. Van Egeraat, A. W. S. M. 1975a. Changes in free ninhydrin-positive compounds of young pea plants as affected by different nutritional and environmental conditions.


