STUDIES ON SOME ECOLOGICAL ASPECTS OF *Cicer Rhizobium* AND THE EFFECTS OF *Rhizobium* INOCULATION METHODS ON CHICKPEAS (*Cicer arietinum* L.)

A Thesis

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Banyong Toomsan

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ABSTRACT

Toomsan, Banyong. Ph.D., The University of Manitoba, October, 1981. Studies on some ecological aspects of *Cicer Rhizobium* and the effects of *Rhizobium* inoculation methods on chickpeas (*Cicer arietinum* L.).

Major Professor: Dr. K.W. Clark.

A technique of dwarfing chickpea plants by cutting the cotyledons off the germinating seedling enabling them to be grown and nodulate in test tube conditions was developed. Chickpea cultivar 850-3/27 growing in a sand medium was chosen as a "trap host" for the entire research program. It proved to be useful in counting chickpea *Rhizobium* in both pure cultures, soils and in unsterilized peat inoculum.

The technique was used to study chickpea *Rhizobium* populations in the fields at ICRISAT (International Crop Research Institute for the Semi-Arid Tropic) Centre and some Indian soils during the dry summers of 1978-1980. In general, the Alfisol fields at ICRISAT Centre that had no chickpea history were low in chickpea *Rhizobium*; once chickpeas had been grown in this soil the population of *Rhizobium* was high. Paddy fields were found to be low in numbers of *Rhizobium* even though chickpeas were grown just 2 years previously. The populations of the chickpea *Rhizobium* were found to change with soil profile depth and growing season.

Chickpea *Rhizobium* populations were highest when soil samples were taken within the plant row, and decreased with increasing distance from the plant. The rhizosphere of chickpea, groundnut, pigeonpea, sorghum and pearl millet were found to be stimulatory or at least not inhibitory to the growth of chickpea *Rhizobium* in pot experiments using an Alfisol and a Vertisol soil.

The survival of two Rhizobium strains inoculated on chickpea seeds using five different stickers was studied in the laboratory conditions. The stickers do not differ in terms of their sticking ability and prolongation of Rhizobium survival.

The effect of different stickers and inoculation methods was also studied under three field conditions. None of the inoculated treatments gave significantly higher yields than the uninoculated control. Liquid inoculation method was found to be superior to the conventional seed slurrying method using methyl cellulose as a sticker in terms of percentage of nodules formed by the inoculated strains under the three field conditions. The inoculated Rhizobium were identified by using a high level streptomycin resistant mutant and a low intrinsic antibiotic resistant character.

FOREWORD

This thesis had been prepared in manuscript format, specified in the 1976 Plant Science Thesis Preparation Guide. It consists of four sections. They are

Section 1: Introduction
Review of literature

Section 2: Results of research in manuscript forms (Manuscripts 1 to 3).

Section 3: Discussion of entire research programme reported in section 2.

Section 4: Bibliography
Appendices

GENERAL INTRODUCTION

The intake of protein in different parts of the world varies widely. The diets of hundreds of millions of people are deficient, and often desperately deficient in protein (Dawson 1970). Protein of vegetable origin is the mainstay of developing countries, whereas the bulk of protein in developed countries is of animal origin. With an increasing price of nitrogen fertilizer in recent years, the tremendous potential of the legume Rhizobium symbiosis is likely to be an important factor in meeting the protein requirement of the people in the developing countries and in increasing total crop productivity in the developed agricultural economies. The International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) was established in 1972. Chickpea is among its five mandate crops. The other four mandate crops are groundnut, pigeonpea, pearl millet and sorghum. Its objectives are improvement of these five SAT (Semi Arid Tropical) crops and development of farming systems to stabilize and increase production.

Chickpea is the major pulse legume with 5.8 million tons produced on 9.5 million hectares. Ninety percent of the world production is in the SAT region. Seventy-four percent of this production occurs in India, followed by 10% in Pakistan and 4% in Ethiopia (Dart and Krantz 1977).

Chickpea is very specific in its Rhizobium requirement (Bhide 1956, Gaur and Sen 1979, Habish and Khairi 1968, Raju 1936, Rasumoskaja 1934). It is also very sensitive to high temperature. Increasing soil tempera-

ture beyond 30°C resulted in nodulation failure (Dart, Day and Islam 1975, Dart, Islam and Eaglesham 1975, Islam 1975).

For legumes to be cultivated vigorously and successfully, we must know the requirements of both symbionts, alone and together. In this context, the ecology of Rhizobium has been neglected sadly as has a proper approach to the production and application of inoculants (Gibson 1981). Control over inoculant quality is essential for the potential of inoculation practice to be achieved, yet few countries have recognized its significance. The study of chickpea Rhizobium ecology and control over inoculated quality has been hampered by the fact the chickpea is very specific for its Rhizobium requirement. Unlike soybeans, groundnut and pigeonpea, there is no alternative test host for this very specific Rhizobium sp. Glycine ussuriensis Regel and Mack has been successfully used in counting Rhizobium japonicum. Macroptillium atropurpureum is used for counting Rhizobium of groundnut and pigeonpea (cowpea group Rhizobium).

The objectives of these studies were to:

1) Develop a technique that can be used reliably in counting chickpea Rhizobium in both pure cultures, soil and peat inoculum.

2) Study chickpea Rhizobium population change during soil storage, fields, locations, seasons, depths, colonization on roots of different crops and dispersal at the end of a growing season.

3) Study the effect of different stickers and inoculation methods on chickpea Rhizobium survival in the laboratory and chickpea yield under field conditions. The success of the inoculation method is measured by yield and percent nodules formed by the inoculated strain. Identification of the inoculated strain was done by using a streptomycin resistant marker (str 200) coupled with a low level intrinsic resistant marker.

LITERATURE REVIEW

Chickpea (Cicer arietinum L.)

Origin and Geographical Distribution

Chickpea (Cicer arietinum L.) is one of the oldest and most widely used grain leguminosae in the Middle and Far East. It has a long history of cultivation, preceding biblical times. The earliest reference is on an Egyptian papyrus-roll, dating between 1500 to 1100 B.C., where chick-pea is called 'falcon-face', a resemblance to its seed shape. Homer mentioned "erebinthos" in his Iliad (1000 - 800 B.C.). The Sanskrit name "chanaka" also points to cultivation preceding the Christian era. The Romans knew chickpea as "Cicer", hence the Latin name for the genus. The specific name arietinum owes its origin again to resemblance to a ram's (aries) head (Krios of the Greeks).

The earliest known occurrence of the chickpea and a specimen probably belonging to a wild species of Cicer were reported from the Hacilar site near Burdur in Turkey. The deposits in these layers were dated by the C^{14} method to about 5450 years B.C. (Helbaek 1970, van der Maesen 1972).

Vavilov (1951) included the chickpea in no less than five centres of origin, now regarded as centres of diversity of cultivated plants:

1) The Indian (Hindustan) centre including Burma, Assam but not N.W. India,
2) The central Asiatic centre including N.W. India, N.

Pakistan, Afghanistan, Tadjikistan, Uzbekistan, W. Tian-Shan,

3) The near Eastern centrewith Asia minor and Transcaucasia,

4) The Mediterranean centre, and

5) The Abyssinian centre (Ethiopia).

There are several opinions about where Cicer originated as a genus. Popov (van der Maesen 1972) visualized Cicer as a comparatively young and incompletely differentiated group with roots in the genera Vicia and Ononis. This would have happened in the Miocene age, which ended 7 million years ago. The chickpea he visualized as an artificial product, created by man, rather than originating in Southern Europe as De Candolle (van der Maesen 1972) had pointed out; possibly in Asia Minor he believed more likely.

The most recent information centres around the findings of wild growing species related closely to chickpea, which itself had never been found truly wild, only as escapes from cultivation. This is the Fertile Crescent, and in particular N. Syria, S. Turkey and adjacent Iran and Iraq (van der Maesen 1972). The nearest relatives, Cicer echinospermum Davis and Cicer reticulatum Ladiz, were newly described in 1975. Hybrids with the chickpea, C. reticulatum Ladiz x C. arietinum L., were obtained and pointed to the possibility of being its progenitor (Ladizinsky and Adler 1976). From the Fertile Crescent the chickpea spread eastward and westward. It is now cultivated in over 31 countries, from Mexico and the USA through Spain and Mediterranean countries, Morocco and North African countries, to West Asia and to India.

The cultivated chickpea can be classified into two types:

1) Kabuli types: These are large seeded, ramshead-shaped

or round seeded chickpeas with more than 26 g per 100 seed, and creamy colour. Plants are medium to tall with large leaflets, no anthocyanin and white flowers and are characteristic of the Mediterranean region.

2) Desi types: These are small seeded types, irregularly shaped and variously coloured, plants are small with small leaflets (6 - 9 mm), sometimes prostrate and mostly with anthocyanin and purplish pink flowers. These are characteristic of East Asia, Ethiopia, parts of Iran and Afghanistan.

Chickpeas can also be grouped into winter (October/November) plantings, mainly of Desi type - from Pakistan eastward, but also in Ethiopia, Sudan, Mexico and Chile; and summer planting, mainly Kabuli type, from Afghanistan westwards into the Middle East, Southern Europe and North Africa. Desi types adapted to summer plantings overlap in Iran and Afghanistan.

Botany

Cicer includes 40 species (van der Maesen 1972). Only Cicer arietinum is cultivated. The wild species occur in West and Central Asia and the Mediterranean. Most have tiny rugose seeds, except C. bijugum, C. echinospermum and C. reticulatum with large seeds but also rough seed coats, even reticulate or spiny. Wild species are occurring in dry conditions, growing in rubble, some occur in the forests of Greece, Turkey and Iran. Perennial species can have tendrils or spines and the leaflets can be reduced to small perules. In India, C. microphylum can be found (Kashmir, Lahaul and Spiti).

Plant Habit. Cicer arietinum L. is a herbaceous annual, branching mainly close to the ground. Some cultivars are semi-erect or erect with only a few main branches; others are semi-spreading with profuse branching. Plant height is normally between 20 - 45 cm, sometimes almost a meter or more. The tap root when pulled up is 15 - 30 cm, but the root can extend to 1 m or deeper. Generally tolerant to drought, the plant usually grows on conserved moisture and dew. Cool conditions are preferred, frost and snow can be withstood in early growth stages. All parts of the plant are covered with glandular hairs, producing a sticky acid secretion.

Stem. The main stem, often not producing flowers, is rounded; the branches are ribbed, straight or flexuous.

Leaves are imparipinnate, with mostly 11 - 13 leaflets, sometimes more and often less in basal leaves. The leaflets vary in size between 6 and 20 mm long and 3 - 14 mm wide; their margin is serrate. Various simple or compound mutants exist. Stipules are toothed or simple. The acid secretion can be collected with the dew.

Flowers are typically papilionaceous, zygomorphic with a campanulate calyx and five petals of which the standard (vexillum) is the largest in the top, orbicular, flanked by the wings half as broad as the standard, the keel consists of two partly jointed petals enclosing the stamens (9+1) and pistils. The flowers are borne on single flowered racemes, jointed to the peduncle by the pedicel. A small arista is a remnant of further flowers. In some types double-flowered racemes occur, but both may not set seed. The flower with the purple pedicel should be selected for crossing.

Fruits are inflated legume of ovate-oblong or squarish shape. These contain one to two seeds attached to the ventral suture, rarely three to four.

Seeds vary in size (4 - 11 mm long or 4 - 75 g/100 seeds) are beaked and round, wrinkled or angular in shape. Seed colour is either brown in various shades, yellowish, orange, cream, green or black. The seed coat can be smooth or rough. The cotyledons are yellow in various shades, or green in the greenseeded cultivars. Germination is hypogeal, the cotyledons remain in the soil.

Chickpea is self pollinated. Flowering habit is indeterminate and flowering is hastened by long day, with large differences in this response between cultivars (Dart, Islam, Eaglesham 1975).

Self-pollination takes place in the bud or half-open flower stage (cleistogamy). Flowers may open on one, two or three successive days, hours depending on the location. Cross-fertilization by bees is very rare.

Production, Use and Agronomy

The world production area of chickpea is about 9.5 million hectares with an output of about 5.8 million tonnes and average yield around 600 kg/ha (Dart and Krantz 1977). Ninety percent of the total production is in the semi-arid tropics. Seventy-four percent of the total world production is in India, followed by 10% in Pakistan and 4% in Ethiopia. Other countries, in decreasing order of production, are Mexico, Burma, Spain, Morocco, Turkey, Iran and Tanzania. The average yields in India are currently about 580 kg/ha (Dart and Krantz 1977) and have declined by 9% over the last 14 years.

Chickpeas are eaten raw or roasted, but are usually consumed after

boiling. In India, Pakistan and Bangladesh this is usually in the form of 'dhal', prepared by splitting the seeds, separating them from the testa, and boiling with spices. Chickpea flour is used in many Indian confectionaries or in biscuits (in Ethiopia). In Arabian countries 'hammas' is prepared and in Ethiopia chickpeas are boiled, ground and mixed with spices to make 'shero wat'.

Chickpeas are grown on a variety of soils from heavy clay Vertisols to silt loams and loess soils, to more sandy soils, e.g. parts of Rajasthan, usually with neutral to alkaline pH. The crop is quite susceptible to saline soil conditions and kabuli types to iron deficiency, producing a leaf chlorosis. Chickpeas are usually grown as a rainfed crop on residual moisture although responses to irrigation, can be obtained. This needs to be carefully controlled and a single irrigation during pod fill is often beneficial (Saxena and Yadav 1975). The response to phosphorous fertilizing has been variable, possibly depending on the soil P status, but this may be related to the location of the added phosphorus in the upper soil layers which usually become progressively drier during the season, and since P's largely immobile in soil, plant roots active in water and nutrient uptake do not have access to the added P.

Heliothis armigera is the only major insect pest (Davies and Lateef 1975). Wilt caused by Fusarium oxysporum, root rot by Rhizoctonia batatiocola, and a stunt disease transmitted by Aphis craccivora are widespread in India and can reduce yields considerably. Ascochyta blight can also be serious under some environmental conditions (Nene and associates 1976).

Cicer Rhizobium Classification

In the 8th edition of Bergey's Manual (Buchanan and Gibbons 1974), Rhizobium was one of the genera which made up the Family Rhizobiaceae within the Order Eubacteriales.

Rhizobium are mainly classified according to their ability to form nodules on plants in cross-inoculation groups. Fred et al (1932) defined cross-inoculation groups as 'groups of plants within which the root nodule organisms are mutually interchangeable'.

Fred et al (1932) classified Cicer Rhizobium in pea group, Rhizobium leguminosarum Frank, but a footnote revealed that Cicer Rhizobium does not belong to this group.

Other workers (Joshi 1920, Rasumowskaja 1934, Raju 1936, Bhide 1956, Habish and Khairi 1968) advocated placing Cicer arietinum rhizobia in a separate group. However, these findings were based either on tests with root-nodule suspensions instead of pure strains of Rhizobium (Rasumowskaja 1934), or on non-reciprocal cross-inoculation tests involving either a single strain of Rhizobium or a host species from different cross-inoculation groups (Joshi 1920, Bhide 1956, Habish and Khairi 1968). Although Raju (1936) conducted reciprocal cross-inoculation tests, only a few strains of Rhizobium and legume species were included.

Gaur and Sen (1979) studied cross-inoculation group specificity in Cicer arietinum L. Seventy-one strains of root nodule bacteria of C. arietinum were examined for nodulation on 87 species of legumes. These species represent all the known cross-inoculation groups and were selected from various tribes and genera of family Fabaceae and Mimosaceae. In a reciprocal cross-inoculation study, 287 strains of root nodule bacteria from 52 of the 87 species, were examined on various genotypes of C.

arietinum. Cicer arietinum and its root nodule bacteria did not show cross-inoculation affinity with any of the members of the known cross-inoculation groups; alfalfa, clover, pea, bean, soybean, lupin, lotus and cowpea miscellany; except for some loose non-reciprocal kinship with Sesbania, which in its turn has strong affinity with the cowpea miscellany. They suggested that Cicer arietinum and its root nodule bacteria should be considered in a separate cross-inoculation group.

Methods for the Examination of Soil and Rhizosphere Populations

The detection, identification and enumeration of strains of Rhizobium in the soil, rhizosphere and nodule can be achieved by the application of various bacteriological principles and techniques (Vincent 1970). The inherent character of the rhizobia to produce distinctive nodules on legumes permits them to be counted in the presence of the natural soil and rhizosphere population by the "plant dilution" technique (Date and Vincent 1962, Brockwell 1963a). Even when Rhizobium are present in the complex soil or rhizosphere environment in high numbers, direct isolation from agar plates is impractical due to the more vigorous growth and higher numbers of other soil micro-organisms. Three methods generally used in enumeration of root nodule bacterial in soil are listed below.

Selective Media

The potential usefulness of a selective medium for Rhizobium spp. has long been recognized (Fred et al 1932), but Rhizobium differ widely in their physiological characteristics (Graham and Parker 1964) and it would be difficult to develop a complete selective medium that would inhibit all micro-organisms except Rhizobium spp. Nevertheless, a medium more selective than those normally used would facilitate the isolation and purifica-

tion of strains. Anderson (1929) added crystal violet to the medium when isolating Rhizobium leguminosarum in an attempt to suppress growth of Bacillus (Agrobacterium) radiobacter. Allen and Baldwin (1931) claimed that they were able to isolate alfalfa and clover nodule bacteria from four Kentucky and two Wisconsin soils using capillary tubes containing selective medium and suspended in a water suspension of soils, the contents of the tubes were plated after 1, 12 and 24 hours on brom-thymol blue, yeast extract mannitol agar. Graham (1969) developed a new medium for selectively isolating strains of Rhizobium from soil consisting of (g/1) mannitol, 5.0; lactose, 5.0; K_2HPO_4, 0.5; NaCl, 0.2; $CaCl_2 \cdot 2H_2O$, 0.2; $MgSO_4 \cdot 7H_2O$, 0.1; $FeCl_3 \cdot 6H_2O$, 0.1; yeast extract, 0.5; agar, 20.0. After autoclaving the above ingredients, cyclohexamide (200 mg), pentachloro-nitro benzene (100 mg), sodium benzyl penicillin (25 mg), chloromycetin (10 mg), sulfathiazole (25 mg) and neomycin (2.5 mg) are added. Congo red may also be added 2.5% of a 1% solution. The pH is then adjusted to 7.0. However, this medium requires further study with Rhizobium showing diverse characteristics from different soils before it can be recommended.

Obaton (1971) used streptomycin to develop mutants in Rhizobium trifolii and it has a potential value in ecological studies. Schwinghamer and Dudman (1973) examined resistance to the antibiotic spectinomycin as a possible marker to supplement streptomycin resistance in ecological or genetic studies with rhizobia. Single step spontaneous mutants resistant to high levels of spectinomycin were isolated from eight effective strains representing four species of Rhizobium. There was no evidence of cross resistance to streptomycin and streptomycin resistant mutants were not cross resistant to spectinomycin. Immunodiffusion showed that there were minor changes in antigenic characteristics for mutants from two strains

but these variants were still identifiable with the parent strains. Partial or full loss of symbiotic effectiveness occurred in about 20% of the spectinomycin resistant mutants and the resistance marker was unchanged through one plant passage. It was concluded that the spectinomycin resistant mutant properly evaluated for possible pleiotropic effects should provide a useful marker system for use alone or in combination with streptomycin resistance in Rhizobium.

Pattison and Skinner (1974) tested the sensitivity of 47 strains of Rhizobium to six antibiotics (chloramphenical, erythromycin, penicillin, streptomycin, sulphafurazole and tetracycline) using Oxoid Multodisks on yeast extract - mannitol agar (YMA); penicillin was the least inhibitory. Growth of selected strains on YMA was also compared with that on YMA containing one or more antimicrobial substances. Penicillin (1 i.u./ml of YMA) only slightly inhibited growth of some strains that were sensitive to it by the Multodisk test but higher concentrations were too inhibitory to be used in a selective medium. YMA containing pentachloronitrobenzene (5 ppm suspended in a 5 ppm solution of Triton X-100), brilliant green (0.5 ppm) and sodium azide (0.5 ppm) did not inhibit 15 of 18 strains of rhizobia but was more inhibitory to the general microflora of four soils than was YMA. This medium (ABPA) was very inhibitory to several Rhizobium strains when supplemented with 1 i.u./ml of penicillin.

Selective media employing antibiotics in various concentrations and combinations, have been used successfully for the enumeration of specific strains of rhizobia, in the presence of other known soil micro-organisms, in model systems of soil and rhizosphere (Trinick 1970 cited in Parker et al 1977). Differentiation between strains of Rhizobium by antibiotic resistance (Kanamycin 2 µg/ml and streptomycin 1.5 µg/ml) has also proven

useful in competition studies between strains in nodule formation in Medicago sativa, Medicago trunculata, Trifolium repens, Trifolium glomeratum and Trifolium subterraneum (Pinto et al 1974).

Incorporation of congo red into medium is known to help differentiate the Rhizobium from closely related bacteria (Fred et al 1932, Hahn 1966, Roughley and Vincent 1967, Vincent 1970). Rhizobium absorb the dye weakly whereas many other bacteria take it up strongly. A tenfold increase in the congo red concentration in agar can enhance the selectivity of the medium for Rhizobium by intensifying the color reaction and inhibiting the growth of gram positive organisms (Hahn 1966).

Fluorescent Antibody (FA)

This technique has been modified for soil and legume rhizosphere counts of Rhizobium spp. Aliquots of a soil or root suspension, allowed to stand for 2 hours, are passed through non-fluorescent membrane filters that are stained by FA and examined by incident light fluorescent microscopy. A comparison of the FA membrane filter rhizosphere count with the conventional plate count, using media containing antibiotics to suppress non-rhizobial organisms showed both methods to be in close agreement. Other organisms and particles did not interfere with the estimation (Trinick 1970 cited in Parker et al 1977).

Plant Dilution Technique

Wilson (1926) initiated the 'plant dilution' method by exposing suitable host legumes to aliquots of a dilution range of soil water, eg. 10^1 - 10^6 and used the nodulation of the test plants to estimate populations of rhizobia in soil. However, dilution methods of counting involve two basic assumptions. That the organisms are randomly distributed throughout

the dilutent, and that one or more organisms will induce nodules on the test seedling. This approach is placed on a quantitative footing by the most probable number theory and numerous versions now exist (Tuzimura and Watanabe 1961a, Date and Vincent 1962, Brockwell 1963a, Ham and Frederick 1966, Elliot and Blaylock 1971, Weaver and Frederick 1972, Brockwell et al 1975, Grassia and Brockwell 1978). The plants are grown in tubes containing agar, sand and vermiculite or Leonard jars or pots containing soil, or plastic bags containing nutrients ("growth pouches"). Date and Vincent (1962) claimed that the dilution method is likely to underestimate the number of viable Rhizobium by a factor not greater than two. When the test plants are grown in certain particulate substrates (eg. sand or vermiculite) the rhizobial population is likely to be underestimated by a factor of 10 to 100 in pure cultures (Thompson and Vincent 1967, Vincent 1970). The difference seems likely to be due to discontinuity between the point of application of the rhizobia and the invadible portion of the root. For counts of non-sterile material there is a much greater chance of non-random distribution of Rhizobium because nodules and aggregates of soil and root gums containing rhizobia may not be completely disrupted during the dilution procedures. Brockwell et al (1975) showed that seedlings grown in tubes containing agar gave a better estimation of the number of Rhizobium than seedlings grown in tubes containing vermiculite. The underestimation was attributed to reduction of the rhizosphere in vermiculite culture compared with agar culture when the rhizosphere effect probably extend throughout the medium. A special calculation is required for the most probable number of Rhizobium when the test plants are grown in vermiculite (Grassia and Brockwell 1978).

Ham and Frederick (1966) compared the relative efficiency of different

laboratory tests in detecting Rhizobium meliloti when alfalfa seeds were inoculated with known numbers of rhizobia. The inverted bottomless bottle (modified Leonard) and crock techniques, which utilize more than one seed per container overestimated the number of rhizobia present on alfalfa seeds due to the transfer of rhizobia among seedlings in the test container. The agar tube technique tended to underestimate the number of rhizobia present perhaps because the anaerobic conditions in the support medium inhibited nodule formation. Using sand as the support medium in the tube gave the most accurate result of direct plate counts.

Methods or selection of the appropriate dilution factor and number of replicate seedlings for the enumeration of rhizobia in the soils have been adequately defined (Vincent 1970). Furthermore, the method may underestimate populations when a concentrated soil suspension, or soil itself is added to the test seedlings (Vincent 1965). Thompson and Vincent (1967) detected viable Rhizobium in soil with very low rhizobial populations by means of the soil core technique; with the same soil no Rhizobium were found by the plant dilution technique. Similarly, viable Rhizobium had been obtained from the agar in which non-nodulated test seedlings had grown Such complications are more likely to occur in agar culture than sand culture and emphasize the value of semi-quantitative, direct methods (eg. growing the legume in a soil core) for estimating Rhizobium in sparsely populated soils (Thompson and Vincent 1967). Interference from microflora can cause skips at lower dilutions in the series of positive tubes in a dilution series and underestimate the number of Rhizobium. This occurs in agar tubes. Robinson (1968) found that the use of antifungals, such as mycostatin, in plant dilution counts, showed some promises in overcoming the underestimation of populations due to interference from microflora

added to the test seedling with the early dilutions. Mycostatin could be added to the medium in the concentration up to 200 ppm. However, actidione was found to be very toxic to the test plants and not recommended.

The Most Probable Number as Estimated by Fisher and Yates Method. In the plant dilution test, a series of suspensions of the rhizobia organisms, five levels in all, is prepared, each of which is a times as dilute as the preceding one. Each suspension is used to inoculate n tubes with a known volume of the suspension, and the plants left to grow before examining for the presence or absence of nodules. It has been shown that 87.7% of the information on the number of organisms per tube at any given level is contained in the total number of positive (X) or negative tubes (Y), counted without regard to level (Fisher and Yates 1963). If λ is the number of organisms per tube at the highest concentration, the values of λ for which the expected average number of sterile plates is equal to the observed number as given by the equation:

$$Y = n\,(e^{-\lambda} + e^{-\lambda/a} + e^{-\lambda/a^2} + \ldots\ldots\ldots + e^{-\lambda/a^{s-1}})$$

Where Y = total sterile or negative tubes

n = number of tubes per dilution step

e = natural logarithm

λ = number of organisms per tube at the highest concentration under test

a = dilution factor

s = total number of dilution steps under test.

Table VIII 2 (W.L. Stevens) in Fisher and Yates (1963) enables the solution of this equation to be obtained expeditiously for twofold, fourfold and tenfold dilution series of any length. The steps involved in

calculating the MPN by Fisher and Yates (1963) are as follows:

1. Assessment of the total number of positive (X) and negative (Y) tubes.
2. Calculation of the mean fertile (x) or mean sterile (y) levels.

$$x = X/n$$

$$y = s - x$$

Where x = mean fertile levels

X = total positive tubes

n = no. of tubes per dilution

y = mean infertile levels

s = dilution steps

3. Enter Table VIII 2 with either x or y value and find the K value according to the number of dilution steps.
4. Find the number of organisms per tube (λ) at the highest concentration under test using the formula:

$$\log \lambda = x \log a - K$$

$$\lambda = \text{antilog } (x \log a - K)$$

5. Calculate the number of organisms/unit weight of original sample using the formula:

$$MPN = \frac{\lambda \cdot d}{v \cdot g}$$

Where λ = number of organisms/tube at the highest concentration in the series under test

d = dilution represented by tube at the highest concentration

v = volume of aliquot

g = weight or volume of sample

6. Calculate the 95% fiducial limits of MPN. This can be done by two different ways:

a) Calculate variance of mean fertile level using the formula:

$$V(x) = \frac{1}{n} \frac{\log 2}{\log a}$$

b) Calculate the variance of log λ using the formula:

$$V(\log \lambda) = \frac{1}{n} \log 2 \log a$$

If V(x) is calculated, the next steps are:

i) Calculate 95% fiducial limits of x using the formula:

95% fiducial limits = $x \pm t\ 0.5,\alpha \cdot S\bar{x}$

Where x = mean fertile level

$S\bar{x}$ = standard error = $\sqrt{V(x)}$

ii) Re-enter the Table VIII 2 with these two calculated figures, repeating steps 4 and 5.

If V(log λ) is calculated, the next steps are:

i) Calculate 95% fiducial limits of log λ using the formula:

95% fiducial limits = $\log \lambda \pm t\ 0.5,\alpha \cdot S\bar{x}$

ii) Change the logarithmic number to actual number and then repeat step 5.

Example: Tests with potato flour containing rope spores (B. mesentericus) gave the following observations using five tubes, each of 1 c.c. of dilutions containing 4, 2, 1 ..... 1/128 g per 100 cc (Fisher and Yates 1963).

g/100 cc	Number fertile	g/100 cc	Number fertile
4	5	1/8	3
2	5	1/16	2
1	5	1/32	2
1/2	5	1/64	0
1/4	4	1/128	0

1. Total positive (X) and negative tubes (Y) are 31 and 19 respectively.

2. Mean fertile level (x) = $\frac{31}{5}$ = 6.20

   Mean infertile level (y) = 10 - 6.20 = 3.80

   or $\frac{19}{5}$ = 3.80

3. Using x = 6.20, y = 3.80, the K value was found to be 0.383.

4. $\log \lambda$ = 6.20 log 2 - 0.383 = 1.483

   = 30.4

5. MPN = $\frac{30.4 \times 100}{1 \times 4}$ = 760

6. a) $V(x) = \frac{1}{5} \frac{\log 2}{\log 2}$ = 0.2

   b) $V(\log \lambda) = \frac{1}{5} \log 2 \log 2$

   = 0.0181

   - When V(x) is calculated:

   i) 95% fiducial limits of x = 6.20 $\pm$ 1.96 x $\sqrt{0.2}$

   = 5.323 and 7.077

   ii) λ = antilog 1.211 and 1.761

   = 16.26 and 57.68

   MPN = 407 and 1440

- When V(log λ) is calculated

i) 95% fiducial limits of log $\lambda = 1.483 \pm 1.96 \sqrt{0.0181}$

$= 1.219$ and 1.747

ii) λ = antilog 1.219 and 1.747

$= 16.56$ and 55.85

MPN = 415 and 1400

Cochran (1950) calculated the 95% fiducial limit factors using another version. These factors depend on a dilution factor and the number of tubes per dilution. The factors are used to multiply or divide the MPN to get the upper and lower limits of the 95% fiducial limits.

The MPN as calculated by using Fisher and Yates method are attached in this paper (Appendices 37 to 40).

Ecology of Rhizobium

One of the main problems in studying Rhizobium ecology in soil is the paucity of methods. If one is to delve into the relationships of these organisms in soil, techniques must be available to assess directly what these interrelationships are. A common procedure is to plant a suitable host legume into the test soil and to observe nodulation, but clearly this technique is inadequate for ecological purposes because of the unknown numbers of organisms required to induce nodulation under soil conditions, the inability of the method to localize the site of microbial action and the prolonged time required for information to be obtained. The results derived from plant testing are of great practical value, but the data frequently cannot be interpreted in useful ecological terms.

The development of the plant-dilution technique by Wilson (1926) enables us to enumerate the number of Rhizobium in contaminated cultures and

in its natural habitats. Many determinations have been made of the populations of Rhizobium in soils that have been deliberately inoculated. Nearly all of these studies were performed by making dilutions of the soil, applying the dilutions to sterile soil or agar with a legume and, following growth, examining the roots for nodules. Summarizing these many studies is very difficult because almost no generalizations are possible.

Wilson (1926) sampled soil from fields of known cropping history and showed a wide variation in the number of nodule bacteria. For alfalfa the number ranges from none per 5 g soil to 10^5 per g. Low pH reduced the number of Rhizobium. The population of nodule bacteria for Trifolium repens was reduced from 10^6 to 50 as the soil pH decreased from 6.8 to 5.4.

Wilson (1930) noted seasonal fluctuation in Rhizobium number. He collected soil samples from 10 (small) plots eight times between October 11, 1928 and June 3, 1929. Except in two plots there was a marked decline in the number of R. trifolii and R. leguminosarum as the winter season advanced. This decrease did not occur at uniform time in the different treatments. As the temperature increased in the spring and the conditions became favourable for growth and multiplication, the bacteria of both species increased until they were in most cases as numerous in June as they had been in October.

Wilson (1931) observed that Rhizobium leguminosarum counts ranged from less than 10 to more than 10^5/g in soils from New York state, whereas the number of Rhizobium trifolii ranged from 2,500 to 10^6/g.

Hely et al (1957) found that the population of Rhizobium trifolii increased during the growing season and then declined. The numbers of Rhizobium trifolii rose from 5/g soil before germination (May) to 75,000/g soil at senescence (November), but fell to 50/g soil by late summer.

Walker and Brown (1935) used Wilson's modification of the dilution method to determine the approximate numbers of Rhizobium meliloti and Rhizobium trifolii in various treated soils at the Agronomy Farm at Iowa State College. They found that, in general, the number of these root nodule bacteria in soils depended upon the previous cropping history of the land and also upon the previous fertilizer treatments. Large numbers of both species were found in the soil of the 3-year rotation plots where mixed red clover and alfalfa were grown every third year (corn-oat-legumes) and few in the soil of the 2-year rotation plots (corn-oat) where legume crops had not been grown for over 20 years. The numbers of Rhizobium meliloti present in soil where alfalfa had been plowed up a month previous to sampling were greater than the fields where alfalfa had not been grown on the land for over a year. Applications of crop residues, manure, limestone and rock phosphate each enabled the soil to support a large number of alfalfa and red clover root nodule bacteria. The largest numbers of these organisms occurred in soils receiving a combination of these treatments.

Nutman and Ross (1969) reported the numbers of Rhizobium trifolii, Rhizobium leguminosarum, Rhizobium meliloti and Rhizobium lupini in some of the arable fields of Rothamsted and Woburn and in selected plots of the Park Grass experiment. All species were widely distributed throughout the arable areas, with Rhizobium trifolii and Rhizobium leguminosarum usually much more abundant than Rhizobium meliloti or Rhizobium lupini, especially in fields cropped by the host. When the host plants were not grown, numbers decreased in a few years from tens or hundreds of thousands per g dry soil to very few or none. Numbers were unaffected or only slightly affected by mineral or nitrogenous fertilizers or by moderate infestation with legumi-

nous or other weeds but were reduced by acidity. Liming increased the numbers of Rhizobium in acid soils.

Tuzimura and Watanabe (1959, 1961b) reported the numbers of Rhizobium meliloti were 1,000 - 16,000 per g of soil under a lucerne stand and 1,000 - 2,500 2 years after the removal of lucerne. Two thousand to 7,000 "genge" bacteria (Rhizobium spp) occurred in cultivated fields in spring and about 20 - 60 in an orchard where genge (Astragalus sinicus) had not been cropped for 5 - 10 years. Numbers of soybean bacteria (Rhizobium japonicum) were 50,000 and 3,000, respectively in parts of a field cropped with soybeans 1 and 2 years before; where soybean was not cropped for the last 20 years the number was less than four. In a neighbouring forest 100 soybean bacteria were counted per g soil. Genge bacteria increased when air-dried and partially sterilized soil was rewetted showed the ability of Rhizobium to multiply saprophytically in competition with other soil micro-organisms.

Jones (1966) investigated the numbers and effectiveness of Rhizobium trifolii in six experimental areas in the vicinity of Aberystwyth, Wales. The numbers of Rhizobium ranged from 100 cells/g soil at pH 3.5 to 1.8 x 10^5 at pH 5.7. There was a high correlation between Rhizobium numbers and soil pH with liming increasing Rhizobium trifolii from less than 10^2 cells per g to 10^5 - 10^6 per g/soil 2 years after the addition of lime to acid soils.

Weaver et al (1972) determined the numbers of Rhizobium japonicum in soil samples taken from 52 fields in Iowa. The numbers ranged from 10 to more than 1 million per g of soil. Presence of soybeans in a cropping history of 13 years accounted for much of the variation in rhizobial population density between fields ($r = 0.71^{**}$). Numbers of

Rhizobium japonicum in rhizosphere samples were not significantly correlated with soil texture, soil pH, soil organic matter or presence of soybeans at sampling. About 80% of the fields that had not grown soybean before sampling contained less than 10,000 Rhizobium japonicum per g of soil, about 90% of the fields that had grown soybeans at least once had 10,000 or more Rhizobium japonicum per g of soil. The probability of finding a soil with less than 1,000 Rhizobium per g of soil was about one in 10 for fields that had grown soybeans and about three in four for fields that had not previously grown soybeans.

These numerous studies have not provided explanations of why some species are abundant while others are rare or absent. Similarly, they do not help to explain why some bacterial populations persist while members of other species of root-nodule bacteria do not maintain high numbers in soils.

Some strains or species of Rhizobium are more abundant in the rhizosphere than in soil at a distance from a plant root. Tuzimura and Watanabe (1962b) studied the numbers of Rhizobium trifolii in the rhizosphere of various crops by the plant-dilution method using crimson clover as the test plant. The growth of Rhizobium was found to be stimulated in the rhizosphere of host plants (ladino clover and crimson clover), non-host leguminous plants (lucerne, common vetch, soybean and peanut) and non-leguminous dicotyledonous plants (rape and tomato). The density of Rhizobium trifolii in the rhizosphere soil of graminaceous crops (upland rice, wheat and Sudan grass) was lower than in other plant rhizosphere soil.

Legumes increase numbers of soil rhizobia through build up and release of rhizobia from plant nodules. Tuzimura and Watanabe (1961c) reported that the population of Rhizobium in the rhizosphere of Astragalus sinicus

was greater in soil remote from the roots. The population increased at fruiting, due to degeneration of the nodules. Rovira (1962) reported that legume exudates contain a variety of substances which undoubtedly can serve as carbon or nitrogen sources for the root-nodule bacteria, or which may provide the growth factors required by auxotrophic rhizobia. Tuzimura and Watanabe (1962a) found that the rhizosphere population of rhizobia under Astragalus sinicus increased from 10^6 to 10^8 per g dry root in 37 days.

Rovira (1961) reported that not only legumes but also non-legumes will occasionally exert a pronounced stimulatory effect on the root nodule bacteria. Rhizobium trifolii was stimulated by paspalum, cotton, wheat, corn, radish, tomato; Urtica urens and Gnaphalium sp. (Rovira 1961), Rhizobium meliloti by radish, wheat and tomato (Tuzimura et al 1966), and Rhizobium leguminosarum by Urtica urens and Gnaphalium sp. (Brown et al 1968 cited in Parker et al 1977). When nodules are intact, rhizosphere population of rhizobia on soybeans are comparable to rhizosphere of non-legumes (Diatloff 1969).

The ability of non-legumes to support rhizosphere and rhizoplane. populations of Rhizobium could be of value in the spread and persistence of rhizobia in the absence of leguminous host plants. Diatloff (1969) has shown that, following the inoculation of cereals, the rhizobia were sufficiently stimulated in the non-legume rhizosphere to provide adequate nodulation of a subsequent soybean crop. He suggested the use of this type of prior inoculation to solve a severe nodulation problem with Glycine max.

Chatel and Greenwood (1973) studied the ability of four strains of Rhizobium trifolii to colonize host root and soil at 5 day intervals up

to 70 days from sowing into a field soil. Strain differences in colonizing ability were demonstrated, with one strain (TA1) consistently inferior to the other three which comprised another introduced strain (UNZ29) and two locally isolated strains.

Although root exudates stimulate the growth of certain rhizobia and inhibit the proliferation of others, the patterns of stimulation and inhibition cannot be correlated with the symbiotic specificities of the rhizobia. Peters and Alexander (1966) reported that strains of Rhizobium nodulating alfalfa and birdsfoot trefoil grew readily in the root environs of their host plants. However, Rhizobium trifolii and Rhizobium leguminosarum proliferated as well in the alfalfa rhizosphere as Rhizobium meliloti, demonstrating that the effect of legume in promoting growth in the root zone is not specific for the bacterium capable of inducing nodulation. No selective influence of the host legume on the ability of its homologous micro-organism to colonize the root surfaces was noted in an examination of seven Rhizobium strains and seven genera of legumes. Legume roots adsorbed large numbers of cells of several strains of the root nodule bacteria, but the extent of adsorption was not correlated with the infective capabilities of the micro-organisms. Root exudates collected from representatives of seven legume genera stimulated growth of certain rhizobia and inhibited the development of others, but the pattern of stimulation and inhibition were unrelated to the symbiotic specificities of the bacteria.

Robinson (1969b) tested the cultures of root-nodule bacteria, isolated from nodules of red clover and subterranean clover growing closely together in the field, for their comparative symbiotic ability (effectiveness) with both red clover and subterranean clover. It was found that test plants of either host species nodulate faster and more effectively when inoculated

with cultures isolated from the homologous host growing in the field than did test plants inoculated with cultures isolated from the heterologous host. Because the hosts had originally been nodulated in the presence of the same field populations of Rhizobium trifolii, it is concluded that the host-legume exerts a selective effect in accepting infections from a mixed population. Robinson (1969a) also reported that the host tended to select the effective strains when compared to ineffective strains. The host selection for specific strains of Rhizobium has also been reported in Trifolium repens L. (Jones and Russel 1972, Jones and Hardason 1979).

The dispersal of bacteria and other micro-organisms has attracted considerable attention for it is the basis for much of epidemiology and is important in problems with spoilage organisms. On the other hand, comparable attention has not been given to the movement of Rhizobium, although it is obviously important for the invasion of new plants growing in an area, for movement from one site or not to another, or to cause nodulation at a point distant from the site of the original habitat.

The movement of the legume root nodule bacteria in soil was first studied by Kellerman and Fawcett (1907) who reported that Bacillus radicicola (Rhizobium) and other organisms moved horizontally at a rate of 2.5 cm in 48 hours at 25°C in sterilized soils which were saturated with water. In barely moist soils, the rate of movement of Bacillus radicicola was reduced to about 2.5 cm in 72 hours. When the temperature was 10°C, Bacillus radicicola moved only 2.5 cm in 3 days in saturated soils.

Frazier and Fred (1922) studied the movement of soybean rhizobia in limed yellow sand in greenhouse conditions. They planted bacteria-free alfalfa in boxes of sterilized soil. Each box was divided longitudinally by a metal wire, the lower part of which was perforated. This divided the

boxes into a main compartment in which the plants were grown and a small side compartment used to receive the water or nutrient solution for the soil. The soils were inoculated at one end of the boxes with a suspension of soybean bacteria. They found that the nodule bacteria travelled at the rate of about 0.25 to 0.5 cm per day.

Griffin and Quail (1968) studied the movement of Pseudomonas aeruginosa Migula in three natural soils and in a particulate system with known pore size. In soils with water tensions controlled at suction pressures ranging from 100 to 1,000 cm H_2O, movement was reduced at tensions below field capacity and prevented at a suction pressure of 500 cm water. Under low suction pressures the organisms moved at 2 cm in 24 hours.

Hamdi (1971) studied the influence of water tension upon the movement of Rhizobium trifolii using coarse and fine sands and a silt loam soil in the laboratory. In these media, movement of the bacteria was slowed with increasing water tension and ceased when water filled pores became discontinuous. Calculations showed that pore sizes were unlikely to be too small to permit Rhizobium movement. Nodulation of legumes sown in partly dry soils could be restricted by failure of the migration of the seed inoculum or of naturally occurring rhizobia, at water tensions which would permit legume seeds to germinate. Hamdi (1974) subsequently found that vertical movement was possible to some degree in soil, the extent of migration being dependent upon the soil particle size and amount of precipitation in a simulated experiment. However, the extent of vertical movement was quite small. These data are to be expected inasmuch as bacteria typically do not show the capacity for extensive movement in soil.

Martin (1971) observed significant differences in the number of bacteria present in water leachates from pots containing different plant

species (wheat, subterranean clover, ryegrass) and within a plant species during growth from seedling to the formation of mature seed. Bacterial number in the leachates reached a peak which coincided with flowering for each plant species. The peak values for wheat, clover and ryegrass respectively were 33, 77 and 99 times the number of bacteria in leachates from control pots without plants. Subsequently, the number of bacteria in leachates from wheat pots decreased until they were not significantly different from the controls. There was a lesser decrease for the clover and no significant decrease for the ryegrass treatments.

Chatel et al (1968) studied the lateral movement of Rhizobium strains in the field. While some strains moved 5 cm in 1 month in soil, others had not moved 2.5 cm in the same period. Brockwell et al (1972) explained the recovery of strains of rhizobia from uninoculated control plots that were serologically indistinguishable from applied strain, as the result of accidental contamination. Rainfall of very high intensity took place and lateral movement of water through the soil and over the ground might be the main reason for this contamination.

The ability to survive deleterious physical and chemical conditions or the ability to colonize the soil is an essential quality in rhizobia. Of great importance is the absolute population size; the higher the initial population before any harmful factors become effective, the greater the probability of some cells surviving. Chatel and Parker (1973a) reported species and strains differ in their capacity to colonize host-plant roots and soil during the growing season in a field showed a nodulation problem in the second year after establishment. Rhizobium lupini was found to reach higher population at a faster rate than Rhizobium trifolii. A sharp drop in the population of Rhizobium trifolii associated with subterranean

clover roots early in the growing season was followed by a recovery to high number. No such phenomenon occurred with Rhizobium lupini. This difference in colonizing ability among the rhizobia must surely be important in maintaining bacterial densities sufficiently high to be useful in nodulating host plants and indeed the colonizing capacity has been related to the failure of second year pastures of annual clovers in Australia (Marshall et al 1963).

There are many factors that control Rhizobium survival and persistence in soil and rhizosphere. Some of these factors will be reviewed as follows.

Temperatures

Work on temperature effects has been mostly confined to high temperature. Vandecaveye (1927) reported that the extreme temperatures of winter and summer did not prove to have any injurious effect on the nodule production of the Rhizobium leguminosarum in certain soil. De-Polli, Franco and Dobereiner (cited in Parker et al 1977) reported little difference was found in the death rates of Rhizobium trifolii and Rhizobium meliloti when added to sterile soil watered to field capacity and held at 35°C. However, a dramatic lethal effect of moist heat on the survival in soil of strains of Rhizobium from pea, clover, lucerne and tropical legumes subjected to 40°C has been demonstrated, as has the survival of rhizobia applied to seed sown into moist soil at 40°C. Bowen and Kennedy (1959) showed that the survival was dependent on strains of bacterium, initial concentration of inoculum and period of exposure to high temperature.

Rhizobia are less affected by dry heat. Wilkins (1967) subjected the soils taken from Western New South Wales and New England tableland to a series of high temperature tests. Rhizobia present in air-dry soils survived temperatures higher than would be experienced under natural conditions.

In moist soils the tolerance of medic rhizobia to high temperature was much lower. Strains of Acacia, Lotus and Psoralea rhizobia from Western New South Wales survived higher temperatures than strains from the New England tableland, but the tolerance to high temperatures of medic rhizobia did not vary with source. Sanderson (cited in Parker et al 1977) found that Rhizobium trifolii in air-dried field soil survived temperatures as high as 90°C for 8 hours. Chatel et al (1968) reported Rhizobium lupini and Rhizobium trifolii survived 80°C for 6 hours in an air-dry sandy soil. However, Marshall (1964) suggested dry heat may accelerate death where annual legumes are grown in areas subject to the hot, dry summer of the Mediterranean-type climate.

Vyas and Prasad (1960) report the death of pea Rhizobium has been related to the low clay content of a problem soil following investigations into the different ability of species of Rhizobium to tolerate high temperatures in India. It has been shown that only certain clays afford protection. Marshall (1964) investigated the survival of root nodule bacteria in autoclaved soil which, after inoculation, were dried at 30°C and subsequently exposed to high temperature. Rhizobium trifolii died in grey and yellow sands heated to 70°C but survived in red sands and soils of heavier texture. Amendment of a grey sandy soil with 5% (W/W) of montmorillonite, illite, fly ash or haematite protected Rhizobium trifolii from the lethal effect of exposing the dry soil to higher temperatures. Kaolinite and goethite did not protect the Rhizobium trifolii. After three successive exposures at 50°C for 5 hours Rhizobium trifolii disappeared in a grey sandy soil, but still survived after four exposures in the presence of montmorillonite. He attributed the greater survival in heavy-textured soils and red sands to the presence of appreciable amounts of illite or

haematite both in these soils. The grey and yellow sand contains only kaolinite and possibly goethite; neither of which are protective. Marshall (1968) suggested that the protective mechanism of a clay envelope around the bacteria may lower the rate of water loss from cells. Vincent (1965) suggested that the heat resistance of dried cells is partly due to reduced protein denaturation under these conditions.

Very little work has been done in behaviour of rhizobia at low soil temperatures. Ek-Jander and Fahraeus (cited in Parker et al 1977) reported work on the adaptation of rhizobia to a cold climate, showed that isolates of clover rhizobia from the subarctic grew faster and nodulated their host earlier at 10°C than isolates from warmer areas. Kunelius (1970) showed that symbiotic nitrogen fixation of Lotus spp. depended on root temperature. N_2 fixation at 9 and 12°C was depressed and growth was poor. Optimum temperature for N_2 fixation was found to be between 18 and 24°C. 30°C root temperature was found to depress N_2 fixation.

Moisture

Vandecaveye (1927) studied the effect of moisture on Rhizobium sp. survival. He carried out pot experiments in the greenhouse and out of doors demonstrating that laboratory cultures of Rhizobium leguminosarum grown in Palous silt loam are capable of surviving unusual exposure to wide extremes of soil moisture without any apparent effect on their ability to produce nodules on the host plants. However, excessive soil moisture to the point of saturation or flooding was found to be much more detrimental to the life of these bacteria than extreme dryness approaching air-dry conditions. Populations of Rhizobium leguminosarum in pots of sterile soil were greatly reduced after 2 weeks flooding. Schroder and Gomensoro (cited in Parker et al 1977) carried out another pot experiment and found

that the reduction of nodule numbers on Centrosema grown in previous flooded soil was attributed to poor survival of the rhizobia. It was claimed that the inoculant strains were more sensitive to excessive water than the native strains.

In Australia (Parker et al 1977), the continued nodulation of certain clovers (Trifolium subterraneum var. 'Yarloop', Trifolium fragiferum) in water-logged soils suggests that harmful effects of water-logging cannot be of great importance to these rhizobia.

Drought is undoubtedly an extremely important factor affecting survival. Fould (1971) studied the changes in population density of rhizobia indigenous to soils. In his experiment, eight samples of soil were taken, air-dried and the reduction in population of Rhizobium meliloti, Rhizobium trifolii and a Rhizobium of the 'Lotus' group was estimated by use of a plant-dilution-infection technique. The cells of Rhizobium trifolii proved to be more tolerant of the severe drought than did the cells of the other two species. The populations reduced from 2.3×10^5 and 2.3×10^2 to 9.2×10^3 and < 10 in 35 days for Rhizobium trifolii and Rhizobium meliloti respectively. Earlier works (Albretcht 1922, Richmond 1926) showed that soybeans and red clover nodule bacteria remain viable in dry soil for many years. However, these early studies of drought resistance were not quantitative. Chatel and Parker (1973b) studied the survival over summer of Rhizobium trifolii and Rhizobium lupini in both the field and laboratory conditions. Dry field soils containing rhizobia were subjected to a range of temperatures in the laboratory. The bacteria were found to survive a 6 hour exposure to temperature as high as 80°C. Populations of rhizobia were estimated at different depth from the end of growing season (October) to early

autumn (April) in plots which had carried dense swards of subterranean clover and serradella. High populations of Rhizobium lupini were maintained in the serradella plots throughout the summer. Populations of Rhizobium trifolii in the subterranean clover were initially much lower, and declines with both time and depth. The problem known as 'second year mortality' is primarily due to low numbers of clover rhizobia in the soil at the end of the growing season. This situation is aggravated over the long hot dry summer, when there is a further decline in numbers.

Salinity

Fred et al (1932) showed that Rhizobium could tolerate sodium chloride concentrations of around 3% in broth. Pillai and Sen (1966) studied salt tolerance of eight strains of Rhizobium trifolii isolated from berseem clover plants. The strains were sensitive to salt. There was a progressive decrease of growth with increase in the salinity of the media. From the regression equations expressing relationships between the growth of the strains and the salinities of the medium, it could be calculated the salinities which completely inhibited the growth of the strains lay between 0.5 and 0.7%. Nodulation of berseem plants in general, was not affected by salinity of the soil. Under uninoculated conditions, yields of berseem plant increased with the salinity of the soil as indicated by positive and significant correlations between the two. The increase in the yields of plants with increase in salinity was also observed in the case of inoculated plants though it was more qualitative. There was a reduction in the efficiency of the strains in saline soils. The reduction in efficiency tended to become less with the increase in the age of the plant.

Bernstein and Ogata (1966) compared the growth, nodulation, and nitrogen content of nitrogen fertilized and nitrogen fixing soybeans and alfalfa of four levels of salinity (0 - 5.4 atm. added NaCl) in gravel culture. Salinity was more inhibitory to the growth of inoculated Lee soybeans than of nitrate fertilized cultures. Nodulation was strongly reduced at 5.4 atm. of added NaCl, and the dry weight percentage of nodules decreased significantly with increasing salinity of the medium. Nodulation of California common alfalfa was only slightly affected by salinity, and relative growth inhibition by salinity was the same for the nitrogen fertilized and the nitrogen fixing cultures.

Yadav and Vyas (1971) studied the influence of some salts and pH characteristics of saline, alkaline and acid soils on Rhizobium spp. for lucerne (Medicago sativa L.), black-gram (Phaseolus mungo Roxb.), green gram (Phaseolus aureus Roxb.), moth bean (Phaseolus aconifolius Jacq.) and pea (Pisum sativum L.). Both salt-sensitive (0.2%) and salt-resistant (3%) strains of lucerne and pea were present. Black gram and moth-bean strains were proportionately sensitive to Cl and SO_4, but green gram was stable. Mg^{++} salts were stimulatory at concentrations lower than 1%. For all rhizobia, 0.4 - 0.6% $NaHCO_3$ was critical. All the strains survived at pH 10, but were inhibited at 3.5.

Subba Rao et al (1972) reported that strains of Rhizobium meliloti nodulating lucerne (Medicago sativa L.) tolerated sodium chloride up to 3%. However, seeds of lucerne did not germinate even at 1.5% concentration of NaCl. At 0.4% concentration, initial nodulation was not only delayed but the number of nodules and leaves were reduced. This effect was accentuated with an increase in the concentration of the salt, and at 0.7% concentration the plants failed to nodulate, indicating that the

levels of salinity inhibitory to symbiosis between the legume and the Rhizobium are different from those inhibitory to the growth of individual symbionts.

Ethirraj et al (1972) tested rhizobial isolates from lucerne (Medicago sativa L.), berseem (Trifolium alexandrium), and daincha (Sesbania aculeata) for their growth and tolerance in the presence of some inorganic salts commonly found in saline soils at various concentrations. Within the range tested sodium chloride did not show much inhibition. However, sodium sulphate, potassium chloride, and potassium sulphate were found to be inhibitory towards berseem isolates but not to lucerne and daincha isolates. Between the isolates from the same host there is great variation towards their salt tolerance. Magnesium chloride and magnesium sulphate were found to be beneficial to the growth of all isolates.

From these works, it can be generalized that salinity tolerances for the host plant, for nodulation, and for the symbiosis, are lower than those for the rhizobia themselves. Some species or some strains are more tolerant to salinity than the others. Magnesium salts at a proper concentration are found to be beneficial for the growth of the Rhizobium.

There has been a claim that pelleting of seed with either lime or gypsum gives some protection against salinity. Chhonkar et al (1971) did pot experiments using a saline alkali soil. It was shown that pelleting of Phaseolus aureus L. seed with lime and gypsum together with Rhizobium inoculation, significantly increased growth, nodulation and nitrogen fixation. However, it is unclear if this was an effect on the survival of rhizobia on the seed, their multiplication in the rhizosphere,

or the infection process. Wilson (1970) showed the evidence of adaptability of the symbiosis between Glycine wightii and its rhizobia to increases in substrate salinity. In his experiment, he subjected well nodulated Glycine wightii plants grown in sand culture to 14 days of salinity ranging from nil to 148 meq. sodium chloride per litre of nutrient solution and compared the response to that of similarly treated nitrogen-fertilized plants. The latter showed less tissue injury and a small reduction in growth rate of high salinity than the inoculated plants. During salinity treatment, the development of new nodules and nitrogen fixation by the existing nodules, were greatly inhibited with the resulting marked decline in plant nitrogen concentration, especially in the laminae and nodules. Despite the severity of the salt effect on the inoculated plants the nodules that developed prior to salt treatment appeared remarkably resistant to stress, and rapidly regained pigmentation and efficiency of nitrogen fixation when sodium chloride was removed from the culture solutions. Salt accumulation in the nodules was limited and sensitivity of symbiosis to salinity appeared primarily dependent on the host. These facts indicate the adaptability of symbiosis to increases in substrate salinity.

Balasubramanian and Sinha (1976) studied the effects of salt stress on the growth, nodulation and nitrogen accumulation during the vegetative phase in chickpea. Growth and nitrogen accumulation were adversely affected by salinity. The larger control plants produced new nodules but the existing nodules on stressed plants grew larger than those of control plants. All plants had similar percentage nitrogen content but the total plant nitrogen was less in stressed plants due to the reduced growth of these plants. Reduced plant growth vigour was the primary

effect of the salt stress and was mediated through processes other than symbiotic nitrogen fixation.

Soil Acidity

A great deal has been written of soil acidity and the ecology of Rhizobium spp. Low pH has been known to have deleterious effects on Rhizobium survival in soil (Richmond 1926, Wilson 1926) and liming has a beneficial effect on Rhizobium survival (Walker and Brown 1935, Vincent and Waters 1954, Jones 1966, Nutman and Ross 1969, Robson and Loneragan 1970a, b).

The slow-growing rhizobia, Rhizobium japonicum, Rhizobium lupini, and the cowpea complex are generally found in acid soils (Fred et al 1932). Cowpea group Rhizobium could survive 3 years in air dry storage conditions in the soil having pH 4.5 - 5.4, while the soybean Rhizobium could not (Richmond 1926). Of the fast grower, Rhizobium meliloti is the least tolerant to acidic conditions (Vincent 1958).

Norris (1965) postulated that acid and alkali production by rhizobia on laboratory media are indicative of similar activity in the rhizosphere of legumes, conferring advantages on the bacteria in alkali and acid soils. The theoretical basis of Norris's hypothesis has been challenged by Parker (cited in Parker et al 1977). Parker suggests that the preferential use of sugars by fast growing rhizobia, and of organic nitrogen compounds by slow growing rhizobia, as their source of energy, results in the production of acid or alkali respectively. In his same study, fast and slow growers were unable to change the pH of the rhizosphere of their hosts or of water extract of the soil. However, Jones and Burrow (1969) supported for Norris's suggested use of acid produc-

tion as a selective character for inoculant bacteria. They tested 90 isolates of Rhizobium trifolii from 36 sites in Wales for acid production in cultures and for symbiotic effectiveness with S.184 white clover. The range in net final pH was from 4.70 to 7.00 from an initial 7.2 in the culture medium. The fact that the great majority of the isolates were acid producers confirm the work of Norris in which he put forward the view that Trifolium repens is adapted to alkali soils.

Antagonistic Microflora and Fauna

Soils are a complex living community consisting of many living organisms interacting with each other. When Rhizobium trifolii and Rhizobium meliloti were added to sterilized and non-sterilized soil, the numbers of both Rhizobium spp. decline more rapidly in non-sterilized soil than in sterilized soil (Danso et al 1973). Many hypotheses have been advanced to account for the failure of the organisms to colonize readily or for the decline of the populations naturally present or those deliberately added to soils. These include the presence of toxin producing micro-organisms, inhibitory agents, bacteriophages Bdellovibrio and protozoa.

Hely et al (1957) reported that the failure of subterranean clover (Trifolium subterraneum L.) in certain areas in Australia was due to an antagonistic effect of certain micro-organisms present in the rhizosphere of the legumes raised in that soil.

Holland and Parker (1966) reported that extracts of certain recently cleared soil in which subterranean clover failed to nodulate were frequently toxic to Rhizobium trifolii, and it was proposed that antibiotic-producing fungi which proliferate on the organic material left after

soil clearing led to nodulation failures.

Begg (1964) proposed that a microbial growth inhibitor was associated with problems in clover establishment in New Zealand, he overcame the problem of soil toxicity by the use of formaldehyde, which presumably destroy the toxin producing organisms. Khan et al (1968) report the influence of partial soil sterilization by either steaming or "Vapam" fumigation resulted in improvement in nodulation and yield of alfalfa. This partial sterilization appeared to have exerted its effect by eliminating endemic microflora which are capable of suppressing development of the introduce strain, but not that of the native Rhizobium.

Robinson (1945) isolated six antagonists of the legume bacteria, representing species of bacteria, fungi and actinomycetes commonly found in the soil. They were tested against the legume bacteria under laboratory conditions. The response of the legume bacteria to the growth of any of these antagonists in association with them was found to vary with the strain of legume bacteria used and the antagonist. This variation in response consisted of the simultaneous occurrence of stimulation and inhibition, or inhibition alone. A greenhouse experiment was also conducted. These six antagonists were added to sterilized soil growing five species of inoculated legumes. Several of these antagonists appeared to interfere with nodulation of legumes. The response seemed to vary with the species of legumes and with the antagonist. This work suggests that the antagonists and their antimicrobial materials, may well be one of the factors which are responsible for the decline of legume bacteria in the soil.

Damirgi and Johnson (1966) tested the susceptibility of eight strains of Rhizobium japonicum to antimicrobial action of 24 isolates

of Actinomycetes on yeast extract mannitol agar. Twenty of the isolates produced no inhibition of rhizobia. Isolate E_1 antagonized only Rhizobium japonicum strain 76 and isolate E_8 were antagonistic to all strains. Two other isolates showed slight inhibition of strains 122 and 123. Infectivity of rhizobia on soybean variety Kent was evaluated in the presence of selected actinomycetes isolates in autoclaved soil. The reduction in nodule numbers produced by rhizobial strains were 35% and 53% when Actinomycete E_8 was introduced into the soil at the time of planting and 28 days before planting respectively. The results suggest that anti-rhizobial soil micro-organisms in a particular soil play a role in the establishment of specific rhizobial strains.

Chatel and Parker (1972) reported soil-water extracts from soils in which clovers nodulated poorly proved inhibitory to Rhizobium trifolii in seed agar plates. The same extracts did not inhibit Rhizobium lupini. The toxic extracts were found mainly in the growing season, but not after heavy rain. Neither the soil nor the soil-water extracts retained their toxicity on storing. Filtered broths from pure cultures of 59 soil micro-organisms, isolated from soil and clover root in problem strands, were tested for their effect on both Rhizobium lupini and Rhizobium trifolii; nine isolates inhibited both species, 19 inhibited Rhizobium trifolii only, and 31 had no inhibitory effect on either species. None inhibited Rhizobium lupini without also inhibiting Rhizobium trifolii.

Sethi and Subba Rao (1975) reported that colonization of soil by Fusarium oxysporum f. pisi (inhibitory towards Rhizobium leguminosarum) and inoculation of soil with Rhizobium leguminosarum resulted in a significant decrease in leghaemoglobin content, root nodule and nitrogen

content of pea plants (Pisum sativum). However, colonization of soil by Penicillium liLaconum (not inhibitory towards Rhizobium leguminosarum but an efficient solubilizer of tricalcium phosphate) and inoculation of soil with Rhizobium leguminosarum resulted in a significant increase in phosphorus status of pea plants.

Bhalla and Sen (1971) isolated 51 bacterial isolates belonging to 10 different genera from the rhizosphere and nonrhizosphere chickpea soil. The antimicrobial effects of these isolates were tested on the Rhizobium in vitro. It was found that bacteria belonging to the same genus influence differently the growth of Rhizobium, i.e. some had a stimulatory effect and some had an inhibitory effect.

Among the rhizobia themselves, different strains may have antagonistic effects to the other. Schwinghammer (1971) examined 41 strains of Rhizobium trifolii and 270 from clover nodules at five localities in Southeast Australia for their inter-strain antagonism in culture. It was found that approximately 35% of the cultures produced dialysalble substances mildly antibiotic towards the six indicator strains used and almost 8% of the cultures were lysogenic or produced bacteriocin-like substances.

Kandaswamy and Prasad (1977) assessed the interrelationship between the altered rhizosphere microflora and rhizobia of the rhizospheres of green gram (Phaseolus aureus), black gram (Phaseolus mungo), and sunn hemp (Crotalaria juncea) following foliar spray with GA (50 and 100 ppm), 2,4-D (5 and 10 ppm) and IAA (250 and 500 ppm). A positive correlation existed between the bacterial and rhizobial populations in the rhizosphere of the three plant species. However, no such correlation was apparent between fungal and Rhizobium populations in black

gram while a positive relationship in green gram and negative correlation in sunn hemp was evident. The interaction between actinomycetes and rhizobia revealed a positive influence in green gram while such relationships did not exist in sunn hemp. This indicated that the influence of the rhizosphere fungi in the Rhizobium population varied with the plant species.

Bdellovibrio and protozoa have been reported to be Rhizobium parasitic and led to the reduction of the Rhizobium population (Keya and Alexander 1975, Danso et al 1975, Alexander 1977).

Keya and Alexander (1975) reported Bdellovibrio in 32 out of 90 soils examined. Bdellovibrio did not initiate replication in liquid media at low host densities, but it did multiply once the Rhizobium numbers increased through growth to about 10^8/ml. From about 10^4 to 6×10^5/ml Rhizobium cells survived attack by the parasite in liquid media. In nutrient-free buffer, no significant increase in vibrio abundance was evident if the rhizobial frequency was low, whereas a Rhizobium population containing 6×10^8 cells/ml were lysed rapidly. The same phenomenon occurred in sterile and non-sterile soils. It is suggested that the major reason for the lack of elimination of the host population in soil by its parasites is the need for a critical host cell frequency, large Rhizobium number being required for Bdellovibrio to initiate replication and low numbers of surviving hosts no longer being able to support the parasite.

The Response of Chickpeas to Rhizobium Inoculation

Chickpea (Cicer arietinum L.) is very specific in its Rhizobium requirement. It will not form nodules with other Rhizobium cross

inoculating groups (Bhide 1956, Habish and Khairi 1968, Guar and Sen 1979). However, the effect of inoculation on growth and yield of chickpea is not very clear.

In 1933, Rasumowskaja (van der Maesen 1972) reported an increase of yield on land planted with seed inoculated with symbiotic bacteria, especially in the second year after inoculation. The chickpea Rhizobium was found to be specific. In 1934, Rasumowskaja established more properties of this Rhizobium species. Other nodule bacteria from vetches, clovers and peas could not form nodules on Cicer. Plants and seeds had higher protein contents after inoculation and final yields were higher. In 1933, Ivanov (van der Maesen 1972) found that the percentages of protein in the seeds varied from 12.6 to 31.2% within the same cultivar. He ascribed this difference to the fact that the crop was new to many stations in the USSR with non-inoculated soils. In 1948, Marcilla Arrazola et al (van der Maesen 1972) reported on field trials in Spain on the influence of a commercial inoculum. After inoculation the root nodules were better developed but the yields were not improved. It is likely, however, that the soil already contained the specific strain of Rhizobium.

Moodie and Vandecaveye (1944) reported that inoculation produced normal plants on a nitrogen free sand culture indicating plants were capable of fixing an adequate amount of atmospheric nitrogen for their requirement. In field trials, inoculated plants were markedly greener and more vigorous than non-inoculated plants. Inoculation produced an average increase of approximately 37 and 74% in the grain yield on the "normal" and "clay" phases of Palouse silt loam respectively. Protein content of the seeds increased by 5.4%. Immature plants increased in

N content (from an average of 1.4% to 2.1%) and the roots increased in N content (from 0.9% to 3.25%). Chickpeas were considered more efficient in fixing atmospherical nitrogen than field peas and were recommended for intercropping in rotations with wheat in the USA.

Gupta and Sen (1962) reported the efficiency of 12 isolates of Rhizobium strains from chickpeas on the protein content of the plants. By inoculation with a suspension in sterilized soil the protein content could be raised with percentages varying from 0.9% to 70.9% depending on strain.

In 1965, Xandri Taguena and Diaz Cala (van der Maesen 1972) reported on the non-effectiveness of the commercial Spanish and American preparations such as Cepar Seccion (liquid) and Nitragin (Rhizobium leguminosarum Frank.) on the yield and protein content of chickpeas in Spain. The soil must have contained Rhizobium of Cicer arietinum. Some earliness in flowering and ripening seemed to be present after inoculation. The nodules of inoculated plants were poorer in nitrogen than those of non-inoculated plants, so that a fast transport of nitrogen that induces earliness is suggested.

Sen (1966) established that the local strains present in the soil were best suited to similar conditions elsewhere. Results of field experiments at Delhi and Karnal and pot experiments at Coimbatore showed that strains isolated from Karnal and Delhi were more effective at Delhi whereas Pusa and Coimbatore strains were more effective at Coimbatore. When seeds were treated with imported strains, the N contents of the whole plant could even decrease. The best strain doubled the N-content in 6-week-old plants compared with the control. A suitable strain increased the yield of grains by 16% in one case, but

no effects were detected in their N-content.

Chopra and Subba Rao (1967) investigated the relationship between the bacteroid-leghaemoglobin and N-content of the root nodules. These were positively correlated, increasing from the 50th to the 138th day. When flowering was underway, the bacteroids and leghaemoglobin content decreased, whilst the N-content remained constant.

Katti (1968) studied the effect of inoculation of chickpeas under various conditions, and found that non-inoculation combined with a rate of 22.4 kg of N per ha gave a higher number of flowers on red sandy loam, while alluvial clay loam produced better plants when inoculation was combined with a rate of 44.8 kg of P_2O_5/ha.

Gupta and Kuar (1969) found abnormally large functional nodules when Cicer was grown on virgin land. Their diameter was 3 - 4 cm. Sundra Rao and Sen (1969) reported an increase of 17% to 34% in grain yield due to Rhizobium inoculation. Rewari (1970) reported a 60% increase on farmer's fields in Mysore. Probably no bacteria were present previously, since chickpea cultivation in this state is less important.

Singh (1971) reported that application of 22.5 Kg N/ha had little stimulatory effect on the growth of plants. The effects on nodulation, nitrogen fixation and yield were comparatively more marked. Responses to phosphate application were very conspicuous, and the growth, nodulation and nitrogen fixation in plants were stimulated significantly. On an average, application of 22.5, 45.0 and 67.5 Kg P_2O_5 per ha increased the yield by 3.81, 5.08 and 6.12 q/ha respectively.

Dolosinskii and Kadyrov (1975) reported the results of a pot experiment. Effective strains of Cicer Rhizobium increased the yield of the aerial mass of chickpea by 25% to 36%, and the protein content by

2 to 6% of the total content of nitrogen in the plants. No correlation was found between the effectiveness of strains and the activity of their dehydrogenase enzymes.

Srirama Raju and Samuel (1976) reported the responses of gram (Cicer arietinum L.), variety BEG482, to seven Rhizobium inoculants along with nitrogen at 10 and 25 Kg/ha under black and chalka soil conditions simultaneously. In black soil, all the inoculants, except IARI culture, gave very good nodulation, which was reflected in higher DM production, higher N-uptake by plant, and its translocation to the seed. These factors contributed to the significantly increased yield which ranged from 63.8 to 134% over control. IARI culture produced a considerable number of nodules but did not contribute in any way for betterment of the crop and was on par with uninoculated nitrogen control.

Bapat and Vaishy (1976) reported different strains of Rhizobium spp. interact differently with different genotypes of Bengal gram.

Agnihothrudu and Tripathi (1976) conducted six trials in Andra Pradesh and Karnataka with Bengal gram during 1974-75. All inoculum treated plots recorded higher yields than control plots. The increase in yield over the control was 10 to 57%. The variations in yield varied from place to place.

Rai et al (1977) studied the effect of inoculation of eight strains of Rhizobium spp. on nitrogen fixing ability and yield of chickpea variety H208 in the field conditions. There was no significant difference among treatments in numbers and dry weight of nodules per plant. However, the yield of the inoculated treatments were significantly increased over the uninoculated control within the range of 14 to 40%. Strain G.E.8 gave the best response of 40%.

Corbin et al (1977) reported a series of glasshouse and field experiments on chickpeas in Australia. The experiments indicated the need for inoculation of this legume species. However, all five strains of rhizobia were effective in their symbiosis with the lines examined and extensive nodulation was observed even with inoculation rates 1/5 normal, with ceresan-treated seeds. The application of solid inoculant (granular) in the rows produced better nodulation.than slurry inoculation of the seeds.

Kadam et al (1977) conducted a field experiment to study the effect of Rhizobium inoculation, nitrogen and simazine application, individually and in combinations, on yield and quality of chickpeas. Application of nitrogen and simazine, and seed inoculation with Rhizobium increased the grain yield significantly. The combined treatment of Rhizobium, simazine and nitrogen increased the grain yield to the extent of 70% over control. Application of simazine increased the methionine content.

Rai and Singh (1979) studied the inoculation effects of nine strains of Rhizobium for their nodulation capacity, leghaemoglobin content, grain yield, crude protein and 16 amino acid content, in chickpea variety C235 grown on a calcareous saline alkali soil. There was no significant correlation between grain yield and number of nodules ($r = 0.37$) or dry weight of nodules ($r = 0.29$), but grain yield was significantly correlated with leghaemoglobin content of nodules ($r = 0.95$). Of the 16 amino acids analyzed in seed samples, aspartic, glutamic, proline and histidine were greatest with strain H45; glycine, leucine and arginine with strain F6; norleucine, tyrosine and phenylalanine with strain KG38, and alanine and valine were greatest with strain KG41. Strain KG38 led to significantly higher grain yield than the

other strains.

Pareek (1979) evaluated the effectiveness of various strains of chickpea in the field for 2 consecutive years. Strain B1 and 6042 recorded respectively about 26 and 141% increases in nodule weight against controls in the 1st and 2nd years. Shoot weight was not benefited by inoculation. Strain 6051 significantly increased grain yield in the 1st year while P21 recorded an appreciable but not significant increase in grain yield in the 2nd year. Nitrogenase activity of intact nodules of strain 6051 was highest in the 1st year and that of 6042 in the 2nd year. Strain N-1 and 6042 fixed highest (104 and 97 Kg N/ha/season) dinitrogen in the 2nd year. Mulching benefited nodulation, grain yield and nitrogenase activity which had been discussed in terms of moisture conservation and raising soil tempera ture and thus became beneficial to nitrogen fixation.

The Responses of Legumes to Inoculation Methods

The legumes, members of the family Leguminosae, because of their importance in soil fertility and sources of protein, have probably received more attention to date than any other nitrogen-fixing group. This beneficial effect on the soil and the importance of green manuring were realized by the ancient Chinese, Greeks and Romans (cited in Stewart 1966) so that the widespread use of legumes in crop rotations was well established long before the reason why they were beneficial was discovered. The first recorded experimental evidence that leguminous plants could utilize nitrogen from the air was obtained by the French scientist Boussingault (cited in Stewart 1966) who, at Bechelbronn in Alsace, observed that when legumes such as peas and

clover were grown under open unsterilized conditions they assimilated more nitrogen than was supplied to them in combined form, whereas cereals such as oats and wheat did not. Unfortunately, he decided to report his experiment using what he considered to be more precise method, which include the use of closed containers and sterilized sand. As a result, there was no rhizobia available to nodulate his plants, his earlier results were, therefore, not confirmed and this, together with the criticism of his studies by Justus Leibig, the eminent agricultural chemist of the day, caused him to abandon his earlier hypothesis, and left Hellriegel and Wilfarth to settle the controversy. Their experiments in which they grew peas, with or without combined nitrogen in 1) sterile sand, 2) non-sterile sand, and 3) sterile sand plus soil extract, were simple but decisive. They showed that good growth occurred in every case when combined nitrogen was supplied. In the absence of added combined nitrogen, the sterile culture did not nodulate and little growth occurred; in non-sterile sand only a few plants which had become nodulated made good growth, while in the presence of unsterilized soil extract all plants formed nodules and showed growth which was often equal to that in the presence of combined nitrogen. They, thus established that only plants bearing nodules fixed nitrogen, and postulated that the nodules were the nitrogen fixing sites, that they were formed as a result of infection of the roots by soil bacteria, and that non-nodulated plants were similar to cereals in that they required combined nitrogen for growth. In 1888 Beijerinck (cited in Stewart 1966) isolated in pure culture a bacterium which caused nodules to form on legume root and termed it Bacillus radicicola and later was given the name Rhizobium leguminosarum by Frank (cited in Stewart 1966).

The practice of inoculating seed with artificial cultures of Rhizobium dates from 1896 (Roughley 1970). In its earliest form the method consisted of growing the bacteria on an agar medium, suspending the cells in water and this suspension was then used to impregnate either the soil directly or to inoculate the seed. It is possible to successfully inoculate legume seed using either agar, freeze-dried, or peat cultures (McLeod and Roughley 1961), the latter form (peat cultures) offers some outstanding advantages. This includes increased protection for the rhizobia when in contact with acid fertilizers (Vincent 1958) and improve survival under a lime pellet (Shipton and Parker 1967).

Peat base inoculum is now widely accepted and used throughout the world, with conventional peat inoculation being the slurrying method, i.e. the peat inoculum slurried in water or sticker solution, the seed then coated with the solution.

Many substances have been used as stickers, i.e. water, 10% sucrose, 40% neutral gum arabic in the suspending fluids, methyl cellulose, skimmed milk, tapioca starch. Date (1970) reported that gum arabic gave better Rhizobium survival than substituted methyl cellulose, especially at a storage temperature of 25°C over long periods. Various methyl, methyl ethyl and methyl hydroxyl propyl celluloses were reported to have been tried and all gave a similar result. Peat slurry (with water) was found to give very poor Rhizobium survival.

Iswaran and Chhonkar (1971) studied the survival of Rhizobium leguminosarum, Rhizobium trifolii and Cicer Rhizobium on inoculated host seed using the plate count method. They found that gum arabic was superior to 10% jaggery (sucrose). The slurry method was inferior to sprinkling

method. Gum arabic was also reported by earlier workers (Brockwell 1962, Date 1970) to give the best survival on seeds.

Davidson and Reuszer (1978) studied the survival of Rhizobium japonicum strain 67A68 on surfaced sterilized soybean using 12 different stickers (including Gum arabic). There was considerable variation in the recovery rates of Rhizobium from one treatment to another at initial plating, indicating that the amount of peat base inoculant sticking to the seed varied considerably. The commercial coating material resulted in a much larger initial population of rhizobia sticking to the seed coat. However, there was no distinct advantage over the control in terms of percentage of the original inoculum surviving at later dates. Mineral oil was quite favourable in terms of numbers of rhizobia held by the seed and percentage of rhizobia surviving. Darco G-60 was exceptional in the percentage of rhizobia surviving at 15°C, however, this was not observed at 22.5 or 30°C. It was found that none of the treatments gave a survival of 200,000 rhizobia per seed after a 3 week storage period.

Waggoner et al (1979) studied the nodulation of white clover (Trifolium repens L.) grown from seed inoculated with a peat based inoculant using water or gum arabic as the adhesive. Inoculation with enough peat to supply 600 rhizobia/seed was adequate when applied with gum arabic, but not with water. Inoculation procedures normally supply approximately 200 rhizobia/seed. There was no significant difference between the uninoculated treatment and inoculated treatment applied with water for dry matter production percent protein or acetylene reduction. Only nodule weight was reported to be different at an early sampling date. When gum arabic was used increasing the number of rhizobia from

600 to 3,000/seed, significant increases in any monitored parameters were not seen.

Occasionally, some fertilizers, e.g. superphosphate, used in association with the sowing of legumes, is deleterious to the survival of rhizobia and prompt nodulation of the legume because of toxic pH levels. This had led to the development of lime-pelleted seed (Cass-Smith and Goss 1958, Roughley et al 1966) and has permitted the sowing of inoculated seed with acid fertilizers, the lime coat acting as a physical buffer between acid fertilizer and inoculum as well as neutralizing the immediate environment of the germinating seed. A number of workers have suggested that pelleting of seeds after inoculation might prolong the survival of the applied root nodule bacteria (Brockwell 1962, Brockwell 1963b). Radcliffe et al (1967) studied the survival of Rhizobium trifolii on inoculated seed pelleted by 13 seed pellet coating materials. A wide variation in survival of organisms was found. Cottrel dust and Gold hill lime proved to be detrimental to Rhizobium. Only one out of six adhesives used did not support Rhizobium growth (i.e. 2% cellulose). A tenfold improvement in rhizobia survival was obtained on pelleted subterranean clover seeds when the rhizobia were suspended in peat rather than broth. Only one of seven pelleting treatments using a peat suspension of rhizobia in 40% gum arabic had adequate numbers after 8 days. Brockwell and Whalley (1970) confirmed that peat inoculant incorporating pellet seeds were superior to broth incorporating pellets.

Iswaran and Jauhri (1969) studied the effect of lime and rock phosphate pelleting on nodulation and nitrogen fixation in soybeans in a pot trial. Lime and rock phosphate pelleting increased nodulation and dry weight over the inoculated but non-pelleted at 8 weeks after planting.

Brockwell and Phillips (1970) reported Rhizobium meliloti inoculant applied to lucerne seed by incorporation within a lime pellet has the ability to tolerate long periods lying in hot, dry soil and is able to survive in sufficient number to form nodules on a large percentage of the host plants. There was little or no survival of Lotus organisms under the same conditions. In the same situation, some Rhizobium trifolii survived but the proportion of clover plants nodulating never exceed 50%. It is concluded that lime pelleted Medicago seed inoculated with peat-borne Rhizobium meliloti can be sown into hot, dry soil with good expectation that the inoculant will survive and the seedling nodulate. No such assurance can be given for other genera of leguminous plants and other groups of Rhizobium.

A lime pellet has been reported to improve nodulation and nitrogen fixation in saline and alkali soil. Chhonkar et al (1971) reported pelleting of Phaseolus aureus L. seed with lime or gypsum together with Rhizobium inoculation, significantly increased growth, nodulation and nitrogen fixation in a pot trial.

Norris (1971a) studied the effect of seed pelleting treatments on Lotus pedunculatus, Desmodium intortum and Desmodium ucinatum in the laboratory using the "growout technique". Two stickers, cellofas A and Methofas were used, and pelleting materials include lime, gypsum, Kaolin, calcium silicate, and rock phosphates. Calcium silicate was quickly lethal to rhizobia. Malt extract was included in the stickers in several experiments and had a protective action on rhizobia. Significant effects of pellet treatments were observed on both seed germination and amount of nodulation.

Norris (1971b) reported the effect of lime and rock phosphate

pellets on nodulation of eight species of legumes in two field conditions. At the sod seeding site there was no beneficial effect in nodulation from lime pelleting. However, at the calcium-deficient site lime pelleting in comparison with cellofas inoculation improved nodulation with eight legume-Rhizobium combinations, had no effect with six combinations, and depressed nodulation with two combinations. Rock phosphate pelleting showed no benefit in nodulation in 12 combinations under sod seeding. At the calcium-deficient site, four combinations showed improved nodulation and 12 combinations no effect, but there were no negative effects. Pelleting treatments gave no yield increases with the exception of Desmodium uncinatum at Beerwah where yield was significantly increased by lime pelleting. No evidence in favour of routine pelleting with either lime or phosphate was provided by these experiments.

Norris (1971c) studied nodulation of Dolichos lablab resulting from lime pelleted and rock phosphate pelleted seed after storage for 1 day and 1, 2, 4, 6, and 8 weeks at 27°C. Cellofas A was used as a sticker and two strains of Rhizobium were compared. Rock phosphate pelleting was superior to lime pelleting in survival of inoculant on the seed, survival of plants in the row, and promotion of nodulation. Lime pelleting depressed yield at 8 weeks of age, but not at 4 months.

Wade et al (1972) reported that doubling the inoculum rate, $CaCO_3$ pelleting, or soil fumigation increased annual dry forage yields 1,200 to 2,000 Kg/ha over that of the normal rate of inoculum. These treatments improved both nodulation and seedling growth. Winter forage yields were increased 200% by pelleting inoculated seeds with $CaCO_3$. Seed pelleting was beneficial in non-fumigated soil, but not in fumigated soil, suggesting that pelleting made conditions more favorable for

seed-borne rhizobia to compete with native soil micro-organisms.

In some crops, for example peanuts, the seeds are generally treated with a fungicide to give protection during the germination period. According to the results obtained in the U.S.A. (Ruhloff and Burton 1951) the nodule bacteria are quickly destroyed by contact with most of the chemicals used as seed protectants, even such as Arasan, Spergon or Phygon which do not contain heavy metals. Various authors have suggested that the inoculant for chemically-protected seed should be mixed with moist inert materials such as bran, saw dust, lime or even earth from the field to be inoculated, adding the mixture to the soil before planting or at the time of planting (e.g. Baur 1944). However, the difficulty of drilling limited amounts of moist material uniformly seems to make these methods impractical. Broadcasting the mixture over the soil surface and then covering it lightly by cultivation, necessitates two special field operations and is not feasible in a hot dry climate, since it is likely to result in rapid drying of the scattered mixture, and a high mortality of bacteria before they are incorporated into the moist soil.

Schiffman and Alper (1968) used a technique of peat base liquid inoculation to sowing groundnuts in Israel. Soil inoculation was tried in field experiments during two seasons using different concentrations and amounts of bacterial suspension and compared with direct seed inoculation of chemically protected and unprotected peanut seed. Soil inoculation gave significantly better results than direct inoculation of chemically protected or unprotected seed. Large yields of high quality peanuts which compared favourably with highly nitrogen-fertilized peanuts were obtained after the application of relatively small amounts

(10-40 gm) of enriched peat inoculant in 5 litres of water per dunam (1/10 ha).

Liquid inoculants have the advantage of adding more cells in the row and thus enable the inoculated Rhizobium to compete with the native strains. Kapusta and Rouwenhorst (1973) showed that the recovery of applied Beltsville serogroup 138 from nodules increased from 18 to 60% when 15 x 10^{10} cells/cm of row were added in a liquid carrier. Boonkerd et al (1978) found increased recovery of Beltsville serogroup 62 from zero to 38% by the addition of 5 x 10^{8} cells/cm of row. The results clearly show that native Rhizobium can be replaced in the nodules by the applied Rhizobium strains.

Hale (1978) found that when white clover seeds were inoculated by slurrying method, less than 30% of the nodules formed at 6 weeks contain the inoculant strain. When a liquid peat inoculum was incorporated in the soils prior to sowing, there was a significant increase in the numbers of nodules containing the inoculated strains (80-90%). Dry matter production was also increased.

Brockwell et al (1978) reported that liquid inoculation gave good nodulation and protected the Rhizobium from chemically treated seeds. A liquid inoculum applicator has been developed for soybeans and lupins (Brockwell et al 1978, Brockwell and Gault 1978).

Granular inoculant is an alternative to liquid inoculant. It has been available to soybean and peanut farmers for several years. In its most common form it consists of granular peat culture, each gram containing about 16,000 granules. American farmers usually apply it via the insecticide hopper attachment to the soybean planter (Brockwell et al 1978). Dean and Clark (1977) reported that granular inoculum gave

better results in faba beans (Vicia faba) than powder inoculum in a low nitrate soil. A granular inoculum appeared to withstand low soil moisture conditions better than the powder form.

High level of inoculation have been achieved with granular soil applied inoculant, which can be applied in the row with the seed. Using this technique, 20 - 50 times more inoculant can be added with seed applied, peat-based inoculants (Nelson et al 1978).

Hale (1978) reported granular inoculant increased clover yield, 80 to 90% of the nodules being formed by the inoculated strain. Brockwell et al (1978) reported good results in nodulation from granular inoculant and it protected the Rhizobium from direct contact with the chemical treated seeds.

Bezdicek et al (1978) reported higher yields and better nodulation of soybeans were obtained with granular than a peat carrier. Beltsville Rhizobium japonicum strains 110 and 138 added as granular inoculum were associated with the highest soybean yield.

Muldoon et al (1979) reported soybean yield in Ontario, Canada did not increase when granular or seed inoculated seeds were grown in land where soybeans had previously grown. However, when the beans were sown in new soybean land, there was an inoculation response. Granular inoculants caused consistently higher yields than the seed applied inoculant. Seed yields increased linearly with rates of granular inoculant which were 1/4, 1/2, 3/4 and 1 times the manufacturers' recommended rates for soybeans grown in 18 cm rows. However, above the lowest rate, the value of the added yield was only equal to the cost of the extra inoculant. Thus, in the narrow row soybean cultural system necessary to maximize yields in short season areas, the manufacturers' recommended rates of

granular inoculant were higher than necessary for maximum economic return.

Methods Used in Identifying Inoculated Rhizobium

When inoculated legume seeds are sown in zero Rhizobium soils, it is expected that 100% of the nodules formed are from the inoculated strain. However, most of the agricultural fields have their indigeneous rhizobia. These rhizobia may be a threat to the success of inoculation. How successful are the inoculated strains able to compete with the native strains in nodule formation? Is the failure to get the response to inoculation due to the strain not competing with the native strains to form nodules on the host or is the inoculated strain inferior to the native populations? These are the tough questions that most of the Rhizobiologists are generally facing. Many techniques have been developed and employed to identify the inoculated strains from the native ones.

Serological Techniques

Serological techniques have long been used for Rhizobium strain identification. Steven (1923) was among the first to report identification of strains of Rhizobium japonicum by serological procedures. He reported the classification of eight strains into three serological groups. Wright (1925a, b) classified eight strains of Rhizobium japonicum serologically and evaluated six of the eight in field inoculation experiments. He observed marked differences in effectiveness in soils where the uninoculated checks produced no nodules. Subsequently, Wright et al (1930) classified 156 isolates of Rhizobium japonicum from soils in which soybeans had grown in Japan, Manchuria, Virginia, Mississippi, and Louisiana into six serological groups and noted a

marked tendency for the isolates to fall into one serological group.

In serology studies, many techniques are used, i.e. agglutination, gel-immuno diffusion, fluorescent antibody and enzyme-linked immunosorbent assay (ELISA). Agglutination and gel-immuno diffusion tests have been commonly used (Vincent 1970). Both methods are time consuming because isolation and subculturing of the rhizobia from nodules are required.

However, the standard agglutination has been used by earlier workers (e.g. Vincent 1941). Read (1953) used this technique to identify the success of the inoculum strains of Rhizobium trifolii in competition for nodulation with indigenous strains. She found that a suitable strain gave rise to 50% or more of the nodules. Vincent and Waters (1953, 1954), Jenkins et al (1954) successfully used this technique in competition studies of Rhizobium trifolii in the laboratory and field conditions. Koontz and Faber (1961) studied the somatic antigens of 25 strains of Rhizobium to determine possible somatic groups. They identified six somatic groups but a seventh appeared possible, since three strains of Rhizobium japonicum did not react with any of the 14 prepared somatic antisera. Škrdleta (1965) studied 62 strains of Rhizobium japonicum from the point of view of their possible relegation to somatic serogroups. Cross-agglutinations were carried out with all strains by the use of 11 antisera prepared against random samples of the studied complex of strains. Twenty-two strains reacted with none of the used antisera even when antisera were diluted by 1:10. It was possible to divide the remaining 40 strains into four somatic groups between which several common strains appeared to exist. No common antigen for all investigated has been found. Johnson and Means (1963) studied serological

groups of Rhizobium japonicum recovered from nodules of soybeans in several field soils. They showed distinctly different populations of bacteria in the nodules of plants grown in each of six different soils.

Since the standard agglutination tests as previously mentioned were done with pure cultures or isolates from nodules, it is more desirable being able to identify the Rhizobium without isolation. Means et al (1964) developed a rapid micro-agglutination test using homogenized suspensions of nodules as an antigen. In this technique, nodules were washed with distilled water to remove adhering soil particles, then a nodule was placed in a 10 ml culture tube and homogenized in approximately 10 times its weight of 0.86% NaCl. Portions of the suspension of each nodule were tested directly for somatic (O) agglutination against the antisera. Heating of the nodular suspension for 30 minutes in a water bath minimized heterogeneous cross reactions by destroying the non-specific (H) antigen, if present. Since then it has been used to identify the Rhizobium japonicum in the soils (Damirgi et al 1967, Caldwell and Hartwig 1970, Bezdicek 1972), competition studies (Johnson et al 1965, Caldwell and Vest 1968, Caldwell 1969, Caldwell and Weber 1970, Weber and Miller 1972, Boonkerd et al 1978, Semu et al 1979). However, this rapid micro-agglutination test using homogenized suspensions of nodules as antigens, is insensitive when small nodules are examined. Only large nodules can be used directly for immuno-diffusion (Dudman 1977).

Another method widely used is the gel immuno-diffusion technique. Dudman and Brockwell (1968) used this technique to study field performance of clover inoculants (Rhizobium trifolii). They examined 456 isolates (between 3 and 42 months after sowing). They found 53.3% of

the isolates were recognized as inoculum strains but one strain, TA1 was recovered more than 11 times as frequently as the other, UNZ29. This technique was essentially the same as the standard serological technique, i.e. preparation of antisera, isolation of Rhizobium. However, instead of doing agglutination tests using tubes, slides or tray, some modification was made. The test was done in agar gels. The wells were made in an agar gel plate in a hexagonally array fashion with one well in the center. The center well served as an antiserum well while the other six wells serve as standard homologous strain and isolate wells. It was essential that the standard strain be put on opposite sides of the hexagons. This enabled the unknown isolates to be adjacent to a standard suspension homologous to the antisera being used. This would distinguish between true reactions of identity and cross-reactions. After putting the antiserum, standard strain, and isolates to their proper wells, the petri dishes were kept at 4°C in tight-lidded boxes over moistened tissue paper. Precipitation bands were visible within 24 hours and could be interpreted with confidence in 48 hours. This method has been widely used in competition studies (Brockwell and Dudman 1968, Gibson 1968, Robinson 1969a, Škrdlela 1970, Brockwell et al 1972, 1977, van der Merwe and Strijdom 1973, Gibson et al 1976, Diatloff 1977). This technique again has the disadvantage of being time consuming because isolation and subculturing of the rhizobia from nodules is required.

The fluorescent antibody (FA) method is another serological method used in Rhizobium strain identification. The development of FA technique in the medical field has provided a means of specifically staining particular micro-organisms in complex environments such as the soil and

rhizosphere. Schmidt et al (1968) reported the use of this technique to identify Rhizobium. The methodology of the FA technique involves the preparation of antisera and conjugation of the antisera with fluorescein isothiocyanate (FITC). The fluorescein-labelled fluorescent antibody was used as a stain to treat glass microscope slides recovered from the tested soils. The microscope slides were then examined by fluorescent microscopy for the presence of bacteria that reacted with the fluorescent antibody stain. Trinick (1969) developed a method of rapid identification of nodule smears using the FA technique. It was found that bacteroids from the nodules of clovers, medics and serradella reacted to FA staining in a similar fashion to cultured cells. Nodule squashes or smears have been used to identify strains of Rhizobium trifolii (Trinick 1969, Jones and Russell 1972, Roughley et al 1976), Rhizobium meliloti (Trinick 1969), Rhizobium lupini (Trinick 1969) and Rhizobium japonicum (Bohlool and Schmidt 1970, 1973) in competitive studies. The use of the FA technique has been reported to be hindered through interference by non-specific adsorption of labelled antisera by plant and soil materials (Trinick cited in Parker et al 1977), but this non-specific fluorescence has been eliminated by treating specimens with gelatin-Rhodamine isothiocyanate (RhIT). However, the disadvantage of this technique is the requirement of expensive microscopic equipment and large amounts of antibody.

A recent serological technique used in identification of Rhizobium is the use of enzyme-linked immunosorbent assay (ELISA). In this test the antigen (A) is added to a specific antibody (Ab) which has previously been adsorbed onto a solid surface of a polystyrene microtiter plate. The immobilized A-Ab is then coated with an enzyme-labelled specific antibody. Further additions of a suitable enzyme substrate

permits the colorimetric detection of the enzyme-labelled antibody that has been complexed with the trapped antigen. If the antigen is not specific to the antibody, the complex cannot be constructed and no colorimetric reaction will occur. Kishinevsky and Bar-Joseph (1978) used ELISA for serological identification of peanut Rhizobium strains both in cell suspension of pure cultures and in single root nodules of groundnut (Arachis hypogaea) plants. Antisera of three peanut Rhizobium strains were tested against eight different Rhizobium isolates. Three serogroups identified by agglutination and immuno-diffusion tests were confirmed by ELISA. It was found that ELISA was more sensitive by four to six orders of magnitude than the agglutination and immuno-diffusion tests and enabled the detection of Rhizobium antigens in cell suspension of 10^4 - 10^5 cell per millilitre. ELISA enabled the precise typing of rhizobial isolates in single small root nodules. The minimum fresh weight of nodule tissue necessary to perform the ELISA test was 0.4 mg crushed in 1 ml of phosphate-buffered saline (PBS). ELISA was also successfully used for strain identification in mixed inoculated plants. One of the strains in each pair formed most of the nodules examined.

Berger et al (1979) used the ELISA technique to identify strains of Rhizobium in culture and in lentil nodules. The test could be used on cells from both fresh and frozen nodules obtained from plants grown in a growth chamber or in the field. Test results were confirmed by immuno-fluorescence. This ELISA technique can be used for field studies and requires less antisera than other serological techniques.

Morley and Jones (1980) made a modification of the ELISA technique using a fluorescent substrate. Comparisons were made between this highly sensitive technique and the conventional method for investigations of

the Rhizobium/legume symbiosis. The technique could be used to detect Rhizobium spp. both from pure culture and from nodules on Trifolium repens at a concentration of 10^4 cells/ml. Tests for cross-reactivity indicated that the technique will facilitate a wide range of experiments which require the identification of Rhizobium strains.

Resistance to High Concentrations of Antibiotics

Many workers have successfully used resistance to high concentrations of antibiotics to identify the inoculated strains. This method involves inoculating the seeds with Rhizobium strains resistant to high concentrations of one or more antibiotics. The seeds are then sown in the field. Nodules are collected, Rhizobium isolated, and exposed to high concentrations of the antibiotics. Obaton (1971) reported the use of Rhizobium meliloti mutants to streptomycin or Kanamycin or both to investigate strain competition and survival in soil. It was found that after inoculation of lucerne seeds, the rhizobia could be isolated from the plant nodules and grown on specific antibiotic containing media even after several years of growth in open soil.

Franco and Vincent (1976) used streptomycin resistance to distinguish between strains of Rhizobium competing for the colonization and nodulation of Macroptilium atropurpureum (DC) VRB (Siratro) and Stylosanthes guianensis (Aubl) Swartz (Stylo, line IRI 1022). They found that related strains and strains of similar growth habit competed more with each other in the colonization of the root surface than did a fast growing strain in association with a typical slow grower. Capacity amongst slow growing strains to dominate a paired competitor in the colonization of the root was a strain characteristic and was not

affected by the host. It was unrelated to effectiveness in the Rhizobium-host association. In five of the seven cases nodulation success could be related quantitatively to root surface representation and a 'competitive index' calculated; in the remainder one of each pair overwhelmed the other over a wide range of inoculum ratios. It was not possible to relate competitive nodulating success to any single feature of the host: Rhizobium symbiosis. In the two most striking cases, a relationship between competitiveness and N_2-fixing effectiveness was reversed, in others competitiveness difference was as great between equally effective as between strains of differing effectiveness. In the case of stylo there was a marked dominance of an ineffective over an effective competitor, which might be attributed to compatibility, as indicated by faster nodulation by the ineffective strain. This last result argues against the use of mixed inocula including any strain ineffective on any of the hosts for which the inoculum is recommended.

Schwinghammer and Dudman (1973) examined resistance to the antibiotic spectinamycin as a possible marker to supplement streptomycin resistance in ecological or genetic studies with rhizobia. Single step spontaneous mutants resistant to high level of spectinomycin were isolated from eight effective strains representing four species of Rhizobium, i.e. Rhizobium meliloti, Rhizobium trifolii, Rhizobium leguminosarum, and Rhizobium japonicum. There was no evidence of cross resistance to streptomycin, and streptomycin resistant mutants were not cross resistant to spectinomycin. Minor changes in antigenic characteristics examined by immuno-diffusion agar were detected for mutants from two strains but these variants were still identifiable with the parent strains. Partial or full loss of symbiotic effectiveness occurred in

only about 20% of the spectinomycin resistant mutants and the resistance marker was unchanged through one plant passage. It is concluded that spectinomycin resistant mutants properly evaluated for possible pleiotropic effects should provide a useful marker system for use alone or in combination with streptomycin resistance in Rhizobium. Holding (cited by Hale 1978) used rifamycin as a supplement to streptomycin resistance in competition studies with Rhizobium trifolii.

Brockwell et al (1977) inoculated subterranean clover seed (Trifolium subterraneum L.) with marked strains of Rhizobium trifolii, distinguished from other strains antigenically and by streptomycin resistance. The inoculated seeds were sown in a field environment having a natural population of Rhizobium trifolii. Isolates from nodules obtained periodically during the following 41 months were classified using both methods of identification in parallel. There was a gradual disappearance of the inoculum strains which occurred more rapidly in plots of cv Woogenellup than in plots seeded with cv Mount Barker. At five harvests, there was 95% (or greater) correspondence between inoculum survival using either method of identification. There was evidence that a small proportion of the progeny of the inocula sustained independent loss of antigenic character and/or streptomycin resistance in the field or, alternatively, that strains occurring naturally acquired these characteristics. A few nodules contained more than one strain of rhizobia. These exceptions occurred at low frequency and did not interfere substantially with identification results. It is concluded that gel immune diffusion serology and the use of streptomycin resistant mutants are both reliable methods for identifying strains of rhizobia re-isolated from field environments.

The isolation of spontaneous mutants of Rhizobium species resistant to antibiotics may be accompanied by change in the ability to form nodules (infectivity) or in the ability to fix nitrogen (effectiveness). Schwinghamer (1967) found that resistance to antibiotics known to inhibit protein synthesis, e.g. streptomycin, spectinomycin and chloramphenicol, was associated with little or no loss of symbiotic effectiveness, whereas resistance to antibiotic affecting cell wall synthesis and permeability was often accompanied by loss of effectiveness.

Pankhurst (1977) isolated mutants resistant to 16 individual antibiotics from two fast growing and two slow growing strains of Lotus Rhizobium. These mutants were evaluated for their effectiveness on Lotus pedunculatus. It was found that resistance to streptomycin, spectinomycin, chloramphenical and tetracycline (inhibitor of protein synthesis) was associated with little or no loss of effectiveness with all four strains but resistance to nalidixic acid and rifampicin (inhibitor of nucleic acid synthesis) and to D-cycloserine, novobiocin and penicillin (inhibitor of cell wall-cell membrane synthesis) was associated with significant loss of effectiveness in 20 to 100% of the mutants. Resistance to viomycin, neomycin, kanamycin and vibramycin was associated with loss of effectiveness with mutants of the two fast-growing strains but not with mutants of the slow-growing strains.

Levin and Montgomery (1973) determined the response of selected strains of Rhizobium japonicum to 50 antibiotics. The most effective antibiotics for this species include kanamycin, streptomycin, triburon, vibramycin and viomycin. Mutants were recovered which were resistant to these drugs individually and in various combinations of two. Single and double

mutants which grew as well as the sensitive parent strains on non-selective media, were tested for infectivity and effectiveness of nitrogen fixation in soybeans. Six weeks after germination, plants were harvested and compared with respect to size, number of nodules, color, number of trifoliate leaves, dry weight and total nitrogen. Their finding indicated no dramatic differences either in infectivity or effectiveness between certain antibiotic-sensitive strains and their resistant mutants. In contrast, Zelazna-Kowalska (1971) reported strains of Rhizobium trifolii became non-infective for red clover after acquiring 100 μg/ml streptomycin resistance by mutation or transformation. His work pointed out that the loss of infectivity of Rhizobium trifolii were streptomycin resistant dependent.

Jones and Bromfield (1978) studied the symbiotic effectiveness of singly or doubly mutant of Rhizobium trifolii resistance to streptomycin and spectinomycin. They found that the majority of them were inferior to the parental strains.

Hale (1978) reported the use of 200 μg/ml streptomycin resistant mutants to study the effect of inoculation methods on nodulation and yield of white clover in problem soil containing a large naturalized population of rhizobia. It was found that when inoculation was done by the slurrying method, less than 30% of the nodules formed at 6 weeks contained the inoculum strain. When either a liquid peat inoculum was incorporated in the soils prior to sowing or granules of the streptomycin resistant strain were sown together with seed, then there was a significant increase in the number of nodules containing the antibiotic resistant strain. In each of the soils tested there was an increase in dry matter production and 80 to 90% of the nodules contained the strep-

tomycin resistant strain. Persistance studies showed a reduction in the number of the streptomycin resistance rhizobia in the soil. This reduction in streptomycin resistance rhizobia is consistent with other workers (Dudman and Brockwell 1968, Brockwell et al 1972, Brockwell et al 1977).

Jones and Hardarson (1979) used mutants of Rhizobium trifolii resistant to streptomycin and spectinomycin to study variations between varieties of white clover (Trifolium repens L.) in their selection or preference for rhizobial strains in nodulation. Significant differences between varieties was found. Significant correlations were found between the preference for rhizobial strains of plants grown from seeds and of stolon lines vegetatively propagated from the former plant, indicating that the preference for rhizobial strains is genetically controlled by the host.

Hardarson and Jones (1979) used antibiotic resistant mutants of Rhizobium trifolii to study the effect of temperature and soil type on the relative success in nodulating cultivars of white clover (Trifolium repens). In aseptic test tube culture, no significant difference was found between the two mutant strains at lower temperatures but temperature x Rhizobium strain interaction was highly significant. In soil, success in nodulation could be altered by temperature and the temperature x bacterial strain interaction was significant. The bacterial strain x variety x temperature was also highly significant.

Hale (1978) suggested that when using antibiotic resistance markers in ecological studies involving Rhizobium it should be borne in mind that resistance can be carried on a plasmid and may be transferred to other bacterial species. However, he commented further that in the soil

environments used for this type of ecological investigation it is unlikely that there will be any selection pressure for antibiotic resistance and consequently the likelihood of transfer of resistance to other bacteria is remote.

Low Intrinsic Antibiotic Resistance

The previous two techniques of Rhizobium identification have been widely accepted. However, they have some advantages and disadvantages. Serological techniques can be very sensitive (Means et al 1964, Krishinevsky and Bar-Josepth 1978) and have been used to monitor the success of introduced strains (Read 1953, Dudman and Brockwell 1968), and to demonstrate that natural populations of rhizobia may be heterogeneous (Hughes and Vincent 1942, Purchase and Vincent 1949, Purchase et al 1951). However, the use of serology is restricted by the limited number of serotypes of Rhizobium which are found and by the fact that the raising of strain specific antiserum is time consuming.

The other main identification techniques involve high level antibiotic resistance markers. This has the advantage of ease of isolation and recognition of inoculant strains from nodules and also from soil. However, such genetic markers may alter symbiotic ability compared with the wild type (Schwinghamer 1967, Zelazna-Kowalska 1971, Pankhurst 1977, Jones and Bromfield 1978). This technique also gives little information concerning the indigenous population of Rhizobium apart from the percentage of nodules which are not formed by the inoculant strain.

Therefore, it is necessary to have a simple technique that can be used to identify the Rhizobium quickly and without altering its symbiotic ability. This is why the technique of strain identification using

intrinsic antibiotic resistance has been developed (Josey et al 1979).

The use of low levels of antibiotic to distinguish the Rhizobium has previously been reported by many workers. Pinto et al (1974) used natural resistance to kanamycin at 2 mg/l and streptomycin at 1.5 mg/l to distinguish strains of Rhizobium meliloti. Graham (1963b) studied the sensitivities of many Rhizobium spp. to nine different antibiotics namely streptomycin, neomycin, aureomycin, chloramphenicin, terramycin, bacitracin, ledermycin, erythromycin, and sodium benzyl penicillin (penicillin G), each at three low concentrations (0.1, 4.0, and 5.0 µg/ sensitivity disk). He showed that strains of several species of Rhizobium had varying resistances to these low concentrations of a range of antibiotics. Mahler and Bezdicek (1978) showed that isolates from a natural population of Rhizobium leguminosarum exhibited variation in their response to quite high concentrations of eight antibiotics.

Josey et al (1979) used the variation in intrinsic resistances to low levels of eight antibiotics as an identifying characteristic for 26 Rhizobium leguminosarum strains. They found that the pattern of antibiotic resistance of each strain was a stable property by which Rhizobium isolated from root nodules of inoculated Pisum sativum could be recognized. The antibiotic test for strain identification with Rhizobium leguminosarum were also applied to Rhizobium phaseoli. It was necessary to include reference cultures in tests with this species, as the test most suitable for the Rhizobium leguminosarum strains showed some variability with Rhizobium phaseoli. Benon and Josey (1980) used two strains of Rhizobium phaseoli, one of which (strain 1234) was resistant to a high level of streptomycin, to inoculate plots of French beans (Phaseolus vulgaris). Bacteria were isolated from nodules and typed

using their intrinsic resistance to levels of seven antibiotics. The inoculant strains were found to be a minority of isolates from inoculated plots. The high level streptomycin resistance character was used in the case of strain 1234 to confirm the accuracy of identification. The resident population of Rhizobium phaseoli was shown to be heterogeneous; 54 different resistance patterns were recorded. Isolates having the same intrinsic resistance pattern, with few exceptions, were uniform in their reaction with anti-serum raised against one of the inoculant strains and in their colony morphology.

MANUSCRIPT I

COUNTING *Cicer Rhizobium* USING A PLANT INFECTION TECHNIQUE

ABSTRACT

A technique for obtaining a chickpea plantlet growing in a test tube has been developed. This is done by surface sterilizing chickpea seeds with 0.2% $HgCl_2$ for 3 minutes, washed thoroughly eight to 10 times with sterilized tap water and then germinated in sterilized plain agar for 3 days in the dark. The cotyledons of the germinating seedling are cut off and the seedling sown in 25 x 200 mm test tubes containing either sand or a sand vermiculite mixture. The plantlet can be used reliably as a 'trap host' for counting the number of specific Cicer Rhizobium in both sterilized and non-sterilized conditions. The value of such a plantlet as a 'trap host' for studying Cicer Rhizobium ecology, strain authentication and inoculum quality control has been demonstrated.

INTRODUCTION

One of the causes of poor nodulation of chickpea (Cicer arietinum L.) in farmers' fields may be that the soil contains too few of the very specific Rhizobium strains that can nodulate chickpea. There is no truly selective medium for Rhizobium which distinguishes it from other soil bacteria, or which differentiates between Rhizobium strains which nodulate different groups of legumes. No selective medium has been selective enough to use in counting Rhizobium although Rhizobium strains selected for high levels of antibiotic resistance can be counted directly from soil suspension by plating on agar containing the antibiotics (Graham 1969, Nutman 1973, Pattison and Skinner 1974), other soil bacteria being inhibited by the antibiotic.

Wilson (1926) developed a method for estimating the Rhizobium population in a sample on the nodulation pattern of plants grown in conditions inoculated by serial dilutions of the sample. Such a test has been widely used and modified (Tuzimura and Watanabe 1961a, Date and Vincent 1962, Brockwell 1963a, Ham and Frederick 1966, Thompson and Vincent 1967, Weaver and Frederick 1972).

There are several tables available which estimate numbers from frequency of positives in a dilution series. Date and Vincent (1962) used Fisher and Yates tables which provide a means of calculating an estimate and its fiducial limits. Brockwell (1963a) and Brockwell et al. (1975) based the estimates on a modified version of Lorenz's table (Lorenz 1941) which provide an estimate of MPN (most probable number).

The test plants are usually grown in test tubes closed with cotton-wool plugs. The media may be agar, sand:vermiculite or sand alone. Small-seeded species grow best in tubes and pose no problem. Where Rhizobium which nodulate large seeded legumes are being investigated, such a legume may be substituted by a symbiotically-related species which has small seeds and can be handled in test tube plant infection tests, e.g. siratro (Macroptilium atropurpureum) for counting the cowpea group of Rhizobium. Wild soybeans (Glycine ussuriensis Regel and Maack) have been used for counting populations of Rhizobium japonicum (Brockwell et al. 1975). Rhizobium nodulating soybean can also be counted by an MPN method using soybean plants grown in growth pouches (Weaver and Frederick 1972).

Even though chickpea Rhizobium was classified as Rhizobium leguminosarum Frank (Fred et al. 1932), it is very specific for Cicer spp. and may nodulate Sesbania ineffectively (Gaur and Sen 1979), although Rhizobium normally nodulating Sesbania spp. do not nodulate chickpea. Chickpeas (Cicer arietinum L.) is a larger seeded legume without a suitable alternative host for the plant infection counting method. Preliminary experiments showed that chickpea does not nodulate normally when grown in test tubes. We report a method of counting chickpea Rhizobium in pure and contaminated materials using chickpea plants which are dwarfed by excising their cotyledons.

MATERIALS AND METHODS

Culture of Chickpea in Test Tubes

Chickpea seeds of cv. 850-3/27 (unless otherwise specified) were

surface sterilized with 0.2% $HgCl_2$ for 3 minutes and then washed thoroughly eight to 10 times with sterilized tap water. The seeds were then placed in 1.2% sterilized plain agar (South Sea Chemical Limited, 131, Hyderguda, Hyderabad 500 029, India) in petri dishes, 30 seeds per petri dish (9 cm in diameter) and kept in the dark for 3 days at 28°C. The whole cotyledons of the germinating seedling were then aseptically excised using scalpel and forceps. The excised seedling was transferred immediately into 2.5 x 20 cm tubes (one seedling/tube) containing either 20 ml washed coarse sand or a 1:1 mixture of sand and vermiculite (V/V) moistened with 9 ml 1/4 strength N-free solution (Readings N-free nutrient or modified long Ashton solution, see Appendix 1). The plants were then placed in wooden racks for 3 to 4 days in the light chamber. The light chamber had been designed for growing the plants in a room where the temperature inside the test tubes is prevented from rising above 30°C. The test tubes are laterally illuminated by fluorescent lights at 40 watts/m^2 (Figure 1).

Inoculation Procedure

Rhizobium cultures were grown in yeast extract mannitol broth (Vincent 1970) in 250 ml conical flasks shaken with rotary action for 5 to 10 days. A required amount of broth was pipetted out and added to an appropriate amount of sterilized tap water blanks to make two-, four- or ten-fold dilution. A dilution series was made up to the level where no Rhizobium was expected to be left. The plants were inoculated with 1 ml of the last six dilutions in the series. The number of replicate tubes per dilution varied from three to 12 depending on the experiment. Uninoculated controls were also kept. The tubes were placed in the light chamber and watered 3 to 4 weeks later with 3 to 4 ml of sterilized 1/4

Figure 1. The light chamber used in the studies.

The light chamber consists of 64 nos. of 5 ft (80 watts) fluorescent tube lights divided into 4 groups of 16 lights each. Each group of 16 lights is fitted on slotted angle trays which are suspended on pulleys to facilitate up and down movement so that the wooden blocks holding the test tubes can be put in position or removed. The starters for the fluorescent lights on the frame holding the tubes, the ballasts are outside the room. The tubes are cooled by blowing conditioned air from the wall AC units over them by two fans. The lights are controlled manually by 4 on-off switches (one for each rack) beside automatic control by a thermostat and a timer. The timer switches on the light at 4:30 p.m. and off at 8:30 a.m. The thermostat further switches the light off when the temperature reaches 30°C and switches them on at the temperature below 30°C.

strength N-free nutrient solution per tube, the amount depending on the growth conditions and plant size. The plants did not require further watering until harvest 6 weeks after inoculation.

The recovery of chickpea Rhizobiumfrom unsterilized soil was tested as follows: Five-day-old broth cultures of Rhizobium strain 9036 and IC-59 were used to inoculate two vertisol soils (1 ml per 100 g soil). The inoculated soil was thoroughly mixed in a plastic bag to ensure adequate dispersal of the Rhizobium. The broth was counted by plates and the plant infection method before adding to the soils. A plant infection dilution count of inoculated and uninoculated soil was done 4 hours after inoculation. This was done by weighing 20 g of the soil, added to 180 ml blank and shaken on a wrist shaker for 15 minutes. This was considered as 10^1 dilution. A serially ten-fold dilution series was made up to the level where no Rhizobium is expected to be left by adding 1 ml of the suspension to 9 ml blanks. The plants were inoculated with 1 ml of the last six dilutions in the series. The number of replicate tubes per dilution was three.

For peat inoculum Rhizobium counting, 10 g of the peat inoculum were weighed, put in 90 ml blank, shaken on a wrist shaker for 15 minutes. This was considered as 10^1 dilution. A serially ten-fold dilution series was made up to the level where no Rhizobium is expected to be left as mentioned above. Plant count and plate count were made to determine the number of chickpea Rhizobium per g peat.

Plate Counts

Three successive suitable dilutions were plated with three replicate plates per dilution. 0.1 ml aliquots of each dilution series were

spread using a glass rod over 30 ml congo red mannitol agar (CRMA) in 9 cm diameter petri dishes. The plates were incubated at 28°C for 6 to 10 days before counting the colonies. The dilutions that had the Rhizobium ranging from 30 to 300 were used to calculate the number of Rhizobium in the original sample.

Calculation of the Most Probable Number and Theoretical Positive Tubes

Plants were harvested 6 weeks after inoculation and scored for nodulation. The total number of positive and negative tubes were then used to calculate the estimates of the number of rhizobia using Table VIII2 of Fisher and Yates (1963). Theoretical positive tubes, based on the plate count were also calculated using the formula given in Fisher and Yates (1963).

RESULTS

The experiment on cotyledon excision was carried out. Different proportions of cotyledons of germinating seedlings were excised, i.e. one, one and one-half and two cotyledons removed. The whole intact seed treatment was also added as the control treatment. The results are shown in Table 1. As the amount of cotyledon removed increased, the accuracy of the plant count also increased. The whole seed and half seed treatment gave significantly lower counts than the plate counts. However, the removal of one and one-half and two cotyledons resulted in greater accuracy in counting. The MPN of the plant and plate count agree well when the 95% confidence limits are calculated. The whole seed and half seed treatments resulted in fast and vigorous growth. The plants grew and coiled in the tubes and tried to push out through the cotton plug. The whole seed and half seed treatments required

TABLE 1. Effect of different proportions of cotyledon removal on the counts of chickpea Rhizobium strain IC-2046[a] in 2:1 sand:vermiculite medium.

Treatments[b]	Log 10 Rhizobium per ml broth (plate count)	Total positive tubes/total tubes	Log 10 MPN per ml broth[c]
1S	9.55	6/36	5.24
½S	9.58	18/36	7.24
¼S	9.53	30/36	9.26
0S	9.48	31/36	9.43

[a]Grown in yeast extract mannitol broth for 10 days, ten-fold diluted. Dilution 10^5-10^{10} were used to inoculate the plants.

[b]1S = whole seed intact; ½S = 1 cotyledon removed; ¼S = 1½ cotyledons removed; 0S = 2 cotyledons removed.

[c]Six dilution steps (10^5-10^{10}) and 6 replicate tubes per dilution were used in the plant counts. The factor for 95% confidence interval on the MPN is ± 0.47.

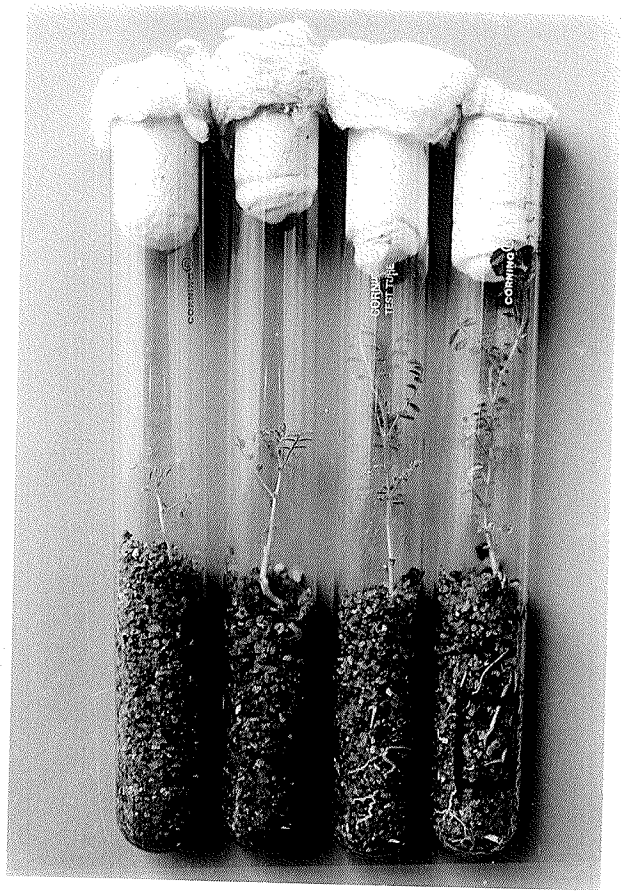

Figure 2. A. Dwarfed chickpea seedling at different ages. From left to right, 3 days, 1, 3 and 6 weeks after transplanting to the test tubes.

B. Close-up picture of a nodule seen from outside of the test tube of a 6 week old seedling.

frequent watering during the 6 week growing period. They were given 5 and 3 ml of 1/4 strength N-free solution respectively at 3 weeks after inoculation. They were also given 3 ml/tube 10 days later. The one and one-half and two cotyledons removal treatments were given only 3 ml of the solution per tube at 3 weeks after inoculation during its 6 week growing period.

Rooting Medium and Harvest Date

Washed sand and sand plus washed vermiculite in two different proportions were tested for their suitability as the rooting medium for plants grown in the test tube. Table 2 shows that all three media gave good plant growth and reliable MPN counts. The time taken to form nodules was examined for the rooting media, 1:1 sand:washed vermiculite and washed coarsed sand, with harvests 3, 4, 5 and 6 weeks after inoculation of the tubes. Table 3 shows that washed sand gave reliable counts 4 weeks after inoculation but nodulation in sand:vermiculite (1:1) was delayed. Secondary roots developed faster in sand than sand:vermiculite and may be related to the earlier nodulation. The other advantage of sand, to sand:vermiculite, is that it is easier to wash. This speeds up nodulation assessment. Nodules can also be seen from outside (Figure 2). Therefore, sand was chosen for use in further studies. An experiment was previously set up to compare agar medium, sand:unwashed vermiculite (1:1), and sand:washed vermiculite (1:1). The results show that agar medium and sand:unwashed vermiculite (1:1) were not suitable for using in MPN counts of chickpea Rhizobium (see Appendix 2). In agar medium it was also found that 50 out of 72 seedling tubes were fungus contaminated. Secondary root formation was also poor in this treatment. Therefore, it was not included in further studies. The temperature in

TABLE 2. Effect of root medium on estimation of chickpea *Rhizobium* numbers in broth cultures (strain IC-128 and IC-2046) by the plant-infection dilution method.

Medium	*Rhizobium* population (log 10)			
	IC-128		IC-2046	
	Plate count	Plant count[a]	Plate count	Plant count[b]
Washed sand	9.68	9.43	9.44	9.63
1:1 Sand:Vermiculite	9.64	9.63	9.51	9.63
2:1 Sand:Vermiculite	9.61	9.84	9.49	9.09

[a,b]Six dilution steps (10^5-10^{10}) and six replicate tubes were used in the plant counts. The factor for the 95% confidence interval on the MPN is $\pm$ 0.47.

TABLE 3. The effect of harvest time on the reliability of counting chickpea *Rhizobium* strain IC-128[a] by a plant infection-dilution method.

Time after inoculation	Rooting medium					
	1:1 Sand:washed vermiculite			Washed sand		
	Total + tubes/ total tubes	Log 10 MPN/ml[b]	Plate count	Total +tubes/ total tubes	Log 10 MPN/ml[c]	Plate count
3 weeks	5/36	5.08	9.48	27/36	7.77	9.64
4 weeks	23/36	8.08	9.48	30/36	9.26	9.64
5 weeks	28/36	8.94	9.48	34/36	9.94	9.64
6 weeks	31/36	9.34	9.48	33/36	9.84	9.64

[a] A 7 day old broth culture.

[b,c] Six dilution steps (10^5-10^{10}) and 6 replicate tubes were used in the plant counts. The factor for the 95% confidence interval on the MPN is $\pm$ 0.47.

the light chamber during the time this experiment was conducted was frequently found to be above 30°C. This might be the reason for poor MPN counts than in plate counts. Table 4 shows that the MPN count differs with the cultivars used as the trap host. Using a ten-fold dilution series of a broth culture of strain IC-128, cultivars 850-3/27, JG-62, BEG-482 and Annegiri gave the count up to 10^9 cells/ml broth, agreeing well with the plate counts when 95% confidence limits are taken into consideration. However, MPN counts for the varieties G-130 and Rabat did not agree well with the plate count. Rabat, a Kabuli cultivar, does not nodulate well in test tube cultures and is also poorly nodulated in the field at the ICRISAT centre. BEG-482, a desi cultivar also forms few nodules in the field but nodulated freely in the test tube. However, the consistency of repeatability of the nodulation pattern for a dilution series is poor with only 10 and eight out of 12 positive tubes at 10^5 and 10^6 dilutions.

Accuracy of the Plant Infection Counts

Broth cultures of strain 9036 were diluted serially (ten-, four- and two-fold) and these dilutions used to inoculate chickpea plants in tubes. Table 5 shows that in the ten-fold dilution series, the theoretical and observed number of positive tubes agreed reasonably well, except at 10^8 dilution. This discrepancy was caused by one tube not receiving enough water and the plant dying. In the four-fold dilution series, theoretical and observed positive tubes agreed well until the dilution 4^6 and 4^7 when the observed positive tubes were less than the number of positive tubes expected. In the two-fold dilution series, the theoretical and observed positive tubes agreed well until the calculated

TABLE 4. The effect of chickpea cultivars on the pattern of nodulation after inoculation from a dilution series of broth cultured chickpea Rhizobium strain IC-128[a] and the calculated most probable number of rhizobia.

	Dilution level						
	10^5	10^6	10^7	10^8	10^9	10^{10}	
	No. of Rhizobium/ml (plate count)						
	50,000	5,000	500	50 ± 3.06	5	0.5	
	No. of tubes tested						
	12	12	12	12	12	12	
	Theoretical + tubes						
	12	12	12	12	11.9	4.8	
Cultivar	Observed + tubes						MPN[b]
850-3/27	12	12	12	12	12	7	8.67×10^9
G-130	9	9	11	10	9	6	5.90×10^8
JG-62	12	12	12	12	12	3	3.37×10^9
BEG-482	10	8	12	12	11	9	2.71×10^9
RABAT	7	7	6	6	6	3	1.44×10^7
Annegiri	12	10	12	12	12	6	4.23×10^9

[a]Grown in yeast extract mannitol broth for 10 days, ten-fold diluted. Dilutions 10^6, 10^7 and 10^8 were plated; the numbers were extrapolated from 10^8 dilution.

[b]Six dilution steps (10^5-10^{10}) and 12 replicate tubes/dilution were used in the plant counts. The factor for the 95% confidence interval on the MPN is $\overset{\times}{\div}$ 1.47.

TABLE 5. Relationship between observed and theoretical* positive plant tube numbers for a ten-, four- and two-fold dilution series of chickpea Rhizobium strain 9036. Appropriate dilutions were plated and inoculated to the plants.

Ten-fold dilution series	Dilution level							
	10^5	10^6	10^7	10^8	10^9	10^{10}	10^{11}	10^{12}
Plate count	32,000	3,200	320	32 ± 3.7	3.2	0.32	0.032	0.0032
Theoretical + tube	12	12	12	12	11.55	3.36	0.35	0
Observed + tube	12	12	12	11	12	3	0	0

Four-fold dilution series	Dilution level							
	4^0	4^1	4^2	4^3	4^4	4^5	4^6	4^7
Plate count	3,147	784	197 ± 19	49	12	3	0.8	0.2
Theoretical + tube	12	12	12	12	12	11.49	6.54	2.13
Observed + tube	12	12	12	12	12	11	6	1

Two-fold dilution series	Dilution level								
	2^0	2^1	2^2	2^3	2^4	2^5	2^6	2^7	2^8
Plate count	450 ± 67	225	113	56	28	14	7	3.5	1.7
Theoretical + tube	12	12	12	12	12	12	12	12	10
Observed + tube	12	12	12	12	12	12	12	12	7

number of Rhizobium added per tube was less than 3.5. When the number of Rhizobium in this two-fold dilution series was less than two, the number of observed positive tubes was again less than the theoretical value.

Counting Rhizobia in Soils and Inoculants

Table 6 shows the recovery of Rhizobium after adding broth cultures to two Vertisol soils with different background populations of Rhizobium. The number of Rhizobium per ml broth was 2.75×10^9 and 1.95×10^9 for strain 9036 and IC-59 respectively. Therefore, the number added per g soil was 2.75×10^7 for strain 9036 and 1.95×10^7 for strain IC-59. The MPN from the plant count agrees well with the plate count and the calculated number of rhizobia added/g soil.

Chickpea Rhizobium inoculants received from various places were checked for Rhizobium number using both plate count and plant infection count in 1978 and 1979. The results are shown in Table 7. The inoculants produced from ICRISAT and Australia were found to be very pure and the plate and plant count agreed well. However, the inoculants received from the other Indian institutes and companies were found to be highly contaminated. The numbers of Rhizobium estimated from plate counts were always higher than plant count. This might be due to the fact that the colonies counted as Rhizobium were not Rhizobium. It is very difficult to distinguish Rhizobium from other similar bacteria visually.

DISCUSSION

The results of different proportions of cotyledon excision showed that nodulation of chickpeas could be improved by cutting off the cotyledons. The excised cotyledon plant growth was retarded; this proved

TABLE 6. Rhizobia count in pure cultures and soils inoculated with known numbers of rhizobia as estimated by plant infection dilution technique.

Strain	Broth plate count	Calculated no. of rhizobia added /g soil	Plant infection dilution-MPN value[a]		
			Broth	Soil[b]	
				A	B
9036	2.75×10^9	2.75×10^7	3.80×10^9	1.74×10^7	3.72×10^7
IC-59	1.95×10^9	1.95×10^7	1.74×10^9	3.72×10^7	9.33×10^6

[a]MPN calculated by Fisher and Yates (1963). Six dilution steps (10^2-10^7) and 3 replicate tubes per dilution were used in the plant count. The factor for 95% confidence interval on the MPN is $\overset{\times}{\div}$ 4.79 (Cochran 1950).

[b]Uninoculated soil A contained 8.61 chickpea Rhizobium/g soil and B, 1.73×10^3 chickpea Rhizobium/g soil.

TABLE 7. Results of chickpea Rhizobium count in peat inoculum received from different sources in 1978 and 1979. Numbers are expressed by Log 10.

Peat	Source	Plate count		MPN/g* peat
		No. of contaminating bacteria/g peat	No. of Rhizobium like colony/g peat	
1978				
IC 2002	ICRISAT, India	Nil	10.02	9.94
Nodulaid	Agricultural Lab, Carlingford Road, Sefton, N.S.W., Australia	Nil	9.82	9.26
Pantnagar culture	Pantnagar Univ. of Agriculture and Technology, India	8.25	8.32	7.08
Rallis Nitrofix	Rallis India Limited, Bangalore, India	9.63	10.26	3.40
Hissar culture	Haryana Agricultural Univ., Haryana, India	9.41	9.35	7.58
H-45	Jabalpur, India	9.56	8.40	5.94
F-75	IARI, New Delhi	8.41	8.44	7.65
1979				
IC-59 Peat	ICRISAT, India	Nil	9.48	10.26
9036 Peat (1978)	ICRISAT, India	Nil	9.57	9.94
9036 Peat (1979)	ICRISAT, India	Nil	9.66	9.94
H-45	Jabalpur, India	8.63	8.03	4.24
F-75 (1979)	IARI, New Delhi	7.59	9.25	8.58
Hissar culture I	Haryana Agric. Univ., Haryana, India	8.84	8.79	8.24
Hissar culture II	Haryana Agric. Univ., Haryana, India	8.78	8.55	8.24
Hissar culture III	Haryana Agric. Univ., Haryana, India	9.26	9.01	8.57
Nitrobact I	Nitrobact Company, Bangalore, India	7.67	8.56	5.24
Nitrobact II	Nitrobact Company, Bangalore, India	7.51	8.43	4.94
Nitrobact III	Nitrobact Company, Bangalore, India	7.88	8.26	4.94

*The factor for 95% confidence interval for 1978 test is ± 0.44 and for 1979 is ± 0.62.

to be very useful because the plant would not try to push the cotton plug out, and required less water. Cotyledons supply the seedling with food materials and some factors necessary for nodulation. Sucrose, glycine, thiamine, pyridoxine, nicotinic acid and mesoinositol seem to benefit nodulation (Raggio et al. 1959). However, we allowed the seed to germinate in the dark for 3 days and this might be enough time to mobilize the essential factors required for nodulation from the cotyledons to the growing seedling. There is no nodulation difference between one and one-half and two cotyledon removal. Both treatments gave reliable counts when compared to plate count. This finding is very useful because not all of the cotyledons need to be removed provided the leftover cotyledon is less than one-quarter of the total cotyledons. The improvement in nodulation after cotyledon excision also implies that we might be able to find a small seeded variety which is promiscuous in nodulation and nodulates freely in a test tube in the light chamber conditions. At the ICRISAT Laboratory, pigeonpea (cv. ICP-1) nodulates freely in a test tube without cotyledon excision. The embryo excision technique could also be applied to larger seeded legumes like groundnut (see Appendix 3).

The nodule number per plant was found to range from one to seven. This technique does not seem to be useful in efficiency of Rhizobium testing. However, it could be useful for strain identification. We adopted this technique for strain identification in our laboratory.

Many varieties could be used as the test plantlets. However, it has to be borne in mind that such varieties must be promiscuous in nodulation and they must not be susceptible to fungal soil borne disease such as Fusarium wilt (Fusarium oxysporum f. sp. ciceri).

Either sand or a sand:vermiculite mixture provided a satisfactory

rooting medium with nodulation being earlier in sand cultures. Secondary roots developed faster in sand than sand:vermiculite and may be related to earlier nodulation. Nodulation of excised roots is reported to be best if the roots developed in sand rather than on agar or in liquid (Bunting and Horrocks 1964, Barrios and Raggio 1964, Cartwright 1967). Subterranean clover (Trifolium subterraneum L. cv. Dwalganup) and serradella (Ornithopus sativus Brot) growing in 15 x 2.5 cm test tubes containing nutrient sand were reported to be successfully used in estimating the number of Rhizobium trifolii and Rhizobium lupini (Chatel and Parker 1973a, b). Small seeded legumes, i.e. "genge" (Astragalus sinicus L.), alfalfa (Medicago sativa L.) and crimson clover (Trifolium incarnatum L.) were reported to be grown in 1.7 x 17 cm test tubes containing a mixture of sand and vermiculite (30 g sand:5 g vermiculite) moistened with 9 ml of nitrogen free nutrient solution. And this assembly was successfully used in the estimation of the number of "genge" Rhizobium, Rhizobium meliloti and Rhizobium trifolii (Tuzimura and Watanabe 1961a). Small see ed wild soybean (Glycine ussuriensis Regal and Maack) growing in 15 x 2.5 cm tubes containing vermiculite were reported to be inferior to agar tubes and need a special calculation method (Brockwell et al. 1975, Grassia and Brockwell 1978). Thompson and Vincent (1967) reported that Trifolium subterraneum L. growing in 15 x 2 cm sand test tubes gave inferior counts of Rhizobium trifolii in pure cultures, however, the use of sand showed (not statistically significant) towards better recovery from soil. We are the first group to use large seeded legumes grown in test tubes in counting Rhizobium population. The 100 seed weight of the chickpea variety 850-3/27 is 25 g.

The excised seedling method of counting gave reproducible results

which were comparable to plate counts of viable rhizobia. Only when the dilution series was calculated to contain less than two cells/ml was there a discrepancy between observed and calculated number of positive tubes. When 95% confidence limits are applied to the MPN, there was no significant differences between plant and plate counts.

The dwarfed seedling method could also be used in counting the number of Rhizobium in unsterile soils. When a known number of Rhizobium was added to the soils, the MPN calculated from the plant infection dilution technique agreed reasonably well with the plate count.

The dwarfed seedling method is also useful in counting the number of Rhizobium in peat inoculum. It is very difficult to distinguish Rhizobium from other similar bacteria. This is true especially in unsterile peat inoculum. Our pure inoculum count using the plate and plant count agreed well except Nodulaid in 1978 and IC-59 in 1979. In the first case, plate count gave higher counts than the plant count but vice versa in the second case. The counts from unsterile peat inoculum generally gave lower counts than the plant count. When a 95% confidence interval is applied to MPN, only Pantnagar cultures gave the same count between plate and plant count in 1978. The good agreement between plate and plant count was found only in three Hissar cultures in 1979.

It is also worth noting the inoculum produced in India contained a heavy load of contaminants. The load of contamination will not be accepted by Australian standards. The plate count always resulted in misleading values where cultures were contaminated. We are the first laboratory to develop and report a method of counting chickpea rhizobia by a dilution plant infection technique where the plant is dwarfed by cutting off the cotyledons. It would seem to be suitable for counting in

most situations likely to be encountered or in ecological studies.

CONCLUSION

The technique of removing cotyledons from a germinating seedling enabled it to be grown and nodulated under test tube conditions. One and one-half and complete cotyledon removal were equally good in terms of nodulation. The reliability of this technique in counting chickpea Rhizobium was shown by the tests using ten-, four- and two-fold dilution series. When the number of Rhizobium in the tube was less than two, there was a discrepancy between the theoretical and observed positive tubes. However, it was concluded that this technique is reliable when 95% confidence limits had been taken into account. Many chickpea cultivars were found to be suitable for use as 'trap hosts', however, a highly nodulating cultivar, 850-3/27, was chosen due to its nodulating capacity. Either sand or sand:vermiculite mixture was found to be suitable as a rooting media. Sand was chosen for use in further studies due to its low cost, ease of handling and early nodulation.

The technique was used to count the number of chickpea Rhizobium in peat inoculum produced in India and Australia. The peat inoculum produced in India were found to be highly contaminated. Plant counts of Indian peat always gave low numbers of Rhizobium per g peat compared to the plate count. The value of the plantlet derived from this technique as a 'trap host' for studying Cicer Rhizobium ecology, strain authentication and chickpea inoculum quality control has been demonstrated.

MANUSCRIPT II

CHICKPEA *Rhizobium* POPULATION STUDY IN SOME INDIAN SOILS

ABSTRACT

The dwarfed chickpea seedling was used as a 'trap host' in the plant infection dilution count of chickpea Rhizobium in some ecological studies. Soil storage conditions, i.e. refrigerator (4°C), room temperature (28°C), and deep freeze (-7°C) were not statistically different. The number of Rhizobium increased with time of storage. Two months storage, in general, did not result in a significant increase in the number.

The population of chickpea Rhizobium in some ICRISAT and Indian soils was studied during 1978-1980 period. In general, the Alfisol fields that had no chickpea history were low in Rhizobium, once chickpeas had been grown in this soil the population of the Rhizobium was high. Paddy fields were found to be low in number of Rhizobium even though chickpeas were grown just 2 years ago. The population of the chickpea Rhizobium were found to change with depths and growing season. The population of Rhizobium in Parbhani soils (19° N latitude) ranged from 4.07×10^2 to 9.55×10^4, Gwalior soils (26° N latitude) ranged from 8.9×10^0 to 8.71×10^3, Hissar soils (29° N latitude) ranged from 2.00×10^2 to 3.95×10^5 Rhizobium/g soil. Failure to detect a difference in Rhizobium populations between fields having chickpea and other crops in summer 1980 was thought to be due to sampling technique which tried to avoid the rhizosphere. Later studies confirmed this belief. Chickpea Rhizobium population was highest when soil samples were taken over the plant and

decreased with increasing distance from the plant.

The rhizosphere of chickpea, groundnut, pigeonpea, sorghum, and groundnut were found to be stimulatory or at least they did not inhibit the growth of chickpea Rhizobium in pot experiments using an Alfisol and a Vertisol soil. The effect is more pronounced in chickpea than other crops.

INTRODUCTION

One of the main problems in studying Rhizobium ecology in soil is the lack of suitable methods. The development of the dilution plant infection technique by Wilson (1926) enabled enumeration of the number of Rhizobium in contaminated backgrounds including natural habitats. Since then this technique has been adopted widely in the study of the ecology of Rhizobium in soil (Chatel and Greenwood 1973, Chatel and Parker 1973a, b, Hely et al. 1957, Nutman and Ross 1969, Thompson and Vincent 1967, Tuzimura and Watanabe 1961a, b, c, 1962a, b, Tuzimura et al. 1966, Walker and Brown 1935, Wilson 1930, 1931).

Chickpea Rhizobium are very specific and will not nodulate other host legumes (Bhide 1956, Habish and Khairi 1968, Joshi 1920, Raju 1936, Rasumowskaja 1934), except a loose, non-reciprocal kinship with Sesbania, which in its turn has strong affinity with the cowpea miscellany (Gaur and Sen 1979). Chickpea nodulation is very sensitive to high temperatures (Dart et al. 1975a, b, Islam 1975). Nodulation and nitrogen fixation are reduced when the temperature exceeds 30°C.

We previously described a most probable number (MPN) technique for counting chickpea Rhizobium using trap host plants dwarfed by cutting off their cotyledons (see Manuscript 1). This paper examines the distribution of chickpea Rhizobium in a variety of soils and locations and in the rhizosphere of some plants. The experiments are divided into four parts, i.e.:

i) The effect of storage of soil on the MPN count of chickpea Rhizobium;

ii) Survey of chickpea Rhizobium in a range of soils and locations and their variation throughout the season;

iii) Rhizosphere effect of different crops on chickpea Rhizobium population;

iv) Rhizobium distribution after chickpea harvest.

MATERIALS AND METHODS

Effects of Soil Storage on Rhizobium Survival

In March 1980, a Vertisol field was sampled in three representative areas. Three kilograms of soil were obtained from each area. The first sample was taken from an irrigated chickpea cv. 850-3/27, 3 days after irrigation. The second sample was taken from a plot of irrigated chickpea cv. Annegiri, 3 days after irrigation. The third sample was taken from a plot of unirrigated chickpea cv. 850-3/27. The moisture content of the three soils were 20.44, 16.44 and 7.30%, respectively. The soils were immediately taken to the laboratory and each manually broken into small pieces on a sterile surface, mixed, divided into three portions and placed in airtight plastic bags. One portion of each replicate was then stored at room temperature (25 to 32°C), refrigerator (0 to 4°C) and in a deep freeze (-7 $\pm$ 3°C). For the samples stored in the deep freeze, they were mixed thoroughly again and sub-divided into four different bags. This was to avoid freezing and thawing of samples when sequential time samples were taken. Only one bag of each of the three soils would be drawn and later discarded after processing. At 0, 2, 8 and 16 weeks after storage, the chickpea Rhizobium population in the soil was counted using a soil dilution plant infection technique.

Twenty grams of soil were added to 180 ml sterilized tap water, shaken for 15 minutes on a wrist action shaker and a tenfold serial dilution series made with tap water to the level where zero Rhizobium was expected (10^6 or 10^7). The last six dilutions were used to inoculate dwarfed chickpea seedlings in test tubes using 1 ml per tube and three plant tubes per dilution. Three replicate samples were taken from each soil sample at each storage condition at each sampling time. After inoculation, the plant tubes were kept in a light chamber, tubes were illuminated at the intensity of 40 watts/m^2 per 16 h day and 8 h dark period. The temperature was maintained below 30°C using the thermostat control. The plant tubes were watered with 3 to 4 ml of one-quarter strength N-free nutrient solution (Summerfield et al. 1977, see Appendix 1), 3 to 4 weeks after inoculation. The plants were assessed for nodulation 6 weeks after inoculation. The number of positive and negative tubes were then used to calculate the MPN using Table $VIII_2$ in Fisher and Yates (1963).

Survey of Chickpea Rhizobium in Different Soils

Five fields at the ICRISAT centre were surveyed during the dry winter season of 1978-79:

a) a paddy field (Fine Mixed Hyperthermic Deep Aquic Ustorthent ?) where chickpeas had not previously been grown;

b) the same field where inoculated chickpeas had been grown 2 years ago;

c) an Alfisol field where chickpeas had not been grown before;

d) the same field where inoculated chickpeas had been grown during the rainy season of 1978;

e) a Vertisol field where chickpea had been regularly grown.

Soil samples were taken with a Viermeyer metal coring tube 6 cm in diameter, having a slit in its side to aid removal of the core. The tube was driven down to the desired level by hammer. Cores remained intact but there was some mixing in the top 5 cm when the soil sample was dry. The core was divided into 0 to 5, 5 to 15 and 15 to 30 cm deep samples. The soils were sealed in new plastic bags and stored at 4°C until the time of counting (usually within 1 month of sampling). Six to 13 samples were analyzed from each field.

The variation in the chickpea Rhizobium population with depth in the soil and over time during the season was studied for field a), d), and e) above. Six to eight samples were taken on March 7 to 19, June 5 to 9, August 14 to 21 and December 7 to 15, 1979 and divided into depths of 0 to 5, 5 to 15, 15 to 30, 30 to 60, 60 to 90, and 90 to 120 cm for the Vertisol (field e) and 0 to 5, 5 to 15, 15 to 30 and 30 to 60 cm for the shallow soils a and d. For the Vertisol field (field e), soil samples were taken with a 6 cm diameter gidding hydraulic coring machine mounted on the back of a Landrover. Soil samples from the other fields were taken with a 6 cm diameter Viermeyer tube driven down the profile with a hand hammer. Since the core could not be lifted out by hand, a 30 x 30 cm pit was dug close to the tube to facilitate its removal intact. The cores were cut into lengths, put in new plastic bags, sealed and then taken to the laboratory; there the soils were broken into small pieces using hands when the samples were wet and motar grinding when the samples were dry. The materials used in grinding including hands were swabbed with 95% ethanol when changing from one sample to another. The soils were subsampled and stored in a refrigerator at 4°C

to await processing. For field a) because of difficulties in taking soil samples in standing paddy, samples were taken in August using a T-tube and were restricted to the top 15 cm. Sampling at the other times was the same as mentioned previously.

During March and April 1980, 22 fields at the ICRISAT centre (latitude 17° N) were surveyed for their chickpea Rhizobium population. Soil samples were taken on a line transect. Each field was roughly divided into three equal areas and one 100 meter line transect laid across each area. Along each line transect, four soil samples from the 0 to 15 cm depth were taken using a Viermeyer tube at 5, 35, 65 and 95 meters. The samples from each treatment were then bulked making three composite soil samples from each field. Care had been taken while sampling not to take the soil sample from the plant rhizosphere. This was to prevent the rhizosphere effect on the population.

During the same period, soil samples were received from other locations in India, i.e. Parbhani (Vertisol soil, latitude 19° N), Gwalior (Entisol, latitude 26° N) and Hissar (Entisol, latitude 29° N). The samples were taken by local co-operators and sent to ICRISAT by mail, where they were stored in a refrigerator until processing within 2 months of initial sampling.

Rhizosphere Effect on Chickpea Rhizobium Population

The experiment was conducted in a glass house during the rainy season of 1980 with an Alfisol and a Vertisol soil. After ploughing, the dry top 15 cm soils were sampled. They were then ground in a mechanical shredder, sieved through 2 x 2 mm sieve to remove small grits and gravels and 4 kg soil placed in an 18 cm diameter pot. The water holding capacity of each pot was determined by watering to excess, allowing to drain

for 24 hours and then weighing again. The difference in weight between the wet and dry soil represented the water holding capacity. For each soil, there were three replicate pots sown with 8 to 10 seed/pot of groundnut (Arachis hypogoea L. cv. TMV-2), pearl millet (Pennisetum americanum cv. NHB-3), sorghum (Sorghum bicolor cv. CSH-6), pigeonpea (Cajanus cajun cv. ICP-1), chickpea (Cicer arietinum L. cv. 850-3/27), and a weed free unplanted control treatment. The Alfisol field had no previous history of chickpea cultivation and a dilution plant infection count showed that the number of chickpea Rhizobium per gram soil was less than one. Each Alfisol pot was inoculated with Rhizobium strain 9036 at the rate of 1.21×10^7 cells/pot at sowing by suspending 1 g of peat inoculum (2.42×10^9 cells/g peat in 1 liter of tap water, shaken vigorously and watering 5 ml of this suspension onto each pot. The plants were thinned to three per pot. The pots were first watered to 80% of their water holding capacity at 3 weeks after planting. From then onward, they were once per week watered to their 80% water holding capacity. The average water change per week in the pot of chickpea, groundnut, pigeon pea, sorghum, millet and control were found to be 434, 426, 407, 464, 426, 400 g in the Alfisol soil and 550, 447, 440, 390, 443 and 292 g in the Vertisol soil, respectively.

The temperature during the growing period varied from 25 to 30°C during the day and 23 to 25°C during the night. The plants were harvested 6 weeks after planting and separated into shoots and roots. The soil was emptied from a pot into an alcohol sterilized tray and the roots were then carefully removed. The soil attached to the root was considered to be the rhizosphere soil and the remainder to be bulk soil. Nodules were carefully removed from the root of chickpea, groundnut and

pigeonpea using a pair of scissors to cut the nodule and part of the root is attached to. For MPN count of chickpea rhizobia using a dilution plant infection technique, all the roots from a pot were put in a plastic bag, 180 ml sterilized tap water added, and shaken in a stomacher for 5 minutes. This was considered to be the 10^{o} dilution. The soil suspension was then diluted up to 10^{10} and 1 ml from each dilution used to inoculate a plant in a test tube. Three replicate tubes/dilution and one dilution series per soil or root sample were obtained. Roots were then separated from the suspension, washed and dried at 70°C. The dry weight of the rhizosphere soil was determined by putting the suspension left in the bag and the water used to wash the roots in an aluminum container on a hot plate and evaporating the bulk of the moisture, with final drying in a 105°C oven for 48 hours before weighing. Forty grams bulk soil was added to 180 ml sterilized tap water (10^{o} dilution), shaken in stomacher for 5 minutes and serially diluted with sterile tap water up to 10^{10}. Each dilution was inoculated to three plant tubes (1 ml/tube). The plants were kept in a light chamber for 6 weeks controlled at 27 to 30°C day temperature, 20°C night temperature. The plants were harvested and assessed for nodulation. The MPN was calculated by using the number of positive and negative tubes (Fisher and Yates 1963). The 95% confidence limits were calculated after Cochran (1950). The MPN of samples from the roots were expressed per gram dry rhizosphere soil or per gram dry root (see Appendix 11).

Rhizobium Distribution in Soil After Chickpea

The experiment was designed to follow the survival and spread of chickpea Rhizobium in soil subsequent to harvesting the crop. Inoculated

chickpea was grown in a paddy soil, devoid of chickpea rhizobia, with two irrigation regimes every 7 to 10 days (irrigated 10 times) and another with an irrigation at sowing and at 45 days after planting. Off-season chickpea was sown on February 19, 1980. The last irrigation for the 10 irrigation chickpea was given when the plant was 60 days old because of the lack of irrigation water. The 10 irrigation soil samples were taken from chickpea cv. CPS-1 inoculated with Rhizobium strain 9036 while the 2 irrigation samples were inoculated with strain IC-59. Chickpeas were grown in a single row on 60 cm ridges with five ridges/plot. Plots were 4 m long. Soil samples were taken on May 6, 1980. Samples from 0 to 15 cm depth were taken with a 6 cm diameter Viermeyer tube at right angles to the ridge at a distance of 0, 15 and 30 cm from the center of the ridge and 0, 15, 45, 75 and 105 cm along the ridge after the last plant in the plot. Each sample analyzed was a bulk sample of five samples taken from the same relative position in each of five ridges/plot. The tube was sterilized by wiping with 95% ethanol every-time it was moved to another position. Soil samples were taken to the laboratory, broken into small pieces, stored in a refrigerator and the MPN of chickpea Rhizobium determined as previously described.

RESULTS

Effects of Soil Storage on Rhizobium Survival

The effect of soil storage conditions and duration in the MPN of chickpea Rhizobium are summarized in the analysis of variance in Table 8. Storage temperatures, i.e. room temperature (28°C), 4°C and -7°C did not have any significant effect on the MPN. Storage duration

TABLE 8. Analysis of variance for MPN count of the effect of soil storage and durations on the MPN count of chickpea Rhizobium.

Source of variation	D.F.	S.S.	M.S.	F. ratio	Level of significance
ain Plot					
Replicates (R)	2	0.2525	0.1262	0.85	N.S.
Soil Samples (S)	2	20.6169	10.3085	69.22	1%
Storage conditions (T)	2	0.8198	0.4099	2.75	N.S.
S X T	4	0.4136	0.1034	0.69	N.S.
Error (1)	16	2.3827	0.1489		
ub-Plot					
Duration of storage (D)	3	3.1657	1.0552	7.99	1%
S X D	6	1.9529	0.3255	2.46	5%
T X D	6	1.0933	0.1822	1.38	N.S.
S X T X D	12	1.6698	0.1392	1.05	N.S.
Error (2)	54	7.1336	0.1321		
Total	107	39.5009			

had a highly significant effect on the MPN. The number of Rhizobium per gram dry soil increased with time. The average logarithmic number of Rhizobium at 0, 2, 8 and 16 weeks were 5.00, 4.89, 5.02 and 5.35. There was no significant difference in Rhizobium number/g dry soil at the first 8 weeks of storage. However, the number at 16 weeks of storage was significantly different from 0, 2 and 4 weeks after sampling. There was a significant interaction between soil samples and duration of storage (see Figure 3). Soil sample no. 2 showed a reduction in Rhizobium number at 8 weeks of storage. However, the number increased again at 16 weeks of storage. For soil sample number 3, the number of Rhizobium per gram dry soil increased with time, reaching its maximum number at 16 weeks of storage. The interaction of soil samples, storage conditions and time of storage (though not significant) are plotted in Figure 4. Soil samples 1 and 2 stored at all the three storage conditions, seemed to be less affected by storage duration. The curves were almost linear. Soil number 3 behaved strangely. When it was stored at 4 and 28°C, the number of Rhizobium seemed not to be affected with storage duration. However, when this soil is stored in the deep freeze (-7°C), the number remained constant at the first two samplings and increased tremendously at 8 and 16 weeks sampling. The moisture content of the three soils were 20, 16 and 7% respectively. Rhizobium multiplication in soil number 3 was unlikely to happen especially since the soil was kept at -7°C. However, it has to be borne in mind that this soil sample was taken from the plant row of an unirrigated chickpea plot. The soil was very dry and it was ground and separated into three portions each for different storage conditions. However, for the deep freeze (-7°C) storage, the soils were further divided into four portions. This was to avoid freezing and thawing of

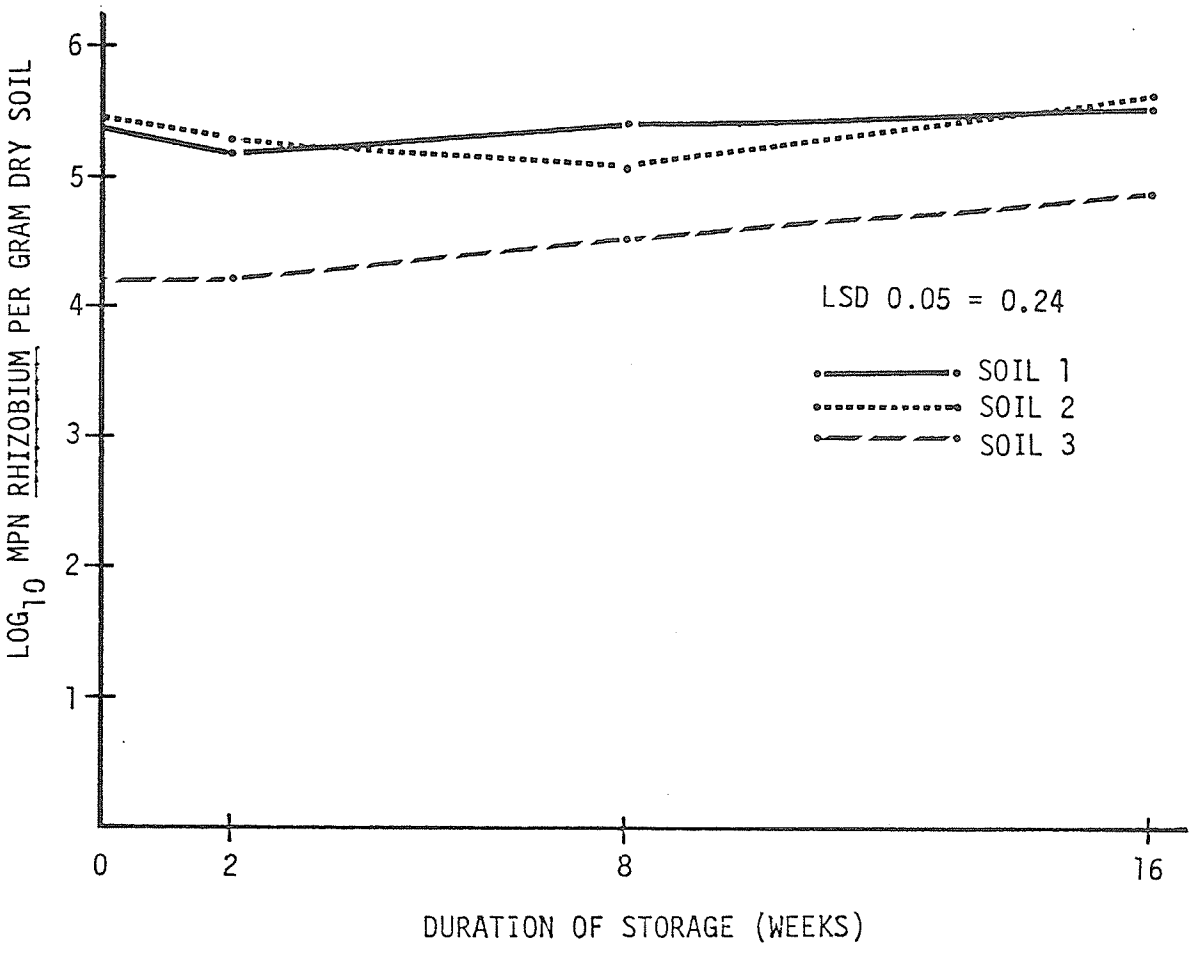

FIGURE 3. THE EFFECT OF STORAGE DURATION ON MPN CHICK-PEA RHIZOBIA. EACH POINT IS THE AVERAGE OVER 3 STORAGE CONDITIONS.

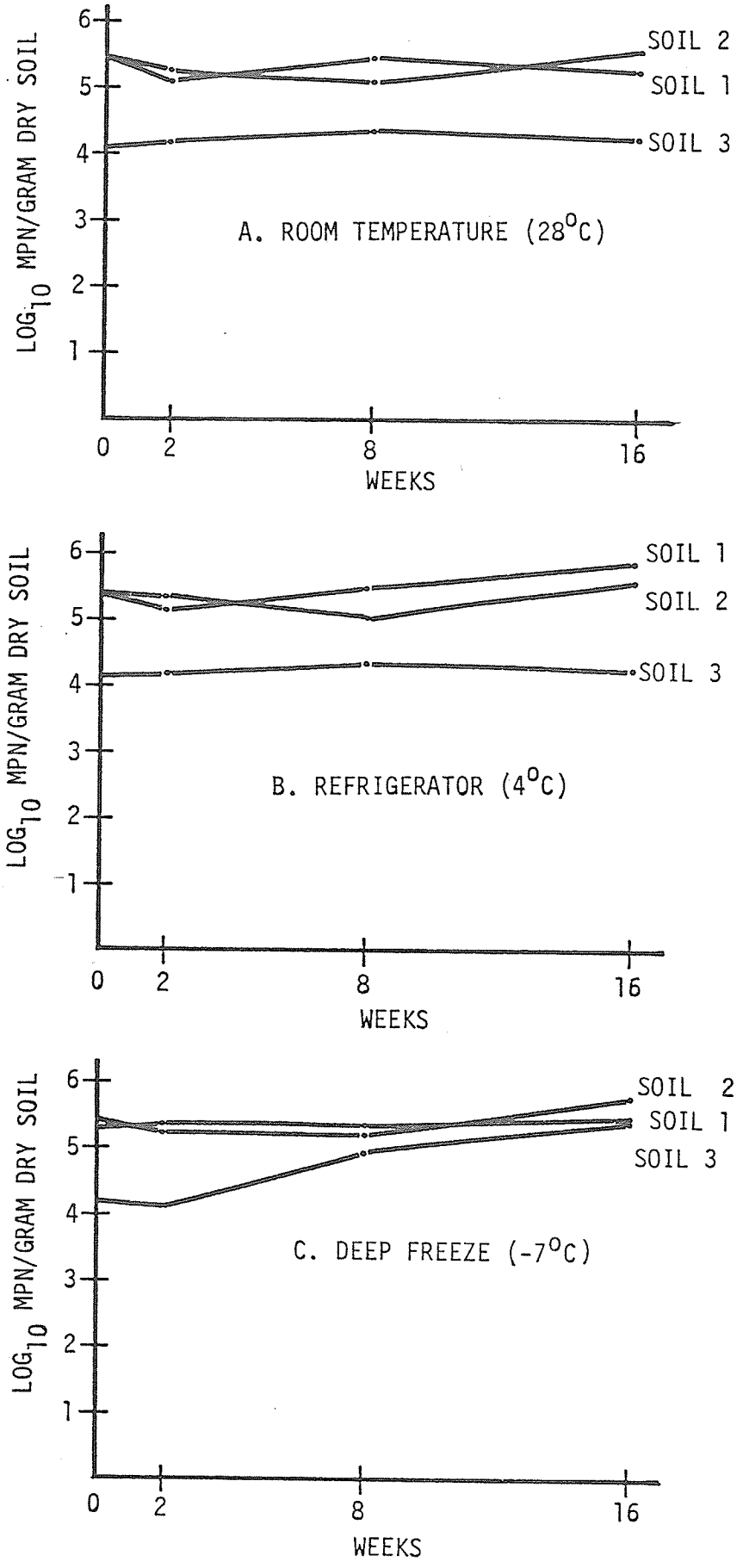

FIGURE 4. THE EFFECT OF STORAGE CONDITIONS, DURATION ON MPN COUNT OF CHICK-PEA RHIZOBIUM IN THREE SOIL SAMPLES.

the samples when they were drawn for processing for MPN. One bag of each sample was drawn each time and discarded after processing. It might be possible that soil sample number 3 was not well mixed enough and the bag sampled from the deep freeze (-7°C) at 8 and 16 weeks storage happened to contain dry nodules and this might result in high Rhizobium number. An alternative explanation for this phenomenon may be that bacteria in dry soil (soil 3) might not be active (lack of moisture) and may be clinging to the soil particle. Ice formation of the thin water film around the soil particle may result in releasing more bacteria from the soil particles. However, in the high moisture content soil, the bacteria are still active. When ice was formed, some Rhizobium might be killed and thus resulted in no increase in Rhizobium population. Another possible explanation is that cold temperature may kill some antagonistic micro-organism resulting in higher Rhizobium counts in the dry soil.

Survey of Chickpea Rhizobium in Different Soils

Table 9 summarizes the results of the survey of soils taken during the dry winter season of 1978/79. Chickpeas are generally grown in Vertisol soils depending on residual moisture in the soil. They are not grown in Alfisol soils where the moisture holding capacity is low. In ICRISAT centre, the Alfisol and Vertisol soils are close together. However, in the Alfisol soil a nodulation response to inoculation has been found (Rupela et al. personal communication). This means the Alfisol soils contain very little or virtually no chickpea Rhizobium. Therefore, uninoculated and inoculated Alfisol soils are included in this survey.

Inoculated Alfisol soil (field 'd') which had chickpea during the 1978 rainy season had the highest number of Rhizobium. A Vertisol field

TABLE 9. Chickpea *Rhizobium* populations (Log 10 MPN/g dry soil) in five fields at ICRISAT centre.

Field	Soil type	History	Soil depth (cm)		
			0 - 5	5 - 15	15 - 30
a	Fine Mixed Hyperthermic Deep Aquic Ustorthent (?)	Never grown chickpea	0.43	0.32	1.25
b	Fine Mixed Hyperthermic Deep Aquic Ustorthent (?)	Chickpea grown 2 years ago	1.78	2.47	2.56
c	Alfisol	Never grown chickpea	2.12	1.30	0.63
d	Alfisol	Chickpea was grown in preceding season	4.87	4.83	4.36
e	Vertisol	Chickpea several times grown	3.49	3.49	3.32

For analysis of variance see Appendix 4.

(field 'e') which chickpea had been grown several times was the second highest. Paddy field that had chickpea 2 years ago (field 'b') had as many Rhizobium as an uninoculated Alfisol (field 'c'). However, it was unlikely that field 'c' soil contained chickpea Rhizobium. The Rhizobium detected in this field might have been moved from field 'd' where chickpeas had been grown in the previous season. The distance between these two plots was only 24 m. In a paddy field where chickpeas had never been grown, the number of Rhizobium was very low. This might be native Rhizobium since no chickpeas had been grown in this field or a nearby plot.

There is a significantly different number of Rhizobium with depth in field 'c'. However, in the other fields there were no significant differences in the number of Rhizobium within the top 30 cm layer. Field 'a' had the lowest number of Rhizobium because chickpeas had not grown in this area before. When the data over depths are pooled and analyzed, there is a significant difference among fields (see Appendix 5). Fields that had grown chickpeas previously had higher numbers of Rhizobium, while those that had no previous chickpea history had low numbers. The numbers in field 'b' were low when compared to the other fields that previously had chickpeas. This reflects the effect of unfavourable conditions in paddy soil to maintain high populations in the soil. Waterlogged conditions had been previously reported by Vandecaveye (1927) to be detrimental for Rhizobium leguminosarum. He reported Rhizobium leguminosarum population in pots of sterile soil were greatly reduced after 2 weeks flooding. The low number of chickpea Rhizobium might be due to the poor survival under waterlogged conditions during the growing season. Analysis of variance shows high cv.; this indicates that there

TABLE 10. Variation in chickpea Rhizobium population (Log 10 MPN/g dry soil) over time at different soil depths in a Vertisol field (field 'e').

Depth (cm)	Time				
	Nov 78 (Harvested corn)	Mar 79 (Harvested chickpea)	June 79 (Fallow)	August 79 (Fallow)	Dec 79 (Standing chickpea)
0-5	3.49 a	4.62 a	3.78 a	3.91 a	3.89 a
5-15	3.49 a	5.34 a	3.55 a	4.34 a	3.99 a
15-30	3.32 a	3.85 b	3.65 a	4.30 a	3.75 a
30-60	-	3.81 b	3.28 ab	3.86 a	3.52 a
60-90	-	2.53 c	2.73 bc	3.31 b	2.74 b
90-120	-	2.13 d	2.10 c	3.02 b	2.23 b

a-d Means in the same column followed by the same letter are not significantly different by Duncan's new multiple range test at $P \leq 0.05$.

For analysis of variance see Appendix 6.

TABLE 11. Variation of chickpea Rhizobium population (Log 10 MPN/g dry soil) over time at different soil depths in an Alfisol field (field 'd').

Depth (cm)	Time				
	Jan 79 (Chickpea in rainy season)	March 79 (Standing groundnuts)	June 79 (Harvested groundnuts)	Aug 79 (Standing pigeonpea)	Dec 79 (Standing pigeonpea)
0-5	4.87 a	4.81 a	4.48 a	4.73 a	4.52 a
5-15	4.83 a	4.61 a	4.00 a	4.55 a	3.89 b
15-30	4.36 a	3.89 b	3.87 b	4.02 b	3.96 b
30-60	-	3.61 b	3.01 c	3.11 c	3.16 c

a-c Means in the same column followed by the same letter are not significantly different by Duncan's new multiple range test at $P \leq 0.05$.

For analysis of variance see Appendix 7.

TABLE 12. Variation of chickpea Rhizobium population (Log 10 MPN/g dry soil) over time at different soil depths in a Fine Mixed Hyperthermic Deep Aquic Ustorthent (?) field (field 'a').

Depth (cm)	Time				
	Jan 79 (Harvested paddy)	Mar 79 (Harvested chickpea)	Jun 79 (Fallow)	Aug 79 (Standing paddy)	Dec 79 (Harvested paddy)
0-5	0.43 a	3.94 a	2.54 a	1.75	2.87 a
5-15	0.32 a	4.06 a	2.19 ab	1.75	2.98 a
15-30	1.25 a	3.57 a	1.42 b		0.92 b
30-60		3.08 a	0.45 c		0.82 b

a-c Means in the same column followed by the same letter are significantly different by Duncan's new multiple range test at $P \leq 0.05$.

For analysis of variance see Appendix 8.

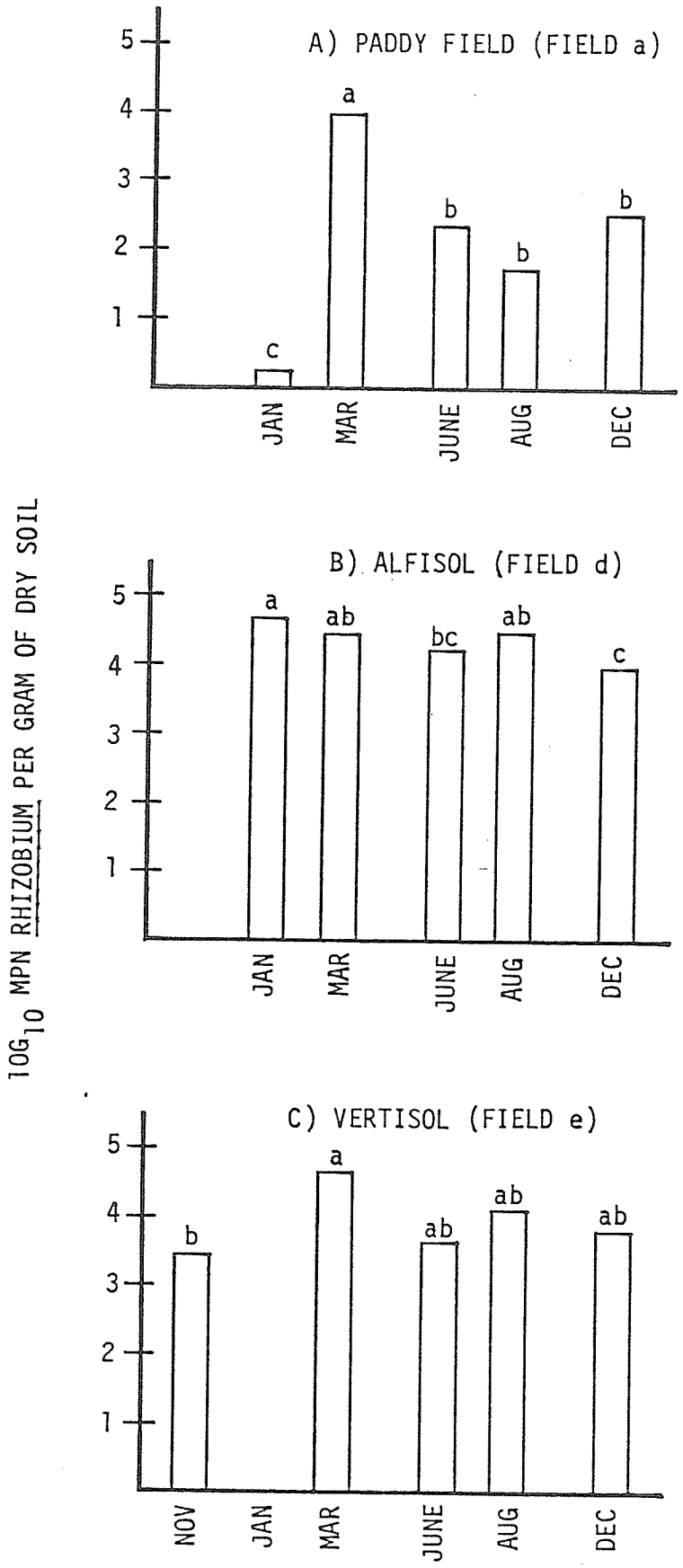

FIGURE 5. POPULATION VARIATION AT THE TOP 15 cm (FIELD a), 30 cm (FIELD d AND FIELD e) DURING 1979.

was a lot of variability within a field (see Appendix 4). It might be explained that the field was heterogeneous. During the paddy growing season, not all the area was fully covered with water. The parts that were not under water, therefore, had a high population but the parts that were under water had a lower population.

The population of Rhizobium through the season was followed in three soils: a paddy field (field 'a'), an Alfisol (field 'd') and a Vertisol (field 'e') (Tables 10, 11 and 12). In all three fields, numbers of Rhizobium declined with depth. For the Vertisol field (field 'e') (Table 10), there was no significant change in the population from 0 to 30 cm when sampled in November 1978 but the numbers decreased with depth beyond 30 cm on other occasions. In most of the cases, except March sampling, there were no significant differences in Rhizobium number in the top 30 cm soil profile. The numbers were lowest at 90 to 120 cm depth.

When the numbers in the top 30 cm of soil were pooled and analyzed to see the effect of time on the MPN of Rhizobium, there was a significant difference between sampling times (see Appendix 9, Figure 5C). The highest number of rhizobia were present in March just after the chickpea harvest. The numbers drop down to the same level as the first sampling in November, which may reflect a summer effect where rhizobia might be killed by the high temperature. There is a slight increase in MPN per gram dry soil during the rainy season but the number declined to the same level as the first sampling in the last sampling in December 1979.

The initial population of the inoculated Alfisol soil (field 'd') (Table 11) was not obtained because the technique of counting chickpea Rhizobium had not been developed. However, if we extrapolate the initial

population of this field from the nearby Alfisol plot (field 'c') where chickpea was not grown previously. We could see that the number of Rhizobium left after growing the crop was very high (10^4 cells/g dry soil) which was even higher than in the Vertisol soil (field 'e') where chickpea had been grown many times. There was no significant difference in MPN between depths in the first sampling in January. However, from March sampling onward, there were significant differences between depths. The first top 15 cm had the highest number of Rhizobium and the lowest depth had the least count.

When the numbers in the top 30 cm were pooled and analyzed to see the effect of time on the MPN of the Rhizobium, there was a significant difference in MPN per g dry soil at different sampling times (see Appendix 9, Figure 5B). The number is highest in January sampling and lowest in the last sampling in December. There was a slight drop (not statistically significant) in MPN count in the June sampling and this might reflect a summer effect. There is a slight increase (not statistically significant) at the August sampling (rainy season). Groundnut has just been harvested in June sampling. The land was cropped with pigeonpeas at the rainy season sampling (August). The number of the Rhizobium was lowest at the last sampling in December 1979. Pigeonpeas were being harvested at this sampling time.

In the paddy field (field 'a') (Table 12), the initial population was very low. However, when chickpeas were grown the number of the Rhizobium significantly increased. There was no significant difference in MPN among depths in January and March sampling. However, the June and December sampling had a significant difference in MPN among the depths. The numbers declined with depth. When the numbers in the top

15 cm were pooled and analyzed, there was a significant difference in MPN per g dry soil at different sampling times (see Appendix 7, Figure 5A). The number was lowest in the January sampling (before chickpea planting) and highest in the March sampling (after chickpea harvest). The number declined during the summer (June sampling where the land was under fallow) and further declined in the rainy season (August sampling where paddy was grown). Waterlogged conditions might have played a great role in this reduction. However, the numbers recovered again; they became as high as summer planting but not to the extent of the March sampling.

Table 13 shows the population in 22 fields at the ICRISAT centre. There was a significant difference in MPN between fields. There were virtually no chickpea rhizobia in the three Alfisol fields R8, RA7 and RW1, where chickpeas had not been grown previously. Field R8 was only a few hundred meters from a Vertisol containing 10^3 to 10^4 Rhizobium/g dry soil. Vertisols had high numbers of chickpea Rhizobium ranging from 5.6×10^1 to 3.89×10^4 cells/g dry soil. The presence of chickpeas during or just before the time of sampling did not seem to increase the number of chickpea Rhizobium. The average of chickpea Rhizobium population in Vertisol soils having chickpea was 1.38×10^3 cells/g dry soil, cereals (maize or sorghum) 4.90×10^3, intercrop pigeonpea and cereals 3.16×10^4, fallow 1.54×10^2 and pigeonpea 2.04×10^3. Chickpea is generally grown in the Vertisol, that is the reason why the population per g dry soil does not differ that much among the field having chickpea and cereals. It has to be borne in mind that the soil samples were taken from non-rhizosphere soil, there it is unlikely that there will be any rhizosphere effect in the number. The numbers that we are presenting

TABLE 13. The numbers of chickpea Rhizobium in 22 ICRISAT fields surveyed in summer 1980.

Field	Crop history	pH (2:1 water)	E.C. (mmho/ cm)	M.C. (%)	Log 10 MPN/g dry soil
M_1	Rabi sorghum	8.35	0.32	12.24	4.03
M_7	Standing chickpeas	8.50	0.24	12.47	3.98
M_{10}	Rabi maize	8.42	0.21	15.04	3.65
M_{13}	Maize and sorghum	8.22	0.20	8.23	3.25
B_1	Standing sorghum	8.27	0.20	10.96	3.14
B_3	Harvested chickpeas	8.37	0.31	7.03	3.33
B_4	Standing pigeonpea	8.50	0.15	11.99	3.36
B_7	Standing sorghum and maize	8.02	0.35	15.86	4.37
BW_1	Pigeonpea-maize intercrop (Kharif)	8.23	0.15	9.82	4.41
BW_2	Pigeonpea-maize intercrop (Kharif)	8.03	0.20	9.61	4.59
BW_3	Chickpeas	7.93	0.19	10.28	4.30
BW_4	Chickpeas	7.93	0.21	8.03	2.74
BW_5	Chickpeas	8.15	0.21	9.07	4.52
BW_6	Chickpeas	8.23	0.19	9.32	2.83
BW_7	Chickpeas	8.13	0.15	8.61	4.12
BW_8	Fallow	8.17	0.15	9.01	1.75
G-5	Fallow	7.93	0.22	10.05	2.62
BA-25	Standing Pigeonpea	8.03	0.15	7.21	3.25
B_9	Chickpea	8.53	0.18	10.84	2.44
R_8	Standing groundnut	7.83	0.22	9.93	0
RA-7	Sorghum	5.67	0.15	1.86	0
RW_1	Fallow	5.53	0.15	1.31	0
	S.E. of mean	0.05	0.01	1.45	0.36
	C.D. at 5%	0.14	0.04	4.15	1.04
	c.v.	1.07	10.91	26.50	20.76

For analysis of variance see Appendix 10.

TABLE 14. Correlation between pH, E.C. and soil moisture content on MPN of chickpea Rhizobium in 22 ICRISAT fields surveyed in summer 1980 (n = 66).

	pH	E.C.	% M.C.
MPN	0.64**	0.31*	0.54**
pH		0.29*	0.63**
E.C.			0.21

now are the populations that were present before chickpeas were planted to the fields. The research reported in the next two sections will give support to this comment.

The correlation among pH, E.C., % M.C. and MPN of the 22 fields are summarized in Table 14. There is a highly significant coefficient of correlation ($r = 0.64^{**}$) between MPN and pH, MPN and % M.C. ($r = 0.54^{**}$). The correlation between MPN and E.C. is also significant ($r = 0.31^{*}$). These correlations were obtained when three Alfisol fields were included but they did not contain any rhizobia. When the Alfisols were excluded from the analysis the coefficients of correlation are -0.13 for MPN and pH, 0.24 for MPN and E.C. and 0.26^{*} for MPN and % M.C. Only the coefficient of correlation between MPN and 1% M.C. was significant.

The results of Rhizobium population survey in Parbhani (Vertisol, lattitude 19° N), Gwalior (Entisol, latitude 26° N) and Hissar (Entisol, latitude 29° N) are summarized in Tables 15, 16 and 17 respectively. For Parbhani soils, the number of chickpea Rhizobium range from 4.07×10^2 cells/g dry soil in field number 4 where chickpea was last grown 8 years ago to 9.55×10^4 in soil number 2 where chickpea had just been grown in 1979-80.

The number of chickpea Rhizobium in the Gwalior soil ranged from 8.9×10^0 to 8.71×10^3 cells/g dry soil. Chickpea has been grown in this field before. Nodulation of chickpeas grown in these fields was found to be ranging from 0 to 20 nodules/plant.

In Hissar, soils were taken from different fields as shown in Table 17. The number of the Rhizobium ranged from 2.00×10^2 cells/g dry soil in field number 9 where peas were being grown to 3.98×10^5

TABLE 15. Chickpea *Rhizobium* numbers in Parbhani soils.

Field no.	Place	History	pH	E.C.	Log 10 MPN/ g dry soil*
1	Central Farm, Marathwada, Agric. Univ.	Sorghum 1978-79 Chickpeas 1979-80	8.10	0.30	3.97
2	Sorghum Research Station, M.A.U.	Wheat 1978-79 Chickpeas 1979-80	8.00	0.65	4.98
3	Farmer's field, Shandra Village	Chickpeas 1977-78 Cotton & Sorghum from 1978-80	7.90	1.35	3.97
4	Farmer's field, Akola Village	Chickpeas 1970-72 Cotton-Sorghum & cotton were rotated later	8.15	0.62	2.61
5	Farmer's field, Akola Village	Chickpeas 1970-72 Cotton-Sorghum & Cotton were rotated later	8.00	1.25	3.28

*95% confidence interval on MPN is ± 0.68 (Cochran 1950).

TABLE 16. Chickpea Rhizobium numbers in Gwalior soils, Madhyapradesh.

Soil no.	Village	Nod./ plant[a]	pH	E.C.	Log 10 MPN/ g dry soil*
1	Kulenth	0(1)	7.60	0.15	3.58
2	"	-	7.55	0.15	2.94
3	"	-	7.50	0.15	3.94
4	Bhatkhedi	16-20(2)	8.10	0.23	1.94
5	Janasi	0-10(2)	8.40	0.18	2.94
6	Jangipur	0(2)	7.65	0.15	2.25
7	Bhagch	0-8(2)	7.60	0.17	2.94
8	Lohgarh	0(2)	8.15	0.27	0.94
9	Kariyawati	0(2)	7.60	0.18	2.27
10	Bagwal	0-6(4)	8.30	0.15	0.95
11	Utila	-	8.70	0.19	1.25

[a]Information extracted from Jabalpur station report in Rabi Pulse Workshop held in September 1979 in Hissar. Figures in brackets are the number of spots observed in the village.

*95% confidence interval on MPN is $\pm$ 0.68 (Cochran 1950).

TABLE 17. Chickpea *Rhizobium* numbers in Hissar soils.

Field no.	Place	History	pH	E.C.	Log 10 MPN/ g dry soil*
1	HAU, ICRISAT area	Chickpea, poor growth (G-130)	8.30	0.15	3.59
2	"	Chickpea, good growth (G-130)	7.90	0.15	3.95
3	"	Chickpea, poor growth (850-3/27)	8.20	0.15	3.60
4	"	Chickpea, good growth (850-3/27)	8.10	0.15	3.95
5	HAU, Microbiol. area	Prepared soil for mung bean	7.65	0.19	3.29
6	"	"	7.95	0.19	4.61
7	HAU, Agronomy Farm	Pea crop	8.15	0.21	3.28
8	HAU, Microbiol. area	Fallow	7.85	0.28	3.26
9	HAU, Agronomy Farm	Pea crop	7.70	0.17	2.30
10	HAU, Microbiol. area	Chickpea	7.70	0.17	5.60

*95% confidence interval on MPN is $\pm$ 0.68 (Cochran 1950).

cells/g dry soil in field number 10 where chickpeas were being grown. The soils examined in overall seemed to have higher chickpea Rhizobium than at Gwalior and Parbhani. These fields are in a general growing area for chickpea and therefore might be expected to contain a high MPN count. The soil samples taken from good (field numbers 2 and 4) and poor (field numbers 1 and 3) growth chickpea plots did not show any significant difference in number of Rhizobium per g dry soil. For example, the number of Rhizobium in field number 1 where chickpea cv. G-130 had a poor growth was 3.89×10^3 cells/g dry soil and field number 2 which had a good G-130 chickpea growth had 8.91×10^3 cells/g dry soil. The same thing happened for chickpea cv. 850-3/27 grown in field numbers 3 and 4. It would seem that factors other than Rhizobium numbers were responsible for the poor growth of the chickpea.

Rhizosphere Effect on Chickpea Rhizobium Population

The effect of root growth on chickpea Rhizobium populations was studied in pot culture using an Alfisol and a Vertisol soil. The plants grew better in the Vertisol soil (Tables 18 and 19) because no N fertilizer was added to millet and sorghum. Crops were N deficient. Nodulation of the three legumes, chickpea, pigeonpea and groundnut was good in both soils. The root weight of the three legumes was similar in both soils but for sorghum and millet the root weight were much greater in the Vertisol.

The numbers of chickpea Rhizobium per g root of chickpea was highest and significantly differed from the other crops in Alfisol soil (Table 20). The number of Rhizobium per g chickpea root was 2.34×10^7 cells which was the highest. The Rhizobium colonized on the root of

TABLE 18. Top weight, root weight, nodule numbers and nodule weight of five crops grown in pots containing a Vertisol soil (6 weeks old).

Crops	Top weight (g/pot)	Root weight (g/pot)	Nodule no.	Nodule weight (mg/pot)
Chickpeas	3.53 c	0.47 b	151 a	116.67 a
Groundnut	5.66 b	0.34 b	173 a	53.33 a
Pigeonpea	3.90 c	0.39 b	121 a	103.33 a
Sorghum	6.37 b	2.40 a	-	-
Pearl millet	8.46 a	1.90 a	-	-

a-c Means within column followed by same letter are not significantly different by Duncan's new multiple range test at $P \leq 0.05$.

For analysis of variance, see Appendix 14.

- Not included in analysis of variance.

TABLE 19. Top weight, root weight, nodule numbers and nodule weight of five crops grown in pots containing an Alfisol soil (6 weeks old).

Crop	Top weight (g/pot)	Root weight (g /pot)	Nodule no.	Nodule weight (g/pot)
Chickpeas	1.84 b	0.50 c	99 a	140.00 a
Groundnut	4.74 a	0.37 cd	114 a	76.67 a
Pigeonpea	2.11 b	0.27 d	115 a	193.33 a
Sorghum	2.21 b	1.13 a	-	-
Pearl millet	2.03 b	0.78 b	-	-

a-d Means within column followed by same letter are not significantly different by Duncan's new multiple range test at $P \leq 0.05$.

For analysis of variance, see Appendix 15.

- Not included in analysis of variance.

other crops were not significantly different. It ranged from 4.17×10^4 cells/g in millet to 4.79×10^5 in sorghum. The numbers per g rhizosphere soil followed the same pattern being highest in chickpea (3.63×10^5 cells/g) and lowest in millet (8.12×10^2 cells/g). The number of Rhizobium in the bulk soil of different crops did not differ significantly from the number in the fallow pot. Chickpea rhizosphere had the highest stimulatory effect which was about 41 times that of the non-rhizosphere soil. Groundnut and pigeonpea, the stimulatory effect of the rhizosphere was about 10 times that of the non-rhizosphere soil. Sorghum's rhizosphere stimulatory effect was five times. However, millet in Alfisol soil did not show much stimulatory effect. Its stimulatory effect was only one time more than the non-rhizosphere soil. This is considered negligible.

In the Vertisol soil, the Rhizobium numbers per g dry root of chickpeas and groundnuts were highest and significantly differed from the other crops, i.e. 7.26×10^6 and 2.69×10^6 cells/g root for chickpea and groundnut respectively (Table 20). For the other three crops there was no significant difference in the MPN number. The numbers per g rhizosphere soil followed the same trend, i.e. chickpea had the highest number (3.98×10^5) and followed by groundnut (1.26×10^5) and the MPN for the other three crops were not significantly different from each other. The number per g dry non-rhizosphere soil of different crops again did not differ from each other and from the fallow pots. All the crops had a stimulatory rhizosphere effect on chickpea Rhizobium. The stimulatory effects were 89, 59, 6, 12 and 22 times for chickpeas, groundnuts, pigeonpeas, sorghum and pearl millet respectively.

TABLE 20. The number of chickpea rhizobia per gram dry root, rhizosphere, non-rhizosphere and fallow soil of five ICRISAT's mandate crops grown in pots containing an Alfisol and a Vertisol soil. The numbers (except R/NR ratio) are expressed as Log 10.

Crop	Chickpea *Rhizobium* (Log 10 MPN/g)							
	Alfisol				Vertisol			
	Root	Rhizosphere soil (R)	Non Rhizosphere soil (NR)	R/NR Ratio	Root	Rhizosphere soil (R)	Non Rhizosphere soil (NR)	R/NR Ratio
Chickpea	7.37 a	5.56 a	3.95 a	41	6.86 a	5.60 a	3.65 a	89
Groundnut	5.39 b	3.86 b	2.86 a	10	6.43 a	5.10 a	3.33 a	59
Pigeonpea	5.53 b	3.96 b	2.95 a	10	5.13 b	4.24 b	3.45 a	6
Sorghum	5.68 b	3.83 b	3.11 a	5	5.44 b	4.09 b	3.01 a	12
Pearl millet	4.62 b	2.91 b	2.89 a	1	5.47 b	4.23 b	2.89 a	22
Fallow	--	--	2.43 a	--	--	--	3.13 a	--

[a,b]Means within column followed by same letter are not significantly different by Duncan's new multiple range test at $P \leq 0.05$.

For Analysis of variance, see Appendices 11 and 12.

Rhizobium Distribution in Soil After Chickpea

The effect of Rhizobium distribution after the end of the growing season is summarized in Table 21. For the 10 irrigation samples, the number was highest when the sample was taken over the plant (1.3×10^6 cells/g dry soil). When the sample was taken 15 cm side way of the ridge the numbers of Rhizobium per g dry soil reduced by 10,000-fold. At the bottom of the ridge, the number was about the same as the numbers at 15 cm from the plant. When samples were taken along the ridges the Rhizobium population was 10,000-fold reduced at 15 cm from the plant and remained constant up to 75 cm from the plant. At 105 cm from the plant, the soil contained approximately 15 Rhizobium per g dry soil.

In the two irrigation soil samples, the number of Rhizobium was lower when compared to the 10 irrigation samples. The population of Rhizobium when the soil samples were taken over the plants were 1.94×10^3 cells/g dry soil. When the sample was taken 15 cm side way across the ridges, the population was found to be two cells/g dry soil. The population was found to be less than one cell/g dry soil at the bottom of the ridges. When the samples were taken along the ridge, the number was three and five cells at 15 and 45 cm from the plant, respectively.

Ten irrigations resulted in better nodulation and better plant growth than two irrigations. The number of nodules, nodule weight, top and root weight/plant of these two plots are shown in Appendix 16. Poor nodulation and poor root growth might be the reasons for low MPN count in the two irrigation soil samples. Root nodules and colonization on the root surface are the main source of Rhizobium supply in the rhizosphere as shown in the previous section. Rhizobium movement in the soil depends on the frequency of irrigations. The chance for the Rhizobium

TABLE 21. *Rhizobium* distribution in a paddy soil (Fine Mixed Hyperthermic Deep Aquic Ustorthent) (?) after growing a chickpea crop.

Distance from plant row	Log 10 MPN/g dry soil	
	10 Irrigations	2 Irrigations
I Right angles to the ridge		
Over the plant	6.10 ± 0.36	3.29 ± 0.00
15 cm away	2.40 ± 0.20	0.21 ± 0.37
30 cm away (in furrow)	2.09 ± 0.17	0.00
II Along the ridge		
Over the plant	6.10 ± 0.36	3.29 ± 0.00
15 cm beyond	2.20 ± 0.21	0.54 ± 0.50
45 cm beyond	2.64 ± 0.35	0.66 ± 0.57
75 cm beyond	2.54 ± 0.40	N.D.
105 cm beyond	1.19 ± 0.17	N.D.

N.D. = Not determined.

to be carried away and persist is greater in the more frequently irrigated soils.

DISCUSSION

Effect of Soil Storage on *Rhizobium* Survival

The temperature at which soil samples were stored had no significant effect in the MPN count of chickpea *Rhizobium*. The MPN count was affected by storage duration with an interaction between soil sample origin and duration of storage. The number of *Rhizobium*/g soil was found to increase with time. However, within the first 8 weeks, the number did not increase significantly. This finding implies that the soil samples, once collected, need to be processed for MPN count within the first 2 months. Soil storage conditions at 28°C (room temperature), 4°C refrigerator did not seem to have much effect on the three soils during the 16 weeks storage. However, soil storage in a deep freeze showed a peculiar increase in MPN in soil number 3. This was thought to be due to many factors as mentioned earlier. Wollum and Miller (1979) reported that *Rhizobium leguminosarum* increased in number over original levels after 14 days of storage up to 120 days. The estimated rhizobial number was generally higher for samples stored at -4°C than comparable samples stored at 5°C. However, this increase in number is unlikely to explain what happened to our soil samples since it was not repeatable in the other two soils. Soil numbers 1 and 2 contained 20 and 16% moisture content. Ice was formed on the samples. However, no ice formation in the third sample where the soil sample had 7% moisture content. It was unlikely that the *Rhizobium* could multiply at the low soil moisture

content. Releasing of Rhizobium from the soil particles or killing of antagonistic micro-organisms or sampling error might be the explanation for this phenomenon.

Survey of Chickpea Rhizobium in Different Soils

The survey of chickpea Rhizobium populations showed that the numbers were high in fields where chickpeas had previously been grown compared with fields where chickpeas had not been grown before, interestingly that some even had no previous chickpea cropping history. Rhizobium can be spread by wind, water and farm implements. Nutman and Ross (1969) reported that the presence of Rhizobium trifolii, Rhizobium meliloti and Rhizobium lupini in arable land without a recent history of legume crops was due to natural agents or farm implements moved from areas of abundance. Tuzimura and Watanabe (1961b) reported counting 100 Soybean Rhizobium per g soil in a forest soil.

Walker and Brown (1935) found that the numbers of Rhizobium meliloti and Rhizobium trifolii depended upon the previous history. The numbers were high when the host plants were present in the cropping rotation system. Nutman and Ross (1969) confirmed that the numbers of Rhizobium trifolii, Rhizobium leguminosarum, Rhizobium meliloti and Rhizobium lupini were high when the fields were cropped by the hosts. When the host plants were not grown, numbers decreased in a few years from 10 or 100's of 1,000 per g of dry soil to very few or none. Tuzimura and Watanabe (1961b) also reported that the presence of the host crops resulted in a higher count of Rhizobium meliloti "genge" (Astragalus spp.) bacteria (Rhizobium spp.), and Rhizobium japonicum. The lower number of chickpea Rhizobium in a paddy field that had grown inoculated

chickpea, 2 years ago, might be due to the fact that inoculation once had little effect on Rhizobium population (see calculation in Appendix 17). Subsequent death due to unfavorable conditions, i.e. waterlogged conditions might be detrimental to Rhizobium (Vandecaveye 1927).

The Rhizobium population in the first three depths (0 to 5, 5 to 15 and 15 to 50 cm) generally showed no significant difference in MPN. However as the depth increased further the MPN count became lower being lowest at the lowest depth. When the numbers of Rhizobium in the top profile were pooled (0 to 15 cm for the paddy field, 0 to 30 cm for the Alfisol soil and Vertisol soil), seasonal variation could be seen.

In the paddy field the number of chickpea Rhizobium was low before chickpea planting (January sampling). However, the number increased tremendously after the chickpea harvest. The reason for higher numbers of Rhizobium after the chickpea harvest in March was the samples were taken in the plant rows. It might be expected that the decayed nodules and roots released the Rhizobium and these Rhizobium would be concentrated only at the rhizosphere region. If the soils were well mixed the number would be lower. We could calculate the numbers of Rhizobium released to the soil based on the finding that the Rhizobium content/nodule ranged from 10^5 to 10^7 Rhizobium/nodule (Toomsan unpublished data) and the Rhizobium colonized on the root was 10^7 Rhizobium/g root (see section 3). Chickpeas grown in this field had approximately three nodules/plant (Rupela and Toomsan unpublished data). Plant population was 1.1×10^5 plants/ha. Assuming that 1 hectare furrow slice (15 cm) weighs 2.5×10^6 Kg. Therefore, the number of Rhizobium added per g soil was found to be 1.36×10^3 cells/g soil (see Appendix 17). The numbers adhering to the root seemed to be negligible when compared to numbers added by

the nodules. However, it must be borne in mind that not all the roots could be recovered. Even if all the roots could be recovered the amount would be negligible. Root mass needs to be increased by 10 times in order to supply the same numbers of Rhizobium. The number at the June sampling depicted the real population in the soil and the reduction in numbers from the March sampling may also be due to high soil temperatures. The further reduction (not statistically significant) in numbers at the August sampling where the paddy plants were being grown showed the detrimental effect of waterlogged condition. However, the soil was not uniform; not all the soils were fully covered with water. This affected the population of Rhizobium being high in non-waterlogged patches and low in waterlogged patches. The number climbed up to the same level as the summer sampling when the paddy was harvested and the soil was dry.

In the Vertisol soil, the tendency is similar to that of a paddy field, i.e. there was a significant increase in MPN at the end of the growing season (March sampling) compared to the count at the beginning of the season (November sampling). The amount of Rhizobium added/g soil at the 30 cm soil depth was calculated using the nodulation and root weight from the previous crop (see Appendix 17). The number of Rhizobium added to the soil was found to be about 10,000 cells/g. However, the actual MPN count was about 4.16×10^4 cells/g soil. This might be due to the fact that samples were taken in the plant row. A slight reduction in the second count in the June sampling (though not statistically different) might be due to: 1) the soils were mixed by ploughing and the Rhizobium were well dispersed, 2) the hot soil temperature might kill some Rhizobium. The reduction in Rhizobium during the hot

summer months has previously been reported (Chatel and Parker 1973b). The field was under fallow at the August sampling, however, good moisture and favourable conditions might result in saprophytic cell multiplication and an increase in numbers. Tuzimura and Watanabe (1961b) reported that "genge" (Astragalus sinicus) bacteria (Rhizobium spp.) increased in number when air dried and partially sterilized soil was rewetted, showing the ability of Rhizobium to multiply saprophytically in competition with other soil micro-organisms.

The summer effect could be seen clearly in the Alfisol field where chickpea was not growing at the time of sampling. The number was slightly lower during the summer months and increased again in the rainy season.

The Rhizobium survey in different fields in the summer of 1980 showed that soil with no previous chickpea history contained less than one Rhizobium/g soil. This was particularly true with the Alfisol soils. In the Vertisol soils where chickpeas were grown, the numbers varied from 5.62×10^1 to 3.89×10^4 cells/g soil. The presence of chickpeas at the time of sampling did not affect the numbers of chickpea Rhizobium. Highly significant coefficiency of correlation between MPN and pH, % M.C. and E.C. were also observed. Low pH had been known to be detrimental to Rhizobium survival (Richmond 1926, Wilson 1926) and liming had a beneficial effect on Rhizobium survival (Walker and Brown 1935, Vincent and Waters 1954, Jones 1966, Nutman and Ross 1969, Robson and Loneragan 1970a, b). Low soil moisture content or drought had been known to affect Rhizobium survival (Fould 1971, Chatel and Parker 1973b). Rhizobium had been reported to be salt sensitive in broth cultures (Pillai and Sen 1966). However, some Rhizobium strains were reported to be salt tolerant

(Fred et al. 1932, Yadav and Vyas 1971, Subba Rao et al. 1972, Ethirraj et al. 1972). Salinity tolerances for the host plant, nodulation and symbiosis are lower than those for the rhizobia themselves (Bernstein and Ogata 1966, Subba Rao et al. 1972).

The results of the Rhizobium population survey in Parbhani showed that the field that had chickpea 8 years ago were low in MPN count when compared to the fields that had just had chickpeas (soil numbers 5 and 6 vs. soil numbers 1, 2 and 3). Soil numbers 3 and 5 had high E.C., however, the Rhizobium numbers were still high. This indicated that the Rhizobium strains in these two fields were resistant to salinity. The surveyed Gwalior soil had chickpea previously with varying degrees of nodulation. The number of Rhizobium per g dry soil varied from nine cells to 8,710 cells/g dry soil. The number of Rhizobium did not seem to agree with the degree of nodulation as reported. It must be borne in mind that the soil samples were not taken at the time of nodulation observation. The field observed might not be the same as the ones the soil sample was taken from. Poor nodulation might be due to the use of poor nodulating cultivars or some other environmental factors.

The MPN of Rhizobium from Hissar soils was generally higher in numbers when compared to the samples from Gwalior and Parbhani. There was no significant difference in Rhizobium number between soil samples taken from good and poor growth of the chickpea crop. The Rhizobium population was not the reason for this poor growth. However, Rhizobium strains in these fields might not be effective, or some environmental factors were playing a greater role here.

In the root colonization study the numbers of chickpea Rhizobium expressed per g dry root, g rhizosphere soil and g non-rhizosphere soil

were significantly higher than most of the crops used in the study except groundnut in the Vertisol soils. All plant species showed stimulatory rhizosphere effects on chickpea Rhizobium. Exudates from legume and non-legume root contain a variety of substances which undoubtedly can serve as a carbon or nitrogen source for the root-nodule bacteria, or which may provide the growth factors required by auxotrophic rhizobia (Rovira 1961). Tuzimura and Watanabe (1962b) reported that the growth of Rhizobium trifolii was stimulated in the rhizosphere of host plants (ladino clover and crimson clover), non-host leguminous plants (lucerne, common vetch, soybean and groundnut) and non-leguminous dicotyledonous plants (rape and tomato). The number of Rhizobium trifolii in the rhizosphere soil of graminaceous crops (upland rice, wheat and sudan grass) was lower than in other plant rhizosphere soil. Other workers also reported the stimulatory rhizosphere effect on the root nodule bacteria (Rovira 1961, Tuzimura et al. 1966). The stimulatory effect of the crops changed with soil type (Tuzimura et al. 1966).

This ability of non-legume to support rhizosphere populations of rhizobia in the absence of leguminous host plants could be of value in the spread and persistence of rhizobia. This helps explain the survival of chickpea rhizobia in soils where chickpeas had not been grown for a long time. Diatloff (1969) showed that fallowing the Rhizobium inoculation of cereals, the rhizobia were sufficiently stimulated in the non-legume rhizosphere to provide adequate nodulation of a subsequent soybean crop.

The effect of Rhizobium distribution after the end of the growing season showed that Rhizobium did not move very far from the plant especially in the less frequently irrigated plot. The reason for high numbers in

the 10 irrigation soil samples was because the irrigation increased nodulation and therefore Rhizobium number. However, most of the Rhizobium concentrated only in the vicinity of the tap root where most nodules were formed. Rhizobium did not move very far especially where there was a lack of soil water. Frequent irrigation resulted in further Rhizobium movement.

In most cases, chickpeas are grown on residual moisture. Very few irrigations are applied. Therefore, the chance of Rhizobium spreading is very rare. Rhizobium have been known to be very slow in spreading through soil in laboratory (Kellerman and Fawcett 1907, Frazier and Fred 1922, Hamdi 1971, 1974) and in the field (Chatel et al. 1968). The movement of the Rhizobium depends on water tension, decrease with increasing water tension and ceases when water-filled pores become discontinuous (Hamdi 1971). Vertical movement of Rhizobium depends on the soil particle size and amount of precipitation (Hamdi 1974).

Since Rhizobium do not move very far especially in the place where little or no irrigation is applied, this might have an impact on a Rhizobium population survey and response to seed inoculation. In a Rhizobium population survey, the method of soil sampling is very important especially when the soils are not ploughed and the crops are standing in the field. The soil samples should be taken from both the rhizosphere and non-rhizosphere soil and from many spots. The soil samples should be ground (or broken into small pieces), well mixed and sub-sampled for soil processing later. Replicate samples from each field are required to see the variation in each field. The main problem of our sampling method using the line transect was that we avoided the rhizosphere sampling. This might not give us the real representative

sample especially when the chickpeas were being grown in the field.

For response of inoculation, the Rhizobium inoculated onto the seed might not be able to move from the inoculated seed to the surrounding soil in fields where chickpeas are grown on residual moisture. This might be particularly true if the seeds are sown and not covered properly leaving an air gap between the soil and seed. Rhizobium cannot move and establish in the surrounding soil, however, the seed might be able to germinate. This gives the native population a better chance to compete for nodulation sites. Therefore, no response to inoculation may be found. Therefore, a method of inoculation to ensure establishment of inoculated inoculum in the surrounding soil is required. The use of liquid inoculation (Schiffman and Alper 1968) and granular inoculation (e.g. Dean and Clark 1977, Brockwell et al. 1978) might be useful. Inoculation of the preceding crop might prove to be useful in establishing the Rhizobium in the problem soil before sowing its host crop (Diatloff 1969).

CONCLUSION

The three soil storage conditions under study were found to be not significantly different. The number of Rhizobium increased with time. The number did not significantly change within the 2 months storage period. This implies that soil samples could be taken and stored in any conditions provided that they are processed within 2 months. Rhizobium population depended on crop history, season and depth. Waterlogged conditions in the paddy field were found to be detrimental to chickpea Rhizobium.

The rhizosphere of chickpea, groundnut, pigeonpea, sorghum and pearl millet were found to be stimulatory or at least not inhibitory to chick-

pea Rhizobium. The stimulation was highest in chickpea. Chickpea Rhizobium did not move very far at the end of the growing season. Most of the Rhizobium concentrated at the roots where nodules were formed. The numbers decreased with increasing distance. This suggests that sampling technique in Rhizobium population study is very important.

MANUSCRIPT III

EFFECT OF STICKERS, INOCULATION METHODS ON CHICKPEA *Rhizobium* SURVIVAL AND CHICKPEA YIELD

ABSTRACT

The effect of five different stickers on the survival of two Rhizobium strains inoculated on chickpea seeds was studied using both plate and plant count methods. All the stickers under study, i.e. 10% jaggery, 1.5% methyl cellulose, 1% guar gum, 5% tapioca and rice starch were found to be equal in terms of sticking ability and prolonging the viability of the Rhizobium. Storage temperature at 4°C prolonged Rhizobium survival even after 7 days of storage. However, increasing storage temperature to 28 and 33°C had a detrimental effect on Rhizobium survival and the count was lowest after 7 days of storage. Rhizobium strain IC-59 was found to survive high storage temperatures better than strain 9036.

The effects of some stickers and inoculation methods on chickpeas were also studied in the field conditions. However, none of the treatments were found to be statistically significant from the uninoculated control in terms of yields and other measured parameters.

To study the success of inoculation methods, strain 9036 (streptomycin resistant mutant) was chosen. Isolates from three treatments, i.e. uninoculated control, conventional inoculation method (slurrying method using methyl cellulose as a sticker) and liquid inoculation, were identified using low level intrinsic antibiotic resistant patterns and high levels of concentration of streptomycin (200 mg l^{-1}) (Str 200). At low competition levels (one Rhizobium/g dry soil), 98 and 100% of iso-

lates from conventional and liquid inoculation method were found to be str 200 resistant, respectively. The recovery of str 200 resistant isolates were 36 and 90% in the medium competition level field (10 to 219 Rhizobium/g dry soil) and 1 and 12% in the high competition level field (4,370 to 20,800 Rhizobium/g dry soil) for the conventional and liquid inoculation method, respectively. One hundred twelve and 204 discrete groups were found in the medium and high competition level fields, respectively. Except in one case, isolates from high level competition field were found to have the same pattern of low intrinsic antibiotic resistant as the standard control strain (9036).

INTRODUCTION

The practice of inoculating seed with artificial cultures of rhizobia dates from 1896 (Roughley 1970). In its earliest form, the rhizobia were grown on an agar medium, suspended in water and this suspension used to impregnate either the soil directly or inoculate seed. It is possible to successfully inoculate legume seeds using either agar, freeze dried, or peat cultures (McLeod and Roughley 1961). Peat based inoculum is now the most widely accepted form. In the conventional slurry inoculation method peat inoculum is mixed with water which may contain a sticker and the slurry is then mixed with the seeds and air-dried to produce a coating on the seed.

Many materials have been used as stickers, e.g. 10% sucrose, 40% gum arabic, methyl cellulose, and skimmed milk (Date 1970, Davidson and Reuszer 1978, Iswaran and Chhonkar 1971, Roughley 1970, Vincent 1970, Waggoner et al. 1979).

Other inoculation methods have been reported as an improvement of the conventional method. This includes lime pelleting (e.g. Brockwell 1962, 1963b, Brockwell and Phillips 1970, Cass-Smith and Goss 1958, Chhonkar et al. 1971, Norris 1971a, b, c, Radcliffe et al. 1967, Roughley et al. 1966, Wade et al. 1972), liquid inoculation (Brockwell and Gault 1978, Boonkerd et al. 1978, Brockwell et al. 1978, Brockwell et al. 1980, Hale 1978, Kapusta and Rouwenhorst 1973, Schiffman and Alper 1968), and granular inoculum (Bezdicek et al. 1978, Brockwell et al. 1978, Dean and Clark 1977, Hale 1978, Muldoon et al. 1979).

However, the materials used as the stickers should preferably be locally available and inexpensive. Inoculation methods should also ensure Rhizobium survival and enhance their competitive ability. This paper investigates the effect of different stickers and inoculation methods, on competition between chickpea Rhizobium inoculant strains and the indigenous soil population, in forming nodules and on yields of chickpea. Inoculant strains were identified using intrinsic antibiotic resistance markers.

MATERIALS AND METHODS

Laboratory Test

The effect of different stickers on survival of Rhizobium on inoculated chickpea seed was studied under laboratory conditions.

Rhizobium. Rhizobium strains IC-59 and 9036 were grown on yeast extract mannitol broth (Vincent 1970) for 7 days. Thirty milliliters of the broth was added aseptically to 40 g peat sterilized by γ irradiation in a sealed polyethylene package. The inoculated peat packages were incubated at 28°C for 2 weeks and then stored at 4°C until use.

Stickers. Five stickers were used in this experiment. They were 10% jaggery (locally available), 1.5% methyl cellulose (Australia), 1% guar gum (locally available), 5% tapioca starch (Thailand) and rice starch (by product from rice cooking).

The stickers were prepared in concentrations as mentioned. Ten percent jaggery was prepared by dissolving 10 g of jaggery to 100 ml deionized water. One point five percent methyl cellulose was prepared

by dissolving 6 g methyl cellulose in 100 ml hot deionized water (80°C), stirring gently until dissolved and then 300 ml of cold deionized water added. One percent guar gum was prepared by adding 1 g guar gum to 100 ml boiling water and stirred vigorously to avoid lumps. Five percent tapioca starch was made by dissolving 5 g tapioca starch in 100 ml deionized water, heating and stirring until the suspension thickened. Rice starch was the solution left after cooking rice and removing the grain.

Inoculation procedure. Four grams of peat inoculum was mixed in a beaker with 20 ml sticker, and the suspension used to coat 1 kg of chickpea seed (cv. 850-3/27). The seeds were air dried for 1/2 h, divided into three groups, packaged and stored at the required temperatures.

Storage temperatures. After inoculation and air drying at room temperature, seeds were stored in 4°C (± 2°C), 28°C (± 1°C) and 33°C (± 1°C).

Counting procedure. The Rhizobium population on the seeds were counted at 0, 1, 3 and 7 days after storage. The seeds were drawn from the package to determine the numbers of chickpea Rhizobium surviving on the seed using both plate count and plant infection dilution technique. One hundred eighty seeds were counted from each treatment, put into 180 ml sterilized tap water, shaken for 15 minutes on a wrist shaker. This dilution was considered as 10^0 dilution. Ten-fold serial dilutions were made up to 10^7. Zero point one milliliter aliquots of the dilution 10^2, 10^3 and 10^4 were put into CRMA (congo red yeast extract mannitol

agar) using the spread plate method. Each dilution was plated in triplicate. The last six dilutions were used to inoculate triplicate plant tubes/dilution. The plants were kept in the light chamber (see manuscript 1). The plates were counted after 5 days growth for strain 9036 and 10 days growth for strain IC-59.

The experiments were repeated three times, on May 21 to 28, June 3 to 10 and July 1 to 8, 1980 and each occasion was considered as a single replicate in the analysis.

Field Test

The effect of different methods of inoculation was also examined in fields. Experiments were conducted during the dry winter season of 1979/80 in three fields, one medium depth, one deep Vertisol field and a paddy field (Fine Mixed Hyperthermic Deep Aquic Ustorthent ?). Analysis of soil samples from the top 15 cm showed that the medium depth Vertisol field had a pH of 8.35, electrical conductivity of 0.16 mmhos/cm and contained 35, 0.5 and 126 ppm available N, P and K respectively (For methods of determination see Appendix 27). Chickpeas had not been grown in this field for at least 10 years. Ammorphos fertilizer (28-28-0) and zinc sulphate were applied at the rate of 75 and 80 Kg/ha after land preparation.

Peat inoculants of two Rhizobium strains, i.e. IC-59 and 9036 were used in conjunction with six different inoculation methods, i.e. 10% jaggery (J), 1.5% methyl cellulose (MC), 1% guar gum (GG), 5% tapioca starch (T), liquid inoculum (L) and lime pellet using 1.5% methyl cellulose as a sticker (LP) and uninoculated control making 13 treatments laid out in a randomized block design with five replications.

The preparation of inocula, sticker and inoculation procedure were carried out as the previous experiment. For liquid inoculation, 1 g peat containing 1.5 to 2.9 x 10^9 Rhizobium cells/g was dissolved in 1 l tap water, stirred vigourously and then used at the rate of 3 ml/seed using automatic syringe or pasteure pipettes.

For lime pelleting, the seeds were inoculated with a suspension of 1.5% methyl cellulose and peat inoculum. After thoroughly mixing, the inoculated seeds were sprinkled with finely ground lime (commercial agriculture lime) and mixed until the seeds were uniformly coated with lime.

The chickpea cv. 850-3/27 was inoculated at the rate of 20 ml sticker solution containing 4 g inoculum/Kg seed. The seed was inoculated at 5 p.m. in the afternoon, air dried, kept overnight at approximately 25°C room temperature and sown the next day. The number of Rhizobium per seed were counted using both the plate and MPN plant count methods.

Thirteen treatments were randomized in five replicates in the field. Seeding was done by hand dibbling, opening a hole in the soil with a metal plunger to 7.5 cm depth, the seed dropped in and the hole closed. Liquid inoculation was done by using an automatic syringe or pasteur pipettes to deliver approximately 3 ml of liquid inoculum over each seed in the open planting hole. The planting hole was then closed over by hand. Sowing was done on November 7, 1979.

Plot dimensions were five ridges (3 m) x 7 m with one ridge (0.6 m) between the plots. Each ridge contained two rows 30 cm apart. The seeds were sown 10 cm apart on the rows. The ridges between the plots were sown to uninoculated chickpea cultivar L-550 to act as guard rows.

Plants were sampled at 6 weeks after planting for nodulation and

nitrogenase activity determined by an acetylene reduction assay (Dart et al. 1972). The plants were removed from a 1 m section at one end of each plot. Twenty plants sampled were separated into roots and shoots. The shoots were put in bags and oven dried at 70°C to determine dry weight and N content. The roots were pooled in 800 ml jam bottles, five plants/bottle, sealed with a Suba-seal and a metal cap. Eighty milliliters of air was evacuated and replaced with the same amount of acetylene. The bottle was then incubated for 1/2 h in the shade in the field. The air temperature in the bottle remained within 25 to 28°C.

The gas samples were analyzed in the laboratory for ethylene production using flame ionization gas chromatography. (For calculation of μ moles C_2H_4 production, see Appendix 28.)

After the assay, the roots were placed in plastic bags, brought to the laboratory to determine the number of nodules and nodule dry weight. One hundred plants per treatment were used for acetylene reduction. Five extra plants/plot were lifted, nodules removed and stored in 20% (V/V) aqueous glycerol and kept in a deep freeze at -7°C until used for isolation and strain identification using intrinsic genetic markers (low level antibiotic resistances) (Josey et al. 1979, Benon and Josey 1980).

The plants were again sampled 10 weeks after planting to determine nitrogen uptake, selecting 10 representative plants at grain filling stage. All plant samples were dried at 70°C in a forced air oven for 48 h before weighing, grinding and the N content determined using a Tecetor block digestion and a Technicon Auto Analyzer (see Appendix 29).

The plants were harvested on March 4, 1980 by cutting at the ground level and weighing, followed by hand threshing. Seed yield was determined on air dried samples and 100 g seed samples from each plot were

ground, sub-sampled and the nitrogen content determined.

A similar experiment was conducted in a deep Vertisol field where chickpeas had been grown regularly. Soil samples from the top 15 cm had a pH of 7.8, electrical conductivity of 0.18 mmhos/cm and contained 34, 2.80 and 255 ppm available N, P and K respectively. No fertilizer was applied the year of sowing.

Treatments consisted of two Rhizobium strains, IC-59 and 9036 used as single strain inoculants with three inoculation methods, i.e. conventional seed inoculation using methyl cellulose as a sticker, liquid inoculation and an uninoculated control.

The chickpea cultivar 850-3/27 was used with inoculation and planting procedures as above, except that 5 ml of the peat suspension was applied per seed in the liquid inoculation method. The five treatments were randomized in four blocks. Sowing was done on November 14, 1979. Plot dimension was five ridges (3 m) x 4 m with one ridge (0.6 m) between the plots. Each ridge contained two rows, 30 cm apart with 10 cm plant to plant in the rows. The ridges between the plots were sown to uninoculated chickpea cultivar 850-3/27 to act as guard rows.

Samples were taken from a 1 m section at one end of the plot, 6 weeks after planting for nodulation and nitrogenase activity. Five extra plants per plot were used for nodule sampling for isolation and strain identification. The plants were sampled again 70 days after sowing for N-uptake. The plants were harvested on March 7, 1980.

A further experiment was conducted in a paddy field after harvest of the rice in December. The field was ploughed immediately. Soil samples were taken just before sowing for counting the population of chickpea Rhizobium. Soil sampled from the top 15 cm shows that the soil

had a pH of 7.75, electrical conductivity of 0.23 m mhos/cm and contained 46, 16 and 175 ppm of available N, P and K respectively.

Rhizobium strain 9036 was used in the study with seven inoculation treatments comparing jaggery, methyl cellulose, guar gum, tapioca as stickers and liquid inoculation and pelleting with calcium peroxide (CaO_2) and with an uninoculated control.

Calcium peroxide (CaO_2) was tried as a seed pellet since results indicated (Interox International) that it increased germination of direct drilled rice and had enhanced nodulation of soybean. The method of pelleting was essentially the same as used previously. Chickpea cultivar CpS-1 was used. The Rhizobium populations on the seed following inoculation were counted by both plate and plant count methods. The seven treatments were randomized in four blocks. Sowing was done by a hand dibbling method on December 26, 1979. Plot dimension was 3 x 7 m with 0.6 m between the plots, with flat planting and 30 cm distance between the rows and 10 cm between plant to plant.

Plants were sampled 6 weeks later for nodulation and nitrogenase activity. Nodules from six extra plants were stored in 20% glycerol at $-7^{o}C$ for isolation and identification of the Rhizobium.

Strain Identification

Isolates of Rhizobium from nodules of treatments inoculated with strain 9036 using liquid inoculation, methyl cellulose stickers and uninoculated control were used for identification. Strain 9036 is a spontaneous str^{+} mutant (resistant to 200 mg l^{-1} of streptomycin) of strain IC-2002 which nodulates chickpea effectively.

For each plot 40 to 50 nodules were removed from storage, surface

sterilized by exposure to 0.1% $HgCl_2$ for 30 seconds to 1 minute, washed 10 times with sterilized tap water, crushed with a glass rod and streaked with a metal loop on congo red yeast mannitol agar plates (Vincent 1970). A single purified colony was used to inoculate an agar slope of YEMA in a 15 x 150 mm cotton plugged tube and incubated for 5 to 7 days. Three milliliters of sterilized 20% V/V glycerol was added to the tube, shaken by using a Vortex mixer to obtain a bacterial suspension and this suspension was used immediately for finger printing.

Strains were authenticated as chickpea Rhizobium by adding 1 ml of the suspension to a plantlet from which the cotyledons had been excised, growing in sand in a test tube (see manuscript 1). The plant tube was kept in a light chamber adjusted to a 16 h day and 8 h night, watered at 3 weeks after inoculation and scored for nodulation 6 weeks after inoculation.

Strains were identified using their pattern of resistances to low levels of 10 antibiotics (Table 22). Isolates were also tested for their resistance to streptomycin (200 mg l^{-1}). The methodology of testing was essentially the same as for low level intrinsic resistance, and was done at the same time using the same suspension of the isolate strain as inoculum.

Antibiotic plates were prepared by pipetting the required amount of stock antibiotic solution to 200 ml of YEMA (Appendix 31). The volume added varied from 10 µl to 600 µl, and was added using a Gibson Pipettman. The flask was hand shaken to mix thoroughly the media and antibiotic. Thirty milliliters of the media was poured into a graduated 50 ml beaker and then into the plate. The plates were kept to solidify on the flat surface. The plates were inoculated with a multiple 25 pin inocu-

TABLE 22. Ten antibiotics used in identification Rhizobium using a low intrinsic resistant level or finger printing technique.

Antibiotic	Supplier	Final concentration (µg/ml media)
Carbenicillin	Pyopen regd. Beecham Research Lab, Brentford	1
	"	2.5
	"	5
Erythromycin	Sigma	1.25
	"	2.50
	"	10
	"	15
Kanamycin sulfate	Sigma	2.5
	"	10
	"	20
Nalidixic acid	Sigma	2.5
	"	10
	"	15
Neomycin sulfate	Sigma	1.25
	"	2.50
	"	10
	"	15
Polymyxin B sulfate	Sigma	5
	"	10
	"	20
Rifampicillin	Sigma	0.25
	"	0.50
	"	2.5
Streptomycin sulfate	Sigma	2.5
	"	10
	"	20
Tetracyclin	Sigma	0.1
	"	0.5
Vancomycin	Sigma	1.25
	"	2.50
	"	10

lator on the same day as pouring (Josey et al. 1979). Stock cultures grown on agar slants for 5 to 7 days (approximately 10^7 rhizobia/slant) were diluted with 3 ml of 20% glycerol and 0.5 ml placed in the wells of the inoculator. Each prong transfers about 10^3 to 10^5 bacteria to the plate (Appendix 30). The number of bacteria transferred was estimated by using the inoculator to transfer the bacteria to wells containing sterile water and dilution plating samples of these wells.

The inoculated plates were incubated in a room where the temperature was maintained between about 28° ± 3°C. There were three replicate plates for each antibiotic concentration. Scoring was done after 6 to 14 days after inoculation depending on growth on control plates. The plates were scored as:

1 - No growth

2 - Some growth

Sometimes there was variability in growth between replicate plates of the same concentration. In these cases the score for the two plates with the same reaction (growth or no growth) was used.

RESULTS

Laboratory Test

Analysis of variance of different treatments on Rhizobium survival on seed as determined by plate and plant count (Tables 23 and 24) shows that there was a significant effect of storage temperature and inoculum strain, but there were no differences between stickers. There was a significant effect of days of storage on the number of Rhizobium surviving. Significant interactions were found between Rhizobium strains x days of

TABLE 23. Analysis of variance for plate count in *Rhizobium* survival on inoculated seed.

Source of variation	D.F.	S.S.	M.S.	F-ratio	Sig. level
Main Plot					
Replications (R)	2	16.8768	8.4384	59.92	1%
Temperature (T)	2	37.2219	18.6109	132.16	1%
Stickers (S)	4	0.9272	0.2318	1.65	N.S.
Inoculum (I)	1	1.3506	1.3506	9.59	1%
T X S	8	0.5120	0.0641	0.45	N.S.
T X I	2	2.1292	1.0646	7.56	1%
S X I	4	0.2165	0.0541	0.38	N.S.
T X S X I	8	0.3272	0.0409	0.29	N.S.
Error (1)	58	8.1676	0.1408		
Sub Plot					
Days (D)	3	48.3291	16.1097	270.66	1%
S X D	12	1.1449	0.0954	1.60	N.S.
I X D	3	3.5869	1.1956	20.09	1%
S X I X D	12	0.3159	0.0263	0.44	N.S.
T X D	6	23.9871	3.9978	67.17	1%
S X T X D	24	0.8813	0.0367	0.62	N.S.
I X T X D	6	1.2950	0.2158	3.63	1%
S X I X T X D	24	0.3850	0.0160	0.27	N.S.
Error (2)	180	10.7137	0.0595		
Total	359	158.3680			

TABLE 24. Analysis of variance for plant count in Rhizobium survival on inoculated seed.

Source of variation	D.F.	S.S.	M.S.	F-ratio	Sig. level
Main Plot					
Replications (R)	2	13.9461	6.9731	29.47	1%
Temperature (T)	2	57.1787	18.5893	73.56	1%
Stickers (S)	4	2.3867	0.5967	2.52	N.S.
Inoculum (I)	1	3.5920	3.5920	15.18	1%
T X S	8	1.4404	0.1800	0.76	N.S.
T X I	2	0.5333	0.2666	1.13	N.S.
S X I	4	0.3283	0.0821	0.35	N.S.
T X S X I	8	0.5992	0.0749	0.32	N.S.
Error (1)	58	13.7246	0.2366		
Sub Plot					
Days (D)	3	50.7473	16.9158	103.59	1%
S X D	12	3.4139	0.2845	1.74	N.S.
I X D	3	1.2064	0.4021	2.46	N.S.
S X I X D	12	5.8435	0.4870	2.98	1%
T X D	6	27.8708	4.6451	28.45	1%
S X T X D	24	2.3926	0.0997	0.61	N.S.
I X T X D	6	3.3182	0.5530	3.39	1%
S X I X T X D	24	1.5927	0.0664	0.41	N.S.
Error (2)	180	29.3919	0.1633		
Total	359	199.5070			

storage, temperature x days of storage, temperature x Rhizobium strains x days of storage.

There was good agreement between plate and MPN plant counts. Analysis of variance of the numbers derived from plate counts and plant counts agreed reasonably well with only a few interaction exceptions. There was a significant interaction between Rhizobium strain x days of storage by using a plate count method but not by a plant count method, and vice versa in the case of the interaction among stickers x Rhizobium strains x days of storage. The correlation coefficient (r) between these two methods of Rhizobium counting is 0.90 (n = 120).

Figures 6 and 7 show the number of Rhizobium/seed at different days of storage. The figures are the average over the two strains, i.e. IC-59 and 9036. All the stickers had no effect on the numbers of Rhizobium sticking on to the seed. The number of Rhizobium sticking on to the seed at 0 day ranged from 2.00×10^6 in jaggery to 3.55×10^6 in tapioca by the plate count method. For plant count method, the numbers of Rhizobium per seed at 0 day ranged from 1.78×10^6 in rice starch to 6.61×10^6 in tapioca. The numbers of Rhizobium reduced with days of storage. However, there is not any one sticker that is superior to the other in enhancing or prolonging Rhizobium survival. The numbers of Rhizobium per seed at 7 days of storage ranged from 3.39×10^5 in rice starch to 3.55×10^5 in jaggery and methyl cellulose (plate count) and ranged from 2.24×10^5 in guar gum to 3.98×10^5 in tapioca (plant count).

The effect of temperature on the survival of the two Rhizobium strains is shown in Figure 8a and b. Plate count indicated an interaction between Rhizobium strains x temperature, however, plant count did

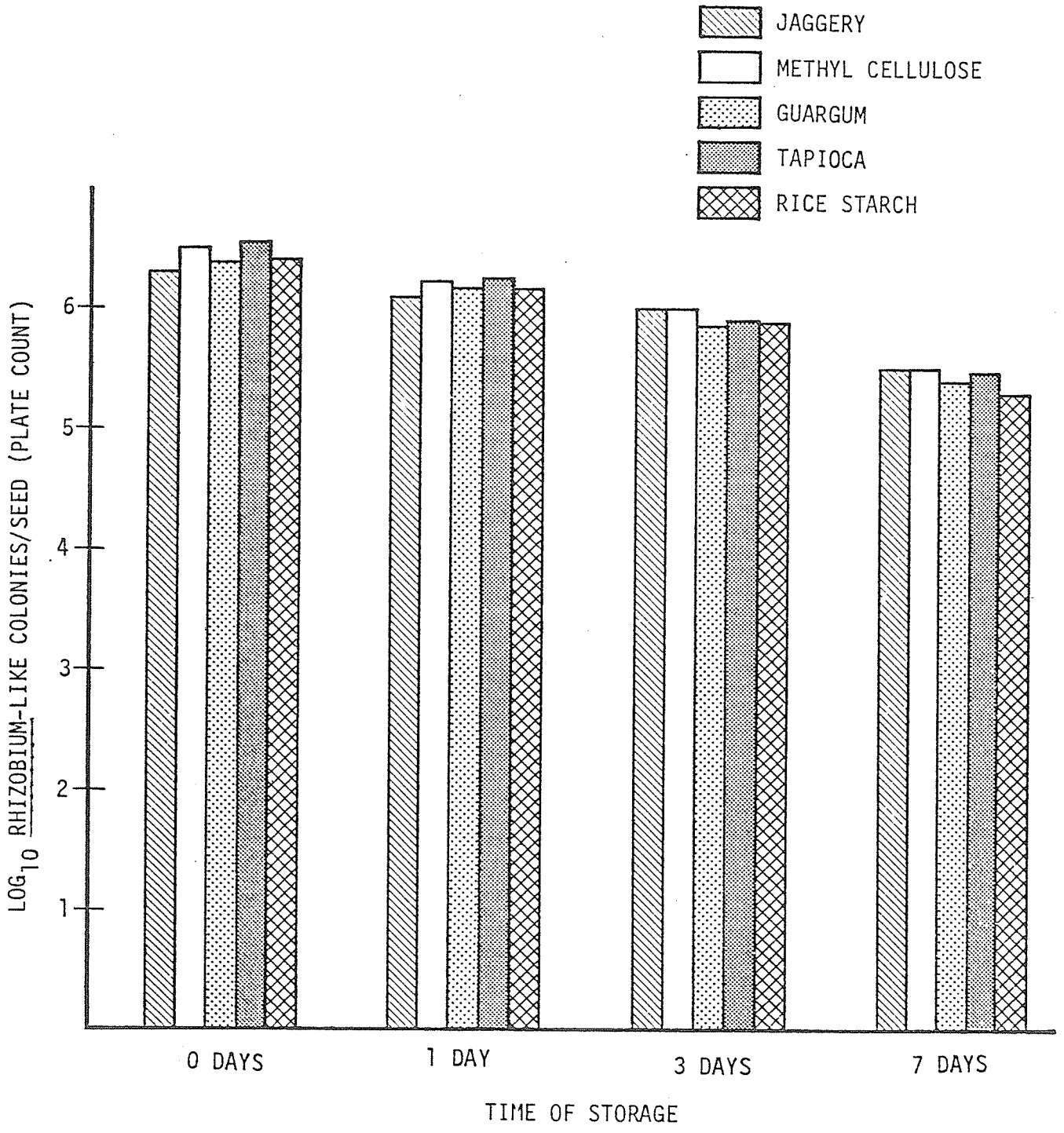

FIGURE 6. LOG_{10} CHICK-PEA RHIZOBIUM-LIKE COLONIES PER SEED AT DIFFERENT DAYS AS AFFECTED BY DIFFERENT STICKERS (PLATE COUNT).

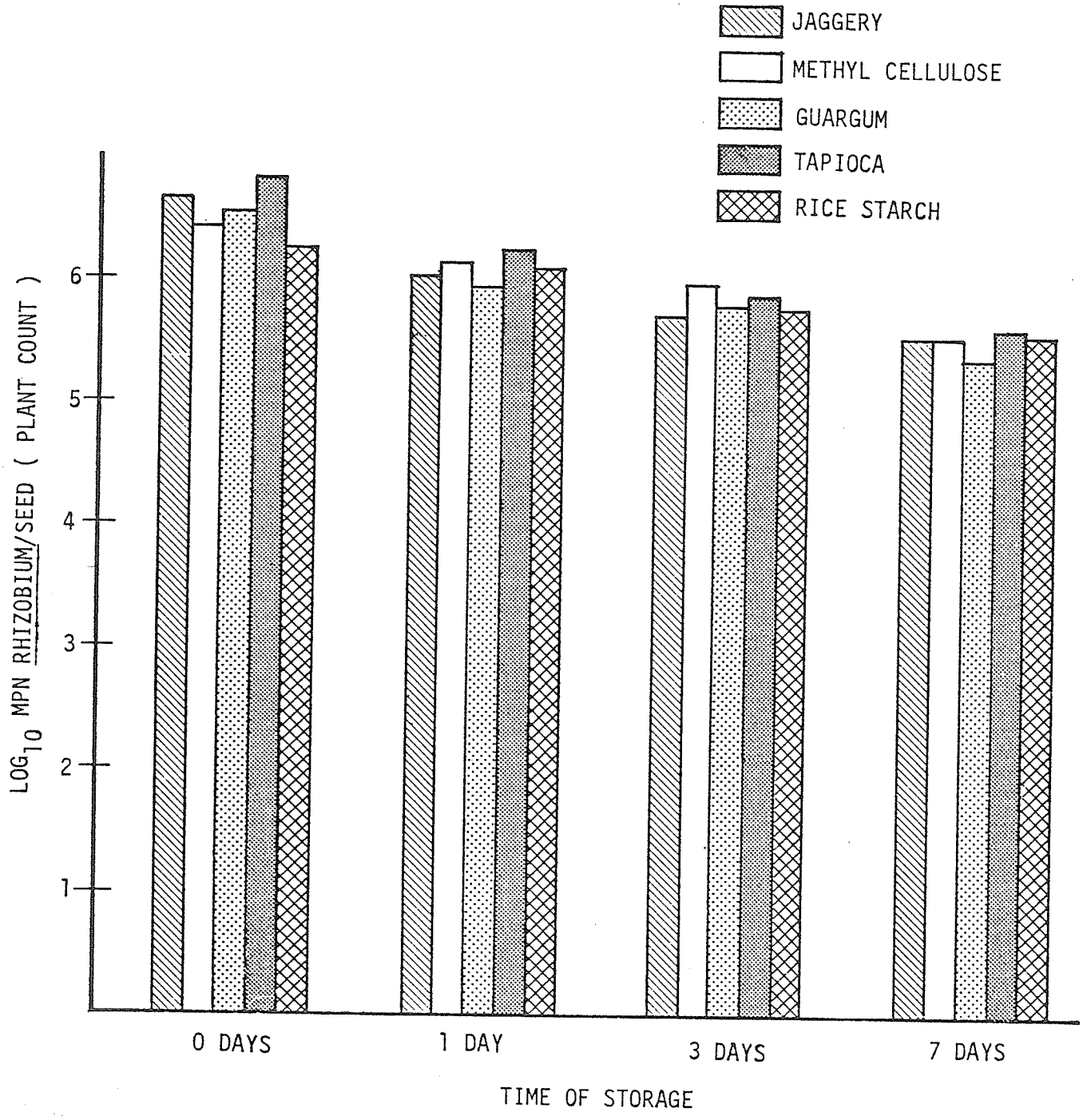

FIGURE 7. LOG_{10} MPN CHICK-PEA RHIZOBIUM SURVIVAL AT DIFFERENT DAYS AS AFFECTED BY DIFFERENT STICKERS (PLANT COUNT).

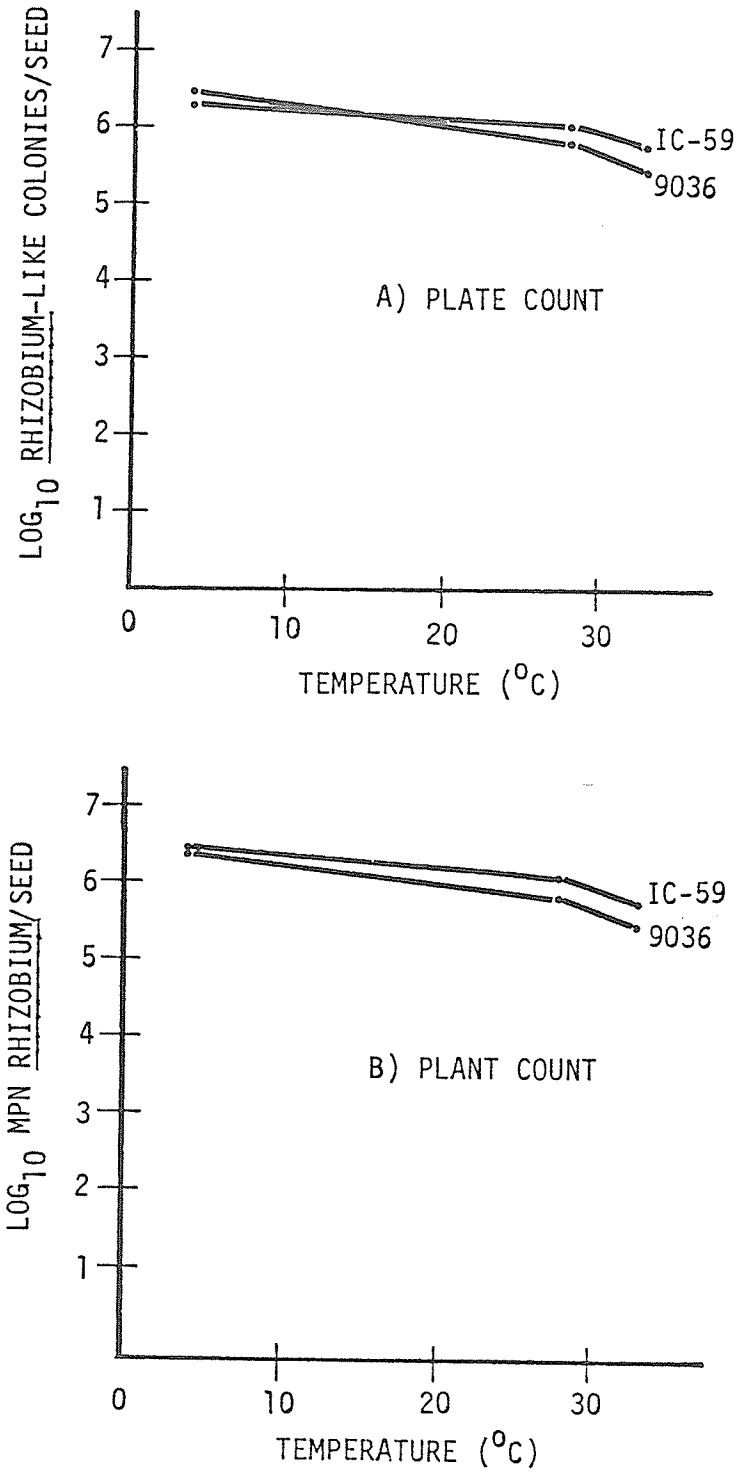

FIGURE 8. THE EFFECT OF TEMPERATURE ON RHIZOBIUM SURVIVAL USING PLATE AND PLANT COUNT METHOD.

not. The number of Rhizobium/seed declined with increasing storage temperatures. Except for plate count at 4°C storage temperature, strain IC-59 was found to be superior to 9036 in survival at different temperatures by both counting methods. The death rate per day was higher in strain 9036 than IC-59 (Appendix 18). The heavy death rate occurred in the first day and later became constant in both counting techniques.

To demonstrate the effect of temperature of storage on Rhizobium survival clearly, an interaction between temperature x days of storage is plotted and shown in Figure 9A and B for both counting methods. 4°C storage temperature had a beneficial effect on prolonging the survival of the Rhizobium. The number of Rhizobium/seed did not significantly change at 7 days after storage at 4°C as measured by plate and plant count. However, as the storage temperature increased, the number of Rhizobium/seed reduced in both counting methods. The death rate/°C increase of storage temperature was always higher for strain 9036 than IC-59 (Appendix 19). Increasing storage temperature from 28 to 33°C resulted in a higher death rate/°C than increasing the temperature from 4 to 28°C.

Field Test

Table 25 summarizes the numbers of background Rhizobium population in the fields where inoculation trials were conducted. The numbers of background Rhizobium population in field A (a medium depth Vertisol field) ranged from 10 to 219 cells/g dry soil, field B (a deep Vertisol field) from 4,370 to 20,800 cells/g dry soil and in field C (a Fine Mixed Hyperthermic Deep Aquic Ustorthent ? field) was less than one cell/g dry soil. Field A had no history of chickpea cultivation since the

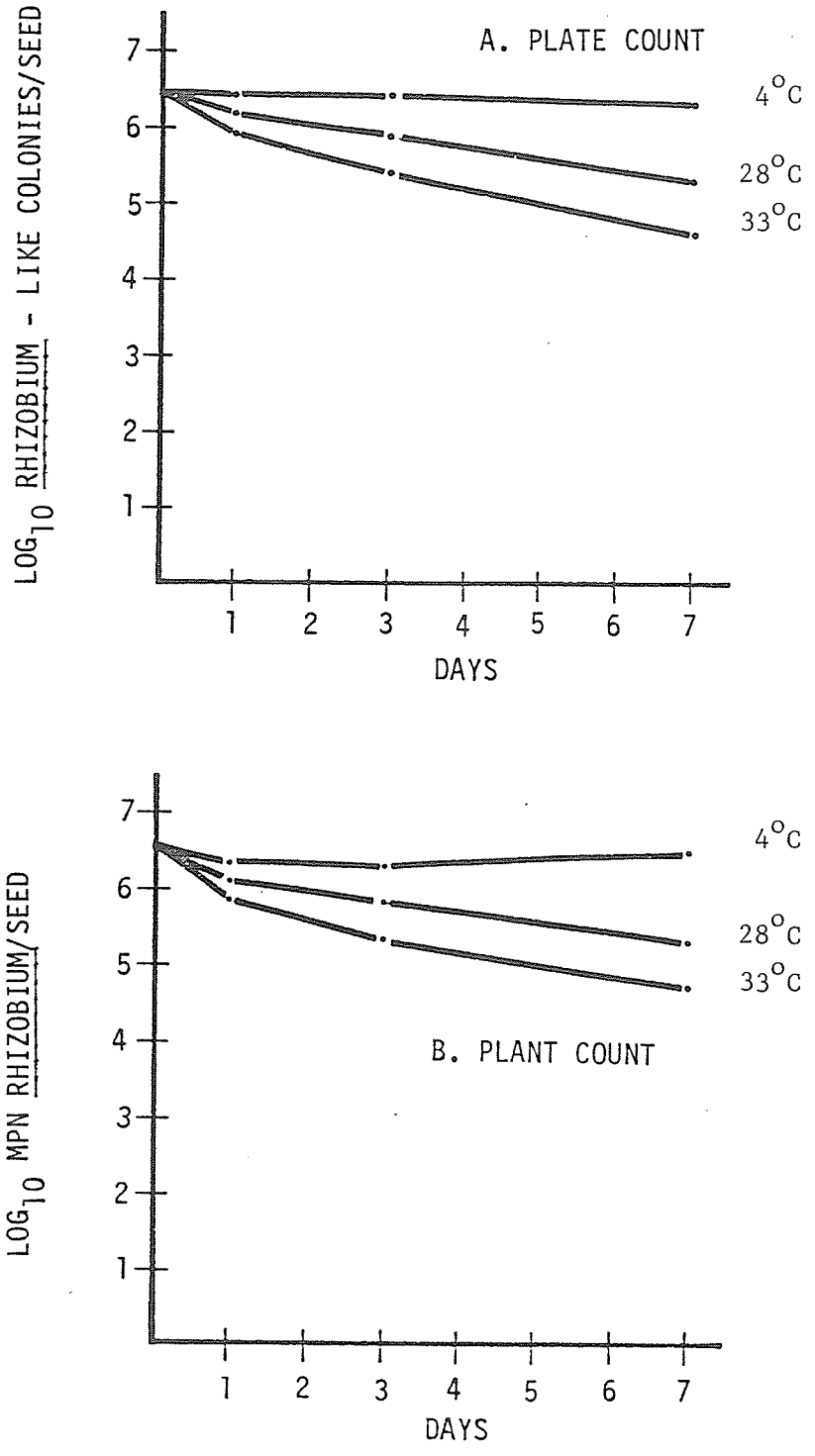

FIGURE 9. EFFECT OF STORAGE TEMPERATURE ON RHIZOBIUM SURVIVAL ON INOCULATED SEEDS (AVERAGE OVER STICKERS AND STRAINS). THE RHIZOBIA WERE COUNTED AT DIFFERENT TIME INTERVALS.

TABLE 25. Background chickpea Rhizobium population in 3 fields. The numbers are expressed as Log 10 MPN Rhizobium/g dry soil.

Replicates	Log 10 MPN/g dry soil*		
	Field A	Field B**	Field C*
1	1.34	4.00	0
2	1.03	3.65	0
3	2.04	3.64	0
4	2.34	4.32	0
5	1.03	(-)	(-)
Mean	1.56	3.90	0

* The factors making the 95% confidence interval on the MPN is ± 0.68 (Cochran 1950).

**Only 4 replicates experiment.

establishment of ICRISAT in 1972. Chickpeas had been grown in field B many times. Field C, which is a paddy field, had no history of chickpea cultivation.

The numbers of Rhizobium inoculated per seed in all treatments are summarized in Table 26. The counts were made using both the plate and plant counts. Liquid inoculum always gave a higher plate count than other treatments (10^7 cells/seed). Lime pelleting or CaO_2 pelleting were found to be toxic to Rhizobium. The Rhizobium were all killed by such treatments. The other treatments contained approximately 10^6 cells/seed except in field C which were about 10^5 cells/seed. Small seeded variety (CpS-1) was used in field C. A large seeded variety (850-3/27) was used in fields A and B.

Table 27 shows the survival of chickpea Rhizobium on inoculated seed recovered from field A at different days after sowing. Lime pelleting treatments killed Rhizobium rapidly. However, the increase in numbers of this treatment reflected the colonization of the Rhizobium on the seed by the native population. The other inoculated treatments showed reduction in numbers per seed with time. Except the lime pelleting treatments, the number of Rhizobium per seed were not significantly different from each other at 1 and 3 days sampling. The numbers of these treatments were reduced by approximately ten-fold within 1 day.

Tables 28, 29 and 30 show shoot, root, nodule weight, nodule number and nitrogenase activity of 6 week old chickpea grown in fields A, B and C respectively. In field A, there was no significant difference among treatments in all the measured parameters. This field was very variable and this could be reflected in the high coefficient of variation in all the measured parameters. The shoot weight/plant ranged from 1.33

TABLE 26. The numbers of Rhizobium inoculated per seed in each treatment in all experiments.

Treatment	Log 10 MPN Rhizobium/seed					
	Field A		Field B		Field C	
	Plate count	Plant count*	Plate count	Plant count*	Plate count	Plant count*
Uninoculated control	0	0	0	0	0	0
9036-J	6.02	6.94	-	-	5.52	5.63
9036-MC	6.79	7.05	6.60	6.63	5.77	5.26
9036-GG	6.52	6.81	-	-	5.65	5.26
9036-T	6.41	6.78	-	-	5.84	5.26
9036-L	7.18	7.64	7.18	7.64	7.43	7.28
9036-LP or CaO_2	0	0	0	0	0	0
IC-59-J	6.18	7.05	-	-	-	-
IC-59-MC	6.62	7.43	5.99	5.58	-	-
IC-59-GG	6.69	7.05	-	-	-	-
IC-59-T	6.29	7.06	-	-	-	-
IC-59-L	7.36	7.95	7.36	7.95		
IC-59-LP	0	0				

*The factors making the 95% confidence interval on the MPN is $\pm$ 0.68.

TABLE 27. *Rhizobium* survival on inoculated seeds at different time intervals after planting in field A. The numbers are expressed as Log 10 MPN *Rhizobium*/seed.

Treatment	0 day		1 day	3 days
	Plate count	Plant count*	(Plant count)*	(Plant count)*
9036-J	6.02	6.94	5.00	5.26
9036-MC	6.79	7.05	6.12	5.63
9036-GG	6.52	6.81	5.48	5.48
9036-T	6.41	6.78	6.12	6.05
9036-LP	0	0	4.32	3.71
IC-59-J	6.18	7.05	6.12	4.80
IC-59-MC	6.62	7.43	6.12	5.63
IC-59-GG	6.69	7.05	6.26	5.81
IC-59-T	6.29	7.06	6.26	6.26
IC-59-LP	0	0	2.71	3.57

*The factors making the 95% confidence interval on the MPN is $\pm$ 0.68.

TABLE 28. Shoot, root, nodule weight, nodule number and nitrogenase activity of 6 week old chickpeas grown in field A.

Treatment	Shoot wt (g/plant)	Root wt (g/plant)	Nodule no. per plant	Dry nodule wt (g/plant)	µmoles C_2H_4/ plant/h	µmoles C_2H_4/g dry nod./h
Uninoculated control	1.96 a	0.14 a	9 a	0.02 a	1.08 a	38.17 a
9036-J	1.97 a	0.12 a	12 a	0.03 a	0.95 a	24.22 a
- MC	2.08 a	0.12 a	8 a	0.01 a	0.29 a	41.47 a
- GG	2.15 a	0.12 a	10 a	0.02 a	0.77 a	25.27 a
- T	1.76 a	0.13 a	10 a	0.01 a	0.45 a	30.13 a
- L	1.91 a	0.12 a	13 a	0.02 a	0.78 a	18.93 a
- LP	1.33 a	0.11 a	9 a	0.02 a	1.04 a	44.46 a
TC-59-J	1.91 a	0.13 a	11 a	0.02 a	1.03 a	35.66 a
- MC	2.11 a	0.13 a	11 a	0.02 a	0.83 a	39.18 a
- GG	1.76 a	0.12 a	9 a	0.01 a	0.22 a	44.49 a
- T	2.13 a	0.14 a	13 a	0.02 a	1.12 a	43.84 a
- L	1.97 a	0.13 a	18 a	0.03 a	1.38 a	40.32 a
- LP	1.79 a	0.13 a	9 a	0.02 a	0.98 a	41.54 a

a Means in the same column followed by the same letter are not significantly different by Duncan's new multiple range test at $P \leq 0.05$.

For analysis of variance see Appendix 20.

TABLE 29. Shoot, root, nodule weight, nodule number and nitrogenase activity of 6 week old chickpeas grown in field B.

Treatment	Shoot wt (g/plant)	Root wt (g/plant)	Nodule no. per plant	Dry nodule wt (g/plant)	µmoles C_2H_4/ plant/h	µmoles C_2H_4/g dry nod./h
Uninoculated control	2.39 b	0.14 a	18 a	0.03 a	0.59 a	19.29 a
9036-L	2.57 ab	0.14 a	19 a	0.03 a	0.67 a	22.55 a
- MC	2.62 ab	0.13 a	19 a	0.03 a	0.74 a	21.56 a
IC-59-L	2.96 a	0.16 a	20 a	0.03 a	0.82 a	22.26 a
- MC	2.33 b	0.13 a	21 a	0.03 a	0.77 a	14.09 a

a-b Means in the same column followed by the same letter are not significantly different by Duncan's new multiple range test at $P \leq 0.05$.

For analysis of variance see Appendix 21.

TABLE 30. Shoot, root, nodule weight, nodule number, N content and nitrogenase activity of 6 week old chickpeas grown after paddy in field C.

Treatment	Shoot wt (g/plant)	Root wt (g/plant)	Nodule no. per plant	Dry nodule wt (g/plant)	N content (mg/plant)	µmoles C_2H_4 per plant/h	µmoles C_2H_4/g dry nod./h
Uninoculated control	1.18 a	0.14 a	0.1 c	0.00 a	36.04 a	0.01 b	0.03 b
9036-J	0.93 a	0.11 a	15.0 a	0.09 a	28.11 a	1.39 a	15.39 a
- MC	1.47 a	0.15 a	18.0 a	0.08 a	48.47 a	1.33 a	19.24 a
- GG	1.25 a	0.12 a	16.0 a	0.07 a	42.07 a	0.90 a	13.71 a
- T	1.32 a	0.14 a	12.0 b	0.06 a	43.71 a	0.90 a	15.44 a
- L	1.32 a	0.15 a	16.0 a	0.08 a	43.72 a	1.65 a	21.36 a
- $CaCO_2$	1.27 a	0.14 a	0.3 c	0.00 a	42.00 a	0.04 b	3.98 b

a-c Mean in the same column followed by the same letter are not significantly different by Duncan's new multiple range test at $P \leq 0.05$.

For analysis of variance see Appendix 22.

to 2.15 g/plant, root weight from 0.11 to 0.14 g/plant, nodule number from eight to 18 nodules/plant and nodule weight from 0.01 to 0.03 g/plant. The nitrogenase activity/plant ranged from 0.22 to 1.38 μmoles/plant/h. Specific nitrogenase activity ranged from 18.93 to 44.49 μmoles C_2H_4/g dry nodule/h.

In field B, shoot weight was significantly different at the 5% level. However, root weight, nodule number, nodule weight, nitrogenase activity and specific nitrogenase activity were not significantly different. The field was uniform. This could be reflected by a lower coefficient of variation in all the measured parameters compared to those in field A. Shoot weight ranged from 2.33 to 2.96 g/plant, root weight from 0.13 to 0.16 g/plant, nodule number from 18 to 21 nodules/plant, nodule weight was 0.03 g. Nitrogenase activity ranged from 0.59 to 0.82 μmoles C_2H_4/plant/h. Specific nitrogenase activity ranged from 19.29 to 24.09 μmoles C_2H_4/g nodule/h.

In field C, nodule number and nitrogenase activity were significantly different at the 5% level. The field contained less than one Rhizobium per g dry soil and this might be the reason for these differences. However, the field was quite variable and this could be seen by the high coefficient of variation of the parameters used in measuring the symbiotic effectiveness. Shoot weight ranged from 0.93 to 1.47 g/plant, root weight from 0.11 to 0.15 g/plant, nodule number from 0.1 to 18 nodules/plant, nodule weight from 0.06 to 0.09 g/plant and N content from 28.11 to 48.47 mg/plant. Nitrogenase activity ranged from 0.01 to 1.65 μmoles/plant/h. Specific nitrogenase activity ranged from 0.03 to 21.36 μmoles/g nodule/h.

Tables 31 and 32 show the amount of nitrogen uptake per plant at

TABLE 31. N uptake by chickpea plant (mg/plant) at 6 and 10 weeks after planting in field A.

Treatment	N uptake (mg/plant)	
	6 weeks	10 weeks
Uninoculated control	74.42 a	135.03 a
9036-J	76.10 a	130.00 a
- MC	86.61 a	186.03 a
- GG	81.93 a	183.84 a
- T	69.47 a	140.56 a
- L	75.93 a	136.82 a
- LP	47.54 a	85.13 a
JC-59-J	74.98 a	146.44 a
- MC	78.61 a	175.07 a
- GG	71.62 a	135.37 a
- T	80.90 a	175.89 a
- L	80.32 a	140.99 a
- LP	76.63 a	158.52 a

a Means in the same column followed by the same letter are not significantly different by Duncan's new multiple range test at $P \leq 0.05$.

For analysis of variance see Appendices 23 and 20.

TABLE 32. N uptake by chickpea plant (mg/plant) at 6 and 10 weeks after planting in field B.

Treatment	N uptake (mg/plant)	
	6 weeks	10 weeks
Uninoculated control	92.87 a	181.84 a
9036-L	101.99 a	183.28 a
9036-MC	101.75 a	213.10 a
IC-59-L	114.29 a	169.39 a
IC-59-MC	89.12 a	156.62 a

a Means in the same column followed by the same letter are not significantly different by Duncan's new multiple range test at $P \leq 0.05$.

For analysis of variance see Appendices 24 and 21.

6 weeks after planting in Fields A and B respectively. At both samplings in field A, the N uptake in different treatments did not show any significant difference. The average N uptake at 6 weeks sampling was 75 mg/plant while at 10 weeks was 148 mg/plant. In field B, the average N uptake at 6 weeks after planting was 100 mg/plant and at 10 weeks after planting was 180.85 mg/plant. Again there was no significant difference among treatments in both samplings.

Tables 33 and 34 show seed yield and seed nitrogen yield of chickpea grown in fields A and B. Seed yields and seed N yield of different treatments were not significantly different in both fields. Since field A was variable, the seed yield ranged from 680 to 1140 Kg/ha and seed nitrogen yield ranged from 23 to 38 Kg/ha. The coefficient of variation for seed yield and seed N yield in this field were 23.2% and 23.7%, respectively. The average seed yield and seed nitrogen yield in field A were 970 and 32 Kg/ha respectively. Field B was more uniform compared to field A. Seed yield ranged from 1,200 to 1,480 Kg/ha and seed N yield ranged from 44 to 56 Kg/ha. The coefficient of variation for seed yield and seed N yield were 11.6 and 17.8% respectively. The average seed yield and seed N yield were 1,370 and 50 Kg/ha respectively.

Rhizobium Identification

The success of the inoculum strains in forming nodules was examined in two treatments, 9036-MC, 9036-L, and compared with the uninoculated control. The *Rhizobium* strain 9036 is resistant to streptomycin (Str 200 resistance). Table 35 shows the recovery of the inoculated strain in fields A, B and C at 6 weeks after planting respectively.

In field C where the soil contained less than one *Rhizobium*/g dry

TABLE 33. Seed and seed nitrogen yield (Kg/ha) of chickpea grown in field A.

Treatment	Seed yield (Kg/ha)	Seed N yield (Kg/ha)
Uninoculated control	901 a	31.30 a
9036-J	901 a	28.00 a
- MC	1038 a	34.08 a
- GG	1075 a	37.77 a
- T	929 a	31.56 a
- L	968 a	34.46 a
- LP	687 a	23.18 a
IC-59-J	993 a	33.07 a
- MC	1145 a	35.65 a
- GG	891 a	30.25 a
- T	1017 a	35.19 a
- L	1081 a	36.79 a
- LP	1034 a	34.15 a

a Means in the same column followed by the same letter are not significantly different by Duncan's new multiple range test at $P \leq 0.05$.

For analysis of variance see Appendix 25.

TABLE 34. Seed and seed nitrogen yield (Kg/ha) of chickpea grown in field B.

Treatment	Seed yield (Kg/ha)	Seed N yield (Kg/ha)
Uninoculated control	1351 a	52.23 a
9036-L	1401 a	49.81 a
- MC	1412 a	52.22 a
IC-59-L	1482 a	56.49 a
- MC	1204 a	43.67 a

a Means in the same column followed by the same letter are not significantly different by Duncan's new multiple range test at $P \leq 0.05$.

For analysis of variance see Appendix 26.

TABLE 35. Percentage recovery of inoculated Rhizobium strain 9036 in 3 selected treatments using str 200 resistant characteristic alone in fields A, B and C.

Field	Population range (Rhizobium/g dry soil)	Treatment		
		Control	Methyl cellulose	Liquid
C	≤ 1	0	98	100
A	10 - 219	0	36	80
B	4,370 - 20,800	0	1	12

For more detail see Appendix 32.

soil, there were no nodules formed in the uninoculated plot. As expected, inoculation using methyl cellulose as a sticker (9036-MC) and liquid inoculation (9036-L) resulted in nodule formation, with recovery of strain 9036 from 98 to 100% of the nodules examined. The identification was done by str 200 resistant characteristic.

When the population of the native Rhizobium in the soil increased from 10 to 219 Rhizobium/g dry soil (field A), the recovery of inoculum strain was 0, 36 and 80% in uninoculated control, methyl cellulose sticker and liquid inoculation treatments, respectively. When the soil population increased further (field B) to 4,370 to 20,800 Rhizobium/g dry soil, the recovery of the inoculum strain in the nodules was 0, 1 and 12% in the uninoculated control, methyl cellulose sticker and liquid inoculation method treatment, respectively.

The pattern of strain distribution in the nodules from fields A and B was also examined by typing the isolates using low intrinsic antibiotic resistant characteristics. Isolates examined were those that were not resistant to str 200 and hence not inoculum strain 9036. On the basis of a unique profile of response to the antibiotics, the 475 isolates from field C fell into 205 groups (see Appendix 33). The number of groups in the uninoculated control, methyl cellulose sticker and liquid inoculation treatments were 90, 82 and 82 respectively. When all the isolates from field B were tested against str 200, only 22 isolates were found to carry a high level resistance marker of the inoculant strain. The low intrinsic antibiotic resistance pattern of these 22 field isolates fell into three groups (see Appendix 35). Samples of the pure culture of 9036 were also added as the control in the experiments and they fell into two groups (see Appendix 35). The discrepancy occurred

in one concentration of Neomycin sulfate, i.e. Neo 10 (see Appendix 35). When an allowance was made for the possibility of an erroneous result for one test out of the complete set, all samples of 9036 fell into one group and 21 of the 22 field isolates fell into the same group (Table 36). The single isolate which did not fall into either of the two major groups again formed a discrete group (see Appendix 35). This isolate was the only isolate from methyl cellulose sticker treatment in field B that was found to be resistant to str 200. The rest of the field isolates that were resistant to str 200 were from liquid inoculation treatment. When one mismatch allowance was made for the 475 field isolates, instead of having 205 discrete groups, they now became 119 groups.

In field A, all the isolates were tested against str 200 first. Only the isolates that were not resistant to str 200 were tested against low intrinsic antibiotic resistance. One hundred twelve discrete groups were found (see Appendix 34). When one mismatch was allowed for grouping, we now had 74 groups. This reflects the variability of the indigenous Rhizobium in this field. It was also assumed that the isolates from field A that were not resistant to str 200 were the indigenous Rhizobium.

DISCUSSION

Laboratory Test

Plant and plate counts corresponded very well. This might be due to the use of pure peat inoculum. Both peat inoculum strains were prepared in the laboratory, the count was found to be up to 10^9 cells/g peat. Plate counts revealed that there was no contamination at the level

TABLE 36. Comparability of two strain identification techniques, i.e. low intrinsic antibiotic resistance vs high level resistance marker (Str 200).

	Technique		
	High antibiotic resistance (Str 200)	Low intrinsic antibiotic resistance	
		0 Mismatch	1 Mismatch
Pure strain (9036 str)	22	13 + 9 (2 groups)	22 (1 group)
Field isolates	22	19 + 2 + 1 (3 groups)	21 + 1 (2 groups)

of 10^6 dilution. Interferences from contaminating micro-organisms has been known to be the reason for discrepancies in plant count (Thompson and Vincent 1967, Robinson 1968, Vincent 1970). Plant counts produce a most probable number with a wide range for the 95% confidence limits when six dilution steps, three replicates/dilution are used. The limits are $\pm$ 0.68 for log 10 MPN or $\times \div$ 4.8 for the actual MPN (Cochran 1950). For example, an MPN of 3×10^9 cells, the 95% confidence limit range will be from 6.3×10^8 to 1.4×10^{10} cells. In terms of log 10 MPN, the range will be between 8.80 to 10.16.

The discrepancy might be due to the decrease of Rhizobium vigour (Wilson and Trang 1980). They reported cowpea rhizobia peat inoculum stored at high temperatures decreased in cell vigour. This resulted in few or no rhizobia detected by a plant count method while the plate count method could still detect Rhizobium. The discrepancy between the two counting methods did not occur at 25°C storage temperature. At 35°C storage temperature, plant count started to decline while the plate count remained unchanged. At 45°C storage temperature, plate count started to decline but plant count decreased rapidly. After 6 weeks of storage at 55°C, there was no detectable viable rhizobia as determined by plant infection counts, but plate count showed approximately 10^4 rhizobia per g even after 15 weeks. However, the storage temperatures used in our study were not as high as the ones used in Wilson and Trang's study. It is unlikely that the discrepancy between the plate and plant count will come from the loss of cell vigour.

The finding that all the stickers under study were equal in terms of sticking ability and prolonging the viability of the Rhizobium has practical value. Cheap and locally available stickers can be used by

local farmers instead of depending on the imported products. Jaggery (home made cane sugar), tapioca and rice starch are available in developing countries like India and Thailand. Guar gum is produced in India. All these stickers are easy to make and use. They are edible products and this eliminates the fear from poisonous effects. Vincent (1958) reported the incorporation of 10% sucrose considerably lessened the death rate of Rhizobium on inoculated seed. Vincent et al. (1962) also found that certain additives such as maltose and gum arabic offered some protection during drying and storage. Burton (1976) indicated that sucrose and maltose as well as some natural and synthetic gums decreased the death rate of rhizobia on seeds. Davidson and Reuszer (1978) studied the survival of Rhizobium japonicum on inoculated seeds using 12 different coating materials. The commercial coating materials of two companies (names unrevealed) resulted in a much larger initial population per seed, however, there was no distinct advantage over the control (seeds + inoculum) in terms of percentage of original inoculum surviving at later dates. None of the treatments induced a survival greater than 200,000 rhizobia per seed after a 3 week storage period.

Our results show a significant difference between the two strains used in their survival at 28 and 33°C when coated on seeds. This finding may have an agronomic importance. This is because chickpeas are generally sown in dry soil in late October when the temperature may still be high. Strains that tolerate high temperature will ensure that there will be enough Rhizobium to produce nodules on the plants. Philpotts (1977a, b) reported that commercially used Rhizobium trifolii strain TA1 was more susceptible to high temperatures than strain CC275e and cowpea Rhizobium strain CB-756.

The storage temperature had a significant effect on the Rhizobium survival. 4°C was found to be the best temperature for Rhizobium survival. The number of Rhizobium per seed remained unchanged during 1 week storage. When the temperature increased to 28 and 33°C, the number of Rhizobium per seed declined and continued to further decline with the number of days in storage. The death rate per day was found to be higher in the first 24 h of storage and later on became constant in both strains and counting technique. Death rate was always higher in 9036 than IC-59. The rapid loss of water during this period might be the reason for this rapid loss of viability (Vincent et al. 1962). Survival at 28 and 33°C after 7 days of storage was about 10^5 and 10^4 cells/seed respectively. This finding has agronomic importance. Inoculated seeds should be sown immediately or stored at low temperature (~ 4°C). Low storage (4°C) could prolong the Rhizobium survival without losing much viability even after 7 days. Storage at high temperature resulted in loss of viability in even just 24 h, however, the number which survived after 24 h is considered to be high and acceptable. Therefore, preinoculated seed stored at room temperature more than 24 h should be reinoculated before sowing.

Field Test

The three fields used in the experiments had different crop history as mentioned earlier. This results in different numbers of native Rhizobium population per g dry soil. Therefore, we had three competitive levels in the studies. The number of Rhizobium inoculated to the seed in various fields, as per our plate and plant count, ranged from 0 to 10^7 cells/seed. Lime pelleting or CaO_2 pelleting were found to be detrimental to the Rhizobium. Many workers had reported that lime pelleting prolonged the survival of applied root nodule bacteria (Brockwell 1962,

1963b, Brockwell and Phillips 1970, Radcliffe et al. 1967), enhanced nodulation and N_2 fixation in grain legumes (Iswaran and Jauhri 1969, Chhonkar et al. 1971) and helped the inoculum rhizobia to compete with native soil micro-organism (Wade et al. 1972). Our lime pelleting and CaO_2 pelleting were detrimental to the Rhizobium inoculum, probably because the lime was too alkaline. Different sources of lime behaved differently in prolonging the survival of inoculated Rhizobium. Cottrel dust and Gold hill lime proved to be detrimental to Rhizobium trifolii (Radcliffe et al. 1967). The lime that we used in this experiment was the lime that we used for liming the field. It might be expected that the lime may contain CaO or some toxic substances that may be detrimental to Rhizobium. CaO reacts with water in an exothermic reaction which may kill the Rhizobium. The pH of the lime used in our experiment was 12.1 which was considered to be very high compared to pure $CaCO_3$ which had the pH of 9.7.

CaO_2 has been claimed by the manufacturer (Interox International) to improve germination of certain seeds such as wheat, rice, sugarbeet and rye during germination in flooded conditions where O_2 is lacking. Increases in nodulation of soybeans in Japan has also been claimed by the manufacturer. The reaction of CaO_2 with water can be summarized in the following equations:

$$CaO_2 + 2H_2O \rightleftharpoons Ca(OH)_2 + H_2O_2$$

$$CaO_2 + OH^- + H_2O \rightleftharpoons Ca(OH)_2 + O_2H^-$$

$$CaO_2 + 2H^+ \rightleftharpoons Ca^{++} + H_2O_2$$

H_2O_2 derived from the reactions may be the cause of Rhizobium death. H_2O_2 has bacteriocidal and bacteriostatic properties. Some chemicals are known to be toxic to Rhizobium and are not recommended as agents for

pH adjustment of peat in the inoculum production (Roughley and Vincent 1967, Vincent 1970). The manufacturer claimed that nodulation of soybeans has been improved in Japan. This might be because they used CaO_2 as the granular form. However, we used CaO_2 in powder form and pelleted it to the inoculated seed coat. Due to the distance from the seed, granular form application might be beneficial because it might kill the native soil organisms and thus indirectly helped the inoculated Rhizobium. But CaO_2 pelleted to the seed was close to the inoculated Rhizobium and might kill all the inoculated Rhizobium.

Rhizobium survival on the inoculated seed planted in field A declined with time after planting. However, the number per seed of the lime pellet treatment increased from less than one cell/seed at the day of planting to 10^3 cells/seed at 3 days after planting. It is suspected that the native population clinging to the soil attached to the seed might be the reason for this increase in number. Our previous laboratory test showed that Rhizobium decreased with the number of days as the seeds were stored in 28 and 33°C, but not at 4°C. The soil temperature at the first 3 days after sowing ranged from 23°C during the nighttime to 33°C during the daytime. Except for the lime pelleting treatments, the survival of Rhizobium per seed was considered quite high. They ranged from 10^4 to 10^6 cells/seed which was within the 95% confidence limits.

There was no significant difference among treatments as measured by different parameters at 6 weeks after planting in field A. There was no response to inoculation over the control. The coefficient of variation of shoot weight was 24%; this depicted the heterogeneity of the field used in this experiment. The soil was patchy at one end of the replicate; the growth of this end was very poor. The coefficient of variation for

root weight, nodule number and nodule weight were 12, 42 and 88%. The coefficient of varitions for C_2H_4/plant/h and C_2H_4/g dry nodule/h were 102 and 69% respectively. The high coefficient of variation of the N_2-ase activity showed high variability between samples.

Except one parameter, i.e. shoot weight, all the parameters used in measuring the effect of different inoculation methods were not significantly different in field B. The soil in this field was uniform and this resulted in low coefficient of variations when compared to field A. The coefficient of variations were 10, 13, 30, 46, 70 and 33% for shoot weight, root weight, nodule number, nodule weight, C_2H_4 production/plant/h and C_2H_4 production/g dry nodule/h. IC-59-L treatment had higher shoot weight/plant (2.96 g) and significantly different from the uninoculated control. However, there was no significant difference among treatments at the later sampling date. Wade et al. (1972) noted that response to inoculation might occur at the early sampling date, but might not at the later ones.

Except CaO_2 pelleting treatment, there was a significant response to inoculation in terms of nodule number/plant, C_2H_4 production/plant/h and C_2H_4 production/g dry nodule/h in field C. As mentioned earlier, field C contained less than one Rhizobium/g dry soil. This might be the reason for the response to inoculation in terms of the previous mentioned parameters. CaO_2 pelleting resulted in killing of the inoculated Rhizobium and, therefore no nodule formation and no nitrogenase activity which was the same as the uninoculated control treatment. This agreed well with our plate and plant count methods that CaO_2 was detrimental to the Rhizobium.

N uptake of chickpeas grown in fields A and B were not significantly

different among treatments at 6 weeks and 10 weeks sampling. N-uptake in field A was found to be 75 and 148 mg/plant at 6 and 10 weeks after sowing. Fifty percent of N had been taken up at 6 weeks after sowing. In field B, N uptake was found to be 100 and 181 mg at 6 and 10 weeks after planting. Fifty-five percent of N was taken up at 6 weeks after sowing.

Seed and seed nitrogen yield among treatments in both fields were not significantly different. Seed inoculation by any of the stickers or methods did not increase seed yield and seed N yield. The coefficient of variation for seed and seed N yield were 23 and 24% in field A, 12 and 18% in field B, respectively. This reflected the heterogeneity in field A and uniformity in field B.

None of the fields under study contained Rhizobium resistant to str 200, hence all isolates resistant to str 200 were presumed to be our inoculum strain. Our data show that both inoculation methods, i.e. conventional slurry inoculation method with methyl cellulose sticker and liquid inoculation method were equally effective when virtually no chickpea Rhizobium population was present. As the soil Rhizobium population increased, the recovery of the inoculum strain declined and conventional slurry of the seed was less effective than liquid inoculation. The reason could be that liquid inoculation provided more Rhizobium per seed at the time of sowing (10^7 cells/seed cf 10^6 cells/seed). The liquid could also carry the inoculum away from the seed so that the developing radicle would pass through a zone of soil containing the inoculum, rather than picking up the rhizobia as the radicle broke through the testa. There was no rain after sowing so presumably Rhizobium moved along the root by swimming in the rhizosphere.

Rhizobium movement in the soil is limited by the soil moisture content (Kellerman and Fawcett 1907, Frazier and Fred 1922, Hamdi 1971, 1974, Worral and Roughley 1976). Movement of Rhizobium slowed with increasing water tension and ceased when water-filled pores became discontinuous (Hamdi 1971). Nodulation of legumes sown in partly dry soil could be restricted by the lack of migration of the seed inoculum strain or of naturally occurring rhizobia, at water tensions which could permit legume seeds to germinate. The Rhizobium inoculated onto the seed by the conventional slurry method might not move that much and thus could form only 36 and 1% of the nodules in fields A and B, respectively. The liquid inoculation method enabled the inoculated Rhizobium strain to move around the vicinity of the seed, become established and ready to compete for nodule formation. This was depicted by the fact that 80 and 12% of the nodules formed in fields A and B were from the inoculated strain respectively. The competitive ability of the inoculated strain by both inoculation methods was reduced as the number of native population increased. However, the percentage of the nodules formed by the inoculated strain in the liquid inoculation method was higher when compared to the conventional slurry method in both fields.

The superiority of the liquid inoculation method over the conventional seed slurry inoculation method in terms of nodule formation has also been demonstrated by others. Kapusta and Rouwenhorst (1973) showed that the recovery of applied Beltsville serogroup 138 from soybean nodules increased from 18 to 60% when 1.5×10^{10} cells/cm row were added in a liquid carrier. The recovery of Beltsville serogroup 62 was increased from 0 to 38% by the addition of 5×10^{8} cells/cm of row (Boonkerd et al. 1978). Hale (1978) reported that when clover seeds

inoculated by the conventional seed slurry method, less than 30% of the nodules formed at 6 weeks after planting contained the inoculated strain. When a liquid peat based inoculum was incorporated in the soil prior to sowing, there was a significant increase in the numbers of nodules containing the inoculated strains (80 to 90%).

Quite a substantial number of nodules were found to contain the inoculated strain by the liquid inoculation treatment in our experiments. Except for top weight/plant at 6 weeks after planting in field B, there was no response to inoculation as measured by other parameters. There was also no response to inoculation in terms of final yield of dry matter, seed and seed N yield. Response in yield due to liquid inoculation has been reported for groundnut (Schiffman and Alper 1968, Nambiar et al. personal communication) and clover (Hale 1978). The lack of response to inoculation in our experiments may be attributed to many factors. Firstly, the native population might be as efficient in N_2-fixation as the inoculum strain. Nodulation and nitrogenase activity in the two Vertisol soils (fields A and B) were not significantly affected by inoculation. Secondly, N_2-fixation in chickpeas is sensitive to high temperature. Nodulation and N_2-fixation at 30°C soil temperature was dependent on Rhizobium strains (Dart et al. 1975a, b, Islam 1975). Daily maximum soil temperature varied from 29 to 41°C at 5 cm depth in the 120 days after sowing and from 26 to 33°C at 15 cm depth (see Appendix 36). Thirdly, chickpea is usually grown in the residual moisture. No irrigation was applied during the growing season in both fields A and B. However, in field C, two irrigations were applied, i.e. before sowing and 35 days after planting. Water stress has been reported to reduce acetylene reduction in field grown soybeans (Mague and Burris

1972), Trifolium repens (Engin and Sprent 1973), Lupinus aboreous (Sprent 1973, Sprent and Silvester 1973) and Phaseolus vulgaris (Sprent 1975). Sprent (1972) reported that acetylene reducing activity of Vicia faba and Glycine max was depressed by drought and the activity could be restored by irrigation. Nodulation, nitrogen fixation and yields of chickpeas were found to be beneficial by irrigation (Rupela et al. personal communication). Chickpeas grown on residual moisture was found to be active in N_2 fixation in a very short period (ICRISAT annual report 1978/79). Under residual moisture condition, N_2 fixation as measured by acetylene reduction technique reached its peak about 7 weeks after planting, declined sharply and showed no activity at 10 weeks after planting.

Low level intrinsic antibiotic resistant character or "finger printing" technique showed that the natural rhizobia population were heterogeneous. One hundred twelve and 204 groups of Rhizobium were recorded in fields A and B respectively (zero mismatch). If one mismatch is allowed into grouping, the number of groups is reduced. However, the antibiotic concentration allowed for mismatching need not be the same. Benon and Josey (1980) grouped 264 Rhizobium phaseoli isolates from their experiments into 54 groups. Isolates having the same intrinsic resistance pattern, with few exceptions, were uniform in reaction with anti-serum raised against one of the inoculant strains and in their colony morphology.

The reason for allowing one mismatch for grouping was due to the fact that our standard control strain (9036) showed variability for resistance to a certain antibiotic concentration, i.e. Neomycin sulfate at 10 mg l^{-1}. Some strains of Rhizobium phaseoli were found to be

variable to resistance to certain antibiotics (Josey et al. 1979, Benon and Josey 1980) and stock culture of the inoculant strains were suggested to be included in every set of printing plates (Josey et al. 1979). We included strain 9036 in every set of printing plates as our standard control strain and noticed that isolates having the same pattern of intrinsic resistance as 9036 behaved the same as strain 9036 at a particular set. Potential sources of error using this technique have been discussed (Rupela et al. 1981). Except in one case, all the field isolates from field B that were resistant to str 200 were found to have the same low intrinsic antibiotic resistant pattern and colony morphology as the 9036 strain. We are not sure whether this exceptional isolate was from the native strains or a cross between a native and a marked strain.

CONCLUSION

Chickpea Rhizobium survival on inoculated seeds was studied by using both the plate and plant count techniques. Ten percent jaggery, 1.5% methyl cellulose, 1% guar gum, 5% tapioca starch and rice starch were found to be the same in terms of sticking ability and prolonging the survival. Therefore, the choice of using the sticker depends on locally available materials. Low storage (4°C) was found to be beneficial and high storage temperatures (28 and 33°C) were detrimental to the inoculated Rhizobium. Therefore, the inoculated seed should be stored at low temperature (4°C). Strain IC-59 was found to survive high temperatures better than strain 9036. This emphasizes the benefitial of a high temperature tolerant strain.

There was no response to inoculation treatments in the three field

conditions under test. Liquid inoculation was found to be superior to the convention inoculation method in terms of enhancing competitive ability of the inoculated strains in nodule formation. It has been discussed that there is a need for a better inoculum strain and a better method to apply liquid inoculum.

Low intrinsic antibiotic resistant patterns were found to be useful in showing that the native Rhizobium are heterogeneous. Except in one case, low level intrinsic antibiotic resistant patterns agree with a high resistant antibiotic resistant (str 200) character.

GENERAL DISCUSSION AND CONCLUSIONS

Small seeded legumes are usually used in Rhizobium MPN counts using the plant infection method because they can be grown aseptically in test tubes. Tumblers (Wilson 1926), modified Leornard's jar assembly (Thompson and Vincent 1967), modified Gibson's seedling tubes (Vincent 1970), assembly developed by Elliot and Blaylock (1971) and growth pouches (Weaver and Frederick 1972) have been used to grow large seeded legumes. However, these techniques are not completely sterile because the plant shoots are exposed to the unsterilized environments and may result in cross contamination. Elliot and Blaylock (1971) reported that a modified Leornard's jar assembly was useless in the dust storm conditions in central Washington State, U.S.A., and this led them to develop another assembly.

Dwarfing the seedling developed from a germinating chickpea by cutting off the cotyledons, enables the seedling to be grown under axenic conditions in a test tube. This axenic culture technique is also suitable for other large seeded legumes, e.g. groundnuts. In chickpeas, many cultivars have been shown to be usable for MPN counts (see Manuscript 1). Therefore, a variety or cultivar can be chosen. The finding that some cultivars gave poor MPN counts indicated that there was a strain x cultivar interaction in chickpeas. Corbin et al (1977) reported there was no host-strain specificity within 29 chickpea cultivars tested in Australia. However, their Rhizobium strains and cultivars

used were different from ours. Not all of the cotyledons need be removed. A quarter of the cotyledons can be left without affecting nodulation and the reliability of the count. Moreover, this technique does not require an expensive rooting medium. Sand was found to be the best rooting medium in our studies.

The root excision technique produced nodules on excised root of Phaseolus vulgaris L. (Raggio and Raggio 1956) and Phaseolus aureus (Yoshida and Yatazawa 1978) provided that the essential organic compounds were supplied in the agar block attached to the cut end of the root. Nodulation in excised Phaseolus vulgaris L. root could be improved if the hypocotyl was left attached to the root (Bunting and Horrock 1964). Sucrose, mesoinositol, indole butyric acid and other organic compounds were found to be essential for nodulation (Raggio et al 1959, Barrios and Raggio 1964, Cartwright 1967, Yoshida and Yatazawa 1978). This technique of root excision of Raggio and Raggio (1956) is very suitable for studying the effect of different compounds on nodulation. However, it cannot be used in ecological studies because high numbers of plants are required in the MPN plant dilution infection technique.

Our technique of dwarfing the germinating seed by cotyledon excision cuts down the food supply to the seedling and thus prevents luxuriant growth. This enables a large seeded legume to be grown under test tube conditions. Hormones and essential compounds required in nodule forming must be sufficient because the whole seedling and some parts of the cotyledons are intact. However, the cotyledons should be left no more than one-quarter seed in chickpeas. Seedlings developed from the whole seed do not nodulate well in the test tube. This might

be due to the effect of the abundant nitrogenous compounds on other substances in the seed that inhibit nodule formation in test tube conditions. Dadarwal and Sen (1969) reported the chickpea Rhizobium survived better on the soaked than the unsoaked chickpea seed. A toxic substance in the seed coat of some legumes was found to be toxic to Rhizobium (Thompson 1960). Gottfred (1981) reported toxic phenolic compounds like tannins found in sainfoin seed (Onobrychis viciifolia L.) inhibit the growth of Rhizobium; the effect is more pronounced in hulled than dehulled seed. Whether chickpea seed contains toxic substances inhibitory to its Rhizobium is another area worthy of study.

Seedlings could be developed from dry embryo excision in groundnut (Nambiar et al, personal communication) but the percent survival and the repeatability was low. The excised embryo was grown in one-quarter strength Jensen's seedling agar (Vincent 1970). No addition of sugars or hormones was added in the medium in the studies. This might be the reason for this failure. Another possibility of getting a seedling is by the conventional tissue culture. This is another area of study that might be beneficial to both microbiological and plant breeding studies.

The method of Fisher and Yates (1963) was used to estimate number of Rhizobium in the sample. The use of the term MPN is not strictly correct (Thompson and Vincent 1967). Only the number of positive and negative tubes (regardless of the dilution) are required in the calculation. The method of Brockwell et al (1975) requires a series of positive tubes to calculate an MPN count and this poses problems especially when tubes with no nodulation appear out of sequence ("skips"). Skips

usually occur at low dilutions when the numbers of Rhizobium are low and competition from other micro-organisms is high. Skips were dealt with by the method of Thompson and Vincent (1967, see Vincent 1970). Brockwell et al's table (1975) does not deal with all the potential positive tube combinations. Fisher and Yate's method handles this problem.

We have shown that the dwarfed seedling method can be used for counting the number of Cicer Rhizobium in pure contaminated cultures, and soils. This implies that the dwarfed seedling method can be used as the "trap host" for enumerating the number of Rhizobium in the soil samples taken from the fields. This will enable us to understand more about the Rhizobium ecology, e.g. population variation with soil types, depth, cropping history and seasons. It can be used to count the background Rhizobium population and thus enables us to predict or explain the performance of an inoculated strain under field conditions. This technique has also been adopted as a routine technique for Cicer Rhizobium identification at ICRISAT. Moreover, this technique proved to be very useful in counting Cicer Rhizobium in peat inoculants and thus is a useful tool in inoculum quality control. Plate counting of unsterilized or contaminated inoculants is very difficult because of the problem of distinguishing between Rhizobium and soil bacteria. Rhizobium are identified as colonies that do not adsorb the congo red incorporated in the medium. However, this could be misleading because some other bacteria also possess this ability. For example, the plate counts of Indian peat inoculants gave a high population of Rhizobium. However, the plant counts showed that they were not chickpea Rhizobium. The commercial peat inoculants from Rallis Nitrofix, H-45 and Nitrobact

company had low plant counts indicating that they were very poor inoculants.

The level of contaminants in the Indian inoculants range from 10^7 to 10^9. This is very high and not acceptable by Australian sterilized peat inoculum standards which state that the inoculum should contain at least 10^9 Rhizobium cells/g peat and the level of contaminants should be less than 10^6 cells/g peat (Thompson 1980). Only few of the Indian samples will pass Australian nonsterilized peat inoculum standards which state that the inoculum should contain 10^7 to 10^8 Rhizobium/g peat. It is, therefore, apparent that the quality of chickpea inoculants produced in India needs be improved and controlled before it reaches farmers. The technique of using dwarfed chickpea as the 'trap' host for counting chickpea Rhizobium is considered to be a breakthrough in chickpea inoculant quality control and may help upgrade the quality of inoculants produced.

We used this technique to study the population of chickpea Rhizobium in different soils, locations, seasons, and depths. In general, the soils that never had a chickpea history did not have or had very few chickpea Rhizobium/g soil. The populations varied with depths and seasons. The rhizosphere of five crops, i.e. chickpeas, pigeon pea, groundnut, sorghum, and pearl millet were found to be stimulatory to chickpea Rhizobium in the two soils used in the studies. Chickpea rhizosphere had the highest stimulatory effect when compared to the other crops. Chickpea Rhizobium population was found to be highest when soil samples were taken over the plant at the end of the growing season, and the numbers reduced when the sample was taken far away from the plant. This implies that soil sampling technique is very

important in chickpea Rhizobium population studies. The failure to detect the differences between the fields that have chickpeas and cereals at ICRISAT centre in the summer of 1980 was due to the fact that samples were taken far away from the plant. Therefore, the recommendation for soil sampling is that samples should be taken from both the rhizosphere and nonrhizosphere, bulked and processed for counting later. The instrument used in sampling at different depths is another aspect that needs to be improved. The instrument should result in the least soil disturbance as possible. It was noticed that when soil samples were taken by using a Viermeyer tube and a hand hammering, more soil disturbance in the first 5 cm resulted. Less soil disturbance at the 5 cm was observed when samples were taken by a 6 cm diameter gidding hydraulic coring machine mounted on the bumper of a landrover. This might be the reason why the populations at the first 5 cm were as high as 5 to 15 cm in depth. The dilution factor of 10, six dilution steps and three replicate tubes/dilution were used in the studies. This might be another reason why we did not get an accurate count. Increasing the number of replicate tubes/dilution or lowering the dilution factors results in more accurate counting of the Rhizobium. However, it has to be borne in mind that either increasing replicate tubes/dilution or lowering the dilution factors requires more space, materials and time.

The effect of different stickers on the survival of chickpea Rhizobium on the inoculated seeds was studied, using both plate and plant count. It was found that all of the stickers under studies were not different in terms of prolonging Rhizobium survival. This implies that locally available stickers can be used to inoculate chickpea seeds instead of depending on imported materials. Seed inoculation by using

different stickers or methods did not increase seed yield when compared to the control. This might be due to the fact that the indigeneous Rhizobium populations were as effective as the inoculated strains. The finding that our lime and CaO_2 pelleting were detrimental to the Rhizobium indicated that other alternatives to pelleting require studying. Plastic coating is suggested but may be expensive. Rhizobium embedded in polyacrylamide gel had been used successfully (Dommergue et al 1978). The recovery of the inoculated strain was found to be higher in the liquid inoculation treatment than the conventional seed slurry method in all the fields under study. Therefore, a more effective strain of inoculum and an efficient inoculation method are required. Liquid inoculation seems to be the answer. However, our method of liquid application was not an efficient one. It required a lot of labour and was not practicable. A machine used for liquid inoculum application has been developed (Schiffmann and Alper 1968, Brockwell and Gault 1978, Brockwell et al 1978, 1980). However, these heavy machines will not be suitable for the poor SAT (semi-arid tropic) farmers. A low cost draught animal drawn implement is required in this part of the world. This is another area that needs studying.

The technique of identification of the Rhizobium using a high antibiotic resistant marker (str 200) and a lower intrinsic antibiotic resistant characteristics proved to be useful. A low intrinsic antibiotic characteristic could differentiate the indigeneous Rhizobium population and at the same time it could confirm the low intrinsic antibiotic resistant pattern of the str 200 resistant strain. Except in one case, all the field isolates from field B that were resistant to str 200 were found to have the same low intrinsic antibiotic

resistant pattern as the parent strain. We are not sure whether this exceptional isolate was from the native strain or a cross between a native and a marked strain.

Regarding the application of low intrinsic antibiotic resistant characteristics in identifying a strain of Rhizobium, it is absolutely essential that every effort is made to maintain rigid control of the experiment conditions. The potential sources of error of this technique are as follows:

1. Medium composition. The concentration of all constituents of the growth medium must be constant and should always be the same grade from the same supplier. Many factors can influence the ability of bacteria to be resistant to any given antibiotic, e.g. the number of ions available in one make of yeast extract may be widely different from those in another brand and consequently it is possible that the growth of a given strain on some antibiotic, will be quite different if two different brands of yeast extract are used on two different occasions.

2. Medium sterilization and melting. Growth medium should always be sterilized and melted in the same way. If the medium is heated for different periods of time or at different temperatures, its composition may also vary, and hence affect the growth as described above.

3. Antibiotics. The same supplier should be used as the strength and formulation of some antibiotics may vary between manufacturers. The potency of nearly all antibiotics will decrease with age particularly when made up into stock solution, so excessive amounts of stock solution should not be prepared. Repeated freezing and thawing of antibiotics should be avoided as much as possible.

4. Mixing of antibiotics in media. When making up antibiotic

plates, agar temperature should be reasonably constant (e.g. on every occasion). Once the plates are poured some antibiotics will start to lose activity, so that plates should be inoculated as soon as the whole set of plates is prepared. Conversely, if the agar is cooled during mixing ($\sim$ 45°C) an even distribution of antibiotic throughout the medium may not be achieved and strict comparisons between plates is not possible. It is very important that the antibiotic concentrations in the medium are accurately reproduced on each occasion.

5. Inoculum condition. Sensitivity to some antibiotics (such as penicillin) may depend on cell growth phase, so it is very desirable to use inocula of a reasonably uniform stage of growth. Inoculations of cells in the stationary phase may result in survival to an exposure of an antibiotic to which they are normally considered sensitive and then commence to grow after the concentration of an antibiotic in the medium has dropped below some threshold value for activity against that particular strain.

6. Thickness of plate. If the thickness of a medium in the plates varies much, then the colony morphology will be influenced, for example slime may only be produced by large colonies and so it may be difficult to assess the difference between control and antibiotic plates, if they are of different thicknesses. Plates with bubbles should also be discarded because of lack of homogeneity.

7. Drying of plate. As plates must be used immediately, drying may not be practical such plates must be stored inverted to avoid cross contamination between isolates, and the same procedure followed on every occasion. If plates are dried this should always be treated the same way each time.

8. Contamination. Contaminated plates should be regarded with suspicion as waste products may be synergistic with, or destroy, the antibiotics and these effects may permeate the whole plate, not just the corner where the contaminant is growing.

9. Fast and slow growing strains should be tested on separate plates so that the fast growers do not out-compete with the slower strains for nutrients.

It had also been noticed that some of the concentrations of the antibiotics used in this experiment could not distinguish between Rhizobium strains. It is recommended such a concentration be eliminated in future studies.

In conclusion, the technique of using intrinsic antibiotic resistance as a means of identifying strains of rhizobia in field trials can be successfully used to examine some of the problems relating to the behaviour of natural population of Rhizobium, and also of introduced strains. Although basically simple, the success of the technique depends very largely on the accuracy with which the procedures are carried out.

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APPENDIX

APPENDIX 1. Composition of modified long Ashton solution as N-free nutrient solution (Summerfield et al 1977).

	g/l
$MgSO_4 \cdot 7H_2O$	0.233
K_2HPO_4	0.175
NaFe EDTA	0.0408
K_2SO_4	0.277
*Trace elements	1 ml/l
Tap water	1 l
Hcl to adjust pH to 6.8	
COMPOSITION OF TRACE ELEMENTS	
$MnCl_2 \cdot 4H_2O$	1.81
$CuSO_4 \cdot 5H_2O$	0.08
$ZnSO_4 \cdot 7H_2O$	0.22
H_3BO_3	2.86
$Na_2MoO_4 \cdot 2H_2O$	0.02
$CoSO_4 \cdot 7H_2O$	0.286
Distilled water	1 l

APPENDIX 2. The effect of root medium and cotyledon excision on estimation of chickpea Rhizobium in broth cultures (strain IC-2046 and IC-128)* by the plant-infection dilution method.

Medium and treatment	IC-2046		MPN**	IC-128		MPN**
	Plate count	Total+ tube		Plate count	Total+ tube	
Unwashed vermiculite:sand (1:1)						
Whole seed	9.66	0/18	4.24	9.94	0/18	4.24
Excised cotyledon	9.66	1/18	4.59	9.94	1/18	4.59
Washed vermiculite:sand (1:1)						
Whole seed	9.66	0.18	4.24	9.94	0/18	4.24
Excised cotyledon	9.66	14/18	8.94	9.94	12/18	8.24
Agar medium						
Whole seed	9.66	0/18	4.24	9.94	0/18	4.24
Excised cotyledon	9.66	3/18	5.24	9.94	11/18	7.94

*Grown for 7 days in yeast extract mannitol broth, ten-fold diluted. Dilution 10^5–10^{10} were used to inoculate the plants (3 tubes/dilution).

**The factor for the 95% confidence interval on the MPN is $\pm$ 0.68.

APPENDIX 3. MPN groundnut *Rhizobium*[a] estimated by dilution-plant infection technique using plants with cotyledons excised growing in sand:vermiculite mixture (2:1), and compared with plate count method.

Weeks after inoculation	Total positive tubes	Total negative tubes	Log 10 no. of plate count	*Rhizobium*/ml broth plant count[b]
5	19	2	7.10	7.59
6	19	2	7.10	7.59

[a]Cowpea group *Rhizobium* strain CB-756 was grown in an agar slant for 7 days. 3 ml of sterilized tap water was added to the slant, shaken; ten-fold diluted to make a serial ten-fold dilution series. Each dilution was inoculated to 6 tubes (1 ml/tube).

[b]MPN calculated from the last 6 dilution steps, 3 replicate tubes per dilution. 95% confidence interval for MPN is $\pm$ 0.68.

APPENDIX 4. Analysis of variance of soil Rhizobium populations surveyed in different fields in 1979/80.

Source of variation	D.F.	S.S.	M.S.	F-ratio	Level of significance
1) Field a (Fine Mixed Hyperthermic Deep Aquic Ustorthent ?) Never grown chickpea					
Replication	5	1.2990	0.2598	0.22	N.S.
Depth	2	3.0780	1.5390	1.29	N.S.
Error	10	11.8960	1.1896		$S\bar{x}$ = 0.45
Total	17	16.2730			c.v. = 164%
2) Field b (Fine Mixed Hyperthermic Deep Aquic Ustorthent ?) Chickpea grown 2 years ago					
Replication	8	42.8720	5.3590	6.69	1%
Depth	2	3.2930	1.6465	2.06	N.S.
Error	16	12.8180	0.8011		$S\bar{x}$ = 0.30
Total	26	58.9830			c.v. = 39.4%
3) Field c (Alfisol - chickpea was grown in preceding season)					
Replication	6	7.6500	1.2750	3.62	5%
Depth	2	1.1200	0.5600	1.59	N.S.
Error	12	4.2280	0.3523		$S\bar{x}$ = 0.22
Total	20	12.9980			c.v. = 12.7%
4) Field d (Alfisol - chickpea was never grown)					
Replication	6	0.9630	0.1605	4.83	5%
Depth	2	7.7380	3.8690	29.05	1%
Error	12	1.5980	0.1332		$S\bar{x}$ = 0.14
Total	20	10.2990			c.v. = 27%
5) Field e (Vertisol - chickpea several times grown)					
Replication	12	6.0090	0.5008	1.96	N.S.
Depth	2	0.2670	0.1335	0.52	N.S.
Error	24	6.1330	0.2555		$S\bar{x}$ = 0.14
Total	38	12.4080			c.v. = 14.7%

APPENDIX 5. Analysis of variance of Rhizobium population surveyed in different fields in 1978/79 (pooled data over depth).

Source of variation	D.F.	S.S.	M.S.	F-ratio	Level of significance
Field	4	73.7801	18.4450	34.79	1%
Error	37	19.6151	0.5301	$S\bar{x}$ = 0.58	
Total	41	93.3952		c.v. = 27.5%	

APPENDIX 6. Analysis of variance for MPN chickpea Rhizobium in a Vertisol soil sampled at different times of the year.

Source of variation	D.F.	S.S.	M.S.	F-ratio	Level of significance
1) November 1978					
Replicates	12	6.0090	0.5008	1.96	N.S.
Depths	2	0.2670	0.1335	0.52	N.S.
Error	24	6.1330	0.2555		$S\bar{x}$ = 0.14
Total	38	12.4080			c.v. = 14.7%
2) March 1979					
Replicates	7	11.3120	1.6160	4.60	1%
Depths	5	72.3380	14.4676	41.17	1%
Error	35	12.2990	0.3514		$S\bar{x}$ = 0.21
Total	47	95.9480			c.v. = 16.3%
3) June 1979					
Replicates	7	7.9880	1.1411	2.66	5%
Depths	5	16.8030	3.3606	7.83	1%
Error	35	15.0210	0.4292		$S\bar{x}$ = 0.23
Total	47	39.8120			c.v. = 20.6%
4) August 1979					
Replicates	7	5.6790	0.8113	4.10	1%
Depths	5	11.1810	2.2362	11.31	1%
Error	35	6.9220	0.1978		$S\bar{x}$ = 0.16
Total	47				c.v. = 11.7%
5) December 1979					
Replicates	7	8.7460	1.2494	2.72	5%
Depths	5	20.1720	4.0344	8.80	1%
Error	35	16.0540	0.4587		$S\bar{x}$ = 0.24
Total	47	44.9720			c.v. = 20.2%

APPENDIX 7. Analysis of variance for MPN chickpea Rhizobium in an Alfisol soil sampled at different times of the year.

Source of variation	D.F.	S.S.	M.S.	F-ratio	Level of significance
1) January 1979					
Replicates	6	7.6500	1.2750	2.84	N.S.
Depths	2	1.1200	0.5600	1.59	N.S.
Error	12	4.2280	0.3523		$S\bar{x}$ = 0.22
Total	20	12.9980			c.v. = 12.7%
2) March 1979					
Replicates	6	16.7890	2.7982	7.79	1%
Depths	3	6.7410	2.2470	6.26	1%
Error	18	6.4650	0.3592		$S\bar{x}$ = 0.23
Total	27	29.9950			c.v. = 14.0%
3) June 1979					
Replicates	6	9.7160	1.6193	7.51	1%
Depths	3	9.6710	3.2236	14.96	1%
Error	18	3.8800	0.2156		$S\bar{x}$ = 0.18
Total	27	23.2670			c.v. = 11.8%
4) August 1979					
Replicates	6	15.9390	2.6565	12.82	1%
Depths	3	11.1620	3.7207	17.96	1%
Error	18	3.7290	0.2072		$S\bar{x}$ = 0.17
Total	27	30.8300			c.v. = 11.1%
5) December 1979					
Replicates	6	12.7300	2.1217	9.01	1%
Depths	3	6.5150	2.1717	9.23	1%
Error	18	4.2370	0.2354		$S\bar{x}$ = 0.18
Total	27	23.4820			c.v. = 12.5%

APPENDIX 8. Analysis of variance for MPN chickpea Rhizobium in a Fine Mixed Hyperthermic Deep Aquic Ustorthent (?) soil sampled at different times of the year.

Source of variation	D.F.	S.S.	M.S.	F-ratio	Level of significance
1) January 1979					
Replicates	5	1.2990	0.2598	0.22	N.S.
Depths	2	3.0780	1.5390	1.29	N.S.
Error	10	11.8960	1.1896		$S\bar{x}$ = 0.45
Total	17	16.2730			c.v. = 163.9%
2) March 1979					
Replicates	5	28.2160	5.6432	13.63	1%
Depths	3	3.3160	1.1053	2.67	N.S.
Error	15	6.2080	0.4139		$S\bar{x}$ = 0.26
Total	23	37.7410			c.v. = 17.6%
3) June 1979					
Replicates	5	12.2780	2.4556	5.38	1%
Depths	3	14.9210	4.9737	10.90	1%
Error	15	6.8440	0.4563		$S\bar{x}$ = 0.28
Total	23	34.0430			c.v. = 40.7%
4) December 1979					
Replicates	5	6.5630	1.3126	7.75	1%
Depths	3	21.9550	7.3183	43.23	1%
Error	15	2.5400	0.1693		$S\bar{x}$ = 0.17
Total	23	31.0580			c.v. = 20.9%

APPENDIX 9. Analysis of variance of seasonal variation of chickpea rhizobia in three fields.

Source of variation	D.F.	S.S.	M.S.	F-ratio	Level of significance
1) B_8 A field 'e'					
Sampling	4	8.0447	2.0112	11.78	1%
Error	40	6.8306	0.1708		$S\bar{x}$ = 0.31
Total	44	14.8752			c.v. = 10.6%
2) R_2 field 'd'					
Replicates	6	10.7750	1.7958	17.61	1%
Sampling times	4	2.1860	0.5465	5.36	1%
Error	24	2.4480	0.1020		$S\bar{x}$ = 0.12
Total	34	15.4090			c.v. = 7.3%
3) Paddy field 'a'					
Replicates	5	3.1920	0.6384	0.85	N.S.
Sampling times	4	43.5350	10.8838	14.50	1%
Error	20	15.0120	0.7506		$S\bar{x}$ = 0.35
Total	29	61.7390			c.v. = 39.8%

APPENDIX 10. Analysis of variance of parameters used in chickpea Rhizobium population survey in summer 1980.

Source of variation	D.F.	S.S.	M.S.	F-ratio	Level of significance
1) MPN					
Replicates	2	2.4000	1.2000	3.03	N.S.
Fields	21	130.9930	6.2378	15.73	1%
Error	42	16.6580	0.3966		$S\bar{x}$ = 0.36
Total	65	150.0510			c.v. = 20.7%
2) pH					
Replicates	2	0.0050	0.0025	0.3425	N.S.
Fields	21	39.0790	1.8609	254.92	1%
Error	42	0.3050	0.0073		$S\bar{x}$ = 0.05
Total	65	39.3890			c.v. = 1.1%
3) E.C.					
Replicates	2	0.0000	0.0000		
Fields	21	0.2010	0.0100	18.98	1%
Error	42	0.0210	0.0010		$S\bar{x}$ = 0.02
Total	65	0.2220			c.v. = 10.9%
4) Moisture content					
Replicates	2	11.2130	5.6065	0.88	N.S.
Fields	21	723.1750	34.4369	5.43	1%
Error	42	266.4170	6.3453		$S\bar{x}$ = 1.45
Total	65	1,000.8050			c.v. = 26.5%

APPENDIX 11. Analysis of variance of chickpea Rhizobium population per gram dry root, rhizosphere and non-rhizosphere soils. The plants were grown in red soil in pots and harvested when they were 6 weeks old.

Source of variation	D.F.	S.S.	M.S.	F-ratio	Level of significance
1) MPN per gram dry root					
Replicates	2	1.7578	0.8789	3.06	N.S.
Crops	4	12.2651	3.0663	10.66	1%
Error	8	2.3010	0.2876		$S\bar{x}$ = 0.31
Total	14	16.3239			c.v. = 9.4%
2) MPN per gram rhizosphere soil					
Replicates	2	2.1381	1.0691	3.06	N.S.
Crops	4	10.9741	2.7435	7.85	1%
Error	8	2.7940	0.3493		$S\bar{x}$ = 0.34
Total	14	15.9062			c.v. = 14.7%
3) MPN per gram non-rhizosphere soil					
Replicates	2	1.1046	0.5523	2.04	N.S.
Crops	5	3.7999	0.7560	2.80	N.S.
Error	10	2.7025	0.2703		$S\bar{x}$ = 0.30
Total	17	7.6070			c.v. = 17.2%

APPENDIX 12. Analysis of variance of chickpea Rhizobium population per gram dry root, rhizosphere and non-rhizosphere soil. The plants were grown in black soil in pots and harvested when they were 6 weeks old.

Source of variation	D.F.	S.S.	M.S.	F-ratio	Level of significance
1) MPN per gram dry root					
Replicates	2	0.1063	0.0532	0.51	N.S.
Crops	4	6.5523	1.6381	15.74	1%
Error	8	0.8330	0.1041		$S\bar{x}$ = 0.19
Total	14	7.4915			c.v. = 5.5%
2) MPN per gram rhizosphere soil					
Replicates	2	0.2892	0.1446	1.69	N.S.
Crops	4	5.3369	1.3342	15.57	1%
Error	8	0.6855	0.0857		$S\bar{x}$ = 0.17
Total	14	6.3116			c.v. = 6.3%
3) MPN per gram non-rhizosphere soil					
Replicates	2	0.8807	0.4404	2.75	N.S.
Crops	5	1.2291	0.2458	1.53	N.S.
Error	10	1.6038	0.1604		$S\bar{x}$ = 0.23
Total	17	3.7136			c.v. = 12.4%

APPENDIX 13. The formulae used to calculate the number of chickpea *Rhizobium* per gram dry root or dry soil.

a) MPN/g dry root $= \frac{\text{MPN estimated from the sample x 180}}{\text{Dry root weight}}$

b) MPN/g rhizosphere soil $= \frac{\text{MPN estimated from the sample x 180}}{\text{Dry rhizosphere soil}}$

c) MPN/g non-rhizosphere soil $= \frac{\text{MPN estimated from the sample x 180 x 100}}{\text{40 x Percentage dry soil}}$

APPENDIX 14. Analysis of variance of shoot weight, root weight, nodule number and nodule weight of the crops grown in pots containing a Vertisol soil.

Source of variation	D.F.	M.S.	S.S.	F-ratio	Level of significance
1) Shoot weight/pot					
Replication	2	3.6394	1.8197	4.97	5%
Treatment	4	47.8246	11.9562	32.67	1%
Error	8	2.9283	0.3660		$S\bar{x}$ = 0.35
Total	14	54.3923			c.v. = 10.8%
2) Root weight/pot					
Replication	2	0.0244	0.0122	0.13	N.S.
Treatment	4	11.4244	2.8561	30.13	1%
Error	8	0.7587	0.0948		$S\bar{x}$ = 0.18
Total	14	12.2075			c.v. = 28.0%
3) Nodule number/pot					
Replication	2	7,982.8889	3,991.4445	9.22	5%
Treatment	2	4,078.2222	2,039.1111	4.71	N.S.
Error	4	1,731.1111	432.7778		$S\bar{x}$ = 12.01
Total	8	13,792.2222			c.v. = 14.0%
4) Nodule weight/pot					
Replication	2	6,688.8889	3,344.4444	4.04	N.S.
Treatment	2	6,688.8889	3,344.4444	4.04	N.S.
Error	4	3,311.1111	827.7778		$S\bar{x}$ = 16.61
Total	8	16,688.8889			c.v. = 31.6%

APPENDIX 15. Analysis of variance of shoot weight, root weight, nodule number and nodule weight of the crops grown in pots containing an Alfisol soil.

Source of variation	D.F.	S.S.	M.S.	F-ratio	Level of significance
1) Shoot weight/pot					
Replication	2	0.4044	0.2022	1.53	N.S.
Treatment	4	17.6236	4.4059	33.84	1%
Error	8	1.0573	0.1322		$S\bar{x}$ = 0.21
Total	14	19.0854			c.v. = 14.1%
2) Root weight/pot					
Replication	2	0.0099	0.0049	0.59	N.S.
Treatment	4	1.4635	0.3659	44.08	1%
Error	8	0.0662	0.0083		$S\bar{x}$ = 0.05
Total	14	1.5396			c.v. = 14.9%
3) Nodule number/pot					
Replication	2	372.2222	186.1111	0.46	N.S.
Treatment	2	461.5556	230.7778	0.57	N.S.
Error	4	1,630.4444	407.6111		$S\bar{x}$ = 11.66
Total	8	2,464.2222			c.v. = 18.5%
4) Nodule weight/pot					
Replication	2	2,466.6667	1,233.3333	0.52	
Treatment	2	20,466.6667	10,233.3333	4.32	N.S.
Error	4	9,466.6667	2,366.6667		$S\bar{x}$ = 28.09
Total	8	32,400.0000			c.v. = 35.6%

APPENDIX 16. Top weight, root weight, nodule number and nodule weight of off-season chickpea grown in a paddy field and receiving 2 or 10 irrigations. The plants were sampled at 6 weeks after sowing.

	10 Times irrigation	2 Times irrigation
Top weight (g/plant)	1.32	N.D.
Root weight (g/plant)	0.12	N.D.
Nodule number/plant	12.00	0
Nodule weight (mg/plant)	84.80	0

N.D. = Not Determined

APPENDIX 17. The numbers of Rhizobium added per g soil after chick-pea crop. The numbers are estimated from nodule and root mass.

Parameters	Paddy	Vertisol
No. of nodules/plant	3	34
No. of Rhizobium/nodule	10^7	10^7
Plant population/ha	1.1×10^5	1.1×10^5
No. of Rhizobium added by nodules	3.3×10^{12}	3.74×10^{13}
Root weight/plant (g)	.0984	0.139
No. of Rhizobium added/g root	10^7	10^7
No. of Rhizobium added by root	1.08×10^{11}	1.5×10^{11}
Total Rhizobium added/ha	3.4×10^{12}	3.75×10^{13}
One hectare furrow slice weight (kg)	2.5×10^6	-
One foot hectare slice weight (kg)	-	3.97×10^6
No. of Rhizobium added per g soil	1.36×10^3	9.45×10^3

APPENDIX 18. Rhizobium survival on inoculated chickpea seed at different days. The data expressed as log 10 Rhizobium/seed.

Storage day	Strain			
	9036	Death rate/day	IC-59	Death rate/day
A) Plate count				
0	6.43		6.37	
1	6.16	0.32	6.18	0.19
3	5.84	0.16	6.01	0.09
7	5.22	0.16	5.64	0.09
B) Plant count				
0	6.49		6.59	
1	6.03	0.46	6.15	0.45
3	5.73	0.15	5.92	0.12
7	5.32	0.10	5.71	0.05

APPENDIX 19. Rhizobium survival on inoculated chickpea seed at different temperatures. The data expressed as log 10 Rhizobium/seed.

Storage temperature (°C)	Strain			
	9036	Death rate/°C	IC-59	Death rate/°C
A) Plate count				
4	6.45		6.36	
28	5.86	0.02	6.04	0.01
33	5.48	0.08	5.75	0.06
B) Plant count				
4	6.36		6.45	
28	5.83	0.02	6.06	0.02
33	5.48	0.07	5.76	0.06

APPENDIX 20. Analysis of variance of different parameters used in measuring N_2 fixation of 6 week old chickpeas grown in field A.

Source of variation	D.F.	S.S.	M.S.	F-ratio	Level of significance
1) Shoot weight (g/plant)					
Replicate	4	2.3920	0.5980	2.78	5%
Treatment	12	2.8880	0.2410	1.12	N.S.
Error	48	10.3000	0.2150		$S\bar{x}$ = 0.21
Total	64	15.5790			c.v. = 24.3%
2) Root weight (g/plant)					
Replicate	4	0.0020	0.0005	2.50	N.S.
Treatment	12	0.0030	0.0003	1.50	N.S.
Error	48	0.0110	0.0002		$S\bar{x}$ = 0.07
Total	64	0.0170			c.v. = 12.2%
3) Nodule number per plant					
Replicate	4	89.4160	22.3540	1.02	N.S.
Treatment	12	421.0550	35.0879	1.61	N.S.
Error	48	1,048.5860	21.8455		$S\bar{x}$ = 2.09
Total	64	1,559.0570			c.v. = 42.0%
4) Nodule weight (g/plant)					
Replicate	4	0.0050	0.0013	4.33	1%
Treatment	12	0.0030	0.0003	1.00	N.S.
Error	48	0.0140	0.0003		$S\bar{x}$ = 0.01
Total	64	0.0220			c.v. = 87.9%
5) µmoles C_2H_4/plant/hr					
Replicate	4	10.5980	2.6495	3.63	5%
Treatment	12	6.9110	0.5759	0.79	N.S.
Error	48	35.0280	0.7298		$S\bar{x}$ = 0.38
Total	64	52.5370			c.v. = 101.8%
6) µmoles C_2H_4/g nodule/hr					
Replicate	4	14,876.2780	3,719.0695	6.09	1%
Treatment	12	4,396.2060	366.3505	0.60	N.S.
Error	48	29,325.2750	610.9432		$S\bar{x}$ = 11.05
Total	64	48,597.7590			c.v. = 68.7%
7) N content (mg/plant)					
Replicate	4	7,489.7010	1,872.4253	3.75	1%
Treatment	12	5,298.4400	441.5367	0.88	N.S.
Error	48	23,985.2610	499.6929		$S\bar{x}$ = 10.00
Total	64	36,773.4020			c.v. = 29.8%

APPENDIX 21. Analysis of variance of different parameters used in measuring N_2 fixation of 6 week old chickpeas grown in field B.

Source of variation	D.F.	S.S.	M.S.	F-ratio	Level of significance
1) Shoot weight (g/plant)					
Replicate	3	0.4490	0.1497	2.07	N.S.
Treatment	4	0.9660	0.2417	3.34	5%
Error	12	0.8690	0.0724		$S\bar{x}$ = 0.13
Total	19	2.2840			c.v. = 10.5%
2) Root weight (g/plant)					
Replicate	3	0.0010	0.0003	1.00	N.S.
Treatment	4	0.0020	0.0005	1.67	N.S.
Error	12	0.0040	0.0003		$S\bar{x}$ = 0.01
Total	19	0.0070			c.v. = 13.5%
3) Nodule number per plant					
Replicate	3	36.8430	12.2810	0.61	N.S.
Treatment	4	22.9690	5.7423	0.29	N.S.
Error	12	241.2090	20.1008		$S\bar{x}$ = 2.24
Total	19	301.0210			c.v. = 23.00%
4) Nodule weight (g/plant)					
Replicate	3	0.0000	0.0000		
Treatment	4	0.0000	0.0000	0.18	N.S.
Error	12	0.0020	0.0000		$S\bar{x}$ = 0.01
Total	19	0.0030			c.v. = 46.5%
5) µmoles C_2H_4/plant/hr					
Replicate	3	0.5510	0.1837	0.72	N.S.
Treatment	4	0.1380	0.0345	0.13	N.S.
Error	12	3.0760	0.2563		$S\bar{x}$ = 0.25
Total	19	3.7650			c.v. = 70.3%
6) µmoles C_2H_4/g nodule/hr					
Replicate	3	264.0480	88.0160	1.64	N.S.
Treatment	4	48.9170	12.2293	0.23	N.S.
Error	12	643.3890	58.6158		$S\bar{x}$ = 3.66
Total	19	956.3530			c.v. = 33.4%
7) N content (mg/plant)					
Replicate	3	712.8051	237.6017	1.45	N.S.
Treatment	4	1,522.0379	380.5095	2.32	N.S.
Error	12	1,965.0349	163.7529		$S\bar{x}$ = 6.40
Total	19	4,199.8780			c.v. = 12.8%

APPENDIX 22. Analysis of variance of different parameters used in measuring N_2 fixation of 6 week old chickpeas grown in field C.

Source of variation	D.F.	S.S.	M.S.	F-ratio	Level of significance
1) Shoot weight (g/plant)					
Replicate	3	0.2590	0.0863	1.05	N.S.
Treatment	6	0.6590	0.1098	1.34	N.S.
Error	18	1.4760	0.0820		$S\bar{x}$ = 0.14
Total	27	2.3940			c.v. = 22.9%
2) Root weight (g/plant)					
Replicate	3	0.0020	0.0007	1.40	N.S.
Treatment	6	0.0050	0.0008	1.60	N.S.
Error	18	0.0090	0.0005		$S\bar{x}$ = 0.01
Total	27	0.0160			c.v. = 16.0%
3) Nodule number per plant					
Replicate	3	58.6270	19.5423	3.98	5%
Treatment	6	141.3700	235.0228	47.90	1%
Error	18	88.3190	4.9066		$S\bar{x}$ = 1.11
Total	27	1,557.0820			c.v. = 20.1%
4) Nodule weight (g/plant)					
Replicate	3	0.0040	0.0013	3.33	5%
Treatment	6	0.0320	0.0053	13.25	1%
Error	18	0.0070	0.0004		$S\bar{x}$ = 0.01
Total	27	0.0430			c.v. = 37.4%
5) µmoles C_2H_4/plant/hr					
Replicate	3	3.5610	1.1870	5.83	1%
Treatment	6	10.1800	1.6967	8.33	1%
Error	18	3.6670	0.2037		$S\bar{x}$ = 0.23
Total	27	17.4070			c.v. = 50.9%
6) µmoles C_2H_4/g nodule/hr					
Replicate	3	184.8710	61.6237	3.70	5%
Treatment	6	1,615.9070	269.3178	16.18	1%
Error	18	299.5250	16.6403		$S\bar{x}$ = 2.04
Total	27	2,100.3030			c.v. = 32.5%
7) N content (mg/plant)					
Replicate	3	712.8051	237.6017	2.16	N.S.
Treatment	6	1,522.0379	380.5095	3.46	N.S.
Error	18	1,965.0349	109.7529		$S\bar{x}$ = 5.24
Total	27	4,199.8780			c.v. = 25.8%

APPENDIX 23. Analysis of variance of parameters used in measuring N_2 fixation of 10 week old chickpeas grown in field A.

Source of variation	D.F.	S.S.	M.S.	F-ratio	Level of significance
1) Top weight (g/plant)					
Replicate	4	31.1034	7.7759	3.23	5%
Treatment	12	69.7975	5.8165	2.42	5%
Error	48	115.4451	2.4051		$S\bar{x}$ = 0.69
Total	64	216.3460			c.v. = 24.5%
2) N content (mg/plant)					
Replicate	4	69,192.7230	17,298.1808	8.76	1%
Treatment	12	45,932.1940	3,827.6828	1.94	N.S.
Error	48	94,826.7220	1,975.5567		$S\bar{x}$ = 19.88
Total	64	209,951.6400			c.v. = 30.0%

APPENDIX 24. Analysis of variance of parameters used in measuring N_2 fixation of 10 week old chickpeas grown in field B.

Source of variation	D.F.	S.S.	M.S.	F-ratio	Level of significance
1) Top weight (g/plant)					
Replicate	3	5.3008	1.7669	2.45	N.S.
Treatment	4	8.5444	2.1361	2.96	N.S.
Error	12	8.6554	0.7213		$S\bar{x}$ = 0.42
Total	19	22.5005			c.v. = 12.8%
2) N content (mg/plant)					
Replicate	3	5,624.5710	1,874.8570	1.96	N.S.
Treatment	4	6,577.9680	1,644.4920	1.72	N.S.
Error	12	11,459.6800	954.9733		$S\bar{x}$ = 15.45
Total	19	23,662.2190			c.v. = 17.8%

APPENDIX 25. Analysis of variance of parameters used in measuring N_2 fixation in chickpeas grown in field A (final harvest).

Source of variation	D.F.	S.S.	M.S.	F-ratio	Level of significance
1) Total yield (Kg/ha)					
Replicate	4	2,076,463.2310	519,115.8078	3.32	5%
Treatment	12	2,646,427.1390	220.535.5949	1.41	N.S.
Error	48	7,497,341.1690	156,194.6077		$S\bar{x}$ = 176.75
Total	64	12,220,231.5390			c.v. = 23.4%
2) Seed yield (Kg/ha)					
Replicate	4	688,571.0860	172,142.7715	3.35	5%
Treatment	12	814,553.4140	67,879.4512	1.32	N.S.
Error	48	2,467,259.5200	51,401.2400		$S\bar{x}$ = 101.39
Total	64	3,970,384.0200			c.v. = 23.3%
3) Seed N yield (Kg/ha)					
Replicate	4	374.9280	93.7320	1.27	N.S.
Treatment	12	897.4130	74.7844	1.02	N.S.
Error	48	3,535.3440	73.6530		$S\bar{x}$ = 3.84
Total	64	4,807.6860			c.v. = 26.2%

APPENDIX 26. Analysis of variance of parameters used in measuring N_2 fixation in chickpeas grown in field B (final harvest).

Source of variation	D.F.	S.S.	M.S.	F-ratio	Level of significance
1) Total yield (Kg/ha)					
Replicate	3	99,709.8770	33,236.6257	0.52	N.S.
Treatment	4	498,485.6780	124,621.4195	1.97	N.S.
Error	12	760,207.5670	63,350.6306		$S\bar{x}$ = 125.85
Total	19	1,358,403.1220			c.v. = 11.5%
2) Seed yield (Kg/ha)					
Replicate	3	50,373.9340	16,791.3113	0.66	N.S.
Treatment	4	172,515.5570	43,128.8893	1.70	N.S.
Error	12	305,098.1350	25,424.8446		$S\bar{x}$ = 79.73
Total	19	527,987.6260			c.v. = 11.6%
3) Seed N yield (Kg/ha)					
Replicate	3	522.6390	174.2130	5.63	5%
Treatment	4	287.1820	71.7955	2.32	N.S.
Error	12	371.1230	30.9269		$S\bar{x}$ = 2.78
Total	19	1,180.9440			c.v. = 10.9%

APPENDIX 27. Procedures used in determining pH, E.C., available N, P and K in soil samples.

1) pH

20 g of soil is weighed into a 100 ml beaker and 40 ml of water added and stirred frequently for half an hour. Then the pH is estimated in the soil suspension in the Elico pH meter. Rating: pH < 6.7 = acid; 6.7 - 8.7 = normal; > 8.7 = alkaline.

2) E.C.

The above soil suspension is allowed to settle and the E.C. is estimated in the clear supernatant liquid with the solubridge.

Rating:

E.C. m.mho/cm	Nature of the soil
<0.8	Normal
0.8 - 1.6	Critical for salt sensitive crops
1.6 - 2.5	Critical for salt tolerant crops
> 2.5	Injurious to all crops

3) Available nitrogen (alkaline permanganate method):

20 g of soil is taken in a 800 ml Kjeldahl flask and 20 ml of water added. Then 20 ml of 0.32% (freshly prepared) K MnO_4 solution and 100 ml of 2.5% NaOH solution are added followed by a few boiling chips or 1 ml of liquid parafin. The flask is connected to the distillation set, and the distillate is collected in 20 ml of N/50 H_2SO_4 till the total volume comes to 50 ml. The excess acid is back titrated with N/50 KOH using the mixed indicator (Bromocresol green in methyl red).

Available N/ha = X x 31.36, where X is the volume of the N/50 acid used in the estimation. Rating adopted is <280 Kg/ha = low; 280-360 Kg/ha = medium; > 360 = high.

4) Available P (Olson's method)

5 g of the soil and a small spoonful of activated carbon (Daxco, which had been previously leached with Olsen's reagent a number of times) are placed in a 100 ml conical flask and 50 ml of Olsen's reagent added. The flask is shaken for half an hour and filtered. A blank is similarly run. After rejecting the first 15 ml of the filtrate, 5 ml is pipetted

APPENDIX 27. (continued)

into a 50 ml volumetric flask. About 25 ml of water is added followed by 5 ml of chloromolybdic acid. Then water is added till the volume comes to almost 48 ml. Then 1 ml of diluted stannous chloride solution (0.5 ml of stock solution diluted to 66 ml) is added and the volume used up to the mark. The colour is allowed to develop for 10 minutes and then estimated at 660 nm in the spectronic 20 colorimeter within another 15 minutes. From the previously prepared calibrating curve with 2 ppm P standard solutions ranging in volumes from 0 - 25 ml, the available P is calculated. The available P (ppm) in the soil = X x 100, where X is the ppm P in the final 50 ml as read from the graph. Rating adopted is: <5 ppm P = low; 5 - 10 ppm P = medium; >10 ppm = high.

Note: Olsen's reagent 42 g of sodium bicarbonate is dissolved in water and made up to a litre. The pH is adjusted to 8.5 with HCl or NaOH.

Chloromolybdic acid 15 g of ammonium molybdate is dissolved in about 100 ml of water kept at 50°C. The solution is filtered if necessary and cooled to room temperature. Then 400 ml of 10 N HCl is added, shaken and made up to a litre and stored in a brown bottle.

Stannous chloride stock solution (40%): 10 g of A.R. Stannous chloride is dissolved in 20 ml of conc. HCl and then stored in a refrigerator. The solution is to be prepared fresh at least once a month.

5) Available potassium

10 g of soil is shaken with 50 ml of 1 N neutral ammonium acetate for 5 minutes and filtered. K is determined in the filtrate using the Flame Photometer.

Available K (Kg/ha) = ppm K in the filtrate x 11.2. Rating adopted is: <113 Kg/ha = low; 113 - 280 Kg/ha = medium; > 280 Kg/ha = high.

APPENDIX 28. Calculation of μmoles C_2H_4 production (modified Dart et al 1972).

$$\mu\text{moles } C_2H_4 \text{ produced/hr} = \left[\frac{S.C_2H_4}{S.C_2H_2} \times \text{Blank } C_2H_2 - \text{Blank } C_2H_4\right] \times \frac{VCF \times BV \times \text{Std VPM} \times 0.06}{22.4 \times \text{Std. } C_2H_4 \times \text{Time (min.)}}$$

where

$S.C_2H_4$	= chart units reading of sample C_2H_4
$S.C_2H_2$	= chart units reading of sample C_2H_2
Blank C_2H_2	= chart units reading of blank C_2H_2
Blank C_2H_4	= chart units reading of blank C_2H_4
VCF	= vacutainer correction factor which is equal to vacutainer volume/ml of gas putting in the vacutainer
BV	= bottle volume (ml)
Std VPM	= C_2H_4 VPM of the standard gas
Std C_2H_4	= chart units reading of C_2H_4 of the standard gas
Time	= incubation time

APPENDIX 29. Plant N content determination using an auto-analyzer.

Dry samples are ground and dried at 70°C for 24 hours. Samples are weighed (250 mg for root, 150 mg for stem, 100 mg for seed and leaves) into a 125 ml Erlenmeyer flask. 4 ml of digestion mixture are added (H_2SO_4 containing 0.5% Selenium) and digestion done at 360°C using a Tecetor Block Digestion. The digested material is cooled down, made up to 75 ml with distilled water, shaken thoroughly to get a homogeneous solution. The solution is then fed to the auto-analyzer. The quantitation of ammonia is achieved utilizing the Berthelot Reaction in which the formation of a blue indophenol complex occurs when ammonia is reacted with sodium phenate followed by the addition of sodium hypochlorite. The complex is measured colorimetrically at 630 nm.

APPENDIX 30. The number of Rhizobium transferred per prong of the 25 multiprong inoculator and the % success of transfer at different Rhizobium concentrations.

Rhizobium concentration (cells/ml)	Rhizobium transferred (cells/prong)	No. of wells tested	No. of positive growth wells	Percentage success
1.55×10^9	1.80×10^5	108	108	100
1.55×10^8	2.10×10^4	108	108	100
1.55×10^7	3.36×10^3	108	108	100
1.55×10^6	2.74×10^2	108	108	100
1.55×10^5	1.14×10^1	108	108	100
1.55×10^4	<1.14	108	83	76.85
1.55×10^3	0	108	18	16.67
1.55×10^2	0	108	0	0

Cowpea Rhizobium strain NC-92 was grown in yeast extract mannitol broth (Vincent 1970) for 6 days. It was then ten-fold diluted and the number of Rhizobium/ml broth determined using the plate count method. Each dilution (from $10^0 - 10^8$) was transferred into the wells of the 25 multiprong inoculator in triplicate. Therefore, 24 holes were occupied by the inoculum. The last well was a sterilized water blank control. The number of bacteria transferred was estimated by using the inoculator to transfer the bacteria to wells of another multiprong inoculator containing sterile water and dilution plating samples of these wells.

The % success of transfer was done by inoculation the multiprong inoculator (after dipping in the wells containing the previous inoculum) to 36 petri dishes containing 30 ml congo red yeast extract mannitol agar. The positive or negative growth was assessed 1 week after inoculation.

APPENDIX 31. The components of YEMA used in low intrinsic antibiotic resistant method (finger printing method).

Compounds	gm/l	Supplier
1) K_2HPO_4	0.5	Sarabhai M Chemical
2) $MgSO_4 \cdot 7H_2O$	0.2	Sarabhai M Chemical
3) NaCl	0.1	Sarabhai M Chemical
4) Mannitol	10	Sarabhai M Chemical
5) Yeast extract	1	Difco
6) Agar	12	Difco Bacto Agar
7) Distilled or deionized water	1 l	

APPENDIX 32. Recovery of inoculation Rhizobium strain 9036 in 3 selected treatments using str 200 resistant characteristic alone in 3 fields.

Replicate	Population (log 10 MPN)	Treatment*		
		Control	Methyl cellulose	Liquid
Field A				
1	1.03	0/49	3/41	44/51
2	2.04	0/24	6/32	17/40
3	2.34	0/18	21/38	48/48
4	1.03	0/21	22/33	33/39
Total	-	0/112	52/144	142/178
%	-	0	36.11	79.77
Field B				
1	4.00	0/29	0/38	5/47
2	3.65	0/53	0/40	2/36
3	3.64	0/25	1/37	7/36
4	4.62	0/43	0/43	6/46
Total	-	0/150	1/138	20/165
%	-	0	0.72	12.12
Field C				
1	0	0/0	44/45	17/17
2	0	0 0	9/9	19/19
3	0	0/0	10/11	24/24
4	0	0/0	18/18	17/17
Total	-	0/0	81/83	77/77
%	-	0	97.59	100

*Number of isolates resistant to str 200/total isolates tested.

APPENDIX 33. Low level intrinsic antibiotic resistance pattern of 475 Rhizobium isolates from field B.

Group no.	Carb 1	Carb 2.5	Carb 5	Ery 1.25	Ery 2.5	Ery 10	Kan 2.5	Kan 10	Kan 20	Nal 2.5	Nal 10	Neo 2.5	Neo 10	Neo 15	Pol 5	Pol 10	Pol 20	Rif 0.25	Rif 0.50	Rif 2.50	Str 2.5	Str 10	Str 20	Tet 0.1	Tet 0.5	Van 1.25	Van 2.5	Van 10	No. of isolates in each treatment			Total
																													Control	M.C.	Liquid	
1	+	+	-	+	+	+	+	-	-	+	+	+	-	-	+	+	+	+	+	-	-	-	-	+	-	+	+	+	-	-	2	2
2	+	-	-	+	+	+	+	+	-	+	+	+	-	-	+	+	+	+	+	-	-	-	-	+	-	+	+	-	2	-	1	3
3	+	-	-	+	+	+	+	-	-	+	+	+	-	-	+	+	+	+	+	-	-	-	-	+	-	+	+	+	-	-	2	2
4	+	+	-	+	+	+	+	+	-	+	+	+	-	-	+	+	+	+	+	-	-	-	-	-	-	+	+	-	6	3	3	12
5	+	+	-	+	+	+	+	-	-	+	+	+	-	-	+	+	+	+	+	-	-	-	-	+	-	+	+	-	-	2	4	6
6	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	-	19	19
7	-	-	-	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	-	-	-	-	-	-	-	+	+	-	2	-	1	3
8	-	-	-	+	+	-	-	-	-	+	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	+	-	-	-	1	2	3
9	-	-	-	+	+	-	+	-	-	+	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	+	+	-	1	3	1	5
10	+	+	-	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	-	-	-	-	-	-	+	+	-	1	-	3	4
11	-	-	-	+	+	-	-	-	-	+	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	+	+	-	-	1	2	3
12	-	-	-	+	+	-	-	-	-	+	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	+	+	-	-	-	1	1
13	+	-	-	+	+	-	+	-	-	+	+	-	-	-	+	+	+	+	+	-	-	-	-	-	-	+	+	-	3	1	-	4
14	-	-	-	+	+	-	-	-	-	+	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	+	+	-	-	1	-	1
15	-	-	-	+	+	-	+	-	-	+	+	-	-	-	+	+	+	+	+	-	-	-	-	-	-	+	+	-	-	1	-	1
16	-	-	-	+	+	-	+	-	-	+	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	+	+	-	1	-	-	1
17	-	-	-	+	+	-	+	-	-	+	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	+	-	-	1	-	-	1
18	+	-	-	+	+	+	+	-	-	+	+	-	-	-	+	+	+	+	+	-	-	-	-	-	-	+	+	-	16	8	11	35
19	+	-	-	+	+	+	+	-	-	+	+	+	-	-	+	+	+	+	+	-	-	-	-	-	-	+	+	-	3	2	5	10
20	+	-	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-	-	-	-	+	-	+	+	+	-	1	1	2

(continued)

APPENDIX 33. (continued)

Group no.	Carb 1	Carb 2.5	Carb 5	Ery 1.25	Ery 2.5	Ery 10	Kan 2.5	Kan 10	Kan 20	Nal 2.5	Nal 10	Neo 2.5	Neo 10	Neo 15	Pol 5	Pol 10	Pol 20	Rif 0.25	Rif 0.50	Rif 2.50	Str 2.5	Str 10	Str 20	Tet 0.1	Tet 0.5	Van 1.25	Van 2.5	Van 10	No. of isolates in each treatment			Total
																													Control	M.C.	Liquid	
21	+	+	-	+	+	+	+	-	-	+	+	-	-	-	+	+	+	+	-	-	-	-	-	-	-	+	+	-	-	1	1	2
22	+	-	-	+	+	+	+	-	-	+	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	+	+	-	-	-	1	1
23	+	-	-	+	+	+	+	-	-	+	+	+	-	-	+	+	+	+	+	-	-	-	-	-	-	+	+	+	1	3	1	5
24	+	-	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-	-	-	-	-	-	+	+	+	-	-	1	1
25	+	-	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-	-	-	-	-	-	+	+	-	-	-	1	1
26	+	-	-	+	+	+	+	+	-	+	+	+	-	-	+	+	-	+	-	-	-	-	-	-	-	+	+	-	3	-	1	4
27	+	+	-	+	+	+	+	+	-	+	+	+	-	-	+	+	-	+	-	-	-	-	-	-	-	+	+	-	4	-	1	5
28	+	-	-	+	+	+	-	-	-	+	+	-	-	-	+	+	+	-	-	-	-	-	-	-	-	+	+	-	1	-	1	2
29	+	-	-	+	+	+	-	-	-	+	+	-	-	-	+	+	+	+	+	-	-	-	-	-	-	+	+	-	2	3	-	5
30	+	+	-	+	+	+	+	+	-	+	+	+	-	-	+	+	+	+	-	-	-	-	-	-	-	+	+	-	1	-	4	5
31	+	-	-	+	+	+	+	+	+	+	+	+	-	-	+	+	+	-	-	-	-	-	-	+	-	+	+	-	2	-	1	3
32	+	-	-	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	-	-	-	-	-	+	-	+	+	-	2	2	2	6
33	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	-	-	-	-	-	-	+	-	+	+	-	-	-	1	1
34	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	-	2	2
35	+	+	-	+	+	+	+	-	-	+	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	+	+	-	-	-	1	1
36	+	+	+	+	+	+	+	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	1	1
37	+	-	-	+	+	+	+	-	-	+	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	+	+	-	2	-	-	2
38	+	-	-	+	+	+	+	-	-	+	+	-	-	-	+	+	+	-	-	-	-	-	-	-	-	+	+	-	2	-	-	2
39	+	-	-	+	+	+	+	-	-	+	+	-	-	-	+	+	+	+	-	-	-	-	-	-	-	+	+	-	1	1	1	3
40	+	-	-	-	-	-	+	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	1	-	-	1

(continued)

APPENDIX 33. (continued)

Group no.	Carb 1	Carb 2.5	Carb 5	Ery 1.25	Ery 2.5	Ery 10	Kan 2.5	Kan 10	Kan 20	Nal 2.5	Nal 10	Neo 2.5	Neo 10	Neo 15	Pol 5	Pol 10	Pol 20	Rif 0.25	Rif 0.50	Rif 2.50	Str 2.5	Str 10	Str 20	Tet 0.1	Tet 0.5	Van 1.25	Van 2.5	Van 10	No. of isolates in each treatment			Total
																													Control	M.C.	Liquid	
41	+	-	-	+	+	+	+	-	-	+	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	+	+	-	2	-	-	2
42	+	-	-	+	+	+	+	-	-	+	+	-	-	-	+	+	+	+	+	-	-	-	-	-	-	+	+	+	10	-	2	12
43	+	-	-	+	+	+	+	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	+	+	-	1	-	-	1
44	-	-	-	+	+	+	+	+	+	+	+	+	-	-	+	+	+	-	-	-	-	-	-	+	-	+	+	-	-	-	1	1
45	+	-	-	+	+	+	+	+	-	+	+	+	-	-	+	+	+	+	+	-	+	-	-	-	-	+	+	-	-	-	1	1
46	+	-	-	+	+	+	+	+	-	+	+	+	-	-	+	+	-	+	-	-	+	-	-	-	-	+	+	-	-	-	2	2
47	+	-	-	+	+	+	+	-	-	+	+	-	-	-	+	+	+	+	-	-	-	-	-	+	-	+	+	-	-	-	3	3
48	+	-	-	+	+	+	+	-	-	+	+	+	-	-	+	+	+	+	-	-	-	-	-	-	-	+	+	-	1	-	2	3
49	-	-	-	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	-	-	-	-	-	+	-	+	+	-	1	-	3	4
50	-	-	-	+	+	+	+	-	-	+	+	+	-	-	+	+	+	+	-	-	-	-	-	+	-	+	+	-	1	1	1	3
51	-	-	-	+	+	+	+	-	-	+	-	+	-	-	+	+	+	-	-	-	-	-	-	+	-	+	+	-	-	-	1	1
52	-	-	-	+	+	+	+	-	-	+	+	-	-	-	+	+	+	+	+	-	-	-	-	+	-	+	+	-	-	-	1	1
53	+	-	-	+	+	+	+	+	-	+	+	+	-	-	+	+	-	+	-	-	+	-	-	-	-	+	-	-	-	-	1	1
54	+	-	-	+	+	+	+	-	-	+	+	+	-	-	+	+	-	+	-	-	+	-	-	-	-	+	+	-	-	-	1	1
55	+	-	-	+	+	+	+	-	-	+	+	+	-	-	+	+	+	+	-	-	+	-	-	+	-	+	+	-	-	-	1	1
56	-	-	-	+	+	+	+	-	-	+	+	-	-	-	+	+	-	+	-	-	-	-	-	+	-	+	+	-	1	-	-	1
57	-	-	-	+	+	+	+	-	-	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+	+	-	1	-	-	1
58	+	-	-	+	+	+	+	-	-	+	+	-	-	-	+	+	-	+	-	-	-	-	-	+	-	+	+	-	1	-	-	1
59	+	-	-	+	+	+	+	+	-	+	+	+	-	-	+	+	-	-	-	-	-	-	-	-	-	+	+	-	-	-	1	1
60	+	-	-	+	+	+	+	+	-	+	+	+	-	-	+	+	+	+	-	-	-	-	-	-	-	+	+	-	2	-	1	3

(continued)

APPENDIX 33. (continued)

Group no.	Carb 1	Carb 2.5	Carb 5	Ery 1.25	Ery 2.5	Ery 10	Kan 2.5	Kan 10	Kan 20	Nal 2.5	Nal 10	Neo 2.5	Neo 10	Neo 15	Pol 5	Pol 10	Pol 20	Rif 0.25	Rif 0.50	Rif 2.50	Str 2.5	Str 10	Str 20	Tet 0.1	Tet 0.5	Van 1.25	Van 2.5	Van 10	No. of isolates in each treatment			Total
																													Control	M.C.	Liquid	
61	+	-	-	+	+	+	+	+	-	+	+	+	-	-	+	+	-	+	-	-	-	-	-	+	-	+	+	-	-	-	1	1
62	+	-	-	+	+	+	+	+	-	+	+	+	-	-	+	+	+	+	-	-	-	-	-	+	-	+	+	-	5	-	1	6
63	+	-	-	+	+	+	+	+	-	+	+	+	-	-	+	+	-	-	-	-	-	-	-	+	-	+	-	-	-	-	2	2
64	+	-	-	+	+	+	+	+	-	+	+	+	-	-	+	+	+	+	+	-	-	-	-	+	-	+	+	+	-	1	1	2
65	+	+	+	+	+	+	+	-	-	+	+	+	-	-	+	+	+	+	+	-	-	-	-	+	-	+	+	-	-	2	-	2
66	+	+	+	+	+	+	+	+	-	+	+	+	-	-	+	+	+	+	-	-	-	-	-	-	-	+	+	+	1	-	-	1
67	+	-	-	+	+	+	+	+	-	+	+	+	-	-	+	+	-	+	-	-	-	-	-	-	-	+	+	+	1	-	-	1
68	+	-	-	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	-	-	-	-	+	-	+	+	-	1	-	-	1
69	-	-	-	+	+	+	+	+	-	+	+	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	1	-	-	1
70	+	+	-	+	+	+	+	+	-	+	+	+	-	-	+	-	-	+	-	-	-	-	-	-	-	+	+	-	1	-	-	1
71	+	-	-	+	+	+	+	+	-	+	+	+	-	-	+	+	+	+	+	-	-	-	-	-	-	+	+	-	2	2	-	4
72	+	-	-	+	+	+	+	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	1	-	-	1
73	+	-	-	+	+	+	+	-	-	+	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	1	-	-	1
74	+	-	-	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	-	-	-	-	-	+	-	+	+	+	-	5	-	5
75	+	-	-	+	+	+	+	-	-	+	+	-	-	-	+	+	+	+	+	-	-	-	-	+	-	+	+	-	3	7	1	11
76	+	-	-	+	+	+	-	-	-	+	+	-	-	-	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	1	-	1
77	+	+	-	+	+	+	+	+	-	+	+	+	-	-	+	+	-	+	+	-	-	-	-	-	-	+	+	-	1	2	-	3
78	-	-	-	+	+	+	+	+	-	+	+	+	-	-	+	+	+	+	+	-	-	-	-	+	-	+	+	+	1	1	-	2
79	+	-	-	+	+	+	+	-	-	+	+	+	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	1	-	1
80	+	-	-	+	+	+	+	+	-	+	+	+	-	-	+	-	-	+	+	-	-	-	-	-	-	-	-	-	-	1	-	1

(continued)

APPENDIX 33. (continued)

Group no.	Carb 1	Carb 2.5	Carb 5	Ery 1.25	Ery 2.5	Ery 10	Kan 2.5	Kan 10	Kan 20	Nal 2.5	Nal 10	Neo 2.5	Neo 10	Neo 15	Pol 5	Pol 10	Pol 20	Rif 0.25	Rif 0.50	Rif 2.50	Str 2.5	Str 10	Str 20	Tet 0.1	Tet 0.5	Van 1.25	Van 2.5	Van 10	No. of isolates in each treatment			Total
																													Control	M.C.	Liquid	
81	+	+	+	+	+	+	+	+	−	+	+	+	−	−	+	+	−	+	+	−	−	−	−	+	−	+	+	−	−	−	1	1
82	+	+	−	+	+	+	+	+	−	+	+	+	−	−	+	+	−	+	+	−	−	−	−	+	−	+	+	−	−	−	1	1
83	+	+	−	+	+	+	+	−	−	+	+	+	−	−	+	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	1	1
84	+	−	−	+	+	+	+	+	−	+	+	+	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	1	1
85	+	+	−	+	+	+	+	+	−	+	+	+	−	−	+	+	+	+	+	−	−	−	−	−	−	+	−	−	−	−	2	2
86	+	−	−	+	+	+	+	+	+	+	+	+	−	−	+	+	+	+	+	−	−	−	−	+	−	+	+	+	6	3	−	9
87	+	−	−	+	+	+	+	−	−	+	+	+	−	−	+	−	−	+	+	−	−	−	−	−	−	+	−	−	−	−	1	1
88	+	−	−	+	+	+	+	−	−	+	+	+	−	−	+	+	+	+	+	−	−	−	−	+	−	+	+	−	3	8	2	13
89	+	−	−	+	+	+	+	−	−	+	+	+	−	−	+	−	−	+	+	−	−	−	−	+	−	−	−	−	−	−	1	1
90	+	−	−	+	+	+	+	−	−	+	+	−	−	−	+	+	−	+	+	−	−	−	−	+	−	+	+	−	−	1	−	1
91	+	−	−	+	+	+	−	−	−	+	+	−	−	−	+	+	−	+	+	−	−	−	−	+	+	+	+	−	−	2	−	2
92	−	−	−	+	+	+	+	−	−	+	+	−	−	−	+	+	+	−	−	−	−	−	−	+	−	−	−	−	−	1	−	1
93	−	−	−	+	+	−	+	−	−	+	−	+	−	−	+	+	+	+	−	−	−	−	−	+	−	+	−	−	1	−	−	1
94	+	−	−	+	+	+	+	−	−	+	−	+	−	−	+	+	+	+	−	−	−	−	−	+	−	+	+	−	1	−	−	1
95	−	−	−	+	+	+	+	−	−	+	+	+	−	−	+	+	+	+	+	−	−	−	−	+	−	+	+	−	1	1	1	3
96	+	−	−	+	+	+	+	−	−	+	+	+	−	−	+	+	−	+	−	−	−	−	−	+	−	+	−	−	−	−	1	1
97	+	−	−	+	+	+	+	−	−	+	+	+	−	−	+	+	+	+	−	−	−	−	−	+	−	+	+	−	−	2	−	2
98	−	−	−	+	+	+	+	−	−	+	−	+	−	−	+	+	+	+	+	−	−	−	−	+	−	+	−	−	−	1	−	1
99	+	−	−	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	−	−	−	−	+	−	+	+	−	−	1	−	1
100	+	−	−	+	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	1	−	−	1

(continued)

APPENDIX 33. (continued)

Group no.	Carb 1	Carb 2.5	Carb 5	Ery 1.25	Ery 2.5	Ery 10	Kan 2.5	Kan 10	Kan 20	Nal 2.5	Nal 10	Neo 2.5	Neo 10	Neo 15	Pol 5	Pol 10	Pol 20	Rif 0.25	Rif 0.50	Rif 2.50	Str 2.5	Str 10	Str 20	Tet 0.1	Tet 0.5	Van 1.25	Van 2.5	Van 10	No. of isolates in each treatment			Total
																													Control	M.C.	Liquid	
101	+	+	-	+	+	+	+	-	-	+	+	+	-	-	+	+	+	+	+	-	-	-	-	-	-	+	+	+	2	-	-	2
102	+	-	-	+	+	-	+	-	-	+	+	-	-	-	+	-	+	+	+	-	-	-	-	-	-	+	+	+	1	-	-	1
103	+	-	-	+	+	-	+	-	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	+	+	+	1	-	-	1
104	+	-	-	+	+	-	+	-	-	+	+	-	-	-	+	+	+	+	+	-	-	-	-	-	-	+	+	+	1	-	-	1
105	-	-	-	+	+	-	+	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	+	+	1	-	-	1
106	+	-	-	+	+	-	+	+	-	+	+	-	-	-	+	+	-	+	-	-	-	-	-	-	-	+	+	-	1	-	-	1
107	+	-	-	+	+	-	+	-	-	+	+	-	-	-	+	+	+	+	-	-	-	-	-	-	-	+	+	-	1	-	-	1
108	+	-	-	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	-	-	-	-	-	-	+	+	+	1	-	-	1
109	+	-	-	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	-	-	-	-	-	-	-	+	+	+	-	1	-	1
110	+	+	-	+	+	+	+	+	-	+	+	+	-	-	+	+	+	+	-	-	-	-	-	-	-	+	+	+	-	1	-	1
111	+	+	-	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	-	-	-	-	-	-	+	+	-	-	-	1	1
112	+	+	-	+	+	+	+	+	-	+	+	+	-	-	+	+	+	+	+	-	-	-	-	+	-	+	+	-	-	-	1	1
113	+	-	-	+	+	+	+	+	-	+	+	+	-	-	+	+	-	+	+	-	-	-	-	+	-	+	+	-	1	2	1	4
114	+	+	-	+	+	+	+	+	-	+	+	+	-	-	+	+	+	+	+	-	-	-	-	-	-	+	+	-	2	1	-	3
115	+	-	-	+	+	+	+	+	-	+	+	+	-	-	+	+	+	-	-	-	-	-	+	-	-	+	+	+	-	1	-	1
116	+	-	-	+	+	+	-	-	-	+	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	+	+	-	-	1	-	1
117	-	-	-	+	+	+	-	-	-	+	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	+	+	-	-	3	-	3
118	+	-	-	+	+	+	-	-	-	+	+	-	-	-	+	+	+	+	+	-	-	-	-	-	-	+	+	-	-	2	-	2
119	-	-	-	+	+	+	-	-	-	+	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	+	+	+	-	3	-	3
120	+	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	+	-	-	1	-	1
																													-	2	-	2

(continued)

APPENDIX 33. (continued)

Group no.	Carb 1	Carb 2.5	Carb 5	Ery 1.25	Ery 2.5	Ery 10	Kan 2.5	Kan 10	Kan 20	Nal 2.5	Nal 10	Neo 2.5	Neo 10	Neo 15	Pol 5	Pol 10	Pol 20	Rif 0.25	Rif 0.50	Rif 2.50	Str 2.5	Str 10	Str 20	Tet 0.1	Tet 0.5	Van 1.25	Van 2.5	Van 10	No. of isolates in each treatment			Total
																													Control	M.C.	Liquid	
121	-	-	-	+	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	+	-	-	2	2	4
122	+	+	-	+	+	+	-	-	-	+	+	+	-	-	+	+	+	+	+	-	-	-	-	-	-	+	+	-	-	2	-	2
123	+	-	-	+	+	+	-	-	-	+	-	-	-	-	+	+	-	+	+	-	-	-	-	-	-	+	+	-	-	1	-	1
124	+	-	-	+	+	+	-	-	-	+	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	+	+	-	-	4	-	4
125	+	+	-	+	+	+	+	-	-	+	+	+	-	-	+	+	+	+	+	-	-	-	-	-	-	+	+	-	-	2	-	2
126	+	-	-	+	+	+	-	-	-	+	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	+	+	-	2	1	-	3
127	+	+	-	+	+	+	+	-	-	+	+	-	-	-	+	+	+	+	+	-	-	-	-	-	-	+	+	+	1	-	-	1
128	-	-	-	+	+	+	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	+	+	+	-	1	-	1
129	+	-	-	+	+	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	+	+	-	2	-	1	3
130	+	-	-	+	+	-	-	-	-	+	-	+	-	-	+	+	+	+	-	-	-	-	-	-	-	+	+	-	1	-	-	1
131	+	-	-	+	+	-	-	-	-	+	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	+	+	-	2	-	-	2
132	+	-	-	+	+	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	+	+	-	1	-	-	1
133	+	-	-	+	+	+	-	-	-	+	+	+	-	-	+	+	+	+	-	-	-	-	-	-	-	+	+	-	1	-	-	1
134	+	-	-	+	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	+	-	1	-	-	1
135	+	-	-	+	+	-	-	-	-	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+	+	-	1	-	-	1
136	+	-	-	+	+	+	+	-	-	+	-	+	-	-	+	+	+	+	-	-	-	-	-	-	-	+	+	-	-	-	1	1
137	+	-	-	+	+	+	-	-	-	+	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	+	+	-	-	-	1	1
138	+	-	-	+	+	+	-	-	-	+	+	-	-	-	+	+	-	+	-	-	-	-	-	+	-	+	+	-	-	-	1	1
139	+	-	-	+	+	+	-	-	-	+	+	-	-	-	+	+	+	+	-	-	-	-	-	-	-	+	+	-	2	1	2	5
140	+	-	-	+	+	+	+	+	-	+	-	-	-	-	+	+	-	+	-	-	-	-	-	-	-	+	+	-	-	1	1	2
																										+	+	-	-	1	-	1

(continued)

APPENDIX 33. (continued)

Group no.	Carb 1	Carb 2.5	Carb 5	Ery 1.25	Ery 2.5	Ery 10	Kan 2.5	Kan 10	Kan 20	Nal 2.5	Nal 10	Neo 2.5	Neo 10	Neo 15	Pol 5	Pol 10	Pol 20	Rif 0.25	Rif 0.50	Rif 2.50	Str 2.5	Str 10	Str 20	Tet 0.1	Tet 0.5	Van 1.25	Van 2.5	Van 10	No. of isolates in each treatment			Total
																													Control	M.C.	Liquid	
141	+	-	-	+	+	+	-	-	-	+	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	+	+	-	-	1	-	1
142	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	1
143	+	-	-	+	+	+	-	-	-	+	+	+	-	-	+	+	+	+	+	-	-	-	-	-	-	+	+	-	1	-	-	1
144	+	-	-	+	+	-	+	+	+	+	+	+	-	-	+	-	-	+	-	-	-	+	-	-	-	+	+	+	-	-	1	1
145	+	-	-	+	+	+	+	+	-	+	+	+	-	-	+	-	-	+	-	-	-	-	-	-	-	+	+	-	1	5	-	6
146	+	-	-	+	+	+	+	+	+	+	+	+	-	-	+	+	+	-	-	-	-	-	-	-	-	+	+	-	-	2	-	2
147	+	-	-	+	+	+	+	+	-	+	+	-	-	-	+	-	-	+	-	-	-	-	-	-	-	+	+	-	-	-	1	1
148	-	-	-	+	+	+	+	-	-	+	-	-	-	-	+	+	-	+	+	-	-	-	-	-	-	+	+	-	-	-	2	2
149	+	-	-	+	+	+	+	+	-	+	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	1
150	+	-	-	+	+	+	+	-	-	+	+	-	-	-	+	+	-	+	-	-	-	-	-	-	-	+	+	-	-	-	1	1
151	-	-	-	+	+	+	+	+	-	+	+	-	-	-	+	+	+	+	+	-	-	-	-	+	-	+	+	+	-	-	2	2
152	-	-	-	+	+	+	+	-	-	+	-	-	-	-	+	+	-	-	+	-	-	-	-	-	-	+	+	-	-	-	1	1
153	+	+	-	+	+	+	+	+	-	+	+	-	-	-	+	+	-	+	+	-	-	-	-	-	-	+	+	-	-	-	2	2
154	+	-	-	+	+	+	+	-	-	+	+	-	-	-	+	+	-	+	+	-	-	-	-	-	-	+	+	-	-	2	2	4
155	+	-	-	+	+	+	+	-	-	+	-	-	-	-	+	+	-	+	-	-	-	-	-	-	-	+	+	-	-	-	1	1
156	+	-	-	+	+	+	+	-	-	+	-	-	-	-	+	+	-	+	+	-	-	-	-	-	-	+	+	-	-	1	1	2
157	-	-	-	+	+	+	-	-	-	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+	+	-	-	-	1	1
158	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	1	1
159	-	-	-	+	+	+	-	-	-	-	-	-	-	-	+	+	-	+	+	-	-	-	-	-	-	+	+	-	-	-	1	1
160	+	-	-	+	+	+	-	-	-	+	+	-	-	-	+	+	-	+	+	-	-	-	-	-	-	+	+	-	-	-	2	2

(continued)

APPENDIX 33. (continued)

Group no.	Carb 1	Carb 2.5	Carb 5	Ery 1.25	Ery 2.5	Ery 10	Kan 2.5	Kan 10	Kan 20	Nal 2.5	Nal 10	Neo 2.5	Neo 10	Neo 15	Pol 5	Pol 10	Pol 20	Rif 0.25	Rif 0.50	Rif 2.50	Str 2.5	Str 10	Str 20	Tet 0.1	Tet 0.5	Van 1.25	Van 2.5	Van 10	No. of isolates in each treatment			Total
																													Control	M.C.	Liquid	
161	+	-	-	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	-	-	-	-	+	-	+	+	+	-	-	1	1
162	-	-	-	+	+	+	-	-	-	-	-	-	-	-	+	-	-	+	+	-	-	-	-	-	-	+	+	-	-	-	3	3
163	-	-	-	+	+	+	-	-	-	+	+	-	-	-	+	-	-	+	+	-	-	-	-	-	-	+	+	-	-	-	1	1
164	+	-	-	+	+	+	+	-	-	+	+	-	-	-	+	+	+	+	+	-	-	-	-	+	-	+	+	+	-	1	1	2
165	+	+	-	+	+	+	+	+	-	+	+	-	-	-	+	-	-	+	+	-	-	-	-	-	-	+	+	-	-	5	-	5
166	+	-	-	+	+	+	+	+	-	+	+	-	-	-	+	-	-	+	+	-	-	-	-	-	-	+	+	-	-	1	-	1
167	-	-	-	+	+	+	+	+	-	+	+	-	-	-	+	-	-	+	+	-	-	-	-	-	-	+	+	-	-	-	1	1
168	-	-	-	+	+	+	+	-	-	+	+	+	-	-	+	+	+	+	+	-	-	-	-	+	-	+	+	+	1	-	-	1
169	-	-	-	+	+	+	+	+	-	+	+	+	-	-	+	+	+	+	-	-	-	-	-	+	-	+	+	-	2	-	-	2
170	+	+	-	+	+	+	+	+	-	+	+	-	-	-	+	-	-	+	-	-	-	-	-	-	-	+	+	-	1	-	-	1
171	+	-	-	+	+	+	+	+	-	+	+	-	-	-	+	+	+	+	-	-	-	-	-	-	-	+	+	-	1	-	-	1
172	+	-	-	+	+	+	+	+	-	+	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	1	-	-	1
173	+	-	-	+	+	+	+	+	-	+	-	-	-	-	+	-	-	+	-	-	-	-	-	+	-	+	-	-	-	1	-	1
174	+	-	-	+	+	+	+	+	-	+	+	+	-	-	+	-	-	+	+	-	-	-	-	-	-	+	+	-	-	1	-	1
175	-	-	-	+	+	-	+	-	-	+	+	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	1	-	1
176	+	-	-	+	+	+	+	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	1	-	1
177	+	-	-	+	+	+	+	-	-	+	+	-	-	-	+	+	-	+	+	-	-	-	-	-	-	+	+	-	-	2	-	2
178	+	-	-	+	+	+	+	-	-	+	+	-	-	-	+	-	-	+	-	-	-	-	-	-	-	+	-	-	-	1	-	1
179	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	1	-	-	1
180	+	+	+	+	+	+	+	+	-	+	+	+	-	-	+	+	-	+	-	-	-	-	-	-	-	+	+	-	1	-	-	1

(continued)

APPENDIX 33. (continued)

Group no.	Carb 1	Carb 2.5	Carb 5	Ery 1.25	Ery 2.5	Ery 10	Kan 2.5	Kan 10	Kan 20	Nal 2.5	Nal 10	Neo 2.5	Neo 10	Neo 15	Pol 5	Pol 10	Pol 20	Rif 0.25	Rif 0.50	Rif 2.50	Str 2.5	Str 10	Str 20	Tet 0.1	Tet 0.5	Van 1.25	Van 2.5	Van 10	No. of isolates in each treatment			Total
																													Control	M.C.	Liquid	
181	-	-	-	+	+	-	-	-	-	+	+	-	-	-	+	+	-	+	-	-	-	-	-	-	-	+	-	-	1	-	-	1
182	+	-	-	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	-	-	-	-	-	-	-	+	+	-	1	-	-	1
183	-	-	-	+	+	+	+	-	-	+	+	-	-	-	+	+	+	+	-	-	-	-	-	-	-	+	+	-	1	-	-	1
184	+	+	+	+	+	+	+	+	-	+	+	-	-	-	+	+	+	+	+	-	-	-	-	+	-	+	+	-	-	-	1	1
185	+	-	-	+	+	+	+	+	-	+	+	-	-	-	+	+	-	+	-	-	-	-	-	-	-	+	+	-	-	-	1	1
186	-	-	-	+	+	+	-	-	-	+	+	-	-	-	+	+	+	+	-	-	-	-	-	-	-	+	+	-	1	2	-	3
187	+	-	-	+	+	+	+	+	-	+	+	+	-	-	+	+	+	+	-	-	-	-	-	-	-	+	+	+	1	-	-	1
188	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	1	-	1
189	+	-	-	+	+	+	+	+	-	+	+	-	-	-	+	+	+	+	-	-	+	-	-	-	-	+	+	-	1	-	-	1
190	-	-	-	+	+	-	-	-	-	+	+	-	-	-	+	+	-	+	-	-	-	-	-	-	-	+	+	-	1	-	-	1
191	+	+	+	+	+	-	+	+	-	+	+	-	-	-	+	-	-	+	-	-	-	-	-	-	-	+	-	-	1	-	-	1
192	-	-	-	+	+	+	-	-	-	+	+	-	-	-	+	+	+	+	+	-	-	-	-	-	-	+	+	-	-	6	-	6
193	+	+	-	+	+	+	+	+	-	+	+	+	-	-	+	-	-	+	+	-	-	-	-	-	-	+	+	-	-	1	-	1
194	-	-	-	+	+	+	-	-	-	+	-	+	-	-	+	+	-	+	+	-	-	-	-	-	-	+	+	-	-	1	-	1
195	-	-	-	+	+	+	-	-	-	+	+	+	-	-	+	+	+	+	-	-	-	-	-	-	-	+	+	-	-	1	-	1
196	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-	-	-	-	+	+	+	+	+	1	-	-	1
197	+	-	-	+	+	+	+	+	-	+	+	+	-	-	+	+	+	+	-	-	+	+	+	-	-	+	+	-	1	-	-	1
198	-	-	-	+	+	+	-	-	-	+	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+	+	-	1	-	-	1
199	+	+	-	+	+	+	+	+	-	+	+	+	-	+	+	+	-	+	+	-	-	-	-	-	-	+	+	-	1	-	-	1
200	+	+	-	+	+	+	+	+	-	+	+	-	-	-	+	+	-	+	+	-	-	-	-	+	-	+	+	-	-	1	-	1

(continued)

APPENDIX 33. (continued)

Group no.	Carb 1	Carb 2.5	Carb 5	Ery 1.25	Ery 2.5	Ery 10	Kan 2.5	Kan 10	Kan 20	Nal 2.5	Nal 10	Neo 2.5	Neo 10	Neo 15	Pol 5	Pol 10	Pol 20	Rif 0.25	Rif 0.50	Rif 2.50	Str 2.5	Str 10	Str 20	Tet 0.1	Tet 0.5	Van 1.25	Van 2.5	Van 10	No. of isolates in each treatment			Total
																													Control	M.C.	Liquid	
201	+	+	-	+	+	+	+	+	-	+	+	-	-	-	+	+	+	+	+	-	-	-	-	-	-	+	+	-	-	1	-	1
202	+	+	-	+	+	+	+	+	-	+	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	+	-	-	1	-	1
203	+	-	-	+	+	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	1	-	1
204	+	+	-	+	+	+	+	-	-	-	-	-	-	-	+	-	-	+	+	-	-	-	-	-	-	+	+	-	-	1	-	1

APPENDIX 34. Low level intrinsic antibiotic resistance pattern of 218 Rhizobium isolates from field A.

Group no.	Carb 1	Carb 2.5	Carb 5	Ery 1.25	Ery 2.5	Ery 10	Ery 15	Kan 2.5	Kan 10	Kan 20	Nal 2.5	Nal 10	Nal 15	Neo 1.25	Neo 2.5	Neo 10	Neo 15	Pol 5	Pol 10	Pol 20	Rif 0.25	Rif 0.5	Rif 2.5	Str 2.5	Str 10	Str 20	Tet 0.1	Tet 0.5	Van 1.25	Van 2.5	Van 10	No. of isolates in each treatment			Total
																															Control	M.C.	Liquid	
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	+	+	+	+	+	+	+	−	−	+	−	+	+	+	1	−	−	1
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	+	+	+	+	+	−	+	−	−	+	−	+	+	+	2	−	−	2
3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	+	+	+	+	+	−	−	−	−	−	−	+	+	−	−	1	−	1
4	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	−	+	+	+	+	+	−	+	+	+	+	+	+	+	+	−	1	−	1
5	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	−	−	+	+	+	+	+	+	+	−	−	+	−	+	+	+	2	−	−	2
6	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	−	−	+	+	+	+	+	−	+	−	−	+	−	+	+	+	1	−	−	1
7	+	+	+	+	+	+	+	+	+	−	+	+	+	+	−	−	−	+	+	+	+	+	+	+	−	−	+	−	+	+	+	3	−	−	3
8	+	+	+	+	+	+	+	+	+	−	+	+	+	+	−	−	−	+	+	+	+	+	−	+	+	+	−	−	+	+	+	1	−	−	1
9	+	+	+	+	+	+	+	+	+	−	+	+	+	+	−	−	−	+	+	+	+	+	−	+	−	−	+	−	+	+	+	3	−	−	3
10	+	+	+	+	+	+	+	+	+	−	+	+	+	+	−	−	−	+	+	+	+	+	−	+	−	−	−	−	+	+	+	1	−	−	1
11	+	+	+	+	+	+	+	+	+	−	+	+	+	+	−	−	−	+	+	−	+	+	−	+	−	−	+	−	+	+	+	1	−	−	1
12	+	+	+	+	+	+	+	+	+	−	+	+	−	+	+	−	−	+	+	+	+	+	−	+	−	−	+	−	+	+	−	1	−	−	1
13	+	+	+	+	+	+	+	−	−	−	+	+	+	+	−	−	−	+	+	−	+	+	−	+	−	−	+	−	+	+	+	1	−	−	1
14	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+	+	+	+	+	−	+	−	−	+	−	+	+	−	1	−	−	1
15	+	+	−	+	+	+	+	+	+	+	+	+	+	+	−	−	−	+	+	−	+	+	−	+	−	−	+	−	+	+	−	1	−	−	1
16	+	+	−	+	+	+	+	+	+	−	+	+	+	+	+	−	−	+	+	+	+	+	−	+	+	+	+	−	+	+	−	1	−	−	1
17	+	+	−	+	+	+	+	+	+	−	+	+	+	+	+	−	−	+	+	+	+	+	−	+	−	−	+	−	+	+	−	2	−	−	2
18	+	+	−	+	+	+	+	+	+	−	+	+	+	+	+	−	−	+	+	+	+	+	−	−	−	−	+	−	+	+	−	3	−	−	3
19	+	+	−	+	+	+	+	+	+	−	+	+	+	+	−	−	−	+	+	+	+	+	−	+	−	−	+	−	+	+	+	2	−	−	2
20	+	+	−	+	+	+	+	+	+	−	+	+	+	+	−	−	−	+	+	+	+	+	−	+	−	−	+	−	+	+	−	1	−	−	1

(continued)

APPENDIX 34. (continued)

Group no.	Carb 1	Carb 2.5	Carb 5	Ery 1.25	Ery 2.5	Ery 10	Ery 15	Kan 2.5	Kan 10	Kan 20	Nal 2.5	Nal 10	Nal 15	Neo 1.25	Neo 2.5	Neo 10	Neo 15	Pol 5	Pol 10	Pol 20	Rif 0.25	Rif 0.5	Rif 2.5	Str 2.5	Str 10	Str 20	Tet 0.1	Tet 0.5	Van 1.25	Van 2.5	Van 10	No. of isolates in each treatment			Total
																															Control	M.C.	Liquid	
21	+	+	-	+	+	+	+	+	+	-	+	+	+	+	-	-	-	+	+	+	+	+	-	+	-	-	-	-	+	+	+	1	-	-	1
22	+	+	-	+	+	+	+	+	+	-	+	+	+	+	-	-	-	+	+	+	+	+	-	+	-	-	-	-	+	+	-	7	-	-	7
23	+	+	-	+	+	+	+	+	+	-	+	+	+	+	-	-	-	+	+	+	+	+	-	-	-	-	+	-	+	+	-	3	-	-	3
24	+	+	-	+	+	+	+	+	+	-	+	+	+	+	-	-	-	+	+	+	+	+	-	-	-	-	-	-	+	+	+	4	-	-	4
25	+	+	-	+	+	+	+	+	+	-	+	+	+	+	-	-	-	+	+	+	+	+	-	-	-	-	-	-	+	+	-	5	-	-	5
26	+	+	-	+	+	+	+	+	+	-	+	+	+	+	-	-	-	+	+	-	+	+	-	+	+	-	-	-	+	+	+	-	1	-	1
27	+	+	-	+	+	+	+	+	+	-	+	+	+	+	-	-	-	+	+	-	+	+	-	+	-	-	+	-	+	+	+	4	-	-	4
28	+	+	-	+	+	+	+	+	+	-	+	+	+	+	-	-	-	+	+	-	+	+	-	-	-	-	+	-	+	+	-	1	-	-	1
29	+	+	-	+	+	+	+	+	+	-	+	+	+	+	-	-	-	+	-	-	+	+	-	+	-	-	+	-	+	+	+	1	-	-	1
30	+	+	-	+	+	+	+	+	+	-	+	+	+	+	-	-	-	+	-	-	+	+	-	+	-	-	+	-	+	+	-	1	-	-	1
31	+	+	-	+	+	+	+	+	+	-	+	+	+	+	-	-	-	+	-	-	+	+	-	-	-	-	-	-	+	+	-	1	-	-	1
32	+	+	-	+	+	+	+	+	+	-	+	+	+	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	+	+	-	1	-	-	1
33	+	+	-	+	+	+	+	+	+	-	+	+	+	-	-	-	-	+	+	-	+	+	-	+	-	-	+	-	+	+	-	3	-	-	3
34	+	+	-	+	+	+	+	+	+	-	+	+	+	-	-	-	-	+	+	-	+	+	-	+	-	-	-	-	+	+	-	-	1	-	1
35	+	+	-	+	+	+	+	+	+	-	+	+	+	-	-	-	-	+	+	-	+	+	-	-	-	-	-	-	+	+	-	3	-	-	3
36	+	+	-	+	+	+	+	+	+	-	+	+	+	-	-	-	-	+	-	-	+	+	-	+	+	-	-	-	+	+	-	-	1	-	1
37	+	+	-	+	+	+	+	+	+	-	+	+	+	-	-	-	-	+	-	-	+	+	-	+	-	-	+	-	+	+	-	4	-	-	4
38	+	+	-	+	+	+	+	+	+	-	+	+	+	-	-	-	-	+	-	-	+	+	-	-	-	-	-	-	+	+	-	-	2	-	2
39	+	+	-	+	+	+	+	+	+	-	+	+	+	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	+	+	-	-	1	-	1
40	+	+	-	+	+	+	+	+	-	-	+	+	+	+	-	-	-	+	+	-	+	+	-	-	-	-	-	-	+	+	-	-	-	1	1

(continued)

APPENDIX 34. (continued)

Group no.	Carb 1	Carb 2.5	Carb 5	Ery 1.25	Ery 2.5	Ery 10	Ery 15	Kan 2.5	Kan 10	Kan 20	Nal 2.5	Nal 10	Nal 15	Neo 1.25	Neo 2.5	Neo 10	Neo 15	Pol 5	Pol 10	Pol 20	Rif 0.25	Rif 0.5	Rif 2.5	Str 2.5	Str 10	Str 20	Tet 0.1	Tet 0.5	Van 1.25	Van 2.5	Van 10	No. of isolates in each treatment			Total
																															Control	M.C.	Liquid	
41	+	+	-	+	+	+	+	+	-	-	+	+	+	+	-	-	-	+	+	-	+	-	-	-	-	-	-	-	+	+	-	1	-	-	1
42	+	+	-	+	+	+	+	+	-	-	+	+	+	-	-	-	-	+	+	+	+	+	+	-	-	-	-	-	+	+	-	-	1	-	1
43	+	+	-	+	+	+	+	+	-	-	+	+	+	-	-	-	-	+	+	+	+	+	-	+	+	-	+	-	+	+	+	-	1	-	1
44	+	+	-	+	+	+	+	+	-	-	+	+	+	-	-	-	-	+	+	+	+	+	-	-	-	-	+	-	+	+	+	-	1	-	1
45	+	+	-	+	+	+	+	+	-	-	+	+	+	-	-	-	-	+	+	+	+	+	-	-	-	-	+	-	+	+	-	3	5	1	9
46	+	+	-	+	+	+	+	+	-	-	+	+	+	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	+	+	-	-	1	-	1
47	+	+	-	+	+	+	+	+	-	-	+	+	+	-	-	-	-	+	+	-	+	+	-	-	-	-	-	-	+	+	-	-	3	-	3
48	+	+	-	+	+	+	+	+	-	-	+	+	+	-	-	-	-	+	-	-	+	+	-	-	-	-	-	-	+	+	-	-	1	-	1
49	+	+	-	+	+	+	+	+	-	-	+	+	-	-	-	-	-	+	+	+	+	+	-	-	-	-	+	-	+	+	-	-	1	-	1
50	+	+	-	+	+	+	+	+	-	-	+	+	-	-	-	-	-	+	+	-	+	+	-	-	-	-	-	-	+	+	-	-	1	-	1
51	+	+	-	+	+	+	+	+	-	-	+	+	-	-	-	-	-	+	-	-	+	+	-	-	-	-	-	-	+	+	-	1	-	-	1
52	+	-	-	+	+	+	+	+	+	-	+	+	+	+	+	-	-	+	+	-	+	-	-	+	-	-	-	-	+	+	-	2	-	-	2
53	+	-	-	+	+	+	+	+	+	-	+	+	+	+	+	-	-	+	+	-	+	-	-	-	-	-	-	-	+	+	-	1	-	-	1
54	+	-	-	+	+	+	+	+	+	-	+	+	+	+	+	-	-	+	-	-	+	-	-	+	-	-	-	-	+	+	-	1	-	-	1
55	+	-	-	+	+	+	+	+	+	-	+	+	+	+	+	-	-	-	-	-	+	-	-	-	-	-	-	-	+	+	-	1	-	-	1
56	+	-	-	+	+	+	+	+	+	-	+	+	+	+	-	-	-	+	+	+	+	+	-	-	-	-	-	-	+	+	-	1	4	3	8
57	+	-	-	+	+	+	+	+	+	-	+	+	+	+	-	-	-	+	+	+	+	-	-	-	-	-	-	-	+	+	-	-	1	-	1
58	+	-	-	+	+	+	+	+	+	-	+	+	+	+	-	-	-	+	+	-	+	+	-	-	-	-	+	-	+	+	-	-	1	-	1
59	+	-	-	+	+	+	+	+	+	-	+	+	+	+	-	-	-	+	+	-	+	+	-	-	-	-	-	-	+	+	-	-	2	1	3
60	+	-	-	+	+	+	+	+	+	-	+	+	+	+	-	-	-	+	+	-	+	-	-	-	-	-	-	-	+	+	-	3	1	-	4

(continued)

APPENDIX 34. (continued)

Group no.	Carb 1	Carb 2.5	Carb 5	Ery 1.25	Ery 2.5	Ery 10	Ery 15	Kan 2.5	Kan 10	Kan 20	Nal 2.5	Nal 10	Nal 15	Neo 1.25	Neo 2.5	Neo 10	Neo 15	Pol 5	Pol 10	Pol 20	Rif 0.25	Rif 0.5	Rif 2.5	Str 2.5	Str 10	Str 20	Tet 0.1	Tet 0.5	Van 1.25	Van 2.5	Van 10	No. of isolates in each treatment			Total
																															Control	M.C.	Liquid	
61	+	-	-	+	+	+	+	+	+	-	+	+	+	+	-	-	-	+	-	-	+	-	-	-	-	-	-	-	+	+	-	4	-	-	4
62	+	-	-	+	+	+	+	+	+	-	+	+	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	+	-	1	-	-	1
63	+	-	-	+	+	+	+	+	+	-	+	+	+	+	-	-	-	-	-	-	+	-	-	+	-	-	-	-	+	+	-	1	-	-	1
64	+	-	-	+	+	+	+	+	+	-	+	+	+	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	+	-	1	-	-	1
65	+	-	-	+	+	+	+	+	+	-	+	+	+	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	+	+	-	-	1	1	2
66	+	-	-	+	+	+	+	+	+	-	+	+	+	-	-	-	-	+	+	-	+	+	-	+	+	-	+	-	+	+	-	-	1	-	1
67	+	-	-	+	+	+	+	+	+	-	+	+	+	-	-	-	-	+	+	-	+	+	-	-	-	-	-	-	+	+	-	1	1	-	2
68	+	-	-	+	+	+	+	+	+	-	+	+	+	-	-	-	-	+	-	-	+	-	-	+	-	-	+	-	+	+	-	1	-	-	1
69	+	-	-	+	+	+	+	+	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	-	2	-	-	2
70	+	-	-	+	+	+	+	+	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	1	-	-	1
71	+	-	-	+	+	+	+	+	-	-	+	+	+	+	-	-	-	+	+	+	+	+	-	-	-	-	-	-	+	+	-	-	1	1	2
72	+	-	-	+	+	+	+	+	-	-	+	+	+	+	-	-	-	+	+	-	+	-	-	-	-	-	-	-	+	+	-	1	-	-	1
73	+	-	-	+	+	+	+	+	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	2	-	-	2
74	+	-	-	+	+	+	+	+	-	-	+	+	+	-	-	-	-	+	+	+	+	+	-	-	-	-	+	-	+	+	+	-	-	1	1
75	+	-	-	+	+	+	+	+	-	-	+	+	+	-	-	-	-	+	+	+	+	+	-	-	-	-	+	-	+	+	-	-	3	1	4
76	+	-	-	+	+	+	+	+	-	-	+	+	+	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	+	+	-	-	27	1	28
77	+	-	-	+	+	+	+	+	-	-	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	+	+	-	1	-	-	1
78	+	-	-	+	+	+	+	+	-	-	+	+	+	-	-	-	-	+	+	-	+	+	-	-	-	-	-	-	+	+	-	-	1	-	1
79	+	-	-	+	+	+	+	+	-	-	+	+	+	-	-	-	-	+	-	-	+	+	-	-	-	-	-	-	+	+	-	-	4	-	4
80	+	-	-	+	+	+	+	+	-	-	+	+	+	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	+	+	-	-	1	-	1

(continued)

APPENDIX 34. (continued)

Group no.	Carb 1	Carb 2.5	Carb 5	Ery 1.25	Ery 2.5	Ery 10	Ery 15	Kan 2.5	Kan 10	Kan 20	Nal 2.5	Nal 10	Nal 15	Neo 1.25	Neo 2.5	Neo 10	Neo 15	Pol 5	Pol 10	Pol 20	Rif 0.25	Rif 0.5	Rif 2.5	Str 2.5	Str 10	Str 20	Tet 0.1	Tet 0.5	Van 1.25	Van 2.5	Van 10	No. of isolates in each treatment			Total
																															Control	M.C.	Liquid	
81	+	-	-	+	+	+	+	+	-	-	+	+	+	-	-	-	-	-	-	-	+	+	-	-	+	-	-	-	+	+	-	-	-	1	1
82	+	-	-	+	+	+	+	+	-	-	+	+	+	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	+	+	-	-	1	2	3
83	+	-	-	+	+	+	+	+	-	-	+	+	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	+	-	1	-	-	1
84	+	-	-	+	+	+	+	+	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	3	-	-	3
85	+	-	-	+	+	+	+	+	-	-	+	+	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	+	+	-	1	-	-	1
86	+	-	-	+	+	+	+	+	-	-	+	+	-	-	-	-	-	+	-	-	+	+	-	-	-	-	-	-	+	+	-	-	1	-	1
87	+	-	-	+	+	+	+	+	-	-	+	+	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	+	+	-	-	1	-	1
88	+	-	-	+	+	+	+	+	-	-	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	+	-	-	1	-	1
89	+	-	-	+	+	+	+	+	-	-	+	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	+	+	-	-	1	-	1
90	+	-	-	+	+	+	+	+	-	-	+	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	+	+	-	-	1	-	1
91	+	-	-	+	+	+	+	+	-	-	+	-	-	-	-	-	-	+	+	-	+	+	-	-	-	-	-	-	+	+	-	-	-	1	1
92	+	-	-	+	+	+	+	+	-	-	+	-	-	-	-	-	-	+	-	-	+	+	-	-	-	-	-	-	+	+	-	-	1	-	1
93	+	-	-	+	+	+	+	+	-	-	+	-	-	-	-	-	-	+	-	-	+	+	-	-	-	-	-	-	+	+	-	-	1	-	1
94	+	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	+	-	-	-	-	1	1
95	+	-	-	+	+	+	+	-	-	-	+	-	-	-	-	-	-	+	-	-	+	+	-	-	-	-	-	-	+	+	-	-	1	-	1
96	+	-	-	+	+	+	-	+	+	-	+	+	+	+	+	-	-	+	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	1	1
97	+	-	-	+	+	+	-	+	+	-	+	+	+	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	+	+	-	1	-	-	1
98	+	-	-	+	+	+	-	+	-	-	+	+	+	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	+	+	-	-	-	1	1
99	+	-	-	+	+	+	-	+	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-	+	-	+	+	-	-	1	-	1
100	+	-	-	+	-	-	-	+	+	-	+	+	+	+	+	-	-	+	-	-	+	-	-	-	-	-	-	-	+	+	-	1	-	-	1

(continued)

APPENDIX 34. (continued)

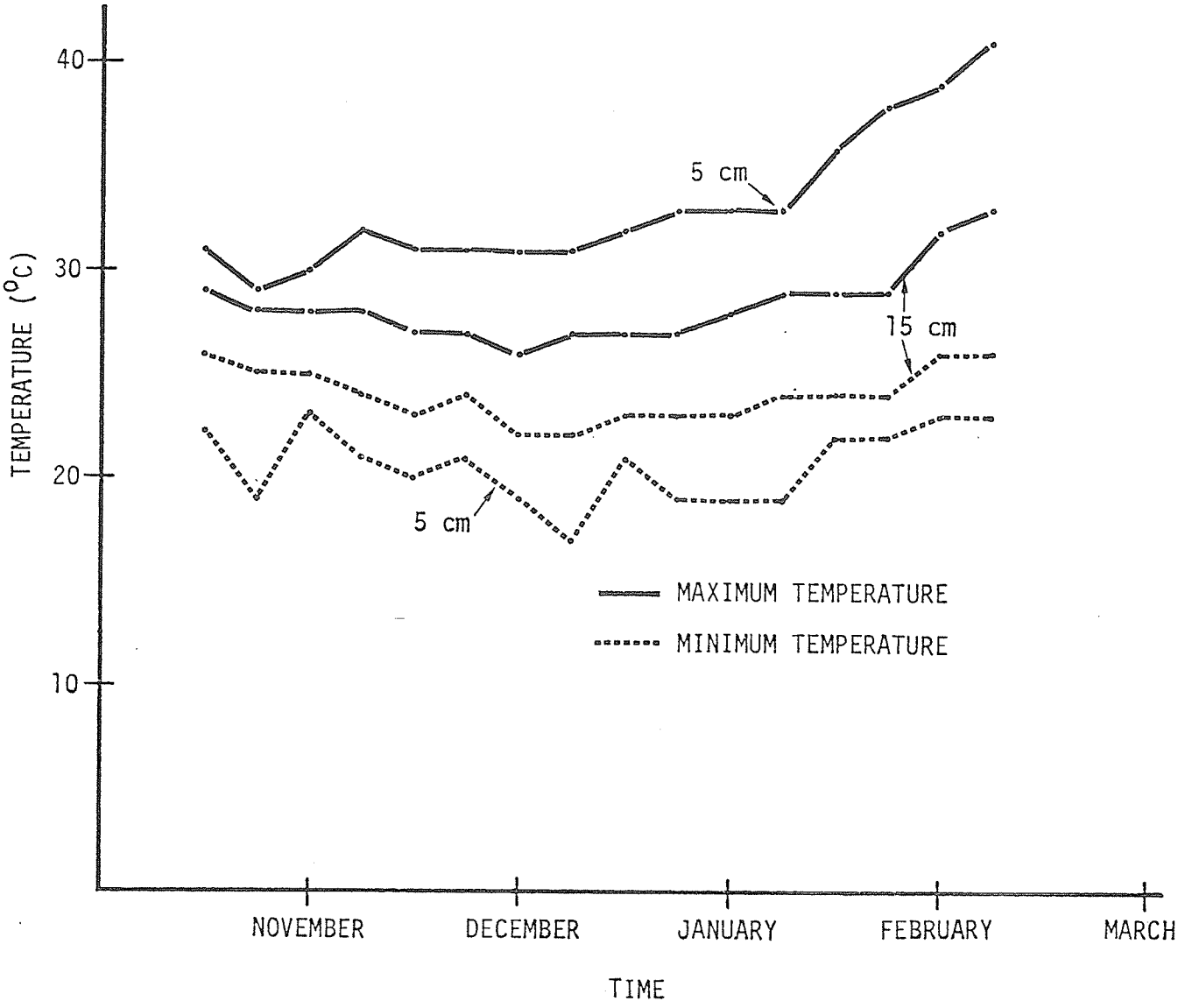
Group no.	Carb 1	Carb 2,5	Carb 5	Ery 1.25	Ery 2.5	Ery 10	Ery 15	Kan 2.5	Kan 10	Kan 20	Nal 2.5	Nal 10	Nal 15	Neo 1.25	Neo 2.5	Neo 10	Neo 15	Pol 5	Pol 10	Pol 20	Rif 0.25	Rif 0.5	Rif 2.5	Str 2.5	Str 10	Str 20	Tet 0.1	Tet 0.5	Van 1.25	Van 2.5	Van 10	No. of isolates in each treatment			Total
																															Control	M.C.	Liquid	
101	+	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	+	+	-	1	-	-	1
102	+	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	1	-	-	1
103	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	+	-	1	-	-	1
104	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	+	-	1	-	-	1
105	-	-	-	+	+	+	+	+	+	-	+	+	+	+	-	-	-	-	-	-	+	+	-	+	-	-	-	+	+	-	1	-	-	1
106	-	-	-	+	+	+	+	+	+	-	+	+	+	+	-	-	-	-	-	-	+	+	-	-	+	-	-	+	+	-	-	1	-	1
107	-	-	-	+	+	+	+	+	+	-	+	+	+	-	-	-	-	+	+	-	+	+	-	+	+	-	+	+	+	+	-	-	1	1
108	-	-	-	+	+	+	+	+	+	-	+	+	+	-	-	-	-	+	-	-	+	+	-	+	+	-	-	+	+	-	-	1	-	1
109	-	-	-	+	+	+	+	+	+	-	+	-	-	-	-	-	-	+	+	+	+	+	-	+	+	-	-	+	+	+	-	-	1	1
110	-	-	-	+	+	+	+	+	-	-	+	+	+	-	-	-	-	+	-	-	+	+	-	-	-	-	-	+	-	-	-	-	1	1
111	-	-	-	+	+	+	+	+	-	-	+	+	+	-	-	-	-	-	-	-	+	+	-	-	-	-	-	+	+	-	-	-	1	1
112	-	-	-	+	+	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	1	2	-	3
																												+	-	-	-	-	1	1

APPENDIX 35. Low level intrinsic antibiotic resistant pattern of 22 isolates from field B (Str 200 resistant) including 22 of 9036 (Str 200 mutant) control.

Pattern	Antibiotics and concentration																											Isolates		
	Carb 1	Carb 2.5	Carb 5	Ery 1.25	Ery 2.5	Ery 10	Kan 2.5	Kan 10	Kan 20	Nal 2.5	Nal 10	Neo 2.5	Neo 10	Neo 15	Pol 5	Pol 10	Pol 20	Rif 0.25	Rif 0.5	Rif 2.5	Str 2.5	Str 10	Str 20	Tet 0.1	Tet 0.5	Van 1.25	Van 2.5	Van 10	9036 control	Liquid	Methyl cellulose
1	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	13	19	-
2	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	9	2	-
3	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	1

APPENDIX 36

WEEKLY MAXIMUM AND MINIMUM SOIL TEMPERATURE MEASURED AT 5 cm AND 15 cm DEPTH (FIELD A)



APPENDIX 37. MPN of *Rhizobium* calculated by using Fisher and Yates' method. The numbers are calculated from 6 dilution steps (10^1-10^6) and 3 replications per dilution.

No. of positive tubes	No. of negative tubes	MPN/unit original sample	Log MPN
0	18	<1.73	<0.24
1	17	3.89	0.59
2	16	8.61	0.94
3	15	1.73×10^1	1.24
4	14	3.75×10^1	1.57
5	13	8.61×10^1	1.94
6	12	1.73×10^2	2.24
7	11	3.75×10^2	2.57
8	10	8.61×10^2	2.94
9	9	1.73×10^3	3.24
10	8	3.75×10^3	3.57
11	7	8.61×10^3	3.94
12	6	1.73×10^4	4.24
13	5	3.79×10^4	4.58
14	4	8.81×10^4	4.94
15	3	1.80×10^5	5.26
16	2	4.23×10^5	5.63
17	1	8.81×10^5	5.94
18	0	$>1.80 \times 10^6$	>6.26

Factor for 95% fiducial limits of MPN = $\overset{\times}{\div}$ 4.18

Factor for 95% fiducial limits of log MPN = $\pm$ 0.62

APPENDIX 38. MPN of Rhizobium calculated by using Fisher and Yates' method. The numbers are calculated from 6 dilution levels (10^1-10^6) and 6 replications per dilution.

No. of positive tubes	No. of negative tubes	MPN/unit original sample	Log MPN
0	36	< 1.57	< 0.20
1	35	2.52	0.40
2	34	3.89	0.59
3	33	5.50	0.74
4	32	8.61	0.94
5	31	1.21×10^1	1.83
6	30	1.73×10^1	1.24
7	29	2.52×10^1	1.41
8	28	3.75×10^1	1.57
9	27	5.81×10^1	1.76
10	26	8.61×10^1	1.94
11	25	1.21×10^2	2.08
12	24	1.73×10^2	2.24
13	23	2.52×10^2	2.40
14	22	3.75×10^2	2.57
15	21	5.81×10^2	2.76
16	20	8.61×10^2	2.94
17	19	1.21×10^3	3.83
18	18	1.73×10^3	3.24
19	17	2.52×10^3	3.40
20	16	3.75×10^3	3.57
21	15	5.81×10^3	3.76
22	14	8.61×10^3	3.94
23	13	1.21×10^4	4.08
24	12	1.73×10^4	4.24
25	11	2.54×10^4	4.40
26	10	3.79×10^4	4.58
27	9	5.90×10^4	4.77
28	8	8.81×10^4	4.94
29	7	1.24×10^5	5.09
30	6	1.80×10^5	5.26
31	5	2.71×10^5	5.43
32	4	4.23×10^5	5.63
33	3	6.95×10^5	5.84
34	2	8.81×10^5	5.94
35	1	1.24×10^6	6.09
36	0	> 1.80×10^6	> 6.26

Factor for 95% fiducial limits of MPN = $\overset{\times}{\div}$ 2.75

Factor for 95% fiducial limits of log MPN = $\pm$ 0.44

APPENDIX 39. MPN of *Rhizobium* estimated by using Fisher and Yates' method. The numbers are calculated from 6 four-fold dilution steps (4^1-4^6) and 4 replications per dilution.

No. of positive tubes	No. of negative tubes	MPN/unit original sample	Log MPN
0	24	<1.18	<0.07
1	23	1.59	0.20
2	22	1.74	0.24
3	21	2.79	0.45
4	20	4.42	0.65
5	19	6.30	0.80
6	18	8.89	0.95
7	17	1.71×10^1	1.23
8	16	1.80×10^1	1.25
9	15	2.54×10^1	1.40
10	14	3.61×10^1	1.56
11	13	5.12×10^1	1.71
12	12	7.25×10^1	1.86
13	11	1.03×10^2	2.01
14	10	1.46×10^2	2.16
15	9	2.08×10^2	2.32
16	8	2.97×10^2	2.25
17	7	4.28×10^2	2.63
18	6	6.15×10^2	2.28
19	5	9.06×10^2	2.96
20	4	1.33×10^3	3.12
21	3	2.03×10^3	3.31
22	2	3.24×10^3	3.51
23	1	4.29×10^3	3.63
24	0	$> 6.34 \times 10^3$	> 3.80

Factor for 95% fiducial limits of MPN = $\overset{\times}{\div}$ 2.61

Factor for 95% fiducial limits of log MPN = $\pm$ 0.42

APPENDIX 40. MPN of Rhizobium estimated by using Fisher and Yates' method. The numbers are calculated from 10 two-fold dilution steps and 4 replications per dilution.

No. of positive tubes	No. of negative tubes	MPN/unit original sample	Log MPN
0	40	<0.18	$<\bar{1}.26$
1	39	0.30	$\bar{1}.48$
2	38	0.54	$\bar{1}.73$
3	37	0.85	$\bar{1}.93$
4	36	1.21	0.08
5	35	1.59	0.20
6	34	2.02	0.31
7	33	2.52	0.40
8	32	3.08	0.49
9	31	3.71	0.57
10	30	4.47	0.65
11	29	5.33	0.73
12	28	6.37	0.80
13	27	7.58	0.88
14	26	9.03	0.96
15	25	1.08×10^1	1.03
16	24	1.28×10^1	1.11
17	23	1.53×10^1	1.18
18	22	1.82×10^1	1.26
19	21	2.17×10^1	1.34
20	20	2.58×10^1	1.41
21	19	3.09×10^1	1.49
22	18	3.69×10^1	1.57
23	17	4.41×10^1	1.64
24	16	5.26×10^1	1.72
25	15	6.30×10^1	1.80
26	14	7.56×10^1	1.88
27	13	9.08×10^1	1.96
28	12	1.09×10^2	2.04
29	11	1.32×10^2	2.12
30	10	1.59×10^2	2.20
31	9	1.95×10^2	2.29
32	8	2.37×10^2	2.37
33	7	2.91×10^2	2.46
34	6	3.62×10^2	2.56
35	5	4.56×10^2	2.66
36	4	5.83×10^2	2.77
37	3	7.67×10^2	2.88
38	2	1.06×10^3	3.03
39	1	1.48×10^3	3.17
40	0	$>2.09 \times 10^3$	>3.32

Factor for 95% fiducial limits of MPN = $\overset{\times}{\div}$ 1.41

Factor for 95% fiducial limits of log MPN = $\pm$ 0.15