EVALUATION OF NON SYMBIOTIC NITROGEN FIXATION BY *BRADYRHIZOBIUM* IN COWPEA

By

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THESIS

Submitted in partial fulfilment of the
requirement for the degree of

Master of Science in Agriculture

Faculty of Agriculture
Kerala Agricultural University

Department of Plant Pathology
COLLEGE OF HORTICULTURE
VELLANIKKARA - THRISSUR

1997

DECLARATION

I hereby declare that this thesis entitled **Evaluation of non symbiotic nitrogen fixation by *Bradyrhizobium* in cowpea** is a bonafide record of research work done by me during the course of research and that this thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title of any other University or Society.

Place : Vellanikkara

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EXTERNAL EXAMINER

Dedicated to
My beloved parents and husband

ACKNOWLEDGEMENTS

I owe my deep sense of gratitude and indebtedness to my major advisor Dr. M.V. Rajendran Pillai, Associate Professor, Dept. of Plant Pathology, College of Horticulture for the erudite guidance, constant support and constructive criticism, and the precious time spared by him during the course of this investigation and preparation of the manuscript.

It is with immense pleasure that I record my deep sense of gratitude to my advisory committee members Dr. James Mathew, Professor and Head, Dept. of Plant Pathology; Dr. Koshy Abraham, Associate Professor, Dept. of Plant Pathology and Sri. S. Krishnan, Associate Professor, Dept. of Agrl. Statistics for their unbounded support, constructive suggestions and valuable guidance throughout the course of this thesis work and for the valuable time they spared in correcting the manuscript.

I respectfully thank Dr. A.I. Jose, Associate Dean, College of Horticulture for his help and interest shown in the study.

I thankfully acknowledge the sincere help and valuable suggestions received from Smt. T.N. Vilasini, Associate Professor, Dept. of Plant Pathology and Smt. S. Beena, Asst. Professor, Dept. of Plant Pathology.

The valuable time spared by Dr. P.J. Joy, Professor, Dept. of Entomology in the statistical analysis work is gratefully remembered.

With all regards I sincerely acknowledge the wholehearted cooperation and gracious help rendered by all my friends and the staff, Dept. of Plant Pathology.

I have no words to express the boundless affection and moral support given by my husband Sri. Rajesh, N.

The encouragement given by my parents, brother, sister and my inlaws during the course of study is remembered with gratitude.

My heartfelt thanks for the unfailing helping hands extended by my friends Sindhu Mol, P., Bindu, N., Lincy Davis, Deepa Sukumar, Sree Kumari, P.K., Haseena Bhaskar and Shalini Manohar.

The award of Junior Research Fellowship by Kerala Agricultural University is gratefully acknowledged.

Above all I bow before '*The Almighty*'

ASHA, S

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INTRODUCTION

Biological nitrogen fixation is an extensively explored field by the scientists all over the world and still it continues to be. Ever increasing cost of fertilizers in terms of hard cash, utilisation of fossil energy etc. demand further research in this field to bring down the cost of fertilisers especially that of nitrogenous fertilisers. Apart from the present high cost, gradual cut in the subsidies on all fertilisers is an additional burden on the farmers. So the need to reduce the use of fertiliser nitrogen by utilising other biological systems is of immense importance.

Utilisation of the established biological nitrogen fixation by the legume-*Rhizobium* symbiosis is now being accepted world wide. This system contributes most nitrogen to the ecosystem. Cowpea is one of the most important legumes which is used both a vegetable and as a grain. Because of its short duration, adaptation to a wide range of soil types and high tolerance to acidity, it forms an important crop in tropics, especially in Kerala. Cowpea-*Rhizobium* symbiosis in fixing atmospheric nitrogen is well established and well utilised in most part of our country. Often the full potential of symbiotic nitrogen fixation is not expressed in acidic soils. In Kerala, most of the soils being acidic, full utilisation of Cowpea-*Rhizobium* symbiosis is difficult by soil inoculation of the bacteria.

The findings of many scientists (Pagan *et al.*, 1975; McComb *et al.*, 1975) that the *Bradyrhizobium* fix nitrogen in defined media and in presence of legume callus (Child and LaRue, 1974) tempted scientists to explore alternate ways for inoculation of *Bradyrhizobium*, other than conventional soil inoculation. Phylloplane

application of *Bradyrhizobium* was tried by many scientists instead of soil application and encouraging results were reported (Nandi *et al.*, 1982; Maiti and Sen, 1990).

If we are able to utilise the beneficial effects of phylloplane application of *Bradyrhizobium* instead of soil inoculation, the acidity problem of Kerala soil can be effectively bypassed. Encouraging results obtained elsewhere, as mentioned above was also inspiring. Thus the present study was taken up as project with following objectives.

1. To isolate, purify and maintain indigenous *Bradyrhizobium* cultures from various agroclimatic regions of Kerala.

2. To procure purify and maintain the known exotic strains and KAU isolate

3. To screen the *Bradyrhizobium* isolates for non-symbiotic nitrogen fixation in cowpea in a pot culture experiment.

4. To select the promising isolates, if any in terms of nitrogen content and biometric characters of cowpea.

5. To find the most suitable method of foliar application of the promising isolates.

Review of Literature

REVIEW OF LITERATURE

2.1 Root Nodule Bacteria

Beijerinck(1885) discovered that the bacteria present in the root nodules of legume now called *Rhizobium*, fix atmospheric nitrogen. Later many workers confirmed the nitrogen fixing ability of *Rhizobium* in association with legume root nodules. Hartwell and Pember(1911), studying the N-fixing phenomenon by legumes, found gains of nearly one tonne of soil nitrogen per acre in a pot culture experiment with cowpea and soyabean. Caroll (1934) established cross-inoculation relationships of forty one leguminous plant species belonging to fourteen genera. Twenty three species of *Crotalaria* and eight species of other genera were placed in the cowpea cross inoculation group. Elkan (1984) classified the fast growing rhizobial strains nodulating cicer, sesbania, leucaena, mimosa and lablab under the genus *Rhizobium* and the slow growing rhizobial strains nodulating on soyabean, lotus and cowpea under the genus *Bradyrhizobium*. Now the nitrogen fixing capacity of *Rhizobium* in symbiosis with legume root nodule is well established (Vincent, 1977).

2.2 Response of legumes to *Rhizobium* inoculation

Ruschel and Ruschel (1975), evaluated the symbiotic nitrogen fixing capacity of beans and reported that inoculation with *Rhizobium phaseoli* increased plant dry weight and total nitrogen content. Bajpai *et al.* (1974) reported a 21 per cent increase in the yield of unshelled groundnut due to *Rhizobium* inoculation. Rai *et al.* (1977) studied the differential response of *Rhizobium* strains of bengal gram and reported that yield increase due to inoculation ranged from 14 to 40 per cent. Inoculation with *Rhizobium* in soyabean plants resulted in an increased number of nodules, plant

height, number of leaves and seed yield (from 0.90 to 1.28 tonnes per ha) and reduced the number of days to flowering (Yazdi-samadi and Zali, 1978).

The symbiotic specificity in three cultivars of soyabean and four strains of *Rhizobium japonicum* was studied by Chamber (1980). He found that all the inoculation treatments showed good nodulation, and increased seed yield ranging from 3.23 tonnes per hectare to 3.6 tonnes per hectare than uninoculated plants. Girija (1982) conducted a study on host varietal specificity for *Rhizobium* for inoculation in groundnut. She used seven varieties of groundnut and isolates of the root nodule bacteria from each of the seven varieties of groundnut and reported a favourable response for all plant growth characters studied in response to inoculation with its respective homologous isolate of *Rhizobium*. Vaishya and Pandey (1983) found that the seed inoculation with *Rhizobium* strain MI increased the nodule number, nodular weight and yield increase by 42.3 percentage in mungbean.

In an experiment, Nambiar and Dart (1983) obtained an increase in the pod yield of 18 to 40 per cent in groundnut inoculated with *Bradyrhizobium*. Namdeo *et al.* (1986) studied the response of pigeonpea cultivars to *Rhizobium* strains. Results showed a significant increase in the nodulation and dry matter production. They also reported that the yield increase ranged from 0.77 tonnes per hectare to 0.82 tonnes per hectare in the first year and 0.96 to 1.0 tonnes per hectare in the second year as compared to uninoculated plants. In a trial, Chowdhary *et al.* (1983) studied the need for seasonal inoculation of soyabean with *Rhizobium* and found significant increase in the grain yield due to inoculation in first year. The highest yield of inoculation treatment was equivalent to the application of 90 kg N per hectare.

Competition between strains of rhizobia can result in reduced or delayed nodulation (Lie *et al.*, 1987). The presence of both nodulating and non-nodulating rhizobial strains in the rooting zone can result infection sites being taken by non-nodulating bacteria, preventing or reducing the establishment of an effective symbiosis. In a trial, Feng (1992) obtained an increase in yield of soyabean due to inoculation of *Rhizobium* ranging from 7.3 to 15.1 per cent. Thurral and Mishra (1993) studied the effect of *Rhizobium* strains on the yield of chickpea and found that the seed yield increased from 4.1 to 15.0 per cent by inoculation with four different strains of Rhizobia. Hoflich and Ruppel (1994) obtained an increase in growth and yield of pea due to the inoculation of effective rhizobial strains.

Ajay Kumar and Malvika Srivastava (1994) studied the efficiency of four exotic strains and three indegenous strains of *Vigna mungo* in nitrogen fixation and biomass production, found that nitrogenase activity, total nitrogen content at different ages in nodules and total biomass per plant were all highest with local strain GKP.3. An indirect competitive, enzyme linked immunosorbent assay (ELISA) was developed to identify styrains (SU391, SU303, WSM937, NZM,5472) of *Rhizobium leguminosarum* bv.viciae in the nodules of *Pisum sativum* and *Vicia faba*. The effect of legume species and indigeneous *Rhizobium leguminosarum* viciae on the nodulating competitiveness of these strains, applied as seed inoculants was also studied. It appears that the indigenous *R. leguminosarum* viciae number of at least 500 g^{-1} soil at sowing may severely reduce the nodulating competitiveness of seed applied inoculant strains with pea; and numbers much >500 g^{-1} soil, the nodulating competitiveness of inoculant strains applied to faba bean. No inoculant was remarkably better than another in competing with indigenous *R. leguminosarum* viciae. It is concluded that

seed inoculation often may be not successful with *V. faba* than *P. sativum*, which is fortuitous because indegenous *R. leguminosarum* viciae are not likely to be less efficient for N_2 fixation with faba bean than that with pea (Evans *et al.* 1996)

2.3 Response of cowpea to *Bradyrhizobium* inoculation

Crofts and Jenkins (1954) reported that inoculation of cowpea with *Rhizobium* resulted in good nodulation and plant growth. In an experiment, Gargantene and Wutke (1960) inoculated cowpea with *Rhizobium* and reported that the inoculated plants fixed nitrogen at the rate of 75 kg per hectare. Nair *et al.* (1970) obtained increased nitrogen content in cowpea plants inoculated with *Rhizobium*. Rao(1972) reported that inoculation of cowpea with *Rhizobium* resulted in 23 per cent yield increase. In an experiment, Summerfield *et al.* (1975) reported that the seed inoculation of cowpea with *Rhizobium* increased the number of pods per plant from 60 to 92. Raju (1977) conducted a study on the effect of inoculation with different strains of *Rhizobium* under different nutrient and soil conditions on cowpea variety New era. He reported that rhizobial inoculation of cowpea seeds resulted in significant increase in the dry weight of nodules, fresh weight of plants and dry weight of shoot and root. Pawar *et al.* (1977) studied the effect of seed inoculation with *Rhizobium* on grain yield of cowpea and reported that the grain yield increased from 0.62 tonnes per hectare to 1.41 tonnes per hectare. Ramachandran (1979) screened 20 rhizobial strains for efficiency in cowpea and found that strain S.17 and S.10 were more effective than others. Fresh weight and dry weight of shoot, nodule number and dry weight of nodules were significantly increased with these strains. In an experiment, Sivaprasad and Sivappashetty (1980) obtained significant increase in yield, shoot dry

weight and leghaemoglobin content of nodules in cowpea inoculated with *Rhizobium*. However, they reported that there was no significant correlation between the nodule number and plant nitrogen content on final yield. Nair and Sivaprasad (1981) reported beneficial effects due to inoculation with different isolates of rhizobia in cowpea. They could correlate symbiotic efficiency with increase in dry weight of cowpea plants. On the contrary, Mathew and Koshy (1982) reported that inoculation had no beneficial effect on plant growth, nodulation and yield of cowpea. Different varieties of cowpea behaved differently when inoculated with the same strain of *Rhizobium*. This was reported by Sohoo *et al.* (1984). In an experiment, Senanayake *et al.* (1987) found that apparent nitrogen fixation per gram of nodule fresh weight in cowpea inoculated with *Rhizobium* reached a maximum at 20 to 30 days after planting. Danso and Owiredu (1988) studied the competitiveness of introduced and indigenous cowpea *Bradyrhizobium* strains for nodule formation in cowpea in different soils. They reported the possibility of increasing nodulation, nitrogen fixation and yield of field grown cowpea through inoculation with selected strains.

Sairam *et al.* (1989) reported that seed inoculation with *Rhizobium* increased nodulation, nodule leghaemoglobin content, nitrogen uptake and dry matter content of cowpea. They also found that the dry yield was 5.14 tonnes per hectare with inoculation and 4.10 tonnes per hectare without inoculation. In an experiment, Awonaike *et al.* (1990) found that the difference in total dry matter yield obtained by *Rhizobium* inoculation was due to large variation in vegetative growth. Significant increase in nodule number, dry weight, yield and percentage nitrogen content in inoculated plants was reported by Beena *et al.* (1990) in cow pea. Gregr (1990) also reported that rhizobial inoculation increased the nitrogen uptake in cowpea. Lowther

and Patrick (1993) studied the spreading behaviour of *Rhizobium* and *Bradyrhizobium* in soil and reported that high population of root nodule bacteria occurred in the soils under established plants and in varied topography.

In an experiment, Sheikh and Tokur (1978) found that the high acidic soil reduces the germination, nodulation and growth of chickpea. In a study, Joe and Allen (1980) reported that the beneficial effects of *Rhizobium* inoculation in cowpea were maximum at pH value between 6.6 and 7.6. Evans *et al.* (1980) found that efficient nodulation and nitrogen fixation did not occur at more acidic soils by peas. Hadad and Loynachan (1985) studied about groundnut *Rhizobium* and reported that the optimum pH for their growth were in general between 6.0 and 8.0. Albrecht (1993) reported that the nodulation by *Rhizobium* in soyabean failed at a pH less than 5.0. The degree of soil acidity is responsible as an environmental factor for nodulation failure on excessively sour soils. He also found that the acidity at which this failure occurred was at pH 5.0 and lower values. In another experiment, Giller *et al.* (1994) studied the origin and diversity of rhizobia nodulating *Phaseolus vulgaris* in African soils and reported that the majority of rhizobia nodulates in a soil pH of 6.8.

2.4 Nitrogenase activity of *Rhizobium* in defined medium

Pagan *et al.* (1975) reported nitrogen fixation by *Rhizobium* cultured on a defined medium. They found that the strains of cowpea rhizobia 32HI, showed nitrogenase activity on a defined medium CS-7 and it was similar for bacteria cultured in presence or absence of host cells. Mc Comb *et al.* (1975) also found nitrogen fixation by pure culture of cowpea *Rhizobium* growing in SCN medium with or

without various supplements. They concluded that genes for nitrogenase are present in *Rhizobium*. Nitrogenase activity of *Rhizobia* in absence of host plants was again reported by Kurz and La Rue (1975). They reported that cowpea *Rhizobia* 32HI reduced acetylene when grown on a defined modified LNB medium containing galactose, arabinose on Xylose together with sucrose. No N2 fixation occurred with *Rhizobium japonicum*; if fixed N was not supplied. Ectosymbiotic N fixation by *Rhizobium* was established in defined medium (Keister, 1975) and in presence of legume callus (Child and LaRue, 1974; Reporter, 1976).

2.5 Phylloplane application of *Bradyrhizobium*

Based on the above findings, a few workers conducted studies to find the effect of foliar application of *Rhizobium* on legumes. Nandi *et al.* (1982) reported an increased nitrogen content and number of nodules on plants sprayed with *Rhizobium*. Similar studies conducted by Sen *et al.* (1985) also endorses this finding. In a trial conducted by Maite and Sen (1986), it was found that the growth and the yield of soyabean plants grown under field conditions were improved by phylloplane application of *Rhizobium*. In another study, Maite and Sen (1990) again reported that the foliar spray of *Rhizobium*, irrespective of cross inoculation groups, on the foliage of legume plants grown in N-less sand culture, improved growth parameters like height of plants, dry weight, number of nodules, dry weight of nodules, chlorophyll content and nitrogen content of the plants and this effect could be favourably compared with the seed inoculated legume. This beneficial effect was further increased to an extent of 30 per cent or more by supplementation of foliar spray with nutrients.

2.6 Foliar application of nutrients on legumes

Several studies were conducted to find the effect of foliar application of nutrients on legumes. Thimmegouda *et al.* (1974) reported higher pod and seed yields on cowpea plants sprayed with nutrients (9 per cent each of nitrogen, P_2O_5 and K_2O plus trace elements) on the foliage. Rose and Felton (1981) studied the response of soyabean varieties to foliar zinc fertilizer. They found that Zn fertilizer increased plant height, foliar Zn concentration and seed oil content but decreased leaf phosphorous content. In another study Singh *et al.* (1988) found that the plant height, chlorophyll content and seed yields of groundnut plants were improved by phylloplane application of nutrients. Abdel -aal and Ebaid (1989) reported that foliar application of some nutrient compounds increased the growth and yield of faba bean. This beneficial effect was further increased by combined application of micronutrients and ***Bradyrhizobium*** japonicum inoculation as reported by Hegazy and Hawary (1990) Ibrahim (1992) studied the effect of phosphate fertilizer and foliar spraying with Zn on growth, yield and chemical composition of soyabean. He obtained the highest seed yield, highest protein and oil yields. The effect of foliar application of boron on crop growth and yield of pigeon pea was reported by Kalyani and Devi (1993). They found that the grain yield increased with increasing boron rates up to 300 ppm and then decreased. Naik and Malvi (1993) studied the effect of irrigation and foliar nutrition on the yield of chickpea and obtained the highest grain, straw yield, pods per plant and grain per plant with two per cent potassium sulphate application and two irrigations. The nodulation and nitrogen fixation as influenced by micronutrients was reported by Bhanavse and Patil (1993). They found that the introduction of molybdenum into the soil as well as foliar application enhances the multiplication of

nodule bacteria, step up infection and improve nodule development. In a pot experiment, Amara and Nasr (1995) inoculated (by foliar application) soyabean cv. crowford with N- fixing bacteria (*Azotobactor chroococcum* and *Azospirillum* sp.) and on phosphate dissolving bacteria (*Pseudomonas flurescens* and *Bacillus megatirium*) or not inoculated and was given (by foliar application) Fe, Mn or Zn singly or combined togather 40 or 70 days after sowing. All the treatments were seed and soil inoculated with *Bradyrhizobium japonicum*. Seed yield was increased by the application of Fe, Mn or Zn and was higher with N fixing bacteria than P-dissolving bacteria except where Zn was applied. The combined inoculation of the N- fixing bacteria and P- dissolving bacteria + Fe + Mn + Zn gave the highest seed yield per plant.

Kalita and Dey (1994) examined the growth and yield characters of green gram as influenced by foliar application of phosphorous and naphthalene acetic acid. The results revealed that P alone or in combination with 50 or 100 ppm NAA was the most effective combination for improving nodular dry weight, leaf nitrogen, chlorophyll content and yield components.

Materials and Methods

MATERIALS AND METHODS

A study on the evaluation of non symbiotic nitrogen fixation by *Bradyrhizobium* in cowpea was conducted during 1993-95 at College of Horticulture, Vellanikkara, Thrissur.

Isolation of *Bradyrhizobium* was done from nodules of cowpea plants collected from twelve different locations of Kerala. Two exotic isolates and one KAU isolate were also used for the study making the total number of isolates, fifteen (Table 1).

3.1 Isolation of *Bradyrhizobium*

Healthy cowpea plants from selected localities in Kerala were used for the isolation of *Bradyrhizobium*. The plants were uprooted carefully causing minimum disturbance to the roots and nodules. Healthy nodules which appeared pink in colour, along with a little portion of root attached to it, were cut and separated. These nodules were thoroughly washed in tap water. Then they were surface sterilized with 0.1 per cent mercuric chloride solution for one minute. Nodules were then washed out in three changes of sterile water. These nodules were crushed in a few drops of sterile water. The suspension from the crushed nodules were streaked on YEMA medium in petri dishes. The composition of YEMA is given below.

Yeast Extract Mannitol Agar (YEMA) (Allen, 1953)

Mannitol	: 10.0g
K_2HPO_4	: 0.5g
$MgSO_4.7H_2O$	: 0.2g

NaCl	: 0.1g
$CaCO_3$	: 3.0g
Yeast extract	: 1.0g
Agar	: 15.0g
Congored (1 per cent aqueous solution)	: 2.5ml
Distilled water	: 1000ml
pH	: 7.0

These plates were incubated at room temperature for four days. The individual bacterial colonies showing the typical characters of *Bradyrhizobium* were later purified by repeated streaking on YEMA medium. Purified cultures were then transferred to YEMA slants without congored and kept under refrigerated condition for further studies. Like this 12 isolates were collected from different localities of Kerala.

3.2 Identity of *Bradyrhizobium*

The different isolates of *Bradyrhizobium* were observed for their cell morphology; Gram reaction and growth on glucose peptone agar media.

3.2.1 Gram staining

Gram staining was done by the following standard procedure.

Bacterial suspension was prepared and a drop was spread on a clean glass slide. It was fixed over a flame by gentle and intermittent heating. It was then stained with ammonium oxalate crystal violet solution for one minute. After

removing excess of the stain the slide was washed under a gentle stream of running tap water. It was then flooded with iodine solution for one minute and washed in 95 per cent ethanol. Then the slide was washed in a gentle stream of tap water. The slide was finally counter stained with safranin for one minute, washed under tap water, dried between folds of filter paper and examined under oil immersion objective of a light microscope for Gram reaction.

3.2.2 Growth on glucose peptone agar

Glucose peptone agar medium with the following composition was prepared.

Glucose	: 10.0 g
Peptone	: 20.0 g
NaCl	: 5.0 g
Agar	: 15.0 g
Distilled water	: 1000 ml
Bromocresol purple (1.6 per cent in ethanol)	: 10 ml
pH	: 7.2

A loopful of *Bradyrhizobium* isolate were streaked on this medium in petri plates and then incubated at 28 °C for seven days in a BOD incubator. The extent of the growth of bacterial isolates and the change of colour of the medium, if any where recorded.

3.3 Exotic cultures

The exotic cultures used in this study were 32HI and CB-756. These cultures were already reported to possess non symbiotic nitrogenase activty. So they were

selected for the present study. 32HI culture was procured from Council of Scientific and Industrial Research, Cunnigham Laboratories, Australia and the culture CB-756 was procured from Rothamstead Experimental Station, Harpenden, England.

3.3.1 Revival of the lyophilised exotic cultures

The ampoules containing the exotic culture were broken at the tip by touching a red hot glass rod. A few drops of YEMA broth was dispensed in the ampoules and the suspension was then poured on the surface of YEMA medium in petri plates. These plates were incubated for four days and the single colonies were picked up and later purified.

EXPERIMENT I

3.4 Screening of *Bradyrhizobium* isolates for non-symbiotic nitrogen fixation in cowpea

This experiment was designed as a 15x2x2 factorial experiment in Completely Randomised Design, replicated thrice. The treatments are as follows,

Factor 1 *Bradyrhizobium* isolates (I)

I_1 : Isolated from cowpea

I_2 : Isolated from cowpea

I_3 : Isolated from cowpea

I_4 : Isolated from sword bean

I_5 : Isolated from cowpea

I_6 : Isolated from cowpea

I_7 : Isolated from lucerne

I_8 : Isolated from cowpea

I_9 : Isolated from cowpea

I_{10} : Isolated from cowpea

I_{11} : Isolated from cowpea

I_{12} : Isolated from cowpea

I_{13} : exotic culture 32HI

I_{14} : exotic culture CB-756

I_{15} : KAU culture

Factor 2 Media used (M)

M_1 : CS-7 medium

M_2 : LNB-5 medium

Factor 3 Method of application (A)

A_1 : Foliar application

A_2 : Seed (soil) inoculation

Control plants were also maintained for comparison.

3.4.1 Preparation of potting mixture

Potting mixture was prepared by mixing soil, sand and powdered farm yard manure in 1:1:1 ratio, and was filled in earthen pots of size 30x15 cm.

3.4.2 Cowpea seeds

Cowpea seeds of the variety 'pusakomal', procured from the Department of Olericulture, College of Horticulture, Vellanikkara were used for the study. Seeds after presoaking for one hour were sown at the rate of three seeds per pot. The plants

were irrigated regularly. All other cultivation practises were done as per package of practice recommendations for crops (KAU, 1993).

3.4.3 Media used for the study

Two media with the following composition were used for the study. They were CS-7 and LNB-5. These media are already reported to support non symbiotic nitrogenase activity.

3.4.3.1 CS-7 Medium (Pagan *et al.*, 1975)

KH_2PO_4	: 0.003g
$CaCl_2.2H_2O$	: 0.00009g
KCl	: 0.00007g
$MgSO_4.7H_2O$	: 0.00002g
Glutamine	: 0.0003g
Myo-inositol	: 0.001g
L- arabinose	: 0.004g
Na-Succinate	: 0.004g
$MnSO_4.4H_2O$	: 0.08g
H_3BO_3	: 0.005g
$ZnSO_4.7H_2O$	: 0.001g
KI	: 0.009g
$CuSO_4.5H_2O$	: 0.001g
$Na_2MoO_4.2H_2O$	: 0.0009g
$CaCl_2.6H_2O$	: 0.0006g
$FeSO_4.7H_2O$	: 0.02g

Na_2 - EDTA : 0.02g

Thiamine : 0.01g

Nicotinic acid : 0.1g

Water : 1000ml

pH : 5.9

3.4.3.2 LNB-5 Medium

KNO_3 : 1.0g

$(NH_4)_2SO_4$: 0.05g

$MgSO_4.7H_2O$: 0.25g

$NaH_2PO_4.H_2O$: 0.15g

$CaCl_2.2H_2O$: 0.15g

Fe : 0.01g

$MnSO_4$: 0.01g

H_3BO_3 : 0.003g

$ZnSO_4.7H_2O$: 0.002g

$Na_2MoO_4.2H_2O$: 0.00025g

$CuSO_4$: 0.00003g

KI : 0.078g

Myo-inositol : 0.1g

Thiamine-HCl : 0.1g

Nicotinic acid : 0.1g

Pyridoxine : 0.2g

Sugar : 30.0g

pH : 5.5

3.4.4 *Bradyrhizobium* inoculation

3.4.4.1 Seed (soil) inoculation

Appropriate media were prepared as broth. A loopful of the isolate were inoculated into the medium and the flasks were incubated at room temperature. The culture was intermittently shaken on a rotary shaker. Six days old culture of *Bradyrhizobium* was used for inoculation. Fifty ml of the culture was poured into each pot. Soil was raked to mix the culture with soil.

3.4.4.2 Foliar inoculation

Foliar inoculation was done on the fifteenth day of sowing using a hand sprayer. Six days old cultures of isolates of *Bradyrhizobium* grown in broth of appropriate liquid media were sprayed on the leaves thoroughly so as to cover the entire surface of leaves. Before foliar application, the soil surface of pots were covered with plastic sheets to prevent the culture being dripped into the soil.

3.4.5 Observations recorded

The plants were uprooted carefully on the 55th day after sowing and observations on height of plants, number of leaves, fresh weight of plants, dry weight of plants, dry weight of roots, number of nodules, fresh weight of nodules, dry weight of nodules, total chlorophyll and nitrogen content were recorded.

The biochemical observations regarding total chlorophyll and nitrogen content were also recorded.

3.4.5.1 Height of plant

The height of plants were taken from soil level to the top most part of the plant before uprooting.

3.4.5.2 Number of leaves

Total number of leaves of individual plants were recorded.

3.4.5.3 Fresh weight of plants

Fresh weight of individual plants including roots were taken soon after harvest, including roots.

3.4.5.4 Dry weight of plants

The uprooted plants were packed separately in labelled paper bags and dried under sun for two days. They were then dried in a hot air oven at 65 °C till constant dry weights were obtained.

3.4.5.5 Dry weight of roots

The roots cut from the plants at soil level were packed air dried, and then dried in a hot air oven at 65 °C till constant weights were obtained.

3.4.5.6 Number of nodules

All the nodules present in the root system of individual plants were separated and their number recorded.

3.4.5.7 Fresh weight of nodules

Nodule fresh weight of individual plants were recorded just after uprooting.

3.4.5.8 Dry weight of nodules

The nodules removed from the plants were packed in labelled paper bags and dried in the hot air oven at 65 °C till constant dry weights were obtained.

3.4.5.9 Chlorophyll content

Total chlorophyll content of the leaves were estimated following the method of Starner and Hardley (1967).

Leaf samples were collected from the plants, cut into pieces and 0.1g of the sample was weighed into a mortar and ground with a pestle to extract the chlorophyll using 80 per cent acetone. The extract was filtered using Whattman No.1 filter paper and made upto 25 ml using 80 per cent acetone. The absorbance of the extract was read at 663 nm and 645 nm wave length in a spectrophotometer. The total chlorophyll content was calculated using the following formula.

Total Chlorophyll (mg g^{-1} of tissue)

$$= 20.0(\text{OD at } 645 \text{ nm}) + \frac{8.02 (\text{OD at } 663 \text{ nm}) \times v}{1000 \times W}$$

OD - optical density

v - final volume of 80 per cent acetone extract

W - fresh weight of tissue in gram

3.4.5.10 Nitrogen content

The nitrogen content of the dried leaves was determined by the modified Micro-Kjeldahl method of Jackson (1967).

Hundred milligrams of the dried and powdered plant sample along with 10g of digestion mixture (potassium sulphate, cupric sulphate, selenium metal powder in the ratio 10:1.0:0.1) was taken in a 100 ml Kjeldahl flask. Three ml of concentrated sulphuric acid of specific gravity 1.84 was added slowly to the digestion mixture and heated for five hours till the material was completely digested. The flasks were allowed to cool down to room temperature before adding 25 ml distilled water to each flask. After cooling, the contents were transferred to 100 ml volumetric flasks and the volume made up with distilled water. Ten ml aliquot of the sample from the volumetric flask was then added to the Kjeldahl's flask along with 10 ml of 50 per cent sodium hydroxide solution and steam distilled till about 10 ml of the distillate was collected in the receiver flask containing 10 ml of 2 per cent boric acid solution with a drop of mixed indicator. The ammoniacal nitrogen content of the distillate was determined by titration with 0.05 N H_2SO_4 and calculations were made using the following formula.

$$\text{Nitrogen content (\%)} = \frac{V \times N \times V_1 \times 0.014 \times 100}{V_2 \times w}$$

V - titre value

N - Normality of sulphuric acid

V_1 - Volume to which the sample was made upto

V_2 - Volume of sample used for distillation

w - Weight of plant sample taken

3.5 Experiment to find out the most suitable method of foliar application of promising isolates

Based on the previous experiment, two isolates were used for the study. They were KAU culture (I_{15}) and CA-756 (I_{14}). The experiment was laid out as a 5x2x2 factorial experiment replicated thrice in C.R.D. The treatments were as follows,

A- Methods of application	I- Isolate	M- Media
A_1 : Soil inoculation	I_1 : CB-756	M_1 : CS-7
A_2 : Spraying *Bradyrhizobium* in water suspension	I_2 : KAU culture	M_2 : LNB-5
A_3 : Spraying *Bradyrhizobium* grown in broth culture with shaking		
A_4 : Spraying *Bradyrhizobium* grown in broth culture without shaking		
A_5 : Smearing *Bradyrhizobium* in slurry form		

Control plants received no *Bradyrhizobial* inoculation was also set up.

Treatment combinations were,

$A_1I_1M_1$	$A_2I_1M_1$	$A_3I_1M_1$	$A_4I_1M_1$	$A_5I_1M_1$
$A_1I_1M_2$	$A_2I_1M_2$	$A_3I_1M_2$	$A_4I_1M_2$	$A_5I_1M_2$
$A_1I_2M_1$	$A_2I_2M_1$	$A_3I_2M_1$	$A_4I_2M_1$	$A_5I_2M_1$
$A_1I_2M_2$	$A_2I_2M_2$	$A_3I_2M_2$	$A_4I_2M_2$	$A_5I_2M_2$

Seeds used and the method of cultivation were all similar as in the previous experiment.

3.5.1 Seed (soil) inoculation

As done in the case of first experiment, soil inoculation was done on the second day of sowing at the rate of 50 ml per pot.

3.5.2 Foliar spray of *Bradyrhizobium* in water suspension

Six days old cultures grown in broth of appropriate media were used for spraying. Fifty ml broth culture was mixed in 100 ml water and were used for spraying in each pot. Soil was well covered with plastic sheets to protect the soil from drippings of the inoculant.

3.5.3 Foliar spray of broth shake culture

Bradyrhizobium cultures grown in broth of the two media which were kept shaking for six days were used for spraying. These cultures were sprayed on the leaves and other aerial parts of cowpea plants at the rate of 50 ml per pot.

3.5.4 Foliar spray of broth non-shake culture

Six days old cultures grown on broth of appropriate media without shaking were sprayed on the leaves and other aerial parts of cowpea. 50 ml was used in each pot.

3.5.5 Application of *Bradyrhizobium* in slurry form

Six days old cultures of *Bradyrhizobium* isolates grown in appropriate solid media were harvested, mixed well in 100 ml water and made it into a slurry form. This was uniformly applied on both sides of the leaves of cowpea plant using a brush.

3.5.6 Observations recorded

The observations recorded were same as in the case of the previous experiment after 55th day of sowing.

3.5.7 Statistical Analysis

The data of the experiment were subjected to Analysis of Variance for CRD with three factor (Snedecor and Cochran, 1967) with PC using the statistical package MSTAT-C.

Results and Discussion

RESULTS AND DISCUSSION

The results of the studies on the evaluation of non-symbiotic nitrogen fixation by *Bradyrhizobium* in cowpea are presented and discussed here.

Isolation of *Bradyrhizobium*

Twelve isolates of *Bradyrhizobium* were isolated from the nodules of legumes collected from different agroclimatic regions of Kerala. Three known isolates were also procured (Table 1).

The suspension prepared from the nodules, when streaked on YEMA medium, yielded typical large, white, gummy colonies of *Bradyrhizobium* on incubation at room temperature for four days. All isolates of the bacterium were found to be Gram negative and rod shaped. On glucose peptone agar, the growth was scanty, without any change in colour of the medium even after seven days of incubation. These characters are in confirmity with those reported for *Bradyrhizobium* by Vincent (1977). The exotic cultures revived also showed the above characters.

4.1 Screening of *Bradyrhizobium* isolates for non- symbiotic nitrogen fixation in cowpea.

The results of the experiment are given below.

4.1.1 Plant height

The variations in plant height of cowpea in response to different treatments are presented in Table 2. Effect of different *Bradyrhizobium* isolates on the height of plants was significant. Top scoring isolate I_{15}, KAU culture recorded a height of

Table 1. Details of *Bradyrhizobium* isolates used in this study

Sl. No.	Isolate	Host	Location and District	Soil type
1	I_1	Cowpea	Vellanikkara, Thrissur	Laterite
2	I_2	Cowpea	Kayamkulam, Alappuzha	Sandy
3	I_3	Cowpea	Mavelikkara, Alapuzha	Red
4	I_4	Sword bean	Kayamkulam, Alappuzha	Sandy
5	I_5	Cowpea	Kannara, Thrissur	Laterite
6	I_6	Cowpea	Vellanikkara, Thrissur	Laterite
7	I_7	Cowpea	Mannuthy, Thrissur	Laterite
8	I_8	Cowpea	Vellanikkara, Thrissur	Laterite
9	I_9	Cowpea	Mannuthy, Thrissur	Laterite
10	I_{10}	Cowpea	Ollur, Thrissur	Laterite
11	I_{11}	Cowpea	Pattambi, Palakkad	Red
12	I_{12}	Cowpea	Vakkom, Trivandrum	Red
13	I_{13}	32HI	CSIR, Cunnigham Laboratories, Australia	
14	I_{14}	CB-756	Rothamstead Experimental Station, England	
15	I_{15}	KAU culture	Kerala Agricultural University	

Table 2. Effect of different isolates, media and methods of application of *Bradyrhizobium* on the height of cowpea (cm)

Isolate	Media			Method of Application	
	M_1	M_2	Mean	A_1	A_2
I_1	29.48	32.33	30.91	28.75	33.07
I_2	32.15	32.47	32.31	32.90	31.72
I_3	32.58	29.68	31.13	31.02	31.25
I_4	34.15	31.46	32.81	33.58	32.03
I_5	35.12	32.25	33.68	33.67	33.70
I_6	33.03	30.93	31.98	33.03	30.93
I_7	29.40	30.53	29.97	29.78	30.15
I_8	31.22	31.28	31.25	31.52	30.98
I_9	34.53	33.20	33.87	34.03	33.70
I_{10}	32.87	31.12	31.99	31.10	32.88
I_{11}	33.68	31.40	32.54	31.93	33.15
I_{12}	31.88	33.85	32.87	31.87	33.87
I_{13}	37.90	37.93	37.92	37.35	38.48
I_{14}	37.78	36.27	37.03	36.78	37.27
I_{15}	40.45	40.73	40.59	40.28	40.90
Mean	33.75	33.03		33.17	33.61
Control			30.50		

Application	Media	
	M_1	M_2
A_1	33.28	33.07
A_2	34.22	32.99

	I	A	IxA	M	IxM	MxA
CD (0.05)	0.91	0.332	1.289	0.332	1.289	0.471

40.59 cm. This was statistically superior to all other isolates. The height of plants inoculated with other isolates varied from 29.97 to 37.92 cm. Excepting I_7, all the isolates recorded the plant height more than that of control plants. In I_7 treated plants, the heights were 29.97cm.

The media in which the isolates were grown also showed significant differences in increasing plant height. The media CS-7 (M_1) was significantly better than the other giving a plant height of 33.75 cm.

Method of application of *Bradyrhizobium* also significantly affected plant height. Soil inoculated (A_2) plants were significantly superior to foliar applied plants (A_1). A_2 and A_1 gave a plant height of 33.61 and 33.17 cm respectively.

The IxA interaction was found to be significant for plant height. The treatment combination $I_{15}xA_2$ showed a maximum plant height of 40.90 cm. The IxM interaction was also found to be significant. Maximum plant height of 40.73 cm was observed in plants inoculated with isolate I_{15} in media M_2. The MxA interaction was also found to be significant. Media M_1 showed superiority when applied by soil inoculation (A_2) resulting in a plant height of 34.22 cm.

4.1.2 Number of leaves

There was significant differences among treatments in the number of leaves of cowpea due to inoculation with different isolates of *Bradyrhizobium* (Table 3). Inoculation with Isoalte I_{15} (KAU culture) showed the maximum number of leaves (28.25) which was on par with isolates I_{14}. Ten isolates were found to be inferior inferior to the control plants in production of leaves.

Table 3. Effect of different isolates, media and methods of application of *Bradyrhizobium* on the number of leaves of cowpea

Isolate	Media			Method of Application	
	M_1	M_2	Mean	A_1	A_2
I_1	21.33	22.17	21.75	20.33	23.17
I_2	20.83	19.83	20.33	19.83	20.83
I_3	18.83	19.33	19.08	15.83	22.33
I_4	20.50	16.50	18.50	17.67	19.33
I_5	22.17	20.83	21.50	20.17	22.83
I_6	21.50	21.50	21.50	19.33	23.67
I_7	19.33	19.67	19.50	17.17	21.83
I_8	25.33	25.33	24.33	19.33	29.33
I_9	24.17	26.00	25.08	20.50	29.67
I_{10}	23.67	23.00	23.33	20.33	26.33
I_{11}	23.33	20.83	21.58	15.67	27.50
I_{12}	23.00	24.17	23.58	18.33	28.83
I_{13}	26.83	25.17	26.00	24.83	27.17
I_{14}	27.33	26.50	26.92	25.00	28.83
I_{15}	28.83	27.67	28.25	26.88	29.66
Mean	23.07	22.43		20.08	25.42
Control			24.00		

Application	Media	
	M_1	M_2
A_1	20.42	19.73
A_2	25.71	25.13

	I	A	IxA	M	IxM	MxA
CD (0.05)	1.92	0.70	2.71	NS	2.71	0.991

There was no significant influence for the two media used in increasing the number of leaves due to the inoculation of isolates.

Between the two methods of application, soil inoculated plants resulted in a significant increase in the number of leaves (25.42) as compared to foliar applied plants (20.08).

The IxA interaction was found to be significant for the number of leaves. Soil inoculation of *Bradyrhizobium* (A_2) with isolate I_{15} resulted in the production of maximum number of leaves (29.66). There was also a significant increase in the number of leaves of plants inoculated with isoalte I_{15} grown in media M_1.

There was significant differences between the different AxM interactions. The maximum number of leaves (25.71) were observed in plants inoculated with *Bradyrhizobium* grown in M_1 media and applied to soil (A_2).

In general, inoculation of *Bradyrhizobium* resulted in an increase in the plant height and number of leaves in cowpea. Maximum plant height and number of leaves were observed in plants inoculated with isolate I_{15} which indicate the superiority of this isolate in the growth of cowpea plants. Isolate I_{13} and I_{14} closely followed I_{15} in terms of plant height and number of leaves (Table 2 and 3). There was about 33 per cent increase in plant height and about 18 per cent increase in the number of leaves in plants inoculated with isolate I_{15} as compared to control plants. A response of increased height of plant and number of leaves in response to inoculation with *Rhizobium* was reported by Yazdi-samadi and Zali (1978) in soyabean. Improved growth in cowpea in response to inoculation with *Bradyrhizobium* was observed by

various workers (Crofts and Jenkins, 1954; Ramachandran, 1979; Sivaprasad and Sivappashetty, 1980; Awonaike *et al.*, 1990).

There was significant improvement in the plant height and number of leaves due to soil inoculation of *Bradyrhizobium* as compared to foliar application. However, the plants received foliar application were also taller than control plants indicating that foliar appliaction is also effective eventhough not as efficient as soil application. Maiti and Sen (1990) reported a similar result. They reported that growtrh characters like plant height was increased in response to foliar application, but soil inoculation was found to be more effective in legumes like soyabean, lentil and pigeon pea. The beneficial effect of foliar application could also be due to the nutrient supplementation by the media which is having enough nutrients like sucrose, phosphates, iron etc. Such an improvement in growth characters was observed by Rose and Felton (1981) in soyabean, Singh *et al.* (1988) in groundnut and Kalita and Dey (1994) in green gram.

4.1.3 Fresh weight of plants

The fresh weight of plants inoculated with different isolates of *Bradyrhizobium* showed significant difference (Table 4). The maximum fresh weight of 23.63 g was observed in plants inoculated with isolate I_{15}. This was significantly superior to all others. Plants inoculated with isolate I_{14} ranked second with a plant fresh weight of 23.03 g. The lowest value of 20.73 g was observed in plants inoculated with isolate I_7 but it was better than control plants (20.03g).

Table 4. Effect of different isolates, media and methods of application of *Bradyrhizobium* on the fresh weight of plants in cowpea (g)

Isolate	Media			Method of Application	
	M_1	M_2	Mean	A_1	A_2
I_1	22.46	22.56	22.51	21.56	23.46
I_2	22.23	22.67	22.45	20.73	24.17
I_3	21.48	22.46	22.22	20.74	23.70
I_4	21.08	21.41	21.25	19.45	23.04
I_5	22.32	22.50	22.41	22.16	22.67
I_6	21.79	21.07	21.43	20.67	22.19
I_7	20.53	20.92	20.73	20.24	21.21
I_8	20.62	21.17	20.90	19.75	22.04
I_9	21.08	21.42	21.25	21.92	20.58
I_{10}	22.67	21.88	22.27	22.08	22.44
I_{11}	21.17	21.83	21.50	21.33	21.67
I_{12}	21.79	20.36	21.08	20.88	21.28
I_{13}	22.32	21.84	22.08	21.42	22.74
I_{14}	23.36	22.69	23.03	22.17	23.88
I_{15}	23.04	24.21	23.63	22.54	24.71
Mean	21.89	21.93		21.18	22.65
Control			20.03		

Application	Media	
	M_1	M_2
A_1	21.13	21.22
A_2	22.66	22.64

	I	A	IxA	M	IxM	MxA
CD (0.05)	0.006	0.0253	0.978	0.0253	0.978	0.357

A significant influence in the fresh weight of plants due to the effect of different media was observed. Media M_2 was found good in giving maximum fresh weight of plants (21.93 g).

Soil inoculation of *Bradyrhizobium* isolates resulted in a significant increase in the fresh weight of plants. Foliar inoculated plants showed a lower value of 21.18 g as compared to 22.65 g in the case of soil inoculated plants.

All the interactions, IxA, IxM and MxA were found to be significant with respect to the fresh weight of plants. A maximum fresh weight of 24.71 g was observed in plants inoculated with I_{15} in soil. Media M_2 was found to be superior with isolate I_{15} with a maximum fresh weight of 24.21 g. A significantly higher plant fresh weight of 22.66 g was observed in soil inoculated plants with isolate grown in media M_1 (Table 4).

4.1.4 Dry weight of plants

The values of dry weigt of plants inoculated with different isolates of *Bradyrhizobium* are given in Table 5. All the inoculated plants showed an increase in dry weight as compared to the control plants. Plants inoculated with isolate I_{15} was observed with a maximum value of 4.95g. Those inoculated with isolates I_{14} and I_{13} were also statistically on par with plants inoculated with isolate I_{15}. The lowest value (3.88g) was observed in plants inoculated with isolate I_4. Even this was better over control plants where the dry weight was 3.23 g.

Dry weight of cowpea plants did not show significant influence due to the effect of different media. Method of application was also not significant. However, soil inoculated plants (A_2) showed a higher value for dry weight (4.82g) than foliar applied plants (3.87g).

Table 5. Effect of different isolates, media and methods of application of *Bradyrhizobium* on the dry weight of plants in cowpea (g)

Isolate	Media			Method of Application	
	M_1	M_2	Mean	A_1	A_2
I_1	4.66	4.53	4.60	4.18	5.12
I_2	4.38	4.35	4.37	3.58	5.16
I_3	4.05	4.37	4.21	3.71	4.71
I_4	3.90	3.86	3.88	3.10	4.66
I_5	4.12	4.53	4.32	4.14	4.50
I_6	4.33	4.63	4.48	3.83	5.13
I_7	3.74	4.12	3.93	3.11	4.75
I_8	4.04	4.83	4.43	3.48	5.39
I_9	4.28	4.75	4.51	4.28	4.74
I_{10}	4.09	3.84	3.97	3.57	4.36
I_{11}	4.10	4.15	4.13	4.08	4.18
I_{12}	4.23	3.68	3.96	3.83	4.08
I_{13}	4.75	4.66	4.70	4.75	4.66
I_{14}	4.98	4.62	4.80	4.14	5.46
I_{15}	4.77	5.13	4.95	4.32	5.58
Mean	4.30	4.40		3.87	4.82
Control			3.23		

Application	Media	
	M_1	M_2
A_1	3.78	3.97
A_2	4.81	4.84

	I	A	IxA	M	IxM	MxA
CD (0.05)	0.31	NS	0.435	NS	0.435	0.158

There were significant difference in IxA interaction with a maximum dry weight of 5.58 g recorded in plants soil inoculated with isolate I_{15}. Isolate I_{15}, grown in M_2 medim was most effective in giving maximum dry weight of 5.13 g. MxA interaction was also found to be significant with a maximum plant dry weight of 4.84 g in soil inoculated plants applied with the bacterium grown in medium M_2.

4.1.5 Dry weight of root

The inoculation of cowpea plants with different *Bradyrhizobium* isolates showed significant influence in the root dry weight. The maximum root dry weight of 0.810 g was observed in plants inoculated with isolate I_{14} which was statistically on par with plants inoculated with isolate I_{13} (Table 6). The other values were in the range of 0.789 (I_{15}) to 0.650 (I_2).

Different media in which *Bradyrhizobium* was grown before inoculation significantly influenced the root dry weight of cowpea plants. Medium M_1 was found superior to medium M_2. Soil inoculated plants (A_2) gave root dry weight of 1.21 g which was significantly higher than foliar applied plants whose root dry weight was 0.278 g.

Interactions among isolates, media and method of application were found to be significant for dry weight of roots in cowpea. Those plants inoculated with isolate I_{15} in soil recorded a root dry weight of 1.295 g. However, plants inoculated with isolate I_{14} and grown in media M_1 recorded the maximum root dry weight of 0.811 g. The MxA interaction was also found to be significant with a maximum root dry weight of 1.21 g in plants inoculated with isolate grown in media M_1 (Table 6) and applied to soil.

Table 6. Effect of different isolates, media and methods of application of *Bradyrhizobium* on the dry weight of root of cowpea (g)

Isolate	Media			Method of Application	
	M_1	M_2	Mean	A_1	A_2
I_1	0.745	0.735	0.740	0.302	1.178
I_2	0.636	0.665	0.650	0.184	1.117
I_3	0.795	0.753	0.774	0.358	1.190
I_4	0.728	0.760	0.744	0.327	1.162
I_5	0.777	0.775	0.776	0.340	1.212
I_6	0.737	0.687	0.712	0.198	1.225
I_7	0.668	0.663	0.666	0.157	1.175
I_8	0.773	0.735	0.754	0.278	1.230
I_9	0.752	0.673	0.713	0.222	1.203
I_{10}	0.747	0.727	0.737	0.307	1.167
I_{11}	0.687	0.725	0.706	0.243	1.168
I_{12}	0.762	0.763	0.763	0.307	1.218
I_{13}	0.792	0.808	0.800	0.331	1.270
I_{14}	0.811	0.808	0.810	0.330	1.290
I_{15}	0.784	0.793	0.789	0.282	1.295
Mean	0.750	0.740		0.280	1.210
Control			0.520		

Application	Media	
	M_1	M_2
A_1	0.28	0.27
A_2	1.21	1.20

	I	A	IxA	M	IxM	MxA
CD (0.05)	0.016	0.0061	0.023	0.0061	0.023	0.008

Biometric observations like total plant fresh weight, dry weight and root dry weight were all found to be improved as a result of inoculation with *Bradyrhizobium* irrespective of isolates used. There was a 56 per cent increase in root dry weight in plants inoculated with isolate I_{14} whereas in I_{15} inoculated plants, the plant dry weight production was increased to 53 per cent over control. However, the total dry matter production of plants inoculated with isolate I_{14} and I_{13} were also on par with that of I_{15} plants. Sairam *et al.* (1989) reported an increased dry matter production n cowpea as a result of soil inoculation with *Rhizobium*. The results of the present study also confirms with the findings of Sivaprasad and Sivappashetty (1980), where an increase in shoot dry weight was observed in response to inoculation with *Rhizobium.* Eventhough, the lowest total plant dry weight was observed in plants inoculated with isolate I_4, these plants showed a 20 per cent increase in the dry weight over control. This indicate that all the isolates tried in the present study had a beneficial effect in the total dry matter production of the cowpea plants over uninoculated plants.

In general, soil inoculation of *Bradyrhizobium* significantly increased the plant growth characters like total plant fresh weight and root dry weight. Total plant fresh weight of soil inoculated plants was increased to the tune of seven per cent as compared to the foliar applied plants which also showed a 5.7 per cent increase over the control plants. This emphasises the fact that foliar application is effective though not as efficient as soil inoculation. Such an improvement in growth characters like root length and plant dry weight as a result of foliar application of *Bradyrhizobium* was observed by Maiti and Sen (1986) in soyabean.

4.1.6 Number of nodules

Inoculation of different isoaltes of *Bradyrhizobium* resulted in a significant increase in the number of nodules (Table 7). The maximum number of nodules (19.33) was observed in isolate I_{15} and it was statistically on par with isolate I_{13} (19.17) and I_{14} (18.25). The lowest number of nodules (14.08) was observed in plants inoculated with isolate I_2. The control plants which received no inoculation, the plants had only eight nodules. The two different media in which the bacteria were grown did not significantly affect the number of nodules formed in cowpea.

Method of application had significant influence in increasing the number of nodules. Number of nodules significantly increased in soil inoculated plants. Foliar application of *Bradyrhizobium* showed a statistically inferior performance in providing the number of nodules (9.49) as compared to the soil inoculated plants (23.53).

The interaction between the isolate and method of application (IxA) was found to be significant. The maximum number of nodules (28.00) was recorded in plants inoculated with isolate I_{15} in soil. In general soil appplication was better than foliar application with respect to IxM interaction. Maximum number of nodules was seen in plants inoculated with isolate I_{15} grown in media M_2. In this combination, the nodule number was 20.67.

MxA interaction was also found to be significant. Maximum number of nodules (23.60) was observed in plants grown in media M_2 and inoculated in soil (A_2).

Table 7. Effect of different isolates, media and methods of application of *Bradyrhizobium* on the number of nodules of cowpea

Isolate	Media			Method of Application	
	M_1	M_2	Mean	A_1	A_2
I_1	16.50	16.50	16.50	9.83	23.17
I_2	14.00	14.17	14.08	7.50	20.67
I_3	17.33	16.83	17.08	10.67	23.50
I_4	15.67	16.00	15.83	11.33	20.33
I_5	17.67	16.83	17.25	10.67	23.83
I_6	16.17	15.17	15.67	7.33	24.00
I_7	14.67	14.83	14.75	7.67	21.83
I_8	16.50	16.00	16.25	8.33	24.17
I_9	16.17	15.00	15.58	7.67	23.50
I_{10}	16.00	15.17	15.58	9.50	21.67
I_{11}	14.83	16.33	15.58	8.50	22.67
I_{12}	17.00	16.50	16.75	10.33	23.17
I_{13}	19.33	19.00	19.17	11.50	26.83
I_{14}	18.00	18.50	18.25	10.83	25.67
I_{15}	18.00	20.67	19.33	10.67	28.00
Mean	16.52	16.50		9.49	23.53
Control			8.00		

Application	Media	
	M_1	M_2
A_1	9.58	9.40
A_2	23.47	23.60

	I	A	IxA	M	IxM	MxA
CD (0.05)	1.44	0.525	2.04	NS	2.04	0.744

4.1.7 Fresh weight of nodule

Inoculation of cowpea plants with different *Bradyrhizobium* isolates showed significant differences in improving the fresh weight of nodules (Table 8). Plants inoculated with isolate I_{13} was found to be statistically superior than the others in giving a nodule fresh weight of 0.272 g. This was followed by plants inoculated with isolate I_{14} (0.258) and isolate I_{15} (0.248). All other isolates were inferior in increasing fresh weight of nodules. Isolate I_7 which gave only 0.175 g of nodule was the lowest.

The effect of different media on the fresh weight of nodules differed significantly. Plants inoculated with the isolate grown in media M_1 had given 0.230 g fresh weight of nodules as compared to 0.224 g in plants applied with *Bradyrhizobium* grown in M_2 media.

Soil inoculation (A_2) of *Bradyrhizobium* resulted in a significant increase in the fresh weight of nodule in cowpea plants. This was significantly higher than the foliar application. A higher value of 0.367g of fresh weight of nodules in soil inoculated plants was observed as compared to 0.086 g of foliar inoculated plants.

Plants with soil inoculated isolates I_{13} and I_{15} resulted in a significant increase in the fresh weight of nodules in cowpea. Both of these isolates on soil inoculation gave 0.418 g of nodule fresh weight. With respect to IxM interactions, those inoculated with isolate I_{14} grown in media M_2 showed maximum fresh weight of nodules, giving a value of 0.268 g.

Table 8. Effect of different isolates, media and methods of application of *Bradyrhizobium* on the fresh weight of nodules of cowpea (g)

Isolate	Media			Method of Application	
	M_1	M_2	Mean	A_1	A_2
I_1	0.227	0.223	0.225	0.103	0.347
I_2	0.182	0.195	0.188	0.052	0.325
I_3	0.217	0.245	0.231	0.120	0.342
I_4	0.210	0.227	0.218	0.095	0.342
I_5	0.255	0.210	0.232	0.108	0.357
I_6	0.250	0.195	0.222	0.055	0.390
I_7	0.175	0.175	0.175	0.037	0.313
I_8	0.247	0.218	0.232	0.082	0.383
I_9	0.243	0.198	0.221	0.055	0.387
I_{10}	0.198	0.202	0.200	0.080	0.320
I_{11}	0.207	0.235	0.221	0.075	0.367
I_{12}	0.260	0.243	0.252	0.117	0.387
I_{13}	0.272	0.268	0.270	0.122	0.418
I_{14}	0.258	0.268	0.263	0.112	0.415
I_{15}	0.248	0.255	0.252	0.085	0.418
Mean	0.230	0.224		0.086	0.367
Control			0.106		

Application	Media	
	M_1	M_2
A_1	0.090	0.082
A_2	0.369	0.365

	I	A	IxA	M	IxM	MxA
CD (0.05)	0.009	0.003	0.012	0.003	0.012	0.004

MxA interaction was significant . The maximum fresh weight of nodule 0.369 g was observed in soil inoculated plants applied with M_1 media (Table 8).

4.1.8 Dry weight of nodules

The dry weight of nodules inoculated with different *Bradyrhizobium* isolates grown in different media and methods of application are given in Table 9. Plants inoculated with isolates I_{15} and I_{14} showed a maximum dry weight of nodules, 0.059 and 0.057 g respectively and were statistically on par. These isolates were superior over control. The lowest nodule dry weight was observed in plants inoculated with isolate I_7.

Dry weight of nodules differed significantly due to the effect of different media in which the isolates were grown. Plants inoculated with isolates grown in media M_2 gave 0.045 g as compared to 0.043 g in plants inoculated with isolates grown in media M_1.

Soil inoculated plants showed a significant increase in dry weight of nodules (0.047 g) as compared to foliar applied plants (0.041 g).

There was a significant increase in the dry weight of nodules of cowpea plants inoculated in soil with isolate I_{14}. Isolate I_{15} in media M_2 resluted in maximum dry weight of nodule (0.062 g).

There was a significant difference between the MxA interaction also. A maximum nodular dry weight of 0.048 g was observed in plants inoculated in soil with *Bradyrhizobium* grown in media M_2.

Table 9. Effect of different isolates, media and methods of application of *Bradyrhizobium* on the dry weight of nodules of cowpea (g)

Isolate	Media			Method of Application	
	M_1	M_2	Mean	A_1	A_2
I_1	0.042	0.037	0.039	0.037	0.042
I_2	0.040	0.038	0.039	0.027	0.052
I_3	0.050	0.0342	0.046	0.052	0.040
I_4	0.033	0.040	0.037	0.025	0.045
I_5	0.047	0.048	0.047	0.040	0.053
I_6	0.047	0.047	0.047	0.040	0.053
I_7	0.032	0.037	0.034	0.033	0.035
I_8	0.043	0.048	0.046	0.038	0.053
I_9	0.047	0.048	0.047	0.043	0.052
I_{10}	0.035	0.038	0.037	0.038	0.035
I_{11}	0.040	0.040	0.040	0.043	0.037
I_{12}	0.035	0.037	0.036	0.035	0.037
I_{13}	0.045	0.056	0.048	0.050	0.045
I_{14}	0.058	0.057	0.057	0.053	0.062
I_{15}	0.057	0.062	0.059	0.058	0.060
Mean	0.043	0.045		0.041	0.047
Control			0.019		

Application	Media	
	M_1	M_2
A_1	0.041	0.041
A_2	0.046	0.048

	I	A	IxA	M	IxM	MxA
CD (0.05)	0.006	0.0019	0.0078	0.0019	0.0078	0.0027

Inoculation of the cowpea plants with *Bradyrhizobium* increased the number of nodules, fresh weight of nodule and nodule dry weight (Table 7,8,9). Maximum number of nodules and nodule dry weight was observed in plants inoculated with isolate I_{15} followed by isolate I_{13} and I_{14}. Inoculation with isolate I_{15} increased nodulation to the extent of 141.6 per cent over control. There was 215.7 per cent increase in dry weight of nodules in plants inoculated with isolate I_{15}. A comparison between I_{15} which is the best performer and I_7 which is one of the poor performer as far as number of nodules and dry weight of nodules are concerned, reveals that the best performer I_{15} is having a high value for dry weight per nodule (0.003 g/nodule) than the poor performer (0.002 g/nodule). This points to the fact that increased dry weight per nodule may be an indicator for efficiency. An increased nodulation and nodular dry weight due to inoculation with *Bradyrhizobium* was reported by several workers (Garg and Beri, 1982; Senanayake, 1987; Sairam *et al.*,1989; Beena *et al.*, 1990). Plants inoculated with isolate I_2 recorded the least number of nodules and nodular dry weight. This could be due to the fact that this particular strain was obtained from a sandy soil (Table 1) which might not be suitable for another soil type. Such poor performance of strains when inoculated in other soil types have been reported by Rossum *et al.* (1994).

4.1.9 Chlorophyll content

Plants inoculated with different *Bradyrhizobium* isolates differed significantly in their chlorophyll content (Table 10). The maximum chlorophyll content was observed in plants inoculated with isolate I_8 (2.145 mg /g). Nine other isolates were also on par with I_8. I_3 was the most inferior isolate recording only 2.030 mg/g of chlorophyll. All other isolates were better than I_3.

Table 10. Effect of different isolates, media and methods of application of *Bradyrhizobium* on the leaf chlorophyll content of cowpea (mg/g)

Isolate	Media			Method of Application	
	M_1	M_2	Mean	A_1	A_2
I_1	2.132	2.140	2.136	2.117	2.155
I_2	2.057	2.095	2.076	2.120	2.032
I_3	2.012	2.048	2.030	2.090	1.970
I_4	2.105	2.105	2.105	2.130	2.080
I_5	2.140	2.140	2.140	2.140	2.140
I_6	2.120	2.120	2.120	2.090	2.150
I_7	2.143	2.137	2.140	2.125	2.155
I_8	2.145	2.145	2.145	2.150	2.140
I_9	2.090	2.085	2.087	2.090	2.085
I_{10}	2.137	2.133	2.135	2.123	2.147
I_{11}	2.110	2.113	2.112	2.123	2.090
I_{12}	2.135	2.137	2.136	2.122	2.150
I_{13}	2.140	2.135	2.135	2.130	2.140
I_{14}	2.147	2.140	2.140	2.140	2.140
I_{15}	2.116	2.138	2.143	2.148	2.137
Mean	2.116	2.121		2.123	2.114
Control			2.090		

Application	Media	
	M_1	M_2
A_1	2.119	2.128
A_2	2.114	2.114

	I	A	IxA	M	IxM	MxA
CD (0.05)	0.013	0.0047	0.017	NS	0.017	0.006

There was no significant influence in the chlorophyll content of cowpea plants inoculated with the bacteria grown in different media. But it differed significantly due to the effect of different methods of application. Foliar inoculated plants showed a maximum of 2.123 mg/g chlorophyll compared to 2.114 mg/g of soil inoculated plants.

IxA interactions were found to be significant. Maximum chlorophyll content was observed in soil inoculated plants with isolate I_{15} and I_{14}. IxM was also signficant with maximum chlorophyll content in plants inoculated with isolate I_{15} in media M_2.

A significantly higher leaf chlorophyll content of 2.128 mg/g was observed in foliar applied plants with isolate grown in media M_2. This combination was also significantly better than other combinations.

Inoculation of *Bradyrhizobium* isolates increased the leaf chlorophyll content of cowpea plant. An increase of 2.9 per cent was observed in plants inoculated with I_8. However, the plants inoculated with I_{15} and I_{14} also showed a 2.4 per cent increase which were statistically on par with I_8 plants. An increase in chlorophyll content could be due to the increased metabolic rates.

The chlorophyll content of foliar applied plants were significantly above that of the soil inoculated plants (Table 10). The increased leaf chlorophyll content in foliar applied plants might be due to the foliar uptake of the nutrient elements from the bacterial metabolites. Such an increased chlorophyll content in response to foliar nutrition was observed by many workers (Singh *et al.*,1988 ; Kalita and Dey,1994). An improved chlorophyll content in response to foliar application of *Rhizobium* in soyabean plants observed by Maiti and Sen (1990) also confirms the findings of the

present experiement.

4.1.10 Nitrogen content

The effect of different isolates of *Bradyrhizobium* on the nitrogen content of the leaves of cowpea plants are given in Table 11. There was significant difference among the different isolates. The highest leaf nitrogen content of 3.107 per cent was observed in plants inoculated with isolate I_1. Isolate I_{15} and I_9 were on par with I_1. The lowest leaf nitrogen content was shown by plants inoculated with isolate I_{11} (2.713 %). This isolate gave a nitrogen content lesser than the uninoculated control plants.

The effect of two different media on leaf nitrogen content shows significant difference. Plants inoculated with isolate grown in media M_1 resulted in a higher leaf nitrogen content (2.980 %) compared to M_2 (2.966%).

The soil inoculated plants (A2) recorded a significant increase in the leaf nitrogen content of cowpea plants. The leaf nitrogen content of soil inoculated plants was 3.069 per cent whereas the foliar applied plants was 2.877.

All the interactions of treatments showed significance in influencing the nitrogen content of leaves. I_6M_2, I_1A_2 and A_2M_1 were the best interactions giving 3.135, 3.240 and 3.080 per cent nitrogen respectively.

In general, the leaf nitrogen content recorded a significant increase in the *Bradyrhizobium* inoculated plants. About 3 per cent was observed in plants inoculated with isolate I_1 which was closely followed by plants inoculated with isolate I_{15} and I_9. An increased nitrogen in I_1 inoculated plants could be due to the fact that indigenous strains are more efficient in fixing nitrogen. The isolate I_1 was from the native soil.

Table 11. Effect of different isolates, media and methods of application of *Bradyrhizobium* on the leaf nitrogen content of cowpea (%)

Isolate	Media			Method of Application	
	M_1	M_2	Mean	A_1	A_2
I_1	3.108	3.105	3.107	2.973	3.240
I_2	2.875	2.943	2.909	2.908	2.910
I_3	3.128	3.002	3.065	3.055	3.075
I_4	3.068	3.017	3.043	2.942	3.143
I_5	2.985	2.960	2.972	3.088	2.857
I_6	3.018	3.135	3.077	3.203	2.950
I_7	3.027	3.105	3.066	2.967	3.165
I_8	2.875	3.000	2.938	2.893	2.982
I_9	3.100	3.073	3.087	2.967	3.207
I_{10}	2.957	2.590	2.773	2.558	2.988
I_{11}	2.682	2.743	2.713	2.328	3.097
I_{12}	3.027	3.028	3.027	2.967	3.088
I_{13}	2.928	2.953	2.941	2.973	2.908
I_{14}	2.817	2.768	2.792	2.362	3.223
I_{15}	3.103	3.072	3.088	2.968	3.207
Mean	2.980	2.966		2.877	3.069
Control			2.730		

Application	Media	
	M_1	M_2
A_1	2.88	2.87
A_2	3.08	3.06

	I	A	IxA	M	IxM	MxA
CD (0.05)	0.022	0.008	0.031	0.008	0.031	0.0113

An increased nitrogen fixation due to indigenous strains was reported by Ajay Kumar and Malvika (1994). Plants inoculated with isolate I_{15} and I_9 also were statistically on par with I_1. Increased nitrogen content in cowpea plants due to inoculation with *Bradyrhizobium* was reported by various workers (Gargantine and Wutke, 1960; Nair *et al.*, 1970; Sivaprasad and Sivappashetty, 1980; Beena *et al.*, 1990).

Soi inoculation of *Bradyrhizobium* significantly increased the leaf nitrogen content by 12.4 per cent above the control as compared to 5.4 per cent increase inthe foliar applied plants. An increased nitrogen content on crop plants sprayed with *Rhizobium* was reported by Nandi *et al.* (1982). Experiments conducted by Sen *et al.* (1985) also confirms the increased nitrogen content due to foliar application. Maiti and Sen (1990) points out that foliar sptray of *Rhizobium* improved the nitrogen content of legume plants and this effect could be favourably compared with soil inoculated legumes. The results suggests that eventhough foliar application is less efficient than soil inoculation with respect to total nitrogen content of plant, foliar application can also be resorted to in improving crop productivity.

From the precceding discussions, it is clear that the difference between control and *Bradyrhizobium* treated plants were highly significant in nitrogen fixation on the leaf surface. A comparison of soil inoculation and foliar application of *Bradyrhizobium* to exploit any possible non-symbiotic nitrogen fixation, confirms the unquestionable superiority of soil inoculation. However, foliar application also improved many of the growth parameters of the plant significantly over control plants. Thus, eventhough less efficient, foliar application can also be advocated in cowpea

Table 12. Top ranking isolates based on their efficiency on foliar application

Sl No.	Growth parameters	Rank		
		I	II	III
1	Height of plants	I_{15}	I_{13}	I_{14}
2	Number of leaves	I_{15}	I_{14}	I_{13}
3	Dry weight of roots	I_3	I_{13}	I_{14}
4	Number of nodules	I_3	I_4	I_{14}
5	Fresh weight of nodules	I_{13}	I_3	I_{12}
6	Dry weight of nodules	I_{15}	I_{14}	I_3
7	Fresh weight of plants	I_{15}	I_{14}	I_3
8	Dry weight of plants	I_{13}	I_{15}	I_9
9	Chlorophyll content	I_{13}	I_{15}	I_9
10	Nitrogen content	I_{15}	I_1	I_{13}

plants. Whether the improvement of plants performance is due to non-symbiotic nitrogen fixation or due to foliar nutrient uptake is yet to be found out. The results summarised in Table 12 shows that the effect of foiar application was remarkable with isolate I_{15}, which showed superior ranking in five out of ten characters studied. In two of the characters, it ranked second. Hence, I_{15} can undoubtedly be selected as a promising isolate. The second best performing isolate was I_{14} which again maintained good ranking. Eventhough, it does not appeared in the superior ranking in any of the plant characters, I_{14} showed better performance in subsequent rankings. Further, this culture is an exotic proven culture in its efficiency in non-symbiotic nitrogen fixation and hence this also was selected. Thus ultimately based on the above mentioned studies, isolates I_{15} and I_{14} were selecetd as the best performing isolates to be tried in Experiment II.

4.2. Pot culture experiment to find the most suitable method of foliar application

Two isolates namely I_{14} and I_{15} selected based on the previous experiment were used for this study designated as isolate I_1 and I_2 respectively. The major objective was to compare the efficiency of different methods of application of *Bradyrhizobium* on cowpea plants. Five methods of application of these isolates grown in two media were tried. Results are discussed here.

4.2.1 Height of plant

All the different methods of applications, resulted in a significant increase in plant height. Soil inoculated plants (A_1) showed maximum plant height with a value of 36.23 cm (Table 13). All the other methods of application A_2, A_3, A_4 and A_5

Table 13. Effect of different methods of application isolates and the media used on the height of cowpea (cm)

Method of Application	Isolates			Media	
	I_1	I_2	Mean	M_1	M_2
A_1	35.90	36.57	36.23	36.43	36.03
A_2	33.03	31.80	32.42	32.67	32.17
A_3	31.92	32.42	32.17	32.10	32.23
A_4	31.78	32.63	32.21	31.83	32.58
A_5	32.75	31.90	32.33	32.35	32.30
Mean	33.08	33.06		33.07	33.06
Control			31.00		

Media	Isolate	
	I_1	I_2
M_1	32.99	33.16
M_2	33.16	32.97

	A	I	IxA	M	IxM	MxA
CD(0.05)	0.488	NS	0.436	NS	NS	NS

showed increase in plant height compared to the control plants.

There was no significant effect of media in which the isolates were grown on plant height. The interaction between the isolates and the media were also not significant. The IxA interaction was significant with maximum values observed in treatment, where soil was inoculated with isolate I_2 (A_1I_2). The MxA interactions did not show any significant differences.

4.2.2 Number of Leaves

The number of leaves observed in response to different methods of application are given in Table 14. There was significant difference in the number of leaves under different methods of application of the bacterium. Maximum number of leaves (30.42) was found in A_1 and A_4 treatment. The isolate I_2 significantly increased the number of leaves to 30.13 per plant. In control plants there were only 24 leaves per plant. The media used and the interactions of media with isolates and media with methods of application were not significant. Isolate I_2 was found to be superior when soil inoculation was done resulting in an increase in number of leaves to the extent of 31.50.

The data presented in Table 13 showed that foliar spray of *Bradyrhizobium* resulted in the growth responses, which although inferior to soil treatment were quite remarkable. Maximum plant height and number of leaves were observed in soil inoculated plants (A_1). Plants inoculated by shake culture of *Bradyrhizobium* (A_3) showed the lowest plant height, but was significantly better than control. Increased plant growth in response to foliar application of *Rhizobium* in soyabean was reported by Maiti and Sen (1986).

Table 14. Effect of different methods of application, isolates and the media used on the number of leaves of cowpea

Method of Application	Isolates			Media	
	I_1	I_2	Mean	M_1	M_2
A_1	29.33	31.50	30.42	30.17	30.67
A_2	28.83	29.83	29.33	28.50	30.17
A_3	29.83	29.17	29.50	29.67	29.33
A_4	31.00	29.83	30.42	31.00	29.83
A_5	28.00	30.33	29.17	29.00	29.33
Mean	29.40	30.13		29.67	29.87
Control			24.00		

Media	Isolate	
	I_1	I_2
M_1	29.20	30.13
M_2	29.60	30.13

	A	I	IxA	M	IxM	MxA
CD(0.05)	1.053	0.666	1.490	NS	NS	NS

4.2.3 Fresh weight of plants.

The fresh weight of plants observed under different methods of application of *Bradyrhizobium* isolates are given in Table 15. The different methods of application significantly influenced the fresh weight of plants. Soil inoculation resulted in maximum fresh weight (24.61g). The values observed for all the other methods of application ranged from 21.96 (A_2) to 20.65 (A_3). Eventhough different methods of application was inferior to soil application they were better than control plants. The fresh weight of plants was not significantly influenced by the effect of different media. However, the fresh weight was significantly increased by isolate I_2 when compared to isolate I_1.

There was no significant interaction with IxA or MxA. But there was significant difference in IxM interactions. The maximum fresh weight of 22.66 g was observed in plants inoculated with isolate I_1 grown in medium M_2.

4.2.3 Dry weight of plants

The dry weight of plants as observed under different methods of application are given in Table 16. All the different methods of application significantly increased the dry weight of plant with the maximum value in A_1 (5.925 g) and minimum in A_3 (4.767 g). A_2, A_3, A_4 and A_5 were statistically on par and superior to control.

There was significant difference in the dry weight of cowpea plants due to the effect of different media. The medium M_2 improved the plant dry weight to 5.20 g, while that with medium M_1 it was 4.98 g. Among the two isolates tried, isolate I_2 significantly was superior in producing plant dry weight (5.28 g).

Table 15. Effect of different methods of application, isolates and the media used on the fresh weight of plants of cowpea (g)

Method of Application	Isolates			Media	
	I_1	I_2	Mean	M_1	M_2
A_1	24.39	24.83	24.61	24.11	25.12
A_2	21.21	22.71	21.96	21.88	22.04
A_3	20.63	20.67	20.65	20.50	20.79
A_4	20.46	21.63	21.04	20.79	21.29
A_5	20.40	20.92	20.66	20.86	20.46
Mean	21.42	22.15		21.63	21.94
Control			19.25		

Media	Isolate	
	I_1	I_2
M_1	21.01	22.24
M_2	21.82	22.06

	A	I	IxA	M	IxM	MxA
CD(0.05)	0.64	0.407	NS	NS	0.576	NS

Table 16. Effect of different methods of application, isolates and the media used on the dry weight of plants of cowpea (g)

Method of Application	Isolates			Media	
	I_1	I_2	Mean	M_1	M_2
A_1	6.183	5.667	5.925	5.667	6.183
A_2	4.908	5.183	5.046	5.075	5.017
A_3	4.842	4.692	4.767	4.683	4.850
A_4	4.467	5.167	4.817	4.808	4.825
A_5	4.508	5.267	4.887	4.675	5.100
Mean	4.980	5.200		4.980	5.200
Control			4.750		

Media	Isolate	
	I_1	I_2
M_1	4.86	5.11
M_2	5.11	5.28

	A	I	IxA	M	IxM	MxA
CD(0.05)	0.28	0.178	0.399	0.178	NS	NS

The IxA interaction was significant with maximum dry weight recorded in soil inoculated plants with isolate I_1 (6.183 g). However, the MxA interaction was not significant. No significant difference was observed in IxM interaction with respect to dry weight of plants.

2.4 Dry weight of Roots

The different methods of application of isolates resulted in a significant difference in the dry weight of roots (Table 17). Soil inoculation resulted in a significant increase in the dry weight of roots (1.171 g) as compared to other methods of application. However all the different methods of application increased the dry weight of roots above the control plants. Among foliar application A_5 was promising.

The dry weight of roots were not significantly influenced due to the effect of different media or isolate (Table 17).

All the interactions among the method of application, isolates or media were not significant in improving dry weight of roots.

From the above results, it is clear that the plant fresh weight, plant dry weight and dry weight of roots were recorded maximum in treatment where the *Bradyrhizobium* was inoculated in the soil. Nevertheless, the foliar inoculated plants also showed marked increase in these parameters as compared to control. Dry weight of roots recorded 77 per cent increase in soil inoculated plants and 20 per cent by foliar application where the bacterium was applied in a slurry form. The lowest dry weight of 0.728 g was recorded in A_2, which again showed 10 per cent increase over

Table 17. Effect of different methods of application, isolates and the media used on the dry weight of roots of cowpea (g)

Method of Application	Isolates			Media	
	I_1	I_2	Mean	M_1	M_2
A_1	1.124	1.218	1.171	1.112	1.230
A_2	0.714	0.728	0.721	0.693	0.749
A_3	0.715	0.810	0.763	0.801	0.724
A_4	0.792	0.729	0.761	0.681	0.840
A_5	0.793	0.794	0.794	0.793	0.794
Mean	0.828	0.856		0.816	0.867
Control			0.663		

Media	Isolate	
	I_1	I_2
M_1	0.723	0.971
M_2	0.968	0.980

	A	I	IxA	M	IxM	MxA
CD(0.05)	0.235	NS	NS	NS	NS	NS

control (Table 17). The total dry matter production was maximum when the bacterium was inoculated to soil showing a 25 per cent increase followed by A_2 and A_5 with six per cent and three per cent increase respectively. An increased plant dry weight was reported in soyabean in response to foliar application of *Bradyrhizobium* (Maiti and sen, 1990). The growth and yield of faba bean were reported to be increased due to the combined foliar application of *Bradyrhizobium* and micronutrients (Abdel -aal, 1989).

4.2.5 Number of Nodules

The number of nodules of cowpea inoculated with *Bradyrhizobium* by dfferent methods of application are given in Table 18. The different methods of application resulted in a significant increase in the number of nodules. The maximum number of nodules (22.92) was observed in plants grown under soil inoculation (A_1). A_5, A_2 and A_3 were statistically on par. The lowest value was observed in A_4 (9.58). Eventhough all the foliar application of the bacteria were less efective in improving the nodule count when compared to soil application, a notable point was that all the foliar application methods were better than control plants where no inoculation was done.

Neither the two media tried nor the two isolates significantly influenced the number of nodules in cowpea plants, eventhough isolate I_2 was slightly better than I_1.

The IxA interaction was found to be significant with maximum number of nodules observed in soil inoculated plants (A_1) with isolate I_1, giving 23.83 nodules per plant. With regard to MxA interaction, soil inoculated plants, inoculated with bacterium grown in media M_1 were promising with a maximum number of nodules

Table 18. Effect of different methods of application, isolates and the media used on the number of nodules of cowpea

Method of Application	Isolates			Media	
	I_1	I_2	Mean	M_1	M_2
A_1	23.83	22.00	22.92	23.33	22.50
A_2	9.67	11.00	10.33	11.00	9.67
A_3	9.83	10.00	9.92	11.67	8.17
A_4	9.50	9.67	9.58	9.67	9.50
A_5	9.17	12.50	10.83	10.00	11.67
Mean	12.40	13.03		13.13	12.30
Control			7.00		

Media	Isolate	
	I_1	I_2
M_1	12.47	13.80
M_2	12.33	12.27

	A	I	IxA	M	IxM	MxA
CD(0.05)	1.048	NS	0.938	NS	0.938	1.483

of 23.33. The IxM interaction was significant with a maximum number of nodules observed in plants inoculated with isolate I_2 grown in medium M_1.

4.2.6 Fresh weight of nodules

The fresh weight of nodules obtained from plants grown under different methods of application of rhizobial isolates are given in Table 19. A significant difference in the fresh weight of nodules was observed with different methods of application. The maximum nodule fresh weight (0.419 g) was obtained from the plants grown under soil inoculation. All the other methods of application A_2, A_3, A_4 and A_5, though higher than control, were statistically on par.

There was no significant difference in the fresh weight of nodules due to the effect different media or isolates. However, isolate I_2 was better than I_1 eventhough not significant, giving 0.244 g of nodule. Similarly media M_1 was slightly better than M_2.

None of the interactions, the methods of application, media and isolates were significant in improving the fresh weight of nodules.

4.2.7 Dry weight of nodules

A significant increase in the dry weight of nodules was observed under different methods of application (Table 20). Soil inoculation resulted in significant increase of the nodule dry weight. All the other methods of application were better the control. The lowest value was observed in A_4 (0.073 g).

Table 19. Effect of different methods of application, isolates and the media used on the fresh weight of nodules of cowpea (g)

Method of Application	Isolates			Media	
	I_1	I_2	Mean	M_1	M_2
A_1	0.417	0.420	0.419	0.430	0.407
A_2	0.183	0.186	0.185	0.183	0.186
A_3	0.176	0.180	0.178	0.182	0.174
A_4	0.191	0.189	0.190	0.187	0.193
A_5	0.192	0.197	0.195	0.198	0.191
Mean	0.232	0.234		0.236	0.230
Control			0.150		

Media	Isolate	
	I_1	I_2
M_1	0.228	0.244
M_2	0.236	0.224

	A	I	IxA	M	IxM	MxA
CD(0.05)	0.032	NS	NS	NS	NS	NS

Table 20. Effect of different methods of application, isolates and the media used on the dry weight of nodules of cowpea (g)

Method of Application	Isolates			Media	
	I_1	I_2	Mean	M_1	M_2
A_1	0.103	0.113	0.108	0.098	0.118
A_2	0.077	0.073	0.075	0.078	0.072
A_3	0.088	0.085	0.087	0.091	0.083
A_4	0.065	0.082	0.073	0.073	0.073
A_5	0.085	0.089	0.087	0.086	0.088
Mean	0.084	0.088		0.085	0.087
Control			0.060		

Media	Isolate	
	I_1	I_2
M_1	0.082	0.088
M_2	0.086	0.088

	A	I	IxA	M	IxM	MxA
CD(0.05)	0.009	NS	0.014	NS	NS	0.014

The different media tried did not significantly influence the dry weight of nodules in cowpea plants. Similar was the case with the different isolates also.

Significant difference was observed in IxA interaction. Maximum nodule dry weight was recorded in plants, soil inoculated with isolate I_1. MxA interaction was also significant with maximum values in soil inoculated plants inoculated with bacterium grown in media M_2. IxM interaction was not significant.

Different methods of application of the selected isolates improved the number of nodules, nodular fresh weight and dry weight in cowpea plants. Soil inoculated plants recorded maximum number of nodule and nodule dry weight. A 227 per cent increase in number of nodules and 80 per cent increase in nodule dry weight over control was observed. Among the foliar inoculation methods, *Bradyrhizobum* in slurry form (A_5) recorded the maximum number of nodules (55 % increase). Nodule dry weight showed maximum values in A_5 and A_3 (45 % increase) (Table 16). The least effective method was foliar application of bacteria grown in media without shaking (A_4) which also resulted in a 37 per cent increase in number of nodules and 22 per cent increase in nodular dry weight over control. Results are in concurrence with the increased number of nodules and nodular dry weight in response to foliar spray of *Rhizobium* as reported by Maiti and Sen (1990).

4.2.8 Chlorophyll content

The chlorophyll content of the plants grown under different methods of application are given in Table 21. Significant increase in the chlorophyll content was observed only in the case of soil inoculation (2.147 mg/g). All the other methods

Table 21. Effect of different methods of application, isolates and the media used on the leaf chlorophyll content (mg/g)of cowpea

Method of Application	Isolates			Media	
	I_1	I_2	Mean	M_1	M_2
A_1	2.150	2.143	2.147	2.150	2.143
A_2	2.135	2.140	2.138	2.140	2.135
A_3	2.135	2.145	2.140	2.145	2.135
A_4	2.138	2.140	2.139	2.140	2.138
A_5	2.133	2.140	2.137	2.137	2.137
Mean	2.138	2.142		2.138	2.142
Control			2.130		

Media	Isolate	
	I_1	I_2
M_1	2.143	2.142
M_2	2.134	2.141

	A	I	IxA	M	IxM	MxA
CD(0.05)	0.0019	0.0014	0.003	0.0014	0.02	0003

of application were statistically on par with control plants.

The leaf chlorophyll content of the plants was significantly increased due to the effect of different media. Medium M_1 resulted in a significant increase in the chlorophyll content as compared to the medium M_2. There was a significant difference in the chlorophyll content due to the effect of different isolates. Inoculation with isolate I_2 resulted in significant increase in chlorophyll content of cowpea plants (2.142 mg/g).

There were signficant difference in the IxA interaction with the maximum leaf chlorophyll content observed in soil inoculated plants with isolate I_1. MxA interaction was also significant and the superior combination was soil inoculation of bacterium grown in medium M_1. IxM interaction showed a significant difference in the leaf chlorophyll content. I_1M_2 combination was significantly inferior than the other three combinations.

The chlorophyll content of the plants recorded significant increase by the different methods of application of *Bradyrhizobium*. A maximum of 0.94 per cent was observed in soil inoculated plants closely followed by a 0.47 per cent increase in foliar appliaed plants (Table 20). Maiti and Sen (1990) also reported a higher chlorophyll content in soyabean in response to foliar application of *Bradyrhizobium*. Increased chlorophyll content due to foliar application of nutrients was reported in groundnut (Singh *et al.*, 1988) and in green gram (Kalita and Dey, 1994). The beneficial effect of foliar application of nutrient compounds was further increased by combined application of micronutrients and *Bradyrhizobium* reported by Hegazy and Hawary. (1990).

4.2.9 Nitrogen content

The leaf nitrogen content of the cowpea plants inoculated by the promising isolates grown in different media and employing different methods of inoculation are given in Table 22. Methods of inoculation significantly influenced the leaf nitrogen content. The maximum leaf nitrogen content of 3.79 per cent was observed in soil inoculated plants while the foliar applied plants did not show much differences. The lowest value of 2.587 per cent was observed in A_2 plants.

The different media used in culturing the rhizobial isolates showed significant difference in influencing the leaf nitrogen content. Highest leaf nitrogen content (2.853%) was observed in plants inoculated with bacteria grown in medium M_2 as compared to 2.826 per cent of plants inoculated with media M_1. There was no significant difference in the leaf nitrogen content of plants inoculated with different rhizobial isolates.

Significant differences were observed in IxA interaction with a maximum leaf nitrogen content in soil inoculated plants with isolate I_2 (3.833 %). The MxA interaction were also significant with media M_1 superior in soil inoculation (3.833 %). Leaf nitrogen content differed significantly in IxM interaction. Maximum leaf nitrogen content of 2.869 per cent was observed in plants inoculated with isolate I_2 grown in media M_2.

The leaf nitrogen was increased by 89 per cent over control due to soil inoculation. Foliar applied A_3 plants showed a 30.3 per cent increase closely followed by a 29.3 per cent increase in plants applied by A_5 and A_4 (Table 21). The

Table 22. Effect of different methods of application, isolates and the media used on the leaf nitrogen content (%)of cowpea

Method of Application	Isolates			Media	
	I_1	I_2	Mean	M_1	M_2
A_1	3.745	3.833	3.789	3.833	3.745
A_2	2.610	2.563	2.587	2.525	2.648
A_3	2.568	2.670	2.619	2.637	2.602
A_4	2.592	2.608	2.600	2.528	2.672
A_5	2.622	2.540	2.601	2.605	2.597
Mean	2.840	2.840		2.830	2.850
Control			2.010		

Media	Isolate	
	I_1	I_2
M_1	2.835	2.817
M_2	2.836	2.869

	A	I	IxA	M	IxM	MxA
CD(0.05)	0.0217	NS	0.031	0.0137	0.019	0.031

differencebetween control and foliar treated plants were significant indicating that nitrogen fixation on the leaf surface was quite appreciable. This suggests that non-symbiotic nitrogen fixation by *Bradyrhizobium* reported by Maiti and Sen (1990) becomes practical to a limited extent. Further, increased nitrogen content in response to phyllosphere application of non-symbiotic organisms like Azetobactor and Klebsella in rice and wheat plants were reported by Nandi and Sen (1985). Non-symbiotic nitrigen fixation by *Bradyrhizobium* is also reported by several workers (Child and LaRue, 1974; Kurz and LaRue, 1975; Pagan *et al.*, 1975; Reporter, 1976). All the observations suggest that there could be nitrogen fixation in phylloplane of cowpea plants and this could be effectively utilised when other usual methods of seed or soil inoculation is not possible.

On the whole, it becomes evident from the results that, general performance of the plant is increased by foliar application by *Bradyrhizobium*. But this method cannot replace the well established soil inoculation method where the bacterium fix nitrigen in association with the root nodule. The findings of Kurz and LaRue (1975) that *Bradyrhizobium* can fix nitrogen outside the host cell seems to have only limilted practical application.

Another notable finding is that in many cases the local selected isolates of the bacterium perform equal to or even better than well known introduced cultures. In the present experiment isolate 1_5 which is a native culture performed better than the proven exotic culture CB-756. Such superiority of local isolates in exhibiting better performance in nitrogen fixation have been reported by several workers (Ajay Kumar and Malvika, 1994; Evans *et al.*, 1996). Eventhough, Pagan *et al.* (1975) reported

nitrogen fixation by *Rhizobium* in defined medium, this effect was not utilised properly. The two defined medium tried, did not influence the plant growth parameters. The reason may be that the proximiy to the medium is lost once the culture is applied to the plants. The medium did not seem permanently bestow the non-symbiotic nitrogen fixation ability to the bacterium.

The second experiment underline the superiority of soil inoculation (A_1) of *Bradyrhizobium*. Most of the foliar application methods were, eventhough less effective, better than uninoculated control plants. Performance of no particular folair application method was consistent. But A_2, foliar application in water suspension was comparatively better in improving height, fresh weight and dry weight of plants. Between the two isolates used, KAU culture (I_2) consistantly performed better than CB-756 (I_1). In increasing only the plant height, CB-756 was better than the others. This again underline the superiority of local cultures in any given locality. The defined medium in which the isolates were grown did not influence the non-symbiotic nitrogen fixation or plant improvement by the bacterium.

Summary

SUMMARY

An investigation was carried out to evaluate the non-symbiotic nitrogen fixation by *Bradyrhizobium* in cowpea at the College of Horticulture, Vellanikkara during the year 1993-95. The highlights of the study are summarised below.

Twelve isolates of *Bradyrhizobium* were collected from the nodules of legumes grown in different agroclimatic regions of Kerala. Two exotic cultures and one KAU culture was also used for the study. The identity of *Bradyrhizobium* was established using routine laboratory tests.

A pot culture experiment was conducted to screen the efficiency of the rhizobial isolates for phylloplane nitrogen fixation in cowpea. Along with it two media and two methods of application were also tried. The results show that no particular isolate was effective in improving any of the parameters observed. However,isolate I_{15} (KAU isolate) ranked first in improving five parameters observed. Between the two method of applications tried, A_2 (seed (soil) inoculation) was superior than foliar application (A_1). The two media which were proven to support non-symbiotic nitrogen fixation did not show any difference in improving the different plant parameters. The interaction among the isolates, methods of application and the media used also varied widely. No particular treatment combination was effective in improving all or majority of the parameters observed. Out of the ten characters studied, I_{15} ranked best in improving five characters namely, plant height, number of leaves, dry weight of nodule, fresh weight of plants and nitrogen content. In characters such as dry weight of plants and chlorophyll content, it ranked second. Thus I_{15} was selected as the promising isolate for the second experiment. Isolate I_{14} (CB-756), which showed next

better performance in improving number of leaves, dry weight of nodules and fresh weight of plants was also selected. It performed third in improving the characters such as plant height, dry weight of root and number of nodules. Moreover, it was one of the known exotic isolates supporting non-symbiotic nitrogenase activity.

Another pot culture experiment was conducted to find out the most suitable method of application of the selected ~~two~~ two isolates. The results of this experiment clearly established the superiority of seed (soil) inoculation (A_1) of *Bradyrhizobium* over different methods of foliar application. All the plant parameters were improved as a result of seed (soil) inoculation of *Bradyrhizobium*. However, foliar applications also showed improved performance over control plants in improving the different parameters observed. Spraying the bacteria grown in broth shake culture (A_3) was found effective in improving the number of leaves, dry weight of roots, chlorophyll content and nitrogen content of plant. Application of the bacterium in slurry form (A_5) and application in water suspension (A_2) were the next best among the foliar application methods. The study proclaimed the superiority of soil inoculation of the *Bradyrhizobium* in improving the growth of cowpea over foliar application. Eventhough less efficient, the foliar application can also be resorted to in situations where soil inoculation is not already done or where it is not possible. This will improve the plant performance better than untreated plants. Among the different isolates tried I_{15} (KAU culture) emerged out comparatively better than others.

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• **Originals not seen**

EVALUATION OF NON SYMBIOTIC NITROGEN FIXATION BY *BRADYRHIZOBIUM* IN COWPEA

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ABSTRACT OF A THESIS

Submitted in partial fulfilment of the
requirement for the degree of

Master of Science in Agriculture

Faculty of Agriculture
Kerala Agricultural University

Department of Plant Pathology
COLLEGE OF HORTICULTURE
VELLANIKKARA - THRISSUR

1997

ABSTRACT

A study was conducetd at the College of Horticulture, Vellanikkara during the year 1993-95 on the evaluation of non-symbiotic nitrogen fixation by *Bradyrhizobium* in cowpea. Twelve isolates collected from different agroclimatic regions of Kerala, two exotic isolates and one KAU isolate were used for the study. First, a pot culture experiment was conducted to screen the above 15 isolates for their efficiency in non symbiotic nitrogen fixation. Two media and two methods of application were also tried. A second pot culture experiment was also conducted to find out the most suitable method of application of the best performer in terms of foliar nitrogen fixation.

The results of the screening experiment showed that no particular isolate was effective in improving any of the parameters observed. However,out of the ten characters studied, isolate I_{15} (KAU isolate) ranked best in improving five characters namely, plant height, number of leaves, dry weight of nodule, fresh weight of plants and nitrogen content. Thus I_{15} was selected as the promising isolate for the second experiment. Isolate I_{14}(CB-756), which showed next better performance in improving number of leaves, dry weight of nodules and fresh weight of plants was also selected. Moreover, it was one of the known exotic isolates supporting non-symbiotic nitrogenase activity. The results of the second experiment clearly established the superiority of seed (soil) inoculation (A_1) of *Bradyrhizobium* over different methods of foliar application. However, foliar applications also showed improved performance over control plants in improving the different parameters observed. Spraying the bacteria grown in broth shake culture (A_3) was found effective in improving the number of leaves, dry weight of roots, chlorophyll content and nitrogen content of

plant. The study show that, eventhough less efficient, the foliar application of *Bradyrhizobium* can also be resorted to in improving the performance of cowpea plants in situations where seed (soil) inoculation is not possible.