ASSOCIATIVE EFFECT OF *Azospirillum* AND *Bradyrhizobium* ON NODULATION AND GROWTH OF COWPEA (*Vigna unguiculata* (L) Walp,

By

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THESIS

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DECLARATION

I hereby declare that the thesis entitled **"Associative effect of *Azospirillum* and *Bradyrhizobium* on nodulation and growth of cowpea (*Vigna unguiculata* (L.) Walp)"** is a bonafide record of research work done by me during the course of research and that the 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.

Vellanikkara,
31-8-1992.

SUNITHA MENON, S.

CERTIFICATE

Certified that this thesis entitled "Associative effect of *Azospirillum* and *Bradyrhizobium* on the nodulation and growth of cowpea (*Vigna unguiculata* (L.) Walp.)" is a record of research work done independently by Miss. Sunitha Menon, S., under my guidance and supervision and that it has not previously formed the basis of award of any degree, diploma, fellowship or associateship to her.

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CERTIFICATE

We, the undersigned members of the Advisory Committee of **Miss. Sunitha Menon, S.**, a candidate for the degree of Master of Science in Agriculture with major in Plant Pathology, agree that the thesis entitled **"Associative effect of *Azospirillum* and *Bradyrhizobium* on nodulation and growth of cowpea (*Vigna unguiculata* (L.) Walp)"** may be submitted by Miss. Sunitha Menon, S., in partial fulfilment of the requirement for the degree.

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SUNITHA MENON S

Dedicated to
my loving parents

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Introduction

INTRODUCTION

Along with the increasing world population, food production and fertilizer consumption have increased gradually during the past several years. The world's demand for nitrogen has been increasing steadily and is expected to reach 139.3 million tonnes by 2000 AD (Verghese, 1977).

Industrial nitrogen fixation, in addition to being expensive and time consuming, is heavily dependant on energy derived from fossil fuel which is getting depleted at a very fast rate. On the other hand, biological nitrogen fixation require approximately half the quantum of energy needed for industrial fixation and is dependant on energy from renewable resources such as products of photosynthesis and soil organic matter. Therefore, the need for less expensive and realistic programmes to improve biological nitrogen fixation becomes more and more important for enhancing our agricultural production. In recent years, several possibilities are being examined to augment biological nitrogen fixation.

In developing countries where the percapita income is low, people depend on vegetable protein, richest source of which are legumes. Even in the developed coutries, the trend is in favour of substituting animal protein by vegetable protein in view of its nutritional qualities.

Majority of Indians depend on vegetable protein, and the grain pulses form an important part in their diet. Eventhough, India is the world's largest producer of grain legumes, the production is not adequate to meet the per capita requirement of 80 g recommended by World Health Organisation and Food and Agricultural Organization. In fact, there is a stagnation in area, production and productivity of pulses over the past three decades as against the increasing demand entailed by a growing population.

Cowpea forms an important component in the tropical cropping systems of India, especially Kerala. It is grown for its long green pods as vegetable, seeds as pulse and foliage as fodder. It is a major source of protein (25 per cent), energy, minerals and vitamins. Its importance is realised on account of its drought tolerance and adaptation to wide range of soil types. The nitrogen fixing ability of cowpea will be an added advantage especially in subsistence agriculture. This can be enhanced by proper exploitation of the property of association of this crop with nitrogen fixing microorganisms.

Inoculation with specific strains of Rhizobium leading to higher nitrogen fixation and yield of legumes is a well established phenomenon. Azospirillum is a soil inhabiting nitrogen fixing bacteria which is found frequently in association with plant roots. The beneficial effects of

Azospirillum inoculation has been proved beyond doubt in many crops. These characters have made *Azospirillum* an organism receiving considerable economic and scientific interest during past several years.

Considering all these facts, the present investigation was carried out to find an efficient way to improve the growth and yield of cowpea, with the following objectives.

1. To isolate strains of *Azospirillum* from the roots of legumes and nonlegumes growing in different agroclimatic regions of Kerala.

2. To test the efficiency of different *Azospirillum* isolates in promoting growth and nitrogen fixation of cowpea and to select promising ones for further work.

3. To procure standard *Bradyrhizobium* culture for cowpea to study its associative effect when inoculated in combination with *Azospirillum*.

4. To study the pH tolerance of selected *Azospirillum* isolates and standard *Bradyrhizobium* under *in vitro* conditions.

5. To find the associative effect, if any, of the selected *Azospirillum* isolate and standard *Bradyrhizobium* isolate on nodulation and growth of cowpea variety 'Pusa Komal'.

Review of Literature

REVIEW OF LITERATURE

Azospirillum was isolated by Beijerinck (1925) from sandy soils in Netherlands and was originally named as *Spirillum lipoferum*. Since its initial discovery as a nitrogen fixing soil inhabitant, *Azospirillum* had been forgotten for about half a century. The search for associative nitrogen fixers led to the isolation and rediscovery of this genus from the roots of *Digitaria* and *Zea mays* (Bulow and Dobereiner, 1975). Tarrand *et al*. (1978) proposed *Azospirillum* as the genus and distinguished two species, *Azospirillum brasilense* and *A. lipoferum* based on the physiological and morphological differences between strains and on DNA homology experiments. Later two additional *Azospirillum* species were identified, *A. amazonense* isolated by Magalhaes *et al*. (1983) from grass roots in the Amazonian area of Brazil and the salt tolerant species *A. halopraeferans* associated exclusively with roots of Kallar grass (Reinhold *et al*., 1987).

2.1 Occurrence of *Azospirillum*

In a survey conducted by Dobereiner *et al*. (1976) it was found that *Azospirillum* was a common inhabitant of the tropics. The occurrence of *Azospirillum* in Indian soils has been reported by Kumari et al. (1976) and Lakshmi et al. (1977). Nair (1981) reported that Azospirillum brasilense could colonize in the root elongation zone and base of root hairs and proliferate in the inner most layer of cortex and conducting vessels in addition to epidermal and other cortical cells in inoculated sorghum plants. The association of Azospirillum with the roots of several annual and perennial crops in coconut based farming systems of Kerala was reported by Ghai and Thomas (1989).

2.2 Characters of Azospirillum

Dobereiner and Day (1975) reported Azospirillum as Gram negative, motile bacteria, generally vibrioid in shape. Day and Dobereiner (1976) found that the growth and nitrogen fixation of Azospirillum was supported best by organic acids such as malate, lactate and succinate as carbon and energy sources. They also reported that the growth of Azospirillum was best under microaerophilic conditions at a temperature between 32 and 38°C and pH between 6.8 and 7.8. Okon et al. (1976a) reported that, when grown in nitrogen free medium, Azospirillum form subsurface pellicular growth indicating that their microaerophilic nature was due to the lack of oxygen protection mechanism for the nitrogenase enzyme. They also reported that Azospirillum can grow as an aerobe only when the medium was supplied with fixed nitrogen.

Unlike for most of the other nitrogen fixing bacteria, sugars are poor carbon substrates for Azospirillum (Okon et al., 1976b). Dobereiner et al. (1976) reported that occurrence of Azospirillum in soil is highly pH dependant with a pH around 7.0 being optimal. The ability of Azospirillum strains to reduce nitrate to nitrite was reported by Neyra et al. (1977) and Baldani and Dobereiner (1980). Charyulu et al. (1980) reported that the nitrogen fixing activity of Azospirillum was high at soil redox potential between 50 and 150 mV. Lakshmi and Dhala (1984) reported that the optimum pH for Azospirillum was between 6.5 and 7.4. Fallik et al. (1988) reported that the activity of Azospirillum was inhibited when the organic matter mixed with sand exceeded one per cent by weight. Tilak et al. (1988) reported that A. brasilense exhibited maximum nitrogenase activity at pH ranging from 6.5 to 8.5.

Hadi and Henis (1989) obtained maximum growth of Azospirillum with no aggregation in a medium containing both fructose and malate as carbon sources. They reported that aggregates formed in media supplemented with organic acid were stable at pH levels above 8.0 and below 6.0 but dispersed at pH 7.1. Singh et al. (1989) reported that a temperature of 35°C gave better growth of Azospirillum and that a 10°C decrease or increase in temperature reduced the bacterial cell number by 91.1 and 35.7 per cent respectively. Maheswari and

Purushothaman (1990) suggested that sugars and amino acids present in the root exudates served as chemo attractants for *Azospirillum*.

2.3 Beneficial effects of *Azospirillum* inoculation

2.3.1 Nitrogen fixation

Tarrand *et al*. (1978) reported that all wild type of *Azospirillum* strains fix atmospheric nitrogen efficiently either as free living bacteria or in association with plants. Cohen *et al*. (1980) obtained increase in nitrogenase activity within the roots of *Zea mays* and *Setaria italica* inoculated with *Azospirillum*. Volpon *et al*. (1980) expressed the efficiency of nitrogen fixation of a strain of *Azospirillum lipoferum* in terms of mg total N fixed in cell and supernatant per g of glucose consumed. They reported that it was 20 at the early log phase and increased to 48 at the late log phase. Rai and Gaur (1982) observed that isolates of *Azospirillum brasilense* and *A. lipoferum* having a greater denitrifying capacity, fixed less nitrogen in a nitrogen free semisolid medium. Rao and Rao (1983) observed an increase in nitrogenase activity in the rhizosphere of upland rice upto 88 days after sowing.

Kapulnik *et al*. (1985) measured the acetylene reduction activity in soil cores containing roots of wheat colonized with Azospirillum and obtained values ranging from 50 to 600 n mol C_2H_4/g dry root/h. Danneberg et al. (1986) reported that the nitrogen fixation and denitrification capabilities of Azospirillum associated with wheat were dependant on bacterial strain, concentration of nitrate and availability of oxygen.

2.3.2 Hormonal effects

Tien et al. (1979) observed that the effect of Azospirillum on the root development and morphology of pearl millet seedlings were similar to the effect of external application of hormones, either synthetic or purified from bacterial cultures. Venkateswarlu and Rao (1983) reported a marked stimulation in the root growth by inoculating pearl millet with Azospirillum strains having low nitrogenase activity but which produced higher quantities of auxins in the culture filtrate. Hartmann et al. (1983) reported that the mutants of Azospirillum resistant to 5-fluoro-tryptophan excreted IAA upto 16 μg/ml which was 30 times higher than the wild type. Govindan and Purushothaman (1984) also reported that the Azospirillum isolates varied in their ability to produce phytohormones like IAA and Gibberellins. Venkateswarlu and Rao (1984) reported that the improvement in plant growth obtained by inoculation with Azospirillum strains was not related to their nitrogen fixing capacity but to phytohormone synthesis.

According to Jain and Patriquin (1985) an *Azospirillum* mutant strain which over produced IAA in culture strongly affected the root morphology of wheat. Similarly Barbieri *et al*. (1986) also reported that *Azospirillum* mutants which failed to produce IAA in culture had no effect on root morphology of wheat. Govindan and Nair (1986) isolated phytohormones from the pure culture of *Azospirillum*. Cacciari *et al*. (1989) reported the production of phytohormone like substance by *Azospirillum brasilense* under diazotrophic conditions. Fallik *et al*. (1989) identified higher amounts of IAA and IBA in inoculated maize roots than in noninoculated roots.

2.3.3 Improvement in mineral uptake and plant water relationship

Lin *et al*. (1983) reported enhancement in the uptake of minerals by roots of *Zea mays* and *Sorghum bicolor* inoculated with *Azospirillum brasilense*. Kapulnik *et al*. (1985) studied on the effect of Azospirillum inoculation in wheat in hydroponic systems and reported enhancement in the uptake of nitrate due to inoculation. Barton *et al*. (1986) reported enhanced uptake of Fe^{2+} in sorghum due to *Azospirillum* inoculation. Similar results were reported on rice under hydroponic conditions by Murti and Ladha (1988).

Sarig *et al*. (1988) attributed the yield increase in *Azospirillum* inoculated sorghum plants primarily to improved utilisation of soil moisture. The inoculated plants were reported to have more water in foliage, high leaf water potential and low canopy temperature than uninoculated plants.

Bashan *et al*. (1990) evaluated the capacity of *Azospirillum* strains to enhance the accumulation of K^+, P^+, Ca^{2+}, Mg^{2+}, Mn^{2+}, Na^+ and Zn^{2+} in inoculated wheat and soybean plants. They reported that a strain capable of accumulation of a particular ion in one plant species or cultivar often lacked the ability to do so in another. Hernandez *et al*. (1990) reported that *Azospirillum* is capable of producing siderophores which can improve iron nutrition of plants by making the nonavailable form of iron into available form.

2.4 Response of crop plants to *Azospirillum* inoculation

It has been already well established that inoculation of many crop plants with *Azospirillum* could result significant change in various plant growth parameters which may or may not affect yield. Inoculation studies have been carried out in cereals, grasses, legumes, vegetables, oil seeds and in many other economically important crop plants.

2.4.1 *Azospirillum* inoculation studies in legumes

Only few reports are there on the response of legumes to *Azospirillum* inoculation alone. Singh *et al*. (1980) studied on the response of cowpea to *Azospirillum* inoculation and obtained fresh fodder yield of 30 t/ha compared to 26.7 t/ha obtained without inoculation. Sarig *et al*. (1986) reported that inoculation of naturally nodulated *Pisum sativum* with *Azospirillum* caused significant increase in nodule number. They also reported that in *Pisum sativum* and *Cicer arietinum*, inoculation had no effect on dry matter yield unlike in *Vicia sativa* where there was significant increase in dry matter yield and percentage nitrogen content. Fayez *et al*. (1988) reported increase in the dry weight of broad bean by *Azospirillum* inoculation. Significant positive effect of *Azospirillum* inoculation on root and shoot growth of chickpea under green house conditions was reported by Delgallo and Fabbri (1990).

2.4.2 *Azospirillum* inoculation studies in nonlegumes

Among the nonlegumes, most of the *Azospirillum* inoculation studies have been done in cereals and grasses. Rai and Gaur (1982) studied the effect of inoculation on the yield and nitrogen uptake of wheat. They reported that the treatment receiving 80 kg N/ha yielded 2.97 t/ha against the yield of 4.15 t/ha in the treatment receiving both inoculant and fertilizer. *Sanoria* *et al*. (1982) obtained significant increase in the plant height of paddy by *Azospirillum* inoculation and reported that use of inoculant alone with no application of fertilizer nitrogen was more desirable. Similar significant increase in growth and dry matter production, both under sterilized and unsterilized conditions was obtained by Venkateswarlu and Rao (1983) by inoculating pearl millet with Azospirillum strains. However, Patriquin et al. (1983) reported that the crucial problem of most of the green house and field experiments with Azospirillum was the inconsistent plant response to inoculation regardless of plant species.

Arunachalam and Venkatesan (1984) reported the possibility of reducing 50 per cent fertilizer nitrogen of sesamum without adversely affecting the yield by the use of Azospirillum. The results obtained by Pahwa and Patil (1984) also indicated the possibility of saving 15-20 kg inorganic N/ha by inoculating forage crops with Azospirillum lipoferum. They reported that simple seed inoculation resulted in increased green yield to the tune of 18.6, 32.6, 30.9, 41.4 and 38.5 per cent in teosinte, maize, bajra, oats and barley respectively. Tanwar et al. (1985) reported 41 per cent increase in crude protein content of oat fodder inoculated with Azospirillum. Increased grain yield and nitrogen content in wheat by Azospirillum inoculation was obtained by Boddey et al. (1986).

Response of cotton plants to *Azospirillum* inoculation was investigated by Fayez and Daw (1987) and they reported a significant increase in plant dry weight and nitrogen uptake following inoculation. Similarly significant increase of 35 per cent in root length, 90 per cent in shoot dry weight, 50 per cent in root dry weight and 90 per cent in total leaf area was reported by Hadas and Okon (1987) in 18 day old tomato plants inoculated with *Azospirillum*. Significant increase in growth and yield of chilli and bhindi variety Pusa Sawani was reported by Amrithalingam (1988) and Balasubramani (1988) respectively. Jeeva (1988) obtained increase in height and girth of pseudostem, leaf production, leaf area and N, P, Ca and Mg content of leaves of banana cultivar Poovan by *Azospirillum* inoculation.

Purushothaman (1988) obtained grain and straw yield increase equivalent to the application of 25 kg N/ha by *Azospirillum* inoculation in rice. According to Porwal and Singh (1989), about 40 kg N/ha could be saved by *Azospirillum* inoculation without significant reduction in grain and straw yield of sorghum. Increase in grain yield of barley in response to *Azospirillum* inoculation was reported by Tilak and Dwivedi (1989).

Beneficial effects of *Azospirillum* inoculation in tomato, egg plant, sweet pepper and cotton was reported by

Bashan et al. (1989). Similar results were obtained in bhindi by Parvatham and Vijayan (1989). They also reported that soil application of Azospirillum was more efficient when compared to seed inoculation. Bopiah and Khader (1989) obtained increase in plant height, shoot dry weight and root dry weight in black pepper by dipping the rooted cuttings in culture solution of Azospirillum. Karunakaran and Palaniappan (1989) reported that seed and soil inoculation with Azospirillum increased capitulum size, number of filled seeds per capitulum, thousand seed weight and seed yield of sunflower.

However, Michiels et al. (1989) reported inconsistent plant response to Azospirillum inoculation as the major barrier constraining its large scale application as a biofertilizer. Rukmani (1990) also observed that eventhough inoculation of bhindi variety Pusa Sawani with Azospirillum resulted in increased yield, fruit length and girth and vitamin C content, the performance of the local isolate was inconsistent.

Vasyuk and Bovkov (1990) reported that seed inoculation of barley with Azospirillum lipoferum increased grain yield from 410 g/m^2 in the control to 495 to 577 g/m^2. Bashan et al. (1990) obtained significant increase in root growth by inoculating wheat with Azospirillum.

Subbiah (1991) reported that soil application of *Azospirillum* improved the yield and nitrogen use efficiency of bhindi variety Co-2 at 50 per cent recommended level of fertilizer nitrogen. Yadav and Kumar (1991) also reported that inoculation of mulberry with *Azospirillum* was more beneficial at low level of fertilizer nitrogen.

2.5 Root nodule bacteria

It was Beijerinck (1888) who discovered that the bacteria present in the root nodules of legumes called *Rhizobium* are the agents responsible for fixation of atmospheric nitrogen. Later, 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 soybean, lotus and *Vigna* under the genus *Bradyrhizobium*.

2.5.1 pH requirement

Albrecht (1933) reported that nodulation by *Rhizobium* in soybean failed at pH less than 5.0. Vincent (1977) reported that all strains of *Rhizobium* grow at pH 5.5 to 7.5. Joe and Allen (1980) reported that the beneficial effects of *Rhizobium* inoculation of cowpea were maximum at pH values between 6.6 and 7.6. Hadad and Loynachan (1985) studied the groundnut nodulating strains of *Rhizobium* and reported that the optimum pH for their growth were in general between 6.0 and 8.0.

2.5.2 Response of cowpea to *Bradyrhizobium* inoculation

Crofts and Jenkins (1954) reported that inoculation of cowpea with *Rhizobium* resulted in good nodulation and plant growth. Gargantini and Wutke (1960) inoculated cowpea with *Rhizobium* and reported that the inoculated plants fixed nitrogen at the rate of 75 kg/ha. Nair *et al*. (1970) and Sahu and Behera (1972) also obtained increased nitrogen content in cowpea inoculated with *Rhizobium*.

The effect of *Rhizobium* on the yield of cowpea was studied by many workers. Rao (1972) reported that inoculation resulted in 23 per cent yield increase over uninoculated control. Summerfield *et al*. (1975) reported that seed inoculation increased the number of pods per plant from 63 to 92. Pawar *et al*. (1977) reported increase in seed yield from 0.62 t/ha to 0.82 t/ha by inoculation. Similarly Bagyaraj and Hegde (1978) also reported increase in seed yield from 0.79 t/ha to 1.22 t/ha in inoculated plants.

However, Mughogho (1978) reported that different strains of *Rhizobium* had no effect on the grain yield of cowpea.

Sivaprasad and Shivappashetty (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 or final yield. On the contrary Mathew and Koshy (1982) reported that inoculation had no beneficial effect on plant growth, nodulation and yield of cowpea.

Neves et al. (1982) obtained differential response in cowpea plants inoculated with different strains of Rhizobium. Sohoo et al. (1984) reported that different varieties of cowpea behaved differently when inoculated with the same strain of Rhizobium.

Senanayake et al. (1987) reported that apparent nitrogen fixation per g of nodule fresh weight in cowpea plants inoculation with Rhizobium reached a maximum 20 to 30 days after planting after which it decreased. Anthoniraj et al. (1989) studied on the nodulation pattern and their relationship with plant biomass in cowpea. They obtained a positive correlation between nodule number and plant biomass and a negative correlation between age of plant and nodule number. Partitioning of biologically fixed nitrogen in cowpea during pod development was studied by Douglas and Weaver (1989). They reported that much of the newly fixed nitrogen must cycle through a nitrogen pool in different plant tissues before reaching the pods.

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 reported that dry yield was 5.14 and 4.10 t/ha with and without inoculation respectively. Awonaike et al. (1990) reported that the difference in total dry matter yield obtained by Rhizobium inoculation was due to large variation in the vegetative growth. Beena et al. (1990) obtained significant increase in nodule number, dry weight, yield and percentage nitrogen content in inoculated plants. However, they reported that there was no significant correlation between nodule number and nitrogen content in plant. Gregr (1990) reported increased nitrogen uptake by cowpea plants following rhizobial inoculation.

2.6 Response of plants to combined inoculation of Azospirillum and root nodule bacteria

Singh and Rao (1979) reported that inoculation of soybean with Rhizobium japonicum and Azospirillum brasilense in combination generally increased the grain yield eventhough not significant. Singh et al. (1980) inoculated cowpea seeds with (a) Rhizobium, (b) Azospirillum and (c) Rhizobium + Azospirillum and obtained fresh fodder yields of 34.0, 30.0 and 35.7 t/ha respectively compared to 26.7 t/ha obtained without inoculation. Borthakur and Sarmah (1983) reported that seed inoculation with Azospirillum stimulated the native strains of Rhizobium for nodule formation in soybean. Iruthayathas et al. (1983) obtained substantial increase in nodulation, nitrogen fixation, shoot dry matter production and nitrogen gain in winged bean and soybean inoculated with both Rhizobium and Azospirillum. However, they also reported that the effect was mainly strain dependant.

Plazinski and Rolfe (1985a) reported that a mixed culture containing Rhizobium and Azospirillum in the ratio 1:200 to 1:2500 inhibited the nodulation in white clover in spite of the significant increase in plant dry weight. Rhizobial strains which nodulates without attacking root hairs are not inhibited by Azospirillum (Plazinski and Rolfe, 1985b). They (Plazinski and Rolfe, 1985c) also reported that all Azospirillum strains vary in their ability to inhibit or enhance nodulation by Rhizobium trifolii strains and that there was a decrease in the effectiveness of R. trifolii strains when an Azospirillum strain caused an increase in nodule number.

Response of cowpea to combined inoculation was studied by Gunawardena and Vlassak (1986) and obtained 134 to 138 per cent increase in plant dry weight over the control. Yahalom et al. (1987) reported that when Azospirillum was applied 24 h before Rhizobium in Medicago polymorpha and Macroptelium atropurpureum, more than two fold increase of nodulation in the non root hair zone was obtained. Increased nodulation following combined inoculation was reported in pigeonpea by Kundu (1988) and in *Medicago sativa* by Schmidt *et al*. (1988). Early nodulation in *Medicago polymorpha*, Siratro and Egyptian clover due to combined inoculation was reported by Yahalom *et al*. (1988).

However, Raverkar and Konde (1988) reported that when *Rhizobium* and *Azospirillum* were inoculated together in peanut, the nodulation, nitrogen content and yield were adversely affected.

Mokadem *et al*. (1989) reported that the beneficial effect of combined inoculation of *Rhizobium* and *Azospirillum* in chickpea was more pronounced in young plants than in mature plants. Galanti *et al*. (1989) reported that stimulation of rhizobial nodulation was induced by only some strains of *Azospirillum* and that also only at the highest concentration of 10^7 and 2×10^7. Agarwala and Tilak (1989) reported that seed inoculation with *Azospirillum brasilense* and *Rhizobium* sp. specific for chickpea and cowpea increased the shoot dry matter content and nitrogen uptake in *Panicum miliare*, *Setaria italica*, *Paspalam scrobiculatum* and *Eleusine coracana*. Raverkar and Konde (1990) reported that the higher number of nodules formed by native rhizobia in *Arachis hypogaea* in the presence of *Azospirillum lipoferum* was not efficient in fixing nitrogen.

Materials and Methods

MATERIALS AND METHODS

A study on the 'Associative effect of Azospirillum and Bradyrhizobium on nodulation and growth of cowpea (*Vigna unguiculata* (L.) Walp)' was conducted during 1990-'92 at College of Horticulture, Vellanikkara, Thrissur. The details of the materials used and the techniques adopted during the course of this investigation are presented below.

3.1 Collection of root samples for isolation of *Azospirillum*

Roots of both legumes and nonlegumes for the isolation of *Azospirillum* were collected from different locations of Kerala. Root samples were collected in polythene bags along with moist ball of earth adhering to it so as to prevent drying of roots and changes in microflora.

3.2 Isolation of *Azospirillum*

Azospirillum was isolated from the collected roots using Nitrogen Free bromothymol blue (NFb) medium (Semisolid malate medium) (Dobereiner *et al.*, 1976) of the following composition.

Malic acid	5.0 g
KOH	4.0 g

K_2HPO_4	0.5 g
$FeSO_4.7H_2O$	0.05 g
$MnSO_4.H_2O$	0.01 g
$MgSO_4.7H_2O$	0.10 g
NaCl	0.02 g
$CaCl_2$	0.01 g
Na_2MoO_4	0.002 g
Agar	1.75 g
Distilled water	1000.0 ml
Bromothymol blue (0.5 per cent alcoholic solution)	2.0 ml

(pH adjusted to 6.6-7.0 using 0.1 N potassium hydroxide)

The medium was transferred to test tubes, 10 ml each, and sterilized in an autoclave at 121°C for 20 minutes.

Fresh root samples collected for the isolation of Azospirillum were washed in tap water to remove the adhering soil particles. The washed roots were cut into bits of 0.5-1.0 cm length and surface sterilized with 0.1 per cent mercuric chloride solution for one minute. These surface sterilized root bits after washing with four changes of sterile water were planted in semisolid malate medium in test tubes and incubated at 37°C for two days. They were then observed for the presence of subsurface, thin, pellicular growth of Azospirillum.

Azospirillum culture commercially sold from Tamil Nadu Agricultural University was also collected for comparison. A small quantity of the inoculant was dispersed in sterile water. A loopful of the suspension was stabbed into the semisolid malate medium in test tubes. After incubation at 37°C for two days, they were observed for the subsurface, thin, pellicular growth.

3.3 Purification of *Azospirillum*

Isolates of *Azospirillum* were purified by successive transfer of thin pellicles into fresh semisolid malate medium for several times. Loopful of the culture was stabbed into the fresh semisolid medium and incubated at 37°C for two days and they were observed for the development of white, pellicular growth.

3.4 Primary characterization of *Azospirillum*

Primary characterization of the collected *Azospirillum* isolates were done by studying the morphology, Gram reaction, cultural characters and physiological characters.

3.4.1 Gram staining

Hucker's modification of Gram staining was employed for studying the Gram reaction of the collected *Azospirillum* isolates. A smear of the bacterial isolate was prepared on a clean glass slide and heat fixed over a flame by gentle intermittent heating. It was stained with Hucker's ammonium crystal violet solution for one minute and then washed in a gentle stream of running tap water. After washing, it was flooded with Gram's iodine solution for one minute and then decolourised with 95 per cent ethanol. After washing again in gentle stream of running tap water the slide was finally stained with saffranin solution for one minute and the excess stain was washed off in tap water. After drying between folds of filter paper, the slide was examined under light microscope for Gram reaction.

3.4.2 Cultural characters

Cultural characters of the collected Azospirillum isolates were studied by growing them in different media and observing for the colony characters such as colour, shape, etc. The different media used and their composition are given below.

3.4.2.1 Okon's medium (Okon et al, 1977) as modified by Kumari et al. (1980)

a.	K_2HPO_4	6.0 g
	KH_2PO_4	4.0 g
	Distilled water	500.0 ml

b.	$MgSO_4 \cdot 7H_2O$	0.2 g
	NaCl	0.1 g
	$CaCl_2$	0.02 g
	NH_4Cl	1.0 g
	Malic acid	5.0 g
	NaOH	3.0 g
	Yeast extract	0.05 g
	Na_2MoO_4	0.002 g
	$MnSO_4 \cdot H_2O$	0.001 g
	H_3BO_3	0.0014 g
	$Cu(NO_3)_2$	0.0004 g
	$ZnSO_4$	0.0021 g
	$FeCl_3$	0.002 g
	Agar	15.0 g
	Distilled water	500.0 ml

The agar was dissolved in 500 ml water by boiling. After cooling it to about 45°C, the remaining components were added to it.

c. Bromothymol blue 2.0 ml
(0.5 per cent alcoholic solution)

Solutions (a) and (b) were mixed, bromothymol blue indicator was added to this mixture and the pH was adjusted to 6.8 to 7.0 using 0.1 N potassium hydroxide solution. The medium was transferred into 250 ml conical flasks and sterilized by autoclaving at 121°C for 20 minutes.

A loopful of culture taken from thin, white pellicle of Azospirillum formed in semisolid malate medium was dispersed in five ml of sterile water. From this suspension, a loopful was taken and streaked on Okon's solid medium in sterile petriplates. After incubation for five days at 37°C, they were observed for the development of thin, dry, slightly convex colonies and change in the colour of the medium to blue.

3.4.2.2 Potato Infusion Agar

Potato	200.0 g
Malic acid	2.5 g
KOH	2.0 g
Sucrose	2.5 g
Agar	15.0 g
Vitamin solution	1.0 ml
Distilled water	1000.0 ml

(Vitamin solution prepared by dissolving 10 mg biotin and 20 mg pyridoxin in 100 ml sterile distilled water).

Potatoes were washed, cut into small pieces and boiled in 500 ml distilled water for 30 minutes. The cooked potato solution was filtered through layers of cheese cloth. Malic acid was dissolved in 50 ml water, two drops of bromothymol blue (0.5 per cent solution in ethanol) was added and pH was adjusted to 7.0. Sucrose, agar and malic acid solution were added to the potato filtrate and made upto 1000 ml with distilled water. The medium was transferred to 250 ml conical flasks and sterilized by autoclaving at 121°C for 20 minutes. The vitamin solution was added to the sterilized medium aseptically before pouring the medium into petriplates.

A loopful of the Azospirillum culture was dispersed in five ml of sterile water. From this suspension, a loopful was taken and streaked on Potato Infusion Agar taken in sterile petriplates and incubated at 37°C for seven days and observed for the pink and wrinkled bacterial colonies.

3.4.2.3 Rojo Congo (RC) medium (Caceres, 1982)

Malic acid	5.0 g
KOH	4.8 g
K_2HPO_4	0.5 g
$MgSO_4.7H_2O$	0.2 g
NaCl	0.1 g
Yeast extract	0.5 g
$FeCl_3.6H_2O$	0.015 g

Agar	20.0 g
Distilled water	1000.0 ml
Congo red	15.0 ml

(1:400 aqueous solution)

pH adjusted to 7.0

The medium was transferred to 250 ml conical flasks and sterilized in an autoclave at 121°C for 20 minutes.

Loopful of Azospirillum culture was taken from thin, white pellicle formed in semisolid malate medium and dispersed in five ml sterile water. From this suspension, a loopful was taken and streaked on RC medium and incubated at 37°C for four days and observed for the development of scarlet bacterial colonies with rugose surface and undulating edges.

3.4.3 Physiological tests

Physiological tests such as acidification of glucose, dissimilation of nitrate and utilisation of carbon sources were carried out.

3.4.3.1 Acid from glucose

Acid production from glucose by the collected isolates under aerobic conditions were tested using the medium having following composition.

Glucose	10.0 g
Peptone	2.0 g
$(NH_4)_2SO_4$	1.0 g
$MgSO_4$	1.0 g
$FeCl_3$	0.002 g
$MnSO_4$	0.002 g
Distilled water	1000.0 ml
Bromothymol blue	2.0 ml
(Five per cent alcoholic solution)	
pH adjusted to 7.0	

Five ml quantities of the medium were taken in test tubes and autoclaved at 121°C for 20 minutes. The sterilized medium was inoculated with 0.1 ml suspension of 48 h old culture of Azospirillum isolates and incubated at 37°C for four days. They were then observed for the change in colour of the medium from green to yellow which indicates acid production.

3.4.3.2 Test for dissimilation of nitrate

The Azospirillum isolates were tested for their ability to dissimilate nitrate. NFb medium was modified by adding five mM ammonium nitrate and 1.5 per cent agar. Five ml quantities of the medium were taken in test tubes and sterilized by autoclaving at 121°C for 20 minutes. These tubes were inoculated by stabbing with a loopful each of the *Azospirillum* cultures and incubated at 37°C for five days. The dissimilation of nitrate was indicated by the shredding of agar blocks.

3.4.3.3 Utilisation of carbon sources

The ability of the *Azospirillum* isolates to utilise different carbon sources were tested. The NFb medium was modified by omitting bromothymol blue and replacing malic acid with one per cent carbon sources such as glucose, lactose, sucrose and mannose. Ten ml quantities of the media were transferred to test tubes and sterilized in an autoclave at 115°C for 20 minutes.

The sterile media in test tubes were inoculated with a loopful each of the cultures grown in semisolid malate medium and incubated at 37°C for three days. They were then observed for the development of thin, white, subsurface pellicular growth of *Azospirillum* and compared with the growth of these isolates in semisolid malate medium.

3.5 Maintenance of *Azospirillum* cultures

After purification and primary characterization, the *Azospirillum* cultures were streaked on Okon's solid medium containing petriplates. The single colonies typical of

Azospirillum obtained were transferred to test tube slants of the same medium. They were then stored in a refrigerator at 4°C. The cultures were maintained with periodic purification and subculturing into fresh test tube slants.

3.6 Isolation of Bradyrhizobium

Healthy cowpea plants from vegetable garden of Department of Olericulture, College of Horticulture, Vellanikkara were selected for the isolation of local strain of Bradyrhizobium. The plants were uprooted carefully, causing minimum disturbance to roots and nodules. Isolation of Bradyrhizobium was done in Yeast Extract Mannitol Agar medium (Allen, 1953) of the following composition.

Mannitol	10.0 g
K_2HPO_4	0.5 g
$MgSO_4 \cdot 7H_2O$	0.2 g
NaCl	0.1 g
$CaCO_3$	3.0 g
Yeast extract	1.0 g
Agar	15.0 g
Congo red (One per cent aqueous solution)	2.5 ml
Distilled water	1000.0 ml
pH adjusted to 7.0	

Healthy, pink coloured nodules along with little portion of root attached to it were separated from the collected cowpea roots. The nodules were thoroughly washed in tap water and surface sterilized with 0.1 per cent mercuric chloride solution for one minute. The surface sterilized nodules after washing in five changes of sterile water were crushed in small quantity of sterile water. The suspension from the crushed nodules were streaked on YEMA medium in petiiplates and incubated at room temperature for four days. Individual bacterial colonies showing the characteristics typical of Bradyrhizobium were purified by repeated streaking on YEMA medium.

Commercial Bradyrhizobium inoculant procured from Soil Testing Laboratory, Pattambi was used to get the standard Bradyrhizobium isolate. A small quantity of the inoculant was dispersed in sterile water and a loopful of this suspension was streaked on YEMA medium. Typical Bradyrhizobium colonies were selected and purified by repeated streaking on YEMA medium.

These two isolates of Bradyrhizobium after Gram staining and microscopic examination were maintained by transferring to YEMA slants in which congo red was excluded and they were stored in a refrigerator. Subculturing of the isolates were done at monthly intervals.

3.7 Screening of *Azospirillum* isolates for their efficiency in promoting growth of cowpea

A pot culture experiment was conducted as a Completely Randomised Design with three replications to find out the efficiency of 25 isolates of *Azospirillum* collected, in promoting growth of cowpea. Potting mixture containing sand, soil and farmyard manure in 1:1:1 ratio were sterilized in an autoclave at 121°C for two hours. After cooling to room temperature, the sterilized potting mixture were filled in plastic pots.

3.7.1 Mass production of *Azospirillum* inoculum

A loopful of each of the cultures was dispersed in five ml of sterile distilled water in test tubes. They were then transferred into 250 ml conical flasks containing Okon's liquid medium. These flasks were incubated at 37°C with periodic shaking for three days. The contents of the flasks after incubation were mixed with sterile potting mixture at the rate of 50 ml inoculum per pot.

3.7.2 Cowpea seeds

Seeds of cowpea variety 'Pusa Komal' procured from the Department of Olericulture, College of Horticulture, Vellanikkara was used. Seeds after presoaking in water for one hour were sown on the same day of soil inoculation at the rate of five seeds per pot.

Thinning was done one week after germination of seeds, retaining one healthy plant per pot. The plants were irrigated regularly with sterile tap water. No additional fertilizer was applied.

3.7.3 Observations

The plants were uprooted carefully on the fifty fifth day after sowing and observations such as plant height, leaf length, leaf breadth, total fresh weight, total dry weight, root length and root dry weight were recorded. Plant height was taken from soil level to the tip of the plant. Similarly, root length was taken from collar region downwards. The dry weights were recorded after drying the plant samples to a constant weight at 60°C in an oven. Nitrogen content was estimated from the dried plant samples.

3.7.3.2 Estimation of percentage nitrogen content of plant top (Jackson, 1967)

One hundred mg of powdered plant sample and one g of digestion mixture (Potassium sulphate, cupric sulphate and selenium powder in 10.0 : 1.0 : 0.1 ratio) were digested with five ml of concentrated sulphuric acid in a digestor till the solution becomes clear. After cooling to room temperature, the contents were transferred into a 100 ml volumetric flask and made up the volume with distilled water. Ten ml of the sample along with five ml of 40 per cent sodium hydroxide solution were steam distilled in a Microkjeldahl distillation system. Ammonia liberated was collected in a receiver flask containing 20 ml of two per cent boric acid, three drops of 0.1 per cent methyl red indicator and six drops of bromocresol green solution.

The distillate was titrated against 0.01 N sulphuric acid, and from the titre value, percentage nitrogen content was calculated using the following formula.

$$\text{Percentage nitrogen content} = \frac{V \times N \times V_1 \times 0.014 \times 100}{V_2 \times W}$$

where,

V - Titre value

N - Normality of sulphuric acid

V_1 - Volume of which the sample was made up to

V_2 - Volume of sample used for distillation

W - Weight of plant sample taken

3.8 Pot culture experiment to study the associative effect of *Azospirillum* and *Bradyrhizobium* in cowpea

A pot culture experiment was conducted to study the associative effect of the four selected *Azospirillum* isolates and two *Bradyrhizobium* isolates on nodulation and growth of cowpea.

Azospirillum treatments

1. A-0 - Without *Azospirillum*
2. A-9 - Isolate from cowpea, Vellanikkara
3. A-10 - Isolate from cowpea, Vellanikkara
4. A-11 - Isolate from cowpea, Vellanikkara
5. A-20 - Isolate from cowpea, Vellanikkara

Bradyrhizobium treatments

1. B-0 - Without *Bradyrhizobium*
2. B-1 - Standard *Bradyrhizobium* isolate
3. B-2 - Local isolate of *Bradyrhizobium*

Design	:	Factorial experiment in C.R.D.
Replication	:	Three
Cowpea variety	:	Pusa Komal

The treatment combinations were as follows:

A-0 B-0	A-0 B-1	A-0 B-2
A-9 B-0	A-9 B-1	A-9 B-2
A-10 B-0	A-10 B-1	A-10 B-2
A-11 B-0	A-11 B-1	A-11 B-2
A-20 B-0	A-20 B-1	A-20 B-2

3.8.1 Preparation of sterile potting mixture

Potting mixture was prepared by mixing soil, sand and powdered farmyard manure in 1:1:1 ratio. It was then sterilized in an autoclave at 121°C for two hours. After cooling to room temperature, the sterilized potting mixture was filled in plastic pots.

3.8.1.1 Estimation of pH

pH of the potting mixture used for pot culture experiment was estimated. For this, a suspension was prepared by mixing it with distilled water in the ratio 1:2.5. It was agitated vigorously for five minutes and the pH was read directly in an ELICO digital pH meter.

3.8.2 Mass production of *Azospirillum* and *Bradyrhizobium* inoculum

Azospirillum was mass produced as in the previous experiment. For mass culturing *Bradyrhizobium*, YEM liquid medium without congo red was prepared in 250 ml conical flasks and sterilized. Forty eight hour old cultures of *Bradyrhizobium* were used for inoculating these broth and they were incubated at room temperature for five days with periodic shaking. The contents of the flasks were used for inoculation of potting mixture at the rate of 50 ml inoculum per pot.

3.8.3 Sowing

Cowpea seeds after presoaking in water for one hour were sown in soil at the rate of five seeds per pot on the same day of inoculation of Azospirillum. The inoculation of Azospirillum was done by mixing the inoculum with the sterilized soil. Bradyrhizobium was inoculated in the same way one day after Azospirillum inoculation. Thinning of the seedlings were done one week after germination of seeds retaining two healthy plants per pot.

The plants were irrigated with sterile water regularly. No additional fertilizers were given. Pea aphids were controlled by spraying 0.01 per cent Rogor.

3.8.4 Observations

From each pot, one plant was used to record the above ground observations on the fifty fifth day after sowing. Growth parameters such as plant height, shoot fresh weight, shoot dry weight, number of leaves, leaf length and leaf breadth were recorded. Dry weights were recorded after drying the samples at 60°C to constant weight in an oven. Nitrogen content of the plant top was estimated by Microkjeldahl distillation method after digesting the samples with concentrated sulphuric acid. Remaining one plant in each pot was retained till pod maturity for taking observations such as root fresh weight, root dry weight, number of nodules, pod fresh weight and pod dry weight.

3.9 Determination of pH tolerance of *Azospirillum* and *Bradyrhizobium* isolates

pH tolerance of the four selected *Azospirillum* isolates viz., A-9, A-10, A-11 and A-20 were studied using Okon's liquid medium. Media of eight different pH, viz., 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5 were prepared. They were taken in test tubes, ten ml each, and sterilized in an autoclave at 121°C for 20 minutes.

A loopful of the culture of each isolate was dispersed in sterile water. One ml of this suspension was used for inoculating the medium in each test tube. After incubation at 37°C for three days with periodic shaking, the turbidity of the media were read in a Spectronic-20 spectrophotometer at 380 nm. The uninoculated media of different pH served as reagent blanks.

Similarly for *Bradyrhizobium*, YEM liquid medium was prepared excluding congo red and the pH was adjusted to 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5 using either 0.1 N hydrochloric acid or 0.1 N potassium hydroxide. Media were taken in test tubes, ten ml each, and sterilized in an autoclave at 121°C for 20 minutes.

The sterilized media in test tubes were inoculated with one ml each of the *Bradyrhizobium* culture suspension and incubated at room temperature for four days with periodic shaking. Turbidity of the media were then read in a Spectronic-20 spectrophotometer at 640 nm, using uninoculated media of appropriate pH as the reagent blanks.

3.10 Statistical analysis

Statistical analysis of data for screening of *Azospirillum* isolates was done as a Completely Randomised Design. The analysis of data obtained in the second pot culture experiment for studying the associative effect of the two microsymbionts was done as a factorial set up with *Azospirillum* at five levels and *Bradyrhizobium* at three levels (Das and Giri, 1979).

Results

RESULTS

4.1 Isolation of *Azospirillum*

Azospirillum was isolated from the roots of legumes and nonlegumes collected from different regions and soil types of Kerala. The formation of thin, white, subsurface, pellicular growth in nitrogen free semisolid medium indicated the presence of *Azospirillum* (Plate I). The pellicular growth of the isolates during serial transfers into fresh semisolid media ensured their purity. Twenty four isolates of *Azospirillum* were thus collected. An isolate from the commercial *Azospirillum* culture procured from Tamil Nadu Agricultural University was also included in the study thus making the total number of isolates twenty five. The details of the collected *Azospirillum* isolates are given in Table 1.

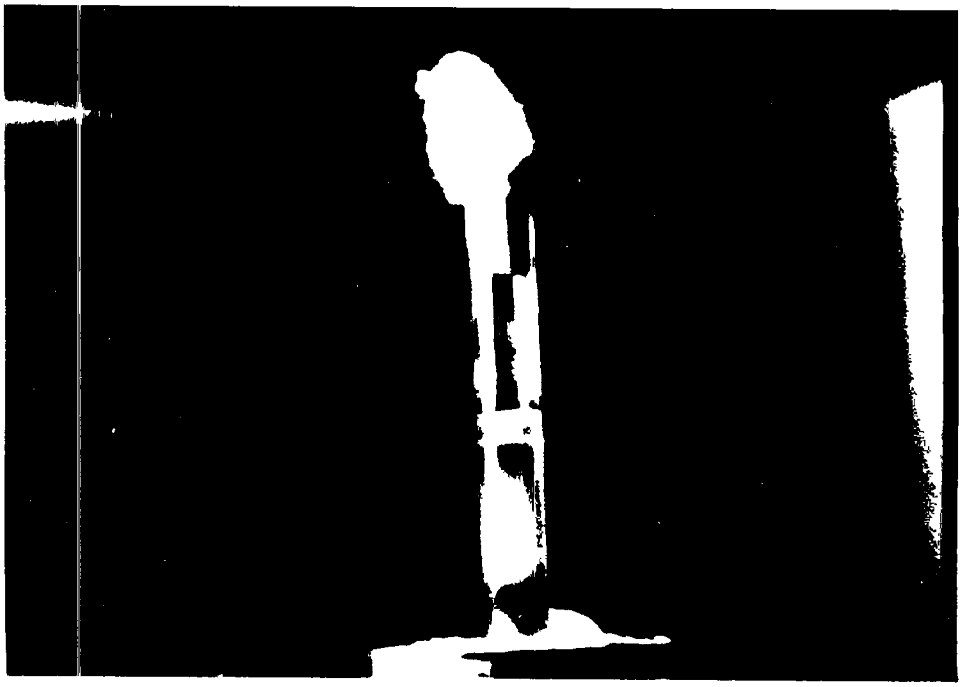
4.2 Primary characterization of *Azospirillum* isolates

On microscopic examination, all the *Azospirillum* isolates were found to be Gram negative and vibrioid. The cultural and physiological characters of the isolates were studied and the results are presented below.

4 2.1 Cultural characters

In Okon's medium after incubation at 37°C for five

Plate I Thin, white, subsurface pellicular growth of *Azospirillum* in semisolid malate medium



[illegible]

Sl [illegible]	[illegible]	[illegible]	Location [illegible]	S[illegible]
1	A 1	Cowpea	Nemmara Palakkad	R[illegible]
[illegible]	A 2	C[illegible]wp[illegible]a	Vellay[illegible] [illegible]	[illegible]
3	A 3	Cow[illegible]	Ch[illegible] Palakkad	Black
4	A 4	[illegible]owp[illegible]a	Valavoode Ernakulam	Sandy loam
[illegible]	A [illegible]	C[illegible]	[illegible]	Laterite
6	A 6	Cowpea	Thar[illegible] Thrissur	Laterite
7	A 7	[illegible]	[illegible]	L[illegible]t[illegible]
8	A 8	C[illegible]	Vellanikk[illegible] Thrissur	Laterite
9	A 9	Cowpea	Vellanikk[illegible] Thrissur	Laterite
10	A 10	Cowpea	Vellanikkara Thrissur	Laterite
11	A 11	C[illegible]wp[illegible]	Vellanikkara Thr[illegible]	Lat[illegible]
12	A 12	Cowpea	Pattikkad Thrissur	Laterite
13	A 13	Cereals	Tamil Nadu Agricultural University Coimbatore	([illegible])
14	A 14	C[illegible]	K[illegible] Alappuzha	A[illegible]
15	A 15	[illegible]wp[illegible]	Palakkad [illegible]	Lat[illegible]ite
16	A 16	Cowp[illegible]	[illegible]	Sandy
17	A 17	[illegible]	[illegible] Alappuzha	[illegible]
18	A 18	Cowpe[illegible]	Changanacherry Kottayam	Laterite
19	A 1[illegible]	[illegible]	Vellanikkara Thrissur	Lat[illegible]
20	A 20	Cowpea	Vellanikkara Thrissur	Laterite
21	A 21	Cowpea	Taliparamba Kannur	Laterite
22	A 22	Digitaria [illegible]	Mannuthy Thrissur	Laterite
23	A 23	Paddy	Vyttila Ernakulam	Saline
24	A 24	Cyn[illegible]	[illegible]	Laterite
25	A 25	Cynodon dactylon	Nemmara Palakkad	R[illegible]

days, all the isolates formed typical thin, dry, slightly convex colonies with a granular wavy surface and undulate margin. The colonies were found to be partially embedded in the medium. The colour of the medium changed to blue, initially as a halo surrounding the individual colonies which later coalesced and spread through the entire plate. The colonies of Azospirillum isolates formed in Potato Infusion Agar were pink, round to irregular, dense and often wrinkled after incubation at 37°C for seven days. In RC medium, the colonies formed after incubation at 37°C for four days were scarlet, dry, round to irregular, with rugose surface and undulating edges. Thus all the collected isolates formed colonies with characters typical of Azospirillum when grown in Okon's medium, Potato Infusion Agar and RC medium.

4 2.2 Physiological tests

Results of the physiological tests conducted for the Azospirillum isolates are presented in Table 2.

4.2.2.1 Acid production from glucose

All the Azospirillum isolates except A-15 and A-19 produced acid from glucose under aerobic conditions. This was indicated by the change in colour of the inoculated medium from green to yellow after incubation at 37°C for four days.

Table Physiological characters of Azospirillum isolates

Isolate	Acid from glucose	Dissimilation of nitrate	Utilization of carbon sources: Malate	Glucose	Sucrose	Lactose	Mannitol
A-1	+		G	S	N	S	N
A-2	+		G	S	N	N	N
A-3	+	+		S	S		S
A-4	+	-	G	S			S
A-5	+	-	G	S	S	S	S
A-6	+	-	G	S	N	N	S
A-7	+	+	G	S	N	N	S
A-8	+	+	G	S	S	N	S
A-9	+	-	G	S	S	N	S
A-10	+	-	G	S	S	S	S
A-11	+	-	G	S	N		S
A-12	+	-	G	S	N	S	S
A-13	+	+	G		S	N	S
A-14	+	-	G	S	S	N	S
A-15		+	G	S	N	N	N
A-16	+	-	G	S	N	N	S
A-17	+	-	G	S	S	N	N
A-18	+	-	G	S	S	N	S
A-19		-	G	S	S	N	N
A-20	+	-	G	S	S	N	S
A-21	+	-	G	S	N	N	N
A-22	+	-	G	N	S	N	S
A-23	+	-	G	N	S	N	N
A-24	+	-	G	S	S	S	S
A 2	+	-	G			N	S

\+ positive reaction G - good growth

\- negative reaction S - scanty growth

N - no growth

4.2.2.2 Dissimilation of nitrate

Among the 25 Azospirillum isolates, five isolates, viz., A-3, A-7, A-8, A-13 and A-15 resulted in shredding of agar block when grown in NFb medium containing ammonium nitrate, showing dissimilation of nitrate.

4.2.2.3 Utilisation of carbon sources

All the 25 Azospirillum isolates had good growth in medium containing malate as the carbon source. Growth in media containing sugars such as glucose, lactose, sucrose and mannose were scanty. After incubation at 37°C for three days, all the isolates except A-22 and A-23 formed scanty, white, pellicular growth in medium containing glucose as the carbon source. Scanty growth was observed for 18 isolates with mannose 16 isolates with sucrose and 8 isolates with lactose as carbon sources. Remaining isolates did not show any growth in media containing these sugars.

4.3 Isolation of Bradyrhizobium

The suspension from the nodules of cowpea when streaked on YEMA medium, large, white, gummy colonies of Bradyrhizobium were formed after incubation at room temperature for four days. Similar colonies were obtained when the suspension from commercial inoculant was streaked on YEMA medium. On microscopic examination they were found to be rod shaped and Gram negative.

4.4 Screening of *Azospirillum* isolates for their efficiency in promoting growth of cowpea

An aseptic pot culture experiment was conducted to screen the 25 *Azospirillum* isolates collected, for their efficiency in promoting growth of cowpea variety 'Pusa Komal'. The results of this experiment are presented below.

4.4.1 Plant height

Generally, inoculation with *Azospirillum* resulted in increase in the plant height of cowpea. The maximum plant height of 34.83 cm was observed in plants inoculated with isolate A-6 (Table 3, Plate II) and it was closely followed by isolates A-13, A-12 and A-10. Plants inoculated with isolate A-3 recorded the lowest plant height. Isolate A-6 was on par with 16 other isolates and significantly superior to control. Eight isolates were on par with control. For the control plants, the mean plant height was only 28.33 cm.

4.4.2 Leaf length

Azospirillum isolates differed significantly in their effect on the leaf length of cowpea. All the isolates except

Plate II Effect of Azospirillum inoculation on plant height of cowpea

Plate III Effect of Azospirillum inoculation on root length of cowpea

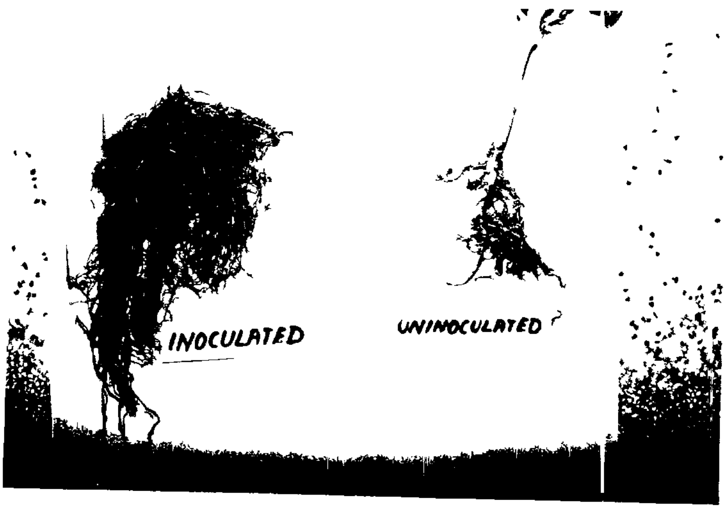

INOCULATED
UNINOCULATED

Table 3 Screening for efficiency of *Azospirillum* isolates - Effect of inoculation on plant height, leaf length, leaf breadth and root length of cowpea

Isolate	Plant height (cm)	Leaf length (cm)	Leaf breadth (cm)	Root length (cm)
A-1	30.83	8.23	5.39	16.67
A-2	31.97	8.51	5.42	16.50
A-3	2[illegible].67	7.64	5.4[illegible]	18.63
A-4	31.10	9.14	6.40	15.90
A-5	31.40	8.99	5.73	13.[illegible]3
A-6	34.83	7.85	5.00	17.63
A-7	30.70	8.30	6.21	17.[illegible]3
A-8	31.63	9.98	5.94	15.00
A-9	32.07	9.92	6.16	20.83
A-10	33.83	9.58	5.98	18.57
A-11	33.33	8.36	5.21	18.17
A-12	33.87	9.81	5.85	18.17
A-13	34.67	8.69	[illegible].36	15.83
A-14	31.50	8.15	5.68	18.40
A-15	31.67	8.36	5.39	21.07
A-16	29.20	8.16	5.11	21.37
A-17	31.67	8.97	5.21	18.37
A-18	30.00	7.34	5.32	1[illegible].67
A-19	28.67	8.83	5.58	14.50
A-20	29.77	8.14	5.39	15.77
A-21	28.60	8.82	5.59	20.60
A-22	28.37	8.07	5.38	16.10
A-23	30.47	7.94	4.77	18.23
A-24	26.[illegible]7	6.57	4.68	15.70
A-25	32.13	8.20	5.37	16.83
Control	28.33	7.25	4.87	13.60
CD (0.05)	4.76	1.66	NS	4.22

NS - Not significant

A-24 resulted in increase in leaf length of cowpea compared to control. A maximum mean leaf length of 9.98 cm was recorded in plants inoculated with isolate A-8 (Table 3). It was on par with 12 other isolates, but significantly superior to control and remaining 12 isolates. The performance of isolates A-9, A-12 and A-10 was almost similar to A-8. Lowest leaf length was recorded in plants inoculated with isolate A-24. It was inferior to control which had a mean plant height of 7.25 cm.

4.4.3 Leaf breadth

Though statistically not significant, Azospirillum treatments in general had an effect in increasing the leaf breadth of cowpea. Plant inoculated with all the Azospirillum isolates except A-23 and A-24 had higher mean leaf breadth compared with the control. Maximum mean leaf breadth of 6.40 cm was recorded in plants inoculated with isolate A-4 and it was closely followed by isolates A-7, A-9 and A-10 (Table 3). In control plants the mean leaf breadth was 4.87 cm.

4.4.4 Root length

There was significant difference between the Azospirillum treatments in their effect on root length of cowpea. Inoculation with all the isolates except A-5 gave higher root length in cowpea compared to control. Maximum mean root length of 21.37 cm was observed in plants inoculated with isolate A-16 (Table 3, Plate III). It was on par with 12 isolates and also significantly superior to control and remaining isolates. Isolates A-15, A-9 and A-21 performed almost equal to isolate A-16 in increasing the root length of cowpea. In control plants the mean root length was only 13.60 cm.

4.4.5 Total fresh weight

The Azospirillum isolates differed significantly in increasing the total fresh weight of cowpea. In general, all the inoculated plants had higher fresh weight compared to control. The maximum mean fresh weight of 71.26 g was recorded in plants inoculated with isolate A-8 (Table 4). It was on par with 10 other isolates among which, isolates A-6 and A-10 performed almost equal to A-8. All the isolates except A-23, A-24 and A-25 were significantly superior to control. The control plants had a mean fresh weight of 36.37 cm.

4.4.6 Total dry weight

Generally, inoculation with Azospirillum isolates resulted in significant increase in the total dry weight of cowpea. The maximum mean dry weight of 11.13 g was recorded

Table 4 Screening for efficiency of *Azospirillum* isolates - Effect of inoculation on total fresh weight, total dry weight, root dry weight and percentage nitrogen content of cowpea

Isolate	Total fresh weight (g)	Total dry weight (g)	Root dry weight (g)	Nitrogen content (%)
A-1	56.82	9.25	3.51	2.77
A-2	52.37	7.8[illegible]	2.41	2.77
A-3	61.34	8.42	3.23	2.72
A-4	68.53	10.1[illegible]	3.41	2.97
A-5	55.35	9.80	3.83	3.39
A-6	70.5	10.56	3.57	3.33
A-7	61.28	9.32	4.1	2.72
A-8	71.26	11.13	3.68	2.87
A-9	62.82	8.76	4.78	3.68
A-10	70.53	10.94	3.62	3.44
A-11	57.37	7.63	2.48	4.10
A-12	55.66	8.13	2.21	3.64
A-13	51.86	8.04	2.28	2.60
A-14	53.33	8.37	2.77	3.64
A-15	52.50	9.75	[illegible].43	2.36
A-16	56.8[illegible]	8.66	3.58	3.[illegible]4
A-17	60.68	9.9[illegible]	3.16	3.69
A-18	56.40	9.18	2.83	3.38
A-19	50.41	8.47	2.97	3.23
A-20	52.87	9.07	3.26	3.80
A-21	58.71	9.04	3.98	2.72
A-22	51.55	7.01	3.03	3.08
A-23	41.28	6.70	2.26	2.88
A-24	42.64	6.80	2.03	2.87
A-25	43.67	7.42	2.52	3.30
Control	36.37	[illegible].27	1.11	3.08
CD (0.05)	11.20	1.4[illegible]	0.52	NS

NS Not significant

in plants inoculated with isolate A-8 (Table 4) whereas, it was only 5.27 g in control. Isolate A-8 was on par with 13 other isolates of which, isolates A-6 and A-10 performed almost equal to it. Remaining 11 isolates were on par and significantly superior to control.

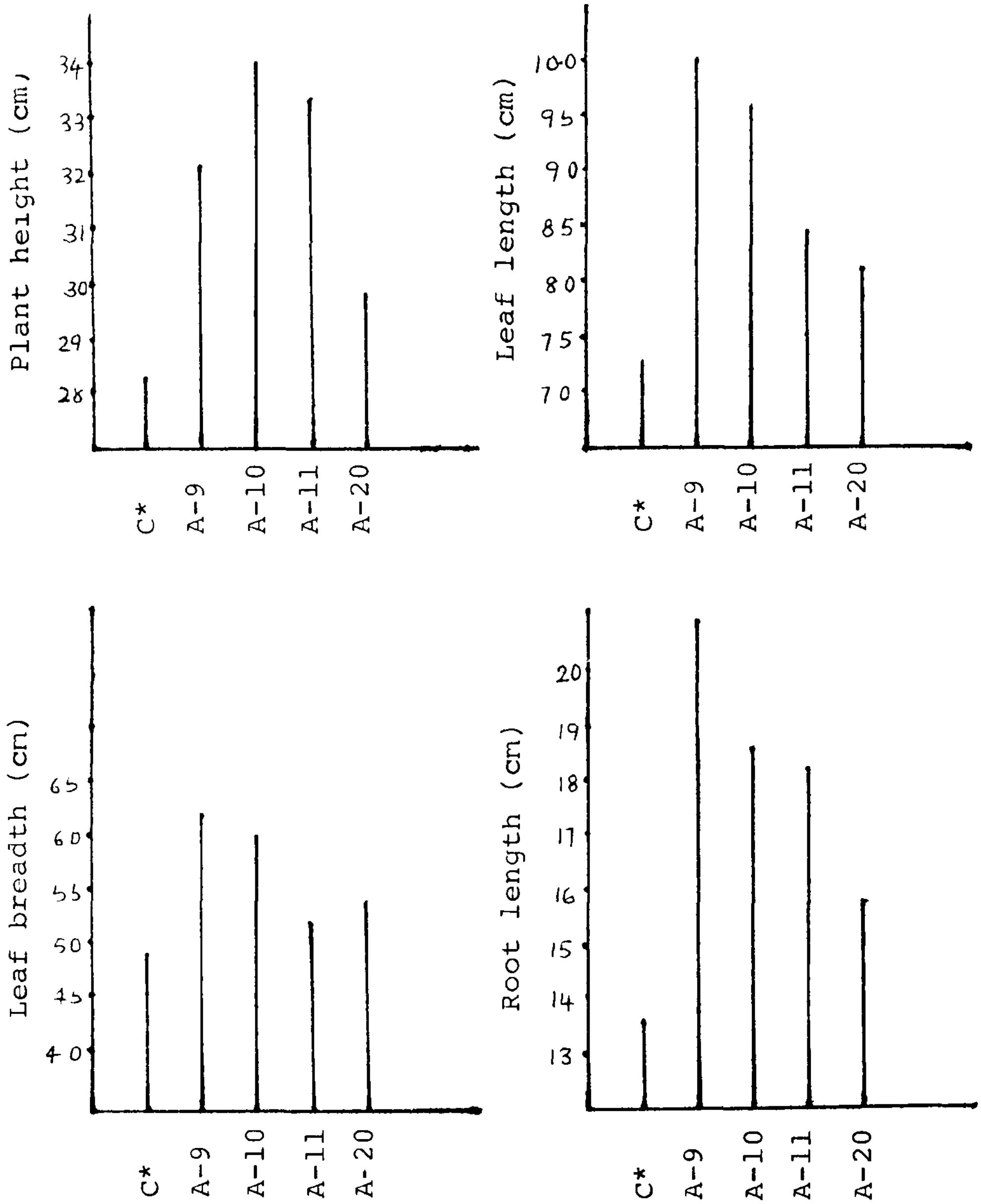
4.4.7 Root dry weight

Cowpea plants inoculated with all the Azospirillum isolates had significantly higher root dry weights compared with the control. The maximum mean root dry weight of 4.78 g was recorded in plants inoculated with isolate A-9 and it was followed by isolates A-7 and A-21. In control plants it was only 1.11 g (Table 4). Isolate A-9 was on par with 11 other isolates and they were significantly superior to remaining 13 isolates.

4.4.8 Percentage nitrogen content

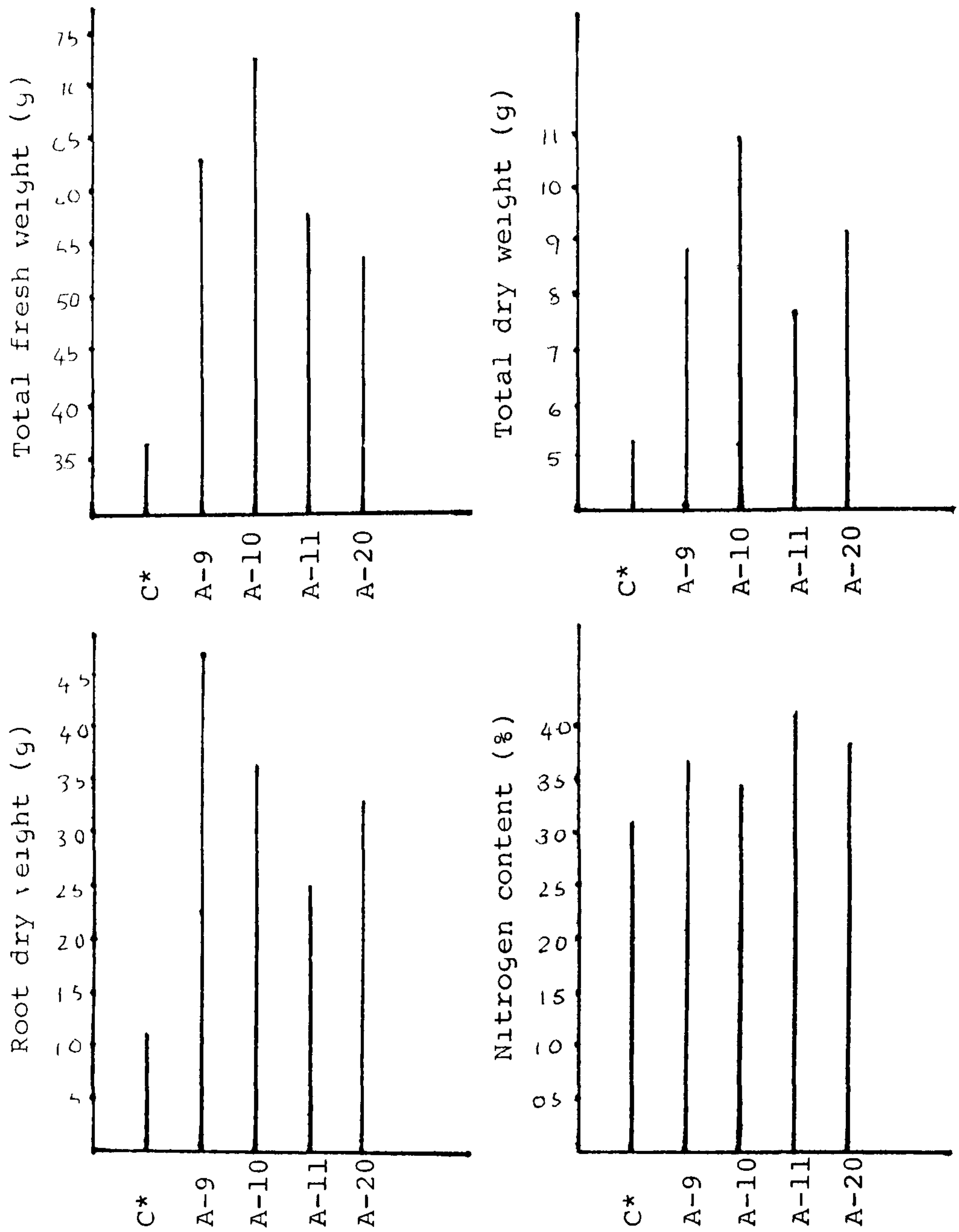
In general, inoculation with Azospirillum had no significant effect on percentage nitrogen content of cowpea. Thirteen isolates gave higher nitrogen content in inoculated plants compared to control and isolate A-11 resulted in the maximum nitrogen content of 4.10 per cent (Table 4). It was followed by isolate A-20 which resulted in percentage nitrogen content of 3.80. The control plants had nitrogen content of

Fig. 1 Effect of the four selected *Azospirillum* isolates on plant height, leaf length, leaf breadth and root length of cowpea



* Control

Fig. 2 Effect of the four selected *Azospirillum* isolates on total fresh weight, total dry weight, root dry weight and percentage nitrogen content of cowpea



* Control

3.08 per cent. Lowest nitrogen content of 2.36 per cent was recorded in plants inoculated with isolate A-15.

4.4.9 Selection of promising isolates

Based on the results of the pot culture experiment for screening the 25 *Azospirillum* isolates, four isolates viz., A-9, A-10, A-11 and A-20 were selected for further work. Isolates A-9 and A-10 were selected based on their better performance in increasing plant height, leaf length, leaf breadth, total fresh weight, total dry weight, and root dry weight of cowpea. Isolates A-11 and A-20 were selected as they gave higher percentage nitrogen content in inoculated plants (Fig.1 and 2).

4.5 Associative effect of *Azospirillum* and *Bradyrhizobium* on growth and nodulation of cowpea

The associative effect of the selected four *Azospirillum* isolates and two *Bradyrhizobium* isolates were studied in a pot culture experiment. The potting mixture used for the experiment had a pH of 6.2. The results of the experiment are presented below.

4.5.1 Plant height

The *Azospirillum* treatments differed significantly in their main effect on the plant height of cowpea. Isolates A-9

Table 5. Associative effect of *Azospirillum* and *Bradyrhizobium* on plant height of cowpea

Bradyrhizobium treatments (B)	*Azospirillum* treatments (A)					Mean
	A-0	A-9	A-10	A-11	A-20	
B-0	29.46	34.70	34.96	28.73	25.30	30.63
B-1	36.60	37.76	39.36	34.46	33.75	36.38
B-2	37.60	39.43	32.46	33.46	29.06	34.40
Mean	34.55	37.30	35.59	32.22	29.37	33.80

CD (0.05) for A - 3.58

CD (0.05) for B - 2.77

A x B - Not significant

and A-10 gave increased plant heights in inoculated plants eventhough not significant when compared with their control. The maximum mean plant height of 37.30 cm was recorded in plants inoculated with isolate A-9 (Table 5). It was on par with isolate A-10 and control. Isolates A-11 and A-20 were inferior to control and the lowest plant height was observed in plants inoculated with A-20. Considering the individual effect of the two Bradyrhizobium isolates, both of them gave significantly higher plant heights compared to their control. Isolate B-1 performed best giving a mean plant height of 36.38 cm. It was on par with isolate B-2.

The interaction between the two microsymbionts had no significant effect on plant height of cowpea. Bradyrhizobium isolate B-1 in combination with Azospirillum isolates A-9 and A-10 gave increased plant height, compared to its inoculation alone. On the other hand, in combination with isolates A-11 and A-20, a decrease in mean plant height was observed. Mixed inoculation of isolate B-2 with all the Azospirillum isolates except A-9 resulted in decreased plant heights compared to its single inoculation. The maximum mean plant height of 39.43 cm was recorded in plants inoculated with A-9 B-2 combination (Fig.3). It was closely followed by A-10 B-1 combination which resulted in a mean plant height of 39.36 cm. Among the isolate combinations, A-20 B-2 gave the lowest mean plant height and it was inferior even to the uninoculated control.

Table 6. Associative effect of *Azospirillum* and *Bradyrhizobium* on number of leaves of cowpea

Bradyrhizobium treatments (B)	Azospirillum treatments (A)					Mean
	A-0	A-9	A-10	A-11	A-20	
B-0	3.31 (11.0)	3.78 (14.3)	3.60 (13.0)	3.58 (13.0)	3.31 (11.0)	3.51 (12.46)
B-1	3.73 (14.0)	3.99 (16.0)	3.87 (15.0)	3.60 (13.0)	3.46 (12.0)	3.73 (14.0)
B-2	3.95 (15.7)	3.73 (14.0)	3.55 (12.7)	3.36 (11.3)	3.50 (12.3)	3.62 (13.20)
Mean	3.66 (13.56)	3.83 (14.77)	3.67 (13.57)	3.51 (12.43)	3.42 (11.77)	3.62 (13.22)

Data transformed. Original values given in parentheses.

CD (0.05) for A - 0.26

B - Not significant

A x B - Not significant

4.5.2 Number of leaves

The main effect of Azospirillum treatment varied significantly with respect to number of leaves of cowpea. Isolate A-9 performed best, giving a mean number of leaves of 14.77 (Table 6). It was on par with isolate A-10 and their respective control, but significantly superior to isolates A-11 and A-20. The Bradyrhizobium treatments did not vary significantly in their main effects in increasing the number of leaves of cowpea. Among the two Bradyrhizobium isolates, B-1 performed best.

There was no significance for the interaction effect of Azospirillum and Bradyrhizobium isolates on number of leaves of cowpea. Isolate B-1 in combination with A-9 and A-10 isolates gave more number of leaves in inoculated plants compared to its single inoculation. However, with isolates A-11 and A-20 the mean number of leaves were less. Mixed inoculation of isolate B-2 with all the four Azospirillum isolates resulted in decrease in the number of leaves compared to its single inoculation. The maximum mean number of leaves of 16.0 was recorded in plants receiving mixed inoculant of A-9 and B-1 isolates (Table 6). Among the isolate combinations, A-11 B-2 gave the least mean number of leaves.

Table 7. Associative effect of *Azospirillum* and *Bradyrhizobium* on leaf length of cowpea

Bradyrhizobium treatments (B)	*Azospirillum* treatments (A)					Mean
	A-0	A-9	A-10	A-11	A-20	
B-0	7.85	8.42	8.74	7.91	6.78	7.94
B-1	9.69	9.60	9.72	9.19	8.99	9.44
B-2	8.76	9.34	8.28	8.70	7.68	8.55
Mean	8.76	9.13	8.91	8.60	7.81	8.64

CD (0.05) for A - 0.87

CD (0.05) for B - 0.67

A x B - Not significant

4.5.3 Leaf length

There was statistical significance for the main effect of both Azospirillum and Bradyrhizobium treatments on the leaf length of cowpea. However, their interaction was not significant. With regard to main effect of Azospirillum treatments, isolate A-9 gave the maximum mean leaf length of 9.13 cm (Table 7). It was on par with isolates A-10 and A-11 and their respective control but significantly higher than isolate A-20. Main effect of Bradyrhizobium isolate B-1 was significantly superior to B-2 and their respective control, giving a mean leaf length of 9.44 cm.

Mixed inoculation leading to increased leaf length compared to individual inoculations was observed only with A-10 B-1 and A-9 B-2 combinations. The maximum mean leaf length of 9.72 cm was observed in plants inoculated with A-10 B-1 combination of isolates (Table 7). Among the plants inoculated with different isolate combinations, those with A-20 B-2 gave the least mean leaf length. It was inferior even to the uninoculated control.

4.5.4 Leaf breadth

The Azospirillum and Bradyrhizobium treatments varied significantly in their main effect on the leaf breadth of cowpea. But their interaction had no significant effect.

Table 8. Associative effect of *Azospirillum* and *Bradyrhizobium* on leaf breadth of cowpea

Bradyrhizobium treatments (B)	*Azospirillum* treatments (A)					Mean
	A-0	A-9	A-10	A-11	A-20	
B-0	4.74	5.65	5.38	4.86	4.14	4.95
B-1	6.24	6.02	6.22	5.44	5.32	5.84
B-2	5.82	5.89	5.17	5.48	4.79	5.43
Mean	5.60	5.85	5.69	5.26	4.75	5.41

CD (0.05) for A - 0.61

CD (0.05) for B - 0.47

A x B - Not significant

Among *Azospirillum* treatments, A-9 performed best giving a mean leaf breadth of 5.85 cm. It was on par with A-10 and A-11 isolates and also with their control, but significantly superior to A-20. Both the *Bradyrhizobium* isolates were significantly superior to their respective control with regard to their main effect on leaf breadth of cowpea. The highest mean leaf breadth of 5.84 cm was observed in plants inoculated with isolate B-1 (Table 8).

Mixed inoculation of B-1 isolate with each of the four *Azospirillum* isolates resulted in decrease in the leaf breadth compared to its inoculation alone. Isolate B-2 also gave lower mean leaf breadth when inoculated in combination with all the *Azospirillum* isolates except A-9. None of the combination of isolates was superior when compared to inoculation with isolate B-1 alone which recorded the maximum mean leaf breadth of 6.24 cm (Table 8) However, A-10 B-1 and A-9 B-1 combinations performed almost similar to isolate B-1 alone giving mean leaf breadths of 6.22 and 6.02 cm respectively. Among the different combination of isolates, A-20 B-2 gave the least mean leaf breadth.

4.5.5 Shoot fresh weight

Eventhough the individual effect of the *Azospirillum* and *Bradyrhizobium* treatments differed significantly with

Table 9. Associative effect of *Azospirillum* and *Bradyrhizobium* on shoot fresh weight of cowpea

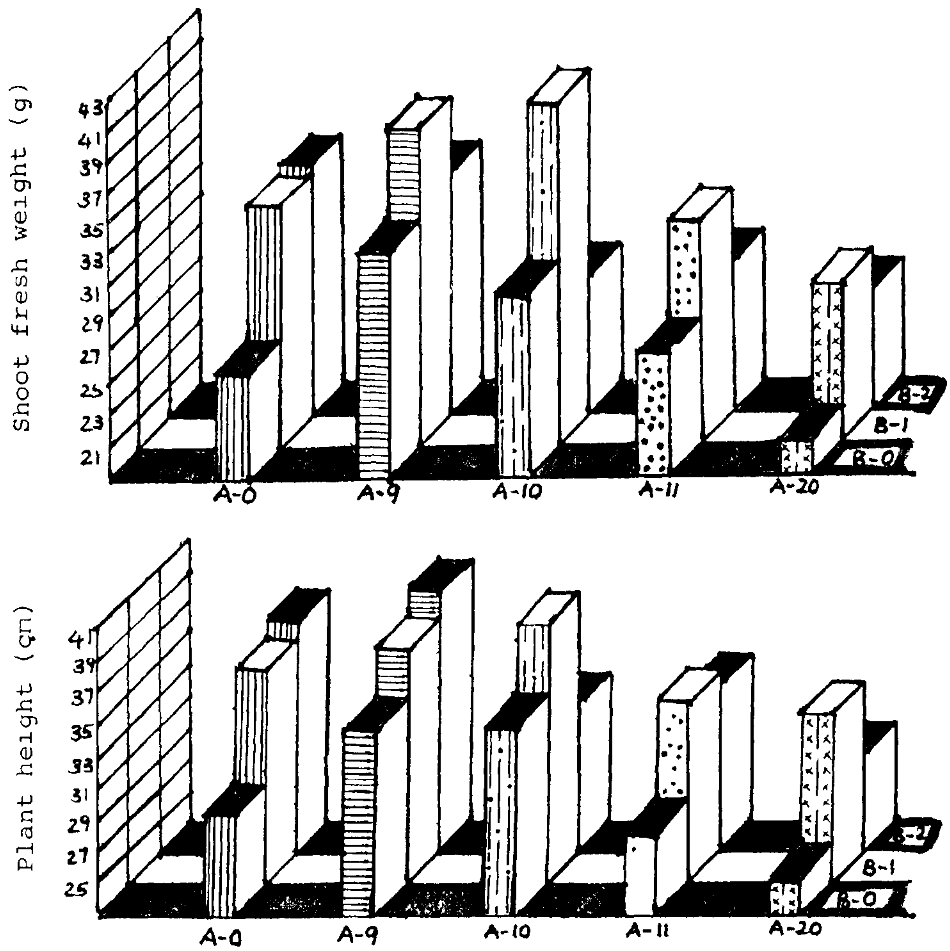
Bradyrhizobium treatments (B)	*Azospirillum* treatments (A)					Mean
	A-0	A-9	A-10	A-11	A-20	
B-0	25.47	32.96	30.25	26.77	21.11	27.31
B-1	34.06	38.96	40.58	32.94	28.80	35.06
B-2	34.65	34.23	27.49	28.20	26.37	30.19
Mean	31.39	35.38	32.77	29.30	25.43	30.85

CD (0.05) for A - 4.56

CD (0.05) for B - 3.53

A x B - Not significant

Fig. 3 Associative effect of _Azospirillum_ and _Bradyrhizobium_ on shoot fresh weight and plant height of cowpea



A-0, A-9, A-10, A-11, A-20 - _Azospirillum_ treatments

B-0, B-1, B-2 - _Bradyrhizobium_ treatments respect to shoot fresh weight of cowpea, their interaction was not significant. Among the Azospirillum isolates, A-9 was the best giving a mean shoot fresh weight of 35.38 g. It was significantly superior to isolates A-11 and A-20 and was on par with isolate A-10 and their respective control. Among the Bradyrhizobium treatments, isolate B-1 performed best in its individual effect on shoot fresh weight giving a mean value of 35.06 g (Table 9).

The mixed inoculation of Bradyrhizobium isolate B-1 with Azospirillum isolates A-9 and A-10 resulted in higher mean shoot fresh weight compared to their single inoculation. But when B-1 isolate was inoculated together with isolates A-11 and A-20, the mean shoot fresh weight was lesser than that obtained by inoculation with B-1 alone. Mixed inoculation of Bradyrhizobium isolate B-2 with all the four Azospirillum isolates resulted in lesser mean shoot fresh weights compared to its single inoculation (Fig.3). The maximum mean shoot fresh weight of 40.58 g was recorded in plants inoculated with both A-10 and B-1 isolates. Among the isolate combinations, the least mean shoot fresh weight was observed in plants inoculated with A-20 B-2 combination.

4.5.6 Shoot dry weight

There was statistical significance for the main effect of Azospirillum and Bradyrhizobium treatments on shoot dry

Table 10. Associative effect of *Azospirillum* and *Bradyrhizobium* on shoot dry weight of cowpea

Bradyrhizobium treatments (B)	*Azospirillum* treatments (A)					Mean
	A-0	A-9	A-10	A-11	A-20	
B-0	3.45	4.48	4.62	3.77	2.95	3.86
B-1	4.75	6.56	5.27	4.71	4.49	5.04
B-2	4.74	4.79	4.02	3.91	3.70	4.23
Mean	4.11	5.28	4.64	4.14	3.71	4.31

CD (0.05) for A - 0.89

CD (0.05) for B - 0.69

A x B - Not significant

weight of cowpea. Isolate A-9 and A-10 among the *Azospirillum* treatments, and isolate B-1 among the *Bradyrhizobium* treatments gave significantly higher mean shoot dry weights compared to their respective controls. The main effect of *Azospirillum* isolates A-11 and A-20 were on par with their control. *Bradyrhizobium* isolate B-2 was also on par with its control.

Eventhough the interaction of *Azospirillum* and *Bradyrhizobium* isolates had no significant effect on shoot dry weight of cowpea, the combination of *Bradyrhizobium* isolate B-1 with *Azospirillum* isolates A-9 and A-10 resulted in higher shoot dry weights compared to their individual inoculation (Table 10). But isolate B-2 gave higher shoot dry weight only in combination with isolate A-9. All other combination of isolates resulted in decrease in the shoot dry weight of inoculated plants. The maximum mean shoot dry weight of 6.56 g was recorded in plants inoculated with A-9 B-1 combination. Inoculation with A-20 B-2 combination resulted in the lowest mean shoot dry weight among all the isolate combinations.

4.5.7 Root fresh weight

Azospirillum treatments did not vary significantly in their main effect on the root fresh weight of cowpea

Table 11. Associative effect of *Azospirillum* and *Bradyrhizobium* on root fresh weight of cowpea

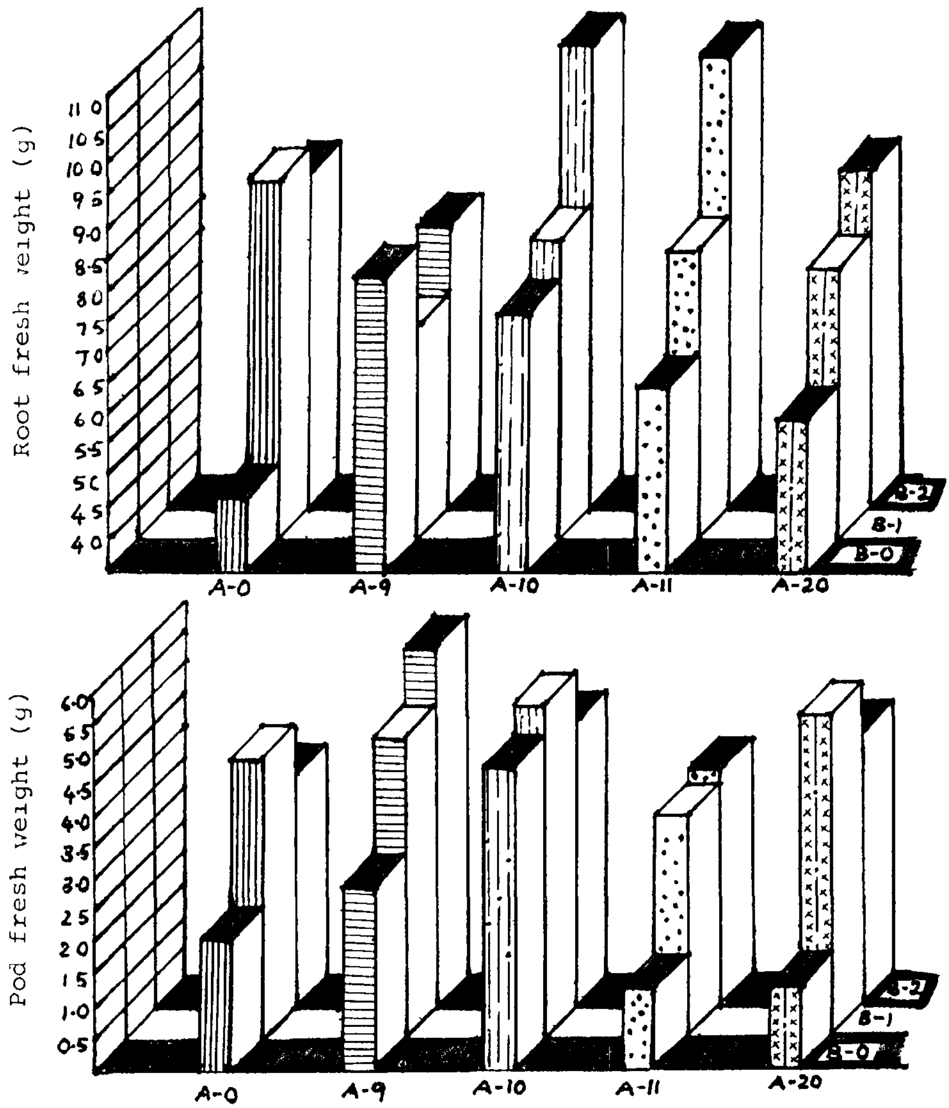
Bradyrhizobium treatments (B)	*Azospirillum* treatments (A)					Mean
	A-0	A-9	A-10	A-11	A-20	
B-0	4.67	8.22	7.58	6.44	5.92	6.57
B-1	9.20	6.88	8.25	8.12	8.28	8.15
B-2	8.75	7.96	10.84	10.62	8.76	9.38
Mean	7.54	7.69	8.89	8.39	7.63	8.03

A - Not significant

CD (0.05) for B - 1.17

A x B - Not significant

Fig. 4 Associative effect of Azospirillum and Bradyrhizobium on root fresh weight and pod fresh weight of cowpea



A-0, A-9, A-10, A-11, A-20 - Azospirillum treatments
B-0, B-1, B-2 - Bradyrhizobium treatments

eventhough all the inoculated plants had higher mean root fresh weights compared to their control. Isolate A-10 performed best giving a mean root fresh weight of 8.89 g (Table 11). Main effect of Bradyrhizobium treatments was significant and inoculation with isolates B-1 and B-2 resulted in significantly higher root fresh weight compared to their control. Among them, isolate B-2 recorded the highest mean root fresh weight of 9.38 g.

There was no significance for the effect of interaction of Azospirillum and Bradyrhizobium on root fresh weight of cowpea. Bradyrhizobium isolate B-1 in combination with the four Azospirillum isolates, gave lesser mean root fresh weight compared to its single inoculation (Table 11 and Fig.4). But mixed inoculation of Bradyrhizobium isolate B-2 with each of the Azospirillum isolates except A-9 resulted in higher root fresh weight than its individual inoculation. The maximum root fresh weight of 10.84 g was obtained by inoculation of isolate combination A-10 B-2 and it was closely followed by A-11 B-2 which resulted in a mean root fresh weight of 10.62 g. Among the different combination of isolates, A-9 B-1 gave the least mean root fresh weight.

4.5.8 Root dry weight

Eventhough inoculation with all the four Azospirillum isolates resulted in increased root dry weight in cowpea,

Table 12. Associative effect of <u>Azospirillum</u> and <u>Bradyrhizobium</u> on root dry weight of cowpea

<u>Bradyrhizobium</u> treatments (B)	<u>Azospirillum</u> treatments (A)					Mean
	A-0	A-9	A-10	A-11	A-20	
B-0	1.09	2.04	1.60	1.69	1.62	1.61
B-1	2.22	1.77	1.86	1.84	1.92	1.92
B-2	2.00	1.80	2.76	2.35	2.04	2.19
Mean	1.77	1.87	2.07	1.96	1.86	1.91

A - Not significant

CD (0.05) for B - 0.39

A x B - Not significant

their main effect had no significance. Isolate A-10 performed best giving a mean root dry weight of 2.07 g (Table 12). The Bradyrhizobium treatments had significant effect on root dry weight. B-2 isolate was the best which resulted in a mean root dry weight of 2.19 g. It was on par with B-1 isolate but significantly higher than their control.

The interaction of Azospirillum and Bradyrhizobium isolates had no significant effect on the root dry weight of cowpea. Inoculation of Bradyrhizobium isolate B-1 in combination with all the four Azospirillum isolates resulted in reduction in the mean root dry weight (Table 12) compared to its single inoculation. On the other hand isolate B-2 gave higher root dry weight in combination with isolates A-10, A-11 and A-20. In combination with A-9 isolate, the mean root dry weight was less compared to its individual inoculation. Inoculation with A-10 B-2 combination resulted in the maximum root dry weight of 2.76 g. All the inoculated plants had higher root dry weight compared to uninoculated control.

4.5.9 Number of nodules

Nodules were produced only in plants in which Bradyrhizobium was inoculated. There was no significance for the main effect of Azospirillum treatments on number of nodules of cowpea. The Bradyrhizobium isolates B-1 and B-2

Table 13. Associative effect of Azospirillum and Bradyrhizobium on number of nodules of cowpea

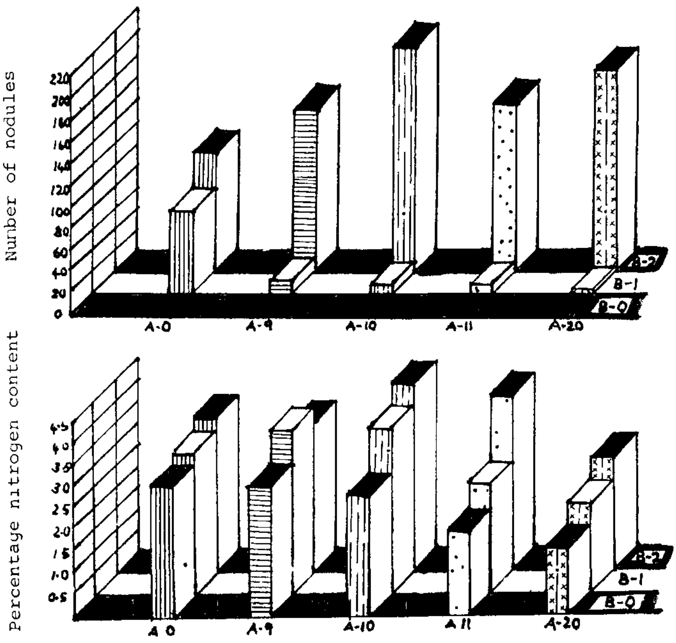
Bradyrhizobium treatments (B)	Azospirillum treatments (A)					Mean
	A-0	A-9	A-10	A-11	A-20	
B-0	0.71 (0)	0.71 (0)	0.71 (0)	0.71 (0)	0.71 (0)	0.71 (0)
B-1	5.18 (26.30)	1.78 (4.00)	1.61 (2.67)	1.79 (3.00)	1.27 (1.33)	2.33 (7.46)
B-2	6.19 (38.00)	6.99 (49.67)	8.10 (67.67)	7.16 (51.67)	7.79 (61.67)	7.25 (53.74)
ean	4.02 (21.43)	3.16 (17.89)	3.47 (23.45)	3.22 (18.22)	3.26 (21.00)	3.43 (20.40)

Data transformed. Original values given in parentheses

A - Not significant

CD (0.05) for B - 0.70 CD (0.05) for A x B - 0.41

Fig.5 Associative effect of <u>Azospirillum</u> and <u>Bradyrhizobium</u> on number of nodules and percentage nitrogen content of cowpea



A-0, A-9, A-10, A-11, A-20 - <u>Azospirillum</u> treatments
B-0, B-1, B-2 - <u>Bradyrhizobium</u> treatments

differed significantly in their main effect in increasing the number of nodules of cowpea. Isolate B-2 performed best giving a mean nodule number of 53.74 (Table 13). It was significantly superior to isolate B-1.

The interaction effect of Azospirillum and Bradyrhizobium was also significant. In general, mixed inoculation with Azospirillum isolates resulted in significant decrease in the number of nodules produced by Bradyrhizobium isolate B-1. On the other hand, the nodulation by isolate B-2 was significantly enhanced when inoculated in combination with Azospirillum isolates. The maximum mean number of nodules of 67.67 was observed in plants inoculated with A-10 B-2 combination of isolates (Fig.5). It was on par with A-20 B-2 and significantly superior to all other isolate combinations. Plants inoculated with A-20 B-1 had the least number of nodules.

4 5.10 Percentage nitrogen content

Eventhough the main effect of Azospirillum and Bradyrhizobium treatments were significant with respect to percentage nitrogen content of cowpea, their interaction was not significant. Among the Azospirillum treatments, A-10 performed best giving mean nitrogen content of 3.63 per cent in inoculated plants (Table 14). It was on par with isolate

Table 14. Associative effect of *Azospirillum* and *Bradyrhizobium* on the percentage nitrogen content of cowpea

Bradyrhizobium treatments (B)	*Azospirillum* treatments (A)					Mean
	A-0	A-9	A-10	A-11	A-20	
B-0	3.03	2.99	2.71	1.87	1.49	2.41
B-1	3.27	3.76	3.83	2.52	2.05	3.09
B-2	3.59	3.45	4.34	3.97	2.57	3.58
Mean	3.30	3.60	3.63	2.79	2.03	3.03

CD (0.05) for A - 0.60

CD (0.05) for B - 0.46

A x B - Not significant

A-9 and their respective control, but significantly superior to isolates A-11 and A-20. Both the *Bradyrhizobium* isolates were significantly superior to their control. Isolate B-2 performed best ,giving a nitrogen content of 3.58 per cent. It was significantly higher to that of isolate B-1.

Mixed inoculation of *Bradyrhizobium* isolate B-1 with *Azospirillum* isolates A-9 and A-10 gave higher percentage nitrogen content ,and with *Azospirillum* isolates A-10 and A-20, gave lesser percentage nitrogen content compared to its single inoculation. Isolate B-2 together with isolates A-10 and A-11 gave higher nitrogen content compared to its inoculation alone. With isolates A-9 and A-20, there was a decrease in the nitrogen content. The maximum nitrogen content of 4.34 per cent was recorded in plants receiving a mixed inoculum of isolates A-10 and B-2 and it was followed by A-11 B-2, A-10 B-1 and A-9 B-1 combinations (Table 14 and Fig.5). Among the isolate combinations, A-20 B-1 gave the least percentage nitrogen content.

4.5.11 Pod fresh weight

The main effect of *Azospirillum* and *Bradyrhizobium* treatments on pod fresh weight of cowpea showed statistical significance. However, their interaction was not significant. Among the *Azospirillum* isolates, A-10 performed best giving a

Table 15. Associative effect of _Azospirillium_ and _Bradyrhizobium_ on pod fresh weight of cowpea

Bradyrhizobium treatments (B)	_Azospirillum_ treatments (A)					Mean
	A-0	A-9	A-10	A-11	A-20	
B-0	2.14	2.86	4.79	1.30	1.31	2.48
B-1	4.53	5.14	5.32	3.46	4.74	4.64
B-2	3.74	5.67	4.72	3.73	4.30	4.43
Mean	3.46	4.56	4.94	2.83	3.46	3.85

CD (0.05) for A - 1.58

CD (0.05) for B - 1.22

A x B - Not significant

mean pod fresh weight of 4.94 g (Table 15). It was on par with isolates A-9 and A-20 and also with their control but significantly superior to isolate A-11. Both the Bradyrhizobium isolates resulted in significantly higher pod fresh weight of cowpea when compared to their control. Isolate B-1 performed best giving a mean pod fresh weight of 4.64 g.

Eventhough the interaction was not significant, Bradyrhizobium isolate B-1 in combination with all the Azospirillum isolates except A-11 resulted in higher pod fresh weights compared to its single inoculation. Similarly Bradyrhizobium isolate B-2 also gave higher pod fresh weights when inoculated together with each of the Azospirillum isolates except A-11 (Table 15). The maximum mean pod fresh weight of 5.67 g was recorded in plants inoculated with both A-9 and B-2 isolates (Fig.4) and it was followed by A-10 B-1 and A-9 B-1 isolate combinations. Mixed inoculation of A-11 and B-1 isolates gave the lowest mean pod fresh weight among the different isolate combinations.

4.5.12 Pod dry weight

There was no statistical significance for the main effect of Azospirillum and Bradyrhizobium treatments and also for their interaction with respect to pod dry weight of

Table 16. Associative effect of _Azospirillum_ and _Bradyrhizobium_ on pod dry weight of cowpea

Bradyrhizobium treatments (B)	_Azospirillum_ treatments (A)					Mean
	A-0	A-9	A-10	A-11	A-20	
B-0	0.68	0.71	1.06	0.63	0.63	0.74
B-1	0.95	1.23	1.22	0.81	1.05	1.05
B-2	0.84	1.89	1.18	0.83	1.09	1.17
Mean	0.83	1.28	1.15	0.76	0.92	0.99

A - Not significant

B - Not significant

A x B - Not significant

cowpea. Isolate A-9 among the Azospirillum isolates and B-2 among the Bradyrhizobium isolates performed best giving mean pod dry weight of 1.28 g and 1.17 g respectively.

Bradyrhizobium isolates B-1 and B-2 in combination with all the Azospirillum isolates except A-11 resulted in higher pod dry weights compared to their single inoculation. The maximum pod dry weight of 1.89 g was recorded in plants inoculated with both A-9 and B-2 isolates (Table 16). Among the different isolate combinations, A-11 B-1 gave the lowest mean pod dry weight.

4.6 pH tolerance of Azospirillum and Bradyrhizobium isolates

All the four selected Azospirillum isolates showed a similar trend in growth when the pH of the medium was increased from 5.0 to 8.5. The growth increased with increasing pH, reached the maximum at pH 7.5 after which the growth declined (Table 17 and Fig.6). Among the four isolates, A-10 showed maximum growth and A-11 showed minimum growth at pH 7.5.

The Bradyrhizobium isolates had scanty growth at both higher and lower pH. Maximum growth was obtained for both the isolates at pH 6.5 (Table 18 and Fig.7). At pH 6.5, the growth of isolate B-1 was more compared to B-2.

Table 17. Growth of *Azospirillum* isolates at different pH levels (optical density)

pH	*Azospirillum* isolates			
	A-9	A-10	A-11	A-20
5.0	0.13	0.10	0.08	0.04
5.5	0.18	0.16	0.10	0.03
6.0	0.20	0.17	0.12	0.09
6.5	0.49	0.26	0.27	0.30
7.0	0.49	0.57	0.45	0.54
7.5	0.70	0.77	0.63	0.71
8.0	0.49	0.55	0.49	0.34
8.5	0.23	0.31	0.25	0.20

Fig.6 Growth of Azospirillum isolates at different pH

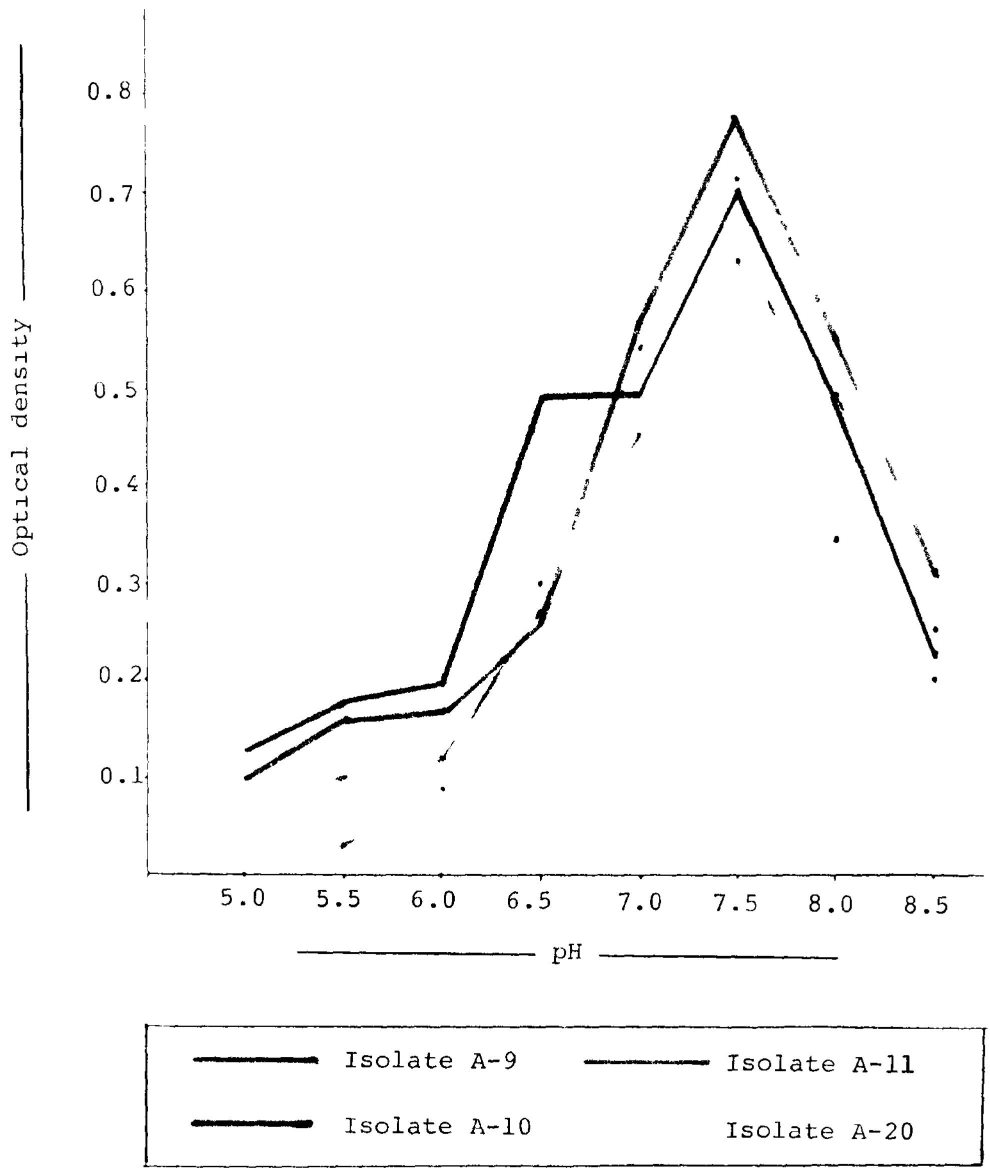
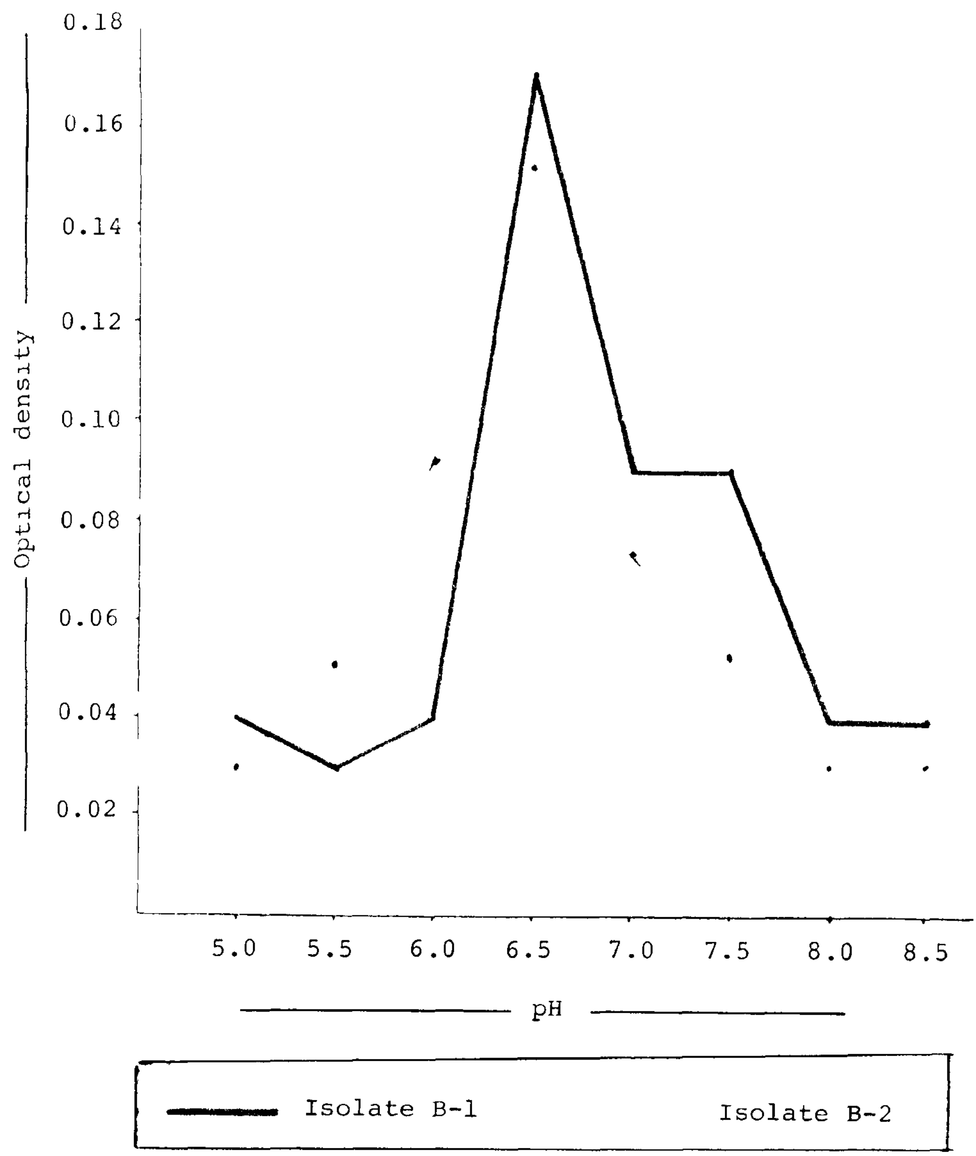


Table 18. Growth of <u>Bradyrhizobium</u> isolates at different pH levels (optical density)

Bradyrhizobium isolates	pH 5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5
B-1	0.04	0.03	0.04	0.17	0.09	0.09	0.04	0.04
B-2	0.03	0.05	0.09	0.15	0.07	0.05	0.03	0.03

Fig. 1 Growth of <u>Bradyrhizobium</u> isolates at different pH



Discussion

DISCUSSION

World wide occurrence, frequent association with plants and beneficial inoculation effects have made Azospirillum, an organism of considerable economic and scientific interest. Results of the field experiments conducted at different regions in India revealed that the response of plants to Azospirillum inoculation varied with location, cultivars and agronomic practices (Wani, 1990). The extreme dependence of Azospirillum on the soil pH for its optimum growth and performance has already been well established (Dobereiner et al., 1976, Dobereiner, 1978 and Tilak et al., 1988). Keeping in view of these facts, the first part of the investigation was carried out with the objective to select local isolates of Azospirillum which are efficient in promoting growth and nitrogen fixation in cowpea under Kerala conditions.

Isolation was done from the roots of both legumes and nonlegumes collected from 24 different locations of Kerala (Table 1). Commercial Azospirillum inoculant from Tamil Nadu Agricultural University was also included in the study. All the 25 isolates used formed white, subsurface pellicles in NFb medium (Plate I). This was reported as the characteristic feature of Azospirillum by Okon et al. (1976a). All these isolates were found to be Gram negative and vibrioid as described by Dobereiner and Day (1975).

In RC medium, all the 25 isolates produced scarlet, dry, round to irregular colonies with rugose surface and undulating edges. Formation of such colonies in RC medium has been reported as the character of Azospirillum by Caceres (1982). The isolates produced thin, dry, slightly convex colonies with granular, wavy surface and undulate margin in Okon's solid medium, and pink, round to irregular, dense and wrinkled colonies in Potato Infusion Agar medium as documented by Okon et al. (1977) and Dobereiner et al. (1976).

All the isolates except A-15 and A-19 produced acid from glucose under aerobic conditions (Table 2). Tarrand et al. (1978) has grouped the Azospirillum into Group I and Group II. One of the characters which formed the basis of this classification was the acidification of glucose. Accordingly, the isolates A-15 and A-19 which did not produce acid from glucose fall in Group II. Among the 25 isolates, only five isolates viz., A-3, A-7, A-8, A-13 and A-15 dissimilate nitrate as indicated by the shredding of agar block when grown in medium containing ammonium nitrate (Table 2). These isolates could be nir^+ and the remaining isolates nir^-. The existence of nir^+ and nir^- strains in Azospirillum has been already reported (Neyra et al., 1977, Baldani and Dobereiner, 1980).

All the 25 isolates showed good growth in medium containing malate as the carbon source. Scanty growth was obtained for 23 isolates with glucose, 18 isolates with mannose, 16 isolates with sucrose and eight isolates with lactose as the carbon source (Table 2). Remaining isolates did not show any growth in media containing these sugars. Thus in general, sugars were found to be poor carbon source for the growth of the isolates. Okon et al. (1976b) has reported that unlike most other nitrogen fixing bacteria, sugars are poor carbon substrates for the growth of Azospirillum.

Thus based on the results of the morphological, cultural and physiological tests conducted, the 25 isolates used in the study were confirmed as Azospirillum spp.

The symbiotic association between cowpea and Bradyrhizobium is a well established phenomenon. Isolation of Bradyrhizobium was done from the nodules of cowpea. On YEMA medium, large, white, gummy colonies were produced by this isolate. The isolate from commercial Bradyrhizobium inoculant also showed similar growth characters. On microscopic examination, these two isolates were found to be Gram negative rods. These characters of the two isolates were in conformity with that of Bradyrhizobium described by Vincent (1977).

Most of the inoculation studies of Azospirillum have been carried out in cereals and grasses (Rai and Gaur, 1982; Pahwa and Patil, 1984, Tanwar et al., 1985 and Bashan et al., 1990). However, there are also reports on the response of legumes to Azospirillum inoculation (Singh et al., 1980 and Sarig et al., 1986). In the present study, the efficiency of the 25 Azospirillum isolates in promoting growth of cowpea was tested, under pot conditions. Cowpea variety 'Pusa Komal' was selected for the study in view of its high yielding potential and increasing popularity in Kerala.

In general, inoculation of Azospirillum isolates resulted in significant increase in plant height, leaf length, root length, total fresh weight, total dry weight and root dry weight of cowpea However, no significant increase was noticed in leaf breadth and percentage nitrogen content of cowpea.

Plants inoculated with all Azospirillum isolates except A-3 and A-24 had increased plant heights compared to control. Isolate A-6 performed best giving the maximum plant height of 34.83 cm. Almost similar values were observed in plants inoculated with isolates A-13, A-12 and A-10 (Table 3). Increase in plant height by Azospirillum inoculation has been reported in rice by Sanoria et al. (1982). Inoculation of all the Azospirillum isolates except A 24 resulted in increase in the leaf length of cowpea. Isolates A-8, A-9, A-12 and A-10 performed better compared to other isolates (Table 3). Similarly, there was increase in the leaf breadth also due to inoculation of the *Azospirillum* isolates except A-23 and A-24. The maximum mean leaf breadth of 6.40 cm was recorded in plants inoculated with isolate A-4 and it was closely followed by isolates A-7, A-9 and A-10 (Table 3). Thus, *Azospirillum* isolates generally had a positive effect on leaf length and leaf breadth of cowpea. A similar increase in leaf area by *Azospirillum* inoculation in tomato was reported by Hadas and Okon (1987).

The positive effect of *Azospirillum* inoculation on root and shoot growth of plants has already been well established (Fayez and Daw, 1987, Hadas and Okon, 1987, Bopiah and Khader, 1989; Delgallo and Fabbri, 1990, Bashan *et al.*, 1990). In the present study, all the *Azospirillum* isolates except A-5 increased the root length of inoculated plants. Isolate A-16 resulted in the maximum root length of 21.37 cm and it was closely followed by isolates A-15, A-9 and A-21 (Table 3). Total fresh weight, total dry weight and root dry weight of all the inoculated plants were higher compared to control (Table 4). Maximum total fresh weight and dry weight of 71.26 and 11.13 g were recorded in plants inoculated with *Azospirillum* isolate A-8. Almost similar results were obtained with isolates A-6 and A-10. With regard to root dry weight, isolates A-9, A-7 and A-21 performed better compared to other isolates.

However, such encouraging results were not obtained with regard to percentage nitrogen content of cowpea by Azospirillum inoculation. Plants inoculated with only thirteen Azospirillum isolates had higher percentage nitrogen content than control (Table 4). Isolate A-11 performed best resulting in the maximum nitrogen content of 4.10 per cent followed by isolate A-20 giving a nitrogen content of 3.80 per cent. Irrespective of the percentage nitrogen content, most of the inoculated plants in general had better root and shoot growth compared to uninoculated control. This suggests that the beneficial effect of Azospirillum inoculation may be due to factors other than nitrogen fixation. Venkateswarlu and Rao (1984) has reported that the improvement in growth of pearlmillet obtained by Azospirillum inoculation was not related to its nitrogen fixing capacity, but to phytohormone synthesis. Production of phytohormones by Azospirillum has been well established (Tien et al., 1979, Govindan and Purushothaman, 1984; Cacciari et al., 1989 and Fallik et al., 1989).

From the results of the present study it is evident that the performance of the Azospirillum isolates from cereals and grasses viz., A-19, A-22, A-23, A-24 and A-25 and the isolate A-13 from commercial culture from Tamil Nadu Agricultural University were poor compared to most of the *Azospirillum* isolates from cowpea (Tables 3 and 4). This indicates that there is some amount of host specificity in *Azospirillum* - plant associations. Such host specific nature of *Azospirillum* - plant associations has been reported earlier by Baldani and Dobereiner (1980). Further, Bashan *et al*. (1989) observed that the response of noncereal crop plants to inoculation with *Azospirillum brasilense* from cereal roots was inconsistent.

Since different *Azospirillum* isolates were found to perform best with respect to different growth parameters of cowpea, selection of efficient isolates were done based on the overall performance of isolates in increasing growth and percentage nitrogen content of cowpea. Isolates A-9 and A-10 which showed better overall performance by giving higher values of plant height, leaf length, leaf breadth, root length, total fresh weight, total dry weight and root dry weight were selected for further studies. Selection of isolates A-11 and A-20 were based solely on their superior performance in enhancing percentage nitrogen content of inoculated plants (Fig.1 and 2).

From Table 1, it can be seen that the four selected *Azospirillum* isolates were those isolated from the roots of cowpea collected from different locations of Vellanikkara itself. This again confirms the location specific and host specific nature of *Azospirillum* - plant associations.

The objective of the second pot culture experiment was to find out the associative effect, if any, of the four selected *Azospirillum* isolates and the two *Bradyrhizobium* isolates on nodulation and growth of cowpea.

The main effect of *Azospirillum* treatments was significant with respect of plant height, number of leaves, leaf length, leaf breadth, shoot fresh weight, shoot dry weight, percentage nitrogen content and pod fresh weight of cowpea. In general, inoculation of cowpea with *Azospirillum* isolates A-9 and A-10 resulted in better shoot and root growth, percentage nitrogen content and yield compared to control. On the other hand, the performance of isolates A-11 and A-20 were poor and the inoculated plants were inferior to control in many aspects. These two isolates were selected based on their superior performance in increasing the percentage nitrogen content of inoculated plants in the first pot culture experiment. In the second experiment, even the percentage nitrogen content of plants inoculated with isolates A-11 and A-20 were less than the control (Table 14). Such lack of consistency of plant response has been reported as the crucial problem of most of the green house and field inoculation studies of *Azospirillum* (Patriquin *et al.*, 1983, Michiels *et al.*, 1989 and Rukmani, 1990). This suggests the need of repeated inoculation tests of *Azospirillum* isolates for their consistent performance.

Bradyrhizobium treatments were also found to be significant in their main effects on plant height, leaf length, leaf breadth, shoot fresh weight, shoot dry weight, root fresh weight, root dry weight, number of nodules, percentage nitrogen content and pod fresh weight of cowpea. Among the two *Bradyrhizobium* isolates, B-1 gave maximum plant height, number of leaves, leaf length, leaf breadth, shoot fresh weight, shoot dry weight and pod fresh weight whereas, isolate B-2 resulted in higher root fresh weight, root dry weight, number of nodules, percentage nitrogen content and pod dry weight of cowpea. Similar differential response in cowpea plants inoculated with different strains of *Rhizobium* has been reported by Neves *et al.* (1982).

The interaction of *Azospirillum* and *Bradyrhizobium* isolates had significant effect only on the number of nodules produced in cowpea. Generally, the response of cowpea to combined inoculation of *Azospirillum* and *Bradyrhizobium* varied with different combination of isolates. With regard to plant height, three isolate combinations viz., A-9 B-1, A-10 B-1 and A-9 B-2 gave higher values compared to their single inoculation. The maximum plant height of 39.43 cm was recorded with A-9 B-2 combination (Table 5 and Fig.3) and it was closely followed by A-10 B-1. Increase in number of leaves of cowpea by combined inoculation of Azospirillum and Bradyrhizobium was observed only in the case of A-9 B-1 and A-10 B-1 isolate combinations. All other combination of isolates resulted in decrease in the number of leaves compared to the single inoculations. The maximum mean number of leaves of 16.0 was recorded in plants inoculated with isolates A-9 and B-1 together (Table 6).

Mixed inoculation resulting in increased leaf length compared to single inoculation was observed only with two isolate combinations viz., A-10 B-1 and A-9 B-2. The maximum mean leaf length of 9.72 cm was observed in plants inoculated with A-10 B-1 (Table 7). Inoculation of all the isolate combinations except A-9 B-2 gave lesser leaf breadth compared to their single inoculation. None of the isolate combinations was better when compared to inoculation with isolate B-1 alone which recorded the maximum mean leaf breadth of 6.24 cm (Table 8). However, A-10 B-1 and A-9 B-1 combinations performed almost similar to B-1 alone giving mean leaf breadths of 6.22 and 6.02 cm respectively.

Eventhough, the interaction of Azospirillum and Bradyrhizobium had no significant effect on shoot fresh weight of cowpea, two isolate combinations viz., A-10 B-1 and A-9 B-1 resulted in increased shoot fresh weight of cowpea compared to their single inoculation. Maximum mean shoot fresh weight of 40.58 g was recorded in plants inoculated with A-10 B-1 (Table 9 and Fig.3). There was no significance for the interaction of Azospirillum and Bradyrhizobium on the shoot dry weight of cowpea also. But isolate B-1 in combination A-9 and A-10 gave higher shoot dry weight compared to their individual inoculation. Similarly, isolate B-2 in combination with A-9 also gave higher values. The best performance was observed with isolate combination A-9 B-1 (Table 10).

Thus, in general, combined inoculation of Azospirillum and Bradyrhizobium isolates resulting in increased shoot growth over single inoculations was observed with three isolate combinations viz., A-9 B-1, A-10 B-1 and A-9 B-2.

With regard to root fresh weight of cowpea, all isolate combinations except A-10 B-2, A-11 B-2 and A-20 B-2 resulted in lesser root fresh weights compared to their single inoculations. The maximum root fresh weight of 10.84 g was obtained by inoculation of A-10 B-2 combination. It was closely followed by A-11 B-2 which gave a mean root fresh weight of 10.62 g (Table 11 and Fig.4). Similarly, in the case of root dry weight also, all the isolate combinations except A-10 B-2, A-11 B-2 and A-20 B-2 gave lesser values compared to their single inoculation. Isolate combination A-10 B-2 resulted in the maximum mean root dry weight of 2.76 g (Table 12). Gunawardena and Vlassak (1986) obtained increase in plant dry weight upto 134 to 138 per cent by inoculation of cowpea with Azospirillum and Bradyrhizobium together.

Combined inoculation of Azospirillum and Bradyrhizobium isolates had significant effect on the number of nodules of cowpea. In general, combined inoculation resulted in significant decrease in the number of nodules produced by Bradyrhizobium isolate B-1 whereas, it significantly enhanced the nodulation by isolate B-2 (Table 13 and Fig.5). The maximum mean number of nodules of 67.67 was observed in plants inoculated with A-10 B-2 combination. Similar increase in nodulation by native rhizobia in the presence of Azospirillum has been reported in soybean by Borthakur and Sarmah (1983). The reasons of decreased nodulation by combined inoculation is evident from the work of Plazinski and Rolfe (1985 b). They found that when white clover was inoculated with Azospirillum and Rhizobium, infectible sites on root hairs were blocked by Azospirillum and that, nodules were produced only by those Rhizobium strains which are able to invade the plant roots via an alternate route.

The study showed that isolate combinations A-9 B-1, A-10 B-1, A-10 B-2 and A-11 B-2 gave higher percentage nitrogen content in inoculated plants compared to the single inoculation of these isolates. The maximum nitrogen content of 4.34 per cent was recorded in plants inoculated with A-10 B-2 and it was followed by A-11 B-2, A-10 B-1 and A-9 B-1 combinations (Table 14 and Fig.5). Thus, from Table 13 and 14 it can be seen that the isolate combinations A-10 B-2 and A-11 B-2 resulted in increased nodulation and percentage nitrogen content of cowpea. Such substantial increase in nodulation and nitrogen gain by combined inoculation of Azospirillum and Rhizobium was reported in winged bean and soybean by Iruthayathas et al. (1983). It must be noted that the nitrogen content of plants inoculated with isolate combinations A-9 B-2 and A-20 B-2 were less in spite of the increase in the number of nodules. On the other hand, isolate combinations A-9 B-1 and A-10 B-1 resulted in higher percentage nitrogen content in inoculated plants eventhough the nodulation was almost completely inhibited. This suggest that there is no correlation between the number of nodules produced and the percentage nitrogen content of cowpea. This is supported by the reports of Sivaprasad and Shivappashetty (1980) and Beena et al. (1990). Moreover, Raverkar and Konde (1990) has reported that the higher number of nodules formed by native rhizobia in the presence of Azospirillum may not be efficient in fixing nitrogen. Plazinski and Rolfe (1985c) also reported decrease in the effectiveness of Rhizobium, when mixed inoculation of it with Azospirillum caused an increase in nodule number.

Plants inoculated with A-9 B-1 and A-10 B-1 isolate combinations showed better shoot growth eventhough the root fresh weight, root dry weight and number of nodules were less. Plazinski and Rolfe (1985 a) also observed that combined inoculation of Azospirillum and Rhizobium resulted in better performance of white clover in spite of the inhibition of nodulation.

All the isolate combinations except A-11 B-1 and A-11 B-2 gave higher values of pod fresh weight and pod dry weight compared to their single inoculations. The maximum mean pod fresh weight and pod dry weight of 5.67 and 1.89 g were recorded in plants inoculated with isolate combination A-9 B-2 (Tables 15, 16 and Fig.4). Increase in grain yield by combined inoculation of Azospirillum and Rhizobium has been reported in soybean (Singh and Rao, 1979).

In general, isolate combinations A-9 B-1, A-10 B-1 and A-9 B-2 performed better in increasing shoot growth and yield of cowpea. Maximum number of nodules, root fresh weight, and root dry weight was obtained by inoculation with isolate combination A-10 B-2. Least performance was observed with isolate combination A-11 B-1 which resulted in decrease of almost all growth and yield parameters of cowpea. These results indicate that the response of cowpea to inoculation of Azospirillum and Bradyrhizobium varied with different combination of isolates of the two organisms. The compatibility between the two microsymbionts thus becomes the most crucial factor for the success of combined inoculation. This is in conformity with the report by Iruthayathas et al. (1983) that the response of winged bean and soybean to combined inoculation of Azospirillum and Rhizobium was highly dependant on the compatibility of the strains involved and that the Rhizobium genotype is more decisive than Azospirillum in expressing the associative effect.

In vitro studies conducted on the pH tolerance of the selected Azospirillum isolates showed that growth of all the four isolates viz., A-9, A-10, A-11 and A-20 was maximum at pH 7.5 (Table 17 and Fig.6). Day and Dobereiner (1976) has also reported a similar trend. They found that the growth of Azospirillum was best at pH 6.8 to 7.8. Maximum growth of the two Bradyrhizobium isolates viz., B-1 and B-2 was observed at pH 6.5 (Table 18 and Fig.7). Vincent (1977) has reported that all rhizobial strains grow at pH 5.5 to 6.5.

The pH of the soil used for the pot culture experiment was found to be 6.2, slightly acidic, which is the characteristic of Kerala soils. Taking into account the earlier reports on high dependance of *Azospirillum* and *Bradyrhizobium* isolates on soil pH (Dobereiner *et al*., 1976, Dobereiner, 1978, Laksnmi and Dhala, 1984 and Joe and Allen, 1980) it becomes evident that the performance of the *Azospirillum* and *Bradyrhizobium* isolates was not their best in soil having a pH 6.2.

In short, this investigation revealed that combined inoculation of *Azospirillum* and *Bradyrhizobium* can result in positive growth and yield response in cowpea. However, careful screening of both *Azospirillum* and *Bradyrhizobium* isolates for compatibility, wider pH tolerance, and consistent field performance will be essential to select an efficient combination of the two microsymbionts suitable for inoculation of cowpea under Kerala conditions.

Summary

SUMMARY

An investigation was carried out at the College of Horticulture, Vellanikkara during 1990-1992 on the associative effect of Azospirillum and Bradyrhizobium on nodulation and growth of cowpea. The results of this study are summarised below.

Twenty four isolates of Azospirillum were collected from the roots of legumes and nonlegumes from different locations of Kerala. An isolate from the commercial Azospirillum inoculant from Tamil Nadu Agricultural University was included. All the 25 isolates formed thin, white, subsurface pellicles in NFb medium. They were Gram negative vibrioids and produced colonies with characters typical of Azospirillum when grown in Okon's medium, RC medium and Potato Infusion Agar medium confirming that all the isolates belong to Azospirillum.

Among the 25 Azospirillum isolates, all except A-15 and A-19 produced acid from glucose under aerobic conditions. Dissimilation of nitrate was observed only in the case of five isolates viz., A-3, A-7, A-8, A-13 and A-15. All the isolates showed good growth in medium containing malate as the carbon source. Scanty growth was observed for 23 isolates

with glucose, 18 isolates with mannose, 16 isolates with sucrose and eight isolates with lactose as the carbon source.

Two Bradyrhizobium isolates were also used in the study, one, a local isolate from the cowpea nodules from vegetable garden, College of Horticulture and the other, an isolate from the commercial Bradyrhizobium inoculant procured from Soil Testing Laboratory, Pattambi.

Initially, a pot culture experiment was conducted to screen the 25 Azospirillum isolates for their efficiency in promoting growth of cowpea. The data revealed that, all the isolates except A-3 and A-24 resulted in increased plant height compared to control. Isolate A-6 performed best and it was followed by isolates A-13, A-12 and A-10. Plants inoculated with all the Azospirillum isolates except A-24 had increased leaf lengths compared to control. Isolates A-8, A-9, A-12 and A-10 performed better compared to others in this aspect. Similarly with regard to leaf breadth also, all the isolates except A-23 and A-24 gave higher values compared to control. Isolate A-4 performed best and it was closely followed by isolates A-7, A-9 and A-10. All the Azospirillum isolates except A-5 increased the root length of inoculated plants. Maximum root length was observed in plants inoculated with isolate A-16 and it was followed by isolates A-15, A-9 and A-21.

Total fresh weight, total dry weight and root dry weight of all the inoculated plants were higher when compared to control. Isolate A-8 performed best with regard to total fresh weight and total dry weight and it was closely followed by isolates A-6 and A-10. Isolates A-9, A-7 and A-21 performed better compared to others in increasing the root dry weight of cowpea. *Azospirillum* isolates did not show significant effect on percentage nitrogen content of cowpea. Plants inoculated with only thirteen isolates had higher nitrogen content compared to control. Isolate A-11 resulted in the maximum nitrogen content in inoculated plants and it was followed by isolate A-20.

Azospirillum isolates A-9 and A-10 which showed better overall performance in promoting growth of cowpea, and isolates A-11 and A-20 which resulted in higher nitrogen content in inoculated plants were selected for the further study. These four isolates were inoculated in combination with the two *Bradyrhizobium* isolates to study their associative effect on nodulation and growth of cowpea.

The results of this experiment revealed that the main effect of *Azospirillum* treatments was significant with regard to plant height, number of leaves, leaf length, leaf breadth, shoot fresh weight, shoot dry weight, percentage nitrogen content and pod fresh weight of cowpea. In general, isolates

A-9 and A-10 resulted in better growth, percentage nitrogen content and yield of cowpea. Isolates A-11 and A-20 performed poor and the inoculated plants were inferior to control in many aspects.

Bradyrhizobium treatments also showed significant effect on plant height, number of leaves, leaf length, leaf breadth, shoot fresh weight, shoot dry weight, root fresh weight, root dry weight, number of nodules, percentage nitrogen content and pod fresh weight of cowpea. Among the two Bradyrhizobium isolates, B-1 resulted in maximum plant height, number of leaves, leaf length, leaf breadth, shoot fresh weight, shoot dry weight and pod fresh weight whereas isolate B-2 resulted in higher root fresh weight, root dry weight, number of nodules, percentage nitrogen content and pod dry weight of cowpea.

The interaction of Azospirillum and Bradyrhizobium had significant effect only on the number of nodules of cowpea. With regard to plant height of cowpea, A-9 B-1, A-10 B-1 and A-9 B-2 combinations gave higher values compared to the single inoculation of these isolates. Among all combinations, A-9 B-2 performed best and it was closely followed by A-10 B-1. Increase in number of leaves of cowpea by the combined inoculation of the two bacteria was observed only with two isolate combinations viz., A-9 B-1 and A-10 B-1, of which

A-9 B-1 performed best. Combined inoculation leading to increased leaf length compared to single inoculation was observed only with isolate combinations A-10 B-1 and A-9 B-2. The maximum leaf length was observed in plants inoculated with A-10 B-1. All the isolate combinations except A-9 B-2 resulted in lesser leaf breadth compared to single inoculation. None of the isolate combinations performed better when compared to inoculation of B-1 alone which resulted in the maximum leaf breadth.

Mixed inoculation resulting in increased shoot fresh weight of cowpea compared to the single inoculation was observed only with two isolate combinations viz., A-10 B-1 and A-9 B-1. Among these, A-10 B-1 performed best. In enhancing shoot dry weight, A-9 B-1, A-10 B-1 and A-9 B-2 gave higher values compared to single inoculation of the isolates. The maximum shoot dry weight was recorded in plants inoculated with A-9 B-1.

The bacterial combinations A-10 B-2, A-11 B-2 and A-20 B-2 resulted in higher root fresh weight and root dry weights compared to single inoculation. The best performance was observed with A-10 B-2 combination. Inoculation in combination with Azospirillum isolates resulted in significant decrease in the number of nodules produced by Bradyrhizobium isolate B-1 whereas, it significantly enhanced the nodulation by isolate B-2. Maximum mean number of nodules was recorded in plants inoculated with A-10 B-2 combination. With regard to percentage nitrogen content, isolate combinations A-9 B-1, A-10 B-1, A-10 B-2 and A-11 B-2 gave higher values compared to the single inoculation of the isolates. A-10 B-2 combination performed best.

All the isolate combinations except A-11 B-1 and A-11 B-2 gave higher pod fresh weight and pod dry weight compared to their single inoculation. Maximum pod fresh weight and pod dry weight were recorded in plants inoculated with A-9 B-2 combination.

Thus in general, isolate combinations A-9 B-1, A-10 B-1 and A-9 B-2 performed better in increasing shoot growth and yield of cowpea. Maximum number of nodules, root fresh weight and root dry weight were obtained by inoculation with A-10 B-2. The least performance was observed in the case of A-11 B-1 combination which resulted in decrease in most of the growth and yield parameters of cowpea, compared to their single inoculation.

In vitro studies on pH tolerance of the selected four *Azospirillum* and two *Bradyrhizobium* isolates indicate that the optimum pH for growth of *Azospirillum* isolates was 7.5 and that of *Bradyrhizobium* isolates was 6.5. The pH of the potting mixture used for the experiment was estimated as 6.2.

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* Originals not seen

Appendices

Appendix - I

Analysis of variance table - Effect of *Azospirillum* inoculation on different growth parameters of cowpea

Source	df	Mean square							
		Plant height	Leaf length	Leaf breadth	Root length	Total fresh weight	Total dry weight	Root dry weight	Percentage nitrogen content
Treatment	25	15.363*	1.944*	0.589	13.007*	257.005*	6.034*	2.263*	0.580
Error	52	8.436	1.066	0.563	6.363	76.237	2.278	0.861	0.410

* Significant at 5 per cent level

Appendix - II

Analysis of variance table - Associative effect of Azospirillum and Bradyrhizobium on plant height, number of leaves, leaf length and leaf breadth of cowpea

Source	df	Mean square			
		Plant height	Number of leaves	Leaf length	Leaf breadth
Azospirillum	4	87.158*	0.230*	2.306*	1.631*
Bradyrhizobium	2	130.154*	0.180	8.378*	3.002*
Azospirillum x Bradyrhizobium	8	16.795	0.100	0.442	0.311
Error	28	13.712	0.700	0.815	0.403

* Significant at 5 per cent level

Appendix - III

Analysis of variance table - Associative effect of *Azospirillum* and *Bradyrhizobium* on shoot fresh weight, shoot dry weight, root fresh weight and root dry weight of cowpea

Source	df	Mean square			
		Shoot fresh weight	Shoot dry weight	Root fresh weight	Root dry weight
Azospirillum	4	126.796*	3.160*	3.101	0.116
Bradyrhizobium	2	230.477*	5.460*	29.986*	1.269*
Azospirillum x *Bradyrhizobium*	8	24.769	0.870	4.828	0.370
Error	28	22.281	0.850	5.558	0.276

* Significant at 5 per cent level

Appendix - IV

Analysis of variance table - Associative effect of *Azospirillum* and *Bradyrhizobium* on number of nodules, percentage nitrogen content, pod fresh weight and pod dry weight of cowpea

Source	df	Mean square			
		Number of nodules	Percentage nitrogen content	Pod fresh weight	Pod dry weight
Azospirillum	4	1.140	3.615*	12.610*	0.158
Bradyrhizobium	2	173.870*	5.140*	10.390*	0.121
Azospirillum x *Bradyrhizobium*	8	4.130*	0.494	1.885	0.164
Error	28	0.880	0.384	2.655	0.166

* Significant at 5 per cent level

ASSOCIATIVE EFFECT OF *Azospirillum* AND *Bradyrhizobium* ON NODULATION AND GROWTH OF COWPEA (*Vigna unguiculata* (L) Walp)

By

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ABSTRACT OF A THESIS

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ABSTRACT

An investigation was undertaken at the College of Horticulture, Vellanikkara during 1990-1992 to study the associative effect of Azospirillum and Bradyrhizobium on nodulation and growth of cowpea.

Twenty four isolates of Azospirillum from the roots of legumes and nonlegumes of different locations of Kerala and one isolate from the commercial Azospirillum inoculant procured from Tamil Nadu Agricultural University were collected. These isolates after purification and primary characterization were screened for their efficiency in promoting various growth parameters of cowpea variety 'Pusa Komal'. Based on the results obtained, four isolates viz., A-9, A-10, A-11 and A-20 were selected for further work.

Two isolates of Bradyrhizobium viz., B-1 from commercial Bradyrhizobium culture recommeded for cowpea in Kerala and B-2 isolated from the nodules of cowpea grown in the vegetable garden of College of Horticulture, were collected. These two Bradyrhizobium isolates were inoculated in combination with the four selected Azospirillum isolates to study their associative effect on nodulation and growth of cowpea.

Interaction of *Azospirillum* and *Bradyrhizobium* had significant effect only on the number of nodules of cowpea. Combined inoculation with *Azospirillum* isolates inhibited the nodulation by *Bradyrhizobium* isolate B-1 whereas it enhanced the nodulation by isolate B-2 Among the different isolate combinations, A-10 B-2 resulted in the maximum number of nodules and nitrogen content in cowpea.

Effect of combined inoculation of *Azospirillum* and *Bradyrhizobium* isolates on root and shoot growth of cowpea varied with different isolate combinations. Plants inoculated with A-9 B-1, A-10 B-1 and A-9 B-2 combinations had better shoot fresh weight and dry weight whereas those inoculated with A-10 B-2, A-11 B-2 and A-20 B-2 combinations had better root fresh weight and dry weight compared to other isolate combinations.

All the isolate combinations except A-11 B-1 and A-11 B-2 increased the pod fresh weight and dry weight of cowpea compared to their single inoculation.

In vitro study on the pH tolerance of the selected *Azospirillum* and *Bradyrhizobium* isolates revealed that the optimum pH for the growth of *Azospirillum* and *Bradyrhizobium* isolates are 7.5 and 6.5 respectively.