STUDIES ON DINITROGEN FIXATION BY *BEIJERINCKIA* ASSOCIATED WITH RHIZOSPHERE OF COCONUT

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MERILYN V. J,

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CERTIFICATE FROM THE GUIDE

This is to certify that the dissertation entitled 'Studies on dinitrogen fixation by *Beijerinckia* associated with the rhizosphere of coconut' submitted in partial fulfilment for the award of the degree of 'Master of Philosophy' in Bioscience (Microbiology) of Mangalore University is a record of *bona fide* research work carried out by Merilyn V.J. under my guidance and supervision. No part of the dissertation has been submitted for any other degree or diploma.

Dated: 28-2-1989.

Thomas VG

(Dr.George V. Thomas)
Scientist S2
Microbiology Division
CPCRI, Kasaragod.
Kerala 670 124.

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I. INTRODUCTION

Coconut (*Cocos nucifera* Linn.) is one of the important plantation crops grown in India. The palm is referred to by such eulogistic epithets as 'Kalpavriksha' (tree of heaven), "tree of life" etc. Since it provides food, livelihood and shelter for millions of people. With an area of 1.2 million hectares and an annual production of 6,620 million nuts, India ranks third on the world map of coconut. Among the different coconut growing states in India, Kerala ranks first with a share of 57.7 per cent of the area, and 49.3 per cent of the total production of coconut. Tall cultivars are largely cultivated for commercial purposes throughout the world. Coconut belongs to the monocotyledonous family Palmae and it is mainly cultivated in coastal areas of the tropics. The palm has an adventitious root system of a monocot and produces about 4000-7000 uniformly thick roots from the base of the stem throughout its life time. The micro-organisms associated with the root zone will have a profound influence on nutrition and productivity of the palm. The nature and activity of micro-organisms associated with perennials such as tree crops are likely to be almost constant but agronomic practices and other inputs can have either beneficial or harmful effect on the micro-organisms.

Nitrogen is a major limiting nutrient for crop production in tropical soils. There is a lot of potential to exploit the nitrogen fixed biologically by certain bacteria and blue green algae as a source of nitrogen to crop plants. Research on biological nitrogen fixation(BNF) is given great impetus in recent years since the manufacture of nitrogenous fertilizers is dependent on petroleum products which are in limited supply. The concern for conservation of energy and foreign exchange and the fact that soil conditions deteriorate under intensive cultivation with chemical fertilizers are some of the other factors responsible for the renewed interest in this topic. Another advantage is that the biologically fixed nitrogen is produced directly at the site of need when it is required. The greatest practical success in the field of BNF is the development of inoculation technology.

Nitrogen fixation studies assume particular significance for plantation crops like coconut due to the cultivation of these crops in tropical soils where major portion of the applied inorganic nitrogenous fertilizers are lost due to leaching, denitrification etc. A number of workers have reported non-symbiotic nitrogen fixation at magnitudes that are of agronomic significance under different agro-climatic conditions (Dart and Day 1975, Charyulu and Rao 1979, Wani 1986). Non-symbiotic nitrogen fixing bacteria are also known to produce growth promoting substances like I.A.A., auxins etc. and show antagonistic properties against plant pathogens. Beijerinckia is a non-symbiotic N_2 fixing bacterium capable of growing and fixing nitrogen at acidic soil conditions in which coconut and other plantation crops are grown (Nair 1974, Subba Rao 1983). It is also known that Beijerinckia produce large quantities of polysaccharides which are very useful in soil aggregation. There have been only very few studies to know the plant-soil-nitrogen fixing bacterial interactions in plantation crops. Therefore, the present investigation has been undertaken with a view to study the association of Beijerinckia with the rhizosphere of coconut in two important coconut growing soil types of Kerala viz. laterite and sandy and to determine the nitrogen fixing potential of the isolates from different locations. A thorough understanding of the extent of association and factors limiting the development of the nitrogen fixing bacterium is essential for deriving maximum possible benefits from these bacteria in plantation crops. Plant protection chemicals are also widely used in the management of diseases of plantation crops, but their influence on this nitrogen fixing bacterium has not been studied so far. For developing inoculation programmes also, it is necessary to evaluate the effects of inoculation in terms of growth response and nitrogen contribution in crop plants.

Keeping the foregoing account in view, the present investigation has been aimed at the following:

1. Enumeration of population of *Beijerinckia* in the rhizosphere of coconut and comparison of rhizosphere to soil ratio of the population in different locations in sandy and laterite soil types.
2. Isolation and testing of the *Beijerinckial* isolates for nitrogen fixing ability.
3. Studies on various factors affecting growth and nitrogen fixation by *Beijerinckia*.
4. Growth promoting effect of *Beijerinckia* using black pepper seedlings as test plants.

II. REVIEW OF LITERATURE

2.1. Initial reports of nitrogen fixation

The notion that microorganisms fix dinitrogen(N_2) was first suggested in 1862 when Jodin reported 'microderms' in nutrients containing only carbon and minerals. Later Berthelot attributed the increase in total nitrogen in soil to bacteria without giving evidence to prove it. Winogradsky (1895) was the first to demonstrate non-symbiotic nitrogen fixation by bacteria in culture. He isolated *Clostridium pastorianum* (later *C. pasteurianum*), capable of growth in the absence of fixed nitrogen. In 1901 Beijerinck isolated and described the aerobic N_2 fixing bacterium, *Azotobacter chroococcum* from soil and *Azotobacter agilis* from water. Lipmann (1904) isolated *Azotobacter vinelandii* and *Azotobacter beijerinckii* from North American soil. Since then a number of aerobic nitrogen-fixing bacteria have been reported from various ecosystems, including cultivated and non-cultivated soils, soils adjacent to the roots (rhizosphere), plant root surfaces and adhering soil (rhizoplane), leaf surfaces (phyllosphere) and aquatic environments.

2.2. Occurrence of Beijerinckia in tropical soils

The genus *Beijerinckia* comprises aerobic heterotrophic N_2 fixing bacteria earlier known as *Azotobacter indicum*. Starkey and De (1939) first isolated *Beijerinckia* from acid soils of the paddy fields in India and gave the name *Azotobacter indicum*. Derx (1950) isolated a similar organism from Indonesia and proposed the name *Beijerinckia*. In addition to the type species *Beijerinckia indica*, Derx (1950) has since added to this genus a new species *B. mobile* and a varient *B. indica* var alba.

Beijerinckia spp. have been isolated from many acidic soils of tropical regions all over the world. They have been found to a lesser degree in subtropical regions and are absent from soils of temperate climates (Tchan, 1953; Becking 1961, Mulder and Brotonegoro 1974, Dobereiner 1968). Suto (1954) described isolation of *Beijerinckia* from acid volcanic soil in Japan. Roy (1958) while studying various soil types in India isolated a new species of *Azotobacter* producing heavy slime and acid, *Beijerinckia acida* from red soils of Pattambi (Kerala). Barooah and Sen (1959) reported the occurrence of *B. indica* var alba in 40% of the different acid soils investigated. The bacteria have been reported from Malaya, West Africa and Madagascar (Altson 1936, Dommergues 1954). Becking (1959) reported unsuspected occurrence of *Beijerinckia* in South African soils. In 1956 Ruinen reported the common occurrence of aerobic N_2 fixing *Beijerinckia* spp. on the leaf surface of vegetation in Java and Sumatra in west tropics of Indonesia.

A survey of *Beijerinckia* distribution in several Brazilian states demonstrated the presence of this organisms in 97% of the soil samples under sugar cane and 60% of the soil samples from other vegetation (Dobereiner 1961).

There have also been reports on the occurrence of *Beijerinckia* spp. in extreme cold conditions. The bacteria were isolated from soils of Koelpura (Lat 30^{o}N) at the base of Himalayan mountains in the Northern India (Becking 1961). Jordan and Patricia (1978) reported the isolation of *Beijerinckia* in the permanently cold soils of circum polar region in the high arctics (Devon Island, North West territories). The studies of Strijdom and Steenkamp (1967) showed that dry spells in summer are more deleterious to *Beijerinckia* spp. than similar periods of desiccation in winter.

The acid tolerance of *Beijerinckia* enable them to compete successfully with most other soil microorganisms but they are unable to compete in neutral soils. Becking (1961) reported the largest percentage (74.2%) of *Beijerinckia* positive soils in the pH range 5.5-5.9 followed by 64.3% in pH range of 5.0-5.4. At higher and lower pH values a gradual decrease in the percentage of *Beijerinckia* positive soils was observed. *Beijerinckia* was absent in soils below pH 4.0 and above pH 7.4. Materassi and Florenzano (1966) isolated a new species of *Beijerinckia* (*B. venezuelae* nov. spp.) from Venezuela from soils in the pH range 7.5-8.0. The combined effect of temperature and desiccation on *Beijerinckia* spp. was studied by Strijdom and Steenkamp. (1967).

Wu (1965) reported the occurrence of *Beijerinckia* in acid red yellow soils of Hupels province. Fernandez (1975) recorded the presence of *Beijerinckia* in soils of Cuba and reported that isolates belonged to two species, *B. indica* and *B. lactinogenes*. Hegazi and Ayoub (1979) developed a technique for estimating the number of *Beijerinckia* and reported the occurrence in Sudanese soils.

2.3. Rhizosphere effect

The term 'rhizosphere' was first introduced by German scientist Hiltner (1904) to denote the region of the soil which is subject to the influence of plant roots. The region is characterised by greater microbial activity than the soil away from plant roots. Studies on the rhizosphere microflora of a number of crop plants have demonstrated higher populations of micro-organisms and increase in physiologically active groups of microorganisms in the root zone of plants as compared to soil away from the roots (Starkey 1929, Katznelson et al. 1948, Rovira 1965, Subba Rao et al. 1961, Thomas 1980). Rovira (1965) reported that the number of microorganisms in the rhizosphere depends on may factors such as the age of the plant, soil conditions, fertilizer doses, mode of cultivation, environmental conditions and foliar sprays. Root exudates play a key role in the selective stimulation of microorganisms in the rhizosphere (Rovira 1965). Organic substances like sugars, amino acids, vitamins and organic acids were reported to be present in root exudates (Andal et al. 1956).

2.4. Beijerinckia in the rhizosphere of crop plants

Dobereiner (1961) demonstrated the stimulating effect of sugar cane vegetation on *Beijerinckia* occurrence in soil samples taken from several Brazilian states. Ishizawa and Toyoda (1964) reported rhizosphere effect on *Beijerinckia* in Japanese rice fields. Forage grasses like *Digitaria* decumbens, Hiparrhenia rufa, Panicum purpurascens, Cynodon datylon and Setaria sphacelata were also reported to stimulate Beijerinckia in the rhizosphere (Ruschel and Dobereiner 1966). Vacura et al. (1965) isolated Beijerinckia from rhizosphere of Vicia faba and Medicago sativa in Egypt.

There are also reports to show that root exudates of C_4 tropical plants, with a more effecient photosynthetic pathway, selectively enhanced the growth of N_2 fixing bacteria at the expense of other groups of the rhizosphere bacteria. Certain forage grasses, rice and sugar cane stimulated Beijerinckia multiplication and depressed the number of amino acids requiring bacteria, molds and actinomycetes (Dobereiner and Campelo 1971). Dey et al. (1986) reported that foliar spraying of Molybdenum at 29 and 40g/ha stimulated proliferation of N_2 fixing organisms like Azotobacter, Beijerinckia and Nostoc in the rhizosphere soils of rice.

Studies on the rhizosphere of plantation crops have added valuable data in this field. Nair and Subba Rao (1977) in a pioneering study on the rhizosphere microflora of coconut reported that mixed cropping of cacao in coconut gardens favoured proliferation of Beijerinckia in the rhizosphere of coconut. In another study in root (wilt) affected tract, Potty (1977) observed higher population of non-symbiotic N_2 fixers in the rhizosphere of coconut under intercropping with forage grasses.

Thomas and Shantaram (1984) also reported higher population of non-symbiotic N_2 fixing bacteria in the root region of coconut when basin management was done with green manure legumes such as *Pueraria phaseoloides*, *Mimosa invisa* and *Calopogonium mucunoides*.

Subba Rao (1983) reported the presence of *Beijerinckia* and *Azotobacter* in the rhizosphere of plantation crops such as coconut, arecanut, cashew, cocoa and pepper. Purushothaman and Govindarajan (1986) reported the occurrence of *Beijerinckia* in the rhizosphere of pearl millet.

2.5. Morphological and taxonomical characteristics of *Beijerinckia*

Morphological and taxonomical characters of *Beijerinckia* have been described by several workers (Dobereiner and Ruschel 1958, Becking 1961, 1962, 1974). Hilger (1965) included the genus *Beijerinckia* in the family *Azotobacteriaceae* and divided it into three species, *B. indica*, *B. fluminensis* and *B. derxii*. *Beijerinckia* cells were reported to be single, slightly curved or peer shaped, Gram '-'ve rods, 0.5-1.5 by 1.7-4.5 um in size with distinctly rounded ends. Becking (1974) described four species of *Beijerinckia* viz. *B. indica*, *B. fluminensis*, *B. mobilis* and *B. derxii*.

The occurrence of B. indica has been reported from soils of Africa, South America, North America and Central and North Brazil (Becking 1961, Dobereiner 1959). Becking (1962) reported persistence of lipoid bodies in aged cultures of B. indica. The production of tenacious slime and slight colouration of colonies on aging were also reported in B. indica. (Becking 1974a)

B. fluminensis was recorded from acidic soils of South America, Africa and Asia (Dobereiner and Ruschel 1958). The name B. fluminensis came from a locality 'Baicada Fluminensi' state Rio de Janerio from which soil it was first isolated. Agar colonies of B. fluminensis were reported to be typically small and granular with irregular rough surface. Presence of characteristic large capsules enclosing two or more individual cells was also reported in this species (Becking 1974a)

B. mobilis was isolated from the soils of Java, Indonesia, South America and tropical Africa. Older cultures of this species on neutral or alkaline agar media were reported to show typical dark amber or deep reddish brown colour (Tchan 1957). Polysaccharide formed by this species was found to be neither elastic nor slimy and the cells possessed conspicous motility (Becking 1961).

Colonies of *B. derxii* were reported to be highly raised and slimy or smooth. Though the colonies were semitransparent or opaque at first, an yellow green water soluble pigment was produced after 2-3 weeks of incubation. (Tchan 1957). Heloiza and Yara (1985) reported production of large quantities of slime by *B. derxii* under culture conditions.

2.6. Nitrogen fixation by Beijerinckia

The measurement of N_2 fixation is of considerable importance and can be determined by various methods. N_2 fixation by free-living N_2 fixing bacteria was earlier evaluated by determining nitrogen increment in culture media by classical Kjeldahl method.

Kluyver and Becking (1950) and Jensen (1954) have shown that *Beijerinckia* fixed atmospheric nitrogen to the extent of 16 to 20mg per gram of energy rich material. Barooah and Sen (1964) studied nitrogen fixation by various strains of *B. indica* in Derx's medium and showed significant positive correlation between the amount of slime (polysaccharide gum) formed by the organism and the intensity of nitrogen fixation in pure cultures.

Dommergues and Mutaftschiev (1965) found that fixation of nitrogen by *B. indica* or *B. fluminensis* increased markedly when an oligonitrophilic yeast *Lipomyces starkeyi* was present in culture medium. Dobereiner (1968) reported N_2 fixation to the extent of 17.2 mg N g^{-1} sucrose by *B. indica*, 12.9 mg N g^{-1} by *B. fluminensis* and 12.5 mg N g^{-1} by *B. derxii*. Dobereiner *et al.* (1973) estimated that as much as 50 kg N ha^{-1} year $^{-1}$ was fixed by *Beijerinckia* spp. Sugar cane association. Potty and Jayasankar (1976) reported nitrogen fixation in the range of 10.6 to 19.6 mg N per 100 ml culture medium by isolates of non-symbiotic N_2 fixing bacteria from the rhizosphere of root (wilt) affected coconut palms.

The use of the stable isotope ^{15}N increased the sensitivity of nitrogen determinations. The ^{15}N enriched N_2 technique was first used during 1940s to measure N_2 fixation (Burris *et al.* 1943). Mallick *et al.* (1988) used this technique to quantify N_2 fixation by Kallar grass after inoculation with *Beijerinckia*.

Acetylene-reduction assay (ARA) is the most widely used test to study N_2 fixation by various systems. The acetylene reduction test arose from independent observations of Dilworth (1966) and Schollhorn and Burris (1966) that nitrogenase preparations reduced acetylene to ethylene. Gas-liquid chromatography with a flame ionisation detector was used as the assay technique and provided speed and sensitivity (Hardy and Knight 1967). Hardy et al. (1968) described applications of this method to various test systems. Spiff and Odu (1973) studied acetylene-reduction by *Beijerinckia* under various partial pressures of oxygen and acetylene. Acetylene-reduction by *B. indica* in shaken liquid cultures increased with increase of C_2H_2 upto 0.74 atmosphere. The oxygen partial pressure also affected acetylene reduction at PO_2 of 0.15 atmosphere for liquid cultures. Mac Rae (1977) reported that acetylene reduction by strains of *B. indica* and *B. laeticogenes* increased with increased partial pressures of acetylene and nitrogen upto 80 k pa. Pena and Dobereiner (1974) studied the effect of NO_3^- and NH_4^+ on nitrogenase activity of *Beijerinckia* spp. *Beijerinckia indica* maintained about 30% of the original nitrogenase activity for 60 hours after addition of 10 mM NO_3^- and 10-20% of the activity after addition of 10mM + NH_4^+ *B. fluminensis* appeared to be unaffected during six hour incubation period. The nitrogenase of *B. derxii* remained active upto 100 hours when incubated in a carbon and nitrogen free medium (Barbosa et al. 1986).

Nitrogen fixation is dependent on the activity of the enzyme, nitrogenase present in the nitrogen fixing microorganisms. The nitrogenase complex consists of two dissimilating proteins one termed nitrogenase reductase or Fe protein, which contain four iron and four acid labile sulphur atoms while the other, the Mo-Fe protein or nitrogenase, which contains two molybdenum, 28-32 iron and approximately 28 acid labile sulphur atoms. Carnahan et al.(1960) published for the first time a method for obtaining cell free extract from Clostridium pasteurianum with consistently high nitrogenase activities. Active cell free extracts for nitrogen fixation have also been obtained from non-symbiotic N_2 fixing bacteria like Azotobacter vinelandii (Bulen et al. 1965) and A. chroococcum (Kelly 1968). Apart from its role in nitrogen fixation, the non-symbiotic N_2 fixing bacteria are also known to produce growth promoting substances. Vancura and Macura (1980) detected I.A.A. in Azotobacter cultures. Cultures of Azotobacter also elaborated considerable quantities of biologically active substances (Lee et al. 1970. Vancura 1961, Brown 1972). They are also known to have antagonistic effect on pathogenic fungi. Lakshmi Kumari et al. (1972) reported that Azotobacter sp. produced thermolabile ether soluble fungistatic substance which inhibited the growth of Fusarium moniliforme in vitro.

2.7. Factors affecting growth and nitrogen fixation by *Beijerinckia*.

Beijerinckia when growth in pure culture under laboratory conditions, tolerates a wide range of pH values. Tehan (1957) observed growth between pH 4-9 and found that the organisms could not grow either at pH 3 or 11. Becking (1961) reported optimum growth and nitrogen fixation by *Beijerinckia* at a pH of approximately 4. There was a sharp drop in growth below pH 4, but there was only slight reduction in growth as pH increased upto 9. Becking (1962) reported that molybedinum requirement of *Beijerinckia* for nitrogen fixation and nitrogen assimilation were larger at low pH than at neutral reactions. Hilger (1964) observed a marked depression of N_2 fixing capacity at pH 5-6 when nitrate was added at concentrations as low as 22.5 ppm.

Becking (1961) observed growth and nitrogen fixation by *Beijerinckia* at temperatures ranging from 20-30°C. Even though there was growth at 16°C *Beijerinckia* failed to grow at 37°C and above. *Beijerinckia* was reported to be fairly cold and frost resistent. Cells of these bacteria suspended in tap water or Nitrogen free mineral medium and stored for 20 months at 4°C in a refrigerator retained same viability (Becking 1959). A variation in resistance to desiccation was observed among the strains, when the effects of two levels of desiccation was tested at 10, 20 and 30°C in a red alluvial soil (Strijdom and Steenkamp 1967). Becking (1961) reported that storage of *Beijerinckia* strains at 4°C for 3-4 months did not affect the viability of cells. *Beijerinckia* strains also varied in their requirement and sensitivity for Fe. *B. indica* from lateritic soils fixed nitrogen in a medium containing 200 ppm Fe.

Charyulu and Rao (1978) observed that application of benomyl, a carbamate fungicide at 10, 20 and 100 ppm concentrations to a flooded paddy soil resulted in significant increase in N_2 fixation, by *Azospirillium* spp. Effect of Gamma BHC was studied by Nayak and Rao (1980) on heterotrophic nitrogen fixation in paddy soils. It stimulated N_2 fixation in alluvial and acid sulphate pokkali soils while considerable inhibition of N_2 fixation was evident in other soils. Ayanaba 1981 observed that incorporation of benomyl and mancozeb (Dithane M-45) at 0.005% in routine media had little or no effect on the number of colonies of *Beijerinckia*.

2.8. Response of crops to inoculation

There are a number of reports available in literature to show the growth promoting effect of *Beijerinckia* in various crop plants. Dobereiner and Ruchel (1962) reported results of a green house experiment in which *Beijerinckia* was inoculated to rice. Inoculation of the seeds brought about a marked change in the rhizosphere equilibrium, stimulating *Beijerinckia* and reducing the number of amino acid requiring microorganisms. In another green house study Souto and Dobereiner(1967) observed significant increase in total nitrogen content of elephant grass (*Pennisetum purpureum*) due to inoculation with *Beijerinckia*.

Balasundaram and Sen (1971) studied the effect of bacterization of rice (*Oryza sativa*) with *Beijerinckia*. Inoculation of *Beijerinckia indica* alone was not effective in increasing grain yield, but in combination with 40 kg N/ha as urea, yield response was comparable to those obtained with 80 kg N/ha alone. Bhat *et al*. (1971) reported increased production of many amino acids from the roots when *Beijerinckia* was inoculated on the shoots of *Dolichos lablab*.

Field trials conducted by Sulaiman (1971) indicated that inoculation of Beijerinckia alone significantly increased the yield of paddy at 5% level of significance. When inoculation was done with Mo application, the yield increase was significant at 1% level of significance. However, Rawat and Sanoria (1976) did not observe any significant response due to inoculation with Beijerinckia in an inoculation trial on Cicer arietinum var. type I with Rhizobium, B. indica and Azotobacter chroococcum. Jagtap and Bhide (1977) studied the effect of Beijerinckia inoculation on growth and yield of paddy cv. IR-8. Inoculation increased paddy yield from 47.5 g/plant in the untreated control to 65.4 g/plant. They attributed the increase in yield to increase in tillering and N_2 fixation. Subramanyan and Prasad (1980) also reported increase in the shoot length and plant dry weight in sorghum due to inoculation with suspensions of Azotobacter and Beijerinckia. Malick et al. (1988) conducted experiments to quantify biological nitrogen fixation associated with roots of kallar grass using ^{15}N isotope dilution method. They reported that 20-26% of the nitrogen in plant was derived from atmosphere when inoculation was done with Beijerinckia sp. and Klebsiella sp.

Combined inoculation of *Beijerinckia* with other microorganisms such as VAM fungi, *Rhizonium* etc. showed synergistic interaction among these organisms. Effect of inoculation with the VA mycorrhizal fungus, *Glomus fasciculatus*, *Beijerinckia* mobilis and a phosphate solubilising fungus *Aspergillus niger*, singly and in combination was studied in onion by Manjunath *et al*. (1986). They observed increase in dry weight and nitrogen content of the plants due to synergistic interaction of the organisms when compared to their individual inoculations. Synergistic interaction was also observed between *Rhizobium* and Beijerinckia in the cover legume, *Pueraria phaseoloides* (Kothandaraman *et al*. 1986). Significant increase in nodule biomass, nodule number and the biomass of the shoot and root was observed when dual inoculation was done with both *Rhizobium* and *Beijerinckia* as compared to inoculation with either one of the bacteria.

III. MATERIALS AND METHODS

3.1 Collection of samples from different locations

3.1.1. Coconut gardens

Rhizosphere and non-rhizosphere soil samples of coconut were collected from different locations in two soil types viz. laterite and sandy in which coconut is largely grown in Kerala. Three locations were selected from each soil type. Kayamgulam, Thakazhi and Quilon were the locations sampled for sandy soil type and Kasaragod, Mavelikkara and Uduma represented the laterite soil type. Farmers' plots having monocropped adult 'West Coast Tall' coconut palms in the age group of 25-40 years were selected from different locations. The selected plots were not receiving inorganic fertilizers and irrigation. The samples were collected during the period from December 1987 to January 1988.

A total of six samples comprising three each from rhizosphere and three from interspaces were obtained from each location. Blocks of soil containing roots were collected at a distance of one meter from bole of the palm from 0-20 cm depth to represent rhizosphere samples. Samples obtained from three directions in the basin were pooled and composite samples prepared. Non-rhizosphere samples were also obtained at the same depth from three locations in the interspaces and pooled. Samples were immediately transferred to clean polythene bags which were then suitably tagged and sealed to avoid any external contamination. The samples were brought to laboratory and stored in refrigerator at 4°C till analysis. Analysis was done at the earliest possible time after collection of samples.

3.1.2. Coconut Nurseries

Soil samples were also collected from four coconut nurseries of Department of Agriculture, Govt. of Kerala viz. Nileshwar, Munderi, Parappanangadi and Thikkodi and one nursery of Central State farm, Aralam. One year old coconut seedlings were selected and samples obtained from rhizosphere region in each nursery. Samples obtained from three directions around the seedlings were pooled and a composite sample was prepared. Four replicate samples were collected from each nursery. The samples were brought to laboratory and stored as described earlier.

3.2. Enumeration of bacteria and *Beijerinckia*

The soil dilution and plate count method(Timonin 1940) was used to estimate the number of micro-organisms in the rhizosphere and non-rhizosphere soils. An estimate of the population refers to the number of viable cells in the same capable of growing on specific agar media used in the test. The different media used for the isolations were soil extract agar and Becking's agar for enumeration of total bacteria and Beijerinckia respectively with the following composition.

3.2.1. Soil extract agar medium

Glucose	1.0g
$K_2H\ PO_4$	0.5g
Soil extract	100ml
Agar	20.0g

Tap water to make 1000 ml.

Sterilized at 121°C for 15 min.

3.2.2. Becking's agar medium

Sucrose	20.0g
KH_2PO_4	0.8g
$K_2H\ PO_4$	0.2g
$Mg\ SO_4 7\ H_2O$	0.5g
$Fe\ Cl_3$	0.1g
$Na_2\ Mo\ O_4$	0.005g
Agar	20.0g

Distilled water to make 1000 ml

p^H 6.5

Blocks of soil containing roots to represent rhizosphere samples and non-rhizosphere samples were weighed in 10g lots and transferred to 250 ml conical flasks containing 90 ml of sterile distilled water and shaken thoroughly to make the solution homogeneous. Suitable dilutions were prepared by pipetting out 1.0 ml of aliquots of the suspension successively into nine ml sterile distilled water blanks. Dilutions were prepared to the order of 10^{-6}. 1.0 ml of the desired dilution was then transferred asceptically into each of the 3 petriplates and about 15 ml of sterile agar media, cooled to just above the solidifying temperature was added to each dish. The dishes were rotated by hand in a broad swirling motion so as to distribute the solution uniformly in the agar medium. Dilutions 10^{-5} and 10^{-6} were plated for estimating total bacteria and dilutions 10^{-2} and 10^{-3} were plated for _Beijerinckia_.

The plates were incubated at $30^{o} \pm 1^{o}C$ for 3 days for bacteria and seven days for _Beijerinckia_ in a BOD incubator. Colonies appearing in both media were counted after the incubation period. For counting purposes dishes containing overlapping bacterial colonies were discarded. The average number of colonies per dish was multiplied by dilution factor and numbers of organisms estimated for one gram dry weight of soil. The data on populations were analysed statistically by analysis of variance (Snedecor and Cochran 1967).

3.2.3. Measurement of rhizosphere effect

Rhizosphere effect is measured in terms of R.S. ratio. R:S ratio is defined as the ratio of the numbers of micro-organisms per gram of rhizosphere soil to the numbers per gram of control soil.

3.3. Dry weight of soil

To determine the dry weight of soil, 10g portions of soil samples were dried in an oven at 110°C till constant weight. The counts of microorganisms were expressed as numbers per gram dry weight of the soil.

3.4. pH of the soil

Ten gram portions of different soil samples were taken and added 25 ml of double distilled water. The solutions were stirred well and allowed to stand for about three hours. The pH was measured using digital pH meter.

3.5. Isolation of Beijerinckia

A loopful of different morphological forms of colonies appeared on the Becking's medium were transferred to tubes containing the same media. For isolating cultures, soil samples were also inoculated on solidified Becking's agar media and incubated at 30 ± 1°C for 10 days. The colonies appeared in the plates were transferred to the tubes. The cultures obtained from different locations were purified by dilution plating technique and repeated streaking on the same media. Single well separated colonies were isolated and maintained on Becking's agar slopes for further studies. These cultures were subcultured every 30 days.

3.6. Morphological and cultural characters of isolates

The isolated cultures were characterised on the basis of cultural, morphological and physiological characteristics suggested in Bergey's Manual of Determinative Bacteriology. The cultural characteristics studied included observations on colony characters such as size, shape, elevation, colour, production of gum on Becking's agar media and the type of growth in Becking's broth. Morphological tests of bacteria were done to study Gram reaction, size, presence of bipolar lipoid bodies and motility as per the methods given in Harrigan (1966).

3.7. Physiological characters.

3.7.1. Growth on N_2 free media

N_2 free Becking's broth was inoculated with 0.2ml of culture broth and incubated for 14 days at $30 \pm 1^{o}C$. Presence of growth was recorded by observing the presence of turbidity in inoculated tubes after inoculation.

3.7.2. Growth at acidic pH

Becking's broth was adjusted to pH 4 using 0.1N HCl. After sterilizing the broth, inoculation was done with the isolated cultures and incubated. Development of turbidity was considered as indication of growth.

3.7.3. Growth with nitrate as N source.

Becking's broth was supplimented with potassium nitrate as nitrogen source and inoculated with cultures. Presence of growth was recorded after incubation.

3.7.4. Growth on Casein agar.

Solidified casein agar (Cote, 1984) in petri plates was streaked with a loopful of the cultures and incubated at optimum conditions for growth. Presence or absence of growth was recorded after incubation.

3.7.5. Catalase test

A loopful of cultures was placed on a clean slide and added one ml of hydrogen peroxide solution. Effervescence, caused by the liberation of free oxygen as gas bubbles indicated the presence of catalase in the culture under test.

3.8. Nitrogenase activity of _Beijerinckia_ isolates

The nitrogen fixing efficiency of isolates was estimated in terms of nitrogenase activity by acetylene-reduction method. The procedure adopted was that of Masterson and

Murphey (1979). To assay the acetylene reduction activity (ARA), 3.0 ml of Becking's broth taken in 15 ml capacity test tubes was sterilized by autoclaving for 15 minutes at 121°C. Inoculation was done using 0.1 ml of inoculum having similar optical density and the cultures were incubated for 14 days at 30 ± 1°C. The cotton plugs were replaced with subaseals following incubation and acetylene was injected (10% of the atmosphere). After incubation for 4-6 h at 30°C ethylene production was assayed by Shimadzu gas chromatograph using flame ionisation detector. The analysis was done with porapack 'T' column using nitrogen as carrier gas at a flow rate of 35 ml per minute. Column and detector temperatures were adjusted to 100°C and 150°C respectively. Peak heights were taken and it is linearly related to concentration of the gas.

<u>Calculation</u>

C_2H_4 produced in uM C_2H_4/h, is as follows:

$$C_2H_4 \text{ sample C.U. X } \frac{\text{vol. gas in sample container}}{\text{vol. injected into G.L.C.}} \text{ X assay time } \frac{1}{h} \text{ X K}$$

$$\text{minus } C_2H_4 \text{ blank C.U. X } \frac{\text{vol. gas in blank container}}{\text{vol. injected into G.L.C.}} \text{ X K}$$

Where C.U. = chart units used to measure peak height.

The blank is a sample container with added C_2H_2 only.

K = conversion factor obtained using a standard mixture to calibrate the chromatograph.

e.g. for 100 vpm C_2H_4 standard, K is derived as follows:

1 ml of 100 vpm C_2H_4 contains 100×10^{-6} ml C_2H_4 and = X C.U.

22.4 l C_2H_4 at S.T.P. = 1 Mole C_2H_4

$$1 \text{ ml of } 100 \text{ vpm } C_2H_4 = \frac{100 \times 10^{-6}}{22.4 \times 10^{3}} \text{ moles } C_2H_4$$

$$= 0.00446 \text{ umoles } C_2H_4 = X \text{ C.U.}$$

$$\text{then K (or 1 C.U.)} = \frac{0.00446}{X} \text{ umoles } C_2H_4$$

3.9. Factors affecting growth and N_2 fixation by *Beijerinckia* isolates.

The effect of various factors such as incubation period, pH, temperature and different carbon sources was studied on growth and N_2 fixation by two cultures of *Beijerinckia* CMF-7 and WCT-12. In addition, the effect of addition of different concentrations of nitrogen and fungicides viz. Bavistin, Dithane M-45 and Aureofungin was also studied. The two *Beijerinckia* cultures were obtained from the culture collection of Division of Microbiology, Central Plantation Crops Research Institute(CPCRI), Kasaragod. They were isolated from rhizosphere soils of coconut from the CPCRI farm and identified as *Beijerinckia*. The cultures CMF-7 and WCT-12 possessed high nitrogenase activity of 109.5 and 122.92 nM C_2H_4 tube^{-1} h^{-1} when tested by acetylene reduction method. Inoculum for these studies was prepared by growing the cultures in 50 ml portions of Becking's broth in 250 ml Erlen Mayer flasks under shake culture conditions for 7 days. All the tests were conducted in 25 ml capacity test tubes having 8.0 ml of Becking's broth. Composition of the broth was altered to test the various factors. Inoculum was added at the rate of 0.2 ml per tube. Incubation was done for 14 days in BOD incubator fixed at $30^{o} \pm 1^{o}C$ in all cases. Three replications were maintained for each test. After incubation optical density of the broth was measured in each test at 500 nm, using spectronic-20. The nitrogen fixed in the broth was estimated by micro-kjeldahl method (Jackson 1973).

3.9.1. Nitrogen estimation

At the end of the incubation period, 5 ml of the culture was carefully transferred to microkjeldahl's digestion tubes to which 2 ml of concentrated sulphuric acid and 0.25 g of digestion mixture were added and digested until a colourless liquid residue was left behind in the digestion tube. After the content had sufficiently cooled down it was transferred to microkjeldahl's distallation set with sufficient washings for distillation. Forty per cent NaOH was slowly added till a brown colour appears. The distillate was collected in 10 ml of boric acid taken in the beaker. The collected solution was then titrated against N/50 H_2SO_4 using mixed indicator (0.066g methyl red and 0.099 g bromocresol green in 100 ml alcohol). The end point was the colour change from blue to pink. The amount of nitrogen fixed by the bacteria in the medium was calculated on the basis of the quantity of N/50 H_2SO_4 used in the titration.

1 ml of N/50 H_2SO_4 O= 0.00028g N

N in the samples = 0.00028 X x

where x is the quantity of N/50 H_2SO_4 used in titration. The quantity of N_2 fixed per gram carbon provided as carbon source in the medium was claculated.

3.9.2. Effect of incubation period

Incubation of the inoculated Becking's broth was done in BOD incubator fixed at $30^{\circ} \pm 1^{\circ}C$ for different periods ranging from 2-18 days. At the end of every two days of incubation, three tubes of each culture were withdrawn and recorded the optical density. The broth was then analysed for N_2 by microkjeldahl's method.

3.9.3. Effect of temperature

After inoculation of Becking's broth, the cultures were incubated at different temperatures viz. 20°, 30°, and $40^{\circ}C$ for 14 days. At the end of incubation, the optical density of the broth and N_2 fixed in the broth culture were estimated as described earlier.

3.9.4. Effect of pH

To find out the effect of pH, the pH of Becking's broth was adjusted to different levels ranging from 3 to 10 using 0.1 N sodium hydroxide and 0.1N hydrochloric acid. After sterilization inoculation was done with the two cultures. The optical density of culture broth was determined and N_2 estimated after the incubation.

3.9.5. Effect of carbon sources

Sucrose is the carbon source used in the Becking's broth at a concentration of 20g per litre. In order to test the effect of different carbon sources, sucrose was replaced in Becking's broth with each of the other sugars. at concentrations to provide the same amounts of carbon provided by the sucrose. The sugars tested included lactose, maltose, malic acid, glucose and mannitol.

3.9.6. Effect of different concentrations of N.

Becking's broth was supplimented with ammonium sulphate to obtain the following concentrations of nitrogen: 0, 25, 50, 100, 500, 1000, ppm. The broth was then autoclaved, inoculated with the cultures and incubated. The optical density of the broth was recorded after incubation.

3.9.7. Effect of different concentrations of fungicides.

Dithane M-45 (Maneb + Zn), Bavistin(Carbendazim) and Aureofungin were the fungicides used in this study at concentrations of 0, 100, 250, 500 ppm. Beckings broth was supplimented with the different concentrations of the fungicides and inoculated with the cultures. The growth and N_2 fixation by the two cultures in presence of the fungicides was determined after the incubation by recording turbidity and amount of nitrogen in the culture broth by microkjeldal's method.

3.10. Effect of inoculation on black pepper.

A pot culture experiment was conducted to test the effect of inoculation with Beijerinckia on the growth and N_2 content of black pepper (Piper nigrum L.) seedlings. The experiment had six treatments with four replications each.

T1 - Control

T2 - Control with urea

T3 - Beijerinckia WCT-12 inoculated

T4 - Beijerinckia WCT-12 + urea

T5 - Beijerinckia CMF-7 inoculated

T6 - Beijerinckia CMF 7 + urea

Pots of 23.5 cm diameter and having a capacity to hold 4 kg soil were used for the experiment. The test soil was sandy loam having a pH of 5.5 and was sterilized in an autoclave at 121°C for one hour on two consecutive days before use. The cultures tested in this study were CMF-7 and WCT-12. The pepper cultivar used as the experimental host was 'Karimunda'.

Beijerinckia inoculum was prepared by growing the cultures for 7 days in Becking's broth under shake culture conditions. The inoculum contained 10^8 cells of Beijerinckia per ml. Pepper plants were raised by planting two noded cuttings of runner wines in sterile soil in polythene bags. Selected rooted cuttings were dipped in culture solutions for one minute and planted in the pots. The initial shoot height of the seedlings were recorded. The cultures were then added at the rate of 20 ml per pot. All the plants were fertilized with 2.5 g super phosphate and 0.67 g potash per pot. Urea was added in treatments T2, T4 and T6 only at the rate of 1.75 g per pot. The plants were arranged in completely randomised design in the green house and were watered regularly.

After five months growth, the plants were depotted and plant height was recorded. The root system was separated from shoot and washed properly to remove soil adhering to it. Samples were dried in an oven at 70°C till constant weight and dry weight of shoot and root determined. The shoot portion was powdered and nitrogen content determined by micro kjeldahl's method (Jackson 1973).

EXPERIMENTAL RESULTS

4.1. Distribution of *Beijerinckia* in coconut soils and rhizosphere

The occurrence of *Beijerinckia* and its population levels in coconut rhizosphere and non-rhizosphere soils were studied in soil samples collected from six different locations under laterite and sandy soil types in coconut growing tract of Kerala. The data on the population in different locations are presented in Table-1. *Beijerinckia* colonies were observed when soil samples from all the six locations were analysed by dilution plating using specific medium recommended for *Beijerinckia*. The rhizosphere soils harboured significantly higher populations than non-rhizosphere soils. The average population was $3.73 \times 10^3 g^{-1}$ soil in rhizosphere compared to $1.61 \times 10^3 g^{-1}$ soil in non-rhizosphere. Comparative study of the populations in the two soil types revealed significantly more numbers of *Beijerinckia* in sandy soils when compared to laterite soils in both rhizosphere and non-rhizosphere samples. The population was maximum ($8.77 \times 10^3 g^{-1}$ soil) in the rhizosphere samples from Quilon in the sandy soil type. Among the non-rhizosphere samples analysed, samples from Thakazhi and maximum population ($3.44 \times 10^3 g^{-1}$ soil) of *Beijerinckia*.

Table 1. Population of *Beijerinckia* in rhizosphere and non-rhizosphere soils from coconut gardens at different locations.

Soil type/Location	Population x $10^3 g^{-1}$ dry soil	
	Rhizosphere	Non-rhizosphere
I Sandy		
Kayamkulam	4.57	1.04
Thakazhi	4.93	3.44
Quilon	8.77	1.72
Mean	6.09	2.07
II Laterite		
Uduma	0.46	0.25
Mavelikkara	1.00	0.82
Kasaragod	2.68	2.40
Mean	1.48	1.16
General mean	3.73	1.61

LSD (P =0.05) for comparison of rhizosphere vs non-rhizosphere	1.69
LSD (P=0.05) for comparison of locations	2.92
LSD (P=0.05) for comparison of soil types	1.69

4.1.1. Proportion of *Beijerinckia* to total bacteria

The relationship between *Beijerinckia* population and total bacterial population in rhizosphere and non-rhizosphere soils in different locations are presented in Fig-1. Total bacterial population ranged from 2.37×10^5 to $7.30 \times 10^5 g^{-1}$ soil in rhizosphere soil whereas it ranged from 1.47×10^5 to $4.58 \times 10^5 g^{-1}$ soil in nonrhizosphere soil. The total bacterial population was also higher in rhizosphere soil when compared to non-rhizosphere soil in all the locations tested. Studies on the distribution of total bacterial population in laterite and sandy soil types revealed more population in sandy soils than the laterite soils.

Proportion of *Beijerinckia* population to total bacterial population was also higher in almost all the rhizosphere samples than non-rhizosphere samples(Table-2) indicating the selective stimulation of *Beijerinckia* in coconut rhizosphere soils. *Beijerinckia* constituted 0.20 to 1.29 and 0.17 to 0.91% of total bacteria in rhizosphere and non-rhizosphere soils, respectively. The percentage of *Beijerinckia* in total bacterial population was particularly more in sandy soils than in laterite soils.

Fig. 1. Relationship between population of total bacteria and *Beijerinckia* in rhizosphere & non-rhizosphere soils

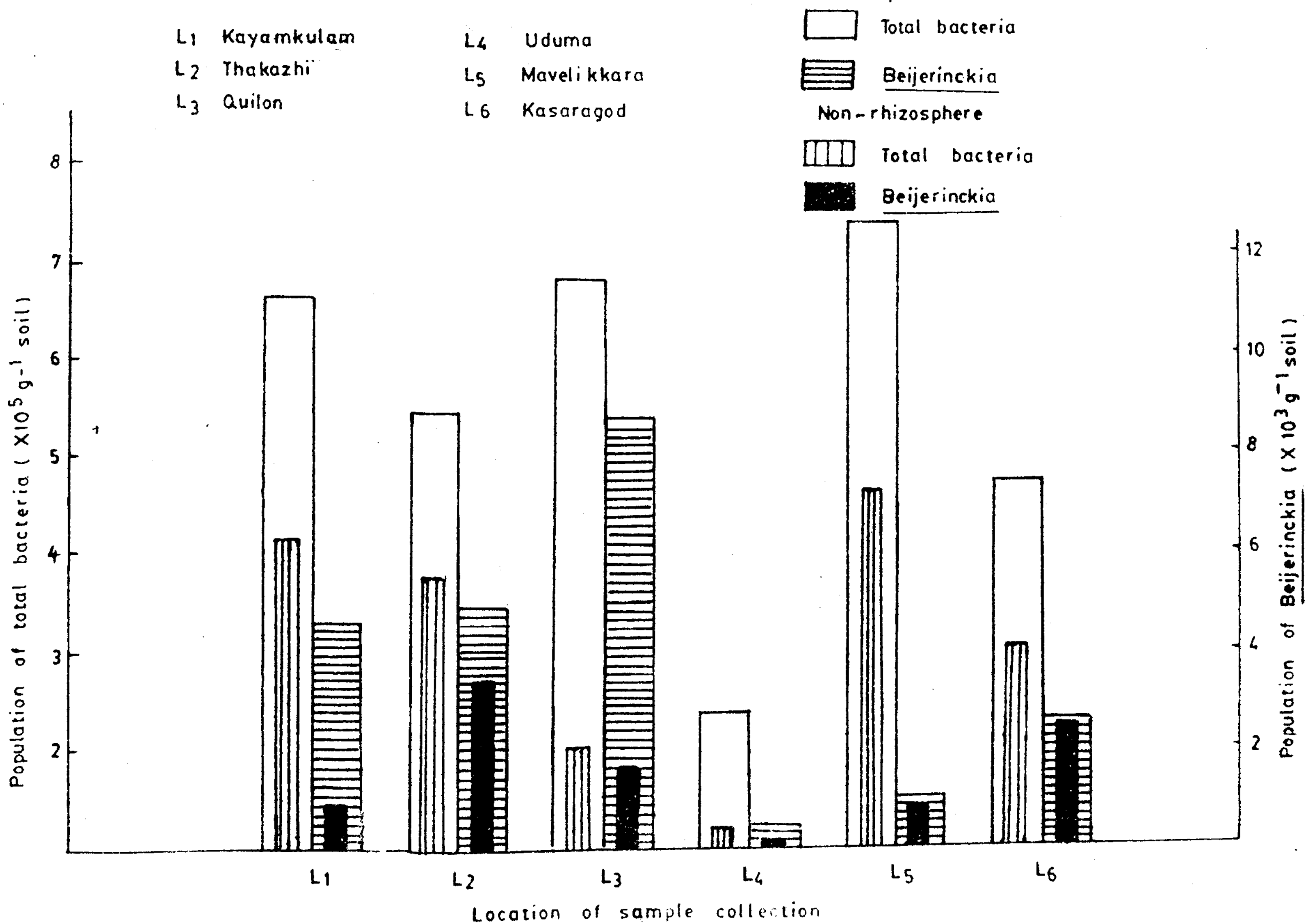


Table-2. Proportion of *Beijerinckia* to total bacteria at different locations.

Soil type/Location	Rhizosphere	Non-rhizosphere
I Sandy		
Kayamkulam	0.69	0.25
Thakazhi	0.90	0.91
Quilon	1.29	0.86
Mean	0.96	0.67
II Laterite		
Uduma	0.20	0.17
Mavelikkara	0.22	0.18
Kasaragod	0.57	0.82
Mean	0.33	0.39
General Mean	0.65	0.53

4.1.2. Rhizosphere effect

The rhizosphere effect was assessed in terms of R:S ratio for both Beijerinckia and total bacteria on the basis of their population in rhizosphere and non-rhizosphere soils. R:S ratio of Beijerinckia was higher than that of bacteria in four out of the six locations. (Table-3). The mean R:S ratio for Beijerinckia and total bacteria were 2.65 and 1.87, respectively. The R:S ratio was particularly high for Beijerinckia in sandy soil when compared to the laterite soil.

4.2. pH of the soils

The data on pH of rhizosphere and non-rhizosphere soils from the six locations are presented in Table-4. There was not much difference in the pH of soil samples from sandy and laterite soil types. It was interesting to note that the rhizosphere soils had a higher pH than non-rhizosphere soils. The mean pH of rhizosphere soil samples were 5.63 and 5.84 in sandy, and laterite soils, respectively. But the non-rhizosphere samples had mean pH of 5.24 and 5.23 only in sandy and laterite soils respectively.

Table-3. R:S ratio of *Beijerinckia* and total bacteria at different locations.

Soil type/Location	*Beijerinckia*	Total bacteria
I Sandy		
Kayamkulam	4.39	1.59
Thakazhi	1.42	1.45
Quilon	5.09	3.38
Mean	3.63	2.14
II Laterite		
Uduma	1.87	1.61
Mavelikkara	1.22	1.59
Kasaragod	1.91	1.61
Mean	1.67	1.60
General mean	2.65	1.87

Table 4. pH of rhizosphere and non-rhizosphere soils from coconut gardens at different locations.

Soil type/Location	Rhizosphere	Non-rhizosphere
I Sandy		
Kayamkulam	5.05	4.93
Thakazhi	5.78	5.15
Quilon	6.05	5.63
Mean	5.63	5.24
II Laterite		
Uduma	5.22	4.72
Mavelikkara	5.33	5.23
Kasaragod	6.98	5.73
Mean	5.84	5.23
General Mean	5.74	5.23

4.3. Occurrence of *Beijerinckia* in coconut nurseries.

Analysis of soil samples from five different coconut nurseries in four northern districts of Kerala revealed the occurrence of Beijerinckia in all the nurseries(Table-5). There was significant difference in the population in different nurseries. Soil samples of Aralam nursery harboured very high population of *Beijerinckia* ($16.35 \times 10^3 g^{-1}$ soil) when compared to those of other nurseries. The total bacterial population was also significantly different in the coconut nurseries studied. It was interesting to note that Aralam nursery soils had low population of total bacteria eventhough it harboured very high population of *Beijerinckia*. *Beijerinckia* constituted 0.[illegible] to 16.19% of total bacteria in the different nurseries. The proportion of *Beijerinckia* to total bacteria was very high in Aralam nursery whereas itwas very low in Parappanangadi nursery.

4.4. Isolation of *Beijerinckia* from different locations

Thirty bacterial cultures were isolated from well separated single colonies appeared on 'Becking's agar from rhizosphere and non-rhizosphere soil samples from six locations. The cultures were purified and their morphological cultural and physical characteristics determined.

Table 5. Population of *Beijerinckia* and total bacteria in different coconut nurseries.

Nursery	Beijerinckia $x\ 10^3 g^{-1}$ dry soil	Total bacteria $x\ 10^5 g^{-1}$ dry soil	Proportion of *Beijerinckia* to total bacteria
Thikkodi	2.41	1.44	1.67
Munderi	3.75	5.05	0.74
Parappanangadi	0.84	20.14	0.42
Nileshwar	5.83	9.08	0.64
Aralam	16.35	1.01	16.19
Mean	5.83	7.34	3.86
LSD(P=0.05) for comparison of nurseries	9.09	9.88	-

4.5. Morphological and cultural characters

The data on various taxonomical characteristics of the isolates are presented in table-6. Gram staining of the bacterial isolates revealed that cells were Gram-negative straight or slightly curved short rods. Young cultures possessed polar lipoid bodies. Test for motility showed that the cultures were motile. All the cultures showed very good growth on nitrogen free nutrient media.

Colonies on Becking's agar were raised with smooth or plicated surface. The photographs of colonies with smooth and plicated surfaces are shown in plates 1 and 2, respectively. Colonies of 22 isolates had smooth surface, while tho remaining ones formed colonies with plicated surface. The young colonies were mucilagenous without pigmentation while older cultures were amber brown or light reddish in colour. All the cultures produced large quantities of slime on agar media. Most of the cultures from Thakazhi, Mavelikkara and Quilon produced slime which was very tough, tenacious and elastic and it was very difficult to remove the slime from agar surface with the help of inoculation loop. On the other hand, the isolates from Kayamkulam, Kasaragod and Uduma produced colonies with granular and viscous slime and it was easy to remove the slime from agar surface.

Table 6. Morphological and cultural characteristics of bacterial isolates.

Sl. No.	Culture No.	Location from which isolated	Source	Gram reaction and shape	Motility	Type of growth in growth	Appearance of colony surface	Type of gum
1	2	3	4	5	6	7	8	9
1.	SKR-1	Kayamkulam	Rhizosphere	G -ve short rod with lipoid bodies	motile	Entire medium viscous	raised smooth	granular
2.	SKN-1	"	Non-rhizosphere	"	"	"	"	"
3.	SKN-2	"	"	"	"	"	"	"
4.	SKN-3	"	"	"	"	"	"	"
5.	SKN-4	"	"	"	"	"	"	"
6.	STR-1	Thakazhi	Rhizosphere	"	"	Pellicle formation on sides at surface	raised plicated	tenacious tough
7.	STR-2	"	"	"	"	"	"	"
8.	STR-3	"	"	"	"	"	"	"
9.	STN-1	"	Non-rhizosphere	"	"	"	"	"
10.	STN-2	"	"	"	"	"	"	"
11.	SQR-1	Quilon	Rhizosphere	"		"	"	"
12.	SQR-2	"	"	"	"	"	"	"

1	2	3	4	5	6	7	8	9
13.	SQN-1	"	Non-rhizos-phere	"	"	"	"	"
14.	SQN-2	"	"	"	"	"	"	"
15.	SQN-3	"	"	"	"	"	"	"
16.	LCR-1	Kasaragod	Rhizosphere	"	"	Entire medium viscious	raised smooth	granular
17.	LCR-2	"	"	"	"	"	"	"
18.	LCN-1	"	Non-rhizos-phere	"	"	"	"	"
19.	LCN-2	"	"	"	"	"	"	"
20.	LCN-3	"	"	"	"	"	"	"
21.	LUR-1	Uduma	Rhizosphere	"	"	Pellicle formation on sides at surface	"	"
22.	LUR-2	"	"	"	"	"	"	"
23.	LUR-3	"	"	"	"	"	"	"
24.	LUN-1	"	Non-rhizos-phere	"	"	"	"	"
25.	LUN-2	"	"	"	"	"	"	"
26.	LUN-3	"	"	"	"	"	"	"

1	2	3	4	5	6	7	8	9
27.	LMR-1	Mavelikkara	Rhizosphere	"	"	"	"	tenacious touch
28.	LMN-1	"	Non-rhizosphere	"	"	"	"	"
29.	LMN-2	"	"	"	"	"	"	"
30.	LMN-3	"	"	"	"	"	"	"

Plate 1. Colony of Beijerinckia on nitrogen free agar having smooth surface

Plate 2. Colony of Beijerinckia on nitrogen free agar having plicated surface

The cultures also formed large quantities of gum in liquid media. The growth produced by the cultures in liquid media was of two types, one type turned the whole medium into homogenous highly viscous transparent mass and the other type formed thick pellicle like structures adhering to the tubes on the surface of liquid broth (Plate-3). The cultures from Kayamkulam, Kasaragod and Uduma were of the former type while the cultures from Quilon and Thakazhi were of latter type.

4.5.1. Physiological characters

The data on physiological characteristics are presented in Table-7. All the cultures showed growth on nitrogen free Becking's broth and were aerobic. The isolated cultures showed positive reaction towards catalase test indicating the presence of catalase in the cultures tested. The isolates were highly acid tolerant as was evidenced by growth in Becking's broth adjusted to pH 4.0. The cultures failed to grow when potassium nitrate was supplimented as nitrogen source in Becking's broth. Majority of the cultures showed poor growth on casein agar medium. Only the five cultures isolated from Kayamkulam and one culture from Mavelikkara)LMN-1) showed growth on this medium.

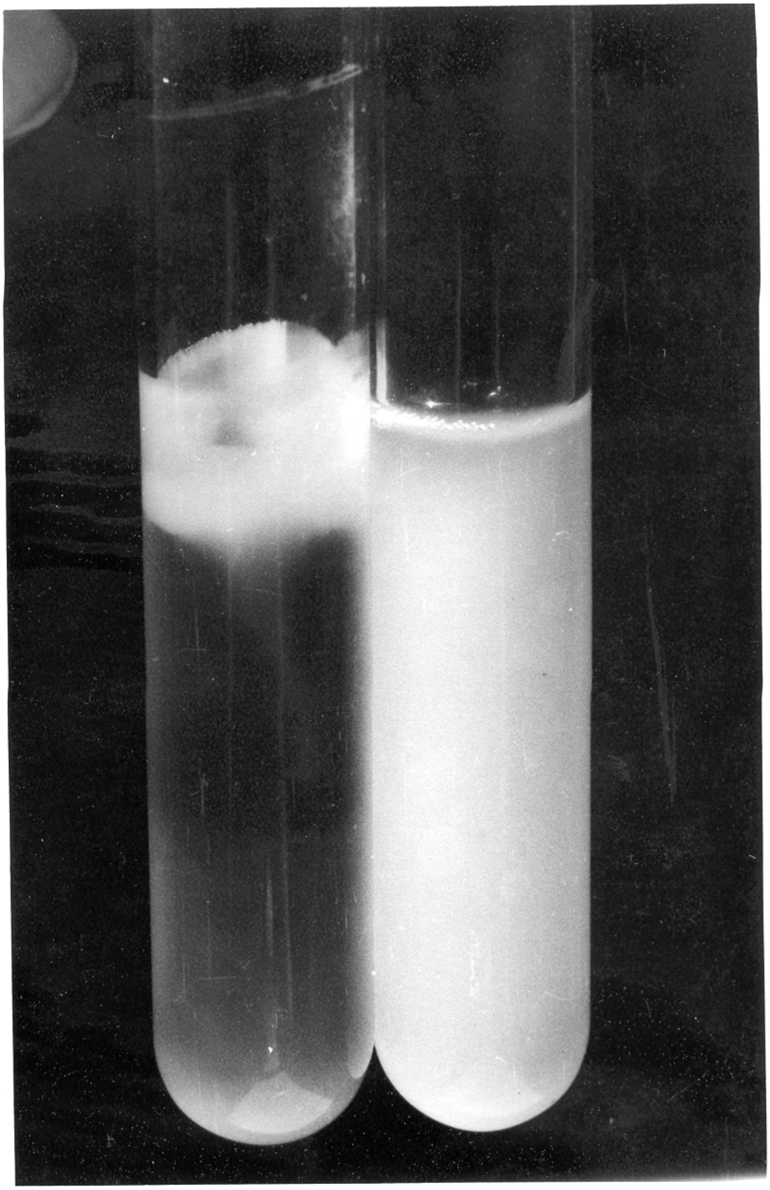


Plate 3. Growth of Beijerinckia cultures in nitrogen free liquid media a) entire medium homogenously viscous b) thick pellicular growth at surface.

Table 7. Physiological characteristics of bacterial isolates.

Culture No.	Growth in N free Beckings broth	Growth at pH 4.0	Catalase test	Growth- using nitrate as N source	Grow- th on cascin agar
SKR-1	+	+	+	–	–
SKN-1	+	+	+	–	+
SKN-2	+	+	+	–	+
SKN-3	+	+	+	–	+
SKN-4	+	+	+	–	+
STR-1	+	+	+	–	–
STR-2	+	+	+	–	–
STR-3	+	+	+	–	–
STN-1	+	+	+	–	–
STN-2	+	+	+	–	–
SQR-1	+	+	+	–	+
SQR-2	+	+	+	–	+
SQN-1	+	+	+	–	+
SQN-2	+	+	+	–	+
SQN-3	+	+	+	–	–
LCR-1	+	+	+	–	–
LCR-2	+	+	+	–	–
LCN-1	+	+	+	–	–
LCN-2	+	+	+	–	–
LCN-3	+	+	+	–	–

4.6. Nitrogenase activity of *Beijerinckia* isolates

Nitrogen fixing efficiency of 30 *Beijerinckia* isolates was tested by acetylene reduction method. The data on nitrogenase activity of the isolates are presented in Table-8. All the cultures showed acetylene reduction activity (ARA). There was large variation in the ARA of different isolates. The ARA varied from 2.71 to 78.94 nM C_2H_4 tube $^{-1}h^{-1}$ in the cultures tested. Majority of the isolates exhibited low ARA while only a small percentage showed relatively higher ARA. Fifty three per cent of the isolates showed ARA of less than 25 nM, 33% showed ARA in the range of 25-50 nM while remaining 13% showed ARA of more than 50nM C_2H_4 tube $^{-1}h^{-1}$.

Isolates obtained from rhizosphere soil exhibited better nitrogen fixing efficiency than those isolated from non-rhizosphere soil. The mean ARA of rhizosphere isolates was 35.86 nM whereas the non-rhizosphere isolates showed ARA of 19.14 n M only. There was not much variation in the ARA of isolates from non-rhizosphere soils in sandy and laterite soil types. The isolates from sandy and laterite soils showed mean ARA of 20.84 and 17.44 nM C_2H_4 tube$^{-1}h^{-1}$, respectively. However, the isolates from rhizosphere samples in sandy soil type showed relatively high ARA when compared to the rhizosphere isolates from

Table 8. Nitrogenase activity of <u>Beijerinckia</u> isolates from different locations.

Sl.No.	Culture No.	Nitrogenase activity nMC_2H_4 $tube^{-1}$ h^{-1}
1	SKR-1	33.26
2	SKN-1	29,67
3	SKN-2	37.93
4	SKN-3	3.65
5	SKN-4	10.63
6	STR-1	51.48
7	STR-2	78.94
8	STR-3	24.70
9	STN-1	13.05
10	STN-2	23.48
11	SQR-1	72.09
12	SQR-2	75.44
13	SQN-1	4.31
14	SQN-2	27.62
15	SQN-3	39.43
16	LCR-1	17.32
17	LCR-2	15.93
18	LCN-1	2.88
19	LCN-2	2.71
20	LCN-3	43.66

1	2	3
21	LUR-1	33.26
22	LUR-2	42.74
23	LUR-3	12.79
24	LUN-1	29.67
25	LUN-2	37.93
26	LUN-3	10,03
27	LMR-1	10.16
28	LMN-1	7.62
29	LMN-2	12.61
30	LMN-3	9.84

laterite soils. The mean ARA of rhizosphere isolates from sandy soils was 52.91nM while those from laterite soils showed low ARA of 18.80 nM only.

4.7. Factors affecting growth and nitrogen fixation.

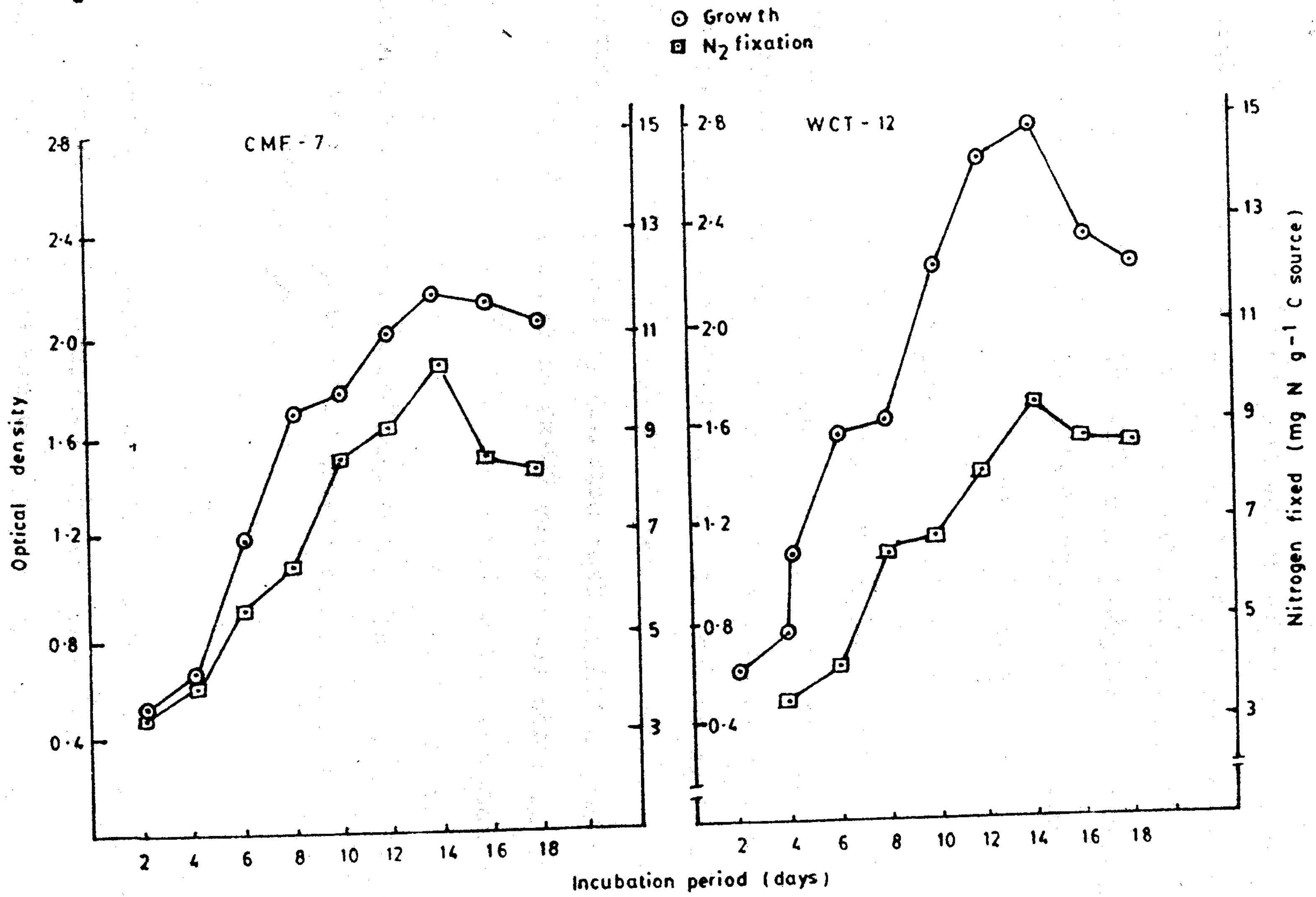
The effect of various physical and chemical factors such as incubation time, pH, temperature and carbon sources on growth and N_2 fixation by two cultures of Beijerinckia CMF-7 and WCT-12 was studied. In addition the effect of different concentrations of combined nitrogen and fungicides was also studied.

4.7.1. Effect of incubation period

To study the effect of incubation period, Becking's broth inoculated with the two cultures was incubated upto 18 days. The growth and nitrogen fixation at different intervals were determined by analysis of the samples from the set of culture tubes withdrawn at two days intervals.

The data presented in Fig.2 indicate that there was gradual increase in growth and N_2 fixation with the increase in incubation period upto 14th day. With further increase in incubation period there was a gradual reduction in growth and the amount of N_2 fixed. The pattern of growth and N_2 fixation was almost same by the two cultures upto 14th day of incubation. The reduction in growth and

Fig. 2. Effect of incubation period on growth and N_2 fixation by <u>Beijerinckia</u>



N_2 fixation was less in case of WCT-12 after 14th day of incubation. There was only 8% reduction in the amount of N_2 fixed at the 18th day of incubation in case of culture WCT-12 when compared to the values at the optimum period of incubation. In contrast to this CMF-7 culture fixed 21% less N_2 than that was fixed at the optimum period.

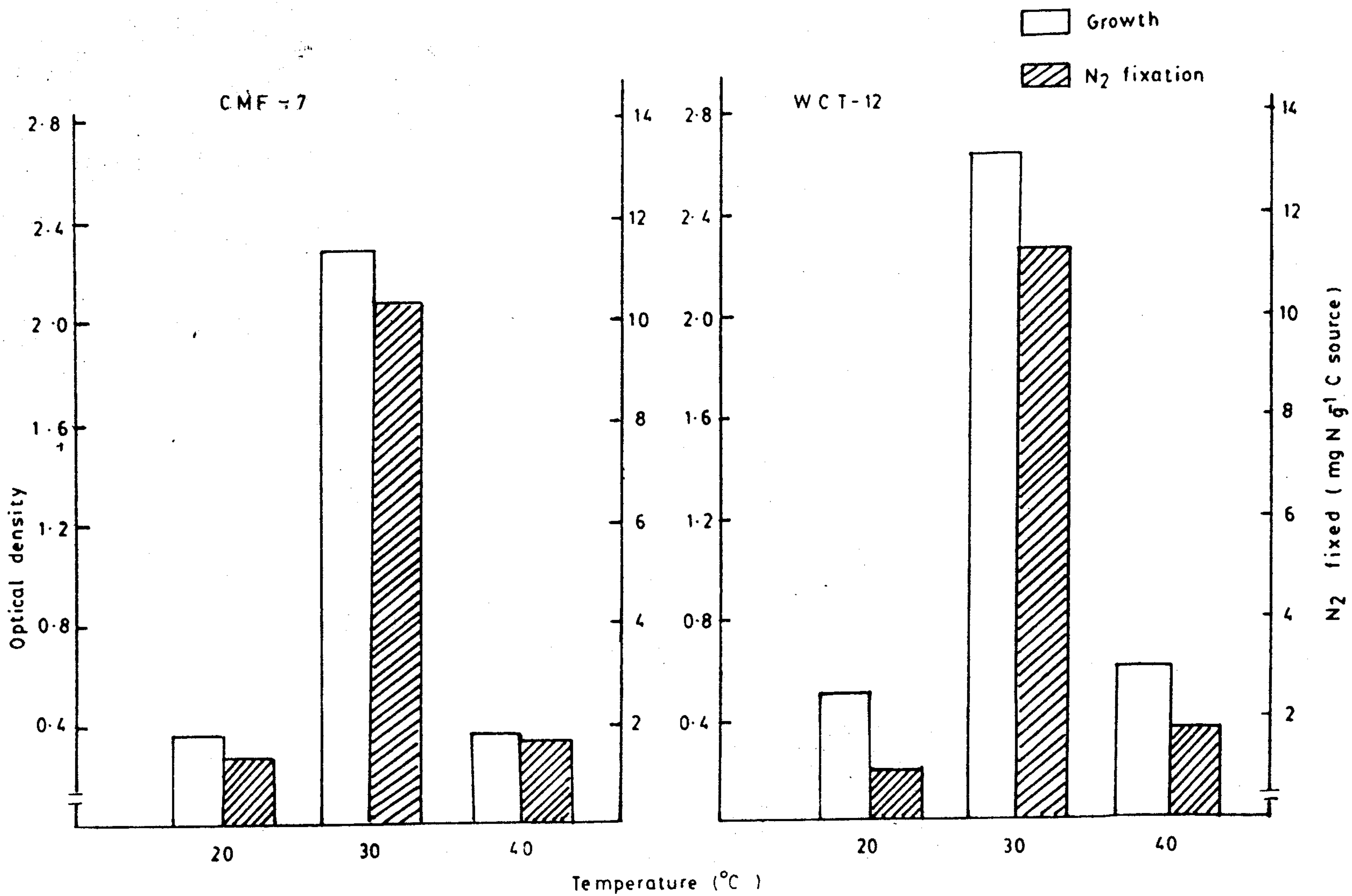
4.7.2. Effect of temperature

The effect of temperature on the growth and N_2 fixation by the two Beijerinckia cultures was studied by analysis of culture broth after incubation at 20°, 30° and 40°C. The cultures CMF-7 and WCT-12 showed maximum growth and N_2 fixation at 30°C. Fig-3. There was drastic reduction in growth and N_2 fixation both at 20° and 40°C. The quantity of N_2 fixed by CMF-7 culture was 88% less at 20°C and 94% less at 40°C when compared to the fixation at 30°C. The reduction in growth in case of CMF-7 was almost same (83%) both at 20° and 40°C. The culture WCT-12 also showed similar pattern of growth reduction and N_2 fixation at 20° and 40°C.

4.7.3. Effect of pH

Becking's broth adjusted to different pH levels ranging from 3.0 to 10.0 was inoculated with the two cultures of Beijerinckia. After the incubation period, growth was recorded in terms of optical density and the quantity of

Fig 3, Effect of temperature on growth and N_2 fixation by <u>Beijerinckia</u>



N_2 fixed in the culture broth was determined by microkjeldahl analysis.

The data presented in Fig.4 indicate that there was gradual increase in growth and N_2 fixation as the pH of the culture broth increased from 3 to 7. Further increase in pH resulted in a decrease in growth and N_2 fixation. Both the cultures exhibited maximum growth and N_2 fixation at p^H 7.0. There was more growth and N_2 fixation at the p^H range of 4 to 7 when compared to lower and higher p^H levels. In the alkaline range the two cultures showed almost the same pattern of reduction in growth and N_2 fixation.

4.7.4. Effect of carbon sources

The effect of different carbon sources was studied by replacing sucrose in Becking's broth with other sugars such as glucose, lactose, maltose, mannitol and malic acid. Both the cultures CMF-7 and WCT-12, were inoculated and analysis was done after the incubation period for growth and N_2 fixation.

All the six carbon sources were utilised by both the cultures (Fig.5). But there was variability in their ability to use the different carbon sources for supporting growth and N_2 fixation. The two cultures showed poor growth and N_2 fixation when malic acid was used as the 'C'source. Glucose, maltose, mannitol and sucrose supported better

Fig. 4. Effect of pH on growth and N_2 fixation by <u>Beijerinekia</u>

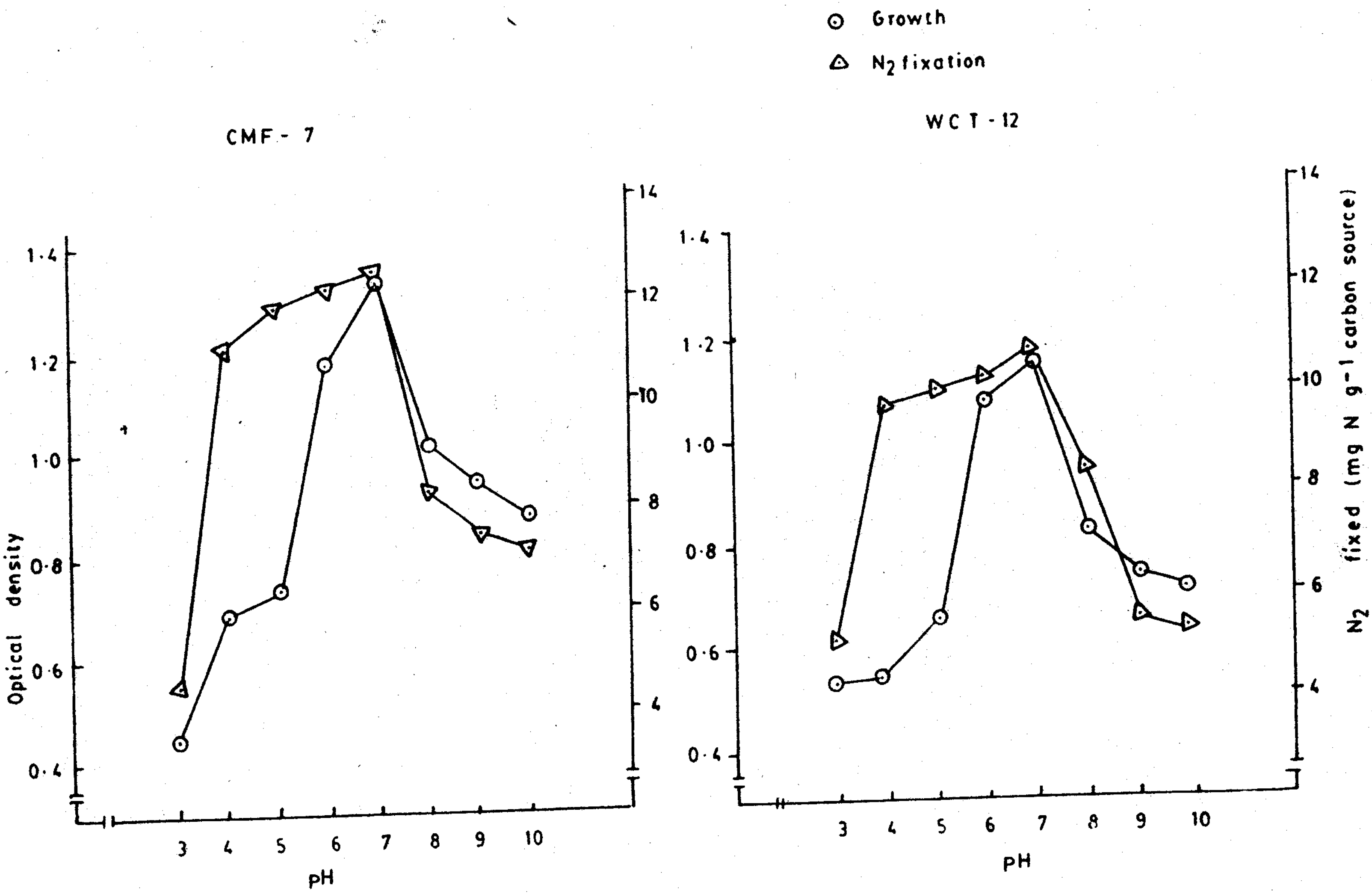
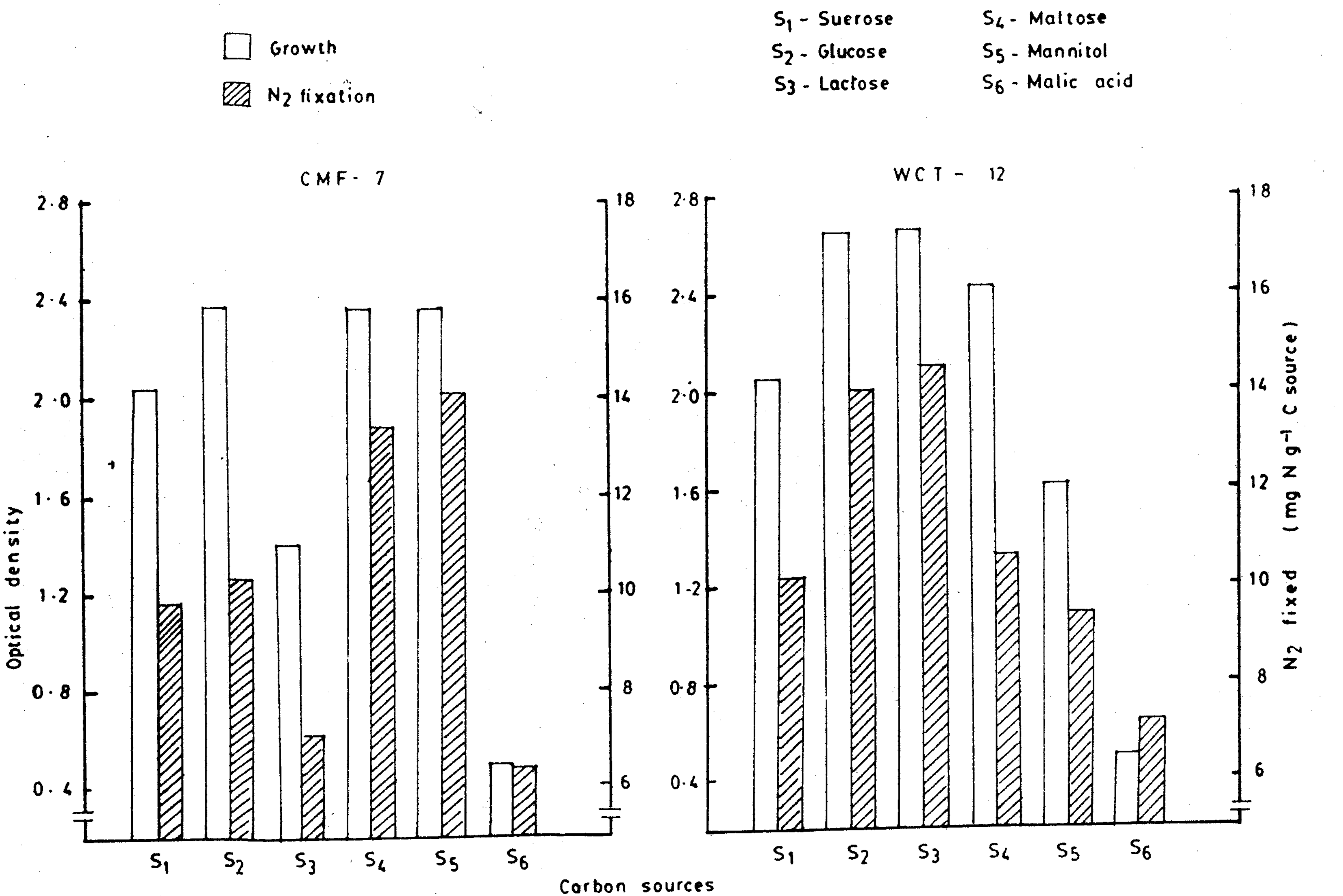


Fig. 5. Effect of different carbon sources on growth and N_2 fixation by **Beijerinckia**



growth of CMF-7. Glucose and lactose were better than others for the culture WCT-12 for supporting growth and N_2 fixation whereas lactose was a poor carbon source for CMF-7.

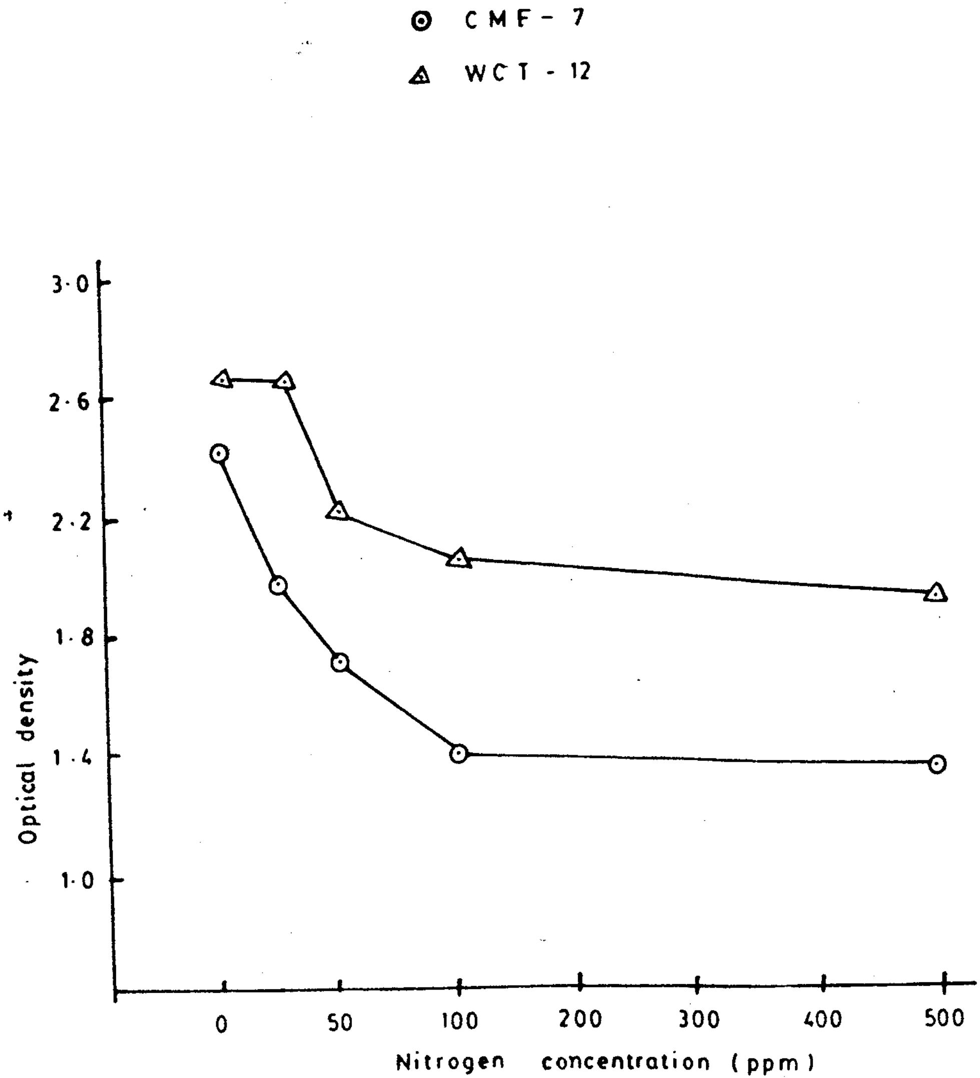
4.7.5. Effect of different concentrations of nitrogen

The effect of different concentrations of nitrogen was studies by supplimenting Becking's broth with ammonium sulphate to provide 'N'at concentrations of 0, 25, 50, 100 and 500 ppm. The optical density of the culture broth was recorded after 14 days of incubation. The data presented in Fig.6 shows that addition of nitrogen resulted in inhibition of growth to different levels at various concentrations of nitrogen. The degree of inhibition increased with the increase in cocentration of nitrogen. The extent of reduction of growth varied in the two cultures. The culturesCMF-7 was more sensitive to nitrogen than WCT-12. A concentration as low as 25 ppm was sufficient to cause inhibition of growth in culture CMF-7 whereas inhibition was observed only from 50ppm N in case of WCT-12. At 500 ppm N 'the inhibition of growth was 45% and 28% in cultures CMF-7 and WCT-12, respectively.

4.7.6. Effect of fungicides

The effect of application of three fungicides viz. bavistin, dithane M-45 and aureofungin was studied at 100, 250 and 500 ppm concentrations on growth and N_2

Fig. 6. Effect of different concentrations of nitrogen on growth of of *Beijerinckia*



fixation by two cultures of Beijerinckia CMF-7 and WCT-12. The data are presented in Figs. 7 and 8. Of the three fungicides only dithane M-45 caused inhibition of growth and N_2 fixation at 100 ppm concentration. There was almost a complete inhibition at 250, and 500 ppm concentrations of the fungicide. On the other hand, addition of bavistin resulted in an increase in growth and N_2 fixation at 100 ppm concentration. But it caused marked reduction in growth and N_2 fixation at 500 ppm concentration. The incorporation of aureofungin at 100 ppm did not cause any change in growth and N_2 fixing capacity of the two cultures when compared to that of control. But there was considerable reduction in growth at 250 and 500 ppm levels. A similar trend was observed in N_2 fixation also. It is important to note that the application of bavistin and aureofungin at 100 ppm concentrations did not cause any adverse effect on survival and N_2 fixing activity of Beijerinckia. The pattern of inhibition stimulation of growth and N_2 fixation observed due to application of the three fungicides was similar in both the cultures.

4.8. Response of black pepper to Beijerinckia inoculation

A pot culture experiment was conducted in sterile soil to test the effect of inoculation with the two cultures of Beijerinckia, CMF-7 and WCT-12 on growth and nitrogen content of black pepper. The cultures were inoculated with and without N_2 application. Controls were also maintained with and without N_2 application.

Fig. 7 Growth of *Beijerinckia* as influenced by different concentrations of fungicides

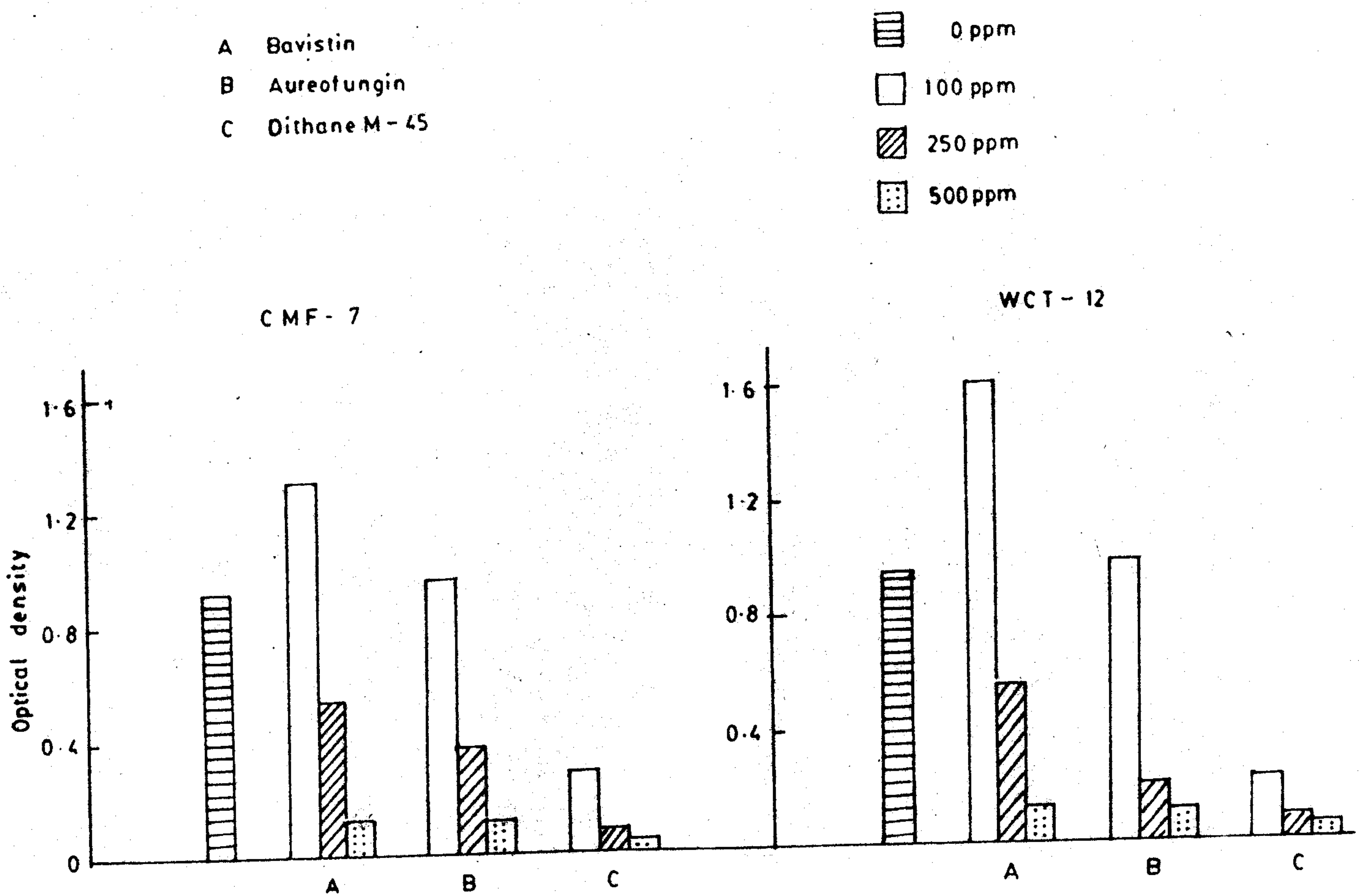
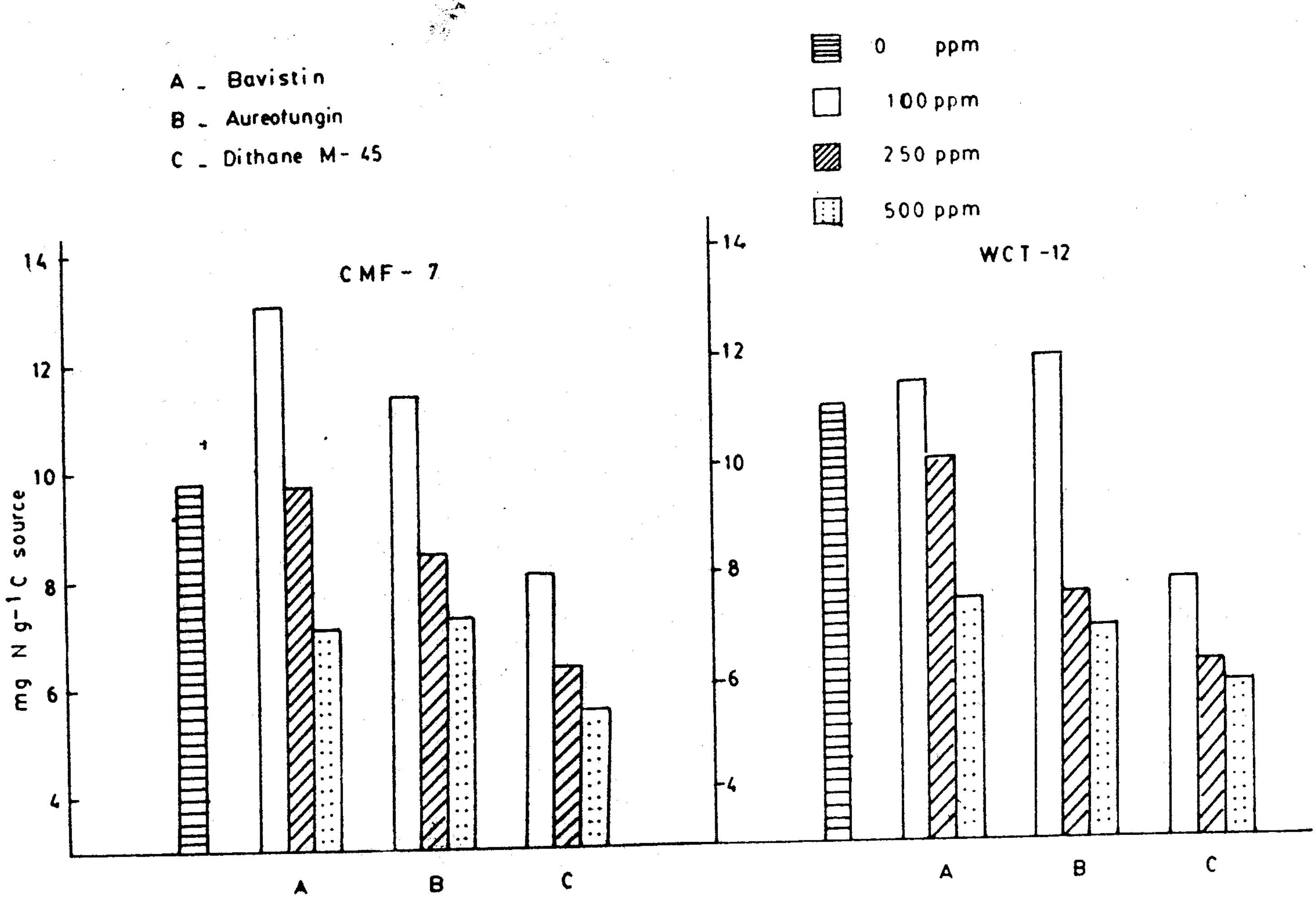


Fig. 8. Effect of different concentrations of fungicides on nitrogen fixation by _Beijerinckia_



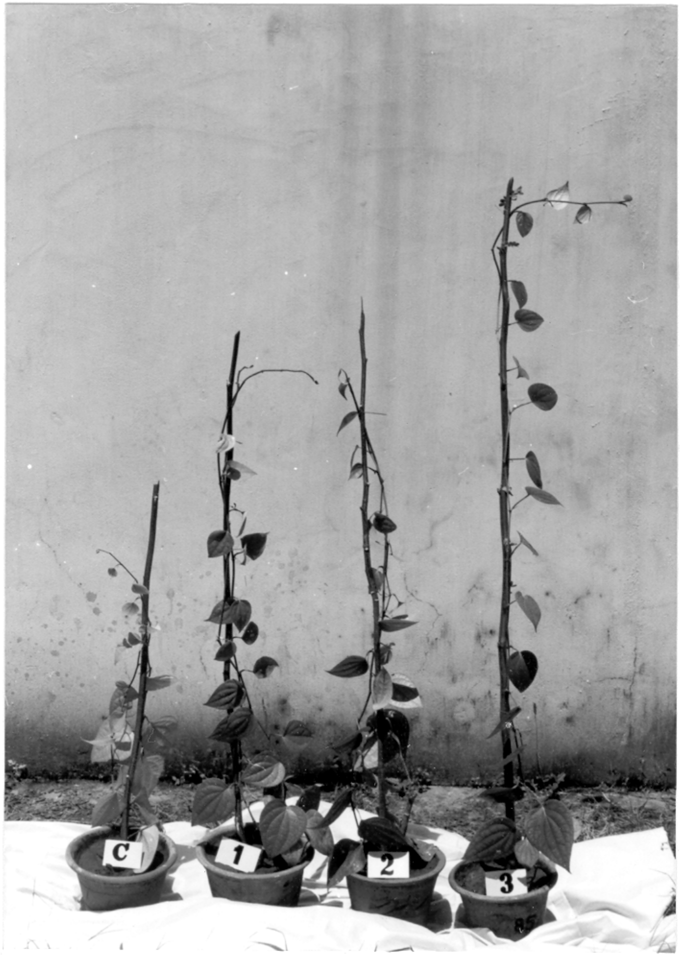


Plate-4. Growth response of black pepper to inoculation with *Beijerinckia* culture CMF-7 and nitrogen application c) Control 1) Nitrogen alone 2) *Beijerinckia* inoculation 3) *Beijerinckia* inoculation + nitrogen application

The effect of *Beijerinckia* inoculation on growth characters(Plate4)such as increase in shoot length, shoot dry weight weight and root dry weight are presented in Table-9 and Fig.9. Inoculation of black pepper with the two cultures of *Beijerinckia* without nitrogen application was effective in increasing growth. Increase in shoot weight was more in treatment of CMF-7 culture when compared to that obtained with WCT-12 culture. There was increase of 31.25, 9.45 and 14.53% in shoot length, shoot weight and root weight in WCT-12 inoculated plants respectively. Inoculation with CMF-7 resulted in 1.82, 23.92 and 13.72% increase in shoot length, shoot weight and root weight, respectively when compared to uninoculated control. Application of urea without bacterial inoculation also increased the growth characters over control. Maximum growth response was observed when *Beijerinckia* cultures were inoculated along with nitrogen application. The shoot weight increased to 11.62 g plant $^{-1}$ when *Beijerinckia* culture WCT-12 was inoculated with N_2 addition as compared to 8.78 g plant^{-1} in uninoculated control. It is interesting to note that root weight was more in all inoculation treatments (3.21 to 3.54 g^{-1} plant^{-1}) when compared to that of nitrogen application alone (3.17 g^{-1} plant^{-1}).

Fig. 9. Effect of <u>Beijerinckia</u> inoculation on plant dry matter yield and nitrogen content of black pepper

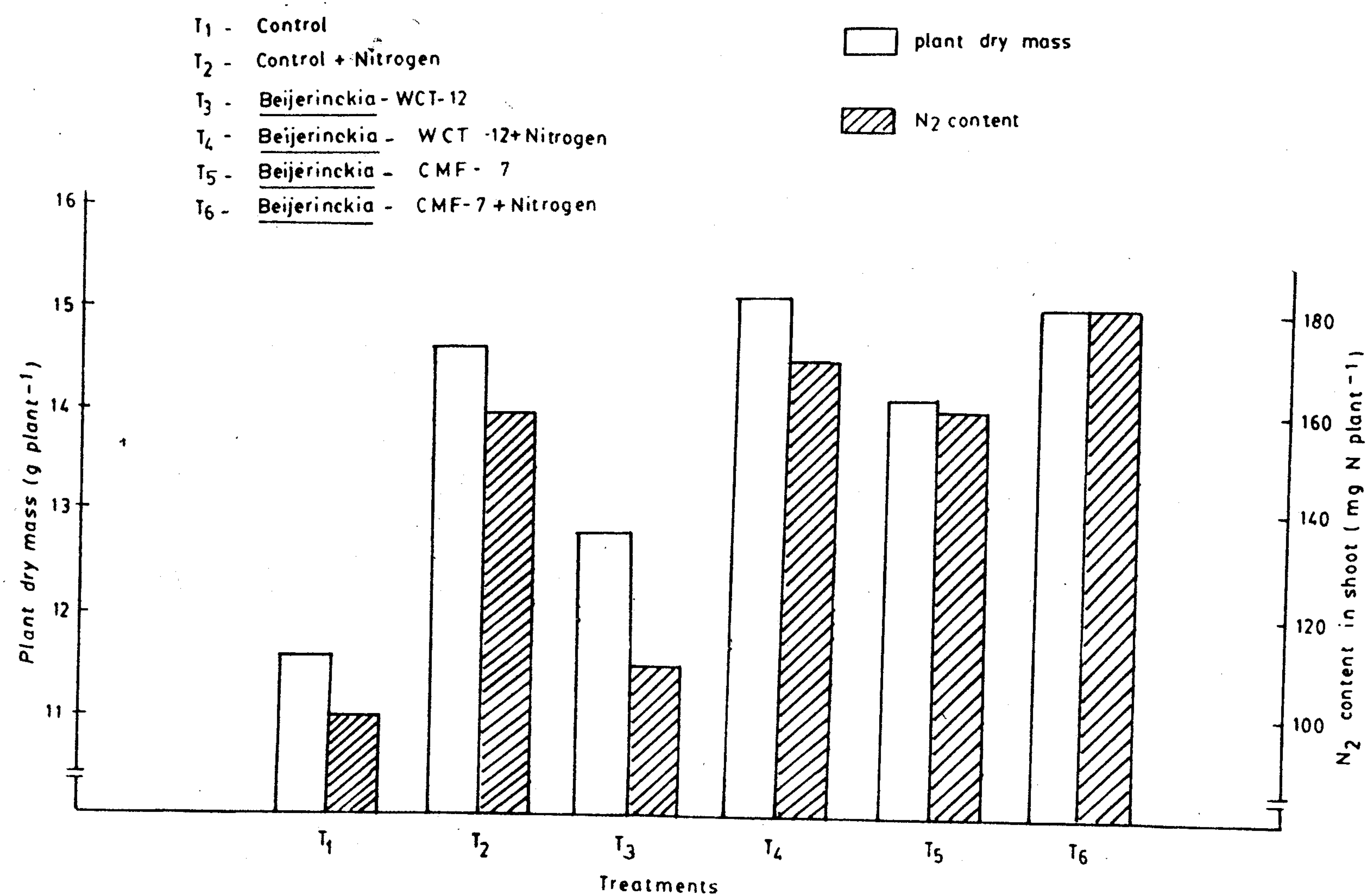


Table-9. Effect of *Beijerinckia* inoculation on growth characters of black pepper seedlings.

Treatment	Increase in shoot length (cm)	Shoot weight g $plant^{-1}$	Root weight g $plant^{-1}$
Control	95.67	8.78	2.82
Control + urea	111.9	11.46	3.17
Beijerinckia WCT-12	125.57	9.61	3.23
Beijerinckia WCT-12+ urea	132.03	11.62	3.46
Beijerinckia CMF-7	97.4	10.88	3.21
Beijerinckia CMF-7 + urea	140.92	11.40	3.54

The data on the nitrogen content of black pepper under the different treatments are presented in table-10. There was increase in nitrogen content in plants inoculated with Beijerinckia without nitrogen application when compared to uninoculated control. The increase in nitrogen content was more with the inoculation of CMF-7 culture as compared to that of WCT-12 inoculation. The nitrogen content was maximum in treatments which received Beijerinckia inoculation along with nitrogen application when compared to treatments which received Beijerinckia inoculation and nitrogen application separately.

Table 10. Effect of *Beijerinckia* inoculation on shoot nitrogen content of black pepper seedlings.

Treatment	Nitrogen content	Nitrogen in shoot mg plant^{-1}
Control	1.09	95.70
Control + urea	1.39	159.29
Beijerinckia WCT-12	1.18	113.39
Beijerinckia WCT-12 + urea	1.51	175.46
Beijerinckia CMF-7	1.43	155.58
Beijerinckia CMF-7 + urea	1.58	180.12

DISCUSSION

Coconut is generally grown on nutritionally poor acidic soils of the tropics. The role of non-symbiotic nitrogen fixing bacteria in restoring nitrogen levels of tropical soils has been stressed by several investigators (Odu and Vine 1968, Dobereiner and Campelo 1971, Yoshida and Ancajas 1973, Jenson 1981). The heterotrophic nitrogen fixing bacteria of the genus, *Beijerinckia* are reported to be present in substantial numbers in acidic tropical soils (Dobereiner and Castro, 1955, Becking 1961, Subba Rao 1983). In contrast to the nitrogen fixing bacteria of the genus *Azotobacter*, there is less information available in literature on the distribution and nitrogen fixation of *Beijerinckia*. In a study on the rhizosphere microflora of coconut and cacao in a mixed cropping experiment, Nair (1974) recorded the presence of *Beijerinckia* in the rhizosphere of both the crops. In the present investigation the extent of association of *Beijerinckia* in the rhizosphere of coconut was studied in different locations in sandy and laterite soil types in the coconut growing tract of Kerala and the nitrogen fixing potential of the isolates determined. The influence of various factors affecting growth and nitrogen fixation of *Beijerinckia* was studied and the growth promoting effect evaluated using black pepper as test plant.

5.1. Enrichment of *Beijerinckia* in coconut rhizosphere

The present investigation revealed the occurrence of *Beijerinckia* in all the six locations in the coconut growing tract of Kerala. The soils from the five coconut nurseries also harboured *Beijerinckia*. Earlier investigations revealed the distribution of *Beijerinckia* mainly in tropical soils of several countries (Derx 1950, Becking 1961, Strydom 1965, Dobereiner and Campelo 1971, Subba Rao 1983). The factors limiting its distribution particularly to tropical soils could be its preference for acidic pH, poor growth at low temperatures and absence of competition from *Azotobacters* in such soils. Strijdom (1967) demonstrated that acid tolerance of *Beijerinckia* enables it to compete successfully with great bulk of soil micro-organisms in acidic soils, while in neutral soils it is inhibited and eventually eliminated.

Investigations on the population levels of *Beijerinckia* in rhizosphere and non-rhizosphere soils of coconut gardens from different locations in the two soil types revealed selective stimulation of *Beijerinckia* in coconut rhizosphere. This was evidenced by a higher R:S ratio obtained for *Beijerinckia* in comparison with that of total bacteria. The proportion of *Beijerinckia* to total bacteria was also high in coconut rhizosphere when compared to the non-rhizosphere soil. Similar rhizosphere effect of *Beijerinckia* has been reported by several investigators (Dobereiner 1961, Ruschel and Dobereiner 1965, Nair and Subba Rao 1977, Subba Rao 1983). Dobereiner (1961) reported stimulating effect of sugar cane vegetation on Beijerinckia occurrence in soil samples taken in several Brazilian states. Nair and Subba Rao (1977) reported rhizosphere effect on Beijerinckia in coconut and cacao under a coconut cacao mixed cropping system.

The types and numbers of micro-organisms present in the rhizosphere were largely determined by energy sources available through root exudates and plant debris (Rovira 1965) and growth promoting compounds in root exudates (Brown 1982). Plant root exudation is affected by plant species, cultivar, plant.age, light, temperature, plant nutrition and soil moisture (Rovira 1965). These factors might also influence the occurrence of nitrogen fixing bacteria in rhizosphere soils. The present study has also shown that Beijerinckia constituted 0.2 to 1.29% of total bacteria in rhizosphere and 0.17 to 0.91% in nonrhizosphere soil. The proportion of Beijerinckia to total bacteria was more in rhizosphere soils. The root exudates of coconut may be providing a better micro-environment for proliferation of Beijerinckia in coconut rhizosphere. It is also possible that the low nitrogen environment available in the root zone enable nitrogen fixing bacteria to compete with great bulk of soil micro-organisms particularly in tropical soils where availability of nitrogen is scarce. It is also important to note that the plots selected in the present study were not receiving chemical nitrogeneous fertilizers.

In the present investigation it was observed that *Beijerinckia* population was comparatively high in rhizosphere and non-rhizosphere samples in locations of sandy soil type as compared to that of laterite soil type. There are two different views regarding the occurrence of *Beijerinckia* in relation to the soil type. Some workers reported that greater percentage of laterite soils contained *Beijerinckia* than the other soils (Becking 1961, Thompson 1968). Such a relation between the occurrence of *Beijerinckia* and laterisation was not corroborated in other investigations (Dobereiner 1959, Meiklejohn 1968). The results of the survey conducted by Dobereiner and Castro (1955) in Brazilian soils revealed highest counts of *Beijerinckia* per gram soil in two of the three poorest soils. Meiklejohn (1968) reported the occurrence of *Beijerinckia* in laterite, sandy, clayey and dark organic soils in Rhodesia. He observed maximum po-population of $38.3 \times 10^3 g^{-1}$ soil in a nutritionally poor soil.

Soil pH is an important factor determining the distribution of Beijerinckia in tropical soils. The pH range of 5 to 6.0 found in most of the coconut soils was highly favourable for the growth and multiplication of Beijerinckia. Becking (1961) reported greater proportion (74%) of Beijerinckia positive samples in the pH range of 5.5 -5.9 followed by 64% in the pH range of 5.0-5.4. Thompson (1968) reported maximum (64 %) Beijerinckia positive samples in the pH range 6.0-6.4 followed by 58% in the pH range of 5.5 to 5.9. The pH of rhizosphere samples analysed in the present study was higher than that of the non-rhizosphere samples in different locations. The higher pH observed in the rhizosphere soil could be due to the buffering action of the root region. Nair and Subba Rao (1977) also reported higher pH in rhizosphere soils as compared to the non-rhizosphere soils.

In general, the population of Beijerinckia and total bacteria recorded in coconut nurseries was similar to the levels of population recorded in adult coconut gardens. But there was large variation in the population in different nurseries. Particular mention is needed about the very high population of Beijerinckia observed in Aralam nursery and very low population in Parappanangadi nursery. The observation of such high variation in population suggest the need to have larger survey of all coconut nurseries to identify the nurseries already having a high level of association with these bacteria. Obviously the seedlings might be benefiting considerably from the nitrogen fixed by these bacteria in such nurseries. The nurseries having low population can be selected for inoculation trials with efficient strains of Beijerinckia.

5.2. Characterisation of isolates

Studies on morphological, cultural and physiological characteristics of the isolated cultures confirmed the identify of the cultures as Beijerinckia. All the cultures were Gram negative short rods and they possessed motility. The young cells of the bacteria possessed characteristics polar lipoid bodies. The cultures also produced large quantities of gum in solid and liquid media. The polysaccharide produced by these bacteria can play a vital role in soil aggregation. Another characteristic feature of the isolates was their ability to grow at the acidic pH of 4. The isolates were catalase positive and they failed to grow when potassium nitrate was provided as nitrogen source. Most of the cultures also failed to grow on casein agar. All these features confirmed the identity of the bacterial isolates as Beijerinckia as described in the VIII edition of the Bergey's Mannual of Determinative Bacteriology.

5.3. Nitrogenase activity of *Beijerinckia* isolates

Nitrogenase activity of the *Beijerinckia* isolates when tested by acetylene reduction method revealed that majority of the cultures showed low ARA while only a small percentage exhibited relatively high ARA. The observation that major portion of *Beijerinckia* isolates possessed low activity indicated the need for introduction of efficient bacteria to increase the contribution of biologically fixed nitrogen in coconut based cropping systems. Kluyver and Becking (1950) and Jenson (1954) reported that *Beijerinckia* fixed nitrogen to the extent of 16 to 20mg per gram energy rich material when analysed by Kjeldahl method. Nair (1974) reported fixation of 9.83 mg of nitrogen per gram of sucrose by a species of *Beijerinckia*. Acetylene-reduction assay is a sensitive method widely used now in the assay for nitrogenase activity of nitrogen fixing bacteria (Subba Rao 1983). Acetylene reduction assay has also been used by many workers to determine nitrogenase activity of *Beijerinckia* (Spiff and Odu 1973, Pena and Dobereiner 1974, Mac Rae 1977).

Comparative study of the nitrogenase activity of *Beijerinckia* isolates from rhizosphere and non-rhizosphere soils from different locations revealed better nitrogen fixing ability by rhizosphere isolates. This has shown that root exudates of coconut stimulated the proliferation of more efficient strains of Beijerinckia in different soils. It is possible that continuous availability of energy sources in root exudates might have helped in maintaining an active group of nitrogen fixing bacteria in the rhizosphere. Non-rhizosphere soil always suffer from non-availability of such energy sources. Another study on Azotobacter by Purushothaman *et al.* (1979) showed that isolates from the rhizosphere of plants having C_4 photosynthetic pathway were better than those from C_3 plants. Certain plants, therefore, possess the potential to stimulate an efficient group of nitrogen fixing bacteria in their rhizosphere through substances elaborated through root exudates.

5.4. Influence of certain factors on growth and nitrogen fixation.

The identification of the factors which limit growth and N_2 fixation under *in vitro* conditions is an important step for any attempt to find agriculturally viable practice which may increase biological nitrogen fixation.

Studies on the effect of incubation period revealed increase in growth and N_2 fixation with the increase in incubation period upto 14th day. With the further increase in incubation period there was a reduction in growth and N_2 **fixation.** The incubation period of 14 days was hence selected to be optimum for further studies. Slow growing nature of *Beijerinckia* was evident from this study. This could be one of the factors limiting actual fixation of nitrogen by these bacteria in soil.

Since pH of soil is an important factor governing the distribution of *Beijerinckia* growth and N_2 fixation was studies in culture media at both acidic and alkaline conditions. It was evident from the study that the two isolates fixed nitrogen at a wide range of pH ranging from 3 to 10. This is one of the characteristic features specific to *Beijerinckia*. Becking (1974) reported maximum N_2 fixation by strains of *Beijerinckia* isolated by him at pH 4.5 and there was no appreciable decline in N_2 fixation with increase in pH to 9 or 10. Tchan (1957) and La Rue (1977) also reported growth of *Beijerinckia* at wide range of pH levels. A comparative study of nitrogen fixation by strains of *Beijerinckia* isolated from acidic and alkaline soils also revealed the same pattern of nitrogen fixatio-n in relation to p^H of the medium(Becking 1961).

Studies on the effect of temperature revealed maximum nitrogen fixation and growth by both cultures at a temperature of 30^0C. But there was a drastic reduction in growth and nitrogen fixation at both 20^0 and 40^0C. Both the cultures showed some growth and fixed small quantities of nitrogen at these two temperatures. Becking (1961) reported an optimal growth range by *Beijerinckia* at lower temperatures of 20° to 30°C. He further reported that most of the strains tested by him failed to grow at 37°C and above. It is interesting to note that the strains tested in this study showed some growth even at 40°C. La Rue (1977) also reported that temperature growth range of Beijerinckia was more limited than that of Azotobacter.

5.5. Effect of combined nitrogen

Addition of ammonium sulphate resulted in inhibition of growth of Beijerinckia cultures. Strainal variation was also observed in their sensitivity to combined nitrogen. There was inhibition of growth of culture CMF-7 at 25 ppm nitrogen application where as inhibition was observed with the culture WCT-12 only from 50 ppm N. Combined nitrogen has been reported to inhibit nitrogen fixation and growth by different nitrogen fixing bacteria. (Dalton and Postgate 1969, Jenson 1981). Balandreau et al. (1975) reported that there was marked decrease in N_2 fixation when ammonium sulphate was added at concentrations above 40 ppm in rice seedlings. In the presence of combined nitrogen, the N_2 fixing bacteria are forced to grow under conditions where they are unable to fix molecular nitrogen. Combined nitrogen is known to interfere directly with the regulatory mechanisms controlling synthesis and activity of nitrogenase (Jenson 1981). It is important to note that only at low levels of combined nitrogen in soil the simultaneous utilization of biological nitrogen fixation and mineral nitrogen fertilizer is possible.

5.6. Effect of fungicides.

Eventhough fungicides are applied for the control of plant diseases, they are known to affect adversely the non-target beneficial micro-organisms in soil. In the present study the effect of three fungicides viz. bavistin, dithane M-45 and aureofungin on growth and N_2 fixation by two _Beijerinckia_ cultures was studied in culture media. These fungicides are used for the control of various diseases of crops in the coconut based cropping systems.

The fungicides dithane M-45 caused significant reduction in growth and N_2 fixation of two _Beijerinckia_ cultures at all concentrations tested. The aureofungin did not cause any reduction in N_2 fixation at 100 ppm level whereas at 250 and 500 ppm levels, it was inhibitory. On the other hand, application of bavistin at 100 ppm levels resulted in increased growth and N_2 fixation of the two cultures while it caused reduction in growth and N_2 fixation at higher concentrations. All the three fungicides caused significant reduction in growth and N_2 fixation at higher concentrations.

A survey of literature showed that the harmful effects of fungicides on free-living nitrogen fixing bacteria depended on the type of fungicide and its concentration. Charyulu and Rao (1978) reported stimulation of Azospirillum population when benomyl was applied at 10, 20 and 100 ppm concentrations in flooded paddy soils. Nayak and Rao (1980) employed ^{15}N tracer technique to study the effect of benomyl on heterotrophic N_2 fixation and reported significant increase in N_2 fixation due to addition of benomyl in alluvial, laterite and acid sulphate soils. Kiran Bala and Rao (1987) observed inhibition of growth and nitrogenase activity of Azospirillum brasilense due to application of bavistin at concentrations of 100, 200 and 300 ppm in culture medium. The present study revealed that only bavistin inhibited Beijerinckia at 100 ppm concentration while all the three fungicides were harmful to the Beijerinckia at 250 and 500 ppm concentrations.

5.7. Effect of inoculation on black pepper.

Growth promoting efficiency of Beijerinckia isolates was studied using black pepper as the test plant.

There are limitations to conduct inoculation trials with coconut seedlings. Since the nutrients required for early growth of the seedlings are provided by the nut itself, the growth response caused by bacterial inoculation may not be reflected in the early growth of the seedlings. Moreover, the slow rate of growth of seedlings makes it difficult to assess any growth response in a short period. Hence a spice crop grown in basins of coconut was selected as test plant to evaluate response to inoculation by Beijerinckia cultures.

Inoculation with the two cultures of Beijerinckia resulted in an increase in growth characters such as shoot height, shoot weight and root weight. The increase was observed when the cultures were inoculated with and without nitrogen application. Maximum response was seen when inoculation was done alongwith nitrogen application. Inoculation trials conducted by various workers in pots as well as in the field have revealed that Beijerinckia inoculation was effective in increasing growth and yield of a number of crop plants (Balasundaram and Sen 1971, Sulaiman 1971, Jagtap and Bhide 1977, Subramanyan and Prasad 1980). Balasundaram and Sen (1971) reported yield response equivalent to those obtained with 80kg N/ha when when rice was inoculated with Beijerinckia along with application of 40 kg N/ha as urea. But the B. indica inoculation without nitrogen application was not effective.

Sulaiman (1971) reported significant increase in yield of paddy when *Beijerinckia* was inoculated with molybdenum application. Combined inoculation of *Beijerinckia mobilis* along with vesicular arbuscular mycorrhizal fungus and a phosphate solubilising fungus on onion also revealed synergistic interaction among these organisms in increasing growth (Manjunath *et al.* 1986).

It was also interesting to note that there was better root development in *Beijerinckia* inoculated plants when compared to plants applied with nitrogen alone. This could be due to the elaboration of growth promoting substances by the bacteria. Other non-symbiotic nitrogen fixing bacteria like *Azotobacter* were reported to produce growth promoting substances like indolacetic acid in culture media (Vancura 1961, Brown 1972). The increased growth obtained in the present study could also be due to the better root development caused by the growth promoting substances elaborated by *Beijerinckia*. Increase in root biomass results in better absorption of nutrients and better growth of plants.

In the present study it was observed that nitrogen content of plants also increased in response to *Beijerinckia* inoculation. Souto and Dobereiner (1967) reported significant increase inntotal nitrogen content of elephant grass (Pennisetum purpureum) in a green house inoculation trial with Beijerinckia. Recently Malick et al. (1988) provided convincing evidence for biological nitrogen fixation by Beijerinckia in roots of Kallar grass using ^{15}N isotope dilution method.

The present study has brought out the occurrence of Beijerinckia in coconut growing soils of Kerala and a selective stimulation of these bacteria in rhizosphere of coconut. Nitrogen fixing potential of the isolates and their growth promoting effect were established. The influence of a number of factors in stimulating/adversely affecting N_2 fixation by Beijerinckia was also reported. It will be worthwhile to undertake further detailed investigations to augment nitrogen fixation by Beijerinckia in coconut soils by introduction of efficient strains and by manipulation of various inputs and agronomic practices.

SUMMARY AND CONCLUSIONS

Nitrogen is a major limiting nutrient for crop production in acidic tropical soils in which coconut is generally grown. In order to exploit biologically fixed nitrogen as source of nitrogen to coconut it is necessary to have an understanding of the bacteria involved in N_2 fixation in such soils. The heterotrophic N_2 fixing bacteria of the genus, *Beijerinckia* are reported to be involved in N_2 fixation in acidic tropical soils. An investigation was undertaken to study the association of *Beijerinckia* in the rhizosphere of coconut in different locations in coconut growing areas of Kerala and to determine the nitrogen fixing ability of the isolates. The factors affecting nitrogen fixation by *Beijerinckia* a and growth promoting effect due to inoculation of these bacteria were also investigated.

The present study revealed the occurrence of *Beijerinckia* in all the six locations in the sandy and laterite soil types. Rhizosphere soils of coconut harboured higher populations than non-rhizosphere soils. The R:S ratio for *Beijerinckia* was higher than that of total bacteria in majority of locations. The proportion of *Beijerinckia* to total bacteria was also high in coconut rhizosphere soils. when compared to that of non-rhizosphere soils. These are more population of

Beijerinckia in sandy soils when compared to laterite soils. pH of the coconut soils was found to be favourable for development of *Beijerinckia*. Rhizosphere soils had higher pH (5.74) than non-rhizosphere soils (5.23). There was significant variation in the population of *Beijerinckia* in different coconut nurseries. The proportion of *Beijerinckia* to total bacteria was very high in Aralam nursery.

Beijerinckia cultures were isolated from rhizosphere and non-rhizosphere soils from the different locations. Studies on the morphological, cultural and physiological characteristics of the isolates confirmed the identity of the cultures as *Beijerinckia*. The cultures produced large quantities of gum in solid and liquid media.

All the *Beijerinckia* isolates possessed nitrogenase activity when tested by acetylene reduction method. The acetylene reduction activity (ARA) ranged from 2.88 to 78.94 nM C_2H_4 tube $^{-1}$h^{-1} in different cultures. The isolates from rhizosphere soils showed higher ARA(35.86 nM) when compared to that (19.14 nM) of non-rhizosphere isolates. Rhizosphere isolates from sandy soils possessed better nitrogen fixing efficiency than those obtained from laterite soils.

The effect of incubation time, pH, incubation temperature and different carbon sources on growth and N_2 fixation by *Beijerinckia* cultures CMF-7 and WCT-12 was investigated under *in vitro* conditions. The incubation period of 14 days was found to be optimum for the both cultures of *Beijerinckia*. The cultures showed growth and N_2 fixation at a wide range of pH ranging from 3 to 10 and a pH range of 4 to 7 was better than the lower and higher levels. An incubation temperature of 30°C was better and there was drastic reduction in growth and N_2 fixation at 20° and 40°C. Glucose, maltose, mannitol and sucrose were the carbon sources which supported better growth and N_2 fixation by CMF-7 where as glucose and lactose were better for WCT-12.

The addition of combined nitrogen in culture broth resulted in inhibition of growth of the two cultures. The degree of inhibition increased with the increase in concentration of nitrogen added.

The effect of different concentrations of three fungicides viz. bavistin, dithane M-45 and aureofungin on growth and N_2 fixation by *Beijerinckia* was studied under *in vitro* conditions. Dithane M-45 caused reduction in growth and N_2 fixation at all concentrations tested. Bavistin and aureofungin were inhibitory only at 250 and 500 ppm concentrations. The application of bavistin at 100 ppm levels resulted in a stimulation of growth and N_2 fixation by both the cultures.

Growth promoting efficiency of two *Beijerinckia* cultures was studied using black pepper as test plant in pot culture trials with and without nitrogen application. Inoculation with the cultures resulted in an increase in growth and nitrogen content of black pepper. Maximum response was observed when inoculation was done with low level of nitrogen application. There was also better root development in *Beijerinckia* inoculated plants when compared to those applied with nitrogen alone.

The occurrence of *Beijerinckia* in different locations in the coconut growing tract of Kerala, proliferation of the diazotroph in the rhizosphere of coconut, nitrogen fixing potential of the isolates and their growth promoting effect were brought out in this study. The observation that majority of *Beijerinckia* isolates possessed low nitrogenase activity indicated the need for introduction of efficient strains to augument nitrogen fixation by these bacteria in coconut soils. Experiments need to be conducted to evaluate response to inoculation with *Beijerinckia* on coconut seedlings particularly in nurseries having low population of these bacteria. But there are limitations for inoculation in existing coconut plantations. This study also indicated the need for judicious use of fungicides in coconut gardens so that nitrogen fixation occurring in rhizosphere soil is not adversely affected. The results obtained on the effect of various factors on nitrogen fixation by Beijerinckia revealed the possibility to improve nitrogen fixation by providing suitable conditions. The present study opens up a further need to develop suitable agrotechniques for maximum exploitation of nitrogen fixing potential of Beijerinckia in coconut based cropping systems.

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