STUDIES ON THE MICROFLORA OF THE ROOT REGION OF PLANTATION CROPS - COCONUT AND CACAO

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STUDIES ON THE MICROFLORA OF THE ROOT REGION OF PLANTATION CROPS - COCONUT AND CACAO

by

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CERTIFICATE

This is to certify that the thesis entitled "Studies on the microflora of the root region of plantation crops - coconut and cocoa", submitted to the Faculty of the Post-Graduate School, Indian Agricultural Research Institute, New Delhi, in partial fulfilment of the requirements for the award of the degree of Doctor of Philosophy in Microbiology, embodies the results of a piece of bonafide research carried out by Mr. Sasi Kumar Nair under my guidance and supervision, and that no part of the thesis has been submitted for any other degree. The help or information as has been availed of during the course of this investigation has been duly acknowledged by him.

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

INTRODUCTION

INTRODUCTION

Ibn Batuta, the fourteenth century traveller wrote of Malabar, Kerala as the land which lies under the shade of trees; where there is not one span free from cultivation and where every body has his own garden with his house 'planted' in the middle of it. This seems to be a well deserved compliment to a state that has about 70 per cent of the country's total area of 10,45,600 hectares under coconut cultivation.

Coconut palm, Cocos nucifera L. is a monotypic genus belonging to the family Palmae. It is unbranched, slow growing, erect and cylindrical covered with heavy scars of old leaves. There is a compact crown of gigantic, feathery, glossy and thick cuticled leaves which are sheathed at the base and oriented at the top with branched inflorescence. Fruits are large, trigonously obovoid or subglobose, single seeded drupes. The palm grows to a height of about 24 meters and has the typical adventitious root system of a monocot and produces about 2500 to 3600 uniformly thick roots from the base of the stem throughout its life time. It grows under varying climatic and soil conditions. A rainfall of about 200 cms per year and a mean annual temperature of about 27°C are the optimal climatic factors for proper growth and maximum yield.

The importance of the coconut palm lies in the fact that

it supplies not only food and shelter but also provides the raw material for a number of important industries. However, the indigenous production of coconut has all along been inadequate necessitating the import of coconut products to varying extents. Clearly there is an urgent need for our plantation industry to catch up with production and attain self sufficiency.

Coconut palms require about ten years to attain normal bearing. In the mean while, in order to obtain some income from the farm, inter and mixed cropping practices are usually adopted in coconut gardens. The Central Plantation Crops Research Institute, Kasaragod - Kerala has been experimenting with several important cash crops such as pine apple, banana, groundnut, chillies, tapioca, cacao, pepper, cinnamon etc. for this purpose. One interesting observation in this connection has been that when cacao (Theobroma cacao L.), was used as a mixed crop with coconut, there was consistent improvement in the yield of coconut*. The increase in yield was about 95 per cent with double hedge of cacao and 65 per cent with single hedge of cacao.

What exactly could be the reason for this increase in yield of coconut ? Mixed cropping of cacao in coconut plantations may provide sufficient soil cover to alter the physico-chemical conditions of the soil. Changes in the physical, chemical and

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* Please see Appendix 1 for yield data.

microbiological status of the soil may be expected to provide ecological conditions conducive for increased yield. The objective of the present work has been to investigate changes in the nature and biological activity of microorganisms in the root region of coconut and cocoa due to mixed cropping. Since this happened to be the pioneering study to be undertaken in this field on the microbiology of the root region of plantation crops, the experiments designed had to be of a fundamental nature to start with so as to obtain a clear-cut idea regarding the various rhizosphere microorganisms of coconut and cocoa. With this objective in mind following experiments were planned.

1. Isolation of bacteria, actinomycetes and fungi from the root region of coconut and cocoa under different treatments.
2. Qualitative and quantitative studies on some of the important physiological groups of soil microorganisms such as nitrogen fixing, phosphate solubilising, auxin and gibberellin producing bacteria, actinomycetes and fungi.
3. Analysis of the soil for organic carbon, total nitrogen and available phosphorus.
4. Interactions among fungi on the surface of the root of coconut and cocoa.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

A statement that roots of higher plants and cells of microorganisms exist together in soil would probably remain unchallenged. In the body of the soil through which plant roots develop, there is a host of microorganisms having the capacity for attacking and transforming a great variety of organic and inorganic materials. The region between the interior of root tissues and the soil appreciably removed from the root surface is of particular interest as regards the activity of microorganisms. In this zone, there appear marked changes in the organism. These changes being initiated by the plant undoubtedly benefit the plant development (Hoffman, 1914-15; Lohnis and Hansen, 1921; Joshi, 1925; Smith, 1928; Graf, 1930; Bhuvaneswari and Sulochana, 1955; Andal et al., 1956; Subba-Rao and Bailey, 1961; Sundara-Rao and Venkataraman, 1963). The term 'rhizosphere' was applied by Hiltner in 1904 to this portion of soil where the microbial population is subjected to the influence of plant roots. Since then studies on rhizosphere have progressed along several important and interesting lines (Starkey, 1929a, b, c; 1931a, b; Krasil'nikov, 1934, 1940, 1954, 1958, 1963; Lochhead, 1940, 1948, 1952; Katznelson, 1946, 1965; Katznelson et al., 1948, 1956a, b, 1962; Clark, 1949, 1969; Tribunskaya, 1955; Rovira, 1956a, b, c, 1965; Briscoe, 1959; Venkata-Ram, 1960; Alexander, 1961; Subba-Rao et al., 1961, Lakshmi Kumari, 1964 and Games et al., 1969).

Microorganisms develop where the supply of organic matter is adequate and environmental conditions are favourable and in the rhizosphere also, their development about roots is controlled principally by the supply of organic matter. In fact, each plant favours the development of microorganisms which are beneficial for its normal growth. The sloughed off root caps and root hairs supply considerable food for the microorganisms as the roots grow into the soil. Some amount of exudates may appear at various regions of the root system. Using C^{14} labelled carbon compounds Rovira (1975) established that in wheat seedlings the major zones of release of carbon containing materials from the roots are the regions of rapid elongation.

The number of microorganisms in the rhizosphere depends on many factors such as the age of the plant, soil conditions, fertiliser doses, mode of cultivation, environmental conditions and foliar sprays (Rovira, 1965). In wheat, for example, Katznelson and Rouatt (1957) showed that the maximum rhizosphere effect was at the end of tillering stage when the ear formation was occuring. Similar results have also been reported by Rodica et al. (1969) in the case of corn and Ramnath and Rangaswami (1971) in the case of rice. Shetty et al. (1970) found that super phosphate with the usual dose of ammonium sulphate and potash in the ratio of 6 : 4 : 18 and 6 : 4 : 36 caused significant increases of microbial population in the soil and

in the rhizosphere of ragi. Ramanath and Rangaswami (1971) have also showed that high doses of phosphate and potash increased the number of actinomycetes and Azotobacter in the rhizosphere of rice. However, higher doses of nitrogen inhibited the Azotobacter population. Anil Kumar and Chakravarti (1970) have made interesting observations on the rhizosphere population of maize seedlings after pretreatment with gibberellic acid, maleic hydrazide and urea. They found that gibberellic acid at 1 ppm stimulated the growth of bacteria, actinomycetes and fungi while at 5 ppm the population was suppressed. Maleic hydrazide at 5 ppm and urea at 0.1 M concentrations stimulated the rhizosphere population. However, at 1 ppm maleic hydrazide stimulated only fungi and actinomycetes.

The nature of the crop and its mode of cultivation, either as an inter or mixed crop or as a relay crop as in crop rotations also influence considerably the rhizosphere population (Rodina et al., 1969). They reported an increase in number of soil microorganisms in the rhizosphere of corn as compared to wheat with a periodic development. Domsch (1969) suggested that repeated cropping with the same plant results in an increase of plant residues in the soil which may build up specific dominance within the rhizosphere population. Mishra and Srivastava (1971) while studying the rhizosphere of different crop plants found that bacterial and fungal population in the rhizosphere of Hordeum

Vulgare L., Pisum sativum L., Linum usitatissimum L., and Brassica nigra L. var. Sarson decreased with increase in soil depth. Maximum bacterial population was observed in the rhizosphere of Pisum sativum L. probably due to bacterial nodules. The microflora of the four crops differed considerably. Specific forms were associated with Hordeum vulgare L. The dicot species always exhibited common fungal flora.

Dudchenko et al. (1973) studied the species composition of microflora in rotation and continuous seeding of perennial grasses and clean cultivated crops. They found that species composition varied within the range of 35 to 40 species. Pseudomonas sp. prevailed under perennial grasses while Pseudomonas sp., Bacillus sp. and Chromobacterium were abundant in the rhizosphere of clean cultivated crops. The species composition was more diverse in fodder crop rotations than under continuous seeding.

Studies on the rhizosphere of plantation crops have recently been added to investigations in this field. Watanabe (1971) while studying the rhizosphere of wilted pineapple plants in Okinawa isolated many genera of fungi such as Trichoderma, Mortierella, Gliocladium, Fusarium, Paecilomyces, Penicillium, Chaetomium, Humicola, Diplodia, and Pythium. A high frequency of Fusarium and Mortierella was observed in the rhizosphere of wilted plants. A hundred fold increase in the number of phosphate

solubilising microorganisms has been reported in the Rubber Board news letter (1973/74) in the case of three cover crops, Centrosema sp., Pueraria sp. and Calopogonium sp.

Radha and Menon (1954) reported greater number of microorganisms in the rhizosphere of healthy coconut palms as compared to wilt infected palms. Joshi (1958) during his investigation on the bud disease of coconut in Maharashtra state observed significantly higher numbers of microorganisms in the soil from the base of healthy palms than in the soil from the base of bud affected ones. Radha and Rawther (1959) have also shown that environmental factors like rainfall and soil moisture can greatly influence the microflora in the coconut rhizosphere. Moderate rainfall was found to be favourable for the microflora whereas heavy rains adversely affected it.

Rhizosphere, thus represents a biologically active milieu in the soil with multifarious effects on higher plants. But the emphasis in rhizosphere research should change from the descriptive type of studies to that of investigating the fundamental factors operating on and around plant roots. More needs to be known about the energy balance in the rhizosphere - how much do the root exudates and sloughed off tissues contribute to the nutrition of microorganisms ? What part does the production of growth factors aminoacids, toxins and antibiotics play in the colonisation of rhizosphere ? Are there any positive

roles for nitrogen fixing, phosphate solubilising, auxin and gibberellin producing microorganisms in the establishment of this unique relationship between plant roots and microorganisms ?

Non-symbiotic nitrogen fixation of Beijerinckia sp.

In its fundamental form, plant growth is a process in which energy and chemical elements are combined together. For higher plants, solar radiation is the source of energy and the chemical elements involved are ultimately of geologic or atmospheric origin. Entry of these elements into the biosphere invariably involves some facet of microbial activity. For example, the lithosphere contains the great bulk of earth's supply of nitrogen largely inaccessible to the biosphere which obtains its nitrogen mainly from the atmosphere. There are several ways for nitrogen to enter the plant soil component of the eco-system. Rain and snow usually contain small amounts of bound nitrogen, but fixation by microorganisms is thought to be responsible for most of the nitrogen in many of the natural eco-systems. Fixation may occur in obvious symbiosis with higher plants or may result from less obvious associations involving organisms such as Azotobacter, Beijerinckia and Clostridium. Among these organisms Beijerinckia is widely distributed in tropical soils (Beeking, 1959, 1961a, b; Vancura et al., 1965; Anderson, 1966; Strydom, 1966; Doberainer

and Campelo, 1971).

Starkey and De (1939) first isolated Beijerinckia from the acid soils of the paddy fields in India and gave the name Azotobacter indicum. Derx (1950) while working in Bogor Botanic Gardens (Java) also isolated similar organisms and proposed that it be classified as a new genus Beijerinckia. Later Hilger (1965) included the genus Beijerinckia in the family Azotobacteriaceae and divided it into three groups B. indica, B. fluminensis and B. derxii.

Döbereiner (1961) observed an increased number of Beijerinckia in the proximity of sugar cane roots. Martin et al. (1965) reported that the mucilage of Beijerinckia is of a polysaccharide nature and decomposes very slowly in the soil, approximately about 19 per cent after 8 weeks and is highly toxic to several soil microorganisms.

Apart from the rhizosphere, Beijerinckia has been identified in the phyllosphere also. Ruinen (1956, 1965) has isolated Beijerinckia from the leaves of many tropical plants. Recently, she has isolated this bacteria from the leaf sheath of tropical grasses, (Ruinen, 1970). In the phyllosphere, Beijerinckia occurs as an epiphyte, utilising sugars and nutrient salts from the cuticular secretions of the leaves and in turn, supplementing the host plant with bound nitrogen.

Kluyver and Beeking (1930) and Jensen (1954) have shown

that Beijerinckia fixes atmospheric nitrogen to the extent of 16 to 20 mg per gram of energy rich material. However, it has also been shown by Jensen (1948) that Beijerinckia makes better use of ammoniacal and nitrate nitrogen and many amino acids than atmospheric nitrogen. Bareeah and Sen (1964) have correlated the production of mucilage by Beijerinckia with the intensity of nitrogen assimilation in pure culture studies.

Solubilisation of inorganic phosphate

Phosphate is widely distributed in nature, both in organic and inorganic forms, but in a bound state not readily available to plants. It is generally supplied to the plants as phosphatic fertilisers. With the demand for phosphatic fertiliser exceeding its production, it will soon become necessary for the huge deposits of low grade rock phosphate to be brought to use as phosphate fertiliser for use in crop production. In fact, it has been shown by many workers, that when rock phosphate is applied 3 to 4 times the amount of superphosphate in acid soils, the uptake of phosphate from the rock phosphate equals to that from superphosphate (Fried and Mackenzie, 1950; Ellis et al., 1955; Kanwar and Grewal, 1958; Motiramani et al., 1963).

Abbott (1923) studied the phosphate transformation by soil fungi and found that Aspergillus flavus, A. fumigatus, Penicillium funiculosum, P. luteum and Cladosporium herbarum solubilised rock phosphate. Waksman (1931) demonstrated the solubilising action

of Penicillium glaucum and P. brevicaule on rock phosphate. Several other workers have also demonstrated the phosphate solubilising property of soil microorganisms (Pikovskaya, 1948; Harley and Brierley, 1954; Goring, 1955; Golebiewska, 1956; Krasilnikov et al., 1957; Sen and Paul, 1957; Das, 1963; Sethi and Subba Rao, 1968; Ahamed and Jha, 1968 and Bardiya, 1970).

Rose (1957) observed that Aspergillus niger, A. terreus and Sclerotium rolfsii dissolved magnesium and ferric phosphates probably by the solvent action of citric and oxalic acids produced in the culture medium which they identified by ascending chromatography. The solubilisation of insoluble phosphates by bacteria and fungi isolated from the soil and the rhizosphere and the implication of organic acids produced by them in this process have been referred to by Schwartz et al. (1954), Schwartz and Martins, (1955), Sperber (1957, 1958), Hoffman (1958), Louw and Webley (1958, 1959), Merentsev (1958), Stevenson and Katznelson (1958), Swaby and Sperber (1958), Bromfield (1959), Moreau (1959), Nikitin (1959), Meyer and Konig (1960), Paul (1966), Tardieux Roche (1966), Chandrasekaran (1969) and Mehta and Bhide (1970).

Konig (1961) showed that acids liberated by fungi dissolved phosphates followed by absorption of phosphate ions by the fungal mycelium which are ultimately released by autolysis of the organisms. He observed that this process took place in

pure culture as well as in soil, irrespective of the soil type and vegetation.

Subba-Rao and Bajpai (1965) and Chhonkar and Subba-Rao (1967) isolated certain fungi associated with root nodules of several leguminous plants and found that Penicillium lilacinum and certain Aspergillus sp. solubilised tricalcium phosphate. Raghu and MacRae (1966) reported the presence of aerobic and anaerobic phosphate dissolving microorganisms from the rhizosphere of rice plants in submerged soils.

Barber (1966) suggested that microorganisms increased the phosphate uptake because plants absorbed more phosphorus in non-sterile soil than in sterile one indicating the possibility of microorganisms playing a part in phosphate dissolution. Gerretsen (1948); Menkina (1950); Johnston (1954); Sen and Paul (1957); Mickowski (1964); Bajpai (1965); Myskow (1966); have all similarly demonstrated that plants take up more phosphate in the presence of phosphate solubilising microorganisms.

Production of IAA and Gibberellin-like substance (GLS) by soil microorganisms

It is well known that the three fundamental factors controlling plant growth are nutritional, hormonal and genetic. In very simple organisms such as bacteria, there is a corresponding simple differentiation of structure. As far as one can understand, growth in these organisms is mainly a matter of replication of

existing cell structures. However, when we consider multicellular plants with distinct correlation of form and function, the need for a control of the direction of growth of these different parts is vital, if a well balanced individual is to result. Actually, the existence of such a system has been known since the early days of plant physiological study in the late 17th century. The original observations that led directly to the first isolation of plant hormone were published by Charles Darwin (1881) in "The Power of Movement in Plants". These substances produced by an organ or tissue reach all parts of the organism and in very minute amount markedly influence the function of other organs.

Naturally, the question that next arises is whether an exogenous supply of these hormones can also bring about similar effects as that of indigenous production within the plant at specific loci ? A knowledge of this is pertinent if one has to implicate any beneficial role to the IAA and GLS producing microorganisms in the rhizosphere. Classic works of the Japanese physiologists Sawada (1912), Kurosawa (1926), Yabuta (1935) and Yabuta and Sumiki (1938) on bakanae or rice wilt disease which led to the isolation of gibberellin from Fusarium moniliforme specially deserves mention here. This was probably the first instance of identification of a microorganism producing excessive amount of a plant hormone which, in turn, cause

visible pathological conditions in the affected plant.

Cultures of Azotobacter also form considerable quantities of biologically active substances. Production of heteroauxin (β-indolyl-acetic acid) has been reported by a number of workers (Bereneva et al., 1958; Bukatsch et al., 1956; Smaly and Bershova, 1957; Pochon and Barjac, 1958; Krupina, 1960; Vancura and Macura, 1960; Petrushenko, 1961; Naumova et al., 1962; Pallet, 1963; Brakel and Hilger, 1965; Kita, 1965; Novikova and Irtuganova 1966; Brown and Walker, 1970; Brown, 1972). Production of gibberellins by Azotobacter sp. has been reported by Vancura (1961); Naumova et al. (1962) and Hutman (1964). Recent investigations on nitrogen-fixing blue green algae have also borne out the fact that the growth promoting substances exuding out of the algal cells may be partially instrumental in improving plant growth (Singh, 1961; Rao et al., 1963; Shields and Durrell, 1964).

In 1935 Hitchcock and Zimmerman found that when very high concentrations of synthetic plant hormones were applied to the roots of intact plants in the soil some of it was absorbed and moved upwards. This was indicated by the response of the aerial parts. Some parts such as the roots and the leaves absorb these chemicals more readily than other parts such as the mature parts of the stem.

The rate of absorption of synthetic hormones has been

studied by the following three methods, (a) by the detection of morphological and histological responses that occur at some distances from the treated area, (b) measurement of the amount of residual growth hormone on the surface of a treated area and (c) by the radioactive tracer method (Parihar, 1964).

The path of translocation of synthetic hormones depends to some extent upon the way the chemicals are absorbed by the plant. Translocation of these substances absorbed by roots apparently occurs in the xylem (Hitchcock, 1935; Hitchcock and Zimmerman, 1935, 1938; Mitchell and Brown, 1945; Weavers and Rose, 1946; Dhillon and Lucas, 1950; Weintraub and Brown, 1950). When absorbed by the leaves, the synthetic hormones are apparently transported downwards to the stem or other parts of the plant through phloem and parenchyma and is probably correlated with the movement of organic metabolites such as carbohydrates (Mitchell and Brown, 1945; Weavers and Rose, 1946; Cerna, 1948; Weintraub and Brown, 1950). If the hormone is applied to the middle of the plant, then it causes a response above and below the treated region but predominantly towards the stem tip (Wurgler, 1947, 1949).

Some of the probable lines of study using synthetic hormones pertaining to the present investigation are, (a) wound healing of the main stem after pruning, (b) initiation of root primordia on stem cuttings, (c) control of abscission of flowers,

fruits and leaves and (d) promoting germination of dormant seeds.

In the process of pruning there is a danger of infection of the wound by pathogenic organisms. Hormones could be used for stimulation of wound healing in trees after pruning operations. The concept of wound substances and wound healing was formulated by Weimer (1892) who proposed that a substance flows out of the wounded cells to become meristematic, resulting in the formation of callus. Shear (1936) reported considerable stimulation in wound healing in terminal shoots of peach, plum, apple and pear by the application of 1 % IAA in lanoline. Jakes and Hexnerova (1939) made similar reports that 1 % IAA in lanoline paste was effective in the wound healing of fruit trees. Parihar (1948) obtained a ring of callus about 3 mm. thick in guava by the application of a lanoline paste containing 0.05 % of IBA.

The promotion of cell division plays a part in the initiation of root primordia on stem cuttings by the stimulation of cell divisions in the pericyclic layer or close to it. Beginning with Duhamel (1758) and Sachs (1880), various workers have explained the root formation on stem cuttings by the accumulation of specific root-forming substances near the basal cut surface. This substance was visulised as being formed in the leaves and from there moving downwards towards the base of the stem. Went (1929) obtained the first unambiguous evidence of the root forming action of a chemical preparation when he

stimulated the development of new roots in defoliated and debudded twigs of Acalypha by applying to the top of the stem an agar jelly containing water extract of papaya. In 1935, Thimann and Koepfli showed that IAA also stimulated root initiation. The discovery that synthetic hormones would initiate rooting in stem cuttings was at once applied by horticulturists for their large scale rooting. Thakurta and Dutt (1941) obtained excellent results in the young marcotts of mango by the application of high concentrations of (1-3%) of IAA in lanoline paste to the ringed portion of the marcotts. Cepper (1944) also obtained favourable response in the marcotts of cocoa and cinchona by using indole-butyric acid (5 mg per ml). Parihar (1948) showed that in Psidium guajava air-layering combined with hormone application could be one of the very easy methods of vegetative propagation. Extensive reviews concerning the relative effectiveness of different hormones and different methods of application have been published by Mitchell and Rice (1942); Avery and Johnson (1947); Pearse (1948); Audus (1972).

Abscission is a common widespread phenomenon in plants. Some of the familiar examples of this phenomenon are the shedding of leaves, shedding of entire flowers or floral parts and dropping of fruits. That hormones can control abscission of various plant parts was first suggested by Laibach (1932) when he demonstrated that Orchid pollinia contain a substance which

prevented leaf abscission. Gardner et al. (1939) suggested the use of synthetic hormones to control preharvest drop in apple. Garns et al. (1961) showed that gibberellic acid retarded abscission in excised cotyledonary nodes of cotton when applied in relatively low concentrations proximal to the abscission zone. Hormone sprays to prevent preharvest fruit drop have been successfully used on apricot and peach (Hesse and Davey, 1942); almond (Serr and Forde, 1952).

Several specific effects of gibberellin in promoting germination and associated processes in Corylus seeds are known. For example, exogenous supply of gibberellin induces embryonic axis growth (Bradbeer and Pinfield, 1967) and cotyledonary cell expansion (Bradbeer, 1968); promotes activity of enzymes which participate in the conversion of fat to sucrose (Pinfield, 1968) and increased labelling of nucleotides (Bradbeer and Pinfield, 1967). Pinfield (1968) suggested that the primary effect of gibberellin action on dormant Corylus seeds may occur in the cotyledons where its role in breaking seed dormancy may be in inducing increased level of enzyme activity especially those concerned with mobilization of cotyledonary oil reserves.

Interaction of fungi on the root surface in the rhizosphere

Fungi, frequently form intimate associations with the surface of plant roots actually penetrating between or into the superficial layers of outer cortex (Subba-Rao and Bailey, 1961; Subba-Rao et al., 1961). Wild and Lafond (1967) pointed out

that these superficial extra material mycelia did not really form a true mycorrhiza or root fungus organs and they proposed that these structures be called 'rhizoclenae' or root mantle. The process of chemotaxis due to root exudates is the primary means by which fungi initiate infection on the host root. The fungi in turn, benefit the host by increasing the solubility of different nutrients and thereby making them more available from difficultly soluble sources (Bowen and Theodrou, 1967). Apart from this, the rhizoclenae fungi can also act as a protective mantle against pathogenic infection of the host roots.

Production of antibiotics by soil fungi, actinomycetes and bacteria has been well recognised for the past several decades. It is generally accepted that the resulting antibiotic concentrations are of sufficient quantity to influence significantly the pattern of saprophytic colonization at these sites by other microorganisms (Garrett, 1960). But, the significance of antibiotic production by saprophytes in reducing the inoculum potential of root pathogens and of subsequent root disease development has to be further investigated. Most attempts at controlling the activities of root pathogens in soil by inoculation with saprophytic organisms which produce antibiotics in the laboratory have failed because the antibiotic production is limited to the immediate substrate or 'ecological niche' of the saprophyte. Antibiosis at such small loci is not of major

significance in reducing the pathogen's inoculum potential outside this site (Garrett, 1960). However, there are definite instances of circumstantial evidence to support the various mechanisms involved in the root protection concept by rhizoclemae and mycorrhizal fungi. Zak (1964) postulated that fungi may furnish protection to the host plant from pathogens by, (a) utilising root carbohydrates and other nutrients which would otherwise be attractive to the root pathogen, (b) providing a physical barrier for the entry of the pathogen as in the case of ectomycorrhiza, (c) secreting antibiotics which will kill or inhibit the pathogen, (d) supporting a protective rhizosphere population of other microorganisms and (f) stimulating the cells of the root during symbiosis to elaborate certain chemicals which will specifically inhibit the pathogens.

One of the most prominant association between plant roots and soil microorganisms is the mycorrhiza. Marks et al. (1968) estimated that the top 6" of soil under Pinus radiata stand contained about half a ton of mycorrhizae, about half of which contained viable associations, the fungal partners of which belonged to basidiomycetous fungi. The full range and extent of fungi which are capable of forming mycorrhizae is still not completely known because the only way in which proof of an association can be established is in an artificial culture, a technique open to many criticisms. Many fungi such as Amanita,

Rhizopogon; Russula, Cantharellus, Chiteophilus, Chitocybe, Cortinarius, Entoloma, Lactarius, Laccata, Paxillus, Russulus and Tricholoma have been shown to form mycorrhizal associations with many host trees (Melin, 1965).

Generally, mycorrhizae form with smaller and slower growing root surfaces of trees (Wilcox, 1968). The rapidly growing large roots, especially those that eventually show secondary growth do not form mycorrhizae. After a root is infected, there is a dramatic fall in its growth rate, possibly as a result of rapid loss of cell wall forming residues to the fungus. In gymnosperms, the tip of the infected roots branch many times and the smaller root is converted into a cluster of short dichotomous branches.

Usually, these fungus root associations are restricted to moderately acidic, well aerated layers of the soil. Thus, in dense compacted soils, mycorrhizae may be found only in the very top most zone, whereas in loose forest soils, they occur at a considerably greater depth. However, the mycorrhizal flora of soils rich in fungal symbionts can be destroyed by prolonged flooding (Wilde, 1954).

Most of the available evidence shows that trees and mycorrhizal fungi exist in symbiotic association in which the tree supplies carbohydrates and other metabolites which are beneficial to the fungus (Lewis and Harley, 1965). In turn, the fungus benefits the tree chiefly by increasing solubility of

phosphate and other nutrients and thereby making them more available from difficulty soluble sources (Baum and Theodorou, 1967). In fact, many field observations on the occurrence of mycorrhizae lead to the conclusion that this form is most frequently seen in infertile soils which implies that these structures have an important function in nutrient mobilization (Melin, 1946).

Although trees can be grown successfully without mycorrhizae under conditions of very high soil fertility, they usually grow much better with mycorrhizae. For example, in Puerto Rico where pines were not native, attempts to introduce pine trees met with recurrent failure until the seedlings were inoculated with leaf mold imported from a pine forest in the United States (Briscoe, 1959). In a forest-tree nursery in Iowa, pine seedlings failed to grow unless and until they developed mycorrhizae. Seedling growth and mycorrhizae development were satisfactory only after the nursery soil was inoculated with duff and humus from a pine plantation (McComb, 1943; McComb and Griffith, 1946).

MATERIALS AND METHODS

MATERIALS AND METHODS

I. General layout of the experimental plot

The different rhizosphere samples of coconut and cacao studied in the present investigation were collected from the Central Plantation Crops Research Institute - Kasaragod, Kerala. Two sets of samples were collected, one in February, 1974 and another in July, 1974. The samples were taken from an experimental plot of one hectare area where cacao was planted as a mixed crop with coconut in 1970. The general layout of the experiment was of randomised block design with the following three treatments :

1. Cultivation of coconut without any mixed cropping of cacao.
2. Cultivation of coconut with single hedge of cacao.
3. Cultivation of coconut with double hedge of cacao.

The average size of each plot was 465 square meters. The coconut palms were planted at a distance of 7.5 meters and cacao at a distance of 3.5 meters from the coconut palm. The distance between the individual cacao plants in a row was 3.5 meters and the distance between the two rows of cacao plants as in double hedge cultivation was 2 meters (Plate 1A and B).

II. Collection of rhizosphere soil samples

A minimum of five rhizosphere samples of coconut and cacao

Plate 1A : Coconut plantation with single hedge mixed cropping of cocoa.

Plate 1B : Coconut plantation with double hedge mixed cropping of cocoa.

PLATE-1A

PLATE-1B

were collected at random from replication II and III of each of the three above mentioned treatments. Blocks of soil containing roots were cut out with as little tearing of the rootlets as possible. The samples were collected from a depth of 3 inches to 5 inches and were transferred into clean plastic bags which were then suitably tagged and sealed to avoid any external contaminations. The soil samples were stored in a cool aerated place and quickly transported for analysis. Both in February and in July samples were collected from the same source to study seasonal variation in the microbial population. Non-rhizosphere samples were collected from an adjacent fallow land.

III. Study of microorganisms in the rhizosphere of sesamum and maize.

The soil dilution and plate count method (Timonin, 1940) was used for counting and isolating pure cultures of different rhizosphere microorganisms. An estimate of the total population of bacteria, actinomycetes and fungi in numbers per gram of dry rhizosphere soil was made. This estimate refers to the number of viable cells or mycelial fragments in the sample capable of growing on specific agar media* used in the test. The different media used for the isolation of bacteria, actinomycetes and fungi were Thornton's Standardised Medium (Thornton, 1922); Gomm's

* Please see appendix III for the composition of different media used.

Glycerol Asparaginate Agar(Conn, 1921) and Martin's Rose Bengal Agar (Martin, 1950), respectively. Jensen's modified nitrogen-free medium was used for the isolation of nitrogen-fixing microorganisms. For cellulose decomposing organisms Omeliansky's Cellulose Mineral Salts Medium (Fred and Waksman, 1938) and for phosphate solubilising organisms, Pikovskaya's modified medium (Rao and Sinha, 1963) were used.

The roots were carefully removed from the blocks of soil with as little tearing of the roots as possible. They were gently shaken to remove soil clumps and cut into small pieces of one inch each using a sterile scalpel. A minimum of twenty such root pieces along with the adhering soil particles were then placed in a weighed flask containing 100 ml of sterile water. After thorough shaking suitable dilutions were prepared aseptically as follows. Ten ml of the suspension was drawn into a sterile ten ml pipette and transferred into a 90 ml sterile water blank and shaken thoroughly for two minutes. Ten ml aliquots were then transferred through successive 90 ml sterile water blanks to get dilutions of the order 10^{-6}. One ml of the desired dilution was then transferred aseptically into each of the several sterile petridishes and 15 ml of sterile agar media cooled to just above the solidifying temperature were added to each dish. The dishes were rotated by hand in a broad swirling motion so that the diluted soil was dispersed uniformly in the agar medium. The different dilutions plated were 10^{-3} for nitrogen-fixing and

cellulose decomposing organisms, 10^{-4} for fungi and 10^{-6} for bacteria, actinomycetes and phosphate solubilising organisms.

The plates were incubated at 28 ± 2°C for 48 hours for bacteria, 4 days for fungi and phosphate solubilisers, 2 weeks for nitrogen-fixing bacteria and actinomycetes and 3 weeks for cellulose decomposing organisms. At the end of the incubation period, the resulting colonies were counted. For counting purposes, dishes containing fungal or bacterial spreaders or large clear zones of antagonisms were discarded. The average number of colonies per dish (platings were done in triplicate) was multiplied by the dilution factor to obtain the number of organisms per gram of the original rhizosphere soil.

Single well isolated colonies of bacteria, actinomycetes and fungi were transferred to tubes containing specific media for further studies. Bacterial cultures were maintained on nutrient agar slants while the actinomycetes and fungi on Czapek-Dox agar slants (Thom and Raper, 1945). Nitrogen-fixing bacteria were maintained on Jensen's modified media for nitrogen-fixers.

In order to determine the weight of rhizosphere soil, the roots were removed from the original dilution flask and washed and the wash water was collected in the same flask itself. The water was then evaporated and the soil residue dried to a constant weight in an oven at 105°C. The flask containing the dried soil was then weighed to determine the weight of the soil. When plate

counts were made, the number of microorganisms per gram of rhizosphere soil was calculated.

IV(a) Identification of nitrogen-fixing bacterium, Beijerinckia SP.

This bacterium was identified by its known cultural, morphological and physiological properties. The various characters studied were size, shape, motility, pigmentation, production of mucilage and nitrogen fixing capacity.

Electron microscopic observation of the bacterium was done using Philips EM-300 series electron microscope. The bacterial cells were initially harvested by centrifuging liquid cultures at 17000 rpm for ten minutes. The pellet was resuspended in ten ml double distilled water and recentrifuged. This procedure of washing was repeated three times to avoid any salt contamination during electronmicroscopic observation.

Formvar film was prepared by placing a glass slide in 0.1 per cent solution of formvar in dioxane. After removal of the glass slide, time was allowed for the superfluous solution to flow off and the film to dry. The film was then separated from the glass slide on to a clean, dust free water surface. The specimen supporting grids were uniformally arranged on the film, which was then retransferred to the glass slide and allowed to dry and cut into square pieces of 3x3 mm around each supporting

grid. A loopful of the bacterial culture after sufficient dilution was placed on each grid, allowed to dry and shadow cast before observing under the electron microscope.

The final magnification of the bacterium on the photograph was computed from the following formula (symbols arbitrary)

$$X = \frac{A \times B \times C}{D}$$

where X = Final magnification on the photograph

A = Projected magnification on the fluorescent screen

B = Ratio of the distance between the screen and the camera

C = Width of the photograph in cms

D = Width of the negative in cms

IV(b) Quantitative estimation of nitrogen-fixing capacity of Beijerinckia sp.

100 ml of sterilised Jensen's nitrogen-free medium for Beijerinckia (in triplicate) was inoculated aseptically with a loopful of freshly grown bacterial culture. The flasks were incubated on a rotary shaker at 28 ± 2°C for 14 days. At the end of the incubation period, the content of each flask was carefully transferred to a Kjeldahl's digestion flask to which 20 ml of Analar concentrated H_2SO_4 and one autodigestion tablet (Kjeltabs auto-L.R. Grade Thompson and Capper Ltd, Manufacturing Chemists, Liverpool) were added and digested for three hours until a colourless liquid residue was left behind in the digestion

flask. After the content had sufficiently cooled down it was transferred to a 50 ml volumetric flask and the volume made up to 50 ml using double distilled water. The amount of nitrogen fixed by the bacteria in the medium was determined by using an autoanalyser (Technicon auto analyser) and by comparing the peak height with the standard prepared with known amount of ammonium chloride.

V(a) Screening of different cultures for their ability to solubilise tricalcium phosphate

The screening of different cultures of bacteria, actinomycetes and fungi was done by inoculating a loopful of the different culture in the centre of sterile, solidified Pikovskaya medium in petri dishes. The cultures were then incubated at 28 ± 2°C for one week. The presence of a clearing zone around the culture due to solubilisation of tricalcium phosphate was taken as a positive indication of the ability of the organism to solubilise inorganic phosphate.

V(b) Quantitative estimation of the solubilisation of tricalcium phosphate

50 ml of sterilised Pikovskaya medium was inoculated aseptically with different cultures of bacteria, actinomycetes and fungi screened initially for their ability to solubilise inorganic phosphate. 25 mg of tricalcium phosphate was incorporated in each flask of different bacteria while 50 mg was

incorporated in each flask of actinomycetes and fungi. The flasks were incubated at 28 ± 2°C from one (bacteria) to three weeks (actinomycete and fungi). At the end of the incubation period, the different cultures were initially passed through seitz filters to make them free of cells, spores and mycelial fragments. They were then decolourised with a pinch of activat charcoal and refiltered through Whatman no. 41 filter paper discs. The culture medium in the control flasks were also similarly treated. 0.2 ml of each of these filtrates was used for determining the amount of available phosphorus by Olsen's method. The intensity of blue colour developed was measured at 610 mu using Hilger's Absorptiometer. The value of available phosphorus obtained after comparison with a standard curve, previously prepared with known quantities of Analar KH_2PO_4 was multiplied by a factor of 2.27 to convert it into P_2O_5 values.

In addition to the above observation, the pH of the medium before and after incubation and the weight of the actinomycete's and fungal mat at the end of the incubation perio were also recorded.

V(e) Identification of different cultures

i) Bacteria (Pseudomonas sp.) : Various cultural, morphological and biochemical properties of the bacterium were studied based on the commonly used routine microbiological tests. Wherever possible references have been made to the various texts

performed. The cultural characters studied were, (a) form, (b) elevation, (c) margin, (d) growth on agar slants, (f) chromogenesis and (g) growth on nutrient broth. Different staining methods were used to study the morphological characters.

Staining methods	References
1. Gram staining	*Text, Chap. 4; *MMM Chap. 2
2. Acid-fast staining	Text, Chap. 4; MMM Chap. 2
3. Spore staining	Text, Chap. 5; MMM Chap. 2
4. Capsule staining	Text, Chap. 5; MMM Chap. 2
5. Flagella staining	Text, Chap. 5; MMM Chap. 2

The various biochemical activities of the bacterium studied were as follows :

Tests conducted	References
1. Fermentation of sugars/ sugar alcohols such as glucose, galactose, rhamnose, arabinose, sucrose lactose, raffinose, mannitol and glycerol	Text, Chap. 10, MMM Chap. 7
2. Hydrolysis of starch	Text, Chap. 10; MMM Chap. 7
3. Hydrolysis of fat	Text, Chap. 10; MMM Chap. 7
4. Liquifaction of gelatin	Text, Chap. 10; MMM Chap. 7
5. Hydrogen sulfide production	Text, Chap. 10; MMM Chap. 7

* Text refers to 'Microbiology' by Michael J. Pelczar, Jr. and Roger D. Reid (McGraw-Hill Book Company, Inc., New York, 1958) and 'MMM' refers to the 'Manual of Microbiological Methods by the Committee on Bacteriological Technique, Society of American Bacteriologists (McGraw-Hill Book Company Inc., New York, 1957).

6. Indole production	Text, Chap. 10; MMM Chap. 7
7. Reaction of litmus milk	Text, Chap. 10; MMM Chap. 7
8. Reduction of nitrates	Text, Chap. 36; MMM Chap. 7
9. Catalase test	Whittenbury (1964)
10. Hydrolysis of casein	Smith et al. (1952) and Chalmers,(1962).

11. Production of ammonia.

Electron microscopic observation was done using Philips EM-300 series electron microscope, the procedure for which has been described earlier.

Bergey's Manual of Determinative Bacteriology VII Edition (1957) was used to compare and compile the characters of the bacteriumstudied.

ii) Fungi : A small fragment of the fungal mycelium was placed on a drop of lactophenol cotton blue on a clean slide and gently pressed with a cover slip and observed under light microscope to study the nature of spores and hyphae. This observation was mainly relied on to classify the different fungi isolated into various genera. "A manual of SoilFungi" by Gilman (1959) was used as a reference book.

VI(a) <u>Screening of different cultures for the production of Indole-acetic acid (IAA)</u>

The ability to produce IAA was tested by inoculating

aseptically different cultures of bacteria, actinomycetes and fungi into 25 ml of sterile tryptone broth. The bacterial cultures were incubated for 48 hours at 28 ± 2°C and the actinomycetes and fungal cultures for 10 days. The production of IAA was tested by adding 2 ml of Kovac's reagent (see appendix III for composition) to 10 ml of bacterial culture and to 10 ml of the culture filtrate of actinomycetes and fungi. The tubes were gently shaken and allowed to stand to permit the reagent to rise to the top. The production of IAA was indicated by a deep red color which developed in the reagent layer (Report, 1958).

VI(b) Quantitative estimation of the production of IAA

100 ml of sterilized tryptone broth (in triplicate) was inoculated aseptically with a loopful of 24 hour old bacterial culture and incubated for 48 hours at 28 ± 2°C. At the end of the incubation period, the content of each flask was divided into four fractions of 25 ml each and centrifuged at 17,000 rpm for ten minutes in order to separate the bacterial cells. The supernatant was used for the quantitative estimation of IAA after extraction with peroxide free ether by Salkowski reaction (Sharma, 1971). The intensity of pink colour developed after incubation at 27°C for 30 minutes in dark was measured in Hilgers absorptiometer using filter no. 52. The amount of IAA present in 25 ml of culture medium was then calculated by compairing the O.D.

values obtained with the standard curve, previously prepared with known quantities of IAA (Indolyl-3-acetic acid, Merck-Germany).

The pellets were, on the other hand, transferred to previously weighed clean, small glass vials and dried to a constant weight at 60°C for 48 hours. The weight of bacterial pellet in each case was then determined using a precision balance (Metler model H-16). The average of twelve such weighings was taken as the weight of bacterial cells present in 25 ml of culture medium. This figure was used for calculating the amount of IAA produced by one microgram dry weight of bacterial cells.

VI(e) Identification of Escherichia sp.

Following tests were done for identifying the Escherichia sp. isolated from the rhizosphere of coconut. (1) Standard count and presumptive test for coliforms (2) Confirmed test and (3) Completed test. These tests were done as per the instructions given in "Standard Methods for the Examination of Water, Sewage and Industrial Wastes", 10th Edn. American Public Health Assoc. Inc., New York, 1955.

VII(a) Screening of different cultures for the production of gibberellin like substances (GLS)

The ability to produce GLS was tested by inoculating

aseptically different bacterial cultures into 25 ml of sterile Thornton's broth and actinomycetes and fungi into 25 ml of sterile Czapek-Dox broth. The bacterial cultures were incubated for 48 hours at 28 ± 2°C and the actinomycetes and fungal cultures for 10 days. A spot test using 0.5 per cent aqueous solution of potassium permanganate and fluorescence in 70 per cent H_2SO_4 were used to detect the production of GLS (Bird and Pugh, 1958). In the former test, a drop of the culture filtrate spotted and dried on a sleam filter paper was sprayed with 0.5 per cent aqueous solution of potassium permanganate. The presence of GLS was revealed as yellow spots on a reddish purple background. The spots were quickly sprayed with more permanganate solution and the filter paper was immediately washed in running tap water to remove the unreacted permanganate. The presence of GLS was detected as permanent brown spots on an almost white background. In the second test of fluorescence in 70 per cent H_2SO_4, a drop of the culture filtrate spotted and dried on a sleam filter paper was dipped in 70 per cent H_2SO_4 and quickly laid flat on a glass plate. The presence of GLS was detected as yellow green fluorescent spots.

VII(b) Chromatographic and bioassay methods used for detecting the presence of GLS in the culture filtrate of Aspergillus flavus and A. fumigatus

100 ml of sterilised Czapek-Dox broth (in triplicate) was

inoculated aseptically with a loopful of freshly grown cultures of Aspergillus flavus and A. fumigatus and incubated for 14 days at 28 ± 2°C. At the end of incubation period, GLS were extracted from the culture filtrate with ethyl acetate after adjusting the pH to 3.0 (Andus, 1972). The eluted fractions were dried at 40°C in dark and the residue redissolved in 5 ml of double distilled water. This was used for both chromatography and bioassay.

In ascending chromatography (Bird and Pugh, 1958) one µ ml. of GLS extracted was spotted on Whatman no. 1 chromatographic paper (22 x 40 cms) along with standard gibberellic acid (Gibberellic acid, serial no. 691104, Phylaxia, Budapest-Hungary) and run at 24°C using benzene-acetic acid-water (10 : 2.5 : 5) solvent systems for 8 hours. The presence of GLS and gibberellic acid were detected as yellow spots on a reddish purple background on spraying with 0.5 per cent aqueous solution of potassium permanganate. The spots were quickly sprayed with more permanganate and the chromatogram was immediately washed in running water to remove the unreacted potassium permanganate. The location of gibberellin-like substances and gibberellic acid were then revealed as permanent brown spots on an almost white background. After the chromatogram was dried, the centre of each spot was marked with a pencil and the Rf values were calculated.

In the bioassay method (Wareing and Phillips, 1970), 48

hour old germinated rice seedlings maintained aseptically either in Erlenmeyer flasks or pots were used to study the elongation of the seedlings after the application of standard gibberellic acid (0.2 µg), gibberellin-like substances extracted from the culture filtrate (2.0 ml) and culture filtrate itself (10 ml). The elongation of the seedlings in flasks was measured after 48 hours, while in pot culture experiments only visual comparison was made to study the elongation of the seedlings.

VIII. Interaction among fungi on the root surface of coconut and cacao

The association of soil fungi with coconut and cacao roots was established by Harley's root washing technique (Harley and Waid, 1955). In this technique, the roots of coconut and cacao were initially removed from the blocks of soil and gently shaken to remove the superfluous soil and cut into small identical pieces of 1.5 mm each using a sterile scalpel. The root pieces were then transferred to sterile screw capped bottles and subjected to a series of twenty five washings in sterile distilled water. Each time, the root washings were collected separately in sterile screw capped bottles. One ml of these washings, 1st, 3rd, 5th, 10th, 15th, 20th and 25th were plated for fungi on Rose Bengal agar medium. The washed root pieces were also plated to culture any fungus associated with the root surface. The number of fungal colonies present in each of these washings was recorded after an

incubation period of 4 days at 28 ± 2°C.

Simple sections of coconut and cocoa roots were also made and observed under light microscope to study the presence of any mycorrhizal association.

Trichoderma lignorum, the most frequently isolated root surface fungus of coconut was used to study possible antagonism over other fungi occurring in the coconut rhizosphere. The fungi tested were, five isolates of Fusarium spp., one species each of Gliocladium, Sepedonium, Cladosporium and Penicillium (Isolate 1), three species of Aspergillus viz. A. niger, A. flavus and A. fumigatus and three isolates of sterile mycelia (red, yellow and grey). Besides these, two known species of Rhizoctonia, R. solani and R. bataticola obtained from the Division of Mycology and Plant Pathology - I.A.R.I., New Delhi and one species of Rhizoctonia isolated from the rhizosphere of coconut during the present study were also used. Spore or mycelial suspension of different fungi was plated on Rose Bengal agar medium. After the media had solidified, the centre of each plate was inoculated with a loopful of the spore suspension of Trichoderma lignorum. The presence or absence of inhibition zone in each case was recorded after an incubation period of 6 days at 28 ± 2°C.

IX. Quantitative estimation of organic carbon, total nitrogen and available phosphorus in the soil

Soil samples from different treatments were studied for

organic carbon by Walkley and Black's rapid titration method, (Walkley and Black, 1934) total nitrogen by Kjeldahl's method and available phosphorus by Olsen's method of estimating available phosphorus in soils by extraction with sodium bicarbonate (Olsen et al., 1954).

X. Determination of pH of the soil.

10 gms each of the different soil samples was taken for this purpose. 50 ml of double distilled water was added to these, stirred well and allowed to stand for 5 hours before the pH was determined by a pH meter (Model LI-10 "ELICO").

XI. Statistical method used for the analysis of data

Correlation study was done as per the following formula :

$$r = \frac{\sum_{i=1}^{n}(X_i - \bar{X})(Y_i - \bar{Y})}{\sqrt{\sum_{i=1}^{n}(X_i - \bar{X})^2 \sum_{i=1}^{n}(Y_i - \bar{Y})^2}}$$

where, r is the correlation coefficient and n is the number of observations. (Snedecor and Cochran, 1967).

EXPERIMENTAL RESULTS

EXPERIMENTAL RESULTS

1.1 Microorganisms in the rhizosphere of coconut and cocoa (Table 1).

The enhancement in the population of bacteria, fungi and actinomycetes in the rhizosphere was conspicuous when compared to non-rhizosphere soil. More interesting was the observation that mixed cropping with double hedge of cocoa led to increased proliferation of microorganisms than in single hedge cultivation. This trend was reflected in the physiological groups such as nitrogen-fixing and phosphate solubilising bacteria. When compared to the coconut, these were more in the rhizosphere of cocoa. In addition, it was also found that nitrogen-fixing and phosphate solubilising bacteria did not occur in the non-rhizosphere soil.

Fungi on the other hand, was more prolific in the rhizosphere of coconut irrespective of the presence of cocoa in single or double hedge. Among the various fungal genera, *Trichoderma* was a dominant form common to the rhizosphere soil of coconut.

1.2 Studies on the distribution of microorganisms (Table 2).

A. Bacteria : The dominant forms of bacteria were isolated and the routine tests for identification carried out. Among the ten isolates of bacteria, cultural tests enabled the

Table 1 : Number* of different types of microorganisms in the rhizosphere of coconut under mixed cropping with cacao.

Characteristics of isolates	Non-rhizosphere soil	CACAO MIXED CROPPING									
		February - 1974					July - 1974				
		Coconut without cacao	Single Hedge		Double Hedge		Coconut without cacao	Single Hedge		Double Hedge	
			Coconut	Cacao	Coconut	Cacao		Coconut	Cacao	Coconut	Cacao
Total bacteria (10^6)	48	104	160	92	212	128	140	152	80	200	104
Total *Beijerinckia* (10^3)	0	2	5	7	6	14	5	5	5	4	9
Total phosphate solubilising bacteria (10^6)	0	1	2	4	5	5	5	4	2	4	8
Total actinomycetes (10^6)	1	2	5	2	5	4	2	2	5	4	5
Total fungi (10^4)	10	15	32	16	59	18	22	76	11	90	14

* Per gram dry rhizosphere soil.

Table 2 : Distribution of microorganisms in the rhizosphere of coconut under mixed cropping with cacao (combined data for February and July, 1974).

Characteristics of isolates	Non-rhizosphere soil	CACAO MIXED CROPPING				
		Coconut without cacao	Single Hedge		Double Hedge	
			Coconut	Cacao	Coconut	Cacao
Bacteria						
Isolate 1 (Cellulose decomposing)	+	+	+	+	+	+
Isolate 2 (Red coloured)	-	-	+	+*	+	+*
Isolate 3 (Cream yellow)	+	+	+	-	+	-
Isolate 4 (Shiny yellow)	-	+	+	-	+	-
Isolate 5 (*Beijerinckia* sp.)	-	+	+	+	+	+
Isolate 6 (*Pseudomonas* sp.)	-	-	+	-	+	-
Isolate 7 (Phosphate solubilising)	-	-	+*	+	+	+
Isolate 8 (Phosphate solubilising)	-	+	-	+	-	+
Isolate 9 (Phosphate solubilising)	-	-	-	+	-	+
Isolate 10 (*Escherichia* sp.)	-	-	+*	+	+	+
Actinomycetes						
Isolate 1 (*Streptomyces* sp.)	+	+	+	-	+	+
Isolate 2 (*Streptomyces* sp.)	+	+	+	+	+	+
Isolate 3 (*Streptomyces* sp.)	-	+	-	+	-	+
Isolate 4 (*Streptomyces* sp.)	-	-	+	-	-	+
Isolate 5 (*Streptomyces* sp.)	-	-	-	-	+	-
Isolate 6 (*Streptomyces* sp.)	-	-	-	+	-	+
Isolate 7 (*Streptomyces* sp.)	-	-	-	+	+	-
Isolate 8 (*Streptomyces* sp.)	-	-	-	-	+	-

Contd.

Characteristics of isolates	Non-rhizosphere soil	CACAO MIXED CROPPING				
		Cocoanut without canna	Single Hedge		Double Hedge	
			Cocoanut	Canna	Cocoanut	Canna
Fungi						
Fusarium sp. (Isolate 1)	+	+	+	+	-	-
Fusarium sp. (Isolate 2)	+	-	-	+	-	+
Fusarium sp. (Isolate 3)	-	-	+	+	-	+
Fusarium sp. (Isolate 4)	+	+	+	+	+	-
Fusarium sp. (Isolate 5)	-	+	+	+	+*	-
Gliocladium sp.	-	+	+*	+	+	+
Sporidesmium sp.	-	-	-	+	-	+*
Gliodesmium sp.	-	+	-	-	+	-
Cylindrocarpon sp.	-	-	+	-	-	-
Trichoderma lignorum	-	+*	+*	+	+*	+*
Rhizoctonia sp.	-	+	+	-	-	-
Penicillium sp. (Isolate 1)(Sclerotial)	-	-	+	+	+*	+
Penicillium sp. (Isolate 2)(Sclerotial)	-	-	-	+	+	+
Penicillium sp. (Isolate 3)(Sclerotial)	-	+	-	+	+	+
Penicillium sp. (Isolate 4)	-	+	-	-	+	+
Penicillium sp. (Isolate 5)	-	-	-	-	+	+
Penicillium sp. (Isolate 6)	+	-	-	-	-	+

Contd.

Characteristics of isolates	Non-rhizosphere soil	CACAO MIXED CROPPING				
		Cocoa without cocoa	Single Hedge		Double Hedge	
			Cocoa	Cocoa	Cocoa	Cocoa
Aspergillus fumigatus	-	-	-	+	+	-
Aspergillus flavus	-	-	-	-	+	+
Aspergillus niger	-	+	+	+	+	+
Aspergillus terreus	-	-	+	+	+	-
Aspergillus sp.	+	-	+	+	+	-
Sterile mycelia (Red)	-	+	-	-	-	-
Sterile mycelia (Yellow)	-	-	+	-	-	-
Sterile mycelia (Grey)	-	-	+	-	-	-

* Isolated from the root surface.

identification of a species of Beijerinckia (Isolate 5) as the dominant nitrogen-fixing bacterium in the different rhizosphere soils. None of the other forms were capable of fixing atmospheric nitrogen. Four of the bacterial isolates (Nos. 6 to 9) proved to be phosphate solubilisers by their capacity to grow on Pikovskaya medium and solubilise the tricalcium phosphate incorporated therein. Isolate 6 was identified as a species of Pseudomonas. Isolate 1 was capable of cellulose decomposition, utilising the filter paper as the sole source of carbon in Omelianesky's medium. Among the other isolates was one belonging to the Coliform group which produced indolyl-acetic acid(Isolate 10). No attempt was made to identify these isolates which did not have the ability either to decompose cellulose, solubilise inorganic phosphate or produce IAA or gibberellin-like substances (GLS).

B. Actinomycetes : Eight isolations (Streptomyces spp.) were made of which isolates 4 to 9 were found to be phosphate solubilisers. None of these isolates was capable of producing either IAA or GLS.

C. Fungi : Among the twenty five isolates of fungi were t the genera of Fusarium, Gliocladium, Sporodinium, Cladosporium, Cylindrosporum, Trichoderma, Rhizoctonia, Aspergillus and Penicillium. Three were unidentified since they remained as sterile mycelia and did not sporulate. The following fungi,

Fusarium spp. (Isolates 3 to 5), Aspergillus niger and A. terreus; Penicillium spp. (Isolates 1 to 5) and sterile mycelia (yellow) were found to be phosphate solubilisers. None of the isolates was capable of producing IAA, while Aspergillus flavus and A. fumigatus produced GLS.

2.1 Cultural characteristics of Beijerinckia sp. (Plates 2, 3A and B).

The cells of this bacterium were oval shaped with their size ranging from 0.88 x 1.67 µm in younger cells and 1.57 x 2.26-2.53 µm in older cells. They were gram negative, capsulated and non-motile. Young colonies on Jensen's nitrogen-free medium were round with regular edges, smooth and mucilaginous without pigmentation. However, older colonies were creamy yellow in colour. Dominant character was the production of abundant gum in liquid cultures.

2.2 Nitrogen fixing ability of Beijerinckia sp. (Fig. 1).

On nitrogen-free Jensen's medium, Beijerinckia sp. fixed 9.83 mg of nitrogen per gram of sucrose utilised.

2.3 Soil characteristics favouring growth of Beijerinckia sp. (Table 3, Fig. 2).

Analysis of the soil for pH, organic carbon and total nitrogen showed the following features. The pH of the non-rhizosphere and rhizosphere soils ranged from 3.5 to 5.2 (acidic

Plate 2 : Gum formation by Beijerinckia sp. on Jensen's nitrogen-free medium (10 day old culture).

PLATE - 2

Plate 3A : Electron micrograph of Reisigrinskia sp. showing the typical cell formation (18,160 x).

Plate 3B : Electron micrograph of a single, isolated cell of Reisigrinskia sp. (18,160 x).

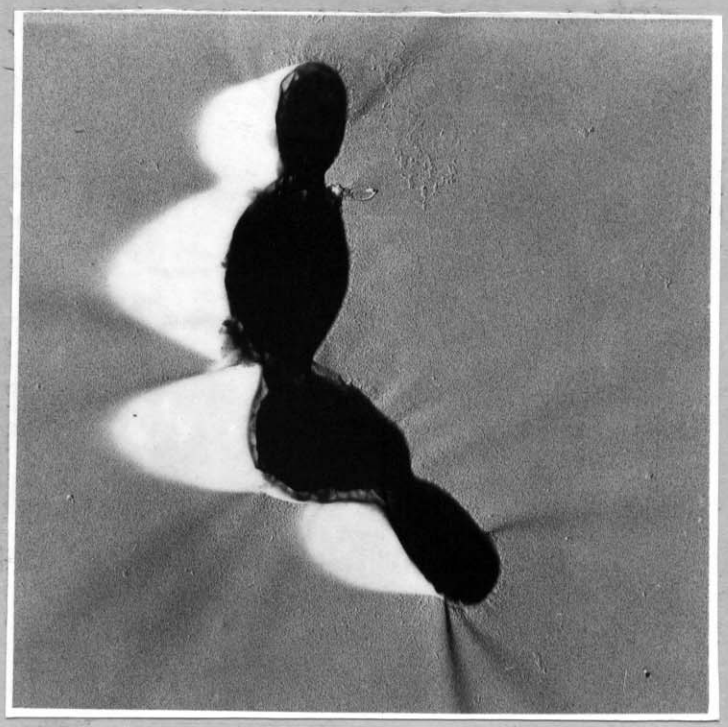


PLATE-3A

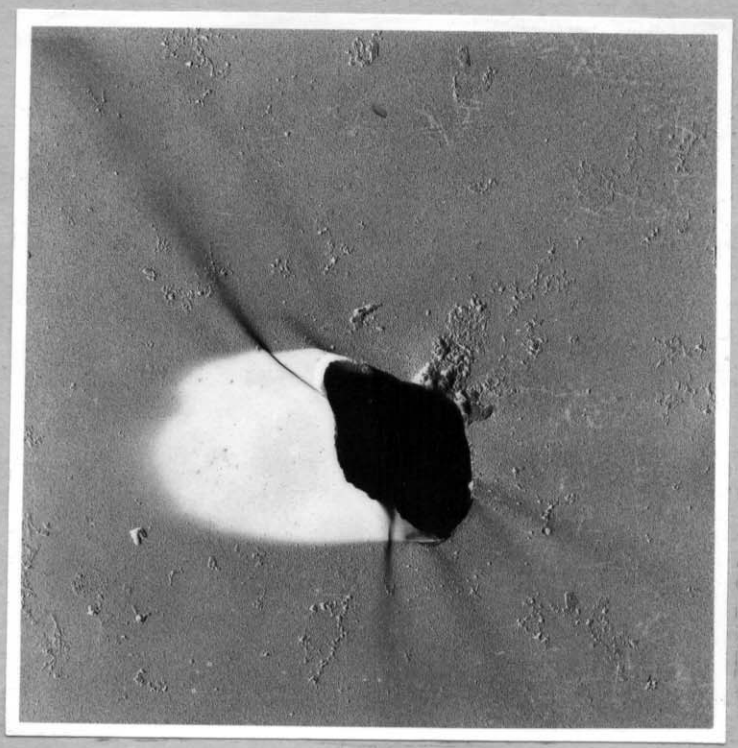


PLATE-3B

Fig. 1. Percentage of Nitrogen Fixed by the *Beijerinckia sp.* along with Ammonium chloride standard

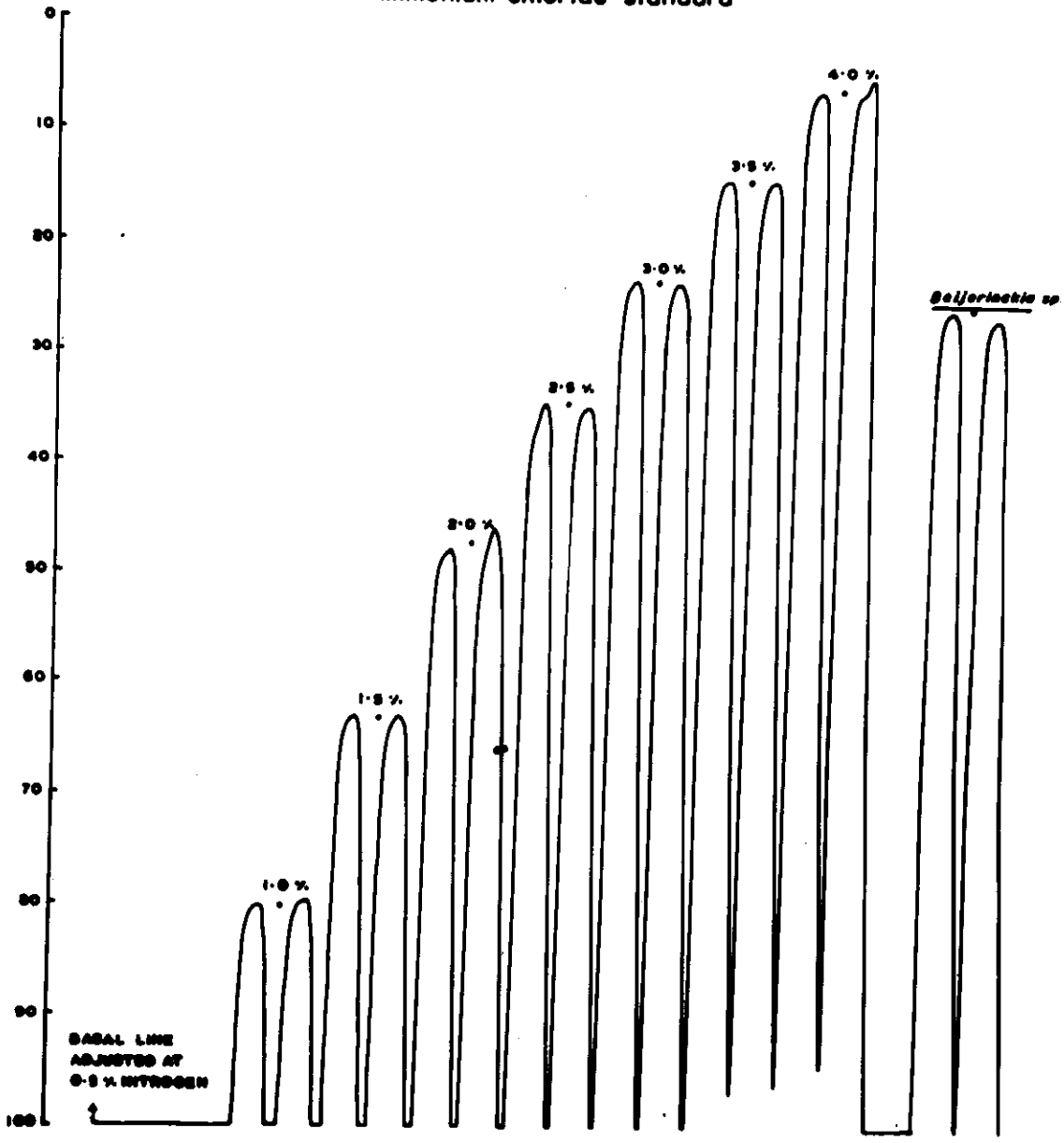
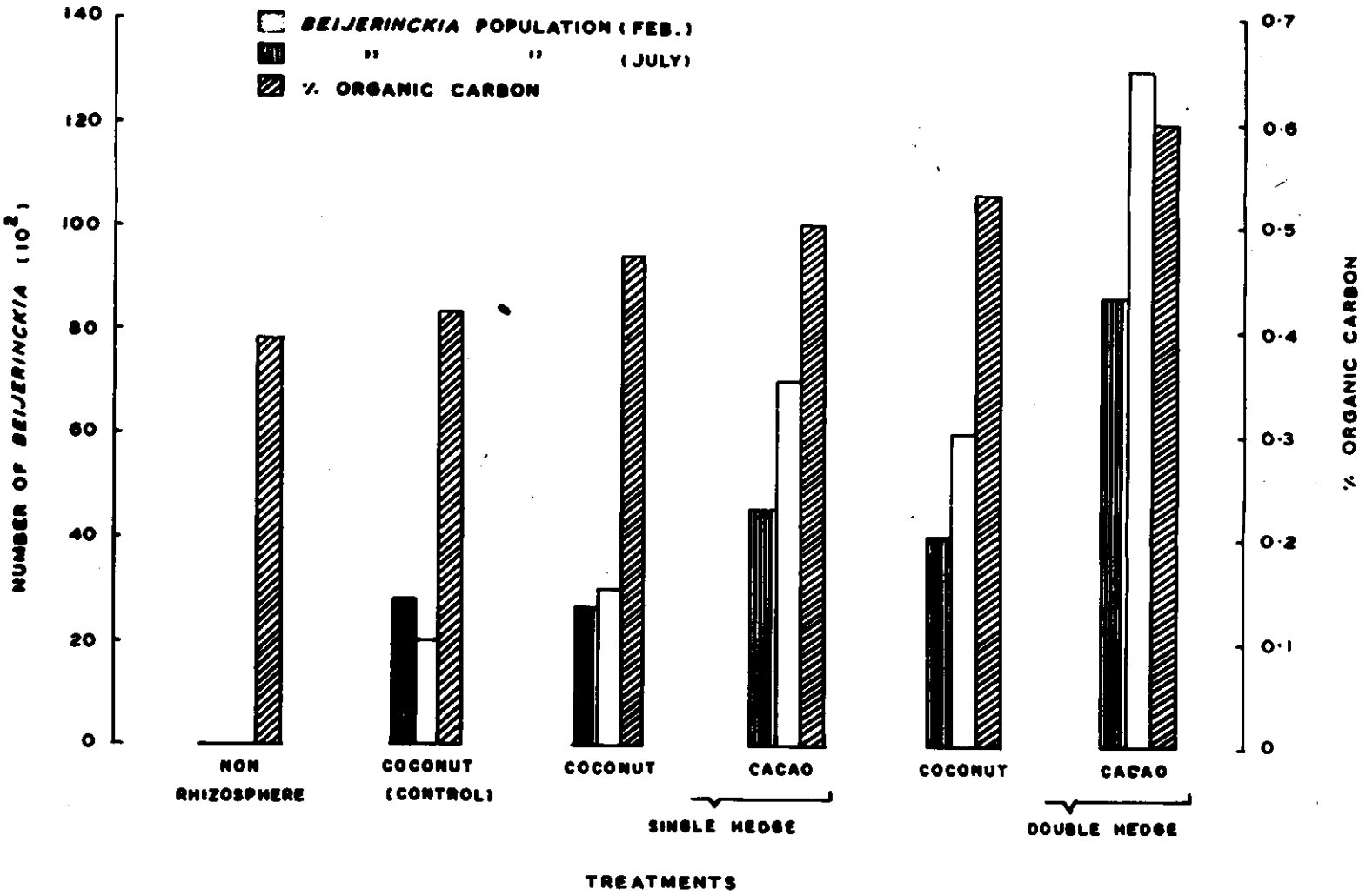


Table 3 : Relation between pH, organic carbon, total nitrogen and the incidence of *Beijerinckia* in soil.

Type of soil	No. of *Beijerinckia*/g rhizosphere soil		Soil pH	% organic carbon	Total Nitrogen mg/g soil
	Feb.'74	July'74			
Non-rhizosphere	0	0	5.5	0.39	0.35
Coconut rhizosphere (control)	2000	3000	5.2	0.42	1.30
Coconut rhizosphere (Single hedge)	3000	3000	4.9	0.48	0.85
Cocoa rhizosphere (Single hedge)	7000	5000	5.1	0.50	1.15
Coconut rhizosphere (Double hedge)	6000	4000	5.0	0.53	0.90
Cocoa rhizosphere (Double hedge)	14000	9000	5.2	0.60	1.10

Fig. 2. Relationship between the number of *Beijerinckia* & Organic carbon in the Rhizosphere Soil samples



range) which was favourable for the growth and proliferation of Beijerinckia sp. It was however, interesting to note that the rhizosphere soil had a higher pH than non-rhizosphere soil, probably due to the buffering action of the root region or due to liming usually practised in coconut plantations.

The percentage of organic carbon in the non-rhizosphere soil was 0.39, while in the coconut and cocoa rhizosphere soils (single hedge) it ranged from 0.42 to 0.50. Under double hedge cultivation of cocoa, organic carbon in the coconut rhizosphere rose to 0.53 % and it was much higher in the cocoa rhizosphere, registering a level of 0.60 %. The general increase in the organic carbon content of the rhizosphere soil was another factor which might have helped the growth and proliferation of Beijerinckia sp.

The total nitrogen content of the rhizosphere soil was more than double when compared to non-rhizosphere soil. This could be attributed to the activity of a large number of Beijerinckia sp. present in the rhizosphere of both the plantation crops, as well as due to the addition of inorganic nitrogenous fertilizers (see appendix II for the recommended dose of fertilizers for coconut and cocoa). Nevertheless, one point that emerged from these studies was that double hedge cocoa cultivation distinctly increased the total number of Beijerinckia especially in the rhizosphere of cocoa.

3.1 Phosphate solubilisation (Table 4, Fig. 3, Plate 4).

Twenty one different microorganisms were quantitatively assayed for phosphate solubilisation. While these organisms had the ability to solubilise $Ca_3(PO_4)_2$ to different degrees, it was clearly evident that Pseudomonas sp. (Isolate no. 6) and Aspergillus niger solubilised the bound phosphate to the greatest extent. Pseudomonas sp. solubilised 48.95 per cent and the fungus, A. niger solubilised it to the extent of 49.69 per cent. This was associated with a fall in the pH of the culture filtrate of these organisms to about 3.0. Assuming that solubilisation of the bound phosphate above 15 per cent is indicative of the organism as "a good solubiliser", different species of Penicillium isolated in the present study may also be considered as efficient phosphate solubilisers.

3.2 The phosphate status of the soil and the incidence of phosphate solubilising microorganisms (Table 5, Fig. 4).

The phosphate status of the soil was generally low - 10 ppm per gram soil, in the non-rhizosphere. In the rhizosphere of coconut and cocoa under double hedge cultivation, the amount of available phosphorus registered a high value of 55 to 65 ppm per gram soil. This may be due to the top dressing of rock phosphate given to individual plants and to the greater incidence of phosphate solubilising microorganisms. Pseudomonas sp. was present in the rhizosphere of coconut under single and double hedge cultivation, while Aspergillus niger was present in

Table 4 : Quantitative studies on the solubilisation of tricalcium phosphate.

Organisms studied	P_2O_5 in mg/50 ml medium (average of triplicate)*	Percentage $Ca_3(PO_4)_2$ solubilised	pH of the culture filtrate**	Mycelial mat wt. (g)
Bacteria				
Isolate 1 (Cellulose decomposing)	0.7638	6.67	6.5	-
Isolate 6 (*Pseudomonas* sp.)	5.5770	48.95	3.5	-
Isolate 7	0.4965	4.39	6.7	-
Isolate 8	0.4965	4.39	6.5	-
Isolate 9	0.4965	4.39	6.5	-
Actinomycetes				
Isolate 4 (*Streptomyces* sp.)	1.6043	6.99	6.0	1.14
Isolate 5 (*Streptomyces* sp.)	0.7638	3.32	6.0	1.13
Isolate 6 (*Streptomyces* sp.)	0.4965	2.18	6.5	1.13
Isolate 7 (*Streptomyces* sp.)	0.2291	1.00	6.5	1.12
Isolate 8 (*Streptomyces* sp.)	0.2291	1.00	6.5	1.80
Fungi				
Fusarium sp. (Isolate 3)	1.2988	5.68	6.5	1.19

Contd.

Organisms studied	P_2O_5 in mg/50 ml medium (average of triplicate)*	Percentage $Ca_3(PO_4)_2$ solubilised	pH of the culture filtrate**	Mycelial mat wt. (g)
Fusarium sp. (Isolate 4)	0.2291	1.00	6.5	1.17
Fusarium sp. (Isolate 5)	0.2291	1.00	6.5	1.16
Penicillium sp. (Isolate 1)	5.2714	23.88	5.7	1.28
Penicillium sp. (Isolate 2)	5.2714	23.01	5.5	1.26
Penicillium sp. (Isolate 3)	2.4448	10.66	6.2	1.24
Penicillium sp. (Isolate 4)	4.2779	18.69	6.5	1.15
Penicillium sp. (Isolate 5)	2.2156	9.69	6.4	1.19
Aspergillus niger	11.3836	49.69	3.0	1.25
Aspergillus terreus	3.0598	13.36	6.5	1.21
Sterile mycelia (Yellow)	0.2291	1.00	6.5	1.29

* Values are expressed after substracting the control values.
** pH of the culture medium after sterilization was 6.5.

Fig. 3. Relationship between the number of Phosphate solubilising Microorganisms & available Phosphorus in the Rhizosphere Soil samples

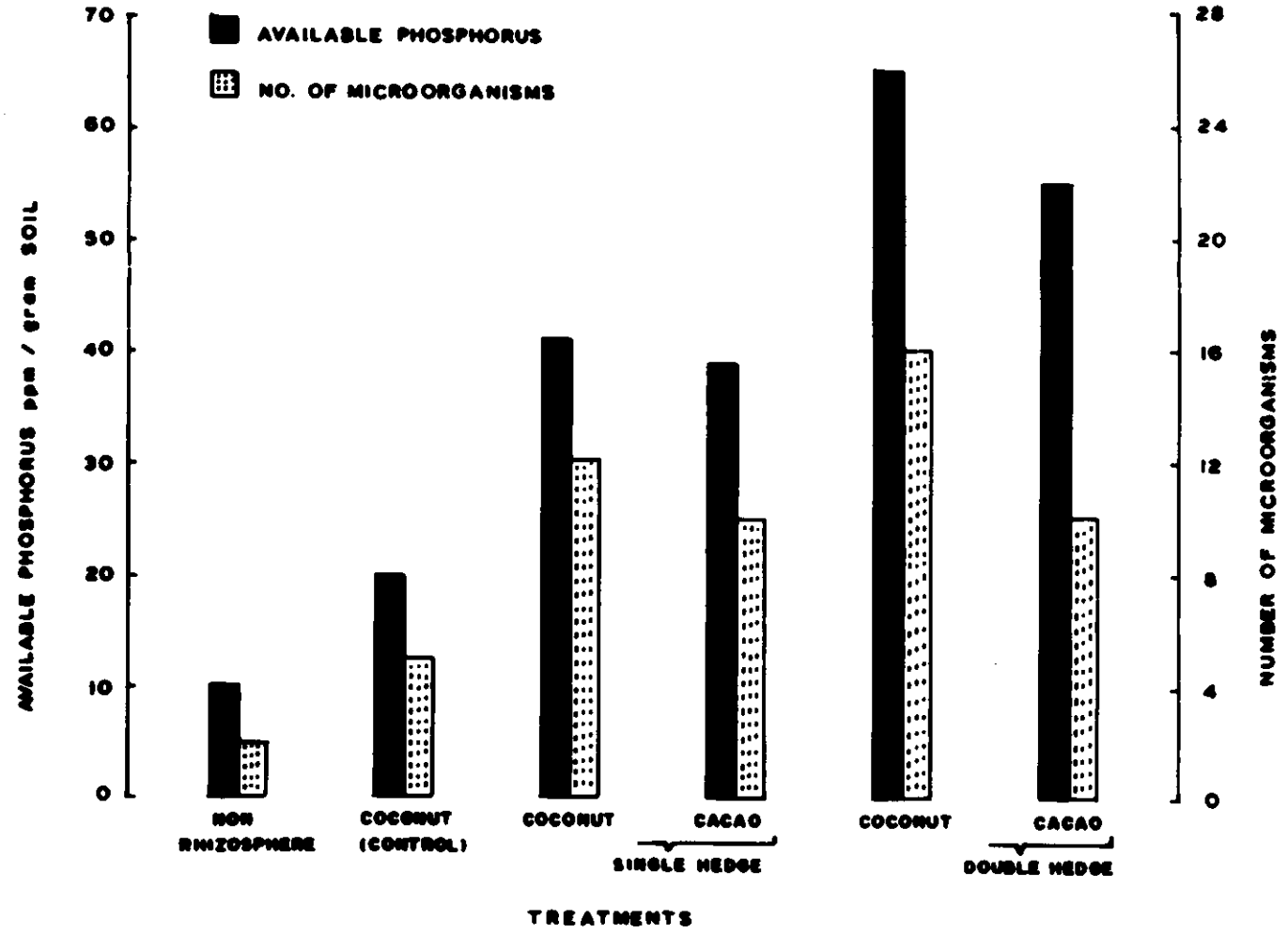


Plate 4 : Aspergillus niger growing on Pikovskaya medium showing the characteristic transparent zone due to solubilisation of tricalcium phosphate.

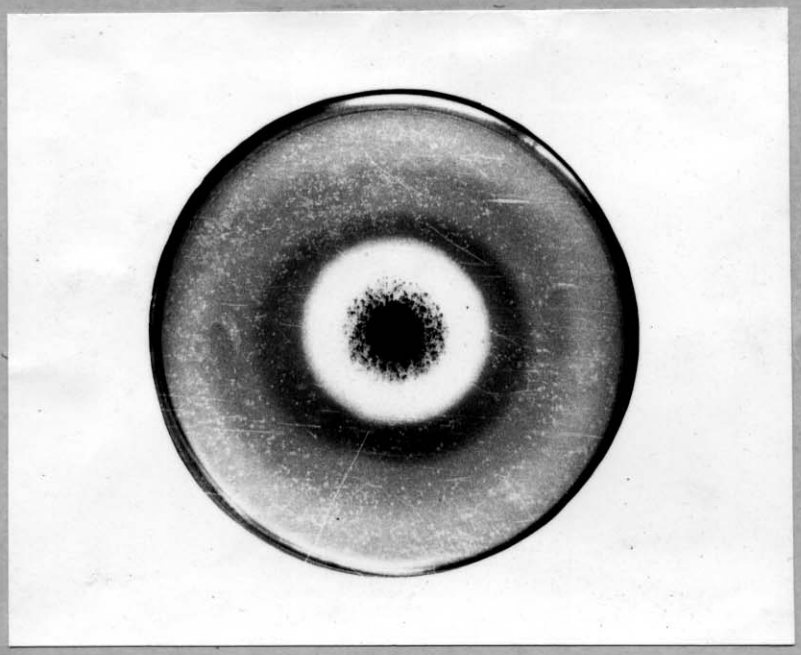
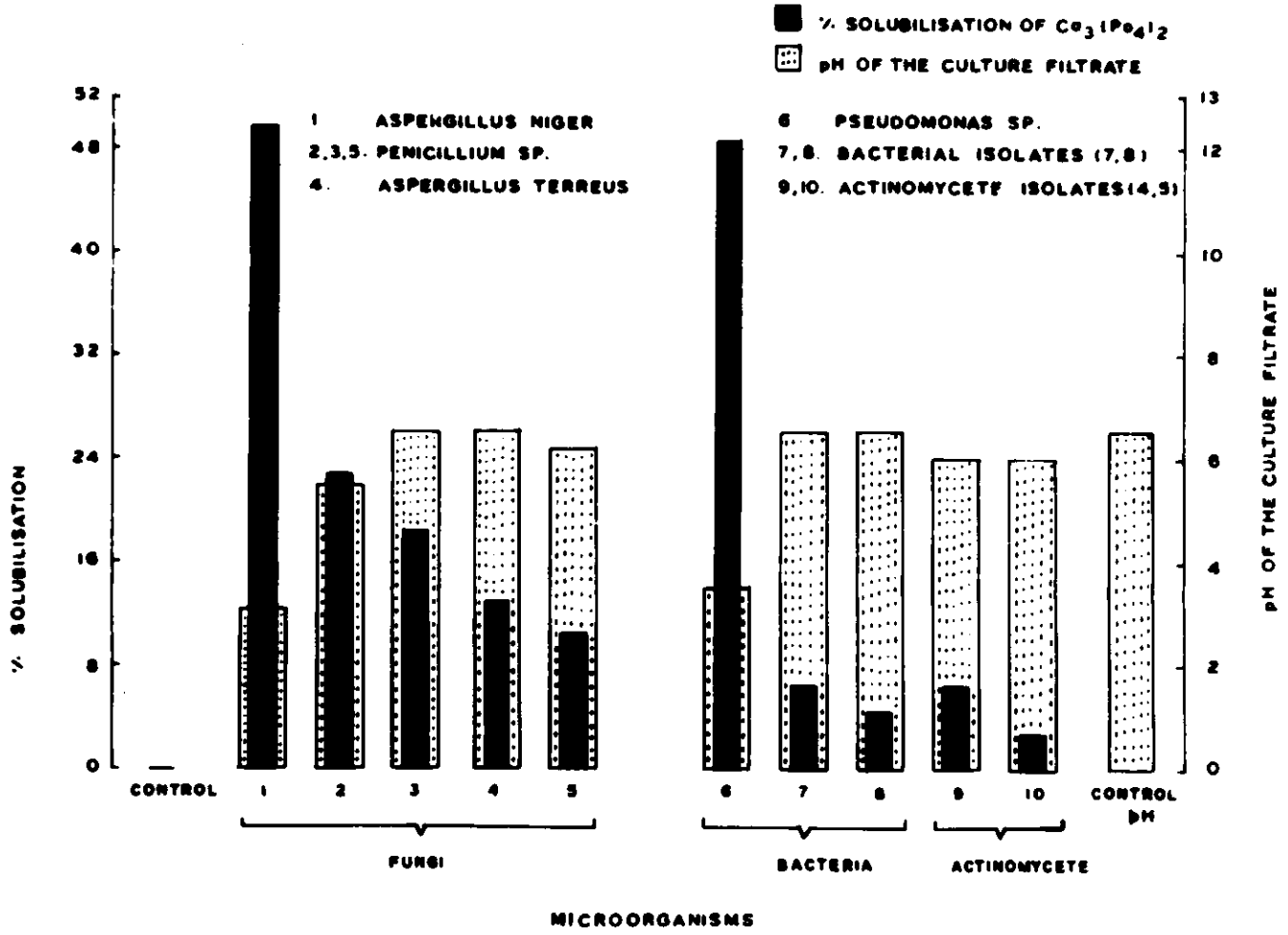


PLATE - 4

Table 5 : Relation between the phosphate status of the soil and the incidence of major phosphate solubilising microorganisms.

Type of soil	Pseudomonas sp.	Aspergillus Niger	Penicillium sp. (iso.1)	Penicillium sp. (iso.2)	Penicillium sp. (iso.4)	Available phosphorus ppm/g soil
Non-rhizosphere	-	-	-	-	-	10.0
Coconut rhizosphere (Control)	-	+	-	-	+	20.0
Coconut rhizosphere (Single hedge)	+	+	+	-	-	41.0
Cocoa rhizosphere (Single hedge)	-	+	+	+	-	38.0
Coconut rhizosphere (Double hedge)	+	+	+	+	+	65.0
Cocoa rhizosphere (Double hedge)	-	+	+	+	+	55.0

Fig. 4 Relationship between Solubilisation of $Ca_3(Po_4)_2$ and pH. of the Culture Filtrate.



the rhizosphere of both coconut and cocoa.

3.3 Cultural characteristics of Pseudomonas sp. (Table 6; Plates 5A and B).

The colonies of Pseudomonas on Pikovskaya medium were circular, convex and entire; growth on agar slants was filiform and flocculent on nutrient broth.

The cells were elongated with a size ranging from 0.98 x 1.29 µm. They were motile by means of a single polar flagellum of approximately 4.12 µm. They were aerobic, Gram negative, non-capsulated and without endospores, producing a water soluble pyocyanin pigment that diffused through the medium. The various biochemical activities of the bacterium are given in Table 6.

4.1 IAA production by Escherichia sp.

This isolate of Escherichia sp. produced IAA, equivalent to 0.2709 µg/µg dry weight of bacterial cells.

Cultural characteristics : The cells were Gram negative non-spore forming rods which fermented lactose with the production of acid and gas (less than 10 % within 24 hours - 91 % confirmation of coliform organisms) and showed the typical colony characteristics on EMB agar plates. The colonies were well isolated, 2 to 3 mm in diameter, slightly raised and concave, revealing a dark, almost black centre in transmitted light. In reflected light, the colonies showed the typical greenish

Plate 5A : Electron micrographs of Pseudomonas sp. Showing the typical single polar flagellum. (18,160 x).

Plate 5B : Electron micrographs of Pseudomonas sp. Showing the typical single polar flagellum (18,160 x).

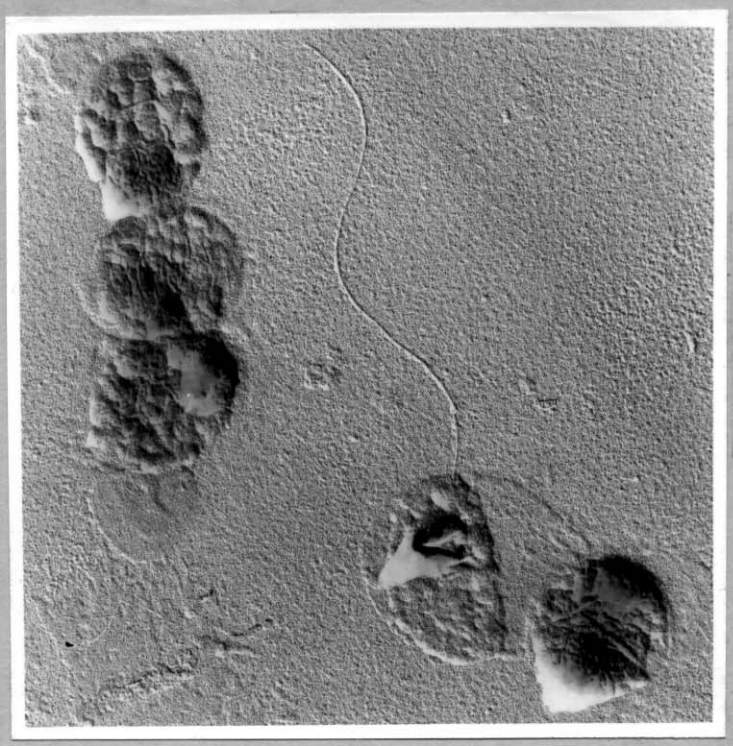


PLATE -5A

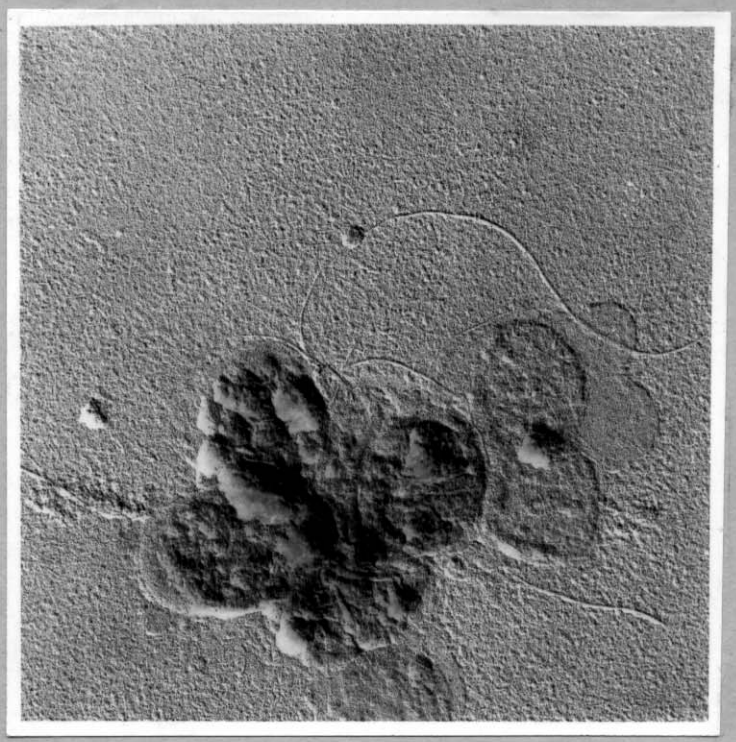


PLATE -5B

Table 6 : Major biochemical characteristics of Pseudomonas sp.

A

Fermentation of different sugars/sugar alcohols.*

Sr. No.	Sugar/ sugar alcohols	Acid production without gas formation	Acid production with gas formation
1.	Glucose	+	-
2.	Galactose	+	-
3.	Rhamnose	-	-
4.	Arabinose	-	-
5.	Sucrose	-	-
6.	Lactose	-	-
7.	Raffinex	-	-
8.	Mannitol	±	-
9.	Glycerol	-	-

* Bacterium possesses oxidative carbohydrate metabolism.

B

Sr. No.	Nature of biochemical tests	Result
1.	Hydrolysis of Gelatin	+
2.	Hydrolysis of Casein	+
3.	Hydrolysis of fat	+
4.	Hydrolysis of starch	-
5.	Production of ammonia	+
6.	Production of IAA	-
7.	Production of H_2S	-
8.	Reduction of NO_3	-
9.	Reaction in litmus milk	-
10.	Catalase test	+

metallic sheen. In the completed test, the bacterium when further inoculated into lactose broth produced acid and gas within 24 hours.

5.1 Production of gibberellin-like substances (GLS) by Aspergillus flavus and A. fumigatus (Table 7, Plates 6, 7A and B).

In ascending paper chromatography the Rf values of pure gibberellic acid was 0.91 and that of GLS from culture filtrate, 0.87. Both the spots reacted to the same spraying reagent and therefore, it was concluded that the substance elaborated by these two fungi was GLS, although not strictly comparable to standard gibberellic acid.

Bioassay data clearly demonstrated the activity of GLS in comparison with standard gibberellic acid. The elongation of the seedlings was evident as a characteristic reaction to the presence of both GLS and standard gibberellic acid. The treated seedlings showed almost twice the height of control seedlings which confirmed the presence of GLS in the culture filtrate of Aspergillus flavus and A. fumigatus.

6.1 Interaction among fungi on the root surface of coconut (Table 8, Plates 8, 9 and 10).

Serial washings of roots of both coconut and cocoa provided an insight into the closely adhering fungal flora of the root

Plate 6 : Chromatographic separation of GLS from the culture filtrate of *Aspergillus flavus*.

1. Ethyl acetate extract of GLS from the culture filtrate
2. Standard gibberellic acid

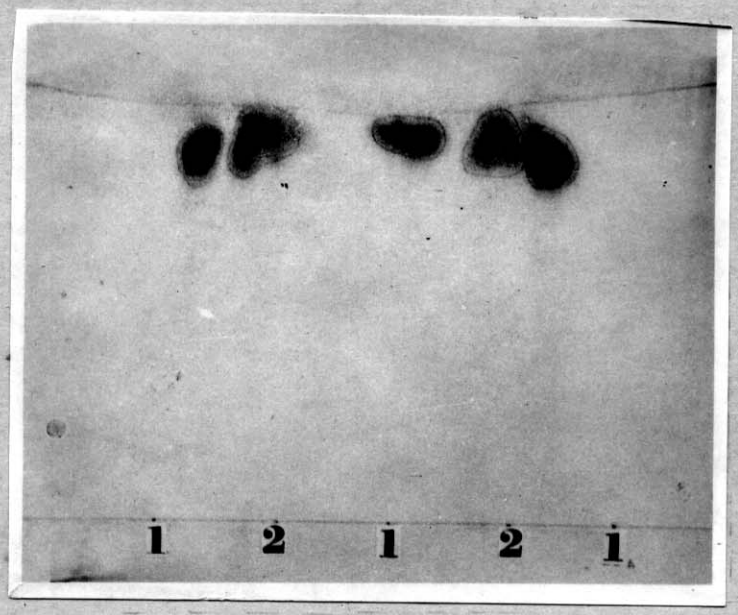


PLATE - 6

Table 7 : Elongation of rice seedlings (cms) in response to gibberellic acid and GLS from the culture filtrates.

Seedling number	Control	Culture filtrate		Ethyl Acetate extract		Standard gibberellic acid
		A. flavus	A. fumigatus	A. flavus	A. fumigatus	
1	2.7	4.8	5.0	6.3	5.4	5.7
2	2.4	5.5	5.8	5.4	6.2	5.7
3	2.7	4.4	4.0	3.4	4.2	7.2
4	2.4	5.8	5.5	4.6	4.0	5.5
5	2.9	5.7	4.8	4.5	4.6	6.1
6	2.6	4.9	5.8	3.5	4.8	5.0
7	3.5	4.3	4.9	4.8	4.6	5.2
8	3.4	3.8	5.7	4.1	3.7	4.2
9	2.5	4.4	4.3	5.6	5.4	4.6
10	3.6	3.8	5.7	4.6	4.3	4.1
11	2.6	4.0	5.5	3.9	4.0	4.2
12	3.2	3.8	4.6	3.6	4.0	3.7
Average length	2.88	4.60	4.80	4.40	4.60	5.10

Plate 7A : Elongation of rice seedlings treated with the culture filtrate of A. flavus and standard gibberellic acid.

1. Control - untreated
2. Treated with standard gibberellic acid
3. Treated with ethyl acetate extract of GLS from the culture filtrate
4. Treated with culture filtrate as such. Note the elongation of seedlings in 2, 3 and 4

Plate 7B : Pot culture experiment demonstrating the effect of GLS from the culture filtrate of A. flavus and standard gibberellic acid.

1. Treated with standard gibberellic acid
2. Control - untreated
3. Treated with cultural filtrate as such. Note the elongation of seedlings in 1 and 3

PLATE-7A

PLATE-7B

Table 2 : Number of fungi in different root washings as per Harley's method.

No. of washing plated	CACAO MIXED CROPPING				
	Coconut without [illegible]	Single Hedge		Double Hedge	
		Coconut	Cocoa	Coconut	Cocoa
1	62	102	80	180	120
3	55	58	60	108	110
5	27	29	28	64	29
10	19	20	13	50	18
15	13	10	2	16	6
20	10	5	-	7	4
25	2	1	-	5	2
Washed root	Fungi detected	Fungi detected	Fungi detected	Fungi detected	Fungi detected

Plate 8 : Occurrence of Sporidesmium sp. in close association with the rhizoplane of cocoa root revealed by Harley's root washing technique.

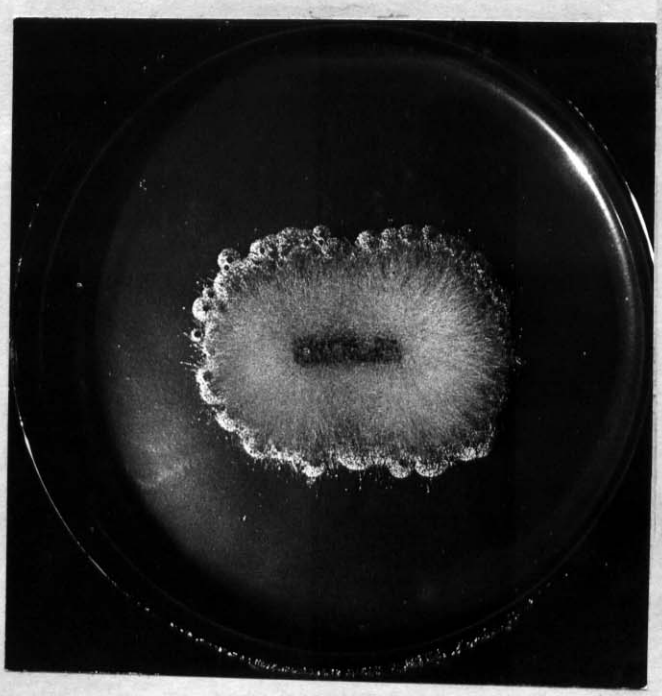


PLATE-8

Plate 9 : Interaction between *Trichoderma lignorum* on the rhizoplane of coconut and *Rhizoctonia* sp.

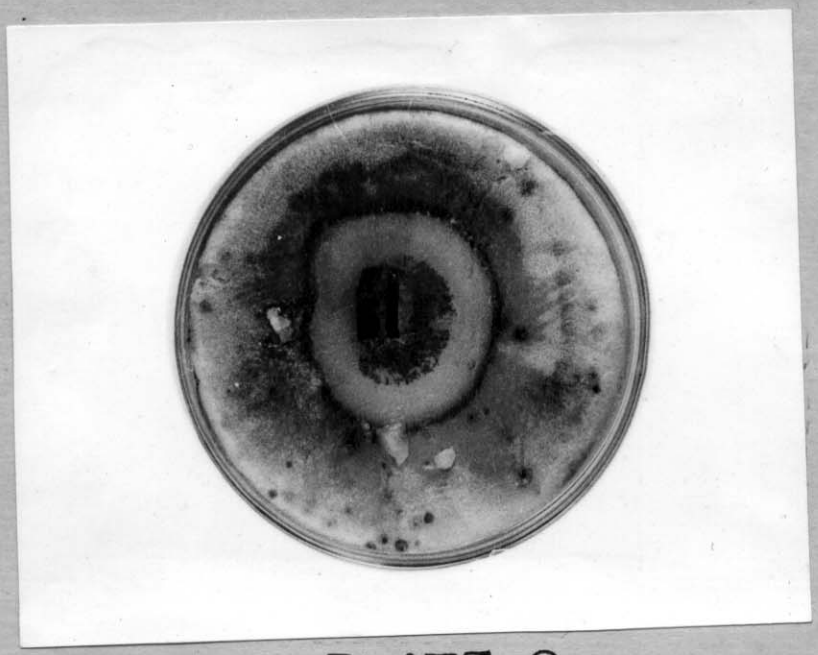


PLATE-9

surface. There was a gradual decline in the number of fungi after successive washings and when finally the washed root pieces were plated single isolates of fungi were obtained. These were identified as Trichoderma lignorum, Fusarium sp. (Isolate 5) and Penicillium sp. (Isolate 1) in the case of coconut and Sepedonium sp. in the case of cocoa. However, the examination of root sections of coconut and cocoa under light microscope did not reveal any mycorrhizal association.

Since it is known that Trichoderma lignorum is a powerful antibiotic producer, it was considered worthwhile to study the interaction of this fungus with other fungi in the root region of coconut. The results revealed that Trichoderma lignorum inhibited Fusarium spp. (Isolates 1, 3 and 4), Gliocladium sp., Sepedonium sp., Cladosporium sp., Rhizoctonia spp. including Rhizoctonia solani and Rhizoctonia bataticola; Penicillium sp. (Isolate 1) and sterile mycelia (red, yellow and grey). It also partially inhibited two isolates of Fusarium (2 and 5). The fungus was not inhibitory to Aspergillus niger, A. flavus and A. fumigatus.

Plate 20: Interaction between *Trichoderma lignorum* from the coconut rhizosphere with *Rhizoctonia* sp.

1. Isolate of *Rhizoctonia* from the rhizosphere of coconut.
2. Known culture of *Rhizoctonia solani*.

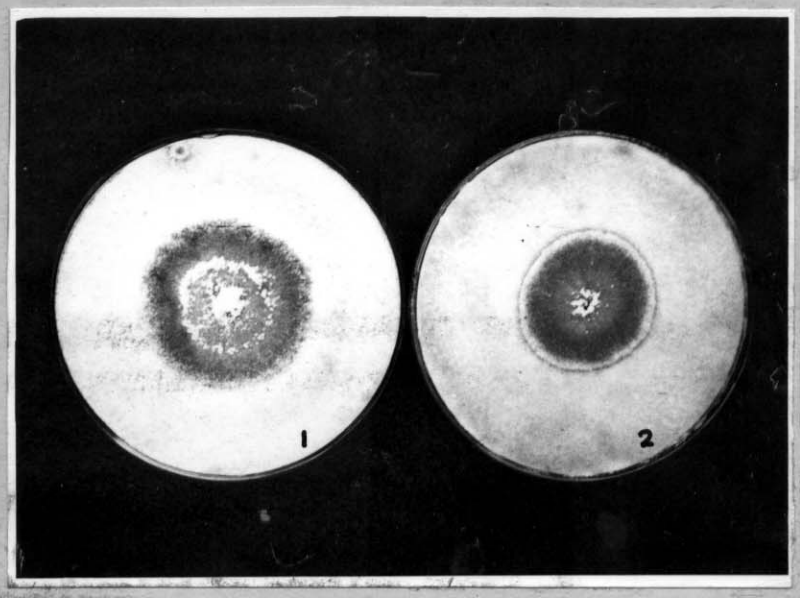


PLATE - 10

DISCUSSION

DISCUSSION

The cultivation of coconut palms, rightly known as 'Kalpavriksha' or the 'Tree of Heaven' is not without its own problems. One of these, is the considerably long initial period, about 10 years, required by the palm to attain the stage of normal bearing only after which the farmer is assured of a regular income from his farm. Therefore, to procure some income during this initial lag period, inter and mixed cropping in coconut gardens are usually adopted. Different types of crops such as pineapple, banana, groundnut, chillies, sweet potato, tapioca, cocoa, pepper, cinnamon etc. have been successfully cultivated for this purpose. However, it was observed that when cocoa was used as a mixed crop in coconut plantations, there was a significant increase in the yield of coconut (Appendix I). The many sided importance of this observation is indeed worth exploring. They are, (1) the additional income to the farmer through increased yield of coconuts without any specific additional input into his farm, (2) establishment of cocoa cultivation on a regular basis in India. This in fact is of considerable importance, as at present India consumes about 1000 tonnes of raw cocoa, almost the entire quantity of which is imported. Our country thus possess an enormous and rapidly increasing consumption potential for this commodity

which again assures the farmer of a regular market and income. The cultivation of cocoa in coconut plantations as a mixed crop does not demand any modified agronomic practices, as the soil and climatic conditions of the west coast of India have been shown to favour the normal growth and pod bearing of cocoa plants.

It is therefore, timely to study the different aspects involved in the increase in yield of coconut as a result of mixed cropping with cocoa. Among the several aspects, the possible role of soil microorganisms was chosen for the present investigation. Experiments were planned in such a way so as to isolate and study as many different types of soil microorganisms as possible, such as nitrogen-fixing, phosphate solubilising and auxin and gibberellin producing bacteria, actinomycetes and fungi. The interaction among fungi on the root surface of coconut and cocoa also formed part of the investigations.

Microorganisms in the rhizosphere of coconut and cocoa.

Greater numbers of bacteria, actinomycetes and fungi were observed in the rhizosphere soil than in the non-rhizosphere soil (Table 1). This was even more pronounced in the case of nitrogen-fixing, phosphate solubilising and auxin producing bacteria and gibberellin producing fungi which were characteristically absent from the non-rhizosphere soil. This

is because the rhizosphere region is a highly congenial habitat for the growth and proliferation of various kinds of micro-organisms. The microflora that responds to the presence of living roots is distinctly different from that characteristic of unplanted soil, since the plant creates a unique subterranean habitat for these organisms (Starkey, 1929a, b, c; 1931a, b; Krasil'nikov, 1934, 1940,1954, 1958, 1963; Lochhead, 1940, 1948, 1952; Katznelson, 1946, 1965; Clark, 1949, 1969; Revira 1956a, b, c and 1965).

On the other hand, within the rhizosphere soil itself, interesting variations in the microbial population were observed, with the maximum number of any group being present in the rhizosphere of coconut and cocoa under double hedge mixed cropping (Table 1). Even though the total number of bacteria and fungi were more in the coconut rhizosphere, more number of nitrogen-fixing and phosphate solubilising bacteria and actinomycetes were observed in the cocoa rhizosphere. Many explanations can be given for these observations, (a) the rapid stimulation of nitrogen-fixing and phosphate solubilising bacteria and actinomycetes due to the increased availability of organic matter in the cocoa rhizosphere resulting from periodical leaf shedding of cocoa (Table 3). It may even be postulated at this stage that augumenting the organic matter status of coconut rhizosphere by the incorporation of cocoa

litter, may lead to a definite improvement in the population of nitrogen-fixing and phosphate solubilising bacteria, (b) the root exudates containing simple sugars and aminoacids specifically available in the cacao rhizosphere may be favouring the proliferation of nitrogen-fixing and phosphate solubilising bacteria, (c) better conservation of soil moisture in the cacao rhizosphere due to shading and litter formation as has been often demonstrated in the plantations favours a rapid growth of these organisms in the rhizosphere of cacao than in the coconut rhizosphere and (d) cacao being a crop newly introduced into the native soil, the already well established microbial flora in the coconut rhizosphere resulting from the cultivation of coconut for many years may not have found a congenial habitat in the cacao rhizosphere. This probably underlines the observation of the natural increase in the total microflora in the rhizosphere of coconut than in cacao rhizosphere. On the contrary, the nitrogen-fixing and phosphate solubilising bacteria, existing in competition with other fast growing bacteria in the coconut rhizosphere, might have found a more amiable niche in the new cacao rhizosphere where the competition from the native flora was considerably decreased. Moreover, the slow growing nature of these organisms might also have restricted their proliferation in the coconut rhizosphere against the competition from the dominant synecgeneous fungal

population which usually thrives on the easily available simple nutrients.

The reduction in the number of different microorganisms observed in July may be more due to climatic factors such as continuous rainfall which leads to partial anaerobiosis in the soil. However, significant variations in the population were not observed indicating the well established nature of the different rhizosphere microorganisms. Taking into consideration the distribution of annual rainfall in Kerala, it is seen that the maximum of 2005 mm is obtained during June to September followed by 549 mm during October to December. The rainfall is 406 mm between March and May while it is only 38 mm in January and February. A direct influence of rainfall on microbial population can not be visulised in the present case because of the assured irrigation to these plantation crops throughout the year. It has been well documented that rhizosphere populations show considerable fluctuations depending on soil moisture, lesser number being obtained from wetter soils than from dry ones (Timonin, 1940; Clark, 1949; Bhuvaneshwari, 1958; Radha and Rawther, 1959; Lakshmi-Kumari, 1964).

Based on the above observations it is even possible to predict a pattern of the incidence of microbial population in this soil with the possibility of maximum microbial population during January and February closely followed by March and May.

The population will be at its lowest level during June and September, the period of maximum rainfall in Kerala. Though a similar trend was observable during the present studies, it has to be verified further by observations on the microbial population at different time intervals, extending over a minimum period of three to five years.

Studies on nitrogen-fixing bacteria, *Beijerinckia* sp.

Among the many ways in which elemental nitrogen enters the biosphere or the plant soil component of the eco-system, the most important is the fixation by microorganisms by the genera *Rhizobium*, *Azotobacter*, *Beijerinckia* and *Clostridium*. Of these, the free living *Beijerinckia* is found widely distributed in the tropical acidic soils (Beeking, 1959, 1961a, b; Vancura *et al*., 1965; Andersen, 1966; Strydom, 1966; Dobereiner and Campelo, 1971).

In the present study, one species of *Beijerinckia* was isolated from the rhizosphere of both coconut and cocoa. The maximum number of *Beijerinckia* occurred in the cocoa rhizosphere and especially under double hedge cultivation (Table 1). The probable role of organic carbon and root exudates in this phenomenon has already been discussed. In addition to these factors, the pH range of 4.9 to 5.2 of the coconut and cocoa rhizosphere soils was also found to be extremely favourable for

its rapid growth and proliferation (Table 3).

Kluyver and Becking (1950) and Jensen (1954) showed that Beijerinckia could fix atmospheric nitrogen to the extent of 16 to 20 mg per gram of energy rich material. The organism isolated in the present case was found to fix only 9.83 mg of nitrogen per gram of sucrose utilised. The rather low fixation capacity of this isolate could probably be attributed to the fact that Beijerinckia makes better use of ammoniacal and nitrate nitrogen and many aminoacids than atmospheric nitrogen (Jensen, 1948). The species under study isolated from a soil getting regular fertilisation has probably got acclimatised to sources of nitrogen other than the elemental form and must have slowly lost its ability to subsist solely on atmospheric nitrogen. This may partially explain why a direct correlation between the number of Beijerinckia and the amount of total nitrogen in different rhizosphere soils could not be obtained (Table 3).

The positive influence of Beijerinckia on plants has rarely been considered fully. Such an assessment is especially difficult in the case of perennial crops such as coconut and cocoa, where, addition of adequate fertiliser is essential to obtain maximum yield and it is difficult to maintain a set of control plants without fertilisation. However, the role of this bacterium in supplementing the nitrogen in its immediate environment, thereby benefiting the microflora and sometimes the

plant itself cannot be ruled out. Several workers have, infact, stressed the probable role of Beijerinckia in restoring the nitrogen level of many tropical fallows (Becking, 1959; Andersen, 1966; Dobereiner and Campelo, 1971). It may further be mentioned here that the possibility of augmenting soil nitrogen by cultivating feeder legumes in between coconut and cacao after inoculating the seed or soil with efficient strains of Rhizobium may also be envisaged.

Finally, the observation of Ruinen (1956, 1965) that Beijerinckia commonly occurs as an epiphyte in the phyllosphere of many forest trees is a matter of additional interest. A study of the phyllosphere of cacao and other plantation crops for the occurrence of Beijerinckia and other related nitrogen fixing bacteria is much warranted because of two reasons. These bacteria in the phyllosphere while utilising the sugars and other nutrient salts in the cuticular secretion of the host plant can supplement the host with bound nitrogen. Secondly, the periodical rainfall in Kerala could be of considerable help in regularly leaching down this bound form of nitrogen from the phyllosphere to the rhizosphere enriching thereby the nitrogen content of the soil.

Solubilisation of inorganic phosphate

For elements like phosphorus orginating in the geologic

substrate, ions may be released by many kinds of reactions, but it is likely that biological weathering induced by soil microorganisms is a dominant reaction. Thus, solubilisation of bound phosphate in soil appears to be a primary function of microorganisms (Abbott, 1923; Waksman, 1931; Pikovskaya, 1948; Harley and Brierley, 1954; Sen and Paul, 1957; Das, 1965; Sethi and Subba-Rao, 1968 and Bardiya, 1970).

In the present study, twenty one different isolates of bacteria, actinomycetes and fungi from the rhizosphere of coconut and cocoa were found capable of solubilising tricalcium phosphate to varying extents (Table 4). Among these, one species of bacteria belonging to the genus Pseudomonas and one isolate of Aspergillus niger was found to possess this ability to a remarkable extent, solubilising respectively 48.95 % and 49.69% of the total $Ca_3(PO_4)_2$ incorporated in their respective media.

Microbial solubilisation of inorganic phosphate is mainly due to the production of organic acids (Schwartz et al., 1954; Sperber, 1957, 1958; Louw and Webley, 1958, 1959; Swaby and Sperber, 1958; Bromfield, 1959; Meyer and Konig, 1960; Paul, 1966; Chhenkar and Subba-Rao, 1967 and Chandrasekaran, 1969 and Mehta and Bhide, 1970). In the present study also, a negative correlation between the pH of the culture filtrate and the amount of phosphate solubilised by different organisms was obtained (Table 4, Fig. 3). Thus, whom the table value for correlation coefficient at 19 D.F. and 1% significance was 0.549,

the calculated value was -0.809 indicating a negative correlation between the pH and the amount of phosphate solubilised. This became even more clear from the specific cases of Pseudomonas and A. niger (Table 4, Fig. 3) where, when the pH of the culture filtrate was at 3.5 and 3.0 respectively, maximum solubilisation of $Ca_3(PO_4)_2$ occurred. This lowering in pH could primarily be due to the production of 2-Keto-gluconic acid by Pseudomonas sp. and citric acid by Aspergillus niger (Louw and Webley, 1959; Bajpai, 1965 and Bardiya, 1970).

Concurrent with the above observation it was noticed that there exists a direct relationship between the incidence of phosphate solubilising microorganisms and the amount of available phosphorus in different rhizosphere soils (Table 5, Fig. 4). The beneficial role of microorganisms in solubilising bound phosphates has been repeatedly reported by Gerretsen (1948); Menkina (1950); Johnston (1954); Sen and Paul (1957); Miekowski (1964); Barber (1966); Myskow (1966), who have demonstrated that plants take up more phosphate in the presence of phosphate solubilising microorganisms.

Production of indole-acetic acid (IAA) and gibberellin-like substances (GLS).

Production of growth promoting substances by rhizosphere organisms is commonly considered to be one of their beneficial

attributes. Indole-3-acetic acid and gibberellin-like substances figure prominently in the list of growth substances of microbial origin. In the present investigation, IAA was produced by one species of bacterium belonging to the genus Escherichia of the family Enterobacteriaceae. It was present in the rhizosphere of both coconut and cacao. The extent of IAA production was approximately 0.2709 µg/mg dry weight of bacterial cells.

Two species of Aspergillus, A. flavus and A. fumigatus produced GLS in their culture filtrate which was detected by chromatographic and bioassay methods. In either case, the characteristics of GLS extracted from the culture filtrate were closely identical with standard gibberellic acid (Table 7, Plates 7A and B).

What could be the probable benefits of the presence of microorganisms capable of producing growth substances in the rhizosphere of coconut and cacao ? That the presence of extraneous sources of growth substances in the root region can tilt the balance of plant well-being is already well established. Production of growth substances by Azotobacter is considered to be one of the factors responsible for increased yield in crops treated to Azotobacterin (Berezova et al., 1958; Bukatsch et al., 1956; Pochon and Barjac, 1958; Krupina, 1960; Vancura and Macura, 1960; Pallot, 1963; Brakel and Hilger, 1965; Hita, 1965; Nevikova and Irtuganova, 1966). Recent investigations

on nitrogen fixing blue green algae have also borne out of the fact that the growth promoting substances exuding out of the algal cells may be partially instrumental in improving plant growth (Singh, 1961; Rao et al., 1963; Shields and Durrell, 1964). On the contrary, Japanese workers have clearly shown that production of gibberellic acid by Fusarium moniliforme in excessive amounts caused the well known bakanae disease in rice (Sawada, 1912; Kurosawa, 1926; Yabuta, 1935 and Yabuta and Sumiki, 1938). It is difficult to draw the line where the beneficial effects cease and harmful effects become manifested in any particular instance. It can, however, be reasonably concluded from the present study that no ill effects due to IAA were evident on the coconut palm even when the IAA producing Enterobacter sp. was found in close association with the roots themselves in the rhizosplane. GLS producing fungi were confined to the rhizosphere only.

In 1935, Hitchcock and Zimmerman found that when very high concentrations of synthetic plant hormones were applied to the roots of intact plants in the soil, some of it was absorbed and moved upwards. The translocation of these substances absorbed by the roots apparently occurs through the xylem (Hitchcock and Zimmerman, 1935, 1938; Mitchell and Brown, 1945; Weavers and Rose, 1946; Dhillon and Lucas, 1950 and Weintraub and Brown, 1950). It can, therefore, be assumed that, whatever

be the amount of IAA and GLS produced by soil microorganisms, they may be absorbed at least to some extent from the soil by coconut and cacao, through their root systems. The extent of the benefit endowed by these organisms on the host plants however, has to be verified and estimated by more studies in this field.

To further these investigations, isolation and purification of these microorganisms and the production of IAA and GLS on synthetic media under controlled conditions were undertaken. The possibility of using such a source of plant hormones to correct and improve any physiological phenomena in coconut and cacao may be envisaged. Some of the probable lines of study can be, (1) prevention of abscission of coconut and cacao flowers and fruits by spraying with low concentrations of GLS (Gardner et al., 1939; Heasy and Davey, 1942; Serr and Ferde, 1952; Carns et al., 1961), (2) initiation of root primordia on cacao stem cuttings as a means of vegetative propagation using IAA (Mitchell and Rice, 1942; Cepper, 1944; Avery and Johnson, 1947; Pearse, 1948; Audus, 1953), (3) rapid wound healing of the main stem of cacao after the removal of unwanted basal 'chupons' (vertical shoots) by applying low concentrations of IAA in lanoline paste (Shear, 1936; Jakes and Hexnerova, 1959; Parihar, 1948) and, (4) promoting germination of cacao seeds with low concentration of GLS (Bradbear, 1968; Bradbear and

Pinfield, 1967; Pinfield, 1968).

Occurrence and interaction of fungi on the root surface of coconut and cocoa.

Another aspect of plant microbe inter relationship currently attracting attention is the frequent formation of a fungal mantle around the root systems of plants often invading into the interior of the root cortex. Whether there exists a true mycorrhizal association or not is often difficult to adjudge as also the role of the fungus in the plants welfare. One distinct possibility repeatedly demonstrated is that these fungi are often instrumental in extracting difficultly available nutrients from the substratum and passing them on to the plant roots (Bowen and Theodorou, 1967 and Wild and Lafond, 1967). Apart from this, the fungi also confer protection to the plant from invading pathogens by virtue of their antibiotic producing abilities (Garrett, 1960; Zak, 1964). Both these possibilities were tested in the present study.

Four different fungi, Fusarium sp. (Isolate no. 5), Aspergillus sp., Penicillium sp. (Isolate no. 1) and Trichoderma lignorum were found closely associated with coconut and cocoa root surfaces (Table 2). The occurrence of Fusarium sp. on the coconut root surface may appear anomalous because of its known pathogenic nature. However, this need not be the case, if one

concurs with the observations of Bilai (1955) and Derekheva (1955). Bilai has reported that Fusaria were beneficial unless the plant is weakened. This was based on her observations on soybeans growing in fumigated soil which was inoculated with various rhizoplane organisms. Fusarium was stimulatory to plant growth unless nematodes were also introduced into the same plot in which case the plants became more stunted than those which received only the nematodes. Derekheva (1955) suggested that beneficial Fusaria were restricted to the root cortex while the harmful forms penetrated the stele. The predominant occurrence of Fusarium and Cylindrocarpon on roots of many plants without any apparent injury made Peterson (1958) to suggest that such fungi may be normal root surface inhabitants. Trichoderma lignorum must also be mentioned here because of its ubiquitous occurrence not only on the rhizoplane but also in the rhizosphere soils of coconut. In many earlier studies this fungus has been reported by be restricted to the rhizoplane only, apparently not entering into the plant roots (Thomas and Parkinson, 1961).

Interactions among the rhizosphere microflora often assume considerable significance in the context of root infections and their biological control. Production of antibiotics by soil fungi, actinomycetes and bacteria has been recognised for the past several decades. It is generally accepted that the resulting antibiotic concentrations are

sufficient to influence significantly the pattern of saprophytic colonisation at these sites by other microorganisms (Garrett, 1960). Zak (1964) postulated that fungi may furnish protection from pathogens by, (a) utilising root carbohydrates and other nutrients which would be attractive to the root pathogen, (b) providing a physical barrier as in the case of ectomycorrhiza for the entry of the pathogen, (c) secreting antibiotics which will kill or inhibit the pathogen, (d) supporting a protective rhizosphere population of other microorganisms and (f) stimulating the cells of the root during symbiosis to elaborate certain chemicals which will specifically inhibit the pathogens.

Investigations into the natural microbial antagonisms existing in the coconut rhizosphere revealed that one of the isolates (Trichoderma lignorum) inhibited the growth of Fusarium spp. (Isolates no. 1, 3 and 4), Gliocladium sp., Cladosporium sp., Rhizoctonia spp. including R. solani and R. bataticola; Penicillium sp. (Isolate no. 1) and sterile mycelia (red, yellow and grey) in vitro. Its inhibition was only partial on two isolates of Fusaria (2 and 5). Isolate no. 5 was associated with the coconut root surface itself.

This behaviour of T. lignorum is understandable in view of its ability to produce a powerful antibiotic viridin well known for its inhibition of several genera of bacteria and fungi. It is highly probable that a good deal of protective influence

was bestowed by this species on coconut plants, as not only their rhizosphere but also the rhizoplane was found to abound with this organism. The antogonistic effect of this fungus was not, however, evident on Aspergillus niger, A. flavus and A. fumigatus which were the active phosphate solubilising and GLS producing fungi isolated from the rhizosphere, revealing thereby the high degree of specificity that exists in plant microbe inter-relationship.

Studies on the occurrence of mycorrhiza in coconut and cacao did not reveal the existence of such an association. In fact, many field observations on the occurrence of mycorrhizae lead to the conclusion that this form is most frequently seen in infertile soils which implies that these structures have an important function on nutrient mobilisation (Melin, 1946). Therefore, the failure to observe any mycorrhizae in coconut and cacao during the present investigation may be due to the adequate fertilisation of the soil in the plantations as a result of which the host plants do not require such an association for increased mobilisation of various nutrients from the soil.

Although trees can be grown successfully without mycorrhizae under conditions of very high soil fertility, they usually grow much better when associated with mycorrhizae. In Puerto Rico where pines were not native, attempts to introduce pine trees met with recurrent failure until the seedlings were inoculated with leaf mold imported from a pine forest in the

United States (Briscoe, 1959). The situation seems to be similar in the case of cloves (Eugenia caryophyllata, Thumb.), another important plantation crop newly introduced to many sites which is not taking on probably because of the absence of a true mycorrhiza.

Instances such as these again reveal some of the fascinating aspects of plant microbe inter-relationships, judicial exploitation of which may yet yield many good dividends.

SUMMARY

SUMMARY

Mixed cropping of cacao in coconut plantations was found to increase the yield of coconut significantly. The probable microbiological factors involved in this phenomenon formed the basis of the present investigation.

It was observed that mixed cropping of cacao favoured a higher incidence of microorganisms in the rhizosphere of both coconut and cacao. This was all the more evident when cacao was cultivated as a double hedge. Favourable pH and high organic matter content of the soil resulting from this mixed cropping have been implicated in this rhizosphere effect.

Conspicuous among the various microorganisms in the rhizosphere was the nitrogen-fixing organism Beijerinckia which on testing was found to be capable of fixing atmospheric nitrogen to the extent of 9.85 mg/g of sucrose. However, the number of Beijerinckia sp. and the amount of total nitrogen in different rhizosphere soils could not be correlated.

Among the different phosphate solubilising microorganisms isolated from the rhizosphere, Pseudomonas sp. (Isolate no. 6) and Aspergillus niger solubilised 48.95 % and 49.69 % of the total inorganic phosphate incorporated in their respective media. A direct relationship between the incidence of phosphate solubilising microorganisms and available phosphorus in different

rhizosphere soils was obtained.

One species of Escherichia isolated from the rhizosphere synthesised IAA in pure culture to the extent of 0.2709 µg/ug dry weight of bacterial cells. Similarly two fungal isolates, Aspergillus flavus and A. fumigatus produced gibberellin like substances in pure culture, which were almost identical with standard gibberellic acid as characterised by ascending paper chromatography.

Observations on the interactions of various fungi on the root surface of coconut and cocoa revealed that Trichoderma lignorum one of the dominant rhizosphere fungi, effectively inhibited the growth of several pathogenic fungi such as Fusarium sp. and Rhizoctonia solani and R. bataticola.

No ecto or endo mycorrhizal associations were seen either in coconut or in cocoa despite several noticeable close associations between various fungi and the host root surface.

It can be rightfully be claimed that the present work has been the first major attempt to work out the microbiological aspects of plantation crops.

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* Original not seen.

APPENDICES

APPENDIX I

Yield performance of coconut palms in the coconut - cocoa mixed cropping experiment (Commenced 1970).

Treatment	Average yield of nuts per palm per year		
	Pre-treatment mean of two years 1969 and 1970*	Post-treatment mean of two years 1972-73 and 73-74	Response (+)
Control	73.25	120.39	47.14
Single hedge	66.50	112.03	45.53
Double hedge	50.45	108.95	58.50

* Yield data for 1970-71 and 71-72 not included as that period is "transition period" for this experiment.

APPENDIX II

Recommendation in terms of the commonly available fertilisers (in grams).

Time and application of fertilisers	Hybrids and high yielding varieties						Others					
	April - May			August - September			April - May			August - September		
	U	U/P or R/P	M/P	U	U/P or R/P	M/P	U	U/P or R/P	M/P	U	U/P or R/P	M/P
3 month after planting				240	190	380				120	120	220
First Year	240	185	370	480	370	740	120	120	220	240	255	440
Second Year	480	370	740	965	740	1480	240	255	445	480	475	890
Third Year	720	555	1110	1445	1110	2220	360	355	665	720	710	1330

Manurial recommendation for adult palms in terms of nutrients (in kgms.)

	N	P	K
For hybrids	1.0	0.5	2
For others	0.5	0.32	1.2
(For cocoa)	0.1	0.04	0.14 (Annually)

U = Urea
U/P = Ultra Phosphate
R/P = Rock Phosphate
M/P = Muriate of Potash

APPENDIX III

Composition of different media and reagents.

1. Conn's glycerol asparaginate agar (Conn, 1921).

K_2HPO_4	-	1.0 g
Sodium asparaginate	-	1.0 g
Calcium carbonate	-	3.0 g
Glycerol	-	10.0 ml
Agar	-	15.0 g
Distilled water	-	1000 ml

2. Czapek - Dox medium (Thom and Raper, 1945).

$NaNO_3$	-	3.0 g
K_2HPO_4	-	1.0 g
Yeast extract	-	1.0 g
$MgSO_4.7H_2O$	-	0.5 g
KCl	-	0.5 g
$FeSO_4.7H_2O$	-	0.01 g
Sucrose	-	30.0 g
Agar	-	15.0 g
Distilled water	-	1000 ml

3. Jensen's modified N_2-free medium.

KH_2PO_4	-	0.8 g
K_2HPO_4	-	0.2 g
$MgSO_4.7H_2O$	0.5	0.2 g

$FeCl_3$	-	0.1 g
Sodium molybdate	-	0.005 g
Sucrose	-	20.0 g
Agar	-	15.0 g
Distilled water	-	1000 ml

4. Martin's Rose Bengal agar (Martin, 1950).

Peptone	-	5.0 g
KH_2PO_4	-	1.0 g
$MgSO_4.7H_2O$	-	0.5 g
Dextrose	-	10.0 g
Rose-Bengal	-	33.0 mg
Agar	-	20.0 g
Distilled water	-	1000 ml
Streptomycin (1% solution)		0.3 ml/100 ml of sterilised medium.

5. Nutrient agar.

Beef extract	-	3.0 g
Peptone	-	5.0 g
Agar	-	15.0 g
Distilled water	-	1000 ml

6. Omelianskiy's nitrogen mineral salts medium (Fred and Waksman, 1928).

$(NH_4)_2SO_4$	-	1.0 g
K_2HPO_4	-	1.0 g
$MgSO_4.7H_2O$	-	0.5 g
$CaCO_3$	-	2.0 g

NaCl	-	0.1 g
Distilled water	-	1000 ml

Sterilised filter paper discs in petri plates served as the source of cellulose.

7. Pikovskaya's modified medium (Rao and Sinha, 1963).

$Ca_3(PO_4)_2$	-	5.0 g
Glucose	-	10.0 g
$(NH_4)_2SO_4$	-	0.5 g
NaCl	-	0.2 g
KCl	-	0.2 g
$MgSO_4.7H_2O$	-	0.1 g
$MnSO_4$	-	Trace
$FeSO_4.7H_2O$	-	Trace
Yeast extract	-	0.5 g
Agar	-	15.0 g
Distilled water	-	1000 ml

8. Peptone water.

Peptone	-	10.0 g
NaCl	-	5.0 g
Distilled water	-	1000 ml

9. Thornton's standardised medium (Thornton, 1922).

K_2HPO_4	-	1.0 g
$MgSO_4.7H_2O$	-	0.2 g
$CaCl_2$	-	0.1 g
NaCl	-	0.1 g

$FeCl_3$	-	Trace
KNO_3	-	0.5 g
Asparagine	-	0.5 g
Mannitol	-	1.0 g
Yeast extract	-	0.25 g
Agar	-	15.0 g
Distilled water	-	1000 ml

10. Tryptone broth.

Tryptone	-	10.0 g
NaCl	-	5.0 g
Yeast extract	-	1.0 g
Distilled water	-	1000 ml

11. Kovac's reagent.

Para dimethyl amino benzaldehyde (PDBA)	-	5.0 g
Amyl alcohol	-	75 ml
Conc. HCl	-	25 ml

12. Salkowski's reagent.

0.5 M $FeCl_3$	-	1.0 ml
35 % $HClO_4$	-	50 ml.