A STUDY OF THE MICROFLORA ASSOCIATED WITH TWO RUBBER CLONES AND THEIR BENEFICIAL ROLES

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BY

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CERTIFICATE

This is to certify that this thesis entitled "A STUDY OF THE MICROFLORA ASSOCIATED WITH TWO RUBBER CLONES AND THEIR BENEFICIAL ROLES" is a bonafide work of Mr Thomas Mathew conducted in the School of Bioscience, Mahatma Gandhi University, Kottayam, under the supervision and guidance of Dr C. Kuruvilla Jacob, Rubber Research Institute of India, Kottayam, during the academic year 1999-2000.

Kottayam
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This is to certify that this thesis entitled "A STUDY OF THE MICROFLORA ASSOCIATED WITH TWO RUBBER CLONES AND THEIR BENEFICIAL ROLES" is an authentic record of the research work carried out by Mr Thomas Mathew, under my scientific supervision and guidance at the Rubber Research Institute of India, Kottayam, in partial fulfilment of the requirement for the degree of Master of Philosophy of the Mahatma Gandhi University, under the faculty of science and no part thereof has been presented for the award of any other degree, diploma or associateship in any University.

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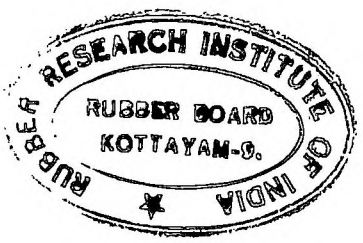
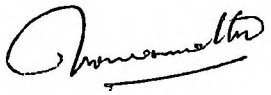
Dr. C. KURUVILLA JACOB
(Supervising guide)



DECLARATION

I hereby declare that this thesis entitled "A STUDY OF THE MICROFLORA ASSOCIATED WITH TWO RUBBER CLONES AND THEIR BENEFICIAL ROLES" has not previously formed the basis of the award of any degree, diploma, associateship, fellowship or other similar titles for recognition.



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..... Dedicated to my students.

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CHAPTER 1

INTRODUCTION

The Para rubber tree, *Hevea brasiliensis* (Muell. Arg) de Juss. is the most important commercial source of natural rubber. The latex obtained from rubber trees is processed into various marketable forms of natural rubber. Among the plantation crops in India, rubber occupies a key position. India is the fifth largest rubber growing country in the world and ranks fourth in natural rubber production. In India, *Hevea* rubber is cultivated in 5,50,000 ha and the annual production currently is around 6,00,000 tonnes. Traditionally rubber cultivation is confined to a narrow belt on the western side of peninsular South India, extending from Kanyakumari district of Tamil Nadu in the South to Kodagu district of Karnataka in the North, a stretch of about 500 kms.

Natural rubber industry in India has achieved tremendous strides in the past five decades, the total production increasing from 16,000 tonnes in 1950-51 to nearly 6,00,000 tonnes in 1998-99. This has been achieved mainly through increased cultivation of new and improved clones. It has been estimated that the present need for natural rubber in India is about 7,00,000 tonnes. As extension of rubber cultivation beyond the present limits in the traditional rubber tracts is constrained by the unavailability of land, increase in productivity would be the most prudent alternative to bridge the gap between the demand and supply of natural rubber. Development of new cost effective agrotechnology is imperative in this context.

Besides productivity, sustainability of agriculture is another major concern. Techniques, which could bring in more production with minimum

ecological impacts are to be evolved. Increase in productivity can be achieved by careful nutrient management, effective crop protection and appropriate harvesting techniques.

The role of soil microorganisms in efficient and sustainable nutrient management has been emphasised by several workers (Heng, 1989; Nealson and Saffarini, 1994; Ikram, 1994; Denorie, 1996). Synthetic fertilizers, which are produced in large factories, cause pollution both to atmosphere as well as to applied soil. These, can to a great extent, be replaced by efficient utilization of soil microorganisms. Atmospheric nitrogen (N) can compliment applied N if properly managed through nitrogen fixing microorganisms. Similarly Phosphorus (P) application can be considerably reduced if the P fixed in soil are made available using P solubilizing organisms.

In rubber, annual dose of 22.5 kg of N per hectare is recommended for young plantations. For mature plantations 30 kg N is recommended per hectare (Rubber Board, 2000). A portion of this can be supplied by nitrogen fixing microorganisms. Conversion of molecular nitrogen in the atmosphere to compounds of NO_2, NO_3, NH_3 and NH_4 are collectively called nitrogen fixation. Many species of cyanobacteria and eubacteria are known to fix atmospheric nitrogen through enzymatic reactions. Such activities are governed by a series of genes, together called Nif genes. Identification of N fixers and their utilization can be of help in reducing N consumption in rubber plantation.

The P requirement for young rubber is 22.5 kg/ha and for matures 30 kg/ha annually (Karthikakuttyamma *et al.*, 2000). After nitrogen, phosphorus is the second important plant nutrient, but more than 95 per cent

of it is not available to plants because it is present in soil in insoluble forms. These are made available by the action of P solubilizers. Many species of bacteria, fungi and actinomycetes are involved in this very important biological phenomenon. An attempt to locate P solubilizers in native soils can pave the way to their effective utilization in soils under rubber. There can be considerable savings in input cost if at least a portion of the N and P requirement is replaced with soil microbial manipulations.

Crop protection is another area where indiscriminate use of chemicals pose threat to sustainability. The annual consumption of copper fungicides in rubber plantation in India is estimated to be over 1000 tonnes. This fungicide is mainly used to protect the trees from *Phytophthora* spp. causing abnormal leaf fall disease leading to heavy crop loss (Jacob *et al.*, 1989). Antagonistic activity of microbes on other microorganisms, plants or animals has been of great interest for many years. Biocontrol activity is simply the utilization of this principle in the natural environment. Nature has been using this phenomenon to fight pathogens from the beginning of life on earth. Bacteria, fungi, yeasts and actinomycetes and even viruses can act as biocontrol agents. Some of these antagonists may be present in the phylloplane, cauloplane or rhizosphere of rubber plants. These could be identified, mass multiplied and used against pathogens if their effectiveness is proved.

Mycorrhizae or fungal roots is another group of microbes seen in symbiotic association with plant roots. It is a very essential phenomenon for the survival of most plants. This association help the plants in many ways such as increase in photosynthesis, N_2 fixation, P absorption and general growth (Joseph, 1997). There has been very few attempts to study the phenomenon in rubber plantations. The plant growth promoting

rhizobacteria (PGPR) present in the rhizosphere also interact positively with plants both directly and indirectly. Through their exudates containing hormones, vitamins, antibiotics, etc. they help to fight pathogens. Indirectly they help in the germination of vescicular and arbuscular mycorrhizal (VAM) spores, in the colonization and nodulation of nitrogen fixers and in the establishment of phosphate solublizing microorganisms (PSM).

A very important feature of these microorganisms is their interactions. Most of these microbes are more effective in presence or in association with some other microorganisms. Such interactions known as rhizocoenosis is more pronounced in the rhizosphere community which may be associative (neutral), harmful or beneficial.

This study aims at enumerating the microorganisms associated with two popular rubber clones namely, RRII 105 and PB 260 at three stages of tree growth from two plantations with a view to locate beneficial microorganisms that could be used or developed into useful tools in sustainable rubber cultivation. The phylloplane, cauloplane and rhizosphere microorganisms are assayed in this context.

The specific objectives of this study are listed below :

1. Analysis and enumeration of microorganisms such as bacteria, fungi, yeasts and actinomycetes from phylloplane, cauloplane and rhizosphere of two popular rubber clones *viz.* RRII 105 and PB 260 grown in two locations.
2. Enumeration and isolation of phosphate solubilizing microorganisms in the rhizosphere of the two clones and assessment of their relative efficiency.

3. Study of the nitrogenase activity in different soil samples from the two rubber growing regions.
4. Enumeration of VAM spores from the two rubber growing soils.
5. Study the extent of mycorrhizal association in root samples of the two clones.
6. Study the antagonistic effect of the different microbes on *Phytophthora meadii.*
7. Study the influence of pH and moisture content of the soils assayed, on the occurrence of the soil microorganisms.

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CHAPTER 2

REVIEW OF LITERATURE

The most important biotic factor that influences life on earth is the seemingly insignificant but omnipresent microflora. Though minute, their number and metabolic rate are so intense that they out-perform every other life forms. It is fascinating to study their mode of life, their interaction with other microbes, plants animals and even the lifeless organic and inorganic world around them. The literature available on the various aspects of their life is voluminous, but only a very brief review of the aspects closely related to the topic of this investigation is attempted here.

Based on the site of their occurrence, the various microbes associated with rubber plants are divided into phylloplane, cauloplane and rhizosphere organisms. Depending on their type, they could be bacteria, fungi, actinomycetes or yeasts. The literature on these organisms are reviewed separately because there is wide variations among the phylloplane, cauloplane and rhizosphere organisms. The various beneficial activities of these microorganisms such as nitrogen fixation, phosphate solubilization, biocontrol activity, VAM association are also discussed. The influence of two important edaphic factors, soil moisture and pH on rhizosphere microflora is also reviewed.

Phylloplane microflora

Phylloplane organisms were known for over a century. The term "phylloplane" was proposed independently by Last (1955) and Ruinen (1956) to the group of organisms residing on leaf surfaces. The beneficial

role of such organisms were recognized only much later (Last and Warren, 1972; Kinkel, 1997).

Phylloplane organisms may be either residents or casuals including bacteria, fungi, actinomycetes and yeasts (Ruinen, 1956; Leben, 1969; Kothandaraman, 1984). The kind and extent of microbial association with plants and the beneficial role of the organism have attracted several scientific investigations. Rao and Mullaiah (1988) listed a number of bacteria from the leaf surface of higher plants. These include *Azotobacter, Beijerinkia* (N_2 fixers) *Pseudomonas, Pseudobacterium, Phytomonas, Erwinia, Sarcina, etc.* Kothandaraman (1984), Rao and Mullaiah (1988) and George (1999) reported the presence of various fungi like *Aspergillus, Rhizopus, Curvularia, Trichoderma, Mucor, Fusarium* and *Alternaria.* Common yeasts are represented by *Torulla, Saccharomyces* and *Candida* (Kothandaraman, 1984; George, 1999). Actinomycetes population on phylloplane of rubber (*Hevea brasiliensis*) consisted of *Micromonospora, Nocardia, Actinomyces* and *Streptomyces* (Joseph *et al.*, 1988). George (1999) observed more bacterial genera like *Serratia, Bacillus* and *Aerobactor* from the phylloplane of rubber.

Phylloplane organisms may vary widely depending on seasons (Thomson, 1993). They do not occur uniformly across the leaf surface, but are localized in particular sites (Leben, 1969; Mariano *et al.*, 1993; Beattie and Lindow, 1999). The phylloplane organisms included both saprophytic and parasitic microbes which interact among themselves and also with the host (Last and Warren, 1972; Gokulapalan and Nair, 1991). The factors that affect the quantitative and qualitative nature of these organisms include type of plant species, age, health of the plants, season, climatic factors, location, agricultural operations like fertilizer application and pests and disease

control (Last, 1955; Dickinson *et al.*, 1976; Varma and Anlakh, 1981; Kothandaraman, 1984).

Nitrogen fixing activity and biocontrol activity of phylloplane organisms have also been reported (Kothandaraman, 1984; Rao and Mullaiah, 1988). There are only very few studies on phosphate solubilization of phylloplane microorganisms.

Cauloplane microflora

Of the three regions studied, cauloplane is the least explored area of plants. The bark surface microfungi was first studied by Bier (1963 & 1964). The term caulosphere was first used by Garner (1967) for the bark surface and the non-living cells within the bark. Some of the cauloplane microflora are antagonistic to parasites (Bier, 1963; Bier and Rowat, 1963). Garner (1967) found that, of the fungal component, many belong to fungi imperfecti. Cauloplane organisms are mainly influenced by environmental factors and vegetation around the trees (Mukerji and Rao, 1982).

Rhizosphere microflora

Clark (1949) suggested the term Rhizosphere as a zone of actual root influence on microbial life. He defined it as the external surface of plant roots along with the closely adhering soil particles. Development of rhizosphere community initiates with the seed germination and increases until the plant growth reaches a peak (Katznelson, 1965). The rhizosphere is rich in microbial colonization. Such concentration of microbial population in the rhizosphere is due to the presence of root exudates consisting of a mixture of aminoacids, sugars, organic acids, mucilage and

other substances, together with sloughed off root cap and other root cells (Griffin *et al.*, 1976; Rovira *et al.*, 1979). The extent of this zone generally depends on the activity of the plant, type of soil, acidity, moisture, nutrient status, electrical conductivity and redox potential of soil (Kothandaraman *et al.*, 1989; Jayaratne, 1995). The total rhizosphere environment is determined by an interacting trinity, the soil, plants and microbes (Jayaratne, 1995).

Enumeration of rhizosphere microorganisms associated with rubber has already been attempted (Joseph *et al.*, 1988; Ikram, 1989; Kothandaraman *et al.*, 1989; Deka *et al.*, 1992; Desai, 1999; Ikram and Yusoff, 1999). Fungal population seems to show higher variations in different seasons, when compared to other types (Deka *et al.*, 1992). All these reports agree that the highest number is for bacteria and lowest for yeasts. Joseph *et al.* (1988) and Deka *et al.* (1992) ranked actinomycetes to be second whereas others ranked fungi in the second place.

Most of the rhizosphere microorganisms belong to the group called Plant Growth Promoting Rhizobacteria (PGPR). Some help in direct growth of rubber plants (Ikram and Yusoff, 1999) while others help to promote the growth of nitrogen fixing leguminous plants like *Pueraria phaseoloides* (Ikram *et al.*, 1989 & 1994). Such activity may be due to the production of siderophores helping to displace deleterious microbes (Ikram *et al.*, 1989) or by the production of flavanoids (Parmar and Dodarwal, 1997).

Nitrogenase activity

Rhizosphere microorganisms associated with rubber trees can accelerate the nitrogenase activity both directly and indirectly. The free

living nitrogen fixers like *Azotobacter* and *Beijerinkia* directly enhance the nitrogenase activity (Wani *et al.*, 1988; Joseph, 1997). Symbiotic nitrogen fixing bacteria like *Rhizobium* also accelerate nitrogenase activity in the rhizosphere (Rangarajan and Hariharan, 1989; Ikram *et al.*, 1994; Denorie, 1996). Symbiotic nitrogen fixation can also be activated by arbuscular mycorrhizal fungi (Ikram *et al.*, 1994; Joseph, 1997).

Nitrogenase is an enzyme complex with two components, neither being active without the other. Component I is nitrogenase and component II is nitrogenase reductase. Component I is known as the MoFe protein and component II is designated as Fe protein which is a smaller molecule. The Fe protein is also called the leg haemoglobin. For nitrogen fixation to take place, a strong reducing agent like feredixin or flavodoxin and ATP are also needed (Pelczar *et al.*, 1993).

N_2 fixation is governed by a series of genes together known as the Nif genes (Pelczar *et al.*, 1993). Using modern tools of recombinant DNA technology and gene cloning, attempts are being made to introduce Nif genes to non-leguminous plants so that the use of chemical fertilizers can be reduced to some extent (Pelczar *et al.*, 1993).

Phosphate solubilization

Phosphate is another major plant nutrient required in large quantities for plant growth. More than 95 per cent of natural phosphates present in soil are in the tightly bound insoluble form and thus unavailable to plants. Plant can use it only in the soluble form such as $H_2PO_4^{-1}$, HPO_4^{-2} and PO_4^{-3}. The phosphate is made available in the soluble forms by phosphate

solubilizing microorganisms (PSM) present in the rhizosphere (Rao, 1981; Muhmed, 1984; Kapoor *et al.*, 1989).

Rock phosphate is the most common source of phosphatic fertilizer. Even though 100 million tons of rock phosphate deposits are available in India, only 1/6 of it is sufficiently enriched with P_2O_5 to be of use. Considering the cost involved in mining, transportation and pulvarization of rock phosphate, its use may not be very economic to farmers (Rao, 1981).

Several microorganisms, particularly those belonging to the genera *Pseudomonas* and *Bacillus* and fungi like *Aspergillus, Penicillium* and *Fusarium* are good phosphate solubilizers (Wani *et al.*, 1979; Rao, 1981; Bopaiah, 1985; Halder *et al.*, 1990; Gupta and Biswas, 1994; Narsion *et al.*, 1994). Many other bacteria are now known to dissolve insoluble phosphates in soil such as *Xanthomonas, Flavobacterium, Brevibacterium, Serratia, Alkaligens, Achromobactor, Acrobactor, Erwinia, Nitrosomonas, Thiobacillus, etc.* (Rao, 1981). Fungal genera include *Curvularia, Humicola, Sclerotium, Pythium, Acrothecium, Phoma, Mortierella, Cladosporium, Rhizoctonia, Cunninghamella, etc.* (Rao, 1981). Yeasts like *Candida* and actinomycetes like *Actinomyces, Micromonospora, Nocardia* and *Streptomyces* are also listed as PSM (Bopaiah, 1985; Ahemad and Jha, 1968).

Within the same genus, many species and within the same species, many strains are known to be phosphate solubilizers and their ability may vary depending on the substrate (Halder *et al.*, 1990). *Bacillus pulvifaciens, B. megaterium, B. circulans, B. subtilis, B. polymyxa, B. mycoides, B. mesentricus* and *B. fluorescens* are phosphate solubilizing bacteria. *Aspergillus niger, A. flavus, A. fumigatus, A. awamori* are fungal phosphate

solubilizers (Rao, 1981). Gupta and Biswas (1994) isolated six P solubilizing strains of *Aspergillus niger* and Narsian *et al.* (1994) succeeded in isolating 27 strains of *Aspergillus* and 7 strains of *Penicillium*..

Phosphate solubilization involves various steps such as acidification, chelation and ion exchange mechanisms. Humic acid (Heng, 1989) and carbonic acid (Kapoor *et al.,* 1989) are actively involved in the process. Optimum pH for bacteria was 7-8 while it was 6-7 for fungi. The optimum temperature was 30-35°C for most P solubilizers except for *Bacillus polymixa* for which it was 35-40°C (Wani *et al.*, 1979). Chelating agents like α–ketoglutaric acid also has an important role in solubilizing phosphates. Phosphate solubilization through composting is another popular method (Kapoor *et al.*, 1989).

Biocontrol activity

Antagonistic activity of one microorganism on another or on higher plants or animals has been of great interest for many years. Antibiotic industry is based on this phenomenon. Biocontrol activity is simply the utilization of this principle in the natural environment. Nature has been practicing this phenomenon to fight pathogens from the beginning of life on earth. Many plant diseases and pests can be effectively controlled using biological means (Andrews, 1992; Heiniger, 1994; Sivasithamparam, 1998; Burr and Otten, 1999; Hoitink and Boehm, 1999). Identification of microorganisms which are predatory or parasitic on pests and weeds (Sikora, 1992; Beast, 1992; Bridge, 1996; Barker and Koenning, 1998) or antagonistic to pathogens (Beast, 1992; Andrews, 1992; Gupta and Biswas, 1994; Sivasithamparam, 1998) have already been attempted.

Rhizobacteria like *Pseudomonas* and *Bacillus* are effective biocontrol agents (Podile *et al.*, 1988; Rangarajan, 1994; Pal and Jalali, 1998; Steddom and Menge, 1999). *Azotobacter* and *Rhizobium*, the best known nitrogen fixers are also known for their synergistic effect in *Cicer arietinum* (Rawat, 1978). Among fungi, *Trichoderma* and *Penicillium* are well known antagonists (Subbaiah *et al.*, 1984; Suneja *et al.*, 1984; Podile *et al.*, 1988; Jacob *et al.*, 1991; Larkin and Fravel, 1999). Many other bacteria and fungi are also proved to be very good biocontrol agents (Podile *et al.*,1988; Krishnamohan, 1994; Rangarajan, 1994; Vanitha *et al.*, 1994, Chakraborty *et al.*, 1998). Actinomycetes are probably the single largest group of potential biocontrol agents with most of their members being antagonistic (Pal and Jalali, 1998). Nematodes and pests can also be controlled using microorganisms (Suneja *et al.*, 1984; Nehru *et al.*, 1991; Sikora, 1992; Bridge, 1996; Barker and Koenning, 1998).

Phytophthora spp. is the most important pathogen on rubber in India causing several diseases namely, abnormal leaf fall, shoot rot, black stripe and patch canker (Edathil *et al.*, 2000; Kothandaraman and Idicula, 2000). *Corynespora, Colletotrichum, Corticium, Ustulina, Pythium, etc.* are other leaf and stem parasites and *Rigidoporus, Phellinus* and *Ganoderma* are important root pathogens (Liyanage and Jacob, 1992). Some strains of actinomycetes from the rhizosphere antagonistic to *Phellinus noxius* have been isolated (Kothandaraman *et al.*, 1991) and *Corticium salmonicolor* (Joseph *et al.*, 1991). Joseph *et al.* (1988) identified an actinomycetes to be antagonistic to *Phytophthora meadii* (Vanitha *et al.*, 1994). Jayasuriya (1996) isolated Trichoderma and *Glyocladium* to check white root disease in rubber. Jacob *et al.* (1991) found *Trichoderma harzianum* to be antagonistic to *Phellinus noxius* causing brown rot of *Hevea*.

Mechanism of action may vary between the various antagonists. Parasitism, predation, competition, secretion of antibiotics or bacteriocins, production of hydrogen cyanide, stimulation of phytolaxin production, production of flavanoid like compounds and other cellular metabolites are some of the mechanisms involved (Jayasuriya, 1996; Pal and Jalali, 1998). Biocontrol activity can also be imparted by increasing the resistance of the host due to better health obtained from other microbial actions such as nitrogen fixation, phosphate solubilization, *etc.* (Rawat and Sanoria, 1978). Siderophore producing organisms are found to be very effective in biocontrol (Podile *et al.,* 1988; Rangrajan, 1994; Krishnamohan, 1994; Jayasuriya, 1998).

Biocontrol organisms are mostly of rhizosphere origin (Subbaiah *et al.,* 1984; Podile *et al.,* 1988; Krishnamohan, 1994; Pal and Jalali, 1998; Hoitint and Boehm, 1999). Many phylloplane organisms are also known to be antagonistic (Andrews, 1992; Chakraborty *et al.*, 1998). Some cauloplane organisms are also proved to be effective in protecting plants from other pathogenic microorganisms (Bier, 1963 & 1964).

Vesicular and arbuscular mycorrhizae (VAM)

About 95% of world's vascular plant species are reported to be mycorrhizal (Lakhanpal and Anandsagar, 1994). VAM are endomycorrhizal associations and their infection rate may vary in different plants (Trimurthulu and Johri, 1998; Schwob *et al.,* 1999). The beneficial effects of VAM fungi may be due to different factors. They are known to increase the rate of photosynthesis, help in phosphate absorption, influence the rhizosphere microflora, augment the uptake of zinc and copper and

increase growth, yield, biomass, nodulation and nitrogen fixation (Ikram, 1985; 1996; Joseph, 1997).

VAM fungi are affected by increased nitrogen and phosphorus content in the soil (Bagyaraj, 1995; Joseph, 1997) and pesticides. Modern high input agriculture practices are detrimental to VAM (Bagyaraj, 1995). Some plants are seen to be mycorrhiza dependent (Pelczar *et al.,* 1993). All plants of Poaceae and Fabaceae are mycorrhizal whereas families like Cruciferae, Chenopodiaceae and Caryophyllaceae are known to be devoid of VAM fungi. Important genera of VAM fungi found in rubber growing soils are *Glomus, Gigaspora, Acaulospora* and *Sclerocystis* (Joseph, 1997). VAM fungi do not lead an independent life, but exist as an interacting community. They interact with nitrogen fixers, phosphate solubilizers, antagonists and with other plant growth promoting rhizobacteria (Saxena and Tilak, 1994).

Edaphic factors

Microbial life in the rhizosphere depends on numerous factors such as moisture, Hydrogen ion concentration (pH), organic and mineral content of the soil, soil texture and other biotic factors (Hashim and Azaldin, 1985; Joseph *et al.,* 1990; Yew and Pushparaj, 1991; Lau, 1992; Samarappuli and Yogaratnam, 1994; Centurion *et al.,* 1995). Of these, moisture content and pH has more direct influence on microbial life. Low pH (4-5) favours fungal life whereas high pH (6-7) promotes bacterial life. Normal pH of rubber growing soils in India is around 5. This is basically a balanced condition for most microorganisms such as fungi, bacteria and yeasts (Hashim and Azaldin, 1985; Samarappuli and Yogaratnam, 1994). As the

life of microorganisms is dependent on the pH and moisture in the soil, an alteration in these shall affect their population.

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CHAPTER 3

MATERIALS AND METHODS

Experimental materials included leaves, bark and rhizosphere soil along with the feeder roots of two popular rubber clones RRII 105 and PB 260, from two locations, Cheruvally estate near Erumely and Malankara estate in Thodupuzha in Kerala State, India. From each location and clone, three replicated samples were collected for three age groups such as 2 to 3 year old trees (1997-98 plantations), 5 to 6 year old trees (1994-95 plantations) and 10 to11 year old trees (1989-90 plantations). For each clone a total of 18 leaf, bark and rhizosphere (soil and root) samples were analysed.

CLONES

RRII 105

This is currently the most popular clone cultivated by both commercial plantations as well as small scale farmers. It is developed by the Rubber Research Institute of India and has an average yield of 2400 kg/ha/yr. The clone is fairly tolerant to abnormal leaf fall disease (*Phytophthora* spp.) but highly susceptible to *Corticium salmonicolor* causing pink disease and *Corynespora cassiicola* causing leaf disease.

PB 260

This is a very vigorous clone with dense canopy and balanced branching. This is also a high yielding clone with an average yield of over 2000 kg/ha/yr. The clone is susceptible to diseases caused by *Phytophthora* spp., *Corticium salmonicolor* and *Pythium* sp.

LOCATIONS

Cheruvally Estate, Erumely

This is one of the oldest plantations in Kerala extending over 360 ha of moderate hills at an altitude of 80 m above msl. The rainfall is nearly 4000 mm per annum distributed in May/June to October/November. In summer months water can be a limiting factor.

Malankara Estate, Thodupuzha

This plantation is established comparatively later on both sides of Moovattupuzha river, extending over 200 ha at an altitude of 106 m above msl and with an annual rainfall of nearly 4200 mm. Moreover, the newly built Malankara dam helps in maintaining the soil moisture as well as the atmospheric humidity. Because of the higher humidity, proneness to diseases is comparatively higher.

COLLECTION OF SAMPLES

Collection of leaves

Mature and healthy leaves from four different rubber trees in an area were collected during February when the leaves became mature after wintering and pooled together for each replication. The samples were labelled and packed in newspaper. The label tag contained the location, clone number, year of planting, replication number and the date of collection. Leaf samples were used immediately for isolation avoiding long storage. Only the leaflets were used for further investigation.

Collection of bark

Bark scrapings from four different rubber trees from an area were collected in polythene bags. Scrapings were collected from an area of 25 cm^2 (5 cm x 5 cm) from each plant and were pooled together to get one replication sample. Eighteen such samples were collected from the two locations and were packed, labelled and stored in the refrigerator until used for culturing and isolation. Scrapings were taken from 150 cm above the ground to avoid contamination from soil microbes.

Collection of rhizosphere samples

Rhizosphere includes both roots and the adjoining soil in which it grows. The approach was to collect the subsoil just under the humus along with the feeder roots that were available in the zone. For each replication a total of 500 g of soil was collected and pooled. It was collected in polythene bags and labelled with tags as described above. As in the case of leaves and bark, eighteen samples were collected from each location and were stored in a refrigerator.

ISOLATION AND ENUMERATION

The various microorganisms present in each sample were isolated for enumeration studies and further investigation of their beneficial roles.

Media

The media used, their pH and the methodology varied with the samples from phylloplane cauloplane and rhizosphere. For isolation of

phylloplane and cauloplane organisms, Leben's medium was used (Leben , 1972).

Leben's Medium

The composition of the basal medium was as follows :

Peptone	:	10.0 g
Casein hydrolysate	:	1.0 g
Glucose	:	5.0 g
Agar	:	20.0 g
Distilled water	:	1000 ml
pH	:	6.5 to 6.8

This basal medium was modified in various ways and was used in the isolation of bacteria, fungi and yeasts.

Medium for phylloplane and cauloplane bacteria

Cyclohexamide (50 mg) dissolved in 12 ml of distilled water and 2,3,5 triphenyl-dihydro tetrazolium chloride (50 mg) dissolved in 12 ml of distilled water were sterilized separately in an autoclave, cooled and added into a litre of the sterilized and cooled basal medium just prior to plating. Cyclohexamide acts as a fungicide whereas the tetrazolium chloride provides colour to bacterial colonies.

Medium for phylloplane and cauloplane fungi

To one litre of the cooled sterilized basal medium 500 mg of the broad spectrum antibiotic tetracycline hydrochloride was added and mixed well just before pouring the plates.

Medium for phylloplane and cauloplane yeasts

To 250 ml of the basal medium amended with 125 mg of tetracycline hydrochloride, 5.5 ml of 0.1 normal sulfuric acid was added (to adjust the pH between 4.4 and 4.8) just prior to pouring into plates.

Preparation of phylloplane inoculum

Leaf samples (100 cm^2) was cut out from three or four leaves selected at random from each sample collected and was made into smaller pieces. It was introduced into the conical flasks containing 100 ml sterile water and was shaken for 30 min on a rotary shaker. From the above inoculum serial dilutions up to 10^{-5} were made and one ml aliquotes were plated with respective media for bacteria, filamentous fungi and yeasts.

Serial dilution technique

One ml of the inoculum (leaf washing, soil sample etc. from the shaker) was pippeted out into a fresh test tube, 9 ml of sterile water was added and shaken thoroughly to get the first level of dilution (10^{-1}). From this another 1 ml was taken, diluted with 9 ml sterile water and shaken well to get the second level of dilution (10^{-2}). This process was repeated until the desired dilutions were obtained. In the present study dilutions up to 10^{-7} were prepared.

Preparation of cauloplane inoculum

The collected bark scraping sample from approximately 100 cm^2 was introduced into 250 ml of sterile water in a conical flask and was shaken on

a rotary shaker for about 30 min. This was used as the stock inoculum for serial dilution to desired levels.

Isolation of phylloplane and cauloplane bacteria

The isolation techniques, the dilution levels of the inoculum and the medium used were the same for both phylloplane and cauloplane bacteria. One ml of the 10^{-5} strength inoculum was pippeted out into a sterile petriplate under sterile conditions. About 20 ml of the medium for either phylloplane or cauloplane bacteria was added into the petridish and was mixed well by rotating the petridishes in both directions. This was repeated in duplicate for all the samples. The plates were incubated at 28±1°C for 4 to 5 days.

Enumeration of phylloplane and cauloplane bacteria

After 4 to 5 days of incubation the petriplates were examined for bacterial growth. Based on the colour and shape of the colonies, bacteria were differentiated into different strains (Plate 1.1). The bacterial strains were counted separately using a colony counter. The total bacterial count was calculated from this, considering the level of serial dilution in each case.

Isolation of phylloplane and cauloplane fungi

One ml of 10^{-3} strength inoculum was poured into a sterile petriplate under sterile conditions. Into this, about 20 ml of the medium was added and mixed well by rotating the petridish. This was repeated with all the samples in duplicate. The plates were incubated at 28±1°C for 4-5 days.

Enumeration of phylloplane and cauloplane fungi

After the incubation period, the petriplates were examined for fungal colonies. Since each fungus has a characteristic growth habit and colour, fungal strains could be easily differentiated (Plate 1.2). The number of each strain of fungal colonies was recorded. The total number of fungi per unit area (1 cm^2) was calculated by considering the dilution.

Isolation and enumeration of phylloplane and cauloplane actinomycetes

No special procedure was adopted for separating actinomycete population from the leaf and bark surfaces. Actinomycetes grow well in the fungal medium to produce smaller thick colony masses which could be easily distinguished from the rapidly spreading fungal colonies. Incubation period was extended up to 7 days at 28±1°C as the growth rate was slow, Actinomycete colonies were identified and counted from the dilution plates for the fungal enumeration.

Isolation and enumeration of phylloplane and cauloplane yeasts

Into 1 ml of the 10^{-1} inoculum 20 ml of the Leben's medium modified specially for yeasts (pH 4.4 to 4.8) was added and mixed well prior to solidification. Plates were incubated at 20°C for 7 days. Colonies appeared as small creamy white spots and were counted and the number of yeast cells present in unit area (1 cm^2) of the leaf and bark surface was calculated considering the dilution factor.

Isolation of rhizosphere microflora

Five different media were used for isolating rhizosphere microflora. They were rose bengal agar (RBA) for fungi, soil extract agar (SEA) for bacteria in general, apetite agar (AA) for phosphobacteria, Ken Knight's agar (KA) for actinomycetes and Jensen's agar (JA) for *Azotobacter*. Composition for each of these media is given below :

1. Rose bengal agar (RBA)

Dextrose (Glucose)	-	10.0 g
Peptone	-	5.0 g
K_2HPO_4	-	1.0 g
$MgSO_4$ ($7H_2O$)	-	0.5 g
Rose bengal	-	30.0 mg
Streptomycin (1% solution)	-	0.3 ml/100 ml (to be added just before use)
Agar	-	15.0 g
Distilled water	-	1000 ml
pH	-	5.5

2. Soil extract agar (SEA)

Soil extract	-	100 ml
K_2HPO_4	-	0.5 g
Glucose	-	1.0 g
Agar	-	15.0 g
Distilled water	-	900 ml
pH	-	7 to 7.2

Soil extract was prepared by autoclaving 1000 g of soil in 1000 ml water for one hour and filtering it through a filter paper. 100 ml of this was used in the preparation of SEA.

3. Apetite agar (AA)

Yeast extract	-	0.2 g
$(NH_4)_2 SO_4$	-	0.5 g
$MgSO_4(7H_2O)$	-	0.1 g
KCl	-	0.2 g
Glucose	-	10.0 g
Soil extract	-	200 ml
K_2HPO_4 (10%)	-	6 ml/100 ml
$CaCl_2$ (10%)	-	4 ml/100 ml
Distilled water	-	800 ml

K_2HPO_4 and $CaCl_2$ were prepared and sterilized separately and added to medium just prior to plating. Soil extract was prepared as explained for SEA.

4. Ken Knight's agar (KA)

K_2HPO_4	-	1.0 g
$NaNO_3$	-	0.1 g
KCl	-	0.1 g
$MgSO_4$ $(7H_2O)$	-	0.1 g
Cellulose source (Filter paper strips)	-	10.0 g
Distilled water	-	1000 ml
pH	-	7 to 7.2

5. Jensen's agar (JA)

Sucrose	-	20.0 g
K_2H PO_4	-	1.0 g
$MgSO_4$ $(7H_2O)$	-	0.5 g
Fe SO_4 $(7H_2O)$	-	0.1 g
$CaCO_3$	-	2.0 g
NaCl	-	0.5 g
Na_2MoO_4	-	0.005 g
Agar	-	15.0 g
Distilled water	-	1000 ml
pH	-	6.5 to 7.0

All media were prepared, sterilized, labelled and stored. When needed it was melted in a microwave oven and allowed to cool down to the desired temperature.

Preparation of rhizosphere inoculum

Into 100 ml of sterile water in a 250 ml conical flask, feeder roots of *Hevea* with attached soil particles were added. The quantity of roots added was such that after dissolving and drying, the final dry weight of the soil was approximately one gram. The flasks with the roots were shaken well on a rotary shaker for 30 min and was allowed to settle slightly. From this inoculum one ml was taken and used for serial dilutions up to 10^{-7}.

After the inoculum was removed the whole content of the conical flask was transferred into a clean petridish whose initial weight was known. All the roots were removed and the water along with the soil was allowed to dry on a water bath. After drying the final weight was determined to confirm the approximate weight of the dry soil.

Isolation and enumeration of rhizosphere fungi

To 100 ml of molten and cooled RBA, 3 ml of 1% streptomycin was added. It was mixed well and about 20 ml of this was poured into a petriplate with 1 ml of 10^{-3} dilution inoculum. The plate was rotated both ways so that the inoculum mixed well with the medium. This was repeated in duplicate with all inoculum samples. Plates were incubated for 4 to 5 days at 28±1°C. Total count and specific count of individual strains were then recorded with a colony counter, from which their number per gram of soil was calculated.

Isolation and enumeration of rhizosphere bacteria

Soil extract agar (SEA) was used for isolating and culturing of soil bacteria. Inoculum diluted to the seventh degree (10^{-7}) was used for isolation. The plates were incubated at 28±1°C for 4 to 5 days and bacterial count was recorded and the total number per gram of soil calculated.

Isolation and enumeration of rhizosphere phosphobacteria

Apetite agar (AA) was the specific medium used for isolating phosphobacteria. To every 100 ml of the molten medium 6 ml of 10% K_2HPO_4 and 4 ml of 10% $CaCl_2$ (both sterilized separately) were added so that the phosphate is precipitated in the medium. Phosphate solubilizing bacteria helps to dissolve this precipitate, indicated by a clear halo around such colonies.

1 ml of 10^{-6} dilution inoculum was taken in a petriplate and about 12 to 15 ml of the precipitated medium was poured on to it and mixed well to form a thin layer. This was repeated with all samples in duplicate. The plates were incubated at 28±1°C for about 4 days. Colonies showing the halo were counted as phosphobacteria. Different strains could be identified by their colour and shape. The total number per gram of soil was calculated.

Isolation and enumeration of rhizosphere actinomycetes

The inoculum (4^{th} dilution) was plated thinly with Kenknight's agar (KA). Since actinomycetes grow very slowly, plates were incubated for about 2 weeks for the colonies to start sporulation making them much

denser. Based on the number of colonies formed, their number per gram of soil was determined.

Isolation and enumeration of rhizosphere yeasts

No special medium or method was used for isolating the soil yeasts. They were identified from the fungal culture plates based on the colour and texture of the colonies and were counted.

Isolation of *Azotobacter* from soil

Azotobactor was isolated from soil by culturing concentrated inoculum (10^{-1}) on Jensen's Agar (JA). Due to the absence of any nitrogen source in the medium only nitrogen fixers can grow well on this medium.

Maintenance of pure cultures

The isolated cultures were maintained on potato dextrose agar medium (PDA) for fungi, actinomycetes and yeasts. Bacteria (including phosphobacteria) were maintained on nutrient agar (NA) medium. The composition and the method of preparation of these media are given below :

Potatoe dextrose agar (PDA)

Pealed potato	-	250 g
Glucose	-	20 g
Agar	-	15 g
Distilled water	-	Enough to make up to 1 litre
pH	-	6 to 6.5

Pealed potatos were cut into small pieces and boiled for half an hour. The extract was strained and collected and was made up to 1 litre with

distilled water. Into this, agar and dextrose were added and heated again in a water bath for 15 to 20 min until the agar was fully melted. This was poured into conical flasks or into test tubes. For making slants 5 to 6 ml was poured into each test tube and plugged with cotton. The conical flasks (with 200 or 250 ml medium) were also plugged tightly with cotton. After plugging the medium was sterilized in an autoclave at 121°C for 30 min. After taking out the test tubes were kept in a slanting position until the medium solidified and were then stored.

Nutrient agar (NA)

Peptone	-	5.0 g
Glucose	-	5.0 g
Beaf extract	-	3.0 g
NaCl	-	5.0 g
Distilled water	-	1000 ml
pH	-	6.8 to 7

All the components were dissolved one by one in distilled water. This was then sterilized in conical flasks or made into slants.

Purification of colonies

Step 1. The isolated colonies were purified using following steps. A small bit of each colony was transferred carefully onto thin clear agar plates in the case of fungi, actinomycetes and yeasts and NA plates in the case of bacteria. Bacteria and yeasts were streaked on the plates and isolated colonies growing from single cells were picked. Actively growing and isolated single hyphal tip of fungi or actinomycetes were carefully removed and introduced at the center of plates with PDA and allowed to grow. The transfer was done very carefully under aseptic conditions to avoid further

contamination while transferring. These were allowed to grow for 4 to 5 days.

Step 2. Once the organisms establish themselves on the above plates in pure form they were subcultured on PDA or NA slants on which they were maintained for longer duration without any contamination or deterioration of vigour.

BENEFICIAL ROLE OF ISOLATED MICROBES

Detailed study was conducted on two beneficial aspects of microorganisms to the rubber trees. These were (1) phosphate solubilizing activity, and (2) biocontrol activity against one of the major pathogens of rubber – *Phytophthora meadii*. The extent of the nitrogenase activity in the soil was also studied to ascertain the effect of nitrogen fixing microorganisms. The percentage of vesicular and arbuscular mycorrhizal (VAM) spores in soils and their colonization in the roots of rubber plants were also studied.

Phosphate solubilization

The phosphate solubilization was assessed by (1) culture plate technique and (2) colorimetric technique.

(1) Culture plate technique

Pure cultures of the identified phosphobacteria were streaked at the middle (single streak) of precipitated AA. Plates were incubated for 4 to 5 days and the extent of the clear zone around the colony was measured. The presence of the halo confirms their phosphate solubilizing activity and the

difference in extent of the clear zones give a qualitative measure of their relative efficiency.

(2) Colorimetric estimation

Selected cultures of fungi or bacteria were grown in 100 ml aliquots of Pikovaskaya's broth (see below) for 12 days at 28±2°C. Colorimetric method of estimation of phosphate solubilization was carried out for selected strains of bacteria as well as a few fungal species isolated. The fungi included *Penicillium, Aspergillus* and *Fusarium*. In the case of fungi, the culture was filtered using Whatman No.42 filter paper. Due to pigments, the filterate was often coloured. In such cases, 1 to 2 g of activated charcoal was added to it and shaken until the filtrate became colourless. Bacterial cultures were filtered through Whatman No.1 paper to remove insoluble phosphates and centrifuged at 10,000 rpm for 10 to 15 min. Filtration and centrifugation were repeated until a clear solution was obtained. This was finally made up to 50 ml with distilled water. To 10 ml of each of the filtrate, 2.5 ml of Barton's reagent was added and the volume made up to 50 ml by adding distilled water. After 10 min, the resultant colour was read in the colorimeter at 430 ηm using a filter.

Preparation of standard graph

Exactly 0.2195 g of KH_2PO_4 was weighed and dissolved in distilled water and this solution was made up to 1000 ml with distilled water. It was further diluted by taking 10 ml of the above solution and making it up to 250 ml. Concentration of phosphorus at this dilution will be 2 ppm/ml (stock solution).

Aliquotes of 0, 2, 3, 4,, 5, 6, 8, 10, 15 and 20 ml of the stock solution were taken in 50 ml volumetric flasks. 2.5 ml of Barton's reagent (see below) was added to each one of the flasks which were later made up to 50 ml with distilled water. Flask with '0' ml stock solution acted as blank. After 10 min, optical density (OD) was read using the colorimeter at 430 ηm. A standard graph was prepared with OD values of the known concentrations.

By plotting the OD values of the unknown sample solutions and extending them down to the X-axis, concentration of dissolved phosphates in each case were determined. OD values for all samples and their concentrations were thus determined.

Pikovskaya's medium

Glucose	-	10.0 g
$Ca_3(PO_4)_2$	-	5.0 g
$(NH_4)_2 SO_4$	-	0.5 g
KCl	-	0.2 g
$Mg SO_4 \ 7H_2O$	-	0.1 g
$Mn SO_4$	-	Trace
$Fe SO_4$	-	Trace
Yeast extract	-	0.5 g
Agar	-	15.0 g
Distilled water	-	1000 ml

For preparing Pikovskaya's broth, agar was avoided. In this experiment, instead of $Ca_3(PO_4)_2$ same amount of rock phosphate was used as a source of insoluble phosphate.

Barton's reagent

Solution A : Ammonium molybdate (25 g) dissolved in 400 ml water.

Solution B : Ammonium metavanadate (1.25 g)was dissolved in 300 ml boiling water. It was allowed to cool and then 250 ml Conc. Nitric acid was added. Later the solutions A and B were mixed together and was made up to 1 litre with distilled water to get Barton's reagent.

Biocontrol activity

All isolated organisms were grown separately on either PDA plates or NA plates depending on the organism. *Phytophthora meadii* also was grown on PDA plates. A total of 92 different isolates of microorganisms were tested (in duplicate) for antagonism. They included 21 fungal isolates, 20 bacterial, 5 actinomycete and 5 yeast isolates from phylloplane/cauloplane sources and 21 fungal, 10 phosphobacterial and 10 other bacterial isolates from rhizosphere sources.

With the help of a sterile cork borrer, a circular bit from a 4 day old *Phytophthora* culture was cut and was inoculated at the middle of a fresh plate with PDA or NA. Four different fungal or actinomycete isolates (bits of mycelia) were inoculated at the four corners of the PDA plates. In the case of yeasts only two were inoculated on both sides of the pathogen bit placed in the middle. Bacteria were inoculated as small streaks on the media on either side of the pathogen.

All the plates were incubated at 28±2°C for five days to study the antagonistic activity which varied from parasitism or predation to secretion of inhibitory materials into the medium. Prospective antagonists were identified and further evaluated using dual culture technique.

Dual culture technique

The experiment was repeated with just one organism along with the pathogen inoculated on opposite sides of a petriplate containing medium. The plates were incubated for 7 days and the width of the clear zone formed in between the growth of the test organisms in each case was measured.

Parasitism and predation was evidenced by an overgrowth of the antagonist over the parasite. Degeneration of hyphae of *Phytophthora* or coiling of the antogonist around the *Phytophthora* hyphae was observed microscopically.

Nitrogenase activity

Principle

Nitrogenase activity in the soil was studied indirectly by measuring the ethylene formed by reaction of gaseous hydrogen released during such activity with acetylene supplied to a closed system (glass tube) using a gas chromatograph. The amount of ethylene assayed is directly proportional to the hydrogen released, which in turn is proportional to the nitrogenase activity. Nitrogenase activity was calculated from the observed values. The procedure adopted is described below in steps :

Step

1. The soil sample was sieved to eliminate stones and pebbles.
2. 20 g of the above soil was weighed and introduced into a special bottle made for this purpose.
3. 0.1% glucose solution was prepared (0.1 g in 100 ml).

4. 5 ml of this solution was poured into each of the tubes with the soil samples and were closed with cotton plugs.
5. The tubes were incubated for 24 hours, so that the glucose helped in activating the free living nitrogen fixers like *Azotobacter* or *Biejerinkia*.
6. The cotton plug was removed and the tubes were closed with sterilized (with alcohol) air tight rubber stoppers that are specially made for this purpose.
7. 5 ml of air was removed from the bottle using a syringe, through the stopper.
8. Acetylene was prepared separately by adding a small piece of calcium carbide in water taken in a bottle closed with a rubber cork through which an injection needle was inserted to allow excess gas to escape.
9. 5 ml acetylene was taken and injected into each bottle containing the samples.
10. The bottles were incubated again for 24 hours at 28±1°C.
11. 1 ml of the gas from each bottle was removed and injected into the gas chromatograph (GC) apparatus and the reading was taken.
12. The nitrogenase activity was calculated from the above reading.

Calculation

Nitrogenase activity in the various soil samples was calculated using the formula :

$$\frac{\text{Sample Ethylene after 1 hr}}{\text{Standard Ethylene after 1 hr}} \times \frac{\text{GV(ml)} - \text{VCF x VPM}}{22.4\,(T_1 - T_0)\ \text{hr}}$$

where sample ethylene after 1 hr is the reading obtained from the GC apparatus
standard ethylene after 1 hr is a constant (6600)
GV is the gas volume of the container (84 ml)

VCF is the vacuum correction factor (∅)
VPM is ethylene concentration
$T_1 - T_o$ is the difference in sampling time (in this experiment it is 24 hrs.)

Vesicular and arbuscular mycorrhyzae (VAM)

The presence of VAM in the rubber growing soils and their association with rubber roots were studied by root infection assay and spore count.

Root infection assay

About 5 g of feeder roots was taken and cleaned thoroughly with tap water, cut into one cm bits, placed in a beaker and 1.8 m (10%) potassium hydroxide solution, added. This was boiled in a waterbath for about 45 min, washed thoroughly in tap water, the excess water drained off and 5 m HCl (1:1) added to neutralize the alkali. The sample was steeped in HCl for about 2 min to clear the roots. The acid was then washed away with tap water, drained and stained with cotton blue in lactophenol by heating on a waterbath for about 3 min. Excess stain was washed off with lactophenol. Roots were mounted on glass slides and examined under a light microscope to observe the stained fungal mycelia, vesicles and arbuscules. Ten bits were examined per sample and the average infection was expressed as percentage.

Spore count

The wet sieving method described by Gerdemann and Nicolson (1963) was followed for the VAM spore count. 20 g of soil sample was

taken in a 500 ml beaker into which about 400 ml of water was added. It was stirred well and allowed to settle for about 30 seconds. The supernatant along with the floating particles was decanted through a series of meshes (710, 250, 105 and 45 μm) with the largest sieve on the top.

The process was repeated four times and the debris collected in the top mesh was discarded. The residue on the three lower meshes were collected using a water jet into a 100 ml beaker and was made up to 100 ml with water. The contents of the beaker was agitated and 1 ml aliquots were drawn into a counting slide and examined under a microscope. The average count from five 1 ml aliquots was recorded. The spore count was expressed as number per gram of soil.

EDAPHIC FACTORS

The moisture content of the soil and soil pH were also recorded to observe their influence on the soil microflora.

Soil moisture content

Approximately 10 g of soil sample was transferred to a pre-weighed weighing bottle and its accurate weight was determined using an electronic balance. The samples in the weighing bottles were kept in an oven at 70°C and dried to a constant weight. The difference between the dry and fresh weights gives the weight of moisture present. This was expressed as percentage. The correlation between the soil moisture content and population of soil microorganisms was worked out.

Soil pH

Soil pH was determined using a pH meter. The pH of a 1:1.5 soil/water solution was measured using glass electrode. 10 g soil was used for each sample and the solution prepared with distilled water.

DATA ANALYSIS

All the data were tabulated and analysed statistically on a computer using appropriate software. A completely randomized factorial design was adopted for the analysis to bring out the main effects and the interaction of the factors under consideration.

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CHAPTER 4

EXPERIMENTAL RESULTS

The results of the various experiments designed under this study are presented below on the basis of the statistical tools applied.

ENUMERATION OF MICROORGANISMS

Phylloplane microflora

(i) **Phylloplane fungi** : There was no significant difference in the total count of phylloplane fungi between the clones, locations and age groups when considered separately (Table 1). However, a significant interaction effect was evident when the clones and age group were considered together. In 2 to 3 year old plantations, the clone RRII 105 harboured significantly higher ($P \leq 0.01$) number of phylloplane fungi (176 x 10^2/cm^2 of leaf area) when compared to PB 260 (105.33 x 10^2/cm^2 of leaf area). The trend remained the same in 10 to 11 year old plantations also, but was reversed in 5 to 6 year old plantations. The difference, however, was not statistically significant in the later cases.

(ii) **Phylloplane bacteria** : Bacterial population in the phylloplane showed significant variation with clones, locations and age of the plantations when considered individually or collectively in different combinations (Table 2). In general, RRII 105 harboured significantly high (77.77 x 10^5/cm^2 of leaf area) population of phylloplane bacteria when compared to PB 260 (35.61 x 10^5/cm^2 of leaf area). Of the two locations phylloplane bacterial population of rubber trees in Malankara estate was significantly higher (95.27 x 10^5/cm^2 of leaf area) than that in

Table 1. Phylloplane fungi (x $10^3/cm^2$ of leaf area)

1.1 Single factor averages

CLONE (A)		LOCATION (B)		AGE GROUP (C)		
RRII 105	PB 260	CHERUVALLY	MALANKARA	2-3 YRS	5-6 YRS	10-11 YRS
153.5	137.56	138/33	152.77	140.67	133.00	162.92

1.2 Two factor interactions

INTERACTION	↓ LOCATION / CLONE (AxB) →	RRII 105	PB 260
CLONE X LOCATION	CHERUVALLY	139.33	137.33
	MALANKARA	167.67	137.78
	↓ AGE GROUP/LOCATION (AxC)→	RRII 105	PB 260
CLONE X AGE GROUP	2-3 years	176.00	105.33
	5-6 years	112.33	153.67
	10-11 years	172.17	153.67
	↓ AGE GROUP/LOCATION(BxC) →	CHERUVALLY	MALANKARA
AGE GROUP X LOCATION	2-3 years	137.50	143.83
	5-6 years	119.83	146.17
	10-11 years	157.67	168.17

1.3 Three factor interactions

(Clone x Location x Age group; AxBxC)

AGE GROUP	RRII 105		PB 260	
	CHERUVALLY	MALANKARA	CHERUVALLY	MALANKARA
2-3 years	159.67	192.33	115.33	95.33
5-6 years	85.33	139.33	154.33	153.00
10-11 years	173.00	171.33	142.33	165.00

Critical difference

Source of variation	CD
A	NS
B	NS
C	NS
AB	NS
AC	42.08
BC	NS
ABC	NS

NS = Not significant

Table 2. Phylloplane bacteria (x 10^5/cm^2 of leaf area)

2.1 Single factor averages

CLONE (A)		LOCATION (B)		AGE GROUP (C)		
RRII 105	PB 260	CHERUVALLY	MALANKARA	2-3 YRS	5-6 YRS	10-11 YRS
77.78	35.61	18.11	95.28	69.17	33.92	67.00

2.2 Two factor interactions

INTERACTION	↓ LOCATION / CLONE (AxB) →	RRII 105	PB 260
CLONE X LOCATION	CHERUVALLY	19.33	16.89
	MALANKARA	136.22	54.33
	↓ AGE GROUP/LOCATION (AxC)→	RRII 105	PB 260
CLONE X AGE GROUP	2-3 years	102.83	35.50
	5-6 years	36.67	31.17
	10-11 years	93.83	40.17
	↓ AGE GROUP/LOCATION (BxC)→	CHERUVALLY	MALANKARA
AGE GROUP X LOCATION	2-3 years	16.67	189.00
	5-6 years	22.67	50.67
	10-11 years	18.67	169.00

2.3 Three factor interactions

(Clone x Location x Age group; AxBxC)

AGE GROUP	RRII 105		PB 260	
	CHERUVALLY	MALANKARA	CHERUVALLY	MALANKARA
2-3 years	16.67	189.00	15.67	55.33
5-6 years	22.67	50.67	17.00	45.33
10-11 years	18.67	169.00	18.00	62.33

Critical difference

Source of variation	CD
A	15.78
B	15.78
C	19.32
AB	22.31
AC	27.33
BC	27.33
ABC	38.65

NS = Not significant

Cheruvally estate (18.11 x 10^5/cm^2 of leaf area). Among the three age groups studied, the 5 to 6 year old plantations had significantly lower phylloplane bacteria (33.92 x 10^5/cm^2 of leaf area) compared to 2 to 3 year old and 10 to 11 year old (69.17 and 67 x 10^5/cm^2 of leaf area respectively), the latter two being at par.

When the interaction of clones and locations was studied together, it was evident that RRII 105 at Malankara estate harboured significantly higher number of phylloplane bacteria (136.22 x 10^5/cm^2 of leaf area) while PB 260 showed almost a steady but low (31.16 to 40.16 x 10^5/cm^2 of leaf area) population irrespective of the age of the plantations (Table 2.2). The mean count was very high (102.83 x 10^5/cm^2 of leaf area) for 2 to 3 year old RRII 105 plantations which was on par with 10 to 11 year old plantations of the same clone. However, in 5 to 6 year old plantations the population was significantly lower (36.67 x 10^5/cm^2 of leaf area for RRII 105 and 35.5 X 10^5/cm^2 of leaf area for PB 260). The population was higher at Malankara estate for all the age groups compared. The highest phylloplane bacterial count was recorded for 2 to 3 year old RRII 105 at Malankara estate (189 x 10^5/cm^2 of leaf area) and the lowest count was recorded for 2 to 3 year old PB 260 at Cheruvally estate (15.6 x 10^5/cm^2 of leaf area).

(iii) **Phylloplane actinomycetes** : When the phylloplane actinomycetes were considered individually, significant difference was noticed among clones, locations and also the different age groups studied (Table 3). RRII 105 harboured significantly higher phylloplane actinomycetes (6.17 x 10^3/cm^2 of leaf area) compared to PB 260 (3.89 x 10^3/cm^2 of leaf area). Among the two locations, the count in samples from Malankara estate at Thodupuzha was significantly higher (6 x 10^3/cm^2 of leaf area) than from Cheruvally estate near Erumely (4.05 x 10^3/cm^2 of leaf area). Between the

three age groups, a steady increase in phylloplane actinomycetes was noticed as the age increases and the difference was statistically significant between 2 to 3 year old plantations and the older plantations studied. But between 5 to 6 year old and 10 to 11 year old, there was no significant difference (In 2 to 3 year old plantations the average count was 3.33 x $10^3/cm^2$ of leaf area, whereas 5 to 6 year old, the count was 5.50 x $10^3/cm^2$ and in 10 to 11 year old, the count was 6.25 x $10^3/cm^2$ of leaf area). The interaction between the factors was not significant.

(iv) **Phylloplane yeasts** : The phylloplane population of yeasts varied significantly with locations and age of the populations, but between the clones, the difference was not significant (Table 4). The population was significantly higher in the Malankara estate (14.33 x $10/cm^2$ of leaf area) compared to Cheruvally estate (14.33 x $10/cm^2$ of leaf area). A comparative analysis showed a gradual increase in phylloplane yeasts with regard to age of the plantations. Significant variation between the age groups studied was evident. The mean count from 2 to 3 year old trees was 20.50 x $10/cm^2$ of leaf area compared to 35.92 x $10/cm^2$ of leaf area for 5 to 6 year old and 89.50 x $10/cm^2$ of leaf area for 10 to 11 year old plantations.

When two factor interactions were considered, significant variations were noticed between clones and age groups as well as locations and age groups, the difference between clone and location being nonsignificant. It was observed that 10 to 11 year old RRII 105 harboured an average of 114.67 x 10 phylloplane yeasts per cm^2 of leaf area compared to 18.33 x 10 for 2 to 3 year old PB 260. Interaction between location and age group showed a maximum for 10 to 11 year old trees in Malankara estate (165.16 x $10/cm^2$ of leaf area) compared to 5 to 6 year old trees in Cheruvally estate (13.50 x $10/cm^2$ of leaf area). In general, the age of the trees had no effect

Table 3. Phylloplane actinomycetes (x 10^3/cm^2 of leaf area)

3.1 Single factor averages

CLONE (A)		LOCATION (B)		AGE GROUP (C)		
RRII 105	PB 260	CHERUVALLY	MALANKARA	2-3 YRS	5-6 YRS	10-11 YRS
6.17	3.89	4.06	6.0	3.33	5.50	6.25

3.2 Two factor interactions

INTERACTION	↓ LOCATION / CLONE (AxB) →	RRII 105	PB 260
CLONE X LOCATION	CHERUVALLY	5.33	2.78
	MALANKARA	7.00	5.00
	↓ AGE GROUP/LOCATION (AxC)→	RRII 105	PB 260
CLONE X AGE GROUP	2-3 years	4.50	2.17
	5-6 years	7.17	3.83
	10-11 years	6.83	5.67
	↓ AGE GROUP/LOCATION (BxC)→	CHERUVALLY	MALANKARA
AGE GROUP X LOCATION	2-3 years	2.83	3.83
	5-6 years	5.17	5.83
	10-11 years	4.17	8.33

3.3 Three factor interactions

(Clone x Location x Age group; AxBxC)

AGE GROUP	RRII 105		PB 260	
	CHERUVALLY	MALANKARA	CHERUVALLY	MALANKARA
2-3 years	4.00	5.00	1.67	2.67
5-6 years	7.67	6.67	2.67	5.00
10-11 years	4.33	9.33	4.00	7.33

Critical difference

Source of variation	CD
A	1.44
B	1.44
C	1.76
AB	NS
AC	NS
BC	NS
ABC	NS

NS= Not significant

Table 4. Phylloplane yeasts (x $10^1/cm^2$ of leaf area)

4.1 Single factor averages

CLONE (A)		LOCATION (B)		AGE GROUP (C)		
RRII 105	PB 260	CHERUVALLY	MALANKARA	2-3 YRS	5-6 YRS	10-11 YRS
53.89	43.39	14.33	82.94	20.50	35.92	89.50

4.2 Two factor interactions

INTERACTION	↓ LOCATION / CLONE (AxB) →	RRII 105	PB 260
CLONE X LOCATION	CHERUVALLY	13.89	14.78
	MALANKARA	93.89	72.00
	↓ AGE GROUP/LOCATION (AxC) →	RRII 105	PB 260
CLONE X AGE GROUP	2-3 years	22.67	18.33
	5-6 years	24.33	47.50
	10-11 years	114.67	64.33
	↓ AGE GROUP/LOCATION (BxC)→	CHERUVALLY	MALANKARA
AGE GROUP X LOCATION	2-3 years	15.67	25.33
	5-6 years	13.50	58.33
	10-11 years	13.83	165.17

4.3 Three factor interactions

(Clone x Location x Age group; AxBxC)

AGE GROUP	RRII 105		PB 260	
	CHERUVALLY	MALANKARA	CHERUVALLY	MALANKARA
2-3 years	13.33	32.00	18.00	18.67
5-6 years	15.33	33.33	11.67	83.33
10-11 years	13.00	216.33	14.67	114.00

Critical difference

Source of variation	CD
A	NS
B	15.78
C	19.33
AB	NS
AC	27.33
BC	27.33
ABC	38.65

NS = Not significant

on the phylloplane yeast population in Cheruvally which remained rather low and steady. The highest phylloplane yeast count was noticed in 10 to 11 year old RRII 105at Malankara estate (216.33 x 10/cm^2 of leaf area) and the lowest mean value was noticed in 5 to 6 year old PB 260 at Cheruvally estate (11.67 x 10/cm^2 of leaf area).

Cauloplane microflora

(i) **Cauloplane fungi** : There was no significant difference between clones, locations or age groups with respect to cauloplane fungal population. But significant variation was noticed when clones and age groups as well as locations and age groups were treated together (Table 5). When the interaction between clones and age groups was studied 2 to 3 year old PB 260 showed significantly lower cauloplane fungi (20.67 x $10^3/cm^2$ of bark area) compared to the cauloplane fungal count for 5 to 6 year old PB 260 (43.33 x $10^3/cm^2$ of bark area). When the interaction between age group and location was considered in the 2 to 3 year old plantations in Cheruvally estate, the fungal count from the bark was significantly lower (21.67 x $10^3/cm^2$ of bark area) than 5 to 6 year old plantations in Malankara (39.83 x $10^3/cm^2$ of bark area), which was at par with 10 to 11 year old trees in Cheruvally estate (39.67 x $10^3/cm^2$ of bark area). Other interactions were not statistically significant.

(ii) **Cauloplane bacteria** : Even though there was no significant difference between the average count of cauloplane bacteria of the two clones, the difference was significant among the locations and age groups (Table 6). Of the two locations, the number for trees in Cheruvally estate was significantly higher (88.17 x $10^5/cm^2$ of bark area) than that in Malankara estate (58.56 x $10^5/cm^2$ of bark area). Of the three age groups, 10 to 11 year old plantation

Table 5. Cauloplane fungi (x $10^3/cm^2$ of bark area)

5.1 Single factor averages

CLONE (A)		LOCATION (B)		AGE GROUP (C)		
RRII 105	PB 260	CHERUVALLY	MALANKARA	2-3 YRS	5-6 YRS	10-11 YRS
31.67	30.78	29.89	32.56	26.17	34.08	33.42

5.2 Two factor interactions

INTERACTION	↓ LOCATION / CLONE (AxB) →	RRII 105	PB 260
CLONE X LOCATION	CHERUVALLY	28.56	31.22
	MALANKARA	34.78	30.33
	↓ AGE GROUP/LOCATION (AxC) →	RRII 105	PB 260
CLONE X AGE GROUP	2-3 years	31.67	20.67
	5-6 years	24.83	43.33
	10-11 years	38.50	28.33
	↓ AGE GROUP/LOCATION (BxC) →	CHERUVALLY	MALANKARA
AGE GROUP X LOCATION	2-3 years	21.67	30.67
	5-6 years	28.33	39.83
	10-11 years	39.67	27.17

5.3 Three factor interactions

(Clone x Location x Age group; AxBxC)

AGE GROUP	RRII 105		PB 260	
	CHERUVALLY	MALANKARA	CHERUVALLY	MALANKARA
2-3 years	26.00	37.33	17.33	24.00
5-6 years	14.00	35.67	42.67	44.00
10-11 years	45.67	31.33	33.67	23.00

Critical difference

Source of variation	CD
A	NS
B	NS
C	NS
AB	NS
AC	10.08
BC	10.08
ABC	NS

NS – Not significant

Table 6. Cauloplane Bacteria (x $10^5/cm^2$ of bark area)

6.1 Single factor averages

CLONE (A)		LOCATION (B)		AGE GROUP (C)		
RRII 105	PB 260	CHERUVALLY	MALANKARA	2-3 YRS	5-6 YRS	10-11 YRS
85.83	60.89	88.17	58.56	47.42	65.92	106.75

6.2 Two factor interactions

INTERACTION	↓ LOCATION / CLONE (AxB)→	RRII 105	PB 260
CLONE X LOCATION	CHERUVALLY	106.56	69.78
	MALANKARA	65.11	52.00
	↓ AGE GROUP/LOCATION(AxC) →	RRII 105	PB 260
CLONE X AGE GROUP	2-3 years	69.11	25.67
	5-6 years	72.83	59.00
	10-11 years	115.50	98.00
	↓ AGE GROUP/LOCATION (BxC)→	CHERUVALLY	MALANKARA
AGE GROUP X LOCATION	2-3 years	49.33	45.50
	5-6 years	59.33	72.50
	10-11 years	155.83	57.67

6.3 Three factor interactions

(Clone x Location x Age group; AxBxC)

AGE GROUP	RRII 105		PB 260	
	CHERUVALLY	MALANKARA	CHERUVALLY	MALANKARA
2-3 years	71.67	66.67	27.00	24.33
5-6 years	57.67	88.00	61.00	57.00
10-11 years	190.33	40.67	121.33	74.67

Critical difference

Source of variation	CD
A	NS
B	26.99
C	33.05
AB	NS
AC	NS
BC	46.74
ABC	NS

NS- Not significant

had significantly higher (106.75 x 10^5/cm^2 of bark area) cauloplane bacteria than the other two age groups (47.42 x 10^5/cm^2 of bark area for 2 to 3 year old and 65.92 x 10^5/cm^2 of bark area for 5 to 6 year old.

When interactions were considered, significant variation was noticed only between locations and age groups. In Cheruvally estate, 10 to 11 year old plants harboured significantly higher cauloplane bacteria (155.83 x 10^5/cm^2 of bark area) compared to 2 to 3 year old plantations (49.33 x 10^5/cm^2 of bark area). Other interactions were not significant.

(iii) **Cauloplane actinomycetes** : Significant variation in cauloplane actinomycete population was seen only among the different age groups when taken individually (Table 7). The mean count from 10 to 11 year old trees were significantly higher (4.08 x 10^3/cm^2 of bark area) compared to 2 to 3 year old trees (1.08 x 10^3/cm^2 of bark area) and 5-6 year old trees (3.08 x 10^3/cm^2 of bark area) respectively. When the interaction between clones and location was studied, it was clear that PB 260 at Malankara estate had higher actinomycetes on their bark (4 x 10^3/cm^2 of bark area) than the same clone at Cheruvally (1.56 x 10^3/cm^2 of bark area), which was a very significant difference. Variation in other interactions were not statistically significant.

(iv) **Cauloplane yeasts** : The population of cauloplane yeasts varied significantly with clone and location, but the difference was not significant in the case of different age groups (Table 8). In general, RRII 105 harboured higher (12.78 x 10/cm^2 of bark area) cauloplane yeast population, when compared to PB 260 (6.72 x 10/cm^2 of bark area). Between the two locations the trees in Malankara estate showed more cauloplane yeasts

Table 7. Cauloplane actinomycetes (x $10^3/cm^2$ of bark area)

7.1 Single factor averages

CLONE (A)		LOCATION (B)		AGE GROUP (C)		
RRII 105	PB 260	CHERUVALLY	MALANKARA	2-3 YRS	5-6 YRS	10-11 YRS
2.72	2.78	2.56	2.94	1.08	3.08	4.08

7.2 Two factor interactions

INTERACTION	↓ LOCATION / CLONE (AxB) →	RRII 105	PB 260
CLONE X LOCATION	CHERUVALLY	3.56	1.56
	MALANKARA	1.89	4.00
	↓ AGE GROUP/LOCATION (AxC) →	RRII 105	PB 260
CLONE X AGE GROUP	2-3 years	1.17	1.00
	5-6 years	3.00	3.17
	10-11 years	4.00	4.17
	↓ AGE GROUP/LOCATION (BxC) →	CHERUVALLY	MALANKARA
AGE GROUP X LOCATION	2-3 years	1.33	0.83
	5-6 years	2.33	3.83
	10-11 years	4.00	4.17

7.3 Three factor interactions

(Clone x Location x Age group; AxBxC)

AGE GROUP	RRII 105		PB 260	
	CHERUVALLY	MALANKARA	CHERUVALLY	MALANKARA
2-3 years	1.67	0.67	1.00	1.00
5-6 years	4.00	2.00	0.67	5.67
10-11 years	5.00	3.00	3.00	5.33

Critical difference

Source of variation	CD
A	NS
B	NS
C	1.56
AB	1.70
AC	NS
BC	NS
ABC	NS

NS = Not significant

Table 8. Cauloplane yeasts (x $10^1/cm^2$ of bark area)

8.1 Single factor averages

CLONE (A)		LOCATION (B)		AGE GROUP (C)		
RRII 105	PB 260	CHERUVALLY	MALANKARA	2-3 YRS	5-6 YRS	10-11 YRS
12.78	6.72	6.28	13.22	8.75	10.50	10.00

8.2 Two factor interactions

INTERACTION	↓ LOCATION / CLONE (AxB)→	RRII 105	PB 260
CLONE X LOCATION	CHERUVALLY	5.56	7.00
	MALANKARA	20.00	6.44
	↓ AGE GROUP/LOCATION (AxC)→	RRII 105	PB 260
CLONE X AGE GROUP	2-3 years	11.50	6.00
	5-6 years	15.83	5.17
	10-11 years	11.00	9.00
	↓ AGE GROUP/LOCATION (BxC)→	CHERUVALLY	MALANKARA
AGE GROUP X LOCATION	2-3 years	4.67	12.83
	5-6 years	6.17	14.83
	10-11 years	8.00	12.00

8.3 Three factor interactions

(Clone x Location x Age group; AxBxC)

AGE GROUP	RRII 105		PB 260	
	CHERUVALLY	MALANKARA	CHERUVALLY	MALANKARA
2-3 years	5.00	18.00	4.33	7.67
5-6 years	5.33	26.33	7.00	3.33
10-11 years	6.33	15.67	9.67	8.33

Critical difference

Source of variation	CD
A	2.93
B	2.93
C	NS
AB	4.15
AC	NS
BC	NS
ABC	NS

NS = Not significant

(13.22 x 10/cm^2 of bark area) as compared to the trees in Cheruvally estate (6.28 x 10/cm^2 of bark area).

When more than one factors were considered together, significant interaction was noticed only between the clones and locations. Maximum cauloplane yeast count of 200/cm^2 of bark area (20.00 x 10) was noticed for RRII 105 at Malankara estate, compared to 55.6/cm^2 of bark area (5.56 x 10) at Cheruvally estate. For PB 260, the counts were almost the same for both the locations.

Rhizosphere microflora

To study the microbial population in the rhizosphere in detail, enumeration of phosphobacteria, regular bacteria, fungi, yeasts, actinomycetes and VAM spores were done for the different soil samples. As the number of N fixers was comparatively low, the nitrogenase activity in soil was studied as an indicator of their activity.

Rhizosphere phosphobacteria : The rhizosphere phosphobacterial population showed significant variation only with the clones and locations when taken individually (Table 9). The mean count of such bacteria was 41.33 x 10^5/g of soil for RRII 105 which was significantly higher than that for PB 260 (27.33 x 10^5/g of soil). Similarly the mean count for Malankara estate was significantly higher (40.28 x 10^5/g of soil) compared to that for Cheruvally estate (28.39 x 10^5/g of soil).

Total rhizosphere bacteria : When the total rhizosphere bacterial count was analysed (Plate 1.1), significant difference was noticed among the two clones (Table 10).

Table 9. Rhizosphere phosphobacteria (x 10^6/g of soil)

9.1 Single factor averages

CLONE (A)		LOCATION (B)		AGE GROUP (C)		
RRII 105	PB 260	CHERUVALLY	MALANKARA	2-3 YRS	5-6 YRS	10-11 YRS
41.33	27.33	28.39	40.28	34.58	34.75	33.67

9.2 Two factor interactions

INTERACTION	↓ LOCATION / CLONE (AxB)→	RRII 105	PB 260
CLONE X LOCATION	CHERUVALLY	32.33	24.44
	MALANKARA	50.33	30.22
	↓ AGE GROUP/LOCATION (AxC) →	RRII 105	PB 260
CLONE X AGE GROUP	2-3 years	45.50	23.67
	5-6 years	35.83	33.67
	10-11 years	42.67	24.67
	↓ AGE GROUP/LOCATION (BxC)→	CHERUVALLY	MALANKARA
AGE GROUP X LOCATION	2-3 years	26.50	42.67
	5-6 years	34.67	34.83
	10-11 years	24.00	43.33

9.3 Three factor interactions

(Clone x Location x Age group; AxBxC)

AGE GROUP	RRII 105		PB 260	
	CHERUVALLY	MALANKARA	CHERUVALLY	MALANKARA
2-3 years	33.33	57.67	19.67	27.67
5-6 years	32.00	39.67	37.33	30.00
10-11 years	31.67	53.67	16.33	33.00

Critical difference

Source of variation	CD
A	8.94
B	8.94
C	NS
AB	NS
AC	NS
BC	NS
ABC	NS

NS = Not significant

Table 10. Rhizosphere Bacteria (x 10^7/g of soil)

10.1 Single factor averages

CLONE (A)		LOCATION (B)		AGE GROUP (C)		
RRII 105	PB 260	CHERUVALLY	MALANKARA	2-3 YRS	5-6 YRS	10-11 YRS
53.39	41.56	52.22	42.72	36.25	52.33	53.83

10.2 Two factor interactions

INTERACTION	↓ LOCATION / CLONE (AxB)→	RRII 105	PB 260
CLONE X LOCATION	CHERUVALLY	51.78	52.67
	MALANKARA	55.00	30.44
	↓ AGE GROUP/LOCATION (AxC) →	RRII 105	PB 260
CLONE X AGE GROUP	2-3 years	35.17	37.33
	5-6 years	63.00	41.67
	10-11 years	62.00	45.67
	↓ AGE GROUP/LOCATION (BxC) →	CHERUVALLY	MALANKARA
AGE GROUP X LOCATION	2-3 years	35.33	37.17
	5-6 years	64.00	40.67
	10-11 years	57.33	50.33

10.3 Three factor interactions

(Clone x Location x Age group; AxBxC)

AGE GROUP	RRII 105		PB 260	
	CHERUVALLY	MALANKARA	CHERUVALLY	MALANKARA
2-3 years	29.33	41.00	41.33	33.33
5-6 years	72.00	54.00	56.00	27.33
10-11 years	54.00	70.00	60.67	30.67

Critical difference

Source of variation	CD
A	10.96
B	NS
C	13.42
AB	15.50
AC	NS
BC	NS
ABC	NS

NS = Not significant

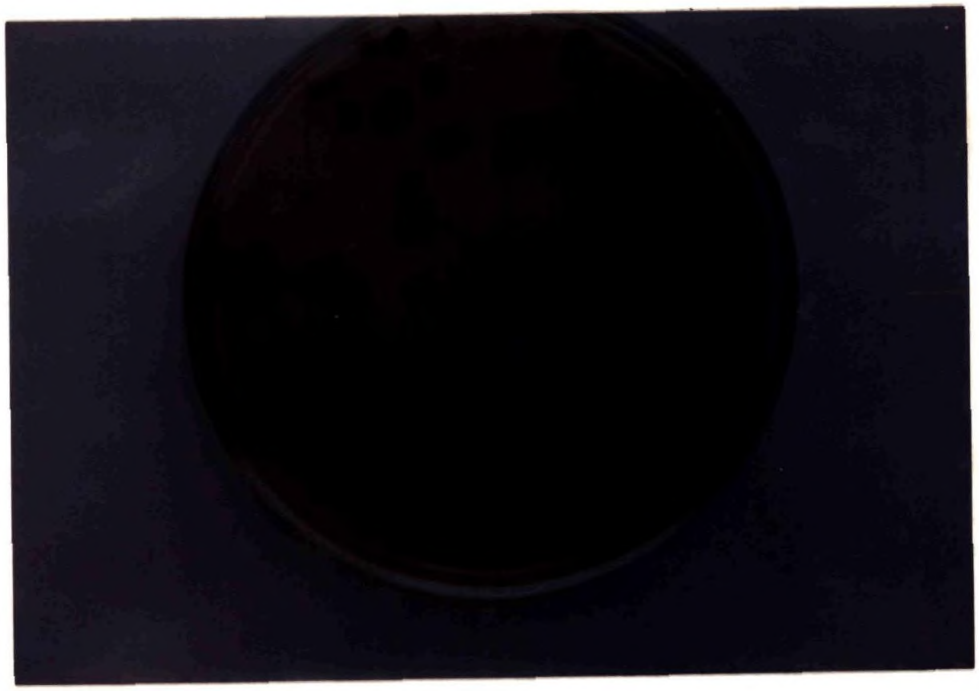

Plate 1.1 Rhizosphere bacterial colonies

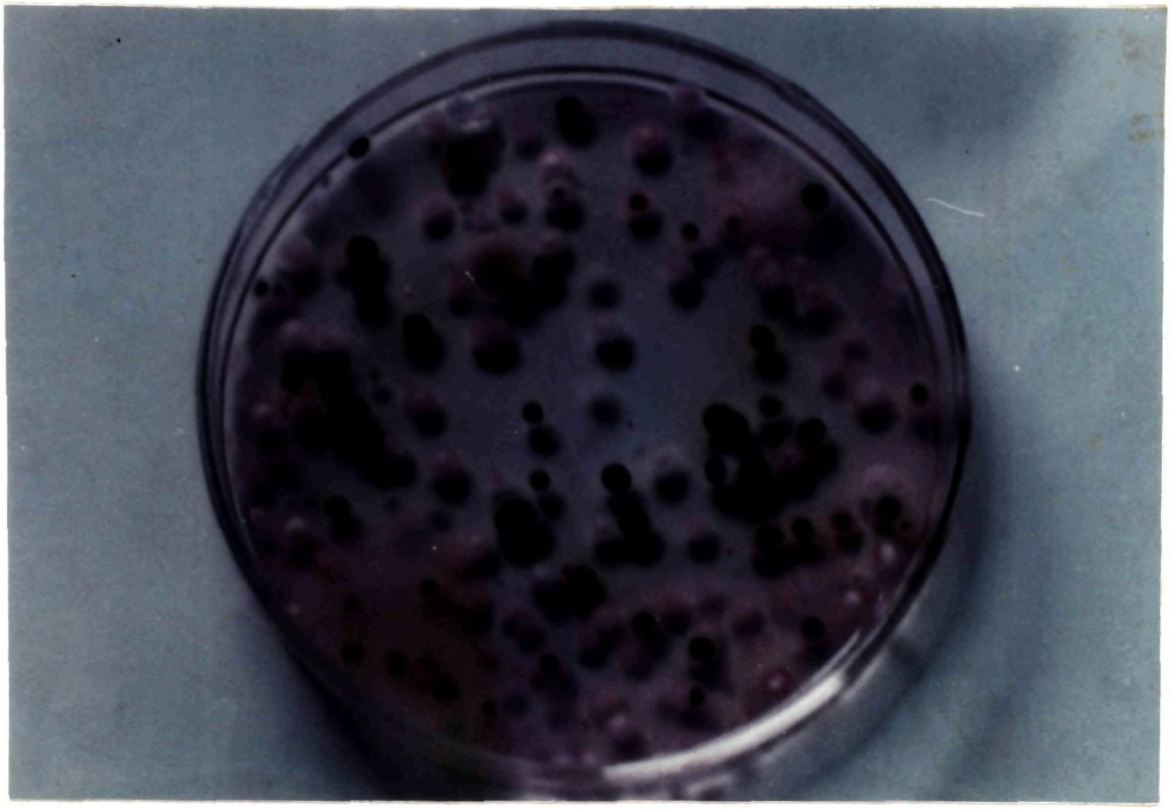

Plate 1.2 Rhizosphere fungal colonies

Higher number was seen in the case of RRII 105 (53.39 x 10^7/g of soil) compared to PB 260 (41.56 x 10^7/g of soil). Significant variation was also noticed among the age groups. Rhizosphere of 10 to 11 year old rubber plantation soils harboured 53.83 x 10^7/g of soil, which was at par with 5 to 6 year old rubber plantation soils (52.33 x 10^7/g of soil) but high compared to 36.25 x 10^7/g of soil for 2 to 3 year old rubber plantation soils.

When the interaction of clones and locations was studied simultaneously, it was clear that RRII 105 at Malankara estate supported a higher rhizosphere bacteria (55 x 10^7/g of soil) while PB 260 at Cheruvally estate supported only a lower number (30.44 x 10^7/g of soil). RRII 105 at Cheruvally and PB 260 at Malankara showed almost identical count of 51.77 x 10^7 and 52.67 10^7/g of soil respectively.

Rhizosphere fungi : Enumeration of rhizosphere fungi (Plate 1.2) revealed that there was significant variation among the different age groups (Table 11). The 10 to 11 year old rubber plantations had a maximum number of 53.75 x 10^3/g of soil, compared to 32.17 x 10^3/g of soil for 5 to 6 year old plantations which was almost at par with 2 to 3 year old rubber plantation soils (35.08 x 10^3/g of soil).

When clones and locations were studied collectively, very significant difference was noticed. A maximum population of 49.33 x 10^3/g of soil was observed for soil under PB 260 at Cheruvally estate compared to the minimum number (32.66 x 10^3/g of soil) for soil under PB 260 at Malankara estate. Interactions between clones and age groups also showed significant variation. The soil under 10 to 11 year old PB 260 harboured a maximum mean population (63.66 x 10^3 g of soil) compared to a minimum of 23.16 x 10^3/g of soil for 5 to 6 year old PB 260.

Table 11. Rhizosphere fungi (x 10^3/g of soil)

11.1 Single factor averages

CLONE (A)		LOCATION (B)		AGE GROUP (C)		
RRII 105	PB 260	CHERUVALLY	MALANKARA	2-3 YRS	5-6 YRS	10-11 YRS
39.67	41.00	41.56	39.11	35.08	32.17	53.75

11.2 Two factor interactions

INTERACTION	↓ LOCATION / CLONE (AxB)→	RRII 105	PB 260
CLONE X LOCATION	CHERUVALLY	33.78	49.33
	MALANKARA	45.56	32.67
	↓ AGE GROUP/LOCATION (AxC)→	RRII 105	PB 260
CLONE X AGE GROUP	2-3 years	34.00	36.17
	5-6 years	41.17	23.17
	10-11 years	43.83	63.67
	↓ AGE GROUP/LOCATION(BxC) →	CHERUVALLY	MALANKARA
AGE GROUP X LOCATION	2-3 years	34.00	36.17
	5-6 years	28.83	35.50
	10-11 years	61.83	45.67

11.3 Three factor interactions

(Clone x Location x Age group; AxBxC)

AGE GROUP	RRII 105		PB 260	
	CHERUVALLY	MALANKARA	CHERUVALLY	MALANKARA
2-3 years	26.33	41.67	41.67	30.67
5-6 years	40.00	42.33	17.67	28.67
10-11 years	35.00	52.67	88.67	38.67

Critical difference

Source of variation	CD
A	NS
B	NS
C	13.56
AB	15.65
AC	19.17
BC	NS
ABC	27.11

NS = Not significant

When all the three factors were pooled together, the highest rhizosphere fungal count was recorded for 10 to 11 year old PB 260 at Cheruvally (88.67 x 10^3/g of soil) and the lowest number for 5 to 6 year old PB 260 at Cheruvally estate (17.67 x 10^3/g of soil).

Based on colony morphology and microscopic examination some of the fungal isolates were identified to the genus level. The more common genera encountered were *Trichoderma, Alternaria, Fusarium, Aspergillus* and *Penecillium* (Plate 2.1 to 2.4).

Rhizosphere actinomycetes : A comparative analysis of the rhizosphere actinomycetes showed significant variation only between the locations (Table 12). Significantly lower mean count (51.7 x 10^4/g of soil) was observed for the samples from Cheruvally estate whereas it was higher (90 x 10^4/g of soil) for the Malankara estate. The difference was not significant for any other factors or interactions studied.

Rhizosphere yeasts : The yeast population in the rhizosphere followed almost a similar pattern to the rhizosphere actinomycetes. Significant variation was noticed only between the two locations (Table 13). The yeast population was higher at Cheruvally estate (14.83 x 10^3/g of soil) compared to Malankara estate (8.33 x 10^3/g of soil).

VAM spore count : Statistical analysis of the VAM spore count per gram of the various soil samples showed no significant difference between them. Their number ranged between 17 and 27 per gram of soil (Table 14).

Plate 2.1 ***Trichoderma*** **sp.**

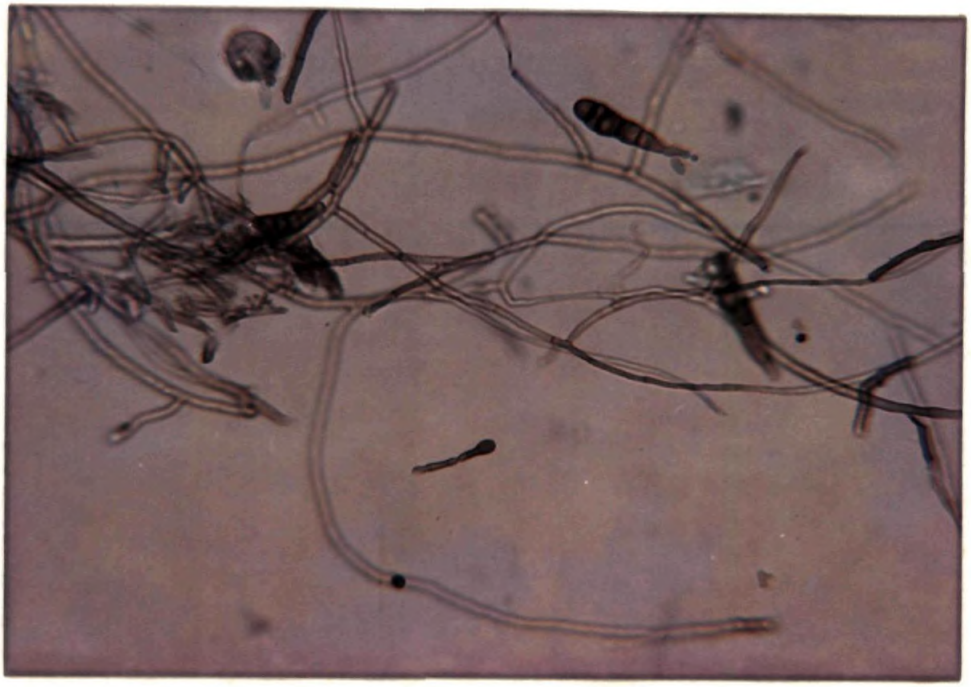

Plate 2.2 ***Alternaria*** **sp.**

Plate 2.3 ***Penicillium*** **sp.**

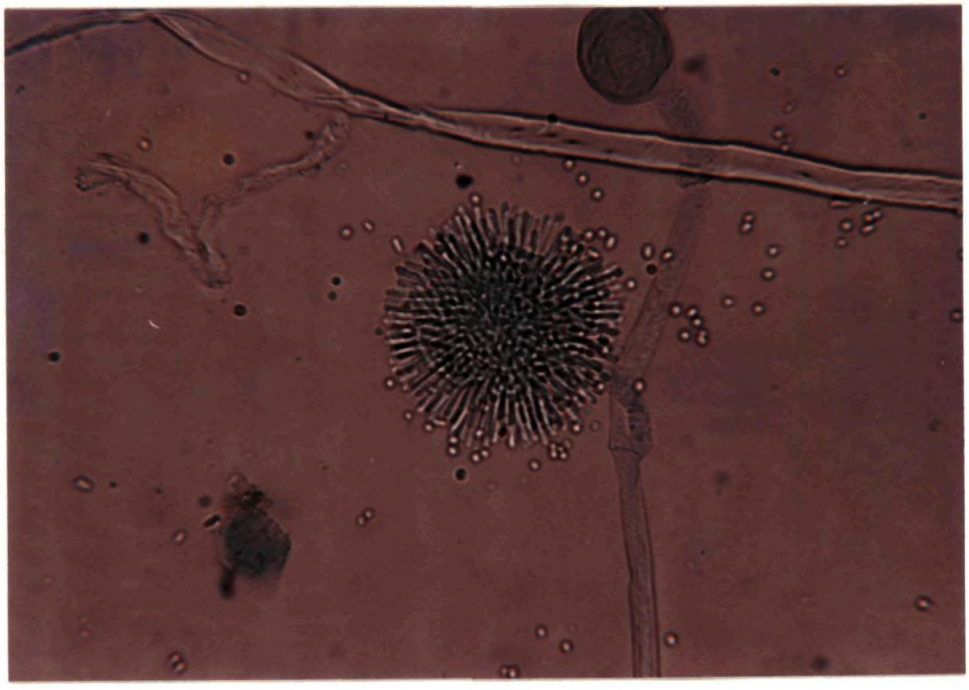

Plate 2.4 ***Aspergillus*** **sp.**

Table 12. Rhizosphere actinomycetes (x 10^4/g of soil)

12.1 Single factor averages

CLONE (A)		LOCATION (B)		AGE GROUP (C)		
RRII 105	PB 260	CHERUVALLY	MALANKARA	2-3 YRS	5-6 YRS	10-11 YRS
61.7	80.0	51.7	90.0	62.5	69.2	80.8

12.2 Two factor interactions

INTERACTION	↓ LOCATION / CLONE (AxB)→	RRII 105	PB 260
CLONE X LOCATION	CHERUVALLY	38.9	64.4
	MALANKARA	84.4	95.6
	↓ AGE GROUP/LOCATION (AxC) →	RRII 105	PB 260
CLONE X AGE GROUP	2-3 years	61.7	63.3
	5-6 years	43.3	95.0
	10-11 years	80.0	81.7
	↓ AGE GROUP/LOCATION (BxC)→	CHERUVALLY	MALANKARA
AGE GROUP X LOCATION	2-3 years	46.7	78.3
	5-6 years	65.0	73.3
	10-11 years	43.3	118.3

12.3 Three factor interactions

(Clone x Location x Age group; AxBxC)

AGE GROUP	RRII 105		PB 260	
	CHERUVALLY	MALANKARA	CHERUVALLY	MALANKARA
2-3 years	33.3	90.0	60.0	66.7
5-6 years	36.7	50.0	93.3	96.7
10-11 years	46.7	113.3	40.0	123.3

Critical difference

Source of variation	CD
A	NS
B	22.89
C	NS
AB	NS
AC	NS
BC	NS
ABC	NS

NS = Not significant

Table 13. Rhizosphere yeasts (x 10^2/g of soil)

13.1 Single factor averages

CLONE (A)		LOCATION (B)		AGE GROUP (C)		
RRII 105	PB 260	CHERUVALLY	MALANKARA	2-3 YRS	5-6 YRS	10-11 YRS
12.06	11.11	14.83	8.33	10.92	10.67	13.17

13.2 Two factor interactions

INTERACTION	↓ LOCATION / CLONE (AxB)→	RRII 105	PB 260
CLONE X LOCATION	CHERUVALLY	14.89	14.78
	MALANKARA	9.22	7.44
	↓ AGE GROUP/LOCATION (AxC) →	RRII 105	PB 260
CLONE X AGE GROUP	2-3 years	11.00	10.83
	5-6 years	10.17	11.17
	10-11 years	15.00	11.33
	↓ AGE GROUP/LOCATION (BxC)→	CHERUVALLY	MALANKARA
AGE GROUP X LOCATION	2-3 years	13.83	8.00
	5-6 years	14.67	6.67
	10-11 years	16.00	10.33

13.3 Three factor interactions

(Clone x Location x Age group; AxBxC)

AGE GROUP	RRII 105		PB 260	
	CHERUVALLY	MALANKARA	CHERUVALLY	MALANKARA
2-3 years	11.67	10.33	16.00	5.67
5-6 years	13.67	6.67	15.67	6.67
10-11 years	19.33	10.67	12.67	10.00

Critical difference

Source of variation	CD
A	NS
B	3.17
C	NS
AB	NS
AC	NS
BC	NS
ABC	NS

NS= Not significant

Table 14. VAM spore count (per g of soil)

14.1 Single factor averages

CLONE (A)		LOCATION (B)		AGE GROUP (C)		
RRII 105	PB 260	CHERUVALLY	MALANKARA	2-3 YRS	5-6 YRS	10-11 YRS
22.56	24.08	22.48	24.17	23.94	24.79	21.23

14.2 Two factor interactions

INTERACTION	↓ LOCATION / CLONE (AxB) →	RRII 105	PB 260
CLONE X LOCATION	CHERUVALLY	22.12	22.83
	MALANKARA	23.00	25.33
	↓ AGE GROUP/LOCATION (AxC) →	RRII 105	PB 260
CLONE X AGE GROUP	2-3 years	22.97	24.92
	5-6 years	24.58	25.00
	10-11 years	20.13	22.33
	↓ AGE GROUP/LOCATION (BxC)→	CHERUVALLY	MALANKARA
AGE GROUP X LOCATION	2-3 years	23.18	24.71
	5-6 years	24.58	25.00
	10-11 years	19.67	22.79

14.3 Three factor interactions

(Clone x Location x Age group; AxBxC)

AGE GROUP	RRII 105		PB 260	
	CHERUVALLY	MALANKARA	CHERUVALLY	MALANKARA
2-3 years	22.77	23.17	23.58	26.25
5-6 years	25.83	23.33	23.33	26.67
10-11 years	17.75	22.50	21.58	23.08

Critical difference

Source of variation	CD
A	NS
B	NS
C	NS
AB	NS
AC	NS
BC	NS
ABC	NS

NS = Not significant

A comparison of the overall mean numbers of microorganisms observed in the phylloplane, cauloplane and rhizosphere are presented in Table 15.

Table 15. Comparative analysis of total microflora

Organism	Phylloplane/cm^2	Cauloplane/ cm^2	Rhizosphere/g, of soil
Fungi	1,455	1,240	40.5×10^3
Bacteria	57,000	2,94,000	47.5×10^7
Actinomycetes	100	108	70.0×10^4
Yeasts	5	4	11.6×10^3
Phosphobacteria	NA	NA	34.0×10^6

BENEFICIAL ASSOCIATIONS

VAM presence

An analysis of the percentage of VAM occurrence in the roots of rubber plant showed that they were not present in all roots and that their percentage of occurrence varied. There was very significant difference between the two clones studied. RRII 105 supported significantly less VAM fungi (65.28%) compared to PB 260 (79.44%) (Table 16). The occurrence also varied significantly between the two locations studied. Trees at Malankara estate harboured more VAM fungi (76.11%) than that at Cheruvally estate (68.61%). Age group of trees as well as various interactions studied did not show any significant difference in the percentage of VAM fungal colonization in the roots (Plate 3.1 and 3.2).

Nitrogenase activity

A study of the nitrogenase activity of the different soils revealed no significant variation between clones, locations or age groups or in any of the interactions studied (Table 17).

Table 16. VAM occurrence (%)

16.1 Single factor averages

CLONE (A)		LOCATION (B)		AGE GROUP (C)		
RRII 105	PB 260	CHERUVALLY	MALANKARA	2-3 YRS	5-6 YRS	10-11 YRS
65.28	79.44	68.61	76.11	69.17	70.42	77.50

16.2 Two factor interactions

INTERACTION	↓ LOCATION/CLONE (AxB)→	RRII 105	PB 260
CLONE X LOCATION	CHERUVALLY	63.33	73.89
	MALANKARA	67.22	85.00
	↓ AGE GROUP/LOCATION (AxC) →	RRII 105	PB 260
CLONE X AGE GROUP	2-3 years	62.50	75.83
	5-6 years	63.33	77.50
	10-11 years	70.00	85.00
	↓ AGE GROUP / LOCATION (BxC)→	CHERUVALLY	MALANKARA
AGE GROUP X LOCATION	2-3 years	65.83	72.50
	5-6 years	66.67	74.17
	10-11 years	73.33	81.67

16.3 Three factor interactions

(Clone x Location x Age group; AxBxC)

AGE GROUP	RRII 105		PB 260	
	CHERUVALLY	MALANKARA	CHERUVALLY	MALANKARA
2-3 years	60.00	65.00	71.67	80.00
5-6 years	65.00	61.67	68.33	86.67
10-11 years	65.00	75.00	81.67	88.33

Critical difference

Source of variation	CD
A	6.00
B	6.01
C	NS
AB	NS
AC	NS
BC	NS
ABC	NS

NS = Not significant

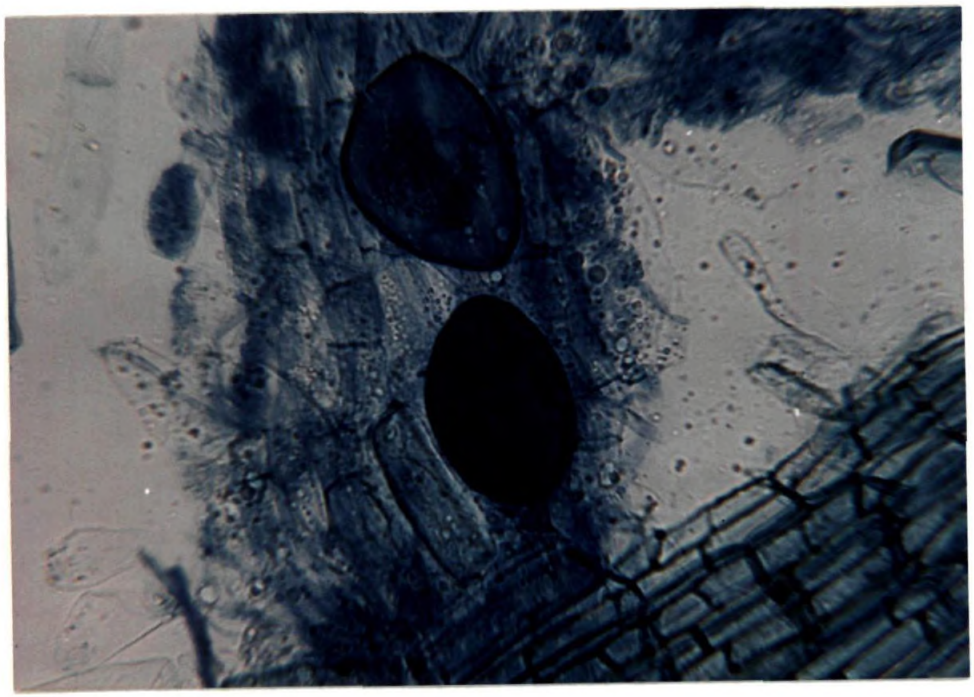

Plate 3.1 VAM spores

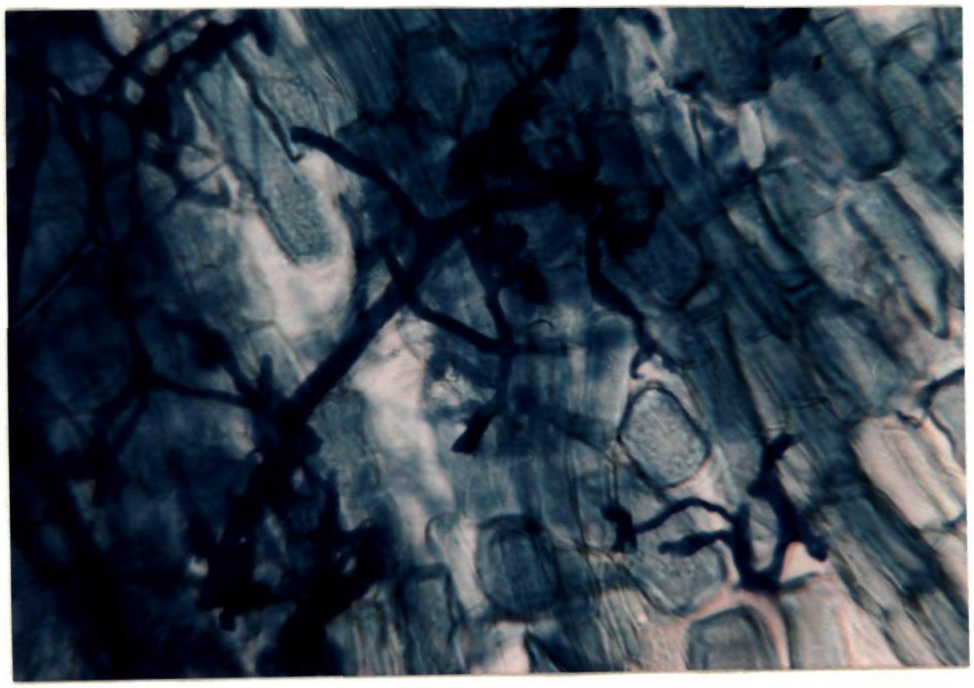

Plate 3.2 VAM infected root smear

Table 17. Nitrogenase activity

17.1 Single factor averages

CLONE (A)		LOCATION (B)		AGE GROUP (C)		
RRII 105	PB 260	CHERUVALLY	MALANKARA	2-3 YRS	5-6 YRS	10-11 YRS
0.48	1.07	0.58	0.97	1.39	0.43	0.51

17.2 Two factor interactions

INTERACTION	↓ LOCATION / CLONE (AxB) →	RRII 105	PB 260
CLONE X LOCATION	CHERUVALLY	0.58	0.59
	MALANKARA	0.38	1.55
	↓ AGE GROUP/LOCATION (AxC) →	RRII 105	PB 260
CLONE X AGE GROUP	2-3 years	0.37	2.40
	5-6 years	0.44	0.41
	10-11 years	0.62	0.40
	↓ AGE GROUP/LOCATION (BxC)→	CHERUVALLY	MALANKARA
AGE GROUP X LOCATION	2-3 years	0.65	2.12
	5-6 years	0.48	0.38
	10-11 years	0.62	0.40

17.3 Three factor interactions

(Clone x Location x Age group; AxBxC)

AGE GROUP	RRII 105		PB 260	
	CHERUVALLY	MALANKARA	CHERUVALLY	MALANKARA
2-3 years	0.40	0.34	0.90	3.90
5-6 years	0.48	0.41	0.48	0.34
10-11 years	0.84	0.40	0.39	0.40

Critical difference

Source of variation	CD
A	NS
B	NS
C	NS
AB	NS
AC	NS
BC	NS
ABC	NS

NS= Not significant

Phosphate solubilization

Detection of solubilization

Culture plate technique : Phosphate solubilizing bacteria dissolved the precipitated phosphate in the medium and formed a clear halo around each such colony. Eleven different strains of bacteria were isolated from various soil samples that was suspected to have phosphate solubilizing property. Since the colonies were crowded in the petriplate all such suspected isolates were obtained in pure cultures and were grown individually on separate precipitated apetite agar plates and their activity was confirmed.

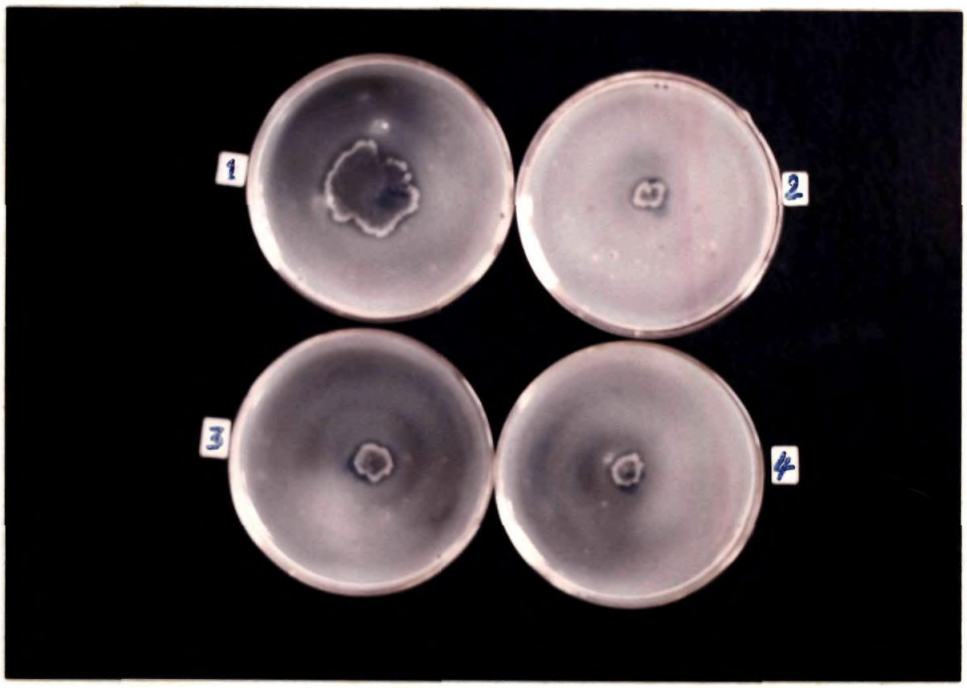

Plate 4.1 Phosphate solubilization
Plate No.1 : RP_1; Plate No.2 : RP_3 ; Plate No.3 : RP_4 ; Plate No.4 : RP_6

Out of the eleven isolates grown, four (Isolate RP_1, RP_3, RP_4 and RP_6) showed well differentiated halo around the colony (Plate 4.1). In RP_1, colony growth and extent of the halo was more than the others. Here the width of the halo was found to be 2 mm whereas RP_6 showed the faintest halo which was less than 1 mm (Plate 4.2).

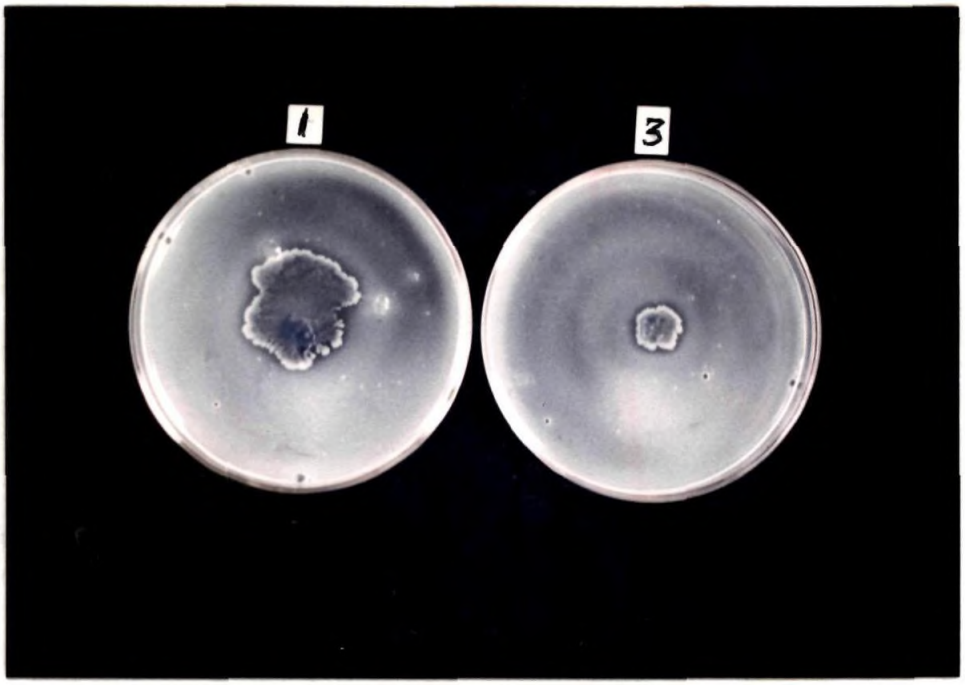

Plate 4.2 Phosphate solubilization
Plate No.1 : RP_1 ; Plate No.3 : RP_6

Colorimetric estimation

In general, the fungal species assayed viz. *Penicillium* sp., *Aspergillus* sp. and *Fusarium* sp. were found to show comparatively higher rate of P solubilization than bacteria. Maximum rate was seen in the case of an *Aspergillus* sp. isolated from phylloplane (PF_{12}) its optical density was found to be 0.175, dissolving 35 ppm of phosphate from rock phosphate in 10 days (Table 18). The second best solubilization was noticed for a

Penicillium sp. (RF_9) isolated from the rhizosphere with an OD value of 0.16 dissolving 32 ppm of phosphate in 10 days. Among the phylloplane isolates a *Penicillium* sp. (PF_9) also had an OD of 1.0 capable of dissolving 20 ppm of phosphate in 10 days. Solubilization capacity for all other fungi tested were lower than this.

Among phosphate solubilizing bacteria isolate RP_{10} from rhizosphere showed maximum efficiency with an OD of 0.14 (28 ppm phosphate dissolution). This was followed by isolate RP_5 (OD value 0.06 with 12 ppm phosphate dissolution) and isolates RP_6 and RP_9 (OD value 0.05). The detailed results are presented in Table 18.

Table 18. Phosphate solubilization of the fungal and bacterial isolates

Fungi				Bacteria		
Isolate No.	Genus	OD	Dissolved phosphate (ppm)	Isolate No.	OD	Dissolved phosphate (ppm)
RF_1	Penicillium	0.50	10	RP_1	0.38	7
RF_2	Penicillium	0.40	8	RP_2	0.003	< 1
RF_7	Penicillium	0.30	6	RP_3	0.028	6
RF_{7a}	Penicillium	0.45	9	RP_4	0.035	7
RF_9	Penicillium	0.160	32	RP_5	0.060	12
RF_{19}	Penicillium	0.030	6	RP_6	0.050	10
PF_9	Penicillium	0.100	20	RP_7	0.033	6
PF_{19}	Penicillium	0.065	13	RP_8	0.033	6
PF_{20}	Penicillium	0.040	8	RP_9	0.50	10
PF_{21}	Penicillium	0.040	8	RP_{10}	0.140	28
RF_{2a}	Aspergillus	0.020	4	RP_{11}	0.40	8
RF_{8a}	Aspergillus	0.035	7			
PF_4	Aspergillus	0.055	11			
PF_{12}	Aspergillus	0.175	35			
RF_5	Fusarium	0.025	5			
RF_{14}	Fusarium	0.055	11			
PF_{14}	Fusarium	0.025	5			

Antagonism to *Phytophthora*

The organisms isolated from phylloplane, cauloplane and rhizosphere were tested for their antagonistic effect on *Phytophthora meadii*. Out of the total 42 isolates of fungi, only 6 were found to have some inhibitory effect on *P. meadii*. Only one (PF_3) out of these six was of phylloplane origin while none of the cauloplane isolates were antagonistic. The inhibitory effect showed wide variation. The width of the inhibitory zone was found to be maximum in the case of RF_4 (0.8 cm) followed by RF_{17} (0.4 cm), RF_{16} (0.3 cm), RF3 and RF_{3a} (0.2 cm each) and PF_3 (0.1 cm) (Plate No.5.1).

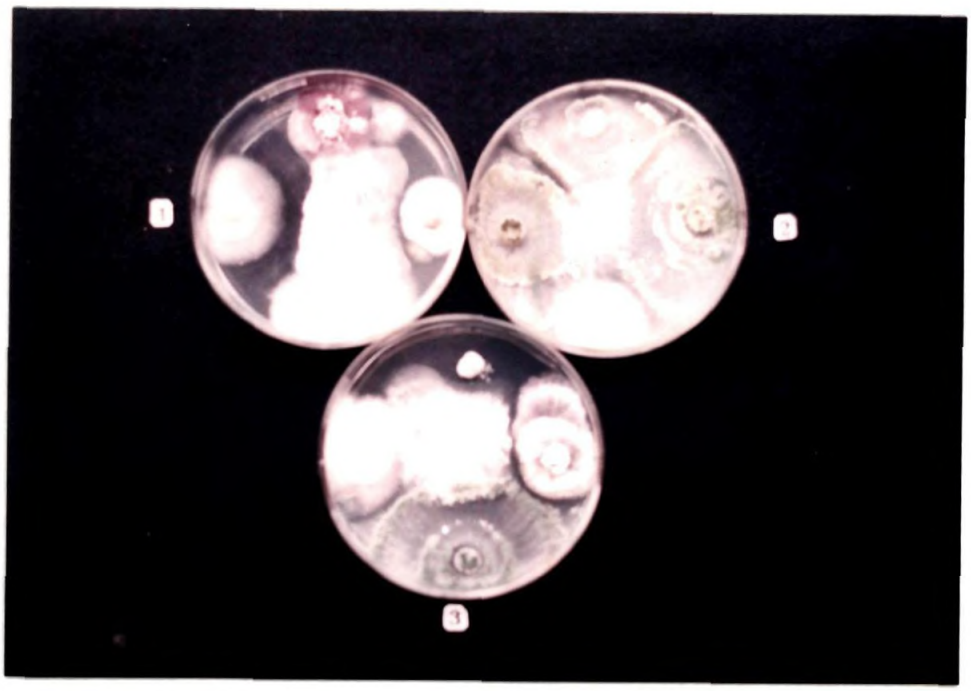

Plate 5.1 Fungal antagonists
Four different fungal isolates in each petriplate showing varying degrees of antagonism to *P. meadii*.

All the five isolates of actinomycetes showed some degree of antagonism. ACT_3 displayed maximum antagonism with an inhibitory zone width of 1.1 cm, compared to 0.7 cm for ACT_1, 0.4 cm for ACT_5, 0.3 cm for ACT_2 and 0.1 cm for ACT_4. All these isolates were obtained from either phylloplane or cauloplane.

Out of the total five isolates of yeasts, only three were found to be antagonistic. They were PY_1 with an inhibitory zone width of 0.7 cm, PY_4 (0.5 cm) and PY_2 (0.4 cm) (Plate 5.2). The yeasts were also isolated from phylloplane or cauloplane sources.

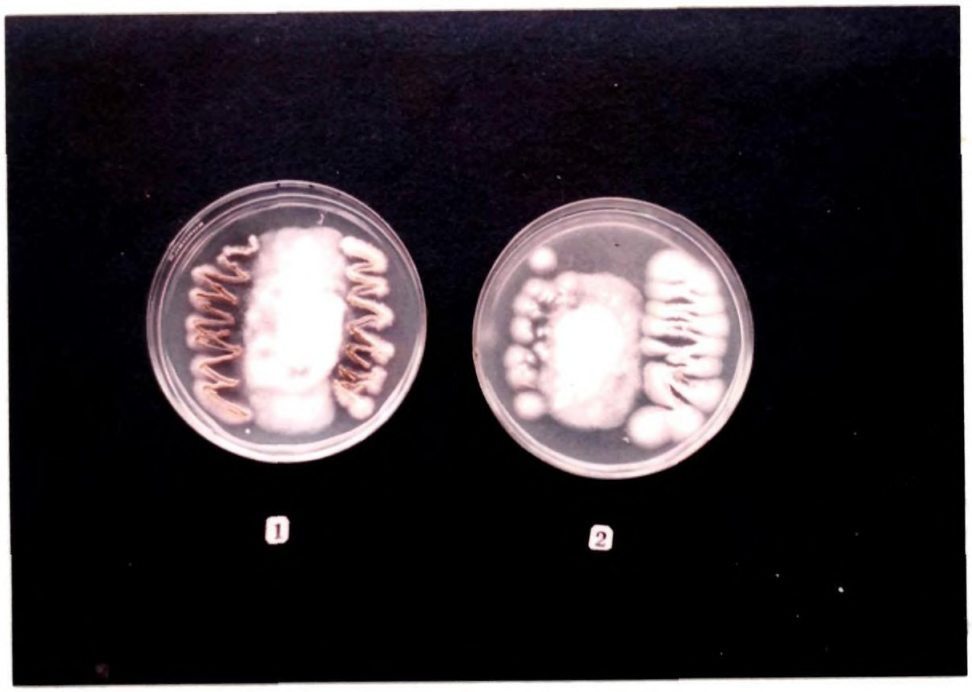

Plate 5.2 Antagonistic isolates of yeast

Bacterial isolates from all the sources showed antagonistic activity. Out of the total 40 isolates of bacteria 17 showed inhibitory effect on *P. meadii* with varying intensities. Table 19 summarizes their effects. Besides

the general rhizosphere bacterial isolates (RB) some of the phosphate solubilizing isolates (RP) also showed antagonism.

Table 19. Antagonistic activity of bacterial isolates

Source	Phylloplane / Cauloplane					Rhizosphere											
Isolate No.	PB_6	PB_8	PB_{15}	PB_{16}	PB_{18}	RB_4	RB_5	RB_6	RB_7	RB_9	RP_1	RP_3	RP_4	Rp_5	RP_6	RP_7	Rp10
Width of inhibition zone (cm)	0.6	1.2	0.2	0.1	0.5	1.0	2.0	0.3	1.1	0.2	0.3	0.4	0.5	0.7	1.3	0.8	0.5

Isolate RB_5 exhibited maximum efficiency with an inhibitory zone width of 2 cm followed by RP_6 with a zone width of 1.3 cm, PB_8 (1.2 cm), RB_7 (1.1 cm) and RB_4 (1.0 cm) (Plate Nos.5.3 & 5.4).

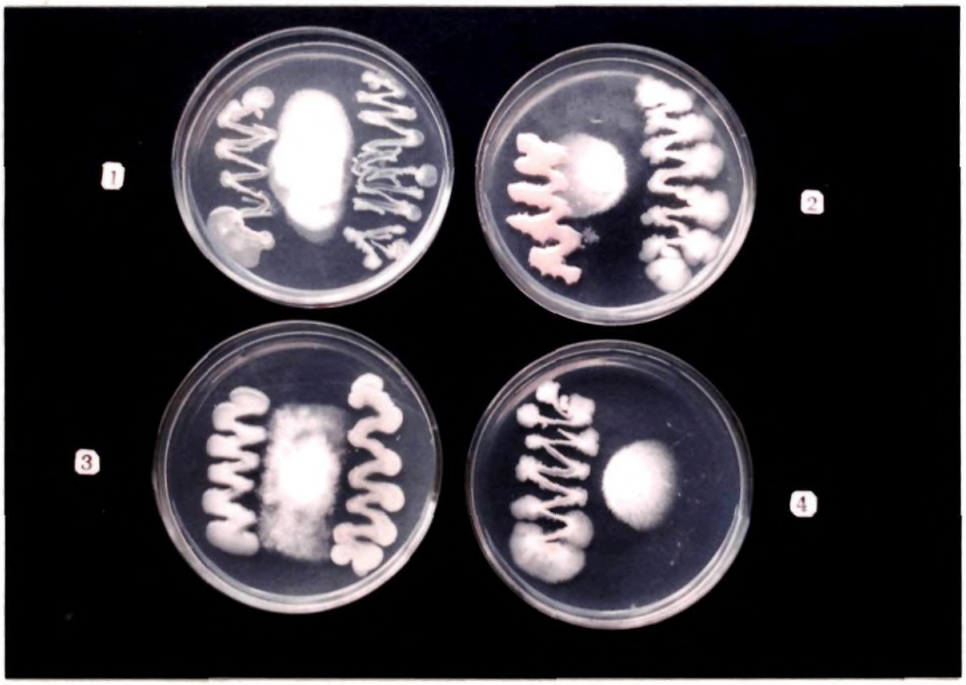

Plate 5.3 Antagonistic bacterial isolates
Plate No.1 : RB_4 ; Plate No.2 : RP_6 ; Plate No.3 : RB_7 ; Plate No.4 : RB_5

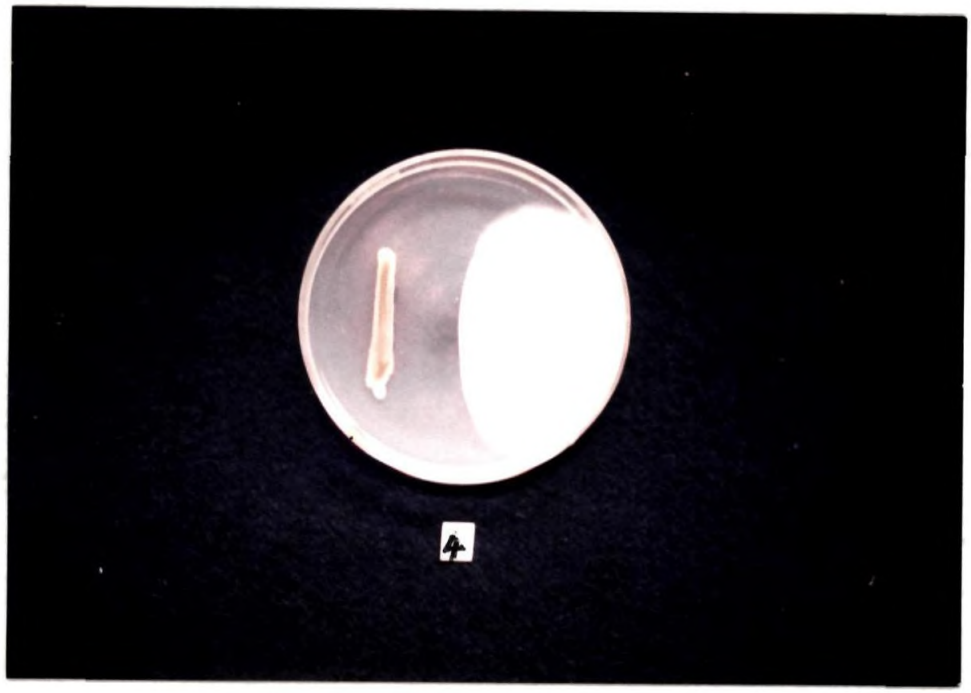

Plate 5.4 A close view of RB_5 showing antagonism against *P. meadii.*

EDAPHIC FACTORS

Moisture content

Moisture content (Table 20) in the soils under the two clones was not found to show any significant variation. But among the locations it showed significant variation (17.60% for Malankara estate compared to 13.59% for Cheruvally estate). Among the age groups also the difference was significant. 5 to 6 year old plantations retained more water in the soil (18.07%) compared to 2 to 3 year old plantations (12.61%) and in 10 to 11 year old plantations the soil moisture content was medium (16.11%).

Table 20. Soil Moisture Content (%)

20.1 Single factor averages

CLONE (A)		LOCATION (B)		AGE GROUP (C)		
RRII 105	PB 260	CHERUVALLY	MALANKARA	2-3 YRS	5-6 YRS	10-11 YRS
14.86	16.33	13.59	17.60	12.61	18.07	16.11

20.2 Two factor interactions

INTERACTION	↓ LOCATION / CLONE (AxB) →	RRII 105	PB 260
CLONE X LOCATION	CHERUVALLY	12.74	14.43
	MALANKARA	16.98	18.22
	↓ AGE GROUP/LOCATION (AxC) →	RRII 105	PB 260
CLONE X AGE GROUP	2-3 years	11.40	13.82
	5-6 years	18.60	17.53
	10-11 years	14.58	17.63
	↓ AGE GROUP/LOCATION (BxC)→	CHERUVALLY	MALANKARA
AGE GROUP X LOCATION	2-3 years	12.07	13.15
	5-6 years	15.30	20.83
	10-11 years	13.40	18.82

20.3 Three factor interactions

(Clone x Location x Age group; AxBxC)

AGE GROUP	RRII 105		PB 260	
	CHERUVALLY	MALANKARA	CHERUVALLY	MALANKARA
2-3 years	12.33	10.46	11.80	15.83
5-6 years	15.90	21.30	14.70	20.37
10-11 years	10.00	19.17	16.80	18.47

Critical difference

Source of variation	CD
A	NS
B	2.10
C	2.57
AB	NS
AC	NS
BC	NS
ABC	5.14

NS = Not significant

Interaction analysis between all the three factors also showed significant variation in soil moisture with a maximum in soil of 5 to 6 year old RRII 105 at Malankara estate (21.30%) compared to the minimum in the soil of 10 to 11 year old RRII 105 at Cheruvally estate (10%). Only the rhizosphere yeast population expressed a significant negative correlation (-0.391) with the soil moisture content. The other soil microorganisms were not affected by the range of variation in soil moisture observed.

Soil pH

The pH of the soils showed only slight variations (Table 21). The soils at Cheruvally estate planted with RRII 105 had a pH of 5.16 which was significantly higher than the pH in RRII 105 plantations at Malankara estate (4.84) (Table 21). The pH of the soils studied did not influence the microflora.

Table 21. Soil pH

21.1 Single factor averages

CLONE (A)		LOCATION (B)		AGE GROUP (C)		
RRII 105	PB 260	CHERUVALLY	MALANKARA	2-3 YRS	5-6 YRS	10-11 YRS
5.00	5.02	5.04	4.98	5.03	4.83	5.17

21.2 Two factor interactions

INTERACTION	↓ LOCATION / CLONE (AxB) →	RRII 105	PB 260
CLONE X LOCATION	CHERUVALLY	5.16	4.91
	MALANKARA	4.84	5.12
	↓ AGE GROUP/LOCATION (AxC)→	RRII 105	PB 260
CLONE X AGE GROUP	2-3 years	4.84	5.22
	5-6 years	4.99	4.68
	10-11 years	5.19	5.16
	↓ AGE GROUP/LOCATION (BxC) →	CHERUVALLY	MALANKARA
AGE GROUP X LOCATION	2-3 years	5.08	4.98
	5-6 years	4.87	4.80
	10-11 years	5.17	5.17

21.3 Three factor interactions

(Clone x Location x Age group; AxBxC)

AGE GROUP	RRII 105		PB 260	
	CHERUVALLY	MALANKARA	CHERUVALLY	MALANKARA
2-3 years	5.05	4.62	5.11	5.34
5-6 years	4.97	5.00	4.76	4.59
10-11 years	5.47	4.90	4.86	5.45

Critical difference

Source of variation	CD
A	NS
B	NS
C	NS
AB	0.32
AC	NS
BC	NS
ABC	NS

NS = Not significant

****

CHAPTER 5

DISCUSSION

Effective utilization of plant microbe inter relationship for higher crop production with minimum interference in the ecosystem is an essential component of sustainable agriculture. Detailed study of such association in nature is imperative to attain this goal. The beneficial role of microorganisms in plant growth and crop production have received scientific attention during the past half a century, the most important among them being the nitrogenase activity, phosphate solubilization activity and biocontrol activity. Even though much work has been done in these fields, due to the high specificity of the microbes to the plants, a generalized study may not help in producing practical results. Specific studies on each crop plant need to be conducted to identify the specific natural microorganism associated with it and to manipulate them for the beneficial purposes.

In the present study an attempt was made to isolate and enumerate the various microorganisms associated with two popular high yielding rubber clones. The beneficial effects of some of the isolated organisms were studied in more detail so that better performers could be identified. Biocontrol activity of the isolates against only one of the major pathogens of rubber, *Phytophthora meadii*, was attempted in this study.

ENUMERATION OF MICROORGANISMS

Phylloplane microflora

Phylloplane provides a favourable substrate for microbial growth. The attached dust particles, atmospheric humidity and the moisture developed due to transpiration makes the leaf surface a very conducive habitat for the

microbes. In the present investigation, different types of microorganisms such as fungi, bacteria, yeasts and actinomycetes were isolated and enumerated. Their number varied widely depending on the type. The number of bacteria was seen to be much higher per unit area (57000/cm^2) followed by fungi (1455/cm^2), actinomycetes (100/cm^2) and yeasts (5/cm^2). When the different interactions were studied independently or in combinations, the same trend was further evident. This conclusively proves that bacterial population dominates on the leaf of rubber plants and yeasts are present only in very low numbers.

The phylloplane microorganisms are very important to the host plant because they influence growth, health and nutrition of the plants (Clark, 1976). Remarkable similarity in the microbial population was observed irrespective of clones, location and age groups with the bacterial count being maximum followed by fungi, actinomycetes and yeasts. While Kothandaraman (1984) reported similar observations from rubber plants, Joseph *et al.* (1988) ranked actinomycetes as second. Ruinen (1961) suggested that the increased bacterial population might be due to slow development of yeasts and filamentous fungi. Yeast colonization depends on the prior activity of bacteria during which some essential nutrients are released. Unlike soil microorganisms, phylloplane organisms depend on the nutrients on the outer surface of leaves, released by the leaves or by other microorganisms on the surface (Sarkar and Sammadar, 1982).

Cauloplane microflora

Like phylloplane, cauloplane also is an aerial substrate for microbial growth. The bark also harbours various types of microorganisms like bacteria, fungi, actinomycetes and yeasts. Although lichens and moses are

also frequently seen on the bark, they were not covered in this study. In general, the population of cauloplane organisms resembled the phylloplane organisms especially in kind. In number also there was similarity except for bacteria whose number was about five times higher than that on the leaf surface. This may be due to the increased availability of substrates like dead tissue, exudates through lenticels, dust particles and higher moisture availability.

Cauloplane bacterial number was found to be 2,94,000/cm^2 compared to 57,000/cm^2 for phylloplane. Fungi stood second with 1240/cm^2 followed by actinomycetes (108/cm^2) and yeasts (4/cm^2). Most of these were saprophytic. Some were beneficial, protecting the plants from parasites (Bier, 1963; Bier and Rowat, 1967). Garner (1963) found that most dominant among cauloplane microorganisms was fungi imperfecti, followed by ascomycetes. Like in the phylloplane, cauloplane organisms may be casuals or residents.

Rhizosphere microflora

Due to better substrate availability and other favourable external factors like the presence of root exudates and longer moisture retention, soil and specifically the rhizosphere is probably one of the best natural substrates for microbial growth. For phylloplane and cauloplane, enumeration was done for one square centimeter (cm^2) whereas for rhizosphere it was done for one gram of soil. Because of this reason a direct comparison of numbers is not possible. Beneficial microorganisms such as phosphate solubilizing bacteria, nitrogen fixing bacteria, biocontrol organisms, *etc.* were identified among the rhizosphere microflora. The activity of nitrogen fixers was

assessed indirectly using gas chromatography and therefore their number is not directly comparable.

The population of bacteria in general and phosphate solubilizing bacteria in particular was found to be very high in all the rhizosphere samples studied. The count of phosphobacteria (34 x 10^6) was nearly 7 per cent of the total bacteria (47.5 x 10^7) Actinomycetes stood next with 70 x 10^4/g of soil followed by fungi with 40.5 x 1010^3/g of soil. Lowest count was found in the case of yeasts which was 11.6 x 10^3/g of soil.

The dominance of bacteria was evident irrespective of the interactions with the factors considered in this study. In the rhizosphere, based on numbers, bacteria dominated all other types and yeasts were dominated by all other microorganisms studied.

Rhizosphere is probably the most studied ecological vicinity of a plant in relation to its microbial associations. Nitrogen fixation, phosphate solubilization, VAM associations, PGPR microflora, rhizosanitizers were all investigated in detail. But many aspects are still unknown mostly due to the high specificity of such organisms. They function individually but more commonly they interact with each other thus influencing their mutual effects (Saxena and Tilak, 1994). Plant type, acidity of soil, moisture, nutrient status, electrical conductivity, redox potential, etc. of the soil may influence the rhizosphere microorganisms (Jayaratne, 1995). The total rhizosphere is determined by an interacting trinity, the soil, plant and microbes. Root exudates also play a significant role. Enhanced production of flavanoids in roots of chick pea seedlings has been observed to help in systemic resistance to pathogenic fungi like *Fusarim oxysporum* and *Rhizoctonia solani* (Parmer and Dodarwal, 1997).

Kothandaraman *et al.* (1989) observed the dominance of bacteria in the rhizosphere of rubber plants in the traditional rubber growing tracts of India. They have reported 55.10^4/g bacteria, $13.14/10^4$/g fungi, 14.14 x 10^4/g actinomycetes and 9 x 10^4/phosphobacteria. Deka *et al.* (1992) also made similar observations from a non-traditional rubber growing region. The population of bacteria was estimated as 38.68 x 10^5/g, fungi as 6.41 x 10^3/g and actinomycetes as 5.93 x 10^5/g of dry soil. In the present study, these numbers were 47.5 x 10^7 for bacteria, 40.5 x 10^3 for fungi, 70 x 10^4 for actinomycetes, 34 x 10^6 for phosphobacteria and 11.58 x 10^3 for yeasts. The higher number may be due to climatic conditions or other agricultural practices that are dominant at the time of collection of samples. Moreover, both the locations included in this study are areas receiving high rainfall.

BENEFICIAL ASSOCIATIONS

Vesicular and arbuscular mycorrhyzae

VAM fungi are more common in higher plants. The most common genera noticed are *Glomus, Gigaspora, Sclerocysts* and *Acaulospora* (Joseph, 1997). About 95% of world's species of vascular plant are reported to be mycorrhizal, but only less than 5% have been examined for mycorrhizae (Lakhanpal and Anandsagar, 1994). The presence of VAM spores in all the soil samples tested in this study indicates their universal association with rubber roots. The lack of significant variation in the spore number, shows that the inoculum of VAM is not a limiting factor in rubber soils. Microscopic examination of the roots of the two clones, belonging to different age groups and locations revealed that the clone PB 260 is more mycorrhizal compared to RRII 105. One of the factors for the higher girthing in PB 260 compared to RRII 105 (RRII, 1997) could be the higher

nutrient absorption efficiency of the roots provided by the mycorrhizae associated with this clone. Higher mycorrhizal infection will also result in better water uptake. Nair and Girija (1988) have observed that *H. brasiliensis* is highly mycorrhizal (71.9% infection) compared to other tree crops of Kerala like *Areca catechu* (21%), *Artocarpus altilis* (breadfruit tree) (70.6%), *Cinnammomon zeylanicum* (46.6%), *Theobroma cacao* (56.3%), *Cocos nucifera* (62%), *Coffee arabica* (62%), *Annona squamosa* (19.9%), *Eucalyptus grandis* (59.4%) and *Mangifera indica* (56.2%). Schwob *et al.* (1999) reported that rubber trees from different sites in Brazil showed wide variation in mycorrhizal infection between sites, but the number was mostly the same in different seasons. The number varied from 38% to 84%.

In the present study the average spore count was 23 per g of dry soil. This is almost double of the estimate by Schwob *et al.* (1999) from Brazilian soil (10 to 15/g). The variation in the number of VAM spores depending on season was also reported by Trimurthulus and Johri (1998) for different types of soils of Uttar Pradesh in Northern India.

Phosphate solubilization

Out of the 92 different isolates of various organisms 28 were found to have phosphate solubilizing property. Of these, 11 were bacteria and 17 were fungi. Fungi included 10 isolates of *Penicillium*, 4 isolates of *Aspergillus* and 3 isolates of *Fusarium*. Among all the phosphate solubilizers an *Aspergillus* species (PF_{12}) isolated from the leaf was the most effective followed by a *Penicillium* species (RF_9) isolated from the rhizosphere. Several workers have reported the effectiveness of *Aspergillus* sp. in phosphate solubilization (Bopaiah, 1985; Gupta and Biswas, 1994;

Narsian *et al.*, 1994). Highest solubilization by bacteria was shown by isolate RP_{10} isolated from the rhizosphere. *Pseudomonas* sp. is reported to be the best phosphate solubilizer among bacteria (Bopaiah, 1985; Gupta and Biswas, 1994).

On the precipitated apetite agar medium RP_1, RP_3, RP_4 and RP_6 were the best solubilizers in the decreasing order of efficiency, while in colorimetric estimation RP_{10} showed the highest efficiency followed by RP_5, RP_6 and RP_9. The difference in the results may be due to the difference in the source of phosphate used. In the first case calcium phosphate was used whereas in the second it was rock phosphate. It could therefore be inferred that different species of the same genus may vary in their efficiency to dissolve different sources of phosphates.

Antagonism to *Phytophthora meadii*

Biological control of plant pathogens by microorganisms has been reported by numerous investigators (Andrews, 1992; TeeBeest *et al.*, 1992; Sivasithambaram, 1998). All isolates were tested for biocontrol activity against *Phytophthora meadii*. Out of the 92 isolates 31 was antagonistic to *P. meadii*. In addition, 5 isolates of *Trichoderma* showed over-growth and sporulation. Of the 31 isolates with antagonistic effects 10 were regular bacteria, 7 were phosphobacteria, 5 Actinomycetes, 3 yeasts and the rest filamentous fungi. Fungi belonged to different genera like *Penicillium, Fusarium*, *Pestalotia* and *Alternaria*. Biological control of soil borne plant pathogens with rhizosphere bacteria (Weller, 1988) and fungi (Adams, 1990) have been reported.

Phosphate solubilizers have not yet been reported to be biocontrol agents. In the present study, out of the 10 phosphate solubilizers studied, 7 were found to be good biocontrol agents as well.

All the five actinomycetes isolated in this investigation were found to be good biocontrol agents against *P. meadii*. This could be attributed to their antibiotic production capacity. Actinomycetes (Joseph *et al.*, 1988; Kothandaraman *et al.*, 1991) and fungi have been used as biocontrol agents against plant diseases and pests (Sunita, 1984; Subbaiah *et al.*, 1984; Jacob *et al.*, 1991; Nehru *et al.*, 1991; Jayasuriya, 1998; Larkin *et al.*, 1999). Plant growth promoting rhizobacteria have been used as antagonist against white root disease (Ikram and Hashim, 1998). Actinomycetes have been demonstrated to be antagonistic against *Phytophthora meadii* and *Phellinus noxius* causing leaf and root disease (Kothandaraman *et al.*, 1991). *Trichoderma harzianum* has been shown to be antagonistic to *Phytophthora meadii* (Vanitha *et al.*, 1994). Some of the microbial isolates now identified could be multiplied and used against *Phytophthora* diseases in rubber after further expermientation.

Nitrogenase activity

Nitrogenase activity in the soil is mainly due to non-symbiotic microorganisms such as *Azotobacter, Beijerinkia* and *Azospirillum* (Kothandaraman, 1984; Wani *et al.*, 1988). Symbiotic nitrogen fixers like *Rhizobium, Bradyrhyzobium, etc.* also fix atmospheric nitrogen with the help of nitrogenase enzyme but its action is mostly limited within the roots (Rangarajan, 1989; Ikram *et al.*, 1994).

In the present investigation maximum nitrogenase activity of 10.98 was shown by a soil sample from Malankara estate where 2 to 3 year old PB 260 trees were grown. This was followed by 2 to 3 year old RRII 105 plantation soil at the same location with an activity of 1.8. All other soils recorded activity below this level.

EDAPHIC FACTORS

Even though this study deals with phylloplane, cauloplane and rhizosphere organisms, more stress is given to rhizosphere. The microbial life in soil is influenced by numerous factors but only two such factors are dealt in this work.

Soil moisture

All microorganisms are dependent on the availability of water. Since in the present study focus was not on the moisture relationship of microorganisms, samples were collected only once in the month of February which marks the beginning of the dry season. Within the soil samples assayed, there were variation in the moisture content, which might have influenced the microbial life. Significant variations in the moisture content of the soil based on locations and age groups was evident. The difference based on location is probably due to higher moisture content and humidity in Malankara estate because of its proximity to the Malankara dam. The difference based on age groups may be due to the difference in the canopy cover. In 2 to 3 year old plantations there is better light penetration leading to higher surface evaporation and less humidity.

The VAM spore count was reported to be significantly different in dry compared to rainy seasons in Brazil. The spore count in one of the sites studied in rainy season was found to be 3139/200 g of dry soil compared to 2367/200 g in dry season and the count was observed to be directly associated with the soil moisture content (Schwob *et al.*, 1999).

Soil pH

pH of soil is a very important factor influencing microbial growth. In the present investigation no attempt was made to study the effect of pH on microbes in detail, but it was seen that soils with RRII 105 at Cheruvally had a significantly higher pH (5.16) than same soil type at Malankara estate (4.84). No correlation was observed between soil pH and microbial population in this study because the soils were in the acidic range with only a narrow variation in pH, not sufficient enough to cause significant changes in the microflora.

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CHPTER 6

SUMMARY

Microbes surround us from all sides for good or evil. This is true in the case of plants as well. Not only they surround the plants but also they reside on plant surfaces and their surroundings. Rhizosphere provide a favourable ecological niche for interacting microorganisms and they abound in number as well as kind in the root vicinity. Phylloplane and cauloplane also support microbial life but only to a lesser extent. An attempt was hence made to enumerate the microorganisms associated with two popular rubber clones, RRII 105 and PB 260.

The various microorganisms associated in the rubber plants such as bacteria, fungi, actinomycetes and yeasts were enumerated in the present study. On all the three plant parts studied namely leaf, stem and rhizosphere the population of bacteria dominated, followed by fungi, actinomycetes and yeast. Among these, the rhizosphere harboured more microorganisms followed by cauloplane and phylloplane. Since there was no significant difference in the pH and moisture content of the soil samples analyzed, difference in microbial flora were not so pronounced based on such edaphic factors.

The study included two popular rubber clones, RRII 105 and PB 260, in two separate plantations. From each clone and location, three age groups such as 2 to 3 year old, 5 to 6 year old and 10 to 11 year old plantations were selected for study.

This investigation also tried to isolate and study some of the beneficial roles of the microorganisms associated with rubber trees. Two such areas studied in detail were phosphate solubilization and biocontrol activity. Nitrogenase activity and VAM presence were also studied.

Phosphate solubilizing organisms were abundant in the rhizosphere. Both bacteria and fungi are involved in solubilizing phosphates in the soil. Out of the 92 organisms tested, 28 were found to be phosphate solubilizing. Of these, 11 were strains of bacteria and the rest were isolates of *Penicillium* (10), *Aspergillus* (4) and *Fusarium* (3). The extent of their ability to solubilize rock phosphate was different for different organisms. Under colorimetric estimation an *Aspergillus* isolate from phylloplane (PF_{12}) showed the maximum ability followed by a rhizosphere bacterial strain (RP_{10}). A bacterial isolate RP_1 showed a wider clear zone around the colony followed by RP_3 when tested for $CaPO_4$ solubilization on apetite agar plates.

Biocontrol activity of all the 92 isolates against *Phytophthora meadii* also was tested and 31 were found to show some degree of antagonism. Maximum effect was seen in the case of RB_5 (inhibitory zone width 2 cm) followed by RB_6 (inhibitory zone width 1.3 cm). *In vitro* experiments gave very promising results. Fungi, Actinomycetes and yeasts also showed some degree of antagonism. However, the isolates should be tried for field effectiveness before exploiting them as large scale biocontrol agents.

All the soil samples tested showed some degree of nitrogenase activity but the rate was not high enough, suggesting the scarcity of free living nitrogen fixers in the soils under study.

All samples showed the presence of VAM in the feeder roots but their percentage of infection varied from 65.28% to 79.44%. VAM spores were commonly seen in all the samples. Their number ranged from 17 to 27 per gram of soil. In general, PB 260 was more mycorrhizal than RRII 105.

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