ASSOCIATION OF MICROFLORA WITH RUBBER

(Hevea brasiliensis)

AND THEIR BENEFICIAL ROLES

Introduction:

The rubber tree (Hevea brasiliensis (Willd. ex A. Juss.) Müll. Arg.), the most important source of natural rubber is constantly under threat from various pathogens that cause severe diseases like abnormal leaf fall, Corynespora leaf disease, brown root disease, pink disease and Colletotrichum leaf disease.

Considerable crop loss due to heavy defoliation in abnormal leaf fall disease has been reported by Jacob et al. 1989, 2006. Manju et al., 2001 has given a description of the severity of Corynespora Leaf Fall (CLF). Maladies of brown root disease has been described by Rajalakshmi et al., 2000. Sripati Rao, 1975, has reported the symptoms and severity of pink disease. Details regarding the Colletotrichum leaf disease has been given by Edathil et al., 2000.

At present, the diseases are controlled by applying chemical fungicides which may have adverse effects on the environment and may deprive the soil of its fertility. Hence the use of biocontrol agents against diseases of rubber needs attention. It is advantageous to search for the biocontrol agents in the vicinity of host plants, as the establishment and survival of such organisms would be better due to their adaptation to the environment. Hence identification of beneficial microorganisms associated with rubber trees of different age groups grown in varying agroclimatic conditions in the south western rubber growing region of India was attempted.

In 1974, Leben proposed that bacteria may survive on dry leaf surfaces in sites that are protected from exposure to harsh physical conditions. Microbial population dynamics on leaves has been studied by Kinkel, 1977. Artificial enhancement in the population of saprophytes on leaf, when such densities do not naturally exist, may help in the biological control of leaf disease in the fields.
Collection of Samples:

In an attempt to recognize the natural microflora which may be used as biocontrol agents, more than 1300 samples from rubber trees growing in three Southern states in India were collected covering six major rubber growing zones in South western India, namely Southern Tamil Nadu, South Kerala, Central Kerala, North Kerala, South West Karnataka and Tropical High Altitudes. Trees belonging to two different clones namely RRII105 and PB 260 and age groups, depending on their availability, were selected and marked in six estates at Nettana (Karnataka), Padiyoor (North Kerala), Palappilly (Central Kerala), Chethackal (South Kerala), Lahai (Tropical High Altitude, Kerala) and Vaikundam (Tamil Nadu). Soil samples were also collected from the virgin forests in the representative areas near to the rubber plantations to enumerate the soil microflora and compare the microflora from rubber plantations with the native flora of virgin soil. The moisture content and pH of soil were recorded to observe their influence on the soil microflora.

The present study aims at enumerating the phylloplane, cauloplane and rhizosphere microflora associated with rubber trees and studying their spatial distribution in rubber plantations in different traditional rubber growing zones of India. The distribution pattern of soil microflora has been studied in a five year old rubber plantation in Tripura by placing fertilizers at varying soil depths (Deka et al., 1998).

The aim of the present research was, to provide insight into the population dynamics of fungi, bacteria and other microbes associated with the two clones of rubber trees, during the different seasons and to check whether the natural microflora residing in the phylloplane, cauloplane and rhizosphere can act as biocontrol agents against the major pathogens causing diseases of rubber trees. The various studies conducted during this research project were as follows:
1. **Seasonal variation in microbial population:**

The isolation and enumeration of microorganisms from the collected samples were done during three seasons, viz. summer, monsoon and post monsoon in order to study the seasonal variations in microflora. The isolated organisms included 144 fungal, 118 bacterial, 7 actinomycetes and 4 yeast isolates from phylloplane, cauloplane and rhizosphere sources and 12 phosphobacterial isolates from rhizosphere sources.

In the present study, it was observed that all the 300 phylloplane samples collected from the six regions harboured a variety of microorganisms. Bacteria and fungi were abundant in all the samples whereas the number of actinomycetes and yeasts were very low or absent in many cases.

Bacterial population was very low in the phylloplane of both mature and immature rubber plants during the summer season and higher during the rainy season. It was most abundant during the post monsoon season.

The phylloplane fungal population was observed to be higher during the post-monsoon season. Although there is a general trend towards increase of fungal population from summer to rainy and finally post-monsoon, the difference was more significant at two regions, namely Nettana and Chethackal.

On statistical analysis it was found that there was significant seasonal variation in phylloplane bacteria on mature rubber trees of clone RRII 105 at Nettana. Immature RRII 105 trees at Nettana also had significant seasonal variation. But seasonal variation was not significant in the case of the clone PB 260 mature trees. Seasonal variation of phylloplane bacteria at Padiyoor on mature as well as immature trees of RRII 105 were significant. At Palappilly, RRII 105 mature and immature and PB 260 mature and immature trees had significant seasonal variation. Seasonal variation of phylloplane bacteria at Chethackal was not significant in mature RRII 105 trees while it was significant in immature trees of RRII 105. Highly significant variation was observed in PB 260 mature trees while lesser significance was observed in the case of immature trees.
Phylloplane bacteria at Lahai had highly significant seasonal variation in RRII 105 mature trees while no significance was observed in immature trees of RRII 105 and mature PB 260 trees. Highly significant variation was present in phylloplane bacteria at Vaikundam in the case of RRII 105 mature rubber trees while it was lower in immature trees. Seasonal variation in phylloplane bacteria on PB 260 mature trees showed only low significance while in immature trees more significant variation was present.

In general, the phylloplane bacterial population was significantly high during post monsoon season and low during the summer season irrespective of the clones RRII 105 and PB 260.

The phylloplane fungi at Nettana showed significant seasonal variation in RRII 105 mature, immature and PB 260 mature trees. At Padiyoor there was significant seasonal variation in RRII 105 mature and immature trees. Seasonal variation at Palappilly was significant in RRII 105 mature and immature and PB 260 immature trees while no significance was observed in mature PB 260 trees. Significant seasonal variation was observed in samples from Chethackal RRII 105 mature, immature and PB 260 mature trees while immature PB 260 trees showed no significant seasonal variation. Slightly significant seasonal variation was observed in mature and immature RRII 105 trees at Lahai while no significant variation was observed in mature PB 260 trees. All the phylloplane fungal samples at Vaikundam namely RRII 105 mature and immature as well as PB 260 mature and immature had significant seasonal variation.

In general, the phylloplane fungal population was high during the post monsoon season and less during summer season irrespective of the clones. The mature trees showed higher population than immature trees.

Highest count of cauloplane bacteria was reported from Padiyoor, from RRII 105 mature tree samples (235.2x10^2) in the post monsoon season while least count was in RRII 105 immature samples (4.0x10^2) from Vaikundam during the summer season.
Statistical analysis of seasonal variation in cauloplane bacteria, revealed that at Nettana, seasonal variation was significant in the case of PB 260 mature trees while mature and immature trees of RRII 105 had no significant variation. RRII 105 mature trees at Padiyoor showed low variation while in immature trees of the same clone it was high. At Palappilly both RRII 105 and PB 260 mature and immature trees showed significant variation. Significant seasonal variation was observed at Chethackal for RRII 105 mature, immature and mature PB 260 trees while PB 260 immature trees showed no significant variation. Mature and immature RRII 105 trees as well as PB 260 mature trees at Lahai had significant seasonal variation. Seasonal variation of cauloplane bacteria at Vaikundam was significant both in mature and immature trees of clones RRII 105 and PB 260.

The cauloplane bacterial population was observed to be highest during the post monsoon season. Between the two clones of rubber trees, RRII 105 harboured higher population of cauloplane bacteria. The population was higher in mature trees.

Cauloplane fungal population also showed significant seasonal variation in RRII 105 mature trees at Nettana while higher significance of variation was seen in RRII 105 immature trees. PB 260 mature trees also showed significant variation. Both RRII 105 mature and immature trees at Padiyoor showed significant seasonal variation. At Palappilly, cauloplane fungi samples had significant variation in the case of mature RRII 105 trees and immature trees. While no significance was observed in the case of mature PB 260 trees, high significance was present in immature PB 260 trees. There was no significant seasonal variation of cauloplane fungi in RRII 105 mature, immature and PB 260 mature trees at Chethackal. But immature PB 260 trees had significant variation. At Lahai, cauloplane fungal population showed slightly significant variation on RRII 105 mature trees while no significant variation was present in the immature trees of the same clone. But mature PB 260 trees showed more significant seasonal variation. Cauloplane fungi samples of RRII 105 mature trees at Vaikundam had significant variation. Mature trees of PB 260 clone had low significance and immature PB 260 trees had no significant variation.
In general, cauloplane fungi was highest in the post monsoon season. Higher cauloplane fungal population was reported in clone RRII 105 than in PB 260. The population was higher in mature trees.

The results of statistical analysis of seasonal variation indicates that rhizosphere bacteria on RRII 105 mature trees had significant variation at Nettana, Padiyoor, Palappilly, Chethackal, Lahai as well as Vaikundam. Immature RRII 105 clones also had significant variation at all the above regions. Rhizosphere bacteria on PB 260 mature trees had significant seasonal variation at Nettana, Chethackal, Lahai and Vaikundam. But at Palappilly the variation was not significant. In the case of immature PB 260 trees, there was significant variation at Palappilly, Chethackal and Vaikundam.

In general, rhizosphere bacterial count was observed to be low during the rainy season. Highest rhizosphere bacterial count was found during the post monsoon season. The rhizosphere bacterial population in mature trees was generally higher in clone PB 260 than in RRII 105.

Seasonal variation in rhizosphere fungi was significant in all the samples from Nettana, Padiyoor, Palappilly, Chethackal and Lahai. Significant variation was present at Vaikundam in mature and immature RRII 105 and immature PB 260 while mature PB 260 had no significant variation.

The rhizosphere fungal population was generally lowest in the rainy season. Between the two clones of mature trees, PB 260 had higher rhizosphere fungal population than in RRII 105.

Rhizosphere actinomycetes were not universally present in all the samples taken during the three seasons. The summer season samples were devoid of actinomycetes while all the samples in the post monsoon and all the samples except one in the rainy season possessed actinomycetes. Highest actinomycetes population was recorded in RRII105 mature samples from Vaikundam during the post-monsoon season.
Seasonal variation of rhizosphere actinomycetes was significant in mature RRII 105 trees at Lahai and immature trees of the same clone at Nettana, Padiyoor, Chethackal and Lahai. In PB 260 trees, seasonal variation was significant in mature trees at Nettana and mature and immature trees at Chethackal.

In general, among the mature trees of the two clones, PB 260 harboured more rhizosphere actinomycetes than RRII 105.

Rhizosphere phosphobacteria were not present in Palappilly RRII 105 immature, PB 260 mature and Vaikundam PB 260 mature soil during the summer season. There was no significant variation between the phosphobacteria samples of summer and rainy seasons in PB 260 mature trees of Lahai. Maximum population was found at Chethackal during the post monsoon season in RRII 105 immature trees.

Statistical analysis of seasonal variation of rhizosphere phosphobacteria showed that the samples from mature RRII 105 trees of Nettana, Padiyoor, Palappally, Chethackal, Lahai and Vaikundam had significant variation. RRII 105 immature rubber trees of Nettana, Padiyoor, Chethackal, Lahai and Vaikundam also showed highly significant variation. In the case of PB 260 mature samples, highly significant variation was found in rhizosphere samples from Nettana and Chethackal. In immature PB 260 trees, significant variation was observed at Palappally, Chethackal and Vaikundam while at Lahai there was no significant variation.

Phosphobacterial colonies on mature RRII 105 and PB 260 clones of rubber trees showed significant clonal variation during the rainy season in samples from Nettana, Palappilly, Chethackal, Lahai and Vaikundam. But during the post monsoon season highest significant clonal variation was shown in samples from Chethackal and Nettana.

Rhizosphere azotobacter count was observed to be low during the rainy season. Highest azotobacter count was found during the summer season in
Nettana and Palappilly, whereas higher count was observed during the post-monsoon season in Chethackal and Lahai regions.

Statistical analysis of seasonal variation of rhizosphere azotobacter have shown that mature RRII 105 trees at Nettana, Palappilly, Chethackal, Lahai and Vaikundam showed highly significant variation while only those at Padiyoor had no significant variation. Similarly rhizosphere azotobacter on immature 105 trees at Nettana, Padiyoor, Chethackal and Vaikundam showed highly significant seasonal variation while those at Lahai showed slightly significant variation and those at Palappilly had no significant variation.

In all the rhizosphere samples of PB 260 from Nettana, Palappilly, Chethackal, Lahai and Vaikundam there was highly significant seasonal variation. PB 260 immature tree samples from Palappilly and Vaikundam also showed highly significant seasonal variation.

In general, rhizosphere azotobacter count was observed to be low during the rainy season. Highest *Azotobacter* count was found during the summer season in Nettana and Palappilly, whereas higher *Azotobacter* count was observed during the post-monsoon season in Chethackal and Lahai.

2. **Studies on VAM**

Root samples of the different clones were processed, stained and examined under the microscope to observe mycorrhizal association. The percentage incidence of vesicular arbuscular mycorrhizae (VAM) in rubber roots was calculated. Spores of VAM were collected by wet sieving and decantation and the spore count was recorded, microphotographs were taken to visualize the presence of vesicles and arbuscular nature of the fungi within the roots.

The seasonal variation of VAM incidence on the roots of rubber trees was not consistent across all the regions. Statistical analysis of the seasonal variation in the incidence of VAM have shown that there was highly significant variation at all the six regions with regard to all the clones belonging to the two age groups.
VAM spore count was consistently high across all the regions during summer and low during the rainy seasons. On analyzing the seasonal variation, it was observed that in all the six regions, the VAM spore count was highly significant in mature and immature RRII 105, PB 260 as well as in the virgin soils.

3. **Studies on influence of edaphic factors on seasonal variation in soil microflora:**

   Moisture content of the soil and pH were determined to observe their influence on the soil microflora. Seasonal variations in microbial population and relationship of microbes with some soil parameters were studied in tea ecosystem of Assam by Gogoi *et al.* (2003). The population was observed to be influenced both by the season and the soil factors like moisture and pH.

   Moisture contents in all regions were dependant on the seasons with highest value recorded during rainy season, followed by post-monsoon and summer seasons respectively.

   Soil pH readings were higher during the post-monsoon season in Nettana, Palappilly and Padiyoor, when compared to readings during summer and rainy seasons. However, higher readings of soil pH were recorded during the summer season in Lahai and Vaikundam regions.

   The correlation between soil moisture and soil pH with the rhizosphere bacteria, fungi, actinomycetes, phosphobacteria and azotobacter were worked out.

4. **Effect of Bio-Chemical Compounds on Microflora:**

   Accumulation of phenols in diseased plant is a known phenomenon in many host-pathogen interactions (Pridham, 1965). Mandavia *et al.* (2000) studied the inhibitory effects of phenolic compounds on fungal metabolism in host pathogen interactions in Fusarium wilt of cumin. Phenol content of tobacco leaves were found to be decreased due to infection by *Corynespora cassiicola* and *Colletotrichum nicotianae* (Oke, 1988).
It has been reported that the reducing sugar content of infected sugarcane plants was higher (Sankpal and Nimbalkar, 1979; Dhumal and Nimbalkar, 1982).

Levels of sugar and phenols in the plant parts were studied in order to study their role in the variation in population of microorganisms.

The leaves from different locations collected during the post monsoon season were subjected to biochemical tests to find out whether there was any correlation between the phenol content and sugar content of the leaves and the presence of microflora on the phylloplane.

A proportional increase in phylloplane fungi was observed with increasing phenol content in all leaf samples from all regions except Lahai.

Reducing sugar content in the leaf samples showed a positive correlation with the fungal count in all the samples except one.

5. Effect of cultural practices on microflora:

Cultural practices and chemical treatments can greatly affect the severity of several soil-borne diseases by directly acting on the pathogen and, in a more complex way, by interfering with microbiological and environmental factors. Rhizosphere microflora has a profound influence on plant growth as it has an important role in making soil nutrients available to plants. The rate and extent of root colonization of soil, type of root system, presence of root exudates, etc. influence the soil microflora. Cultural practices like type and method of fertilizer application, soil disturbances, etc. also influence microbial population in the rhizosphere of cultivated plants.

In order to study the effect of different cultural practices on the population of microflora associated with rubber trees, plots with different cropping systems at Central Experimental Station of RR II at Chethackal were selected. Five rubber trees each from plots with two inter-cropping systems [banana planted along with rubber trees and coffee planted with rubber trees] and plots where no inter-crop were grown, were selected. Collected samples during the summer and rainy seasons.
It was observed that there was an increase in bacterial population during the rainy season in both phylloplane and cauloplane of rubber trees. Fungal population in phylloplane and cauloplane significantly increased during the rainy season irrespective of crop type.

The bacterial population in rhizosphere and soil was significantly high in areas where intercrop of banana with rubber was practised. The fungal population in rhizosphere and soil was significantly high in areas where intercrop of coffee with rubber was done.

The population of actinomycetes was highest in virgin soil, while there was marginal increase in areas with intercropping.

The phosphobacterial count in non intercropped areas was higher than in areas with intercrop during the rainy season. Also there was an overall increase in phosphobacterial count in soil during the rainy season with virgin soil recording the highest count.

_Azotobactor_ count in intercropped areas was higher than non-inter crop areas during the rainy season. However there was an overall increase in _Azotobactor_ count in soil during the rainy seasons, with intercropped areas with coffee recording the highest count.

6. **Studies on the beneficial effects of natural microflora:**

A wide range of microorganisms including bacteria, fungi, mycorrhiza, etc., are present in the environments of rubber plants. These may be pathogenic or non-pathogenic to the plants. Some of the microbes produce certain antibiotic chemicals or toxins which may affect the growth of other microbes residing along with them and thus may become beneficial to the plants. Phylloplane fungi were used as biocontrol agent against _Colletotrichum_ leaf disease of rubber, _Hevea brasiliensis_ Muell. Arg. (Evueh and Ogbebor, 2008). In the present study, such microbes were isolated from the leaf, bark and root surfaces, and studies were conducted on their antagonism, to determine whether they could be used as bio-control agents for major diseases of rubber.
Detailed studies were conducted on the beneficial aspects of microorganisms to the rubber trees. These were (1) *In vitro* antagonism studies (2) Production of HCN (3) Siderophore (4) Volatile Compounds against five major pathogens of rubber namely, *Corynespora cassiicola*, *Phytophthora meadii*, *Phellinus noxius*, *Corticium salmonicolor* and *Colletotrichum acutatum* and (5) Phosphate solubilization.

It was observed that the bacterial isolate (B54) showed high level of antagonism against *Corynespora cassiicola* and *Colletotrichum acutatum* while isolate (B61) showed overall effectiveness against all the five pathogens. Another isolate (B24) was observed to be antagonistic against the pathogens, *Phytophthora meadii*, *Phellinus noxius* and *Corticium salmonicolor*.

Out of 144 fungal isolates tested, 12 isolates showed antagonism against all the five major pathogens of rubber. Among these, isolate F65 was most effective against the pathogen *Colletotrichum acutatum*. However, overall effectiveness against all the five pathogens was shown by the fungal isolate F90.

*In vivo* studies were conducted using young healthy leaves of RRII 105 rubber seedlings grown in poly bags. The antagonist inoculum was applied on to the leaves followed by inoculation with the pathogen and development of disease lesions observed at specific intervals. The results indicated that the isolates that showed antagonism in *in vitro* studies were also effective in *in vivo*.

7. **Studies on Fungal Enzymes:**

In order to correlate enzyme production and antagonism of fungi against the five pathogens, the fungal antagonists were selected and their cellulase enzyme was assayed by employing appropriate substrates.

The isolates F90, F97 and F65 produced higher quantity of Endo β-1, 4 glucanase, when compared to the other isolates under study. The isolate F65 showed more enzyme activity as is evident from the release of more reducing
sugar. Negligible quantity of Endo β-1, 4 glucanase was produced by these isolates in the non-enzyme filtrates.

**Conclusion:**

Studies on seasonal variation in phylloplane microorganisms of rubber trees showed that the microbial number in summer was much less than that in the other seasons. The highest number was recorded during the post monsoon season. The reason for lesser bacteria in summer may be the lower moisture content on the leaves. As leaf surfaces dry, the number of bacteria in the leaf washings decreases. Often, only a fraction of the populations that develop on leaves in the absence of free water can be washed off.

Cauloplane bacteria and fungi also showed remarkable variation during the three seasons. But rhizosphere microflora did not show this pattern of variation. However, pH and moisture content of soil in the different locations influenced the microbial population. Seasonal variation in bacterial and actinomycetes population could be correlated with moisture regimes and pH of the soil.

Actinomycete population was very low or absent in phylloplane and cauloplane samples across all the six locations. Actinomycetes may require high organic matter content, such as soil, to survive.

VAM population was observed to be higher during summer and low during rainy season irrespective of locations.

The biochemical studies indicated that sugar and phenol content had an influence on phylloplane microflora. The population of microorganisms were influenced directly by these biochemical contents in the leaves.

Intercropping of rubber with banana was found to improve the rhizosphere bacterial population while intercropping rubber with coffee improved the rhizosphere fungal population.
Several microorganisms which were isolated from the phylloplane, cauloplane and rhizosphere proved to be antagonistic to the five known major pathogens of rubber trees.

Studies on siderophore, HCN and volatile compounds produced by antagonistic bacteria have indicated that these may be responsible for their antagonistic property.

Enzyme assays have suggested the presence of cellulase in the antagonistic fungi which may play a key role in their antagonistic property.

Bacterial isolates B_{54}, B_{61} and B_{24} and isolates of fungi F_{90}, F_{97} and F_{65} from phylloplane, cauloplane and rhizosphere of rubber trees were identified as beneficial organisms for biocontrol of major diseases of rubber. PB_{8} and PB_{9} isolates of bacteria were detected as phosphate solubilizers which can improve the P availability.