CHAPTER - 1

INTRODUCTION

One of the most important discoveries in the domain of Agricultural Microbiology, which has completely revolutionised our ideas regarding the plant microorganism relationship and which opened up new avenue for the utilization of microflora for the benefit of crop performance, had been the concept of 'Phyllosphere'. The workers in this field of Applied Microbiology are interested in microorganisms that improve crop productivity by studying those as found on the leaf. This may give hope to gain a better understanding of how those microorganisms and plants come in close contact resulting in the establishment of a unique and dynamic ecological environment. The environment is unique in the sense that both the plant leaf system and microorganisms exude and absorb organic materials and, thus facilitate a two-way movement of easily assimilable nutrients. The environment is dynamic since shifts in the quality and quantity of every potential material in the leaf surface offer plenty of scope for a variety of biological activity. This queer ecological niche of the external surface of leaf, a layer in contact with, and consequently, under the influence of the leaf and atmosphere, as a habitat for microorganisms had been characteristically termed, independently by Last (1955) and Ruinen (1956), as 'Phyllosphere'.

The term 'Phyllosphere' of plant leaves is analogous to 'Rhizosphere' of roots. Of late, the term 'Phylloplane' is preferred to 'Phyllosphere' (Mukherji and Subba Rao, 1982). Nevertheless, to be more precise, phylloplane is the layer in direct contact with the leaf and phyllosphere is the leaf surface comprising the microbial layer with the air (Ruinen, 1974). So, for wider perspective, the term 'Phyllosphere' is usually used, which for reasons of usage and priority will be adhered to including the 'Phylloplane'. Thus, the phyllosphere is an ecological microhabitat of the microorganisms, which is subject to the regulating influence of both the plant leaves and surrounding aerial environment. The inhabiting
micropopulation avail of its water and dissolved gases from the atmosphere and its nutrients from the exudates and leachings from the living leaf. The exudation and leaching of the minerals and organics are regulated by the nutritional status of the plant, mainly under the governance of the soil. As such, soil has to be included among other factors conditioning the phyllosphere into a suitable environment for a microbial community with widely different potentialities for establishment, survival and benefaction (Ruinen, 1974).

1.1. **Phyllosphere and its atmosphere**:

As phyllosphere is in direct contact with the atmosphere, the whole ecosystem comprising the plant and the phyllosphere population is very well influenced by the environmental factors - light, temperature, and humidity. These factors fluctuate seasonally and diurnally between physiologically wide extremes, and are dependent on geographical and climatic zone (Ruinen, 1974). Precisely, the dependence of the plant microclimate on the exchange of energy, and vice-versa, having profound implications on the phyllosphere microbial performance, has been well documented (Geiger, 1961; Slayter and McIlroy, 1961; Rose, 1966; Burrage, 1971; Monteith, 1973). Effects of temperature, leaf wetness, leaf bacteria and leaf and bacterial diffusates on production of *Rhynchosporum secalis* spores has been well documented (Rotem et al., 1976). Although liable to modification by the leaf or the plant surface, the exchange of energy is comprehensively determined by the climatic factors mentioned above (Raschke, 1960; Gates, 1968; Jamil and Satyakala, 1989).

Like roots, leaves are bounded by an environment which is rich in microbial propagules. However, air, unlike soil, is not conducive to the growth of most of the microorganisms and as such, microbiologically less complex as an inoculum for developing leaves. Despite being, to a great extent, microbiologically inert, the aerial atmosphere does exhibit striking diurnal, seasonal and random fluctuations in its physical, chemical and microbiological characteristics (Dickinson, 1971).
A succession of wet and dry, bright and gloomy, stormy and calm, hot and cool periods make the phyllosphere a specialised habitat where the occurrence, proliferation and activity of a diverse population of microflora fluctuate in a dramatic manner. Sometimes the microflora are active and sometimes in regression, as influenced by the physiological and biochemical status of leaf, until senescence (Ruinen, 1974). The earliest colonizers on newly formed leaves do not have to encounter any competition due to absence of any preoccupant and, in fact, they receive a potential supply of surface nutrients through leaf exudates and leachates. But as they get established they come across a relatively hostile environment due to wide fluctuating temperatures and the incidence of ultraviolet radiation (Pugh and Buckley, 1971; Pugh, 1980) They may immediately grow utilizing the fresh supply of the substances present on the leaf surface or lie dormant and inactive until leaf becomes senescent (Mukherji and Subba Rao, 1982).

Rise in temperature with the day gradually increases evaporation which enhances exudation of the leaf in the water flow through palisades and epidermis. The subsequent dew deposit on the leaf surface starts to dissolve the dried exudates of the day, leaches the surplus photosynthate and the metabolic products of leaf and phyllosphere population including the microbial decomposition material (Ruinen, 1974). Microbial ecosystems have been found (Simon et al., 1994) on the leaves of *Atriplex halimus*, a salt excreting plant in the central Negev highlands of Israel. Because of the occurrence of dew at this location during night the leaves undergo a diurnal wetting so that phylloplane microorganisms experience large fluctuations in salinity and water activity and tolerate repeated desiccation. During the dry season, in the late spring and summer, a significant amount of salts and organic matter coats the leaf surface. At this period the salt concentration on the leaf surface was calculated to be 0.4 M, and the bacterial population on leaf surface by direct count ranged from 1.06 X 10^4 to 5.06 X 10^5 per sq cm. The leaf bacteria were desiccation tolerant when on the leaf surface or when directly washed off the leaves, but much less in isolated culture. A major component of the tolerance to desiccation is probably related to the compound on
the leaf surface. As a consequence, the materials present in the phyllosphere is a resultant effect of the metabolism of plant and the resident microorganisms in this locale. In this respect, the contribution of the plant is the leaf exudates and leachates and that of phyllosphere microflora, the metabolized products of the leaf exudates and leachates and their own exudates. Two-way exchange of metabolites between the phyllosphere bacteria and host plant leaves has been demonstrated (Nandi and Sen, 1982). It is very difficult to distinguish between exudation and leaching. Probably for this reason, Tukey (1970) included all the processes involving the phenomenon of the export of plant metabolites at the leaf surface under 'leaching' which was defined as the loss of water soluble inorganic and organic metabolites from the plant (Bunster et al., 1989).

The inorganic compounds include all the essential minerals, both macro- and micro-Among the organics, the major quantity is the carbohydrates comprising sugars, pectic substances and sugar alcohols; all the amino acids found in the plants, organic acids; phenolics; alkaloids; vitamins, phytohormones; phytoalexins and volatiles (Tukey, 1970, 1971; Jones, 1976). The overall composition varies with the species and age of the plant, being ultimately governed by the climatic factors (Tukey, 1971), which exert an enormous influence on the microbial population of the phyllosphere (Blackman, 1971, Godfrey, 1976).

Phyllosphere at seedling stage entertains the lowest number of microorganisms which increases with the age of the plants. The highest count is attained when the leaves start yellowing at maturity (Dickinson, 1967; Sharma and Mukherji, 1976). The first colonizers are bacteria followed by actinomycetes, fungi, lichens, and arthropods in succession (Rumon, 1956, 1961). Pugh and Buckley (1971), however, observed that the leaves were colonized by fungi from the first unfolding of the bud. The youngest leaves of Sesamum and Gossypium harboured actinomycetes at first, which were gradually replaced by bacteria, yeast and filamentous fungi (Sharma and Mukherji, 1972, 1974). On the whole, the most commonly found microorganisms are a number of oligonitrophiles and nitrogen
fixing bacteria, actinomycetes, yeasts, fungi, and algae including nitrogen fixing blue-green algae, which showed a considerable variation in thickness and species composition on the upper and lower leaf surfaces (Ruinen, 1956, 1961, 1974). As moisture is a major factor enabling the exchange of metabolic products, the growth of the organisms in the lower surface of leaves being exposed to only the condensation of ground mist, is less conspicuous than the adaxial side, the upper surfaces, as these are exposed to dew, mist condensate and rain (Ruinen, 1974). Nutrients, supplied by the leaf exudates and the combined nitrogen supplied by the nitrogen fixers, provide an adequate substrate for the inhabiting microbes, particularly the successive ones (Ruinen, 1974).

Mainly depending on the plant species and environment, a gradual change in the distribution and abundance of phyllosphere microorganisms occurs during physiological ageing of the leaves. Ecolani (1991) isolated a number of mesophilic heterotrophic, aerobic or facultatively anaerobic bacteria, which grew on yeast extract tryptone glucose agar, from the surface of olive leaves of different ages. Unweighted average linkage cluster analysis on either the Jaccard coefficient or the simple matching coefficient recovered 1,701 representative strains in 32 phyla defined at the 70% and 80% similarity level, respectively. Of these, 25 were identified to genus or lower level. Bacterial communities on leaves of a given age at a given time during any one year displayed a very similar structure but differed significantly from those on the leaves of the same age at a different time during any one year. Communities on the leaves of a given age at a given time of the year were invariably dominated by one or another. The climax population of the phyllosphere was maintained on the mature and fully active leaf. Later, the microvegetation became more autonomous especially as metabolism of leaf and exudation into the phyllosphere decreased during senescence (Ruinen, 1961), when the fungi predominated (Ruinen, 1974). The nutritional requirements were met by mutual exchange between the various organisms of the phyllosphere and from leaching from leaves, particularly from those situated at higher levels of plant community (Ruinen, 1974). Jacques et al. (1995) found that greater bacterial population sizes
were quantified on leaves of different age groups of broad bean endive during field cultivation from leaf emergence until harvest. At harvest, a linear gradient of decreasing densities of epiphytic bacteria from outer (older) to inner (younger) leaves of the head was significant. Leaf age influenced the frequency distribution and variability of bacterial population sizes associated with leaves of broad bean endive.

1.2 Distribution of microorganisms in the Phyllosphere

In the phyllosphere, the most abundant non-diazotrophic bacteria present, in general, on different agricultural plants, belong to the genera *Pseudomonas*, *Mycobacterium*, *Chromobacterium*, *Sarcina* and *Bacillus* (Klincare et al., 1971, Lambert et al., 1990). *Achromobacter*, *Lactobacillus Xanthomonas*, *Micrococcus*, *Corynebacterium* and *Macrocyclus* were also found in the foliage of various plants (Voznyakovskaya, 1959; Stout, 1960, 1961, 1964; Bhat et al., 1971; Bessems, 1973; Ruinen, 1974). On the other hand, Murty (1984) documented Murty, (1984) documented that phyllosphere of cotton plant was a good habitat for the nitrogen fixing bacterium, *Beyernchina*. Among the actinomycetes, most common are the species of *Nocardia* (Ruinen, 1974). The predominant yeasts are *Cryptococcus*, *Candida*, *Torulopsis*, *Bullera*, *Rhodotorula* and *Sporobolomyces* (Di Menna, 1958, 1959, 1966, Ruinen, 1963). The most common nonpathogenic fungi are *Alternaria*, *Cladosporium*, *Aureobasidium*, *Epicoccum*, *Stemphylium Asochyeta*, *Phoma*, shoozy molds and species of *Chactosbyntaceae* (Dickinson, 1976) Among the green algae *Treinethia*, *Phycopelites* and species of *Protococcaceae* are dominant (Harrelson, 1969).

1.3 Relationship between plant leaves and microorganisms

More important for understanding the interrelationship between plant leaves and microorganisms in the phyllosphere is the appreciation of the physiological activities of the concerned non-pathogenic microorganisms inhabiting the phyllosphere. Some have been found to fix atmospheric nitrogen for the benefit of the higher plant; degrade plant surface waxes and cuticles, produce plant growth
regulators, compete with plant parasites and cause plants to synthesize phytoalexins and influence the growth behaviour and root exudation of plants (Last and Warren, 1972, Sharma and Mukherji, 1976). Among the microorganisms those capable of fixing nitrogen, and inhibiting the growth of plant pathogens have received more attention during recent years. Leaves of some terrestrial plants (Kenerley and Andrews, 1990) supported a diverse assemblage of microbes, predominantly filamentous fungi, yeasts and bacteria. Population densities fluctuated over several orders of magnitude and were influenced by many variables: climatic and seasonal factors, host species, air borne spore and leaf position within the canopy. The development of successful bioherbicides will, thus, require a thorough understanding of the ecology of potential agents and their interactions with other microbes within the phylloplane environment. In the present investigation, apart from the general observations, emphasis has been proposed to be given on the abundance and performance of those fixing atmospheric nitrogen, producing phytohormones and solubilizing insoluble phosphates.

1.4 Role of nitrogen fixing microorganisms:

Free-living nitrogen fixing microbes find a preferential habitat for their growth and activity on the leaf surface because of the nutrients and energy source supplied by the assimilating plant and the presence, although temporary, of water. Ruinen (1956, 1961, 1974) observed the frequency of nitrogen fixing bacteria to the tune of $10^7$ cells per sq cm on the leaf surface of various plants in tropical countries. In Eastern India, a wide variety of plants, including agricultural and horticultural crop plants, harbour nitrogen fixing microorganisms, with a higher frequency in nitrogen-poor environments, e.g., seashores and arid regions (Sengupta and Sen, 1976; Pati and Chandra, 1980; Sengupta et al., 1981; Sen et al., 1985). In the tropics, the common leaf inhabiting nitrogen fixing bacteria identified are Beijerinckia, Azotobacter, Derxia, Azomonas, Methanobacterium, Pseudomonas, Clostridium, Aerobacter, Klebsella, Desulfovibrio, Achromobacter, Xanthobacter, Cellulomonas, Chlorobium, and Rhodospirillum, (Bhurat and Sen, 1968; Bhat et al., 1971; Ruinen, 1974, Sen et al., 1985) Blue-green algae identified are

Widespread occurrence of the nitrogen fixers on the leaf surface led to the factual idea that the foliage, in the climatic condition of humid tropics, acts as a 'factory' for the production of organic nitrogen, which might contribute to the economy of the system (Ruinen, 1971). Formation of a loose symbiotic system, beneficial for the plant, has been proposed for the association between nitrogen fixing microorganisms and the leaves (Ruinen, 1961). Notwithstanding a lesser rate and amount of nitrogen fixation by most of the phyllosphere isolates as compared to their rhizosphere counterparts, the phyllosphere associates have been found to fix dinitrogen at a considerable rate ranging from 6-48 μg N/mg leaf/day with detached coffee, cotton and Phaseolus leaves floating on nitrogen free mineral salt solutions under illumination for several days (Ruinen, 1965). Working with various plants, Sadykov and Umarov (1980) observed highest rates of nitrogen fixation in the phyllosphere of the plants belonging to Compositae amounting to 434 μg N/m²/hour, corresponding to about 0.1 to 10% of the nitrogen assimilated by the plants. That dinitrogen is actually fixed on the leaf surface is substantiated by the observation that active nitrogen fixing bacteria in association with IR-579 rice leaves reduced acetylene at the rate of 664-816 n moles/g leaf/hour. (Sengupta et al., 1981).

The nitrogen fixed in the phyllosphere can easily be taken up by and benefit the concerned plant. It is evident from the redistribution of phyllosphere fixed ¹⁵N in the plant within 48 hours (Edmisten and Kline, 1968) Nitrogen fixation in the phyllosphere has been found to enhance the growth and dry matter yield of different plants (Ruinen, 1974). In effect, the apparent omnipresence of the nitrogen fixers as a component of a leaf surface vegetation suggests an important relationship between their nitrogen fixing properties and the luxuriant growth of tropical vegetation on nitrogen poor soil through an association with the living leaves (Bhar et al., 1971, Ruinen, 1974).
The magnitude of beneficial effect bestowed by the leaf diazotrophs suggest their possession of an additive property, like elaboration of growth factors, apart from dinitrogen fixation. The phyllosphere nitrogen fixing bacteria have been found to produce growth regulating substances, viz., auxins, cytokinins, and gibberellins which cause growth promotion of the respective plants (Sen et al., 1985). This property is also possessed by other leaf epiphytic microflora (Wichner and Libbert, 1968 a, b).

1.5. **Role of phosphate solubilizing bacteria**

Reports on the proliferation and activity of the phosphate solubilizing organisms in the phyllosphere are essentially lacking except those of Datta and associates (Banik and Datta, 1988; Datta et al., 1992; Datta and Banik, 1994, 1997) who visualized higher yield of crop on foliar spraying of phytohormone producing phosphate solubilizing bacteria, inspired by the fascinating results obtained on inoculation of the same in the rice rhizosphere (Datta et al., 1982).

1.6. **Manipulation and utilization of phyllosphere diazotrophs and phosphate solubilizers**

Using the information gathered over the years on activity of the phyllosphere microflora, attempts have been made and are being made to manipulate the microflora for some selected properties that benefit plants either by the direct effect or indirectly by controlling the harmful organisms. This can be achieved by introducing various beneficial microorganisms, particularly those isolated from the phyllosphere, into the leaf surface of the crop plants. In this regard, nitrogen fixers can be used, by spraying culture suspensions, to increase their frequency per unit area, for the betterment of the crops. This may participate directly in the nutrition of the crops through leaves, by fixing dinitrogen, or indirectly by increasing rhizosphere effect due to inoculation of the culture or the fixed product into the soil by run off. Fixed nitrogen retained and recycled by the microflora could eventually reach the soil where, together with the directly leached fixed nitrogen, it will be available for uptake by the roots of the plants (Runen,
Nitrogen fixing bacteria isolated from the phyllosphere of water hyacinth (Sen 1975), and rice and jute (Pati and Chandra, 1981) when sprayed on the foliage of wheat brought about higher yield. Similar results were also obtained with rice (Sen, 1975; Iswaran et al., 1978). Pati et al., (1995) compared the performance of two diazotrophic bacteria Azotobacter Chroococcum and Corynebacterium sp. isolated from rice and potato phyllosphere, respectively, in increasing dry weight and total nitrogen content of wheat seedlings when applied in vitro. An average increase in dry weight nearly by 35 - 50% and total nitrogen content by 56 - 58% was obtained which was near to that obtained with nitrogenous fertilizers. Yield increase of rice due to leaf inoculation of phytohormone producing phosphate solubilizing bacteria has been mentioned earlier in this text.

1.7. Objectives

Considering the facts stated above, it has been felt that a study of the phyllosphere organisms, especially those carrying out beneficial activities, like fixation of nitrogen, solubilization of insoluble phosphorus and production of phytohormones, and the effect of the use of the most efficient ones as foliar inoculants on the microbiological composition of the phyllosphere and rhizosphere together with the nitrogen content, phosphorus uptake, and yield of crop plants will be very interesting in regard to nutrition of the crops. Hence, the present investigation has been undertaken on the following aspects

1) Enumeration, isolation and identification of the bacteria, actinomycetes, fungi and blue-green algae in general, and nitrogen fixing, and phosphate solubilizing bacteria, in particular, of the leaves of two non-leguminous plants - water hyacinth (Eichhornia crassipes) and money plant (Scindapsus officinalis)

2) Assessment of the nitrogen fixing and phosphate solubilizing bacterial isolates, elaboration of phytohormone by nitrogen fixers and phosphate solubilizers and production of organic acids by the phosphate solubilizing
ones in the specific broths for securing the most efficient strains of nitrogen fixing and phosphate solubilizing bacteria for further experimentation.

3) Enumeration, isolation, identification and characterization, in regard to respective efficiency, of nitrogen fixing bacteria and phosphate solubilizing bacteria of the phyllosphere, and enumeration of the cited microflora together with the nitrogen content and nitrogen fixing and phosphate solubilizing power of the rhizosphere soils of a staple cereal crop rice (Oryza sativa L.) and an oil seed crop, sunflower (Helianthus annuus L.) as affected by the leaf inoculation of the most efficient strains of the nitrogen fixing and phosphate solubilizing bacteria isolated from water hyacinth leaves in order to assess their indirect benefit to the said crops with and without soil amendments.

Bacterial isolates from water hyacinth leaves were used in this experiment, for inoculation, because it was found, in the investigation, that the nitrogen fixing bacteria isolated from the said leaves were more efficient than those isolated from money plant leaves and phosphate solubilizing bacteria were only present on the leaves of water hyacinth plants.

4) Estimation of nitrogen content and phosphorus uptake, and growth and yield attributes of rice and sunflower crop plants as influenced by the foliar inoculation of the same, above mentioned, selected isolated strains of nitrogen fixing, and phosphate solubilizing bacteria of water hyacinth leaves in order to obtain information regarding the direct benefit of inoculation of the physiologically efficient leaf bacteria to the said crop plants in presence and absence of soil amendments.