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Colonization of Epiphytic Nitrogen Fixers and Associate Microbes on Forest Phyllosphere

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ABSTRACT

Phyllosphere or leaf surface of plantis an ecological habitat for many microorganisms. In this study, microbial population on the phyllosphere of tropical forest plant was performed. Both bacterial and fungal colonies were observed from the leaf surface of Simul (*Bombax ceiba* L.), Gamhar (*Gemelina arborea* Roxb.), Bahera (*Terminalia belerica* (Gaertn.) Roxb.), Haldu (*Adina cordifolia* (Roxb.) Brandis), Bijayasar (*Pterocarpu smarsupium* Roxb.), Mahua (*Madhuca latifolia* (Roxb.) A. Chev.), Jam (*Garuga piñat a*Roxb.), and Piyal (*Buchanania cochinchinensis* (Lour.) Almeida) plant. Leaf impression technique was used to assess the approximate density of microbial population and leaf washing method was adopted for isolation of the organisms. Nutrient agars with myconazole nitrate powder and potato dextrose agar with antibacterial antibiotic streptomycin were used for isolation of bacteria and fungi respectively. Burk's nitrogen free agar media was used for the isolation of nitrogen fixers. Mature leaves contain more propagules in comparison to young leaves. Total bacterial flora was quite high on the phyllosphere of plant like Bahera, Haldu, Bijayasar, Jam, and Simul. Total bacterial count always outnumbered the fungal propagules. With regards to the density of nitrogen fixers as compared to the total bacterial population, it was found that Mahua, Jam and Gamhar leaf surfaces contained very poor number of nitrogen fixing organism among the forest plant. Whereas in Simul, Bijayasar, Bahera and Haldu, their numbers were high. Among the fungal population, maximum number was recorded only on Haldu leaves. Based on colony characteristics and individual cell morphology 06 strains of nitrogen fixers differentiated. All the isolates were grown at 30°C for 72 hours to see their growth performance and at the same time nitrogen fixing capacity was also observed through micro Kjeldahl technique and Acetylene reduction assay in all the organisms. In order to nitrogen fixation capability these strains can be arranged in the following order SML2> BR1> BS2> HLD3> JM2> PL1.

Keywords: Nitrogen fixers, acetylene reduction test, diazotrophs

INTRODUCTION:

Ecology of phyllosphere started five decades ago,in this regards the term phyllosphere was coined by Last (1972) and first used by Ruinen (1956). They defined the phyllosphere as the thin layer in contact with the leaf and the atmosphere. In tropical countries like India, both the surfaces of leaves can be ocupied by a great number of different species of blue-green algae (cyanobacteria), fungi, bacteria, green algae, lichens and bryophytes **(**Ruinen 1961, Natacha 2014). Many studies have revealed that environmental conditions and leaves microclimates can have important effects on microbial community structure of phyllosphere (Lindow and Brandle 2003) (Redford and Fierer. 2009). The surface characteristics of different leaves differ widely due to variations in surface architecture like pattern of venation, trichome density, wax deposits and silicification. The epidermal cells of leaves are covered with cuticle, composed mostly of cutin. Above the cuticle a thin layer of wax is present which makes the leaf surface almost water proof. The wax is a simple lipid, composed of a long chain alcohol and a fatty acid. The alcohol may contain 12-32 carbon atoms. Wax controls the wettability of leaves which again modify the population density of microbes on leaf surfaceas wax sprays against microbial pathogen (Campbel 1985, Redford and Fierer. 2009). Besides theabove two main components wax and cutin, leaf surface also contains lipid and silicified material forming anindiscriminate complex layer. The phyllospheric microbes are very important to the plant itself. Some of them are able to fix atmospheric nitrogen and produce plant growth regulators (Murty1983); and many of themproduce antibacterial compounds (Mc Cormack *et al*., 1994). The microbes on leaf surface derive their water and dissolved gasses from the atmosphere and nutrients from the oozing and leaching materials of the living leaf. Leaching oozing out of different substances from the leaf surfaces are actually regulated by the nutritional and environmental status of the host plant (Freiberg 1998).

Agriculture and horticulture scientists are now searching some eco-friendly and sustainable alternative source of nitrogenous nutrients for the growing crop plants as well as horticultural plants. Some selected strain of micro-organisms, which fix atmospheric nitrogen, is the most suitable of all the alternatives. Those nitrogen fixers are also the unavoidable alternative to synthetic fertilizers. Uses of rhizospheric nitrogen fixers as biofertilizer are now well-known agricultural practice. In this aspect the phyllospheric nitrogen fixers also have a great potentiality to increase crop productivity.Soquantification of nitrogen fixers on leaf surface and their fixation potentiality ensure the contribution of them in agriculture. For quantification of nitrogen fixation capability, acetylene reduction assay (Giri and Pati, 2004) is the most popular method which depends on the howmuch amount of acetylene (C_2H_2) reduced to ethylene (C_2H_4) by nitrogenase enzyme, present in the nitrogen fixers instead of reducing $N_2$to NH_3. The role of phyllosphere nitrogen fixers on the forest plants is however not properly explored, basically in red lateritic soil like our forest area. Search of more beneficial micro-organisms from the leaf surface may give away the clear information of mutualism between the phyllosphere and the host plant. Following the above idea, we have tried to find out some phyllospheric nitrogen fixers and their nitrogen fixing potentialities from forest plants.

MATERIALS AND METHOD:

Media: isolation of the various microorganisms was carried outthrough dilution plating technique using nutrient agar, potato dextrose agar and Burk's agar media. During isolation of bacteria on nutrient agar media, antifungal antibiotic was added (2µg/ml) to suppress fungal growth, while in PDA antibacterial antibiotic were added (20µg/ml) to the media to suppress bacterial growth.

Leaf impression: Fresh leaves were collected from different forest area. After preliminary dust removal they are pressed on suitable sterile media inside petridishes. Plates were then incubated as a whole for 2 to 4 days. Appearing colony on the petri plate gave an idea about the density of the organisms present on the leaf surface.

Dilution plating: After preliminary washing with sterile water freshly collected leaves are cut in to small pieces and placed in 250ml Erlenmeyer flasks containing 50ml buffer solution together with several glass beads. The flasks were then shaken on a rotary shaker (200 rpm.) for 5 to 10 minutes. The solution are diluted in same buffer and used as inoculum. This solution then poured on Burk's agar media and incubated at 32°C for 90h. Large colonies appeared on the nitrogen free agar media were selected and stored in same media at 4°C for further work.

Nutritional condition for optimum growth:

To study the effect of different nutrients composition on growth, Selected organisms were grown in a basal liquid medium (Burk's broth) which supplemented with single or mixed nutrient elements. Temperature adjusted 30°Cat pH 6 for better growth. To find out the suitable carbon source for optimum growth and nitrogen fixationdifferent carbohydrates at variable concentration were tested. In this way optimum concentration of sole carbon source and additional carbon source were determined. To determine the effective phosphate sources, we studied by adding K_2HPO_4 and KH_2PO_4 at different concentration in single as well as mixture to the basal medium.

Efficiency of individual cells to the total number of organisms presents per ml. of sample was determined by using a haemocytometer.

Nitrogen fixing ability of the bacteria:

Nitrogen fixing potentiality of the selected organisms was carried out through microkjeldahl method (Pati 1992) as well as acetylene reduction assay (Weaver et.al. 1994). Acetylene reduction assay or nitrogenase activity was measured in Hewlett Packard gas chromatograph (HP48908), fitted with hydrogen FID detector, N2 as carrier gas and HP-PLOT-Q column. The amount of ethylene produced from acetylene was determined from the peak area and reference standard curve. The values were corrected for change in attenuation. Acetylene gas was generated from calcium carbide pellet in the laboratory.

RESULT AND DISCUSSION:

In the present study a comprehensive work of microbial population on the phyllosphere of forest plants was carried out (Table 1). Leaf impression method gave an idea about the degree of dilution necessary for quantification of bacteria. Both bacterial and fungal colonies were grown in large numbers on nutrient agar plates and PDA plate respectively. Leaf Impression onnitrogen free Burk's agar medium showed the nitrogen fixing organism on both the surfaces. Different colour forming bacterial colonies were abundant on both the surfaces of almost all the forest plant leaves. Upper surface of leaves showed more number of micro-organisms than the lower surface. Mature leaves contain innumerable microbes in comparison to young leaves. Then dilution plating technique also gave us the quantitative data about the bacteria and fungi, present in leaf surface. Different workers used different technique for accessing phylloplane microbes but we adopted dilution plating which provides statistically significant result (Yadav et al 2010).

Observation of microbial population from the leaf surfaces of different forest plants has been shown (Table-2). Total bacterial flora was quite high on the plant like Bahera, Haldu and Bijayasar. Total bacterial count always outnumbered the fungal population. In this study, the variation of microbial flora on the leaf surface is due to leaf topology (Monier et al. 2004) and wide variety of leaf exudates released by the host plants (Werker 2000, Zhao et al. 2016).

It was found that the density of nitrogen fixers as compared to the total bacterial population,leaf surfaces of Jam and Gamhar plant contained very poor number of nitrogen fixing organism among all the forest plant. In Gamhar, out of total 144.64 bacterial populations, only 08.32 were the nitrogen fixer but in Bijayasar, Simul, Haldu and Bahera, the numbers of nitrogen fixer were high.

About 22 nitrogen fixing bacterial colonies were isolated from different dilution plates during the period of isolation. Then on the basis of colony characteristics and individual cell morphology06 strains of nitrogen fixers were selected. Growth of those selected bacteria in Burk's broth and their nitrogenase activity including total nitrogen content is tabulated in Table-3. Growth performance of all the isolates were observed at 30°C for 72 hours and at the same time nitrogen fixing capacity was also recorded. According to nitrogen fixation capability these isolated strains can be arranged in the following order SML2> BR1> BS2> HLD3> JM2> PL1.

In the present study, it was found that leaf surface of all the forest plant shows a large number of both nitrogen fixer and other microbes. This may be due to qualitative and quantitative availability of nutrient present on the leaf surfaces (Brighigna et.al. 1992, Michael et. al. 2008). To estimate the nitrogen fixation, modern trend is to assay the activity of the nitrogenise enzyme present in bacteria through acetylene reduction assay (Giri and Pati, 2004). The differential rate of nitrogen fixation by the various nitrogen fixers is supposed to be due to variation in their metabolic system and the amount of nitrogenase present within them. It has also been observed that the rate of acetylene reduction is directly proportionate with the rise of cell mass. It can be explain as that generally nitrogen fixing enzymes, basically the nitrogenise are present in the cell cytoplasm of the microororganism, thereby amount of these enzymes are increased with the rise of cell number and showed variation in the nitrogen fixing potentiality.

CONCLUSION:

Phyllosphere or leaf surfaces are the habitat of many microorganism. Nitrogen fixers are the alternatives of plant nitrogenous sources. In this context we have isolated few nitrogen fixers from forest phyllosphere which are more potential to nitrogen fixing capabilities.

Table- 1: Plants on which the phyllosphericmicrobes have been observed.

Common	Scientific	Family	Locality
Asan (ASN)	**Terrminalia tomentosa**	Combretaceae	Vidyasagar University campus, Midnapore, West Bengal, India
Kendu (KD)	**Diospyros melanoxylon**	Ebinaceae	Arabari forest (Paschim Medinipur, West Bengal, India)
Mahua(MH)	*Madhuca latifolia*	Sapotaceae	Vidyasagar University campus, Midnapore, West Bengal, India.
Bahera (BR)	*Terminalia belerica*	Combretaceae	Abas firm(Midnapore, West Bengal, India)
Gamhar (GM)	*Gemelina arborea*	Lamiaceae	Gop Gar forest (Midnapore, West Bengal, India)
Jam (JM)	*Garuga pinata*	Burseraceae	Arabari forest (Paschim Medinipur, West Bengal, India).
Piyal (PL)	*Buchanania cochinchinensis*	Anacardiaceae	Abas firm(Midnapore, West Bengal, India)
Haldu (HLD)	*Adina cordifolia*	Rubiaceae	Arabari forest (Paschim Medinipur, West Bengal, India).
Bijayasar (BS)	*Pterocarpus marsupium*	Fabaceae	Arabari forest (Paschim Medinipur, West Bengal, India).
Simul (SML)	*Bombax ceiba*	Malvaceae.	Arabari forest (Paschim Medinipur, West Bengal, India).

Table- 2. Record of Microbial population from the leaf surface of different plants*.

Population in $10^3/cm^2$					
Name of the plants	Nitrogen Fixers	Total Bacteria	Ratio of total Bacteria/ N2-fixer	Fungi	Ratio of total Bacteria/ Fungi
Bahera (*Terminalia belerica*)	123	1650.22	133.84	51.42	32.09
Haldu (*Adina cordifolia*)	134	1572.06	11.73	190.80	79.40
Bijayasar (*Pterocarpus marsupium*)	388.52	1160.03	3.00	22.03	52.65
Jam (*Garuga pinata*)	10.58	678.07	64.08	2.33	291.01
Simul (*Bombax ceiba*)	354.07	540.09	1.52	14.38	37.55
Piyal (Buchanania latifolia)	55.45	214.82	3.87	14.52	14.80
Mahua (Madhuca latifolia)	23.02	211.32	9.18	06.09	34.80
Gamhar (*Gemelina arborea*)	08.32	144.64	17.38	03.17	45.62

* The above data represent an average of five sampling. From all samples 10 replicate experiments were performed and averages of those were taken in to consideration.

Table - 3 . Growth and Nitrogen fixation by the different isolated nitrogen fixing strains.

Strains	Growth (mg/ml)	Nitrogen fixation	
		A*(mg of N fixed l^{-1} culture)	B*(n moles of C_2H_2 reduced 10^8 $cells^{-1}$ h^{-1})
BS2	0.164 ±0.01	22.32 ±2.11	78 ± 1.50
SML2	0.335 ± 0.01	56.33 ±2.26	167± 2.14
BR1	0.178 ± 0.01	36.40 ±1.52	57 ±1.23
HLD3	0.44 ±0.01	34.24 ±2.13	58 ±1.40
PL1	0.23 ± 0.01	24.40 ±2.22	43 ±2.34
JM2	0.26 ± 0.01	18.34 ± 1.13	37 ± 1.5

*A= Microkjeldahl method *B= ARA technique.

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