



RESEARCH ARTICLE

DISTRIBUTION OF NITROGEN FIXING BACTERIA AND ASSOCIATED MICROORGANISM ON THE PHYLLOSHERE OF SOME TROPICAL FOREST PLANT

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ABSTRACT

A study on microbial population on the phyllosphere of forest plant was carried out. Both fungi and bacteria were isolated from the leaf surface of sal (*Shorea robusta*), teak (*Tectona grandis*), sissoo (*Dalbergia sissoo*), kosi (*Bridelia Sp.*), arjun (*Terminalia arjuna*) and sandal wood (*Santalum album*) plant. Leaf washing method was adopted for isolation of the organisms although leaf impression techniques was used to assess the approximate density of microbial population. Mature leaves contain innumerable propagules in comparison to young leaves. Chromogenic bacterial colonies were abundant on both the surfaces of almost all the forest plant leaves. Total bacterial flora was quite high on the phyllosphere of plant like Teak, Sissoo and Arjun. 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 revealed that Sal and Kosi leaf surfaces contained very poor number of nitrogen fixing organism among the forest plant. In Sal, out of total 280 bacterial populations, only 03 were the nitrogen fixer whereas in Teak, Arjun, sissoo and sandalwood, their numbers were high. Among the fungal population, maximum number was recorded only on teak leaves. Negligible number of fungus was recorded on the leaf surfaces of other plants. Population ratio of total bacteria/fungi was rather high on Sissoo leaf compared to those of other leaves. Based on colony characteristics and individual cell morphology 6 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 Acetylene Reduction Assay as well as Microkjeldahl method. In order to nitrogen fixation capability these strains can be arranged in the following order TK3>AN2>KS3>SL3>SIS1>CN2.

Key words: Leaf surface microorganism, Diazotrophs, Nitrogen fixation, Phyllosphere bacteria.

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INTRODUCTION

Study of Leaf surface ecology started five decades ago when the term phyllosphere was coined by Last (1955) and adopted by Ruinen (1956). Phyllosphere has been defined as the thin layer in contact with the leaf and with the atmosphere. Phyllosphere or plant leaf surface is a habitat for many microorganisms (Ruinen 1961, Vorholt, 2012). Many studies have shown that environmental conditions can have important effects on phyllosphere community structure (Lindow and Brandl, 2003). The surface characteristics of leaves differ widely due to variations in wax deposits, silicification, venation and distribution of trichomes. The wall of epidermal cells are covered with a cuticle composed mostly of cutin which is a polymer of fatty and hydroxyfatty acids. The cuticle makes the leaf surface almost water proof and this is enhanced

further by a surface coating of wax. The wax is composed of alkanes, alkyl esters and aldehydes, primary and secondary alcohols and long chain monobasic organic acids. Wax affects the wettability of leaves which is most important in the efficiency of sprays against microbial pathogen (Redford and Fierer, 2009). Besides the two main components wax and cutin leaf surface also contain lipid and silicified material forming a heterogenous complex. The leaf surface microbes are important in several ways. Some of them are known to fix atmospheric nitrogen (Favilli and Messini, 1990), (Giri and Pati 2002) produce plant growth regulators and can control plant parasites either by stimulating plants to synthesize phytoalexins or by producing antibacterial (Mc Cormack *et al.*, 1994) and antifungal compounds. Nitrogen fixation rates of those diazotrophs and their growth depends on different microclimatic conditions of leaf surface and light exposure (Furnkranz *et al.*, 2008). They also found the dominant diazotrophs on the leaf surface of tropical lowland rain forest of Costa Rica are *Nostoc spp.* The epiphytic microflora are also known to produce sugars, aminoacids, peptides, enzymes,

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vitamins, organic acids and nucleotides which exert influence on the plant growth (Chandramohan and Mahadevan, 1968). The microbial population derives its water and dissolved gasses from the atmosphere and nutrients from the exudates and leaching of the living leaf exudation and leaching of different substances from the leaf surfaces are regulated by the nutritional status of the host plant. In tropical regions, the upper surface of leaves can be populated by a great number of different species of bacteria, blue-green algae (cyanobacteria), fungi, green algae, lichens and bryophytes (Ruinen 1974; Lucking 1995). From the study of the phylloplane organisms of both temperate and tropical regions it has been demonstrated that some of the phylloplane fungi are cosmopolitan in their distribution (Campbell, 1985). Where as a study on three tree species in Hawaii (*Metrosideron collina* var *Polymorpha*, *Acacia koa* var *hawaiiensis* and *Cheirodendron trigynum* var *trigynum*), showed that they had very different, though extensive, fungal floras (Baker *et al* 1979). Madhaiyan *et al.* (2015) found that diazotrophs like *Methylobacterium* is the dominant species on the leaf surface of *Jatropha curcas* and those bacteria are significantly contribute to *Jatropha's* tolerance to low soil nutrient. So the phyllosphere has many features that make it an excellent habitat in which to study microbial ecology. Leaves are clean, and microbes can be observed directly on leaves, enabling the use of powerful new microscopic techniques to measure microbial identity, activity and gene expression (Lindow and Brandl 2003).

Agriculture scientists have now concentrated more in finding out some alternative source of nitrogenous nutrients for the growing crop plants to compensate inevitable shortage and increase in price of synthetic nitrogenous fertilizers from some selected strain of micro-organisms, which fix atmospheric nitrogen, is the most perspective of all the alternatives. Rhizospheric nitrogen fixers are now widely used for better production of different crop plants. In this aspect the phyllospheric diazotrophs have a great potentiality and can be used as foliar spray on the growing crop plants. The role of phyllosphere nitrogen fixers on the higher plants is however not properly understood due to the dearth of comprehensive studies on phyllosphere micro-organisms. Search of more beneficial micro-organisms from the leaf surface may reveal the complete picture of mutualism between the phyllosphere and the host plant. From the above idea I have tried to explore the phyllosphere of some forest plants from the surrounding areas of Paschim Medinipur district of west Bengal, India.

MATERIALS AND METHOD

Media

Isolation of the various organisms was made through plating technique in nutrient agar, potato dextrose agar and Burk's agar. During isolation of bacteria on nutrient agar, in order to suppress fungal growth, 2µg/ml antifungal antibiotic was added, while in PDA antibacterial antibiotic were added at a concentration 20µg/ml to the media to suppress bacterial growth.

Leaf impression

Collected leaves were pressed on suitable sterile media inside petridishes after removal of superficial dust by agitation. Plates were then incubated as a whole for specific period. This gave

an idea about the density of the organisms present on the leaf surface.

Dilution plating

Leaves after preliminary washing with sterile water 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 (200rpm.) for 10 minutes. The solution are diluted in same buffer and poured on Burk's agar media and incubated at 32°C for 90 hrs. Large colonies appeared on the nitrogen free agar plate were selected and stored in agar media at 4°C for further work.

Nutritional condition for optimum growth

To study the effect of different nutrients on growth, organisms were grown in a basal liquid medium (Burk's broth) that supplemented with single or mixed nutrient elements. Temperature adjusted 30°C at pH 6 for better growth. Different carbohydrates at different concentration were tested to find out the suitable carbon source for optimum growth and nitrogen fixation. In this way optimum concentration of sole carbon source and additional carbon source were determined. Effects of phosphate sources were studied by adding K_2HPO_4 and KH_2PO_4 at different concentration in single as well as mixture to the basal medium. In order to determine the efficiency of individual cells the total number of organism present per ml. of sample was determined by using a haemocytometer.

Nitrogen fixing ability of the bacteria

Nitrogen fixing ability of the organisms were tested through acetylene reduction assay (Murty, 1983) and microkjeldahl method (Pati 1992). Acetylene reduction assay or nitrogenase activity was measured in Hewlett Packard gas chromatograph (HP48908), fitted with hydrogen FID detector, using N_2 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 was generated from calcium carbide in the laboratory.

RESULT AND DISCUSSION

In the present study impression of leaf on Burk's agar medium showed the nitrogen fixing organism on both the surface. Lower surface showed less number of micro-organisms than the upper surface. Leaf impression technique gave an idea regarding the degree of dilution necessary for quantification. Both fungal and bacterial colonies were grown in large numbers on PDA plates and nutrient agar plate respectively. Mature leaves contain innumerable propagules in comparison to young leaves. Chromogenic bacterial colonies were abundant on both the surfaces of almost all the forest plant leaves. Record of microbial population from the leaf surfaces of different plants has been shown in (Table-1). The dearth of comprehensive studies on diazotrophs had seriously hindered on phyllosphere microorganisms. Ruinen (1956), first established the presence of nitrogen fixing bacteria on the surface of leaves of tropical plants, and their potentialities experimentally proved by several workers (Pati 1992). Most of the initial workers reached in a point that various physico-chemical factors can affect the growth and survivability of diazotrophs on leaf surface (Lindow 2003, Kim *et al.*, 2012).

Forest plants from which the nitrogen fixers have been isolated

Common name	Scientific name	Family	Locality ,Logitude:87°10'- 87°21' Latitude:22°30'-22°56
Sal (SL)	Shorea robusta	Dipterocarpaceae	Jhargram(W.Mid.)
Teak (TK)	Tectona grandis	Verbenaceae	Arabari forest (W.Mid')
Sissoo(SIS)	Dalbergia sissoo	Papilionaceae	V.U. campus,
Kosi (KS)	Bridelia spp	Euphorbiaceae	Abas firm(W.Mid')
Arjun(AN)	Terminalia arjuna	Combretaceae	GopGar forest(W.Mid')
Sandalwood (CN)	Santalum album	Santalaceae	Arabari forest(W.Mid')

Table 1. Record of Microbial population from the leaf surface of different plants

Name of the plants	Population in 10 ³ /cm ²				
	Nitrogn fixers	Total bacteria	Ratio of total Bacteria/ N2-fixer	Total Fungi	Ratio of total bac./fungi
Sal (Shorea robusta)	2.6	280.31	107.81	21.42	13.08
Teak (Tectona grandis)	420	1806.06	4.3	190.80	9.46
Sissoo (Dalbergia sissoo)	106.52	1390.03	13.04	3.17	123.03
Kosi (Bridelia spp)	10.58	239.71	22.65	2.33	10.58
Arjun (Terminalia arjuna)	117.07	541.09	4.62	23.38	23.14
Sandalwood (Santalum album)	55.45	193.82	3.5	14.52	13.35

* 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 2. 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 ₂ H ₂ reduced 10 ⁸ cells ⁻¹ h ⁻¹)
SL3	0.183 ±0.01	19.37 ±2.10	47 ± 1.42
TK3	0.256 ± 0.01	51.76 ±3.20	132 ± 3.55
SIS1	0.223 ± 0.01	16.55 ±1.36	43 ±1.23
KS3	0.09 ±0.01	20.45 ±2.25	58 ±1.40
AN2	0.221 ± 0.01	34.32 ±2.68	88 ±2.52
CN2	0.055 ± 0.01	15.20 ± 2.51	38 ± 1.5

*A= Microkjeldahl method *B= ARA technique.

Depending on the dimension of work some scientist have been enriched the nitrogen free media by addition of some growth factors for the better growth and nitrogen fixation (Madhaiyan *et al.*, 2015) and they also observed that vigorous growth attained after 4 to 5 days of incubation. With regards to the density of nitrogen fixers as compared to the total bacterial population, it was revealed that Sal and Kosi leaf surfaces contained very poor number of nitrogen fixing organism among the forest plant. In Sal, out of total 280 bacterial populations, only 03 were the nitrogen fixer whereas in Teak, Arjun, sissoo and sandalwood, their numbers were high. Among the fungal population, maximum number was recorded only on teak leaves. Negligible number of fungus was recorded on the leaf surfaces of other plants. Population ratio of total bacteria/fungi was rather high on Sissoo leaf compared to those of other leaves. Total bacterial flora was quite high on the plant like Teak, Sissoo and Arjun. Total bacterial count always outnumbered the fungal propagules. About 35 nitrogen fixing bacterial colonies were randomly collected from different dilution plates during the period of isolation. Based on colony characteristics and individual cell morphology 06 strains of nitrogen fixers differentiated. Growth in Burk's broth and amount of nitrogen fixed including nitrogenase activity of isolated nitrogen fixer is tabulated in Table-2. 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 in all the organisms. In order to nitrogen fixation capability these strains can be arranged in the following order TK3>AN2>KS3>SL3>SIS1>CN2. In the present study, it was found that the forest plant harbour a huge number of both nitrogen fixer and other microbes. This might be due to availability of nutrient varying both qualitatively and quantitatively on the leaf surface (Lindow 2003).

The topography and micro climate of leaves are a major factor in the distribution of microorganisms, mainly the plant cuticular wax composition can affect the community composition of phyllosphere bacteria (Reisberg *et al.*, 2013). Most of the initial workers reached in a point that various physico- chemical factors can affect the growth and survivability of diazotrophs on leaf surface (Pati and Chandra, 1993). Depending on the dimension of work some scientist have been enriched the nitrogen free media by addition of some growth factors for the better growth and nitrogen fixation (Steven *et al.*, 2014) and they also observed that vigorous growth attained after 4 to 5 days of incubation like our observation. To estimate the nitrogen fixation, modern trend is to assay the activity of the enzyme nitrogenase through acetylene reduction assay. 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 (Giri and Pati, 2002). It can be explain as that generally nitrogen fixing enzymes are present in the cytoplasm of the organism, thereby amount of these enzymes are increased with the rise of cell number and showed higher nitrogen fixing activity.

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