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Phyllosphere Microflora of Muga Silkworm Host Plant *Persea bombycina* Kost (Som) Leaves in Jorhat District of Assam, India

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Abstract

Phyllosphere microorganisms influence the growth of their host plants, either negatively as pathogens or positively by increasing the stress tolerance and disease resistance. Persea bombycina Kost is the primary host plant of golden silk producing muga silkworm Antheraea assamensis. In this study, silkworm fed and non-fed leaf samples of Persea bombycina was collected from Jorhat District, Assam, India towards the isolation, enumeration and characterization of phylloplane microflora by culture dependent techniques using NA, Luria, Czapek-Dox, PDA and RBC Agar media. The average fungal and bacterial population was recorded more in non-fed leaf then silkworm fed leaf samples throughout the year. There was significant positive correlation between temperature and microbial population, whereas negative correlation was observed against relative humidity. Characterization of bacterial isolates was carried out by Gram's staining method and according to Bergey's Manual of Systematic Bacteriology. Out of eight isolates, two were Gram positive cocci, three Gram positive rod, two Gram negative rod and one Gram negative cocci. Fungal isolates were identified on the basis of their colony morphology, mycelium, sporangiophore and spore morphology. It was noticed that the Penicillium species is dominant among all the isolated fungal species. Other isolates were identified as Aspergillus sp., Fusarium sp. and Yeast.

Keywords: *Antheraea assamensis*, *Persea bombycina*, phyllosphere microflora, host, epiphyte.

Introduction

The interaction of microbial communities in phyllosphere influences the safety and survivability of the host plant and the productivity of agricultural crops for human and animal consumption. Phyllosphere is one of the major microbial habitat on the earth[1], that provides shelter to diverse and complex microbial communities like bacteria, yeast, fungi, actinomycetes, algae, protozoa etc[2]. Phyllosphere microorganisms influence the growth of their host plants, either negatively as pathogens or positively by increasing the stress tolerance and disease resistance[3]. The leaf surface contains different types of stimulatory and inhibitory substances that regulate the microbial colonization on phyllosphere[4]. The filamentous fungi are present predominantly as spore, whereas rapidly sporulating species, bacteria and yeasts colonize this habitat more actively.

Muga silkworm *Antheraea assamensis* Helfer is endemic to Assam and adjoining areas of North-Eastern India, and produces natural golden coloured exquisite silk, which is avenue for rural livelihood to more than 50000 families[5]. Muga silkworm is semi-domesticated in nature and rearing is carried out in outdoor condition on wide range of perennial food plants[6,7,8] such as Som (*Persea bombycina*), Soalu (*Litsea polyantha*), Dighloti (*Litsea salicifolia*) and Mejankari (*Litsea citrata*).

Persea bombycina Kost. is the primary host plant of muga silkworm that belongs to the family Lauraceae is a perennial tree with grey warty bark, lanceolate leaves, small flowers and globose berry fruits. The phyllosphere microbes of *P. bombycina* may have manifold interactions with the host plant as well as the silkworm fed on its leaves. As the report on phyllosphere microflora in *P. bombycina* is very limited, the aim of the current study is to isolation and characterization of epiphytic microbial communities during different seasons in Som phyllosphere by culture-dependent methods.

Material and Methods

Sample collection: Both silkworm fed and non-fed leaf samples of *P. bombycina* were collected from four farms during different muga silkworm rearing seasons like *Jathuwa* (Spring), *Bhodia* (Summer), *Katia* (Autumn) and *Jaruwa* (Winter) of 2012-13. Three samples (10 gm per plant/sample) from each farm were collected in sterile poly-bags and taken back to laboratory for isolation of epiphytic phylloplane microflora. The sampling host plants were selected randomly and leaves were collected from 4 different branches at the height of about 5 meter above the ground throughout the year.

Isolation: The collected leaf samples were detached aseptically from the branches and washed separately by shaking for one hour with 100 ml sterile distilled water and the suspension was treated as stock. Serial dilutions of the stock solution was prepared up to 10^{-5} and an aliquot of 200 µl from the dilutions

were plated separately in Nutrient agar (Hi-media Ltd., Mumbai) and Luria agar (Casein 10 g/L, yeast extract 5 g/L, NaCl 5 g/L and agar 15 g/L) media for isolation of bacteria. Fungal colonies were isolated by using Rose Bengal Chloramphenicol agar (Peptone 5 g/L, dextrose 10 g/L, KH_2PO_4 1 g/L, $MgSO_4$ 0.5 g/L, rose bengal 0.05 g/L, chlorampenicol 0.1 g/L and agar 15.5 g/L) and Czapek-Dox agar (Sucrose 30 g/L, $NaNO_3$ 2 g/L, K_2HPO_4 1 g/L, $MgSO_4$ 0.5 g/L, KCl 0.5 g/L and $FeSO_4$ 0.01 g/L) media. The bacterial petriplates were incubated at 37 ^{0}C for 24 hrs, whereas for fungi incubation was done at 25 ± 2^{0}C for 72 hrs.

Characterization and identification: Total viable count of the bacterial colonies was carried out as per the method of Al-Jasass[9] and single colonies were isolated after 24-36 hrs of incubation. The isolates were tested with respect to Gram reaction and biochemical characteristics[10] and further identified on the basis of pigment, colony form, elevation, margin, texture and opacity[11]. Biochemical tests performed for both Gram positive and negative bacteria were Citrate utilization, Lysine utilization, Ornithine utilization, Urease, Phenylalanine deamination, Nitrate reduction, H_2S production, Glucose, Adonitol, Lactose, Arabinose, Sorbitol, Malonate, Voges proskaur, ONPG, Catalase, Arginine, Sucrose, Mannitol, Trehalose by using Biochemical Test Kit (Hi-media Ltd., Mumbai)[12].

Fungal colonies were isolated after 3-4 days and pure cultures were transferred to Potato Dextrose Agar (Hi-media Ltd., Mumbai) slant. The mycelial and spore characters of the fungi were studied under microscope (Leica, Germany) by cover slip insertion method. Sterile cover slips were inserted at 45^0 angles on to solidified PDA medium inoculated with fungal isolates on petridisc. The petri-dishes were incubated at 23-25^{0}C for 7 days. The mycelial growth with sporangiophore/spores extended over the coverslips were removed carefully and semi permanent slides were prepared using lactophenol cotton blue. Fungal isolates were identified on the basis of cultural, morphological[13,14] and microscopic characteristics viz. mycelium, sporangiophore, spore bearing organ, spore structure etc. The colonization frequency (CF%) of the fungal isolates were calculated by using the formula CF = (N_{col}/N_s) x 100, where, N_{col} no of unit leaf samples colonized by the fungus and N_s is the total number of unit leaf samples taken for the experiment[15,16].

Statistical analysis: All experimental are the average of independent replications including the environmental parameters i.e. temperature, humidity and rainfall during 2012-13. Statistical analysis of the data was carried out by Statistical Analysis System (SAS) and with the help of statistical software programme 'SPSS version 16'.

Results and Discussion

Altogether 12 unit (10 gm/unit) of muga silkworm fed and non-fed *Persea bombycina* leaf samples were collected randomly from four different farm. The average bacterial population of *P. bombycina* phyllosphere was more in silkworm fed samples than the non-fed, throughout all the muga silkworm rearing seasons. As shown in table-1, bacterial colony (cfu) were enumerated more in the silkworm fed leaf samples collected during *Bhodia* crop (284.4), which is followed by *Jethuwa* (270.6), *Katia* (266.2) and *Jaruwa* (183.8), respectively. There is positive co-relation between temperature and assemblage of bacterial colonies in Som phyllosphere, whereas negative co-relation was observed between relative humidity and bacterial population (table-2).

Non-fed *P. bombycina* phyllosphere anchoraged three bacterial isolates, whereas the silkworm fed leaf harboured five different bacteria isolates. Out of total eight isolates three were Gram negative and rest of the five were Gram positive bacteria with different size, shapes and pigmentation. The bacterial population was more in silkworm fed leaf sample than the non-fed one. All the bacterial isolates exhibited diverse biochemical properties during the analysis for the biochemical tests as per Bergy's Manual of Determinative Bacteriology (table-3).

In comparison to silkworm fed *P. bombycina* phyllosphere, the fungal population (in cfu) was less in non-fed leaves. Silkworm fed phyllosphere harboured more fungal population during *Jethuwa* (86.4) and followed by *Bhodia* (72.2), *Katia* (60.8) and *Jaruw*a (41.8) crops (table -1). Correlation analysis of the fungal populations indicated positive correlation with the environmental temperature and negative correlation with relative humidity (table-2)

Altogether, seven fungal species of different genera were isolated and identified on the basis of colony morphology, mycelia, sporangiophore and spore structure. Most of the fungal isolated were belongs to the class Ascomycetes having septate hyphae and asexual spores like conidiospore (micro and macro). Determination of colonization frequency (CF%) indicated that the dominant fungal genera on *P. bombycina* phyllosphere were the members of *Penicillium*, *Aspergillus* and *Fusarium*. The cultural, morphological and microscopic study revealed the characteristics of vegetative and reproductive structure of the fungal isolates (table -4).

Microbial biodiversity become an integral part of the human welfare because of their significant role in agriculture, industry, medicine, food industry, textiles, biotransformation and bioremediation[16]. The aerial part of plants is dominated by leaves that harboured a wide range of microbial communities with manifold interactions to the host.

In the present study total 8 bacterial strains were isolated from the muga silkworm host plant *P. bombycina*. Bacteria assemblage in Som phyllosphere is more during hot and humid condition i.e. during summer season. All the isolates were of diverse shapes and sizes with different Gram staining property. They exhibited wide range of biochemical characteristics that

are indication for their manifold functionability. The bacterial population size fluctuates depending upon season, climate, geographical position, age and health of host plant, physical and nutritional conditions of the phyllosphere[17,18]. The results clearly indicated significant co-relations among environmental temperature and relative humidity with microbial population. Bacterial communities occur in large groups particularly at the depressions formed at the junctions of epidermal cells, along the veins of leaf[19] and at the bases of trichomes[20].

So far as fungal community is concern, 7 fungal species were isolated and identified, most of which are belong to the class Ascomycetes. Although bacterial community numerically dominated the leaf surface[21], different types of filamentous fungi, molds, yeast and other sporulating fungal species belongs to the class Ascomycetes and Deuteromycets also actively colonize on *P. Bombycina* phyllosphere. The silkworm fed leaf have got more microbial population and it may be due to contamination made by silkworm during the rearing activities or accumulation of their saliva and faecal exudates on the leaf surface.

Healthy *P. bombycina* leaf has got different types of carbohydrates, crude fibres and polyphenols that provide nutritive value for better muga silk production[22,23] as well as the suitable environment for phyllosphere microbial community. The plant species identity and leaf constituents have an significant influence on the structure of phyllosphere community[24]. The phyllosphere microorganisms have either neutral, negative or positive influence on the host plant by serving as pathogen[25] or preventing leaf colonization by plant[26] or silkworm pathogen. The epiphytic phyllosphere microbes involve in carbon cycle[27], nitrogen cycle by nitrification of ammonium pollutant intercepted by plants[28,29] and atmospheric nitrogen fixation[30] that may affect the health of the host plant and eventually the silk productivity. In addition, nutrient recycling efficiency of microorganisms from organic compounds to decomposers is a key parameter to stabilize an ecosystem[31].

Table-1
Seasonal variations in number of bacterial and fungal colonies in muga silkworm fed and non-fed *P. bombycina* leaves during 2012-13

Seasons	Temperature (^{0}C)		Humidity (%)	Nos. of bacterial colony		Nos. of fungal colony	
	Max.	Min.		Feed	Non-feed	Feed	Non-feed
Jethuwa (Spring)	34.5	15.4	66.00	270.6 (± 26.4)	184.4 (± 17.5)	86.4 (± 12.2)	51.3 (± 10.6)
Bhodia (Summer)	36.2	24.0	86.00	284.4 (± 31.8)	203.2 (± 21.6)	72.2 (± 16.6)	46.7 (± 12.2)
*Koti*a (Autumn)	36.7	18.8	88.25	266.2 (± 18.8)	170.6 (± 22.6)	60.8 (± 15.4)	16.2 (± 4.8)
Jaruwa (Winter)	27.7	6.4	84.50	183.8 (± 23.6)	106.4 (± 15.2)	41.8 (± 11.2)	10.4 (± 2.6)

Table-2
Correlation coefficient and significance among temperature, humidity and microbial population on *P. bombycina* phyllosphere during 2012-13

Parameter	Correlation/Significance	Nos. of bacterial colony		Nos. of fungal colony	
		Feed	Non-feed	Feed	Non-feed
Temperature (^{0}C)	Correlation	0.959*	0.943	0.650	0.570
	Significance (Level: 0.05)	0.041	0.057	0.350	0.430
Humidity (%)	Correlation	-0.180	-0.206	-0.685	-0.634
	Significance (Level: 0.05)	0.820	0.794	0.315	0.366

*…Correlation is significant at 0.05 levels.

Table-3

Morphological and biochemical characteristics of the bacterial strains isolated from phyllosphere of muga silkworm host plant *P. bombycina*.

Bacterial isolate	Silkworm fed/ non-fed leaf	Gram stain	Shape	Biochemical test (Gram positive bacteria)											
				Citrate	Catalase	Nitrate	Voges proskaur	Malonate	ONPG	Aginine	Sugar utilization tests				
											Glucose	Sucrose	Mannitol	Arabinose	Trehalose
PB-01	Fed	'+' ve	Cocci	-	+	+	+	-	-	+	-	+	-	+	-
PB-03	Fed	'+' ve	Cocci	+	+	-	-	-	+	-	+	+	+	+	+
PB-04	Fed	'+' ve	Rod	+	-	+	+	+	-	+	+	+	-	-	+
PB-06	Non-fed	'+' ve	Rod	-	-	+	-	-	+	+	+	-	+	+	-
PB-08	Non-fed	'+' ve	Rod	+	+	-	-	+	+	-	+	-	+	+	+
Bacterial isolate	Fed/ Non-fed leaf	Gram stain	Shape	Biochemical test (Gram negative bacteria)											
				Citrate	Lysine	Ornith-ine	Urease	Phenylaline	Nitrate	H_2S	Sugar utilization tests				
											Glucose	Lactose	Arabinose	Sorbitol	Adonitol
PB-02	Fed	'-' ve	Rod	+	+	+	-	+	+	-	-	+	+	-	-
PB-05	Fed	'-' ve	Cocci	+	-	+	+	+	-	-	+	-	+	-	+
PB-07	Non-fed	'-' ve	Rod	-	+	-	+	-	-	+	+	+	-	+	-

Table-4

Morphological and biochemical characteristics of the bacterial strains isolated from phyllosphere of muga silkworm host plant *P. bombycina*.

Fungal isolate	Class	Silkworm fed/ non-fed leaf	CF%	Cultural characteristics					Microscopic Observation		
				Colour	Margin	Reverse	Growth rate	Texture	Mycelium	Conidiophore	Conidia
Aspergillus niger PF-01	Ascomycetes	Non-fed	16.7%	Dark green	Smooth	Black	Fast	Powdery	Septate, branched, hyaline	Erect, un-branched, single and club shaped	Conidia are round shaped arranged in a long chain, single celled, green coloured
Yeast sp. PF-02	Ascomycetes	Fed	8.3%	White	Smooth	Pale white	Fast	Slimy	Unicellular	No	Oval shaped, unicellular
Penicillium sp. PF-03	Ascomycetes	Fed	33.3%	Gray green	Smooth	Off white	Fast	Flat & Velvety	Septate, branched, hyaline	Erect, un-branched, septate, monoverticillate	Conidia are round shaped arranged in a long chain, single celled, green coloured
Penicillium sp. PF-04	Ascomycetes	Non-fed	25%	Olive green	Smooth	White	Fast	Powdery	Septate, branched, hyaline	Erect, branched, septate, bi-verticillate	Conidia are round shaped arranged in a long chain, single celled, green coloured
Fusarium solani PF-05	Ascomycetes	Fed	16.7%	Pale white	Irregular	Off white	Medium	Cottony	Septate, branched	Erect, un-branched, septate	Microconidia in cluster, shape pyriform, 2-3 celled, hyaline, straight
Trichoderma sp. PF-06	Deuteromy-cetes	Non-fed	8.3%	Green	Smooth	Pale white	Fast	Powdery	Septate	Branched with flask shaped phialides.	Conidia are ellipsoidal, typically smooth, green coloured and smooth walled
Alternaria sp. PF07	Deuteromy-cetes	Fed	8.3%	Dark grey	Irregular	Dark brown	Fast	Powdery	Septate and branched	Erect, sort, branched or un-branched, septate	Conidia multicelled, obclavate with a short conical beak. Smooth walled and pale brown in colour.

Conclusion

Based on the above finding it can be concluded that seasonal variation and silkworm feeding practise changes the phylloplane environment of *P. bombycina* that eventually influence the population structure of phyllosphere microflora. The quantitative and qualitative characterization of Som phyllosphere microflora may also play a crucial role in forewarning and forecasting of upcoming diseases of muga silkworm and its host plant.

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