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Responses of Phyllosphere Microbial Population of Some Common Road Trees In Relation To Dust Deposition – A Case Study in Kolkata, India

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Abstract:*In the present paper, the effect of urban roadside air pollution on the epiphytic micro flora was studied on three most common plants of urban area i.e. Ficus religiosa, Ficus benghalensis and Anthocephalus cadamba at Kolkata. The increasing rate of vehicles day by day simultaneously increases the pollution level in air and the dust settled down on the phylloplane of those plants. In this study, samples were collected from Dunlop (direct roadside) and Rajarhat (from at least 500-750 meter away from direct roadside or non-roadside sites).Results showed a marked reduction of the microbial count on the roadside environment than the non-roadside environment. Bacterial and fungal population was higher on the low polluted site. Analysis of four different trace metals was carried out from the phylloplane of the aforesaid trees. Correlation coefficient showed significant reduction of microbial community in Dunlop due to the higher concentration of lead, zinc, copper and Cadmium. Presence of traffic generated pollutant might be a cause of decrease in growth for microbes.Aspergillusochraceus,Penicilliumfrequentaus, Trichodermaviridewere the most abundant species in roadside environment, whereas absence of fungi like Penicilliumcitrinum, Aspergillusnigerwere observed in roadside environment. The deviation of fungal population diversity might be occurred due to its sensitivity or resistance to the pollutants with those fungi.*

Keywords:*Air pollution, diversity index, microbial population, phyllosphere, SEM.,*

I. Introduction

Phyllosphere, being a rich and complex habitat for microorganism, is a larger space for both agricultural and environmental important microbes. According to a group of researchers [1-3] a large array of culture dependent and independent bacteria, fungi, yeast and algae are found on the phylloplane, known as epiphytic microbes. Different studies have been revealed that bacteria are the most dominant microbes on phyllosphere, average population is 10^6-10^7 CFU cm^{-2} (up to 10^8 CFU per gram) of leaf [4,5]. It is also noticed that the presence of culturable bacteria is ranging between 10^2 and 10^{12} cells per gram of leaf in arid conditions of Mediterranean summer [6-8]. The growth of phylloplane microbes generally depends on various biotic and abiotic factors.

In today's world industrialization and urbanization are the most important factors of air pollution. The main sources of urban air pollutants are coming from fossil fuel driven motor vehicle emission and other sources are workshops, and residential heating etc. These problems are increased day by day not only for the population growth but also for narrow and congested road and old poorly maintained vehicles. A wide assortment of toxins like benzene, carbon monoxides, organic compounds, nitrogen and sulfur dioxides discharged each day from vehicles. Leaf surface of plants which are located beside the high roads are generally trapped the dust and air borne pollutants. Different metal of air pollutant leaches on the leaf surface and interact with the microbes. The interaction between them can alter and affect the activity of microorganisms on phyllosphere. In Kolkata metropolitan city traffic induced aerosols consist of huge number of elements, which are K, Ca, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Se and Pb.It also shows that the concentration of metals are generally ranges in the order of Zn>Pb> Ni> Cu> Cr>Co[9]. Previous study [10] showed different plants like *Azadirachtaindica*, *Terminaliacatapa*, *Ficus religiosa* and *Ficus benghalensis* aregood indicator of pollution level in urban and peri urban environment by their leaf surface in Jalgaon city. It was reported [11] that *Ficus benghalensis* and *Polyathialongifolia* can capture dust ranging between 0.12 mg/$cm^2$to 1.89 mg/cm2 in urban areas of Kolkata mainly in Salt Lake region on both adaxial and abaxial surfaces.

Disturbances on plant-microbe association can obstruct different type of non-pathogenic activity i.e. nitrogen fixation, degradation of waxes, production of growth hormones and biological control of pathogenic bacteria [12-14]. The alteration of leaf surface by microbial population isexplained by a group of researchers [2,15,16,]. Interaction with the air contaminants can causes direct effect on the biological control of leaf pathogen, when antagonist is very much sensitive with the contaminants [17]. Chronic exposure of SO_2 in polluted areas causes loss of phyllosphere fungi and in short term exposure no significant loss was observed by studies [18].

Pollutant like gaseous and atmospheric aerosols directly alters microbial biodiversity [19]. The bacterial population decreases with high metal concentration of air pollutants, near to the traffic congested sites. However, in case of fungi, total count may be higher sometimes near the roadside for the low pH concentration [20].A Study in North Eastern region of India revealed that the number of fungal and bacterial significantly decreased with the increasing amount of roadside pollution level in *Alnusnepalensis*and *Pinuskesiya*. Microbes are generally negatively correlated with different metal like Zinc, Lead Copper, Cadmium and Sulfur [21].The growth of bacteria, filamentous fungi and yeast on the leaf surface of trees like mango [22] are also affected by different chemical sprays.

Not many studies have been done in India particularly in Kolkata to understand the interaction between the phyllosphere bacteria and fungi with air particulate in the phylloplane of roadside plant [23-26]. The present study is therefore to investigate the trend of microbial population on the road side plant phyllosphere, which are affected by vehicular dust and also to investigate the impact of pollutants over the population dynamics of the microbes.

II. Materials and Methods

Kolkata (22°82'N and 88°20'E) is one of the oldest metropolitan cities of India, densely populated and immensely polluted. The study was conducted on the month December 2013. Roughly 16 million people are to be found on the city of joy and this number of inhabitants is increasing day by day. Increasing amount of high rise, roads, bridges as well as growing transportation system causes rapid urbanization on this city, which are the major contributor of particulate emission in air. The analysis was done in two sites i.e. Dunlop (site $1/S_1$) (22°38'N and 88°61'E), and Rajarhat (site $2/S_2$) (22°35'N and 88°28'E). The air quality of the study sites was obtained from the available data in the website of West Bengal Pollution control board (WBPCB) as they have monitoring station in both polluted sites described here. In Dunlop, heavy load of traffic and other activities were seen during the busy day time (5000-6000 vehicles/hour). Anothersitewas less affected by traffic vehicle as it was a constructional area and plants are more than 500 meters away from the actual roadside, mainly covered by tall buildings (non-roadside environment). This site was considered as less polluted and non-roadside location. Three dominant, broad leaves, evergreen plants,*Ficusreligiosa linn*(Peepal) indicated as P_1, *Ficus benghalensis linn*indicated as P_2 (Banyan), *Anthocephalus cadamba(Roxb.) Miq. Syn. Neolamarckia cadamba var A. chinensis* (Kadam) indicated as P_3were selected for this analysis in both sites.

Full mature, green leaves of both plants were collected from the sites from an elevation of 5ft approx. Samplings ware done in the early morning. Five mature leaves samples were collected from P_1, P_2 and P_3 at the two sites in sterilised zipper bags and transported for microbial assay in between twenty-four hours to laboratory. Microbial analysis by isolation of micro-fungi and bacteria was done by serial dilution method as described by Joshi [21]. One gram of leaf sample was weighed and taken in a 250-ml conical flask containing 100 ml of sterilised distilled water. Then it was shaken rapidly for about half an hour for remove the phylloplane or leaf surface microbes. Then from the original wash, suitabledilutation of the suspension was prepared. 0.5 ml suspension was plated aseptically in to sterile petriplate containing nutrient agar medium and czapec-dox media for bacteria and fungi respectively. For each sample three replicas were done and the petridisheswere incubated at 37±1°c and 27±1°c for bacteria and fungus respectively. Total CFU (colony forming unit) of fungi and bacteria at per gram of leaf were calculated, depending on the dilution factor.

2.1 Calculation for relative abundance (1), Shannon index (2) and species richness (3) in sites

The formula for calculating the relative abundance of fungi is:

Relative abundance = (Total number CFU of individuals/Total no of CFU of all the species) X 100….(1)

The formula for calculating the Shannon diversity index of fungi is: [27]

$$H = \sum_{i=1}^{s} - (P_i * \ln P_i)$$

where:

H = the Shannon diversity index

Pi = fraction of the entire population made up of species i

S = numbers of species encountered

$\sum$ = sum from species 1 to species S……. (2)

Species richness index is calculated by

Species richness =(S-1)/log N

Where, S= number of fungal species.

N=Total number of Fungi……. (3)

2.2 Analysis of metal in the leaf samples (Pb,Cu,Zn,Cd)

At first leaf sample was dried and powdered.0.5 gram of leaf dust was taken in triplicate ata digestion tube, then it was kept for overnight and digested with 20 ml of concentrated Nitric acid. Then the extracts were filtered and volume made up to 50 ml with distilled water. Then the sample concentration (ppm) was measured on Perkin Elmer (A400) atomic absorption spectrophotometer.

2.3 Direct examination of leaf Surface

Imageries were taken by scanning electron microscope as described by Ram et al.[28]. Small pieces of leaves (1 cm2) were cut from undisturbed sample by a sharp blade. Then it was air dried in a clean and close chamber and small strips were sliced from each leaf from the middle and marginal area. Each leaf strip was mounted on aluminium stab and coated by thin layered gold (about 200°A), which was conductive in nature, in an ion sputter coater (GIB2). Then the image of the coated sample was taken in scanning electron microscopy (Hitachi, S530).

2.4 Statistical Analysis

The correlation coefficient was calculated using Pearson's correlation coefficient. These analyses weredone by using SPSS for windows. (SPSS Statistics v21)

III. Result and discussion

The air quality of the study sites ware shown in "Table1". Itwas seen that levels of SPM and RSPM were quite higher than the national standards of air quality at both sites. But in case of NO_2 only Dunlop showed high values than the national standards, whereas in case of SO_2 it was lower than the standards in both instances.

Table 1: Pollution levels in study sites and standard parameters of air pollutants.

Sites	SO_2	NO_2	SPM	RSPM
Dunlop	6.825±0.88	61.975±5.40	205±41.32	100±12.16
Rajarhat	4.975±0.499	42.775±4.18	177±26.73	88±8.26
Standard*	60	60	140	60

All values in µg per m^3

*Air quality standard values of West Bengal pollution control board.

Plant canopy acts as a nature's absorbing surface with a suitable surface area for dust deposition. Dust particles were found to attach on both the surfaces, adaxial and abaxial surfaces of all the three plants. Scanning electron microscopy of the leaf sample phylloplane, collected from Rajarhat (S_2), revealed small growth of fungal conidiophores emerged out near the stomatal pore and different fungal thread like hyphae, surrounded by atmospheric dust and leaf trichomes (figure 1a), but no such association is found from S_1 samples, roadside environment "Fig.1". Maximum dust particle was seen on the upper surface of leaves. Stomata are acting like key passages for the transport of moisture and nutrient around the cuticle layer of the leaf surface, which helps to harbour the microbial community. Theresuspended dusts usually block the stomata on the roadside plants and act as a barrier. Hence, absence of fungal growth was seen on the roadside plant of these study and a small emerging conidiophore as well as fungal hyphae was seen to start near the stomata of non-roadside plant. No microbial association could not be find on the phylloplane of Roadside samples. This might be because ofstomatal pore of the plant situated on the roadside environment was blocked by the dust particles (Fig.2a), whereas the upper surface of the phylloplane was also covered by metal contaminated dust particles (Fig.2b). Microbial colony distribution was mainly seen on the lower surface of phylloplane by the direct microscopy. All these three plants in the roadside area showed the ability to capture the re suspended dust. A study by Prajapati and Triphathi [29] suggested that dust deposition generally depend on various factors like leaf size and structure, their epidermal traits, and on their leaf surface characteristics like trichrome, hair, cuticles etc. *Ficus Benghalensis*had greater efficiency of dust adsorption so there are chances to use it as passive air pollution monitor. Mafia et al. [30] was observed the occurrence of fungal community on the lower surface of leaf of Eucalyptus spp. They also observed the growth of fungal colony at close to leaf stomata. Theabaxial surface of leaf generally consist more number of trichrome, stomata [31].Even another colonizing microbes, bacteria tends to colonize at the lower surface of bacteria, near the stomata and trichrome. This observation was supported by a group of researchers [32-33]. The large flux of UV radiation, water and nutrient availability were the major factors for microbial growth in phylloplane. It was seen that microbes of leaf habitat were very much affected by such rapid fluctuations of different physical conditions than that of other habitats.

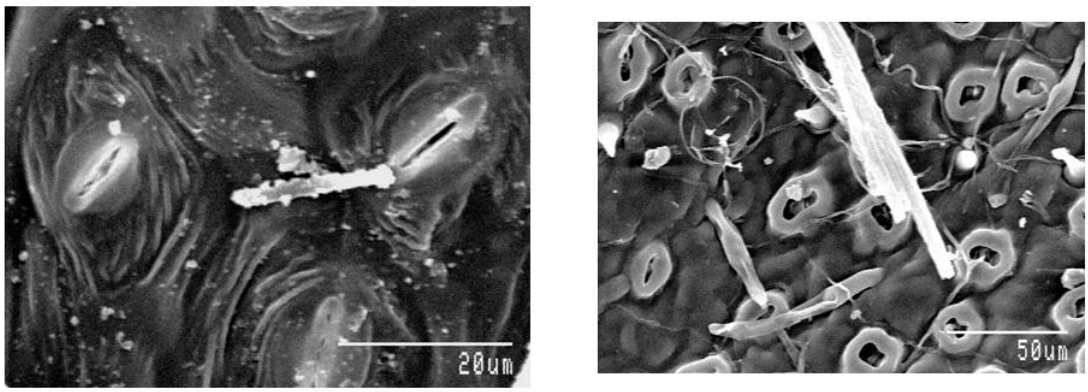
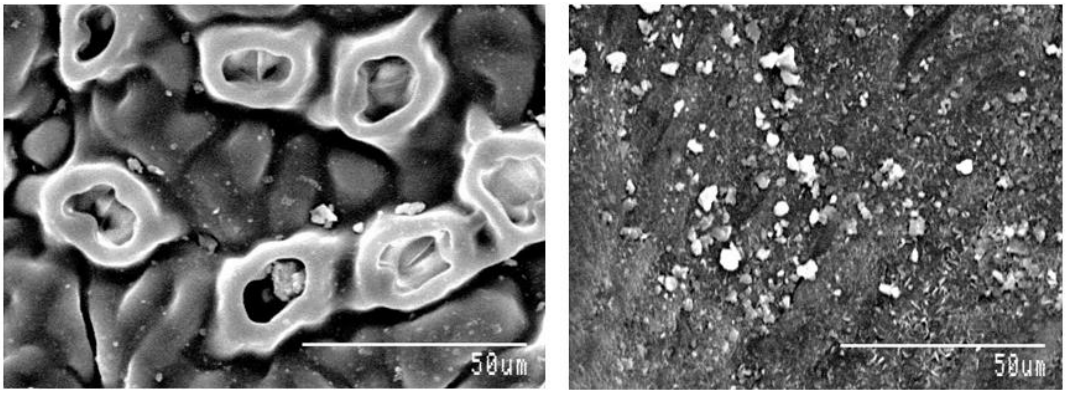


Figure 1: SEM micrograph showing the conidiophore emerges out near the stoma and hyphal growth of fungi near the stoma of *F. benghalensis.*ofsite S_1.



(A) Stomata blocked by dust particles (B) upper surface of leaf covered by dust particles.

Figure 2: Scanning electron micrograph of stomatal dust deposition on the phylloplane of roadside plant of site S_1

The size of microbial population (S_1 and S_2) of the three plant's phylloplaneis represented on "Fig 3". In our studyphylloplane microbial population showed a reduction of number in roadside location (S_1) than the non-roadside environment (S_2). Number of bacteria and fungi were decreased at polluted or roadside environment in compare with non-roadside environment. A considerable difference in the level of bacterial and fungal colony in the phylloplane of the two sites was noticed. Due to vehicular pollution of roadside site a significant decrease in bacterial and micro fungal community of phylloplane was occurred. Presence of different vehicular exhausting metals was greater in the roadside site in compare to the non-roadside site (Fig 4).The fluctuation of CFU of bacteria and fungi clearly showed a negatively significant correlation with the variation in labels of Pb, Zn, Cd and Cu in polluted site but in non-roadside sampling site elevated count of microbial populations wasfound, which was not significant between those trace metals and samples collected from the sampling site of Rajarhat (S_2).Dust deposition on the phylloplane of the three plants left a damaging effect on the microbial community of the phyllosphere shown by the coefficient correlation data analysis "Table 2". These findings were supported by Joshi [21]. That study reflected decrease of bacteria and fungi with increase of pollution at roadside site. Again, De Jageret al. [22] reported the loss of microbial community due to chemical spray on leaf surface. Even lichen was also adversely affected by air pollution as described by Monge-Najeraet al.[34]. Similarly, a study by Sandhuet al. [35] also indicated the detrimental effect of the air pollutants over the other group of bacteria than some air borne phenol tolerant microbes. CFU fluctuation and fungal species variation was also indicated the study bacterial population on dusty hemlock leaves [36]. Another study by Mohamed and Abo-amer [20] revealed that higher heavy metal concentration caused lower in bacterial counts in both phylloplane and soil;however, the fungal count might be higher in some roadside areas because of the acidic condition as it was favourable for fungal growth.

Figure 3: Bacterial and fungal population of the phyllosphere of three plants situated on the roadside environment and non-road sideenvironments. Bar represents the mean values of CFUx10^4

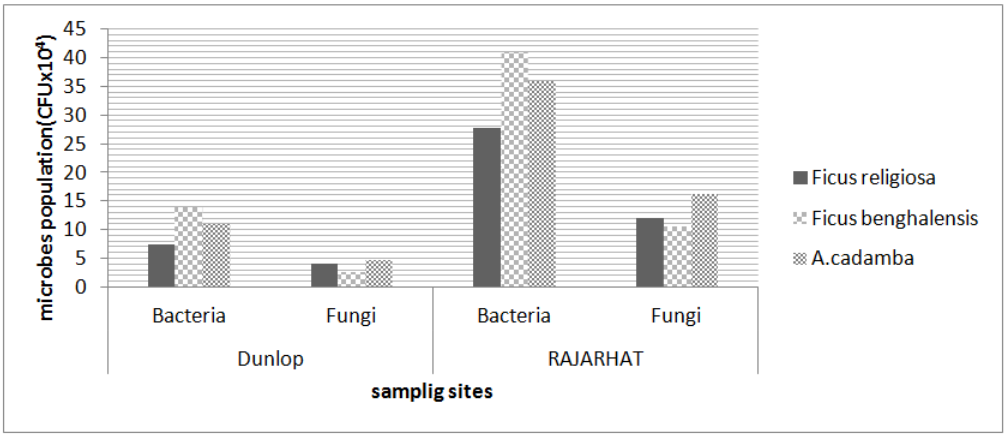


Table 2 : Correlations coefficients for Pb, Cu ,Zn ,Cd,fungal and bacterial population on phyllosphere of trees at roadside site.

Sources of variation	DF	Roadside site		Non-roadside site	
		Bacterial population	Fungal population	Bacterial population	Fungal population
Pb	9	-.872**	-.901**	-.699	-.310
Cu	9	-.879**	-.787*	-.654	-.315
Zn	9	-.861**	-.749	-.525	-.204
Cd	9	-.939**	-.900**	-.590	-.112

** Correlation is significant at the 0.01 level (2-tailed).
* Correlation is significant at the 0.05 level (2-tailed).

Total number of eight fungal species was identified out of twenty-three fungal types the leaf surface of those trees from S_1 and S_2. From these, six species were isolated from the site S_1, the roadside location and eight species were recorded from S_2, the non-roadside location. The isolated strains from S_1were *Aspergillusglacus, Aspergillusflavus, Penicilliumfrequentaus, Aspergillusochraceus, Penicilliumcitrinum, Trichodermaviride. Aspergillusniger*.*Penicilliumcitrinum*was specific in non-roadside environment only. No fungal colony of above said strain could be found on S_1, which indicated its tendency of sensitivity to the pollutant site. CFU of *Aspergillusochraceus* and *Penicillumfrequentaus* were more dominant in roadside environment.In case of non-roadside locationS_2,*Trichodermaviride*was showed the highest abundance (shown in Table 3). Shannon diversity index, similarity index of fungal population was also observed and demonstrated in table 4 for the three plants in both locations (shown in table 4). The fungal population in terms of number of CFU and species richness was higher in the non-roadside environment, which might cause due to little lesser deposition of dust particle on the leaf phylloplane (Table 4). The predominance of *Penicilliumfrequentaus* showed its resistance power against the pollutants and modification of its spore germinating capacity. Fluctuation of CFU might be occurred due to change of relative humidity, temperature, rainfall. These factors directly affect the pollutants or the dust depositions on the leaf surface. Mowllet al. [37]showed a distinct difference of fungal count between polluted and non-polluted sites, affected by vehicular air pollutants. A significant reduction of fungal colony with increasing toxicity was seen in different studies [38]. Different element like Na, Mg, Al, Si, Cl, K, Ca, Cr, Mn, Fe, Ni, Cu and Zn were the major component in the dust deposition [11]. This deposit above a specific range turned into toxic substance and hampered the colonization of microbes. It was the one of the reasons of less number of population densities of microbes in the roadside environment.

Table 3: Relative abundance (%) of fungal species on leaf surface of three plants (P_1, P_2 andP_3) at roadside and non-roadside location (S_1 and S_2)

	Dunlop (S_1)			Rajarhat (S_2)		
Fungal strains	P_1	P_2	P_3	P_1	P_2	P_3
Aspergillusglacus	16.66	13.33	9.52	10.31	13.46	14.85
Aspergillusflavus	15	16.66	13.12	21.58	9.76	11.01
Aspergillusniger	—	—	—	9.91	8.62	15.11
Penicillumfrequentaus	17.77	40	17.85	14.76	23.68	19.64
Aspergillusochraceus	32.77	23.33	21.46	6.66	9.76	—
Penicilliunmpallidum	11.11	6.66	—	—	14.42	11.33
Penicilliumcitrinum	—	—	—	13.09	5.59	9.18
Trichodermaviride	6.66	—	38.12	23.64	14.68	18.83

Table 4: Index of diversity, species richness and similarity index of two sites (S_1 and S_2)

	Dunlop		Rajarhat		
plants *	Species richness	Shannon index	Species richness	Shannon index	Similarity index
P_1	2.48	1.55	5.05	1.871	76.92
P_2	2.96	1.56	7.12	2.011	76.92
P_3	2.5	1.57	4.54	1.92	83.33

*P_1, P_2 and P_3represent three plants *F.religiosa,F.benghalensis* and *A.cadamba* respectively.

IV. Conclusion

Our findings revealed that reduction of total microbial population in roadside environment than the non-roadside environment. Fungal association was directly seen by microscopy near the stomata at the lower surface of the phylloplane of less polluted zone, which indicated its sensitivity to the dust as well as toxic metal contaminated with dust particles. Stomata generally act as a direct passage for water transportation. Covering of stomata with dust causes the disturbance between the plant and microbe interaction, it affects adversely on the microbial population. An extensive and further research is needed in this area to describe the proper mechanism of the interaction of air pollutants and microbial association. Among which the complex area of heavy metal pollution and microbial association is need to be exposed, to identify the potentiality of those microbes as bio remediation agents.

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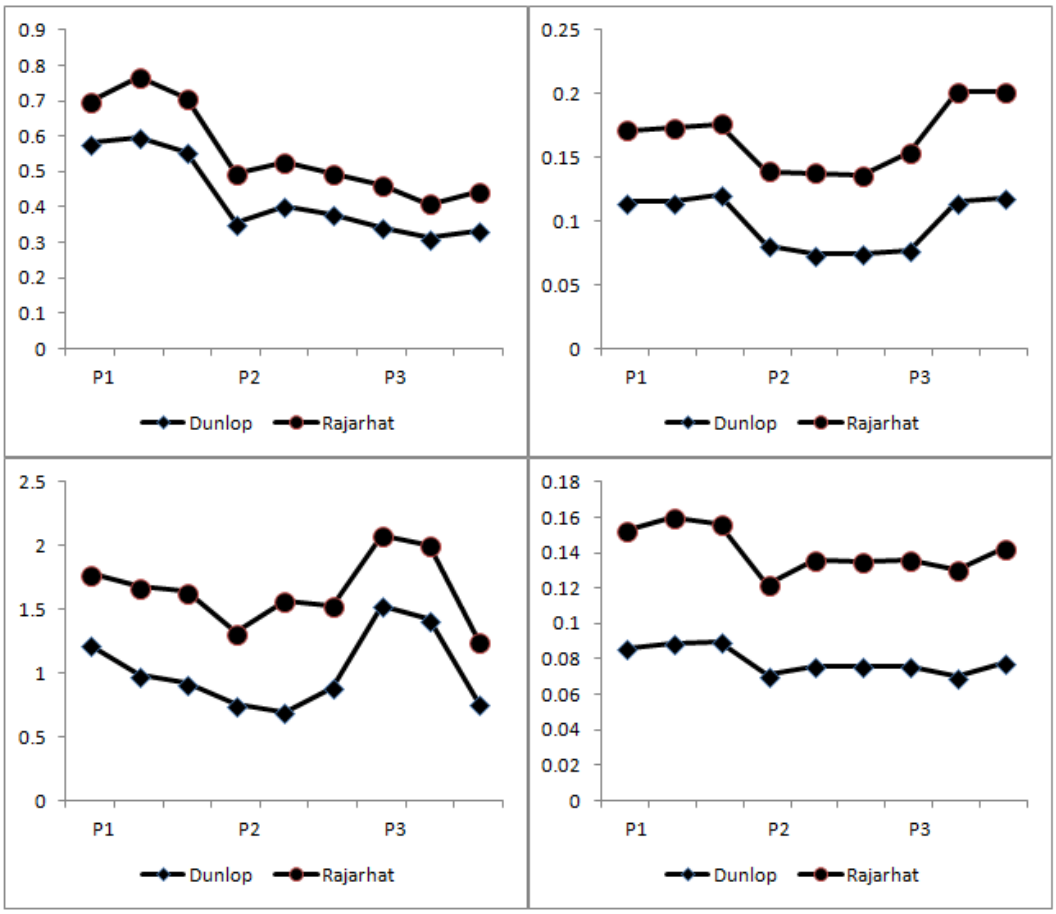

Figure 4.Variation of the concentration (ppm) of Pb,Cu, Zn and Cd in the phylloplane of roadside(S_1) and non-roadside(S_2) plants. P_1, P_2 and P_3represent three plants *F.religiosa,F.benghalensis* and *A.cadamba* respectively.

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