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Seasonal Population Dynamics of Rhizosphere and Non-rhizosphere Soil Microorganisms of Chir Pine Seedlings (*Pinus roxburghii* Sarg.)

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Authors' contributions

Author AY designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Author KY managed the analyses of the study. Both the authors read and approved the final manuscript.

Research Article

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ABSTRACT

Seasonal population dynamics of bacteria, actinomycetes and fungi was studied in rhizosphere and non-rhizosphere soil of chir pine (*Pinus roxburghii* Sarg.) seedlings growing in polybags and in situ (forest). In greenhouse experiment bacteria and actinomycetes were present in higher numbers and their populations fluctuated with season. Fungal population, although lower in numbers, remained stable throughout the year. Population fluctuations with lower numbers were more prominent in rhizosphere and non-rhizosphere soils of forest plants. Differential bacterial population characteristics *viz.* sporeformers, fluorescent colony producers, methylene blue reducers, ammonifiers and glucose fermenters were also taken into account. The population of sporeformers was comparable with methylene blue reducers, which was higher than fluorescent colony producers, ammonifiers and glucose fermenters, respectively. The rhizosphere soil bacterial count of nursery seedlings ranged from $4.36 \times 10^6 - 6.37 \times 10^6$ g^{-1} dry soil weight and from $8.8 \times 10^5 - 2.64 \times 10^6$ g^{-1} soil on dry weight basis in forest plants during various seasons. Sporeformers were a magnitude lower than total bacterial population and fluorescent colony producers were magnitude lower than sporeformers. Actinomycetes count ranged from $6.0 \times 10^5 - 3.02 \times 10^6$ g^{-1} dry soil weight in the nursery plants and from $6.4 \times 10^5 - 1.17 \times 10^6$ g^{-1}dry soil weight in forest plants. Fungal population was a magnitude

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lower than bacterial and actinomycetes population, which ranged from $9.0 \times 10^4 – 2.9 \times 10^5$ g^{-1} dry soil weight in the nursery plants and from $9.0 \times 10^4 – 3.1 \times 10^5$ g^{-1} soil in forest plants. A similar trend of microbial population fluctuation but with lower numbers was observed in forest non-rhizosphere soil.

Aims: To compare the seasonal population fluctuations of rhizosphere and non-rhizosphere soil for better understanding of population dynamics of soil microorganisms.

Study Design: The observations were taken from nursery grown and forest grown seedlings. The microbial populations of pine seedling rhizosphere and non-rhizosphere soil were seasonally enumerated for one year at the intervals of three months for four times.

Place and Duration of Study: The study was conducted at Sardar Bhagwan Singh (PG) Institute of Biomedical Sciences and Research (S.B.S.P.G.I.), Dehradun, Uttarakhand, India, situated at foothills of central Himalayas, for one year during January to December, 2005.

Methodology: The seeds of *Pinus roxburghii* Sarg. were collected from natural chir pine forest from one healthy plant to minimize genetic variability in the experiment. The seeds were germinated on water agar medium and saplings were planted in polybags and kept in greenhouse nursery. Microbial colony forming units (CFUs) of the rhizosphere and non-rhizosphere soil of nursery grown and forest seedlings were enumerated for one year. Their populations were correlated with the meteorological data of the Dehradun valley.

Results: The total bacterial population in terms of CFUs was comparatively higher in all seasons followed by actinomycetes, both these populations fluctuated with season. Fungal population, although lower in numbers, was consistent throughout the period. Microbial populations were found to be dependent on environmental factors like soil and air temperature, relative humidity and precipitation. The population of each microbial type reached maximum during third trimester, just after the end of monsoon season.

Conclusion: The microbial population of chir pine rhizosphere soil and non-rhizosphere soil fluctuates seasonally. Microbial populations were found to be dependent on soil temperature, air temperature, precipitation and relative humidity.

Keywords: Rhizosphere; chir pine; microflora; population.

1. INTRODUCTION

The *Pinus roxburghii* Sarg. (known as chir pine) is a species of pine native to the Himalayas. It is widely planted for timber for house construction, fuel wood extraction, charcoal formation, resin tapping, fuel briquetting, cattle bedding and for manufacturing organic manure, etc. It generally occurs at lower altitudes (500 – 2,000 m) than other pines in the Himalayas. Some patches of *P. roxburghii* Sarg. are also present in Dehradun forest. It has been successfully planted in New forest, Forest Research Campus, Dehradun [1]. Remote sensing analysis of Forest Research Institute, Dehradun showed that the area of chir pine plantation had abruptly been reduced from 784.4 hectare to 162.84 hectare within 25 years from 1976 to 2001 [2]. These lowland forests have mostly been cleared for agriculture, but a few pockets remain [3]. Measures on reforestation in the forests of Dehradun with chir pine require continuous efforts and patience because the plant has slower growth rate [4] and it often suffers from plantation failures [5]. The successful reforestation efforts require proper understanding of the regional climate and plant rhizosphere microflora. The rhizosphere represents one of the most diverse habitats on the planet and is important in ecosystem functioning [6]. Maximum microbial population occurs in rhizosphere because of secretion of high amount of organic substrates in this region [7]. The ecology and dynamics of rhizosphere soil microorganisms vary temporally and spatially due to the

rhizosphere effect [8].The rhizosphere microorganisms contribute essentially to the protection of plants against unfavorable soil and environmental conditions [9]. There is an increasing need to understand rhizosphere population dynamics to harness its potential benefits.

To face the range of biotic and abiotic stresses, plants interact with many different members of the soil microbial community, interacting in many ways and in a range of trophic cascades [10]. Microbial community structure in the soil shows enrichment gradient towards roots, with uniform distribution of sporeformers and sporadic distribution of fluorescent pseudomonads [11]. Such associations between plants and soil biota include positive and negative feedbacks between soil organisms, their chemical environment and plants and require in depth study of rhizosphere microbial community and it's effect on plant growth.Interactions in rhizosphere are also influenced by environmental and climatic factors [12]. Seasonal studies of populations of rhizosphere microorganisms is important for better understanding the rhizosphere dynamics. The present study was designed to provide insight in the seasonal variations of rhizospheric microbial community. Population study of microbes (bacteria, actinomycetes and fungi) was conducted in the nursery and forest plants. Differential bacterial populations *viz*., fluorescent colony producers, glucose fermenters and methylene blue reducers were also enumerated. Meteorological data of Dehradun was recorded throughout the year and correlated with microbial population.

2. MATERIAL AND METHODS

2.1 Study Site

The experiment was performed at S.B.S.P.G.I., Dehradun, situated at the foothills of central Himalayas, during March to December, 2005. The Doon valley is uniformly oriented in NW–SE direction, with the lesser Himalayas in the northeast and the Shiwalik range in SW direction. The soil is usually udic with a thermic to a hyperthermic temperature regime [13]. The valley falls under sub-tropical to temperate climate due to its variable elevation. This region receives most of the rainfall during June to September.Two independent observation sets, one of seedlings growing in greenhouse and another of forest seedlings (of same height) were seasonally studied by enumerating microbial populations of rhizosphere and non-rhizosphere soil. The observations were taken in five replicates. Nursery seedlings were grown in S.B.S.P.G.I. campus and randomly collected for sampling. Forest growing seedlings, equivalent to the height of nursery growing seedlings, were randomly collectedfrom the forest of pine trees from higher slopes of Shiwalik forest range, Dehradun, India (30°17'26"N 78°13'53"E).

2.2 Greenhouse Experiment and Field Observations

2.2.1 Soil: The soils of Dehradun, Shiwalik were developed on the deep alluvial deposits with parent material derived from the Doon alluvium. It consists of accumulated beds of clays, boulders, pebbles and sand with the mixture of water-borne small to big size stones in the subsoil in varying proportions [14]. The same soil was used in nursery polybags.

2.2.2 Seed treatment and greenhouse experiments: For greenhouse experiment, chir pine seeds were surface-disinfected in 1.5% sodium hypochlorite solution for 25 min, washed with sterile distilled water and dried. The seeds were germinated on Petri plates containing sterile water agar medium (1% agar) during the first week of January. Visibly healthy and non-infected saplings were transferred to nursery polybags (one per polybag) with 100 g soil and

used in the study. The saplings were kept in greenhouse nursery and watered every week or when required.

2.3 Estimation of Microbial Populations in Pine Rhizosphere

The microbial populations were enumerated from pine rhizosphere at the intervals of three months for one year from March to December (Fig. 1). Polybags containing seedlings were torn and soil was carefully separated from it. Some of this soil was collected in sterile Petri plates for non-rhizosphere soil (bulk soil) sampling. The rhizosphere soil sticking to the roots of seedlings was separated with fine sterile paintbrush and collected in sterile Petri plates. One gram of the collected soil was diluted to 10^{-4} to 10^{-6} dilutions and 1 ml of dilution was spread onto Petri plates containing respective medium. The readings were expressed as CFUs x dilution factor g^{-1} dry soil weight. Total bacterial population was enumerated using nutrient agar medium. Differential populations of rhizosphere bacteria was determined on the basis of bacterial sporulation and nutrition utilization patterns. Bacterial sporeforming population was determined by pasteurizing the sample dilutions at 80^0C for 10 min and plating 1ml of diluted solution (10^{-4} dilution) onto nutrient agar plates. Fluorescent colony producers were identified using trypticase soy agar plates [15]. The test for ability to reduce methylene blue was done with a liquid medium containing culture medium-B [16] with 0.02% aqueous solution of methylene blue, decolourization of medium provided a positive result. The ability of ammonification was examined in a liquid medium prepared according to Rodina [16] and NH_3 was detected with Nessler's reagent. Glucose fermenting bacteria were enumerated on a basal medium that contained the equivalents of 2 g l^{-1} glucose (used as a carbohydrate source); 0.5 g l^{-1} of trypticase and yeast extract; 0.5 g l^{-1} of cysteine HCl (used as a reductive agent); 0.1 g l^{-1} of sodium acetate; 0.005 g l^{-1} of resazurine; 20 ml of Widdel mineral solution [17]; and 1 ml of Widdel trace element solution [17]. Actinomycetes population was enumerated using glycerol yeast extract agar medium containing equivalents of 5 ml l^{-1} glycerol, 2 g l^{-1} yeast extract, 1 g l^{-1} dipotassium hydrogen phosphate, 0.01 g l^{-1} aureomycin. For the isolation of bacteria and actinomycetes Petri plates containing respective medium and soil dilutions were incubated at 30^0C for two days. Fungal population was enumerated using potato dextrose agar medium by incubating Petri plates containing soil dilutions at 30^0C for one week.

2.4 Statistical Analyses

Each population value was the mean of five replicates with standard error (SE). Statistical analyses *viz.* regression and principal components analysis (PCA) of meteorological data were done using XLStat-Pro software [18].

3. RESULTS AND DISCUSSION

Several types of microorganisms thrive in rhizosphere soil but bacteria are chief among total microbial populations. Bacteria studied in this category are called 'rhizobacteria', which are mainly fluorescent pseudomonads, bacilli, *Azospirillum* sp. etc. [7,19,20]. Such type of bacteria may either reduce or facilitate the establishment of symbiosis [21].

In the current study the seasonal population dynamics of rhizosphere and non-rhizosphere soil of nursery and forest growing seedlings of *P. roxburghii* Sarg. was studied. Overall each population type was highest in September and lowest in March. Bacterial population was comparatively higher in all seasons followed by actinomycetes, both these populations

fluctuated with the season. Fungal population, although lower in numbers, was consistent throughout the period.

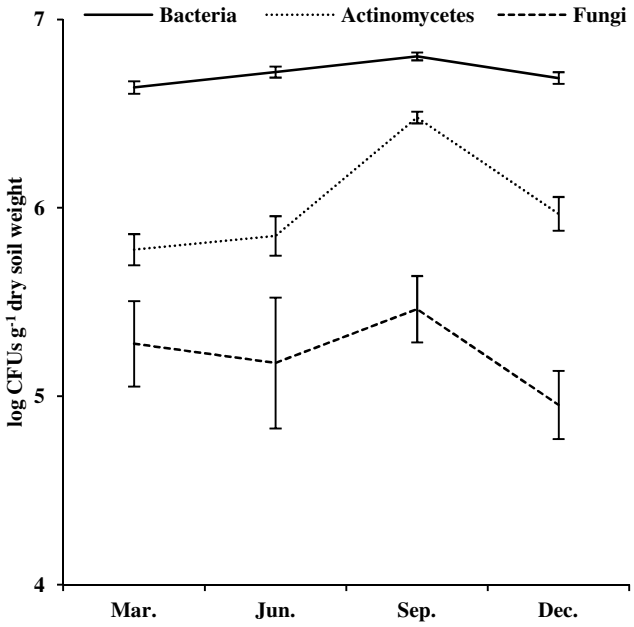

Fig. 1. Seasonal microbial populations (g^{-1}dry soil weight) of rhizosphere soil from nursery grown chir pine seedlings. Each value is a mean of five replicates, vertical bars represent SE.

3.1 Seasonal Microbial Population of Pine Rhizosphere Soil

From rhizosphere of nursery seedlings, maximum number of bacterial CFUs were recorded in September (6.37 x 10^6 g^{-1} soil) and minimum in March (4.36 x 10^6 g^{-1} soil)(Fig. 1). Maximum CFUs of actinomycetes were recorded in September (3.02 x 10^6 g^{-1} soil) and minimum in March (6 x 10^5 g^{-1} soil). Maximum fungal CFUs were recorded in September (2.9 x 10^5 g^{-1} soil) and minimum in December (9 x 10^4 g^{-1} soil). A similar trend of microbial populations with lower CFUs was recorded from forest rhizosphere soil (Fig. 2). Overall higher bacterial and actinomycetes populations were recorded from nursery and forest seedlings,which fluctuated with season. Fungal population with lower CFUs, on the other hand, remained stable throughout the year.

3.2 Seasonal Microbial Population of Non-rhizosphere (bulk) Soil

From nursery seedlings maximum bacterial CFUs were recorded in September (3.38 x 10^6 g^{-1} soil) and minimum in December (1.91 x 10^6 g^{-1} soil) (Fig. 3). Rhizosphere bacterial population ranged one to two magnitude higher than non-rhizosphere soil. The results are in accord with previous study done on rhizosphere microflora associated with mycorrhiza of Douglas-fir (*Pseudotsuga menziesii* var. menziesii)[22]. Bacterial population was comparable to actinomycetes. Actinomycetes and fungal populations were maximum in September (2.95 x 10^6 g^{-1} soiland6.18 x 10^5 g^{-1} soil, respectively) and minimum in March (8.3 x 10^5 g^{-1} soil) and

June (15.8 x 10^4 g^{-1} soil), respectively. A similar trend of populations but with lower CFUs was recorded from non-rhizosphere soil of forest seedlings (Fig. 4).

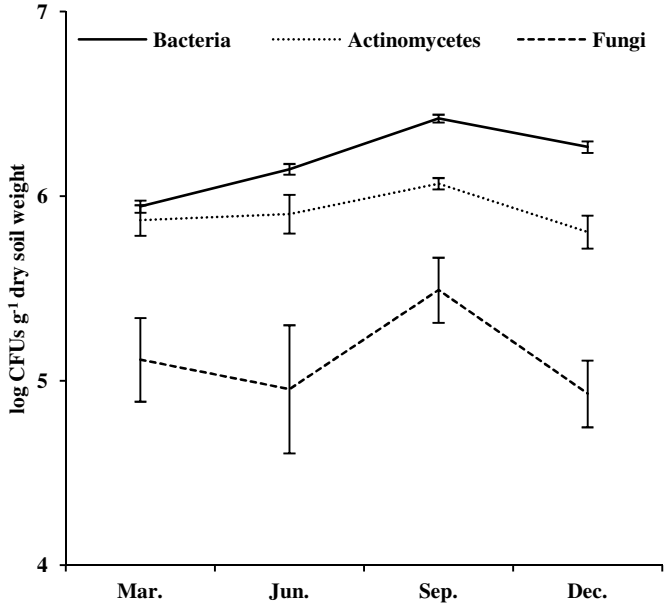

Fig. 2. Seasonal microbial populations (g^{-1}dry soil weight) of rhizosphere soil from forest growingchir pine seedlings. Each value is a mean of five replicates, vertical bars represent SE.

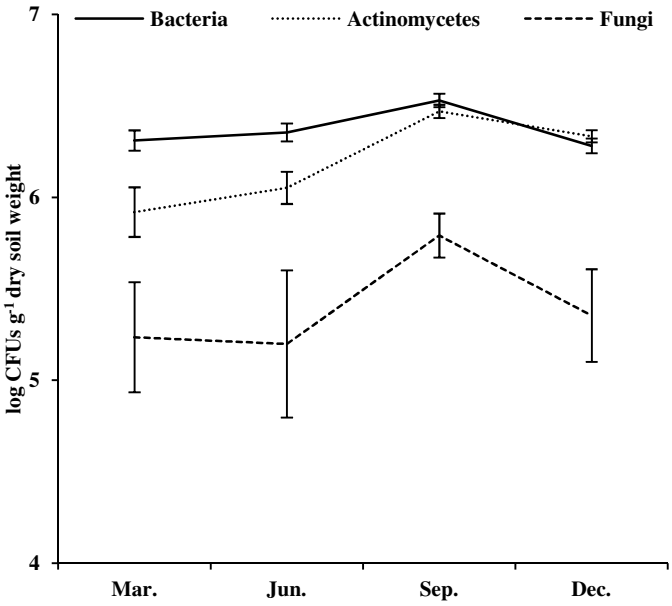

Fig. 3. Seasonal microbial populations (g^{-1}dry soil weight) of non-rhizosphere soil from nursery grown chir pine seedlings. Each value is a mean of five replicates, vertical bars represent SE.

3.3 Differential Microbial Population of Pine Rhizosphere Soil

In nursery seedlings, sporeforming population was a magnitude lower than bacterial population. Maximum sporeforming population (7.8 x 10^5 g^{-1} soil) was recorded in September and minimum (9.8 x 10^4 g^{-1} soil) in March (Fig. 5). In forest seedlings also sporeforemers were maximum in September (11.4 x 10^4 g^{-1} soil) and minimum in March (3.2 x 10^4 g^{-1} soil) (Fig. 6). Maximum CFUs (4.4 x 10^4 g^{-1} soil) of fluorescent colony producers were recorded in June and minimum (2.2 x 10^4 g^{-1} soil) in March. Fluorescent bacterial population in forest seedlings was maximum in September (2.8 x 10^4 g^{-1} soil) and minimum in March (1.6 x 10^4 g^{-1} soil). Fluorescent bacterial number was always lower than sporeformers during each sampling months. In nursery seedlings,ammonifiers were highest in December (6.2 x 10^4 g^{-1} soil) and lowest in March (4 x 10^4 g^{-1} soil). Ammonifying bacterial number in forest seedlings was maximum in June (3.8 x 10^4 g^{-1} soil) and minimum in March (2.2 x 10^4 g^{-1} soil).Ammonifier population in each season was comparable to fluorescent colony producing bacterial population. Ammonifiers are responsible for converting organic N compounds into inorganic forms (NH_4^+ and NO_3^-), which is made available to plants. Methylene blue reducers in the nursery seedlings were highest in September (3.54 x 10^5 g^{-1} soil) and lowest in December (1.24 x 10^5 g^{-1} soil). In forest plants they were highest during September (1.48 x 10^5 g^{-1} soil) and lowest in March (3.8 x 10^4 g^{-1} soil). Their population was consistently comparable to sporeformers. In nursery seedlings maximum number of glucose fermenters (8.8 x 10^4 g^{-1} soil) were recorded in December and minimum (3 x 10^4 g^{-1} soil) in March. Population of glucose fermenters from the rhizosphere of forest seedlings was maximum in September (2.2 x 10^4 g^{-1} soil) and minimum in June (1.7 x 10^4 g^{-1} soil). The population of glucose fermenters was comparable to fluorescent colony producers and methylene blue reducers. The rhizosphere bacteria show specificity towards glucose utilization and remain present over root surface.

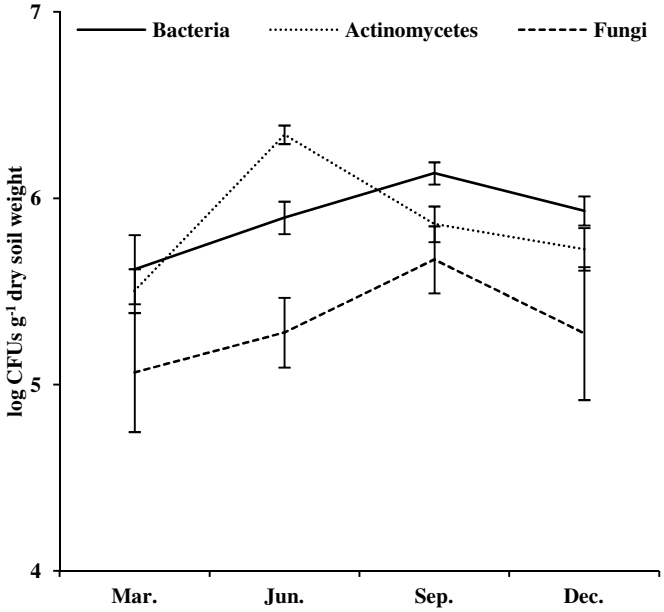

Fig. 4. Seasonal microbial populations (g^{-1}dry soil weight) of non-rhizosphere soil from forest growingchir pine seedlings. Each value is a mean of five replicates, vertical bars represent SE.

In the rhizosphere of nursery grown *P. roxburghii* plants the bacterial and actinomycete population reached maximum in September (monsoon) and minimum in March (spring). Reduced bacterial population in March can be attributed to the early period of intense cold during winter, which might had led to lower microbial growth rate (Fig. 7). Current study proves the negative effect of lower temperature on rhizosphere and non-rhizosphere soil microflora (Fig. 1 to Fig. 7).

The average soil temperature at the depth of 0.1 m was higher during June, July and August (21^0C, 21.5^0C and 20^0C, respectively, Fig. 7), which moderately dropped to 18.8^0C in September. Monsoon season in Doon valley starts from mid of June and continues till mid-September [23]. Enhanced precipitation leads to faster growth rate of microorganisms due to increased water activity in soil. Months of July, August and September received maximum precipitation and relative humidity (Fig. 8, Fig. 9). In September the precipitation reduced considerably but relative humidity remainedat higher level (84.9%). The higher average soil temperature and relative humidity during September, preceded with even higher values during July and August, might had resulted in maximum populations of all microbial types in the sampling of September.

Fungal population was minimum in June and December due to extended climatic desiccation (Fig. 8). With a similar trend, rhizosphere of forest grown seedlings depicted comparatively lower CFUs of all microbial types. The forest soil experiences extremes of climate and lower nutrition, making the survival and multiplication of microbes difficult. The higher bacterial population of nursery soil can be correlated with better growth conditions in greenhouse.

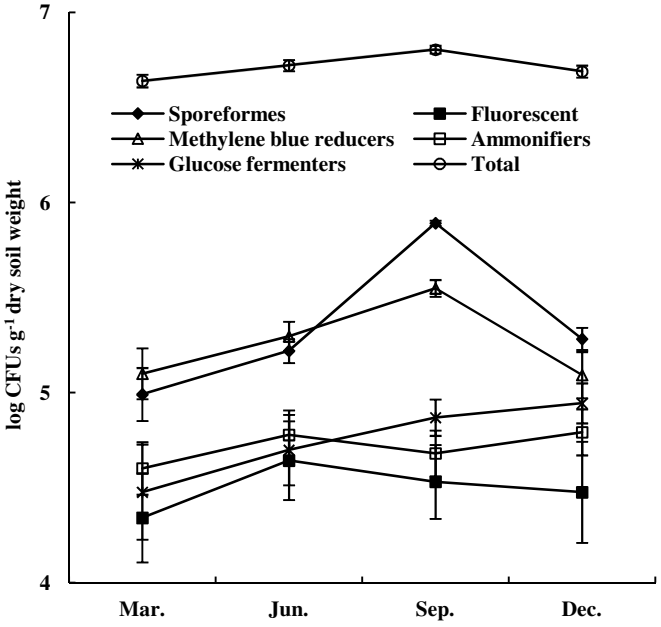

Fig. 5. Seasonally differential bacterial populations ofrhizosphere soil (g^{-1}dry soil weight) from nursery grown chir pine seedlings. Each value is a mean of five replicates, vertical bars represent SE.

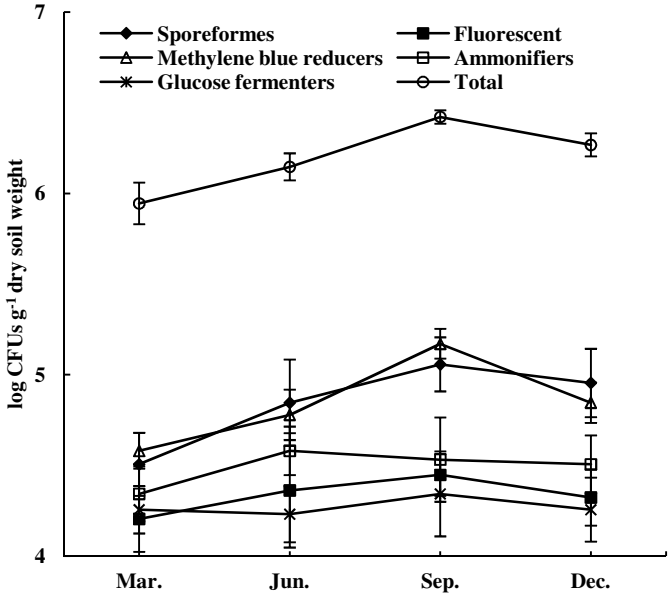

Fig. 6. Seasonally differential bacterial populations ofrhizosphere soil CFUs (g^{-1}dry soil weight) fromforest growing chir pine seedlings. Each value is a mean of five replicates, vertical bars represent SE.

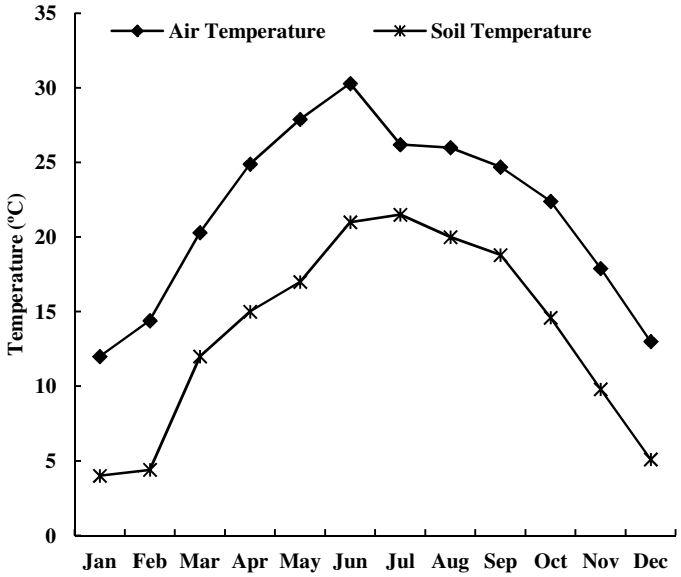

Fig. 7. Average forest air and soil temperature (0.1 m depth) of Dehradun during 2005.

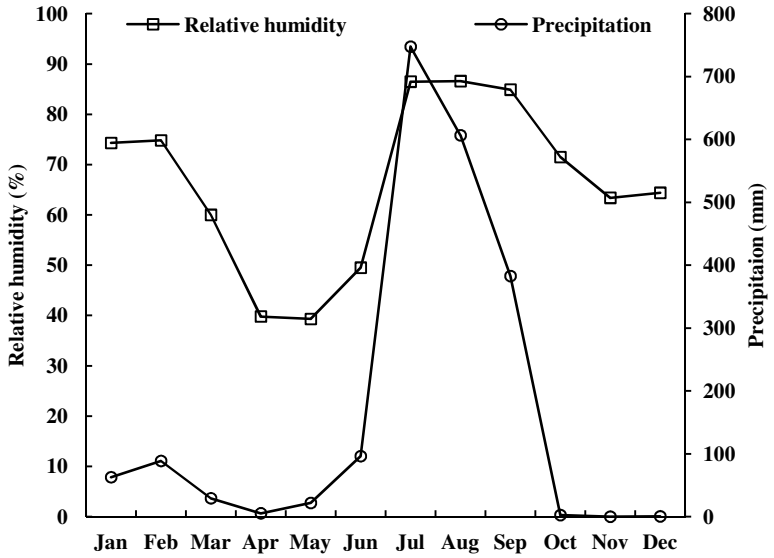

Fig. 8. Relative humidity and precipitation of Dehradun during 2005 (Source: weather station- 421110, Dehradun, India).

Actinomycetes are important rhizosphere inhabitants of many plants where they enhance plant growth and protect the plant roots against attack by phytopathogens [24]. In non-rhizosphere soil, comparatively lower actinomycetes CFUs were obtained. The population of actinomycetes, although lower, was comparable to bacterial population. Fungi were a magnitude lower than bacterial population. The microbial populations of non-rhizosphere soil from forest seedlings had overall lower CFUs but followed the same seasonally fluctuating trend.Differential population of each bacterial type in the nursery grown seedlings was higher than forest seedlings. The sporeformer population was maximum followed by methylene blue reducers and glucose fermenters. The CFUs of each microbial type was minimum during winters (December and March). During winters Himalayan region receives severely lower temperatures. The soil temperature also drops severely (Fig. 7) causing lower microbial metabolism.

The PCA between study months, humidity, air temperature and soil temperature shows that July, August were the most humid months with maximum precipitation. Month of June received highest air and soil temperature, whereas January, February and December were coldest months with least precipitation and relative humidity (Fig. 9). The summer (sampling of June) depicted the overall microbial population drop possibly due to decrease in relative humidity, which dropped to 49.5%, preceded with even drier period of April (R.H. 39.8%) and May (R.H. 39.3%) (Fig. 8).Summer receives lower and infrequent precipitation leading to lowest relative humidity. Lower relative humidity leads to lower soil water activity, which is responsible for causing stress on soil microorganisms [25]. A strong linear regression was obtained between air temperature and soil temperature at the depth of 0.1 m (R^2=0.921) (Fig. 10). Populations from forest sampling plots, although lower than nursery plots, depicted the same seasonally fluctuating trend. Himalayan mountains face desiccation in summers leading to lower water activity in soil. Populations of all soil microbial types were maximum during monsoon with highest species diversity (data unpublished). The months of monsoon (July, August and September) received higher relative humidity and precipitation. High precipitation, however, favor high and permanent loss of the elements beyond the rooting

zone by leaching and/or runoff, making surface as well as sub-surface soils impoverished in the basic cations and anions [13]. During next two trimesters comparatively lower microbial population was observed due to overall lower temperatures, leading to lower metabolism.

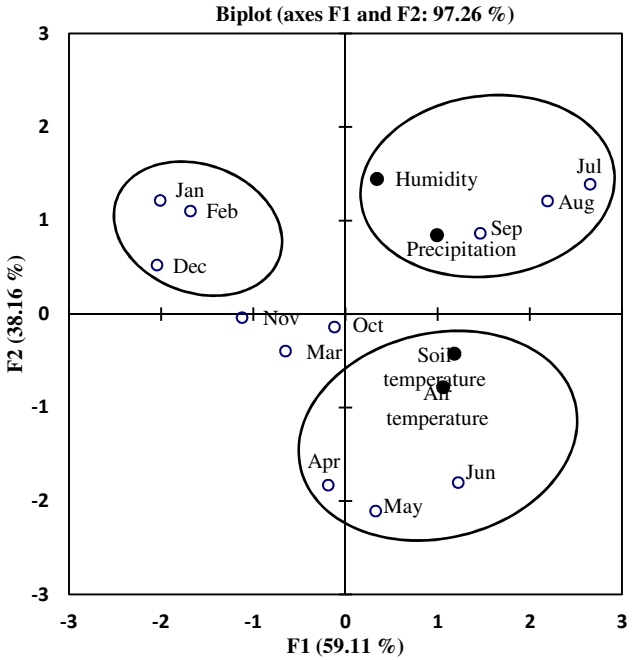

Fig. 9. PCA betweenstudy months, humidity, soil temperature and air temperature (hollow circles represent study months, solid circles represent four climatic variables).

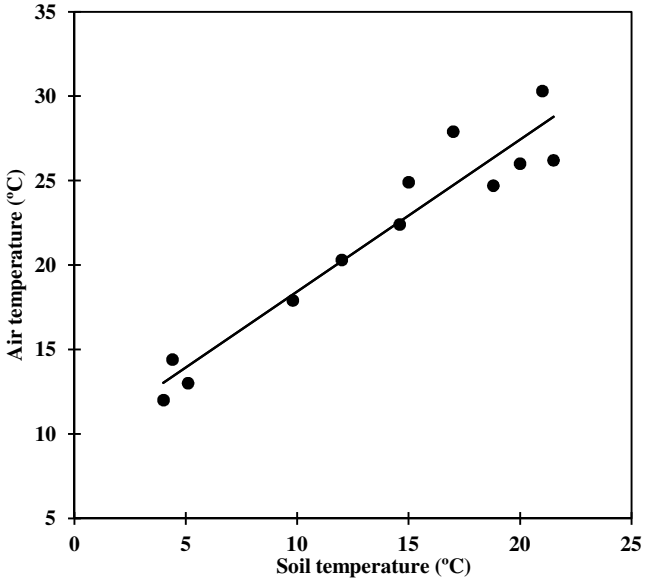

Fig. 10. Regression of air temperature by soil temperature (R^2=0.921).

Previous studies done on *Quercus leucotrichophora* rhizosphere from Kumanu Himalayas revealed maximum bacterial CFUs (1.41 x 10^8 g^{-1} soil) during rainy season and minimum (9.87 x 10^7 g^{-1} soil) during summer [26]. From the same sitetheauthor 1 isolated four bacterial isolates *viz.*, *Bacillus subtilis*, *Pseudomonas fluorescens*, *Vibrio* sp.1 and *Xanthomonas* sp., respectively from *Q. leucotrichophora* rhizosphere and reported total bacterial CFUs of 1.65 x 10^6 g^{-1} dry root weight and fluorescent CFUs of 3 x 10^4 g^{-1} dry root weight [27]. Fig. 5 shows differential bacterial populations of rhizosphere soil from nursery growing pine seedlings per trimester. In nursery seedlings, sporeformers and methylene blue reducers were present in higher numbers with seasonally fluctuating population. Glucose fermenters, ammonifiers and fluorescent colony producers, were although lower in numbers, but with stable population. Nonsporulating rods, pseudomonads, and actinomycetes have been reported to be the most common bacteria in the soil [28]. In nursery growing seedlings maximum population fluctuation was observed in sporeformers during June to September. Their population of March (9.8 x 10^4 g^{-1} soil) reached maximum (7.8 X 10^5 g^{-1} soil) in September and dropped tolower value (1.92 X 10^5 g^{-1} soil) in December. In forest plants (Fig. 6) the fluorescent pseudomonads and glucose fermenters had stable population but their number was negligible compared to total bacterial population.Populations of sporeformers and methylene blue reducers were comparatively in higher numbers with seasonally fluctuating population, which shows their abundance in rhizosphere soil.

4. CONCLUSION

The microbial populations of chir pine rhizosphere soil and non-rhizosphere soil fluctuates seasonally. The microbial populations were found to be dependent on soil temperature, air temperature, precipitation and relative humidity. Bacteria were present in higher numbers followed by actinomycetes. Both these populations fluctuated with season. Fungal population was stable throughout the year. Differential bacterial populations also varied with season. Population of fluorescent colony producers, although much lower than sporeforming population, was stable throughout the period, suggesting their possible contribution in plant growth promotion on chir pine. Microbial composite population, which was lower during first trimester, reached lowest during second trimester (summer) but shoot to maximum during third trimester and dropped to lower level in the last trimester of the year. Population of sporeforming bacteria was always higher than fluorescent colony producers but lower than total bacteria population by a magnitude. This study suggests the favorable period for pine plantations in Dehradun forest. The third trimester of the year is the best period for chir pine plantations. The plant during monsoon season receives maximum water in the form of precipitation, optimum relative humidity and temperature. During this period the plant may receive maximum growth support due toincreased population of native rhizosphere microflora.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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