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Application of Microbial Inoculants Enhanced Microbial Population Load in Himalayan Cypress and Blue Pine Rhizosphere

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ABSTRACT

A pot experiment was carried out during 2018-2019 to study the impact of microbial inoculants on microbiological characteristics of Himalayan cypress and Blue pine soil. The experiment was laid in Completely Randomized Design with three replications which comprised forty-two treatment combinations of seven inoculants (*Azotobacter* sp., *Azospirillum* sp., *Pseudomonas fluorescens, Bacillus subtilis, Pisolithus tinctorius, Laccaria laccata* and control). The microbial inoculants increased the soil microbial populations significantly. *Pseudomonas fluorescens* and *Bacillus subtilis* resulted in a significant increase in total viable bacteria, followed by *Azotobacter* sp., *Azospirillum* sp., *Pisolithus tinctorius, Laccaria laccata* and control, respectively. However, for total viable fungal count, *Pisolthus tinctorius* and *Laccaria laccata* proved significant and it was followed by *Pseudomonas fluorescens, Bacillus subtilis, Azotobacter* sp., *Azospirillum* sp. and control. Thus the treatments viz.,*Bacillus subtilis, Pseudomonas fluorescens, Pisolithus tinctorius* and *Laccaria laccata* performed exceptionally well and proved to be superior for all the studied parameters.

Keywords Blue pine, Himalayan cypress, Microbial inoculation, *Azotobacter, Azospirillum.*

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INTRODUCTION

Pinus wallichiana Jackson, commonly known as kail, blue pine or Bhutan pine, is an evergreen large conifer tree which has bluish feathery foliage. At young age, it is one of the most beautiful pines in the world. In the Himalayan region, kail is frequently found between 1500-3000 m. However, sometimes it may grow upto 3600 m in the upper reaches. They are largely found in

areas where rainfall is 1000-2000 mm annually. From Afghanistan in the west, the kail region extends upto Bhutan and Arunachal Pradesh in the east, although it is absent in considerable portions of Kumaon and Sikkim. Other important places where kail grows abundantly in the sub-continent are from Garhwal through Jaunsar, the Shimla hills, Kulu, Chamba and Muree hills. Kail requires well-drained moist, fresh and deep soils; preferably derived from mica-schist which decomposes in moist fresh soil. In certain cases, the species also grows on deep limestone soils. It sometimes grows up in great abundance on bolder and gravel deposits in the beds of streams owing to its preference for porous soil with a fair amount of sub-soil moisture (Troup 1921).

The increasing pressure of human and livestock population, indiscriminate extraction of forest produce, regular forest fires and mining activities have resulted in soil erosion, loss of fertility and moisture content and decreasing productivity of forests which pose manifold problems to restore the ecosystem. Thus, microbial inoculants present in the soil form a strong and important component of our soils, mainly owing to their role to promote plant growth by providing access to the nutrients, nitrogen fixation, mobilization of some unavailable nutrients and production of antifungal antibiotics.

The Himalayan cypress belonging to the family Coniferae is a large evergreen tree with a pyramidal crown and drooping branchlets. Trees upto 47 m height and 7.15 m in girth have been measured in Tehsil Garhwal (Troup 1921). Bark greyish brown, peeling off in long thin strips; leaves small, scale like; seeds compressed with an orbicular wing, light reddish brown. The tree has a local distribution in the western Himalayas from Chamba to Nepal between 1800-2750 m elevations. The tree is naturally found on limestone. In its natural habitat the absolute maximum shade temperature is probably about 90^{o}F, the absolute minimum about 15^{o}F and the normal rainfall varies from 1000 to 2400 mm per annum.

The indiscriminate use of inorganic fertilizers and pesticides is neither environmentally safe nor economically feasible. There is pressing demand for microbial inoculants for quality seedling production in nursery and also the establishment of plantation to increase the forest productivity. Bioinoculants are cost effective, ecofriendly, cheaper and renewable sources of plant nutrients and play a vital role in maintaining long-term soil fertility and sustainability. Moreover, they form an important component of organic farming practices. Thus, to meet the challenges like poor regeneration, deforestation and spread of wastelands, introduction of microbial inoculants at the nursery stage of forest trees has become inevitable. Although various aspects of mycorrhizal impact of the forest trees have been studied, no work has been done on the impact of other microbial inoculants on the regeneration of forest trees.

MATERIALS AND METHODS

The present investigations were undertaken at the Faculty of Agriculture, SKUAST-Kashmir, Wadura, Sopore during 2018-2019. Microbial inoculants isolated from rhizosphere of blue pine and Himalayan cypress forest stands were used in the studies.

Mass production of microbial inoculants

The two free living aerobic nitrogen fixing bacteria viz., *Azotobacter* sp. and *Azospirillum* sp. were mass cultured using nutrient medium enriched with glucose and peptone. Plant growth promoting rhizobacteria (PGPR) viz., *Pseudomonas fluorescens* and *Bacillus subtilis* were mass propagated in King's B nutrient broth. The two ectomycorrhizae viz., *Pisolithus tinctorius* and *Laccaria laccata* were mass multiplied in Melin Norkran's nutrient broth and Potato Dextrose Agar, respectively. Field operations for the microbial inoculation, one year old seedlings of blue pine of uniform heights and collar diameter growing in polyethylene bags (9 × 7) containing 1 kg potting material of soil and sand mixture in the ratio of 1:1 were selected.

Isolation and characterization of microbial inoculants

A field survey of two districts of Kashmir valley viz.

Kupwara and Bandipora was carried out during 2018-2019 for the collection of rhizosphere soil samples of Blue pine and Himalayan cypress stands. The collected rhizosphere soil samples of both the species were brought directly to the laboratory for isolation of bacterial and fungal inoculants. The fungal inoculants were isolated by dilution plate method (Johnson *et al.* 1957) on potato dextrose agar medium. The soil samples were thoroughly homogenized. Ten grams of soil was placed in 90 ml distilled sterile water and different dilutions made. One ml of each 10^4 and 10^5 dilution was pipetted out and poured into sterile Petri-dishes. Later, 15 ml molten PDA medium was poured in petriplates which were gently rotated and incubated at 26±2°C for 36 h. The cultures obtained were purified by single spore/hyphal tip method and maintained for further studies. The identification of isolated fungal inoculants was done on the basis of cultural and morphological characteristics viz., growth, color and shape of the colonies, color, shape and size of hyphae, basidiospores, cap, mycelia spines, gleba, conidiosphores and conidia (Arx JA von 1981).

The bacterial inoculants were isolated from the rhizosphere soil samples of blue pine and Himalayan cypress stands by serial dilution technique. One gram of rhizosphere soil sample was transferred to 250 ml conical flask containing 100 ml sterile water. After thorough shaking for 15 minutes in a shaker, serial dilutions upto 10^{-7} were prepared. One ml of each 10^{-6} and 10^{-7} dilution was pipetted out and poured into the sterile petri-dishes. Fifteen ml molten King's B medium (KB) (King *et al.* 1954) was poured in plates which were rotated gently and incubated at 28±2°C for 24 hours. The bacterial growth developed was purified by the dilution plate technique. The bacterial cultures were maintained on King's B medium in culture tubes at 4°C. Characterization of the isolated bacteria was done according to the methods recommended in the laboratory guide for the identification of microbial inoculants (Schaad 1992). The biochemical and physiological tests viz. gelatine liquefaction, arginine dihydrolase, H_2S gas production, catalase, levan production, oxidase, indole production, starch hydrolysis, urease test and pigment production on various growth media were used for characterization of microbial inoculants viz. *Azotobacter* sp., *Azospirillum* sp., *Pseudomonas fluorescens* and *Bacillus subtilis,* respectively (Gopalakrishnan and Meena 2004).

Statistical analysis

The data was statistically analyzed by using O.P Stat software developed by Haryana Agriculture University, Hisar, India.

RESULTS AND DISCUSSION

Perusal of the data presented in Table 1 shows that

Table 1. Impact of microbial inoculation on total viable bacteria (× 10^8 CFU g^{-1}) of blue pine soil (*Pinus wallichiana* A.B. Jackson) at nursery stage. Initial total viable bacterial count = 2.10 ×10^8 (cfu). Figures in parenthesis indicate CD of individual months.

Treatment	2018				2019		
	April	June	August	October	December	February	Mean
Control	2.40	2.90	3.80	4.30	4.00	3.60	3.50
Azotobacter sp.	2.90	3.50	4.80	5.50	5.09	4.70	4.41
Azospirillum sp.	2.80	3.40	4.70	5.40	5.00	4.60	4.31
Pseudomonas fluorescens	3.20	4.00	5.50	6.70	6.10	5.70	5.20
Bacillus subtilis	3.10	3.90	5.40	6.60	6.00	5.60	5.10
Pisolithus tinctorius	2.60	3.10	4.30	4.80	4.30	4.00	3.85
Laccaria laccata	2.50	3.00	4.20	4.70	4.20	3.90	3.75
Mean	2.78	3.40	4.67	5.42	4.95	4.58	
	(0.173)	(0.249)	(0.361)	(0.537)	(0.492)	(0.476)	
	Treatment (T)		Month (M)		T x M		
CD ($p \leq 0.05$)	0.019		0.018		0.048		
SEm	0.007		0.006		0.017		

Table 2. Impact of microbial inoculation on total viable bacteria (x 10^8 CFU g^{-1}) of Himalayan cypress soil (*Cupressus torulosa* Don) at nursery stage. Initial total viable bacterial count = 2.10 x 10^8 (cfu). Figures in parenthesis indicate CD of individual months.

Treatment	2018				2019		
	April	June	August	October	December	February	Mean
Control	2.50	3.00	3.90	4.40	4.10	3.80	3.61
Azotobacter sp.	3.00	3.60	4.90	5.60	5.20	4.90	4.53
Azospirillum sp.	2.87	3.50	4.80	5.50	5.10	4.80	4.42
Pseudomonas fluorescens	3.30	4.10	5.60	6.80	6.20	5.90	5.31
Bacillus subtilis	3.20	4.00	5.50	6.70	6.10	5.80	5.21
Pisolithus tinctorius	2.70	3.20	4.40	4.90	4.40	4.10	3.95
Laccaria laccata	2.60	3.10	4.30	4.80	4.30	4.00	3.85
Mean	2.88	3.50	4.77	5.52	5.05	4.75	
	(0.175)	(0.249)	(0.361)	(0.537)	(0.492)	(0.470)	
		Treatment (T)		Month (M)	T x M		
CD ($p \leq 0.05$)		0.014		0.013	0.036		
SEm		0.005		0.004	0.012		

there was a significant increase in total viable bacteria of Blue pine soil with the application of microbial inoculants than control. Application of *Azotobacter* and *Azospirillum* sp. exhibited 20.63 and 18.79% increase in bacterial count respectively over control. Similarly with the application of *P. tinctorus* and L. *laccata*, there was an increase of 9.09 and 6.66% over control. However, the application of *Pseudomonas fluorescens* and *Bacillus subtilis* gave the highest viable cell count of 37.69 and 31.37% over control. Moreover the total bacterial count registered a significant gradual increase from April to October and a decrease was observed from October onwards till February.Table 2, contains data on impact of microbial inoculants on total viable bacteria of Himalayan cypress soil. The data reveals that the viable cell count was significantly improved by the application of various microbial inoculants as compared to control. *Pseudomonas fluorescens* inoculation resulted in higher viable count of bacteria than other inoculants and was the best treatment resulting in an increase of 32.01% over control. Similarly it was followed by *Bacillus subtilis* which gave 30.71% more viable bacterial count than control. Application of *Azotobacter* and *Azospirillum* sp. gave 70.30 and 18.32 % more over control. However, *P. tinctorius* and *L. laccata* treatments proved least effective in case of total viable bacterial count increase and resulted in 8.60 and 6.23% over control, respectively. Moreover, viable bacterial count showed a significant increasing trend upto October and a decreasing trend in the winter months of December and February. The increase in bacterial population can be attributed to the secretion of certain root exudates which serve as chemoattractants from rhizosphere bacteria and production of growth promoting substances by the microbial inoculants (Jackobsen *et al.* 1994, Singh *et al.* 1998). The increase in bacterial population may also be due to the presence of suitable soil moisture and temperature conditions. However the decrease in bacterial population from December to February may be ascribed to unfavourable climatic conditions (Vander 1963).

Perusal of the data presented in Table 3 indicated that there was a significant increase in total viable fungi, with the application of various microbial inoculants as compared to control. The microbial dose of *Pisolithus tinctorius* resulted in a significantly higher total viable fungal count which was 15.64% more over control and thus proved superior over other inoculants. It was followed by *Laccaria laccata* (14.61%), *Pseudomonas fluorescens* (6.03%), *Bacillus subtilis* (4.75%), *Azotobacter* sp. (2.77%) and *Azospirillum* sp. (1.40%), respectively over control. Further total viable fungal count registered a significantly increasing trend upto October and a declining trend in the last winter months.As evident from the data contained in Table 4, the total viable

Table 3. Impact of microbial inoculation on total viable fungi (x 10^6 CFU g^{-1}) of blue pine soil (*Pinus wallichiana* A.B. Jackson) at nursery stage. Initial total viable fungal count = 4.9 x 10^6 (cfu). Figures in parenthesis indicate CD of individual months.

Treatment	2018 April	June	August	October	2019 December	February	Mean
Control	5.10	6.00	7.20	8.30	7.90	7.60	7.01
Azotobacter sp.	5.30	6.20	7.40	8.50	8.10	7.80	7.21
Azospirillum sp.	5.20	6.10	7.30	8.40	8.00	7.70	7.11
Pseudomonas fluorescens	5.50	6.40	7.70	8.80	8.40	8.00	7.46
Bacillus subtilis	5.40	6.30	7.60	8.70	8.30	7.90	7.36
Pisolithus tinctorius	6.40	7.20	8.70	9.50	9.20	8.90	8.31
Laccaria laccata	6.30	7.10	8.60	9.40	9.10	8.80	8.21
Mean	5.60	6.47	7.78	8.80	8.42	8.10	
	(0.305)	(0.319)	(0.355)	(0.368)	(0.365)	(0.361)	
	Treatment (T)		Month (M)		T x M		
CD ($p \leq 0.05$)	0.019		0.018		0.049		
SEm	0.007		0.006		0.017		

fungal count of Himalayan cypress got increased significantly with the application of various microbial treatments over control. Amongst the various treatments *P. tinctorius* proved best over other treatments and resulted in 15.45% more viable fungal count over control. It was followed by *L. laccata* which gave 14.44% more viable fungal count over control. Microbial doses of *Azotobacter* sp., *Azospirillum* sp., *Pseudomonas fluorescens* and *Bacillus subtilis* resulted in 2.73, 1.38, 6.20 and 4.94% more viable fungal count over control. Moreover viable fungal count demonstrated a significant increase upto October and a non-significant decrease in the winter months. The increase in fungal count could be attributed to the production of growth promoting substances secreted by soil microbes (Tien *et al*.1979) and secretion of certain root exudates. Further the presence of suitable soil moisture and temperature conditions might have increased its population. Moreover, the unfavorable weather conditions in winter months might have accounted for its decrease (Vander 1963).

Table 4. Impact of microbial inoculation on total viable fungi (x 10^6 CFU g^{-1}) of Himalayan cypress soil (*Cupressus torulosa* Don) at nursery stage. Initial total viable fungal count = 4.9 x 10^8 (cfu). Figures in parenthesis indicate CD of individual months.

Treatment	2018 April	June	August	October	2019 December	February	Mean
Control	5.20	6.10	7.30	8.40	8.00	7.70	7.11
Azotobacter sp.	5.40	6.30	7.50	8.60	8.20	7.90	7.31
Azospirillum sp.	5.30	6.20	7.40	8.50	8.10	7.80	7.21
Pseudomonas fluorescens	5.60	6.50	7.80	8.90	8.50	8.20	7.56
Bacillus subtilis	5.50	6.40	7.70	8.80	8.40	8.10	7.46
Pisolithus tinctorius	6.50	7.30	8.80	9.60	9.30	9.00	8.41
Laccaria laccata	6.40	7.20	8.70	9.50	9.20	8.90	8.31
Mean	5.70	6.57	7.88	8.90	8.52	8.22	
	(0.309)	(0.327)	(0.367)	(0.391)	(0.387)	(0.379)	
	Treatment (T)		Month (M)		T x M		
CD ($p \leq 0.05$)	0.013		0.012		0.032		
SEm	0.007		0.004		0.011		

REFERENCES

Arx JA Von (1981) The genera of fungi sproulating in pure culture. VADUZ Press, Germany, pp 424.

Gopalkrishnan J, Meena C (2004) Biochemical characterization of *Pseudomonas fluorescens* and its antibacterial metablites. In: Sharma P, Aggarwal R, Gopalkrishnan J, Singh DV (eds). Biocontrol Strategies for Management of Plant Pathogens. Centre of Advanced Studies in Plant Pathology, IARI, New Delhi, pp 56-59.

Jackobsen I, Joiner EJ, Larsen J (1994) Hyphal phosphorus transport, a keystone to mycorrhizal enhancement of plant growth. In : Gianinazzi S, Schuepp H (eds).Impact of AM on Sustainable Agriculture and Natural Ecosystem.Birkhaus er Verlag, Switzerland, pp 133-146.

Johnson TR, Case L, Whipps JM (1957) Laboratory Experiments in Microbiology. 4th edn. Benjamin/Cummings Pub. Co. Inc.

King EO, Wood MK, Reney DE (1954) Two simple media for demonstration of pyocyanin and fluoresein. *J Laboratory Clinic and Med* 44: 301-307.

Schaad NW (1992) Laboratory guide for Identification of Plant Pathogenic Bacteria. International Book Distributing Co Lucknow, India, pp 60-88.

Singh AK, Ram H, Maurya BR (1998) Effect of nitrogen and phosphorus on microbial population. *Ind J Agric Chem* 31: 90-94.

Tien TM, Gaskins MH, Hubell DH (1979) Plant growth substances produced by *Azospirillum brasilense* and their effect on the growth of pearl millet (*Pennisetum americanum* L.). *Appl Environm Microbiol* 37: 1016-1024.

Troup RS (1921) The silviculture of Indian trees. Vol. III, Clarendra Press, Oxford, pp 1195.

Vander D (1963) The disappearance of litter in mull and more in connection with weather conditions and the activity of macro fauna. Soil organisms (eds Dooeksen and D. Vander.) North Holland Publication, Amsterdam, pp 125-133.