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Field performance of Himalayan cypress (*Cupressus torulosa*) seedlings inoculated with selected species of microbial inoculants under temperate conditions of Kashmir

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Abstract
A pot experiment was carried out to study the field performance of Himalayan Cypress seedlings inoculated with selected species of bio-inoculants under temperate field conditions. The experiment was laid in Completely Randomized Design with three replications which comprised of seven inoculants (*Azotobacter* sp., *Azospirillum* sp., *Pseudomonas fluorescens, Bacillus subtilis, Pisolithus tinctorius*, *Laccaria laccata* and control). Various growth characters viz., shoot height, collar diameter, root length, and seedling survival at various intervals responded significantly to all the microbial inoculants. Among microbial inoculants the two mycorrhizae viz., *Pisolithus tinctorius* and *Laccaria laccata* proved beneficial for all growth parameters than rest of the inoculants. It was followed by *Azotobacter* sp., *Azospirillum* sp., *Pseudomonas fluorescens* and *Bacillus subtilis*. However for root length *Pseudomonas fluorescens* and *Bacillus subtilis* gave best results than *Azotobacter* sp. and *Azospirillum* sp. Microbial inoculation of *Pisolithus tinctorius* and *Laccaria laccata* gave best results with respect to per cent decrease in seedling mortality rate of the species. Thus the two treatments viz; *Pisolithus tinctorius* and *Laccaria laccata* proved superior for all the studied growth parameters. Our findings show that the application of bio-inoculants improve the growth attributes of Himalayan Cypress seedlings under natural field conditions.

Keywords: Himalayan cypress, inoculation, seedlings, Kashmir

Introduction
The growing stock of commercial forests of Jammu and Kashmir is 132.9 million m^3 with average annual yield of 1.65 m^3 ha^{-1} (Anonymous, 2009) [2]. With this productivity annual yield of timber from commercial forest area alone must be 27.25 million cubic feet. Contrary to this fact, we presently import timber. Due to timber mining more than 60 per cent of our demarcated forests have been declared as uncommercial/degraded (Anonymous, 2005) [1]. Natural regeneration does not practically take place in forests where crown density is less than 40 per cent. Relying on natural succession, it will take us hundreds of years to regenerate the degraded forests to climax stage with species like *Pinus wallichiana* Jackson (kail), *Cedrus deodara* (Roxb.), G. Don (Deodar), *Abies pindrow* Spach (silver fir), *Picea smithiana* Wall. (spruce) and *Cupressus torulosa* Don (Himalayan cypress) which dominate vegetation of our forests. To meet the huge demand and supply of timber, fuelwood and firewood, raising of Himalayan cypress forests on degraded forest patches can be a good and viable option in future.

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. 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 90ºF, the absolute minimum about 15ºF and the normal rainfall varies from 1000 to 2400 mm per annum. The heartwood is light brown with dark streaks, moderately hard, suitable for making furniture and building materials. It is an excellent timber for making railway sleepers. The timber of cypress shapes smoothly; as compared to teak. Its working quality index is 116 (Pant *et al.*, 1989) [29]. In modern landscaping cypress is preferred over other conifers especially in temperate belts. Due to limited availability, its uses are not explored fully.

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. 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. Therefore, the present study was undertaken to evaluate the field performance of Himalayan cypress inoculated with selected bioinoculants under natural temperate conditions.

Materials and methods

The present investigations were undertaken at the Forest Nursery of Department of Forestry, Regional Research Station, Sher-e-Kashmir University of Agricultural Sciences and Technology, Wadura, Kashmir-India during 2009-2010. A field survey of two districts of Kashmir valley viz. Kupwara and Bandipora was carried out during 2008-09 for the collection of rhizosphere soil samples of Himalayan cypress stands. The collected rhizosphere soil samples 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) [18] 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±2ºC for 36 hours. 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, colour and shape of the colonies, colour, shape and size of hyphae, basidiospores, cap, mycelia spines, gleba, conidiosphores and conidia (Arx von, 1981) [3].

The bacterial inoculants were isolated from the rhizosphere soil samples of 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) [20] was poured in plates which were rotated gently and incubated at 28±2ºC 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 4ºC. Characterisation of the isolated bacteria was done according to the methods recommended in the laboratory guide for the identification of microbial inoculants (Schaad, 1992) [31]. 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 characterisation of microbial inoculants viz. *Azotobacter* sp., *Azospirillum* sp., *Pseudomonas fluorescens* and *Bacillus subtilis*, respectively (Gopalakrishnan and Meena, 2004) [11]. The identification of isolated inoculants was also got confirmed from Division of Mycology and Plant Pathology, IARI, New Delhi.

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 Himalayan cypress of uniform heights and collar diameter growing in polyethylene bags (9″ x 7″) containing 1 kg potting material of soil and sand mixture in the ratio of 1:1 were selected.

Microbial inoculation

For inoculation, the different broth cultures of N-fixers, P-solubilizers and ectomycorrhizal inoculants isolated from local forest stands were applied to the potting material (25 ml/seedling) in the month of March, 2009, without disturbing the root system of the seedlings.

Nursery operations

The seedlings were irrigated with rose-cans as and when needed and maintained virtually weed free by manual weeding.

Plant growth measurement

All the growth parameters viz., plant height (cm), collar diameter (mm), root length (cm) and seedling survival (%), were measured at an interval of 2 months upto 12 months. All the growth parameters of the seedlings at the initial stage of the experiment were recorded.

Statistical analysis

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

Results and Discussion

Plant height

Perusal of the data presented in Table-1, Fig 1 shows that the application of various microbial inoculants significantly enhanced the mean plant height of the Himalayan cypress seedlings as compared to control. Amongst various microbial inoculants, *Pisolithus tinctorius* resulted in maximum increase in plant height over control (38.27 %). It was followed by *Laccaria laccata* (35.66%), *Azotobacter* sp. (28.29%), *Azospirillum* sp. (25.91%), *Pseudomonas fluorescens* (21.76%) and *Bacillus subtilis* (19.36%), respectively. Treatment of seedlings with ectomycorrhizal fungi viz., *Pisolithus tinctorius* was significantly superior over all other treatments. Plant height revealed a significant increase from April to October and from October on wards till February there was a slight increase. Moreover, the interactions between inocula and month's were significant till October and thereafter it was non-significant. The increase in shoot height by *P. tinctorius* and *L. laccata* could be attributed to the

production of growth promoting substances like auxins (Dehn, 1982) [8] and enhancement of water absorption and nutrient mobilization (Dar *et al*., 1997) by vastly increased surface area network of the fungal mycelia (Myer, 1992) [24]. In case of *Azotobacter* and *Azospirillum* sp. Inoculation, the increase in shoot height could be ascribed to nitrogen fixing ability, synthesis of growth promoting substances like cytokinens, gibberellins, auxins (Reynders and Vlassak, 1979; Hartmann *et al*., 1983; Jain and Patriquin, 1985) [30, 13, 16] and production of antifungal antibiotics (Chahal and Chahal, 1988) [6]. However, increase in shoot height by inoculation with *Pseudomonas fluorescens* and *Bacillus subtilis* could be through iron chelating siderophores (Schippers, 1988) [32] by releasing phytohormones, solubilizing P and reduction in population of deleterious microorganisms (Weller, 1988) [37]. Further our findings are in close conformity with the results of Oh and Park (1989) [27], Jeffries and Dodd (1991) [17], Natarajan *et al*. (1995) [25] who reported that *P. tinctorius* and *L. laccata* inoculation resulted in enhancement of plant height of *Acaccia nilotica, Quercus serrata, Eucalyptus camaldulensis* and *E. deglupta* seedlings respectively. similarly, the enhancement in plant height with respect to *Azotobacter* and *Azospirillum* sp. has also been reported in *Quercus serrata* (Pandey *et al*., 1986) [28] in peach (Awasthi *et al*., 1996) [4]. Moreover, the inoculation of clover plants with *Pseudomonas putida* has also been reported to enhance the plant height (Meyer and Linderman, 1986) [23]. Further, the maximum increase in shoot height of Himalayan cypress seedlings lies in the fact that Himalayan cypress being a fast growing species, has got an efficient root system. Moreover, the gradual decline in plant height of the specie in the laterhalf of study period could be due to below freezing soil temperatures and short growing season of conifers.

Collar diameter

Perusal of the data presented in Table-2, Fig 2 indicates that the application of various microbial inoculants significantly enhanced the collar diameter of Himalayan cypress seedlings as compared to control. Amongst various microbial inoculants, *Pisolithus tinctorius* resulted in maximum increase (31.82%) in collar diameter. It was followed by *Laccaria laccata* (28.74%), *Azotobacter* (24.56%), *Azospirillum* (21.84%), *Pseudomonas fluorescens* (16.78%) and *Baccillus subtilis* (14.74%), respectively. Application of *Pisolithus tinctorius* was significantly superior over rest of the treatments. Moreover, collar diameter registered an increasing trend from April to October and from October onwards there was a slight increase, however, the interactions between inocula and months were significant till October and from October onwards it was non-significant. Enhancement in collar diameter could be due to the release of plant growth substances and increase in the nutrient availability in the root zone (Jackobsen *et al*., 1994) [14]. Further, the gradual decline in collar diameter of seedlings in the winter months may be due to low fluctuating soil temperatures which might have stopped the growth of microbial inoculants. Similar observations have been recorded by other workers in various plants (Lee and Koo, 1985; Kumar and Lakhanpal, 1990; Tam and Griffiths, 1994) [21, 19, 39].

Root length

Perusal of the data presented in Table-3, Fig 3, depict a significant increase in root length of Himalayan cypress seedlings due to application of various microbial inoculants. Application of *P. tinctorius* resulted in a significant increase in root length which was 25.11 per cent more than control and proved best over all other inoculants. It was followed by *L. laccata* (23.82%), *Pseudomonas fluorescens* (21.86%), *Bacillus subtilis* (21.00%), *Azotobacter* (17.37%) and *Azospirillum* (18.06%), respectively. Further, the data revealed that the two treatments viz., *Pseudomonas* and *Bacillus* were at par with each other. Plant root length registered a significant increase between April and October and from October onwards there was a slight increase. Interaction effects between inocula and months were also significant between April and October and from October onwards there was a non-significant effect. The increase in root length due to microbial inoculants could be attributed to their capability to synthesize biologically active substances (Jackson, 1964) [15] and increased uptake of essential macronutrients (Bowen, 1973) [5]. Further the increase in root length with these microbial inoculants has also been reported by several workers (Tien *et al*., 1979; Navratil and Rochon, 1981; Ljungquist and Stenstrom, 1983; Pandey *et al*., 1986; Gaudin *et al*., 1994) [36. 26, 28, 19].

Seedling survival

The data contained in Table-4, f Fig 4 on impact of microbial inoculation on seedling survival percentage of Himalayan cypress at nursery stage clearly indicate that the survival percentage of the seedlings got significantly improved by the application of various microbial inoculants. Amongst various microbial inoculants, the application of *Pisolithus tinctorius* resulted in maximum seedling survival percentage, which was 17.40 per cent more than control and thus proved to be the best over rest of the treatments. Similarly *Laccaria laccata* inoculation resulted in 15. 95 per cent more survival percentage as compared to control. It was followed by *Pseudomonas fluorescens* (12.97%), *Bacillus subtilis* (11.19%), *Azotobacter* (9.10%) and *Azospirillum* (7.90%), respectively. Seedling survival percentage revealed a declining trend in the last months of December and February. Enhancement in seedling survival could be attributed to the ability of microbial inoculants to secrete antifungal antibiotics, uptake of nutrients by converting them into available forms and greater access to water (Stribley, 1987) and production of growth promoting substances (Jackobsen *et al*., 1994) [14]. The decrease in survival percentage in winter months could be attributed to low and below freezing soil temperatures which might have stopped the growth of inoculants and other soil microflora present there.

Table 1: Impact of microbial inoculation on plant height (cm) of Himalayan cypress (*Cupressus torulosa* Don) at nursery stage

Treatment	2009					2010	Mean
	April	June	August	October	December	February	
Control	18.33	21.40	24.26	26.46	26.48	26.48	23.90
Azotobacter sp.	22.66	27.23	32.30	39.26	39.28	39.28	33.33
Azospirillum sp.	21.75	26.17	31.10	38.18	38.19	38.19	32.26
Pseudomonas fluorescens	20.58	25.10	29.20	36.13	36.15	36.15	30.55

Bacillus subtilis	19.58	24.23	28.43	35.20	35.21	35.21	29.64
Pisolithus tinctorius	24.40	31.06	38.13	46.23	46.25	46.25	38.72
Laccaria laccata	23.15	30.06	36.96	44.23	44.25	44.27	37.15
Mean	21.49	26.46	31.48	37.95	37.97	37.97	

	Treatment (T)	**Month (M)**	**T x M**
CD ($p \leq 0.05$)	0.067	0.062	0.016
SEm	0.024	0.022	0.058

Initial plant height = 17.00 cm

Table 1: Impact of microbial inoculation on collar diameter (mm) of Himalayan cypress (*Cupressus torulosa* Don) at nursery stage

Treatment	**2009**					**2010**	**Mean**
	April	**June**	**August**	**October**	**December**	**February**	
Control	3.10	3.23	3.42	3.68	3.70	3.70	3.47
Azotobacter sp.	3.40	3.91	4.53	5.24	5.26	5.26	4.60
Azospirillum sp.	3.35	3.80	4.37	5.05	5.06	5.06	4.44
Pseudomonas fluorescens	3.27	3.63	4.10	4.66	4.68	4.68	4.17
Bacillus subtilis	3.23	3.57	4.02	4.54	4.55	4.55	4.07
Pisolithus tinctorius	3.54	4.21	5.02	5.92	5.94	5.94	5.09
Laccaria laccata	3.49	4.09	4.80	5.61	5.62	5.62	4.87
Mean	3.34	3.77	4.32	4.95	4.97	4.97	

	Treatment (T)	**Month (M)**	**T x M**
CD ($p \leq 0.05$)	0.010	0.009	0.026
SEm	0.037	0.035	0.091

Initial collar diameter = 2.81 mm

Table 2: Impact of microbial inoculation on root length (cm) of Himalayan cypress (*Cupressus torulosa* Don) at nursery stage

Treatment	**2009**					**2010**	**Mean**
	April	**June**	**August**	**October**	**December**	**February**	
Control	19.42	22.21	26.34	30.61	30.62	30.62	26.63
Azotobacter sp.	23.62	27.45	33.48	36.28	36.29	36.29	32.23
Azospirillum sp.	23.15	27.12	33.21	37.18	37.19	37.19	32.50
Pseudomonas fluorescens	24.52	28.37	34.22	39.12	39.13	39.13	34.08
Bacillus subtilis	24.10	28.14	34.08	38.65	38.66	38.66	33.71
Pisolithus tinctorius	25.70	29.81	35.62	40.75	40.76	40.76	35.56
Laccaria laccata	25.10	29.20	35.14	40.10	40.11	40.11	34.96
Mean	23.65	27.48	33.15	37.52	37.53	37.53	

	Treatment (T)	**Month (M)**	**T x M**
CD ($p \leq 0.05$)	0.237	0.219	0.581
SEm	0.084	0.078	0.206

Initial root length = 15 cm

Table 4: Impact of microbial inoculation on seedling survival (%) of Himalayan cypress (*Cupressus torulosa* Don) at nursery stage

Treatment	**2009**					**2010**	**Mean**
	April	**June**	**August**	**October**	**December**	**February**	
Control	82.32	82.32	82.32	80.21	78.25	74.64	80.01
Azotobacter sp.	89.12	89.12	89.12	88.10	87.52	85.13	88.02
Azospirillum sp.	88.13	88.13	88.13	87.11	86.44	83.01	86.88
Pseudomonas fluorescens	93.10	93.10	93.10	92.12	91.13	89.16	91.94
Bacillus subtilis	91.31	91.31	91.31	90.29	89.21	87.51	90.15
Pisolithus tinctorius	98.20	98.20	98.20	97.19	96.31	93.13	96.87
Laccaria laccata	96.70	96.70	96.70	95.67	94.27	91.67	95.20
Mean	91.26	91.26	91.26	90.00	89.01	86.32	

	Treatment (T)	**Month (M)**	**T x M**
CD ($p \leq 0.05$)	0.059	0.055	0.146
SEm	0.021	0.019	0.051

Initial seedling survival = 100 %

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