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Growth response and nutrient uptake of blue pine (*Pinus wallichiana*) seedlings inoculated with rhizosphere microorganisms under temperate nursery conditions

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Abstract. Microbial inoculants (*Trichoderma harzianum, Pseudomonas fluorescens, Laccaria laccata*) inoculated either individually or in combination significantly improved the growth and biomass of blue pine seedlings. The ECM fungus *Laccaria laccata,* when inoculated individually, showed significantly higher plant growth, followed by *Pseudomonas fluorescens* and *Trichoderma harzianum.* The combined inoculation of rhizosphere microorganisms showed synergistic growth promoting action and proved superior in enhancing the growth of blue pine than individual inoculation. Co-inoculation of *L. laccata* with *P. fluorescens* resulted in higher ectomycorrhizal root colonization. Uptake of nutrients (N, P, K) was significantly improved by microbial inoculants, tested individually or in combination. Combined inoculation of *L. laccata* with *T. harzianum* and *P. fluorescens* significantly increased in N, P and K contents in blue pine seedlings as compared to control. Acid phosphatase activity in the rhizosphere of blue pine seedlings was also enhanced by these microorganisms. L. laccata exhibited higher acid phosphatase activity followed by *P. fluorescens.*
Keywords *Pinus wallichiana*, rhizosphere microorganisms, nutrient uptake, growth.

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Introduction

Blue pine (*Pinus wallichiana* A. B. Jackson), is propagated mainly through nursery-raised seedlings, which like most of the forest trees face severe problems in successful regenera-

tion under existing natural conditions in soils having low nutrient content. Pines are mycotrophic in nature. Symbionts such as mycorrhizal fungi and free living organisms form integral components of pine rhizosphere, an area showing all kinds of antagonistic, parasitic and growth promoting interactions (Zhang et al. 1991). The ectomycorrhizal symbiosis alters the physicochemical and biological conditions in the surrounding soil and creates a highly specialized environment called 'ectomycorrhizosphere', wherein selected microbial communities in association with mycorrhiza play vital role in higher biochemical activities and nutrient cycling (Christophe et al. 2007, Meyer et al. 2010). Microbial activity in the plant rhizosphere has substantial effect on plant productivity (Schroth & Weinhold 1986).The free-living rhizosphere microorganisms such as *Trichoderma, Gliocladium, Penicillium, Pseudomonas*, *Bacillus, Azotobacter, Azospirillum* etc. favourably influence the plant growth directly or indirectly. The direct promotion of plant growth by these organisms occurs either through release of plant growth promoting compounds or by facilitating the uptake of certain nutrients from the environment. Further, they indirectly promote plant growth by reducing or preventing the deleterious effect of one or more phytopathogenic organisms (Glick 1995, Hayat et al. 2010). Many plant growth promoting rhizobacteria are free living diazotrophs and can convert molecular nitrogen into ammonia by virtue of nitrogenase enzyme complex (Postgate 1982). Plant growth promoting rhizobacteria (PGPR) stimulate plant growth by facilitating the mineral uptake particularly phosphates in plants (Kloepper et al. 1989, Rashmi Awasthi et al. 2011). *Trichoderma* species are opportunistic avirulent plant symbionts as well as mycoparasites. Root colonization by *Trichoderma* species also frequently enhances root growth and development, improves crop productivity, induces resistance to abiotic stress and increases uptake and use of nutrients (Harman et al. 2004).

Mycorrhizae improve plant growth through increased availability of phosphorus from non-labile sources (Jayachandran et al. 1989, Dar et al. 2007). The ectomycorrhizal fungal hyphae by growing away from the roots may detect zones of higher phosphorus availability and hasten root proliferation in these zones (Harley & Smith 1983, Christophe et al. 2006). The other factors which contribute to enhance uptake of phosphorus by mycorrhizal plants include: (i) the possibility of fungi having a lower threshold concentration for uptake of phosphorus, (ii) the production of organic acids and enzymes which increase the availability of phosphorus and (iii) the ability of fungi to store and efficiently translocate phosphorus into the host (Grove & LeTacon 1993). A major portion of phosphorus in forest soils is in inorganic form. Ectomycorrhizal fungi catalyze hydrolysis of inorganic phosphorus and thereby enhance phosphate uptake by host roots (Gerlitz & Werk 1994). It has been suggested that organic acids secreted by ectomycorrhizal fungi may release phosphorus from organic and sparingly soluble inorganic forms either by lowering the soil pH or by chelation of metal ions (Malajczuk & Cromack 1982, Gray & Dighton 2009). Ectomycorrhizal symbiosis also facilitates the access of plant partner to soil nitrogen. This is of paramount importance in ecosystems where primary production appears to be restricted by nitrogen availability (Linder 1989). In addition to provide access to organic nitrogen resources, ectomycorrhizae through improved root systems help to counteract the effects of nitrogen depletion zones around roots (Finlay et al. 1988). The dominant form of nitrogen in forest soils is NH^+_4-N and ectomycorrhiaze through their mycelial network increase the uptake of NH^+_4-N (Reid et al. 1983). Inorganic nitrogen absorbed into hyphae is assimilated and translocated as amides and amino acids in the fungus utilizing metabolic pathways (Martin & Botton 1993).

The present investigation was under taken

to assess the impact of microbial inoculants on nutrient uptake and growth of blue pine in nursery under temperate conditions of Kashmir.

Materials and methods

Ectomycorrhizal fungus (*Laccaria laccata* Broome & Berkely) was isolated from sporocarps collected from the canopy of blue pine plantations in Lidder Forest Division, Kashmir on Modified Melin-Norkran's medium (Marx 1969). *Trichoderma harzianum* Rifai. and *Pseudomonas fluorescens* Migulla were isolated from the soil collected from the rhizosphere of blue pine seedlings. The fungal antagonist was isolated by dilution plate method on potato dextrose agar medium and the culture was purified by single spore/hyphal tip method and maintained for further studies. Rhizobacteria were isolated by dilution plate method on King`s B medium (King et al. 1954) and maintained in culture tubes at 4°C. Mycelial inoculum of ectomycorrhizal fungus was prepared in a vermiculite-based carrier according to the method of Marx & Bryan (1975). Ectomycorrhizal fungus was grown aseptically in 1 litre Erlenmeyer flasks containing 750 ml of vermiculite moistened with 375 ml of MMN liquid medium. The flasks were incubated at 26 ± 2°C in the dark for 10 weeks. After incubation, inoculum was removed from flasks just before inoculation and leached with distilled water to remove unused nutrients.

Pseudomonas fluorescens was multiplied in talc formulation with prior growth on King's 'B' broth medium using the procedure given by Vidhyasekaran & Muthamilan (1995). The product with final bacterial population of 3 x 10^8 cfu/g was shade-dried to reduce the moisture content to 20 per cent, packed in polypropylene bags, sealed and stored at 4° C for further study. A talc based formulation of *Trichoderma harzianum* was prepared as given by Rudresh et al. (2005) with inoculum load of 1 x 10^9 c fu/g.

Potting mixture was sterilized at 1.4 kg/cm^2 for one hour for three successive days. One kg of sterilized potting mixture [organic carbon (%) = 0.7, pH = 6.8, available N = 281.2 kg/h, available P = 15.8 kg/ha, available K = 243.0 kg/ha] was put in each plastic bag of 1.5 kg capacity. The growth promoting microorganisms were inoculated separately and in combination in desired quantities into the upper 8-10 cm of potting mixture and mixed properly. Ectomycorrhizal fungal inoculum prepared in a vermiculite based carrier was added 15 days before sowing at the rate of 15 ml/kg. Prepared talc based formulation of *Trichoderma harzianum* with inoculum load of 1 x 10^9 cfu/g, was added at 5 g/kg of potting mixture, while as *Pseudomonas fluorescens* multiplied on talc formulation with inoculum load of 2.5 x 10^8 cfu/g, was added at 5 g/kg of potting mixture five days before seed sowing.

Healthy seeds of blue pine were surface sterilized in 30% hydrogen peroxide for 30 minutes, washed thoroughly with sterile distilled water and stratified for 48 hours at 4^0 C in dark. Five seeds were sown per bag and after germination seedlings were thinned out to one per bag. The inoculated and uninoculated seedlings were arranged in a completely randomised design, with each treatment replicated 12 times. The seedlings were grown under greenhouse conditions at 25 ± 3°C and irrigated with sterile distilled water as and when required. No fertilizers or protective chemicals were applied throughout the study. The seedlings were out planted gently and observations on plant biomass, seedling height, root length, ectomycorrhizal root colonization and nutrient uptakes were recorded at 30, 60, 90 and 120 days after seedling emergence. Dry weight of seedlings was determined by drying the plants at 60° C for 48 hours in hot air oven.

For the estimation of per cent ectomycorrhizal infection in roots, standard method of Daughtridge et al. (1986) was followed. Seedlings were gently uprooted and their roots

washed under running tap water to remove adhering soil debris. The roots were later stained for 15 minutes in 10 per cent solution of glacial acetic acid (v/v) which contained 0.1 per cent Ponceau S (w/v). Upon removal, the roots were immediately rinsed in 10 per cent acetic acid to remove excessive stain and then washed with distilled water. To evaluate the accuracy of Ponceau S staining technique, the roots were stained for 5 minutes with trypan blue in lactophenol. The root systems were examined under a stereomicroscope to count the number of mycorrhizal short roots as suggested by Beckjord et al. (1984).

$$\text{Degree of mycorrhization}\,(\%) = \frac{\text{No. of ectomycorrhizal lateral rootlets observed}}{\text{Total number of lateral rootlets examined}} \cdot 100$$

Nutrient content (NPK) of pine seedlings collected at different growth stages was estimated as per standard procedures of Jackson (1973). The nutrient uptake of plant was worked out on dry weight basis by using following formula:

$$\text{Nutrient uptake (g/plant)} = \frac{\text{Nutrient content (\%) x dry matter (g)}}{100}$$

The acid phosphatase activity of rhizosphere soil was measured by the method of Tabatabai & Bremner (1969). One g of soil sample, drawn from the rhizosphere of plant in each treatment, was put in 50 ml volumetric flasks. Then 0.2 ml toluene, 4 ml modified universal buffer (prepared by dissolving 2.42 g tris, 2.32 g malic acid, 2.8 g citric acid and 1.26 g boric acid in 100 ml IN NaOH and volume made upto 1 litre with distilled water) and 1 ml of 0.025 M P-nitrophenyl phosphate was added. The flasks were incubated at 37 ± 1° C for 1 hour. Enzymatic reaction was stopped by adding 4 ml of 0.5 M NaOH and 1 ml 0.5 M $CaCl_2$. The contents were filtered through Whatman`s filter paper No. 42, and the absorbance of yellow colour (p-nitrophenol released) estimated by spectrophotometer at 460 nm wave length. The amount of p-nitrophenol released was calculated with reference to a standard curve prepared by using different concentrations of p-nitrophenol. The enzyme activity was expressed as µM p-nitrophenol (PNP) released g^{-1} soil hr^{-1}.

Results

The study on the impact of microbial inoculants on growth and nutrient uptake of blue pine seedlings revealed that microbial inoculants (*Trichoderma harzianum, Pseudomonas fluorescens, Laccaria laccata*) inoculated either individually or in combination significantly improved plant growth (seedling height and root length) and biomass (fresh and dry plant weight) as compared to uninoculated control. Among the microbial inoculants tested individually, the ectomycorrhizal fungus *L. laccata* showed significantly higher plant growth followed by *P. fluorescens* and *T. harzianum.* The blue pine seedlings inoculated with *L.laccata* showed 24.6, 50.6, 45.4 and 30.11 per cent increase in shoot height, root length, fresh and dry plant weight, respectively, as compared to uninoculated control (Table 1). The highest increase in growth and biomass of blue pine seedlings was observed due to combined inoculation of all the three microbial inoculants (*L. laccata* + *T. harzianum* + *P. fluorescens*). The seedling height, root length, fresh weight and dry weight of blue pine seedlings were increased by 61.0, 95.8, 105.8 and 61.33 per

Table 1 Influence of microbial inoculants on growth and biomass of blue pine seedlings

Treatments	Seedling height (cm)	Root length (cm)	Plant fresh weight (mg/plant)	Plant dry weight (mg/plant)
Laccaria laccata (LL)	9.60	11.0	791.00	350.00
Trichoderma harzianum (TH)	8.30	9.2	690.00	322.00
Pseudomonas fluorescens (PF)	8.80	10.2	740.00	330.00
LL + TH	9.80	11.5	885.00	361.00
LL + PF	11.00	12.6	954.00	383.00
TH + PF	9.00	10.4	854.00	358.00
LL + TH + PF	12.40	14.3	1120.00	434.00
Control	7.70	7.3	544.00	269.00
Mean	9.50	10.8	822.20	250.80
CD ($p = 0.05$)	0.48	0.4	8.02	6.04

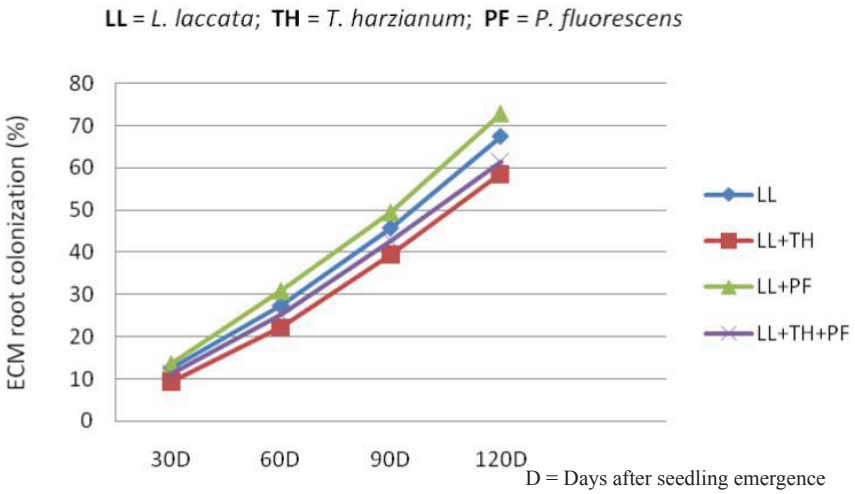

Figure 1 Mycorrhizal root colonization of blue pine seedlings by *L. laccata*, also combined with *P. fluorescens* and *T. harzianum*

cent, respectively as compared to uninoculated control.

Roots of pine seedlings were colonized by the ectomycorrhizal fungus *L. laccata*, inoculated as vegetative inoculum. Generally co-inoculation of *L. laccata* with *P. fluorescens* increased the total number of mycorrhizal short roots as compared to individual inoculation (Fig. 1).

The higher uptake of macronutrients (N, P, K) was recorded in blue pine seedlings inoculated with microbial inoculants (*L. laccata*, *P. fluorescens* and *T. harzianum*) either individually or in combination as compared to uninoculated control. The combined inoculation proved superior over individual inoculants. *L. laccata* proved most efficient in improving the uptake of nutrients followed by *P. fluorescens* and *T. harzianum*. *L. laccata* and caused 99.2, 241.9 and 145.2 per cent increase in nitrogen, phosphorus and potassium uptake respectively, as compared to uninoculated control (Table 2). The combined inoculation of *L. laccata* + *T. harzianum* + *P. fluorescens* resulted in significantly higher uptake of N, P and K by blue pine seedlings as compared to individual

Table 2 Influence of microbial inoculants on nutrient uptake (NPK) of blue pine seedlings

Treatments	Nitrogen uptake (mg/plant)	Phosphorus uptake (mg/plant)	Potassium uptake (mg/plant)
Laccaria laccata (LL)	6.475	0.465	3.759
Trichoderma harzianum (TH)	4.733	0.332	2.737
Pseudomonas fluorescens (PF)	5.214	0.363	3.003
LL + TH	6.931	0.504	3.971
LL + PF	7.660	0.612	4.289
TH + PF	6.479	0.455	3.710
LL + TH + PF	9.461	0.824	5.685
Control	3.250	0.136	1.533
Mean	6.200	0.461	3.585
CD ($p = 0.05$)	0.557	0.026	0.477

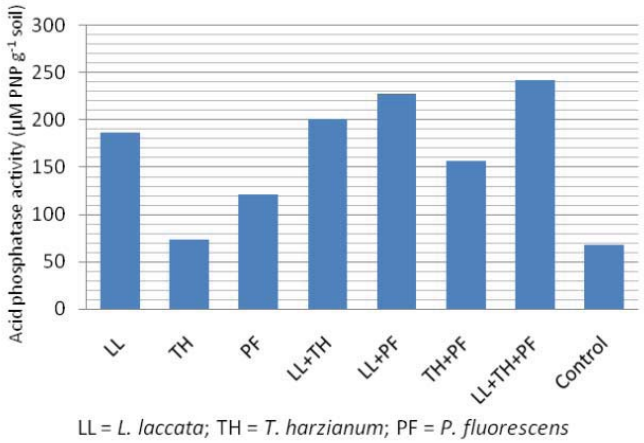

Figure 2 Impact of microbial inoculants on rhizosphere acid phosphatase activity of blue pine seedlings

inoculation and control.

Acid phosphatase is solely of extra cellular enzymatic origin and is involved in the mineralization of organic phosphates. Among the rhizosphere microorganisms inoculated individually, *L. laccata* exhibited higher acid phosphatase activity followed by *P. flourescens* and *T. harzianum* (Fig. 2). The combined inoculation of *L. laccata* + *T. harzianum* + *P. fluorescens* caused highest increase in acid phosphatase activity in the rhizosphere of blue pine seedlings as compared to uninoculated control. This was followed by the coinoculation of *L. laccata* + *P. fluorescens*.

Discussion

The impact of ectomycorrhizal fungi or rhizosphere bacteria on seedling growth and nutrient uptake is well known. However, few studies have combined these microorganisms in one experiment to clarify their relative contribution and interactions in nutrient acquisition. In the present study ectomycorrhizal fungus *L. laccata* , mycorrhizosphere bacterium *P. fluorescens* and mycorrhizosphere fungus *T. harzianum* were monitored for the respective contributions, on nutrient uptake and plant growth of pine seedlings. *L. laccata, P. fluorescens* and *T. harzianum* are considered to be opportunistic avirulent plant symbionts, benefiting

their host by frequently enhanced root growth, resistance to abiotic stress and the uptake and use of nutrients. These organisms have the ability to increase plant growth and productivity by solubilization of otherwise unavailable mineral nutrients.

ECM fungus *L. laccata* showed significantly higher plant growth as compared to uninoculated control. The seedlings with considerable ectomycorrhizal colonization rapidly regenerate new lateral roots, create more new sites for ectomycorrhizae and thereby utilize available nutrients more efficiently than non-mycorrhizal seedlings (Marx & Hatchell 1986). Positive effects on seedling growth and survival in the field environment after nursery inovulation with different mycorrhizal fungi have reported by various researchers (Diaz et al. 2009, Alves et al. 2010, Dalong et al. 2011). The favourable influence of ectomycorrhizal fungus on plant growth and health may be attributed to the excretion of growth promoting substances by mycorrhizae (Duchesne et al. 1987, Strzelezyk et al. 1985) or indirectly, by alteration in root physiology, uptake of minerals and pattern of exudation into the mycorrhizosphere (Leyval & Berthelin 1990). Sudhakara & Natarajan (1997) reported that 38.0 and 186.5 per cent increase in *Pinus patula* seedling height and shoot dry weight, respectively due to *L. laccata* after 8 months of inoculation.

Application of plant growth promoting rhizobacterium *Pseudomonas fluorescens* significantly improved seedling height and root length as well as fresh and dry biomass as compared to uninoculated control. The improvement in plant growth and biomass seems to be a consequence of intensive root colonization by bacteria which may have promoted the synthesis of growth promoting compounds as well as facilitated more nutrient supply in rhizosphere. The positive effects of plant growth promoting bacteria on plant growth are always correlated with remarkable changes in root morphology (Pacovsky 1990, Okon & Vanderleyden 1997, Bertrand et al. 2000). It is generally assumed that these developmental responses are triggered by phytohormones produced by bacteria (Bloemberg & Lugtenberg 2001, Persello-Cartieaux et al. 2003). Significant increase in plant height, basal area and crown breadth was observed in *Bacillus* and *Pseudomonas* inoculated *Pinus massoniana* plants ten months after their inoculation (Yang et al. 2002). *Pseudomonas fulva* significantly stimulated the growth of *Pinus sylvestris* seedlings in a pasteurised soil and increased the number of mycorrhizal roots in seedlings inoculated with *Suillus luteus* (Pokojeska et al. 2004).

In present study *T. harzianum* showed stimulatory effect on growth and biomass of pine seedlings. *Trichoderma* species reportedly produce hormone like metabolites and release nutrients from soil or organic matter thereby facilitate better plant growth (Windham 1986). *Pinus sylvestris* seedlings inoculated with *T. virens* produced a significantly higher biomass of needles, trunks and root than uninoculated plants (Werner 2002).

In combined inoculation of all the three microbial inoculants (*L. laccata* + *T. harzianum* + *P. fluorescens*) steep increase in plant growth may be ascribed to the synergistic growth promoting action of microbial inoculants as well as more solubilization of mineral nutrients. *P. fluorescens* seems to be a good candidate for optimizing the efficiency of ectomycorrhizal mycelium inoculum. Garbay & Duponnois (1992) reported that *P. fluorescens* promoted mycorrhizal formation of *L. laccata* with Douglas-fir and oak seedlings, thereby increased the growth of seedlings. Co-inoculation of ectomycorrhizal fungus, *S. citrinum* with the *Collimonas* sp. bacterial strain significantly improved the *Pinus sylvestris* biomass as compared to non-inoculated pine plants (Koele et al., 2009). Werner (2002) observed that mycorrhizal *Pinus sylvestris* seedlings inoculated with *T. virens* produced significantly higher plant growth and biomass.

Roots of pine seedlings were colonized by

the ectomycorrhizal fungus *L. laccata*, inoculated as vegetative inoculum. Generally co-inoculation of *L. laccata* with *P. fluorescens* increased the total number of mycorrhizal short roots compared with individual inoculation (Fig. 1). The degree of colonization of *L. laccata* varied among the treatments with highest root colonization observed due to co-inoculation of *L. laccata* with *P. fluorescens*. It seems that *P. fluorescens* might have stimulated the mycorrhizal development of *L. laccata* with the roots of pine seedlings. Our results are supported by the observations of Garbaye *et al.* (1992) where as *P. fluorescens* isolate BB_C6 promoted mycorrhiza formation with *L. laccata* with four conifer species. The mycorrhizal helper bacteria seem to have acted in early stage to improve the receptivity of the root to the fungus before the first mycorrhizas were formed.

L. laccata proved most efficient in improving the uptake of nutrients (N, P, K) followed by *P. fluorescens* and *T. harzianum*. Increased nutrient uptake in *L. laccata* inoculation seedlings could be attributed to the solubilization of unavailable nutrients and presence of more root absorbing surfaces. Ectomycorrhizal fungi catalyse hydrolysis of inorganic phosphorus and thereby enhance phosphorus uptake by roots (Gerlitz & Work 1994). Organic acids secreted by ectomycorrhizal fungi may release phosphorus from organic and inorganic forms either by lowering the soil pH or by chelation of metal ions (Melajeuk & Cromack 1982).

Ectomycorrhizal symbiosis facilitated the access of plant partner to soil nitrogen. Ectomycorrhizae through improved root systems help to counteract the effects of nitrogen depletion zones around roots (Finlay et al. 1988) and through their mycelial network increase the uptake of NH^+_4-N (Reid et al. 1983). The ectomycorrhizal fungal hyphae growing away from the root may detect zones of higher phosphorus availability and hasten the proliferation of roots in these zones (Harley & Smith 1983). It has been suggested that organic acids secreted by ectomycorrhizal fungi may release phosphorus from organic and sparingly soluble inorganic forms either by lowering the soil pH or chelation of metal ions (Malajczuk & Cromack 1982). Improved uptake of nutrients in *P. fluorescens* plants might have attributed due to their mineralization activity in the rhizosphere. Plant growth promoting rhizobacteria are free living diazotrophs that can convert molecular nitrogen into ammonia in a free state by virtue of nitrogenase enzyme complex (Postgate 1982). Brimecombe et al. (1998) reported increased mineralization of organic residues by the two strains of *Pseudomonas fluorescens*. Several reports have suggested that PGPR stimulate plant growth by facilitating the mineral uptake particularly phosphates in plants (Kloepper et al. 1989). *Trichoderma* spp. also frequently enhances root growth and development, and increase uptake and use of nutrients (Harman et al. 2004). *T. harzianum* (T-22) was found to solubilize phosphate and other micronutrients that could be made available to provide plant growth (Altomare *et al.*, 1999). Combined inoculation of *L. laccata* + *T. harzianum* + *P. fluorescens* resulted significantly higher uptake of N, P and K by blue pine seedlings as compared to individual inoculation and control. The inoculation of two or more beneficial microbes have synergistic effect on host plants. The significant increase in nutrient uptake is due to more nutrient availability in the soil, which ultimately enhanced the plant growth and biomass. Present findings are supported by the work of Singh & Singh (1998) & Singh et al. (2001). Deepa et al. (2003) reported that *L. laccata. T. harzianum* and *Pseudomonas corrugata* influenced the soil microflora, nutrient status of rhizosphere soil and that of different parts of *Cedrus deodara* seedlings with increased uptake of N, P and K.

Among the inoculants, *L. laccata* exhibited higher acid phosphatase activity followed by *P. flourescens* (Fig. 2). The phosphatases produced by ectomycorrhizae, solubilize insolu-

ble forms of phosphorus and other nutrients not readily available to uninfected plant roots (Louche et al. 2010, Bartlett & Lewis 1973). The expression of phosphorus cleaving enzymes (mainly phosphomonoesterases and phosphodiesterases) by mycorrhiza and ECM fungi represents an important mechanism to increase phosphorus availability for plant symbionts (Van Aarle et al. 2001).

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