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Management of Root Rot Caused by *Rhizoctonia solani* and *Fusarium oxysporum* in Blue Pine (*Pinus wallichiana*) Through use of Fungal Antagonists

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ABSTRACT

The present study was aimed to identify root rot pathogens of blue pine (*Pinus wallichiana*) in Kashmir and develop appropriate eco-friendly disease management strategy. During nursery surveys, *Fusarium oxysporum*, *Rhizoctonia solani* and *Macrophomina phaseolina* were found root rot incitants with isolation frequency of 47.3, 29.7 and 13.0%, respectively. Locally isolated antagonists inflicted 33.0-73.3 and 29.5-70.8% mycelial growth inhibition in *F. oxysporum* and *R. solani*, respectively, with *Trichoderma harzianum* and *T. viride* proving most effective. The mycorrhizal fungi, *Pisolithus tinctorius* and *Laccaria laccata,* significantly inhibited the growth of *R. solani* and *F. oxysporum* by 46.2 and 45.4 and 44.7 and 43.7%, respectively. Bioagents significantly improved seedling biomass and root/shoot length. Mycorrhizal plants showed 5-13 fold higher rhizosphere phosphatase activity than non-mycorrhizal ones. Four effective fungal bioagents, inoculated individually and in combination with pathogen under nursery conditions, significantly improved seedling biomass and height with maximum gain by *P. tinctorius* and *L. laccata*. *Rhizoctonia* infection decreased biomass and seedling height by 32.6 and 35.4%, whereas bioagents mitigated the pathogenic effect. The bioagents in *R. solani*/*F. oxysporum*-infected soil significantly improved seedling biomass and height over pathogen treatments alone. *P. tinctorius* and *L. laccata* exhibited 44.2 and 39.1% root colonization in comparison to 19.5-24.2% in presence of pathogens. The study revealed that bioagents, especially mycorrhizae, effectively mitigate root rot in blue pine and can be efficiently exploited in integrated disease management module.

Key words: Antagonists, *Fusarium oxysporum,* mycorrrhiza, *Rhizoctonia solani,* root rot

INTRODUCTION

The Himalayan mountainous ranges harbour four out of the six indigenous pine species of Indian subcontinent, viz., *Pinus wallichiana, P. roxburghii, P. gerardiana* and *P. kesiya*. Of these, blue pine (*Pinus wallichiana* Jack.) has its native habitat spread over Eastern Afghanistan, South-eastern Tibet and China to North Burma and is mostly found in Himalayas at an elevation of 1500 to 3500 m m.a.s.l. The tree is widely distributed in Jammu and Kashmir, Himachal Pradesh and Gharwal hills in the West, spreading towards Bhutan with sporadic distribution in Arunachal Pradesh in the east. Of the total 20,230 km^2 area under forests in Jammu and Kashmir State, conifers cover 40.87% area out of which blue pines occupy 9.73% area (Beig *et al*., 2008a).

Blue pine, locally known as Kail pine, faces several constraints in its successful regeneration in field. The plants are often exposed to persistent pathogenic attacks, particularly those inciting root rot and wilt diseases, at primary stages of plant establishment. Root rot, wilt and die-back on container-grown conifers such as spruce and pine is major problem since 1990's in Europe (Lilja *et al*., 2010). The root rot fungi which pose serious threat to forest nurseries include the species of *Fusarium, Phytophthora, Pythium, Rhizoctonia, Macrophomina* and *Cylindrocladium* (Huang and Kuhlman, 1990; Asiegbu *et al*., 1999; Wafaa and Haggag, 2002). These pathogens often invade terminal unsuberized roots of young seedlings and cause late damping off or root rot/wilt thereby kill the host. The fungi penetrate into root epidermal cell wall, grow intercellularly, decompose cell wall constituents and persist by metabolising cell contents. Root rot is world-wide a serious problem in pine seedlings and serious losses due to this disease have been reported from Ontario, Canada, USA and many European countries (Greifenghagen *et al*., 1991; Lilja and Rikala, 2000). In view of highly devastating nature of root rot pathogens, effective disease management is essential to raise healthy pine seedlings for successful implementation of reforestation and afforestation programmes.

Several approaches involving fungicide use and cultural measures have been adopted by the nursery growers to reduce the root rot incidence; yet the disease continues to assume serious threat to conifers (Shah *et al*., 1999). The use of biocontrol agents is presently gaining momentum as a supplement to chemical treatment in integrated disease management module. The effective use of antagonistic bacteria, actinomycetes and fungi as biocontrol agents against several soil-borne pathogens have been demonstrated in several field and horticultural crops (Yobo *et al*., 2010). The free-living rhizosphere micro-organisms such as *Trichoderma, Gliocladium, Penicillium, Pseudomonas*, *Bacillus, Azotobacter, Azospirillum,* etc., favourably influence the plant growth directly or indirectly; whereas mycotrophy benefits the host plant by enhancing the root capacity to absorb nutrients, extracting the nutrients beyond depletion zones in soil and protecting the root from pathogenic invasions (Eziashi *et al*., 2006; Gacitua *et al*., 2009; Lu and Huang, 2010; Heydari and Pessarakli, 2010). The fungal antagonists may compete for an ecological niche by consuming available nutrients and by secreting a spectrum of biochemicals effective against various fungal pathogens. These biochemicals may include cell wall-degrading enzymes, siderophores, chelating iron, a wide variety of volatile and non-volatile antibiotics, etc. (Susi *et al*., 2011). Widyastuti *et al*. (2003) and Benitez *et al*. (2004) speculated that indirect and direct defensive mechanisms of *Trichoderma* species act coordinately. Accordingly the success in biocontrol process depends on the antagonisitic strain involved, the antagonized fungus, the crop plant and the environmental conditions, including nutrient availability, pH, temperature and iron concentration. Root drench of potted *Pinus radiata* with *Trichoderma atroviride* isolate R32 not only enhanced seedling root biomass and stem diameter but also induced systemic resistance to *Diplodia pinea* and reduced dieback incidence by 20% compared to untreated controls (Reglinski *et al*., 2011).

Symbionts, such as mycorrhizal fungi and free living organisms form integral components of pine rhizosphere, an area exhibiting all kinds of antagonistic, parasitic and growth promoting interactions (Taylor and Alexander, 2005). Mycorrhiza reportedly aid young seedlings in their early survival and establishment through intricate and complex system of hyphal networks thereby not only ensures the sustained nutrient supply but also provides protection against invading pathogens (Podila and Douds, 2001; Jha *et al*., 2008). The culture filtrate of mycorrhiza, *Suillus cdlinius Hebeloma mesophaeum* and *Paxillus* sp., reportedly exhibit antagonistic effect on the mycelial growth and spore germination of *Fusarium oxysporum* and *Pythium vexans* and the antifungal

activity has been attributed to oxalic acid production (Quarraqi *et al.*, 2005; Yamaji *et al.*, 2005). Inoculation of *Pisolithus tinctorius* in *Pinus densiflora* significantly increased dry matter and stem diameter when compared to non-inoculated seedlings (Choi *et al.*, 2005). *Paxillus involutus* effectively controlled root rot caused by *Fusarium oxysporum* and *Fusarium moniliforme* in red pine (Pal and Gardener, 2006). Efforts are underway to improve the quality of forest nursery seedlings through inoculation of suitable mycorrhizal strain in association with other compatible bioagents.

The perusal of literature reveals that some work has been done on the management of root rot diseases in conifers other than blue pine (Enebak *et al.*, 1990). From India, meagre information about the causal pathogens responsible, symptomology and management of diseases is available for conifers (Kaushik *et al.*, 2002; Bisht *et al.*, 2003). In view of the importance of root rot disease in blue pine and the damage inflicted by it, there is urgent need to develop a suitable disease management strategy which may include biological control as an important component. Therefore, the present investigation was undertaken to assess the bio-efficacy of fungal antagonists in the management of root rot disease on blue pine under temperate conditions of Kashmir.

MATERIALS AND METHODS

The root rot pathogens were isolated from diseased roots, collected during the survey of blue pine (*Pinus wallichiana*) nurseries in years 2007 and 2008. Three to four surface-sterilized diseased root bits of 3-5 mm size were aseptically transferred to Potato Dextrose Agar (PDA) medium and plates incubated at 25±2°C for mycelial growth. The cultures were purified by hyphal tip method (Dasgupta, 1988). Various cultural and morphological characteristics of isolated fungi were recorded by visual and microscopic examinations. Morphological characteristics of isolated fungi were compared with standard descriptions given by Nelson *et al.* (1983) and Sneh *et al.* (1998). The fungal/bacterial antagonists and ectomycorrhiza were isolated from the rhizosphere of blue pine trees by dilution plate method on PDA and Modified Melin Norkan's agar (MMN) media, respectively (Marx, 1969; Rangeshwaran and Prasad, 2000). The cultures were purified by single spore/hyphal tip method. The identification of isolated fungi was done on the basis of cultural and morphological characteristics (Arx, 1981). The isolated ectomycorrhiza were identified on the basis of culturo-morphological characteristics and identified with the help of standard descriptions (Godbout and Fortin, 1985; Lakhanpal, 1988).

***In vitro* assessment of biocontrol agents:** The antagonistic activity of isolated rhizosphere fungi/bacterium and mycorrhizae against root rot pathogens was assessed by dual culture technique using PDA and MMN agar media, respectively (Dhingra and Sinclair, 1985). Treatments were replicated four times in a completely randomized design and incubated at 25±2°C for 8 days. Radial growth was recorded on 8th day of incubation and mycelial inhibition calculated as per Vincent (1947).

The morphology of hyphae in interaction zone was observed under light (10x) microscope. Clear and characteristically parasitized hyphae were examined under high magnification power (40x). Based on the growth and mycoparasitic nature, biocontrol agents were grouped into various categories as per the scale given by Munshi and Dar (2004). The antifungal activity of bacterial antagonist was assessed by dual culture technique according to the method given by Dennis and Webster (1971), using PDA medium. Control plates inoculated with pathogen alone were also

maintained. The treatment plates, replicated four times, were incubated at 28±2°C in completely randomized design. Mycelial growth of pathogen and inhibition zone was measured after 72 h of incubation.

***In vivo* evaluation of antagonists:** The efficacy of antagonist under *in vivo* conditions was assessed as per the method described earlier (Ahangar *et al.*, 2011). The treatments were replicated 10 times and arranged in a completely randomized block design in a glasshouse at 26±3°C. The seedlings were gently uprooted after 3 months to assess fresh plant biomass and mycorrhizal development. The entire root systems were examined under a stereomicroscope to count the number of mycorrhizal short roots (Daughtridge *et al.*, 1986). The mycorrhizal colonization was assessed by counting the total number of mycorrhizal short roots formed by inoculated fungi against the total number of short roots observed. Root and shoot length of seedlings was measured and alkaline acid phosphatase activity of rhizosphere soil was estimated as per the method of Tabatabai and Bremner, 1969. The amount of ρ-nitrophenol (PNP) released was calculated in reference to the standard curve prepared by using different concentrations of PNP. The enzyme activity was expressed in terms of μM PNP released g^{-1} soil h^{-1}.

Nursery studies on pathogen-antagonist interaction: The most effective five fungal antagonists, including two ectomycorrhiza, were evaluated individually and in combination with major pathogens. The pathogenic inoculums of non-mycorrhizal fungus were incorporated @ 10 g kg^{-1} mixture to ensure sick soil formation. Biocontrol agents, multiplied on wheat bran, were added @ 10 g kg^{-1} potting mixture (with an inoculum load of 1×10^{9} cfu g^{-1}) as per treatment. Ectomycorrhiza were multiplied in vermiculite-peat moss based carrier added to sick soil @ 15 mL bag^{-1}. The antagonist additions to sick soil were made 15 days before seed sowing. Non-sick soil and sick soils without antagonist inoculation served as controls. The surface sterilized and stratified healthy seeds of blue pine were sown 3 per bag of sterilized potting mixture of one kg in a 1.5 kg capacity plastics bag. After germination seedlings were thinned out to one per bag. Each treatment replicated 10 times was arranged in a CRD in a greenhouse at 25±3°C and irrigated with sterile water as and when required. No fertilizers or protective chemicals were applied throughout the study. Ectomycorrhizal infection in roots was estimated and roots examined under a stereomicroscope to count the number of mycorrhizal short roots as suggested by Beckjord *et al.* (1984).

The fresh plant biomass and seedling height were estimated 90 and 180 days after seedling emergence. Root colour intensity and root rot index was measured on the basis of root area affected according to the root rot index rating scale described by Purkayastha *et al.* (1981). The data were subject to analysis of variance and means compared using Duncan's new multiple range test (Gomez and Gomez, 1984). Arcsine and square transformations were employed wherever applicable. The data analysis was carried out using Statistical Packages for Social Sciences (SPSS ver. 11.5 Chicago USA for windows).

RESULTS AND DISCUSSION

Root rot pathogens of pines: During preliminary survey the roots of blue pine seedling showing root rot and wilt symptoms, collected from forest nurseries in Anantnag and Baramulla districts of Jammu and Kashmir, were found infected by three root rot causing fungi viz., *Fusarium oxysporum* f.sp. *pini* Schlecht. Synd. and Hans., *Rhizoctonia solani* Kuhn. and *Macrophomina phaseolina* (Tassi.) Goid. Besides these, some saprophytic fungi were isolated from the affected root

portions. *Fusarium oxysporum* was isolated from all the surveyed nurseries with an overall isolation frequency of 47.3% which was followed by *Rhizoctonia solani* and *Macrophomina phaseolina* with overall isolation frequencies of 29.7 and 13.0%, respectively and the rest 10% were the species of *Mucor, Rhizophus, Penicillium, Aspergillus* and *Trichoderma*. These observations are in line with Pinto *et al.* (2006) who recorded 44.6% isolation frequency of *F. oxysporum* from colonized roots of *Pinus sylvestris* seedlings and 0.3% isolation frequency of *R. solani* from the diseased roots of *Picea abies* seedlings from Uppsala Sweden. Lilja *et al.* (1995) and Stepniewska-Jarosz *et al.* (2006) frequently isolated the above pathogens from diseased conifer roots, cone scales and seeds of Scots pine from Florida and Poland forest nurseries, respectively. *Fusarium* species, especially *F. oxysporum,* reportedly is highly potential root rot pathogens in many forest nurseries (Enebak *et al.*, 1990).

Antagonistic isolates: Three non-mycorrhizal fungal antagonists *Trichoderma viride, T. harzianum* and *Gliocladium roseum* and four mycorrhizal fungi *Pisolithus tinctorius, Laccaria laccata, Boletus* spp. and *Suillus granulatus* were isolated from blue pine rhizosphere. Their identification was made on the basis of morpho-cultural characteristics. *L. laccata* and *S. granulatus* have previously been isolated from the basidiomata collected from *Pinus patula* plantations by Reddy and Natarajan (1997) from Nilgiri hills, Tamil Nadu, India and Yamada *et al.* (2001) from Ibaraki, Japan. Dar *et al.* (2009) have reported the presence of *Pisolithus tinctorius, Laccaria laccata, Boletus* spp. and *Suillus granulatus* from Gulmarg, Bandipora and Baramulla conifer forests of Kashmir with high rhizosphere phosphatase activity and root colonizing potential.

Pathogenic growth inhibition by antagonists: *In vitro* evaluation of antagonists against *F. oxysporum* and *R. solani* in dual culture revealed that all the tested biocontrol agents significantly inhibited the mycelial growth of pathogens (Table 1). The mycelial inhibition on 8th day ranged from 33.0 to 73.3% and 29.5 to 70.8% for *F. oxysporum* and *R. solani*, respectively, with *T. harzianum* proving more effective against both the pathogens, followed by *T. viride* (70.5 and 64.8%) and *Gliocladium roseum* (50.0 and 54.7%). *Trichoderma* species proved highly antagonistic

Table 1: *In vitro* effect of various antagonists on mycelial growth of *Fusarium oxysporum* and *Rhizoctonia solani* in dual culture

	Fusarium oxysporium			Rhizoctonia solani		
Antagonists	Inhibition over control (%)	Degree of antagonism	*Zone of inhibition	Inhibition over control (%)	Degree of antagonism	*Zone of inhibition
Trichoderma viride	70.5 (48.07)	HA	+	64.8 (46.09)	HA	+
T. harzianum	73.3 (49.00)	HA	-	70.8 (48.17)	HA	-
Gliocladium roseum	50.0 (40.48)	MA	+	54.7 (42.34)	MA	+
Pisolithus tinctorius	45.4 (38.57)	MA	-	46.2 (38.91)	MA	-
Laccaria laccata	43.7 (37.84)	MA	-	44.7 (38.28)	MA	-
Suillus granulatus	33.7 (33.23)	SA	-	36.4 (34.54)	SA	-
Boletus spp.	33.0 (32.89)	SA	-	29.5 (31.09)	SA	-
Pseudomonas fluorescens	42.4 (37.30)	A	+	40.6 (36.50)	A	+
CD (p = 0.05)	-2.09			-3.97		

HA: Highly antagonistic, MA: Moderately antagonistic, A: Antagonistic, SA: Slow antagonistic, * +: Inhibition zone present, -: inhibition zone absent

and exhibited strong mycoparasitic activity. They completely overgrew the host mycelia once in contact with pathogens and formed hyphal coils on pathogenic colonies. *G. roseum* and *Pseudomonas fluorescens* grew slowly and developed zone of inhibition against the pathogen. Amongst the mycorrhizal fungi *Pisolithus tinctorius* and *Laccaria laccata* inflicted significantly higher mycelial growth inhibition of 45.4 and 46.2 and 43.7 and 44.7% in *F. oxysporum* and *R. solani,* respectively. Further, *L. laccata* and *P. tintorius* were observed to be moderate antagonists as they caused rupture and twisting of pathogenic hypha followed by their gradual desiccation, protoplasm shrinkage and ultimately cell lysis. *Boletus* spp. and *Suillus granulatus* inflicted only 33.0 and 29.5 and 33.7 and 36.4% inhibition in mycelial growth of *F. oxysporum* and *R. solani*, respectively, so were categorized as slow antagonists. The growth inhibitive effects of antagonists are in agreement with Rudresh *et al.* (2005) who observed 72.1 and 77.0% growth inhibition in *R. solani* and *F. oxysporum*, respectively, by *T. harzianum* and *T. viride* which also exhibited strong mycoparasitic activity and completely overgrew the host mycelia once in contact with them. Dubey (1998) observed hyphal coil or hook or appresoria formation by *T. harzianum* on fungal colony of *Rhizoctonia solani*. *Trichoderma* species reportedly produce chitinase and β1-3, glucanase enzymes which degrade cell wall and cause hyphal lysis of pathogens (Wu *et al*., 1986).

The formation of inhibition zone by *T. viride, G. roseum* and *P. fluorescens* suggests the involvement of strong antibiosis mechanism, possibly due to the production of volatile metabolites and diffusible chemicals produced by antagonist. Munshi and Dar (2004) noticed inhibition zone formation by *Gliocladium* sp. against *Fusarium pallidoroseum*. The mycoparasitic activity of *Laccaria laccata* against *R. solani* and *Fusarium* sp. has earlier been suggested by Zhao and Kuo (1988).

Plant growth improvement by antagonists: Preliminary *in vivo* evaluation of mycorrhizal and non-mycorrhizal fungal bioagents in improving pine seedling growth was assessed in pot culture experiments. All the test biocontrol agents significantly improved root and shoot length of pine seedlings and yielded higher biomass than uninoculated control, as observed 90 days after seedling emergence (Table 2). *L. laccata* and *P. tinctorius* depicted significantly higher fresh plant biomass of 0.99 and 0.97 g plant^{-1}, respectively, followed by *T. harzianum* (0.85 g plant^{-1}) and *T. viride* (0.83 g plant^{-1}) in comparison to un-inoculated control (0.62 g plant^{-1}). Similar trend was noticed

Table 2: *In vivo* effect of fungal antagonists, including ectomycorrhiza, on plant growth, root colonization and rhizosphere acid phosphatase activity in blue pine seedlings

Fungal antagonists	Fresh plant biomass (g plant^{-1})	Root length (cm)	Shoot length (cm)	Mycorrhizal root colonization (%)	Acid phosphatase activity (μM PNP g^{-1} soil)
Laccaria laccata	0.99	10.50	9.40	42.0	212.0
Pisolithus tinctorius	0.97	9.80	8.90	40.5	214.5
Suillus granulatus	0.76	6.40	6.80	35.7	206.0
Boletus spp.	0.74	6.20	6.50	33.9	204.0
Trichoderma viride	0.83	6.70	7.00	0.0	38.0
T. harzianum	0.85	7.00	7.40	0.0	42.0
Gliocladium roseum	0.70	6.50	6.80	0.0	23.0
Control	0.62	5.90	6.30	0.0	16.0
CD (p = 0.05)	0.05	0.09	0.05	-	10.3

Observations taken 3 months after inoculation

in case of root and shoot length with significantly higher length of 10.5 and 9.4 cm, respectively, in *L. laccata* in comparison to the respective values of 5.9 and 6.3 cm in un-inoculated control.. This was followed *P. tinctorius, T. harzianum* and *T. viride*. The findings are in agreement with Villeneuve *et al.* (1991) who observed 40% growth increase in Douglas-fir seedlings due to the inoculation of *L. laccata and* Reddy and Natarajan (1997) who reported 38.0 and 186.5% increase in seedling height and shoot dry weight of *Pinus patula* due to *L. laccata* inoculation after 8 months. It appears that mycorrhizal fungi imparted protection, induced resistance or released antimicrobial compounds to favour plant growth. Fungal sheath around the roots seemed to have restricted the fungal advancement into mycorrhizal cortex. Protection of conifer seedlings against *Fusarium* spp. due to *L. laccata* has been attributed to the possible production of antifungal phenol compounds by the host in presence of mycorrhizal species (Chakravarty *et al*., 1991). Farquhar and Peterson (1990) showed that *Pinus resinosa* seedlings inoculated with *Paxillus involutus* had induced resistance to *F. oxysporum*.

Mycorrhizal colonization and rhizosphere phosphatase activity: The mycorrhizal root colonization and rhizosphere phosphatase activity was higher in mycorrhiza inoculated treatments. The tested ectomycorrhizal fungi colonized 33.9-42.0% roots in 90 days, developed symbiotic association with pine seedling roots and improved mycorrhizal short root formation (Table 2). Significantly high mycorrhizal root colonization was observed in pine inoculated with *L. laccata* (42.0%) with better rhizosphere phosphatase activity of 212.0 μM PNP g^{-1} soil. However, *P. tinctorius* was at par with *L. laccata* with root colonization of 40.5% and rhizosphere phosphatase activity of 214.5 μM PNP g^{-1} soil. In case of soils inoculated with non-mycorrhizal bioagents and untreated control the phosphatase activity was significantly very low ranging from 16.0 to 42.0 μM PNP g^{-1} soil. Mycorrhizal plants exhibited 5-13 fold higher rhizosphere phosphatase activity than non-mycorrhizal ones. Acid phosphatase is solely of extracellular origin and is involved in the mineralization of organic phosphates. The phosphatase enzymes solubilize insoluble forms of phosphorus and other nutrients not readily available to plant roots. Dunabeitia *et al.* (2004) and Beig *et al.* (2008b) observed greater acid phosphatase activity in the rhizosphere of mycorrhizal plants than non-mycorrhizal ones. *T. harzianum* has also the ability to solubilize many plant nutrients including rock phosphate from their solid phase compounds by their enzymatic activity (Altomare *et al*, 1999). The synergistic effect of biocontrol agents in combined inoculation resulted higher phosphatase activity in the rhizosphere of blue pine seedlings.

Plant growth improvement in presence of pathogens: Under field nursery conditions four effective fungal biocontrol agents including 2 effective ectomycorrhiza were inoculated individually and in combination with root rot pathogen (*F. oxysporum* or *R. solani*). All the test bioagents significantly improved plant growth in terms of biomass and seedling height in blue pine whereas presence of root-rot pathogens reduced the overall plant growth and biomass (Table 3). The bioagent inoculated plants had higher biomass of 0.51-0.60 and 0.98-1.15 g $plant^{-1}$ on 90 and 180th days growth, respectively, in comparison to 0.46 and 0.84 g $plant^{-1}$ in uninoculated control. Maximum gain was observed in case of *P. tinctorius* followed by *L. laccata*. After 90 days growth, the use of biocontrol agents individually, significantly improved seedling height (5.9-6.5 cm) over uninoculated control (4.8 cm), with maximum gain in blue pine seedlings inoculated with *P. tinctorius,* followed by *L. laccata, T. viride* and *T. harzianum*. With the advancement in growth period, the shoot height depicted almost similar trend with inoculated plants having height of 9.2-10.6 cm as compared to 7.5 cm in unioculated control after 180 days growth.

Table 3: *In vivo* interaction of fungal antagonists with wilt pathogens, *Rhizoctonia solani* (*R*) and *Fusarium oxysporum* (*F*) on blue pine seedlings (pooled data of 2 years)

	Fresh plant biomass (g plant^{-1})		Shoot height (cm)				
Treatments	90 day	180 day	90 day	180 day	ECM (%)	Root rot index* (%)	Root colour intensity
Trichoderma viride	0.51	0.98	6.00	9.60	0.0	0.0	-
T. harzianum	0.54	1.06	5.90	9.20	0.0	0.0	-
Pisolithus tinctorius	0.60	1.15	6.50	10.60	44.2	0.0	-
Laccaria laccata	0.57	1.10	6.20	10.10	39.1	0.0	-
Rhizoctonia solani (R)	0.31	0.56	3.10	5.00	0.0	38.3	+++
R. solani+T. Viride	0.39	0.69	3.70	6.10	0.0	26.7	+
R. solani+T. Harzianum	0.42	0.74	3.50	5.70	0.0	25.2	++
R. solani+P. Tinctorius	0.46	0.80	4.10	6.60	24.2	23.0	+
R. solani+L. laccata	0.44	0.77	4.00	6.30	19.5	22.6	+
Fusarium oxysporum (F)	0.22	0.41	2.40	4.10	0.0	47.2	+++
F. oxysporum+T. Viride	0.30	0.55	3.20	5.40	0.0	35.4	+
F. oxysporum+T. harzianum	0.32	0.60	3.10	5.20	0.0	33.6	++
F. oxysporum+P. Tinctorius	0.28	0.58	2.60	4.80	21.4	31.0	+
F. oxysporum+L. Laccata	0.29	0.52	2.80	5.10	18.9	29.4	+
Control	0.46	0.84	4.80	7.50	0.0	0.0	-
CD (p = 0.5)	0.06	0.09	0.15	0.12	-	-	-

* Root rot scale: No root rot = 0; <10 percent root area affected = 0.10; 11-25 percent root area affected = 0.25; 26-50 percent root area affected = 0.50; 51-75 percent root area affected = 0.75; 76 percent root area affected = 1.00

Rhizoctonia-infection resulted in less plant biomass (0.31 g plant^{-1}) and seedling height (3.1 cm) after 90 days growth, with almost similar trend noticed after 180 days. However, in presence of bioagents the effect of *R. solani* was significantly reduced and seedling biomass and shoot height in pine was 0.39-0.46 g plant^{-1} and 3.5-4.1 cm, respectively, on 90th day of growth. The seedlings biomass and height was 0.69-0.80 g plant^{-1} and 5.7-6.6 cm, respectively, in *Rhizoctonia* + bioagent treatments as compared to 0.56 g plant^{-1} and 5.0 cm in *R. solani* treatment alone on 180th day of growth with maximum increase in *R. solani* + *P. tinctorius* followed by *R. solani* + *L. laccata* treatment. The bioagents in presence of root rot pathogen *Fusarium oxysporum* also promoted plant growth and mitigated the influence of pathogen (Table 3). *Fusarium oxysporum* infected blue pine seedlings had less biomass and shoot height of 0.22 g plant^{-1} and 2.4 cm, respectively, after 90 days growth and 0.41 g plant^{-1} and 4.1 cm after 180th days growth in comparison to unicoculated control. The bioagents in *F. oxysporum*-infected soils remarkably had higher plant biomass and seedling height ranging from 0.28 to 0.32 g plant^{-1} and 2.6 to 3.2 cm, respectively, on 90th day of growth and from 0.52-0.60 g plant^{-1} and 4.8-5.4 cm, respectively, on 180th day of growth with maximum improvement in biomass in *F. oxysporum+T. harzianum,* followed by *F. oxysporum+P. tinctorius* and maximum increase in plant height in *F. oxysporum+T. viridi* treatment followed by *F. oxysporum+T. harzianum*. The overall improvement in plant biomass and seedling height by bioagents may be attributed to the growth promoting and protective effects of biocontrol agents. Werner *et al.* (2002) observed that mycorrhizal *Pinus sylvestris* seedlings inoculated with *Trichoderma virens* produced significantly higher plant growth and biomass of needles, trunks and root than uninoculated plants. *T. harzianum* showed stimulatory effect on seedling growth and biomass of pine. *Trichoderma* species reportedly produce hormone like metabolites and release nutrients from soil or organic matter thereby facilitate better plant growth (Windham *et al.*, 1986; Yobo *et al.* 2010).

Disease control by antagonists: The antagonists remarkably decreased root rot disease index and improved root colour intensity in pathogen-infected treatments (Table 3). High root rot index was observed in pine seedlings raised in either *R. solani* or *F. oxysporum* infested soil as compared to control (non-sick soil). The bioagents significantly reduced root rot from 38.3% in *R solani* treatment to 22.6-26.7% with maximum decrease in *L. laccata* followed by *P. tinctorius, T. harzianum* and *T. viridi*. In case of *F. oxysporum* infection, the bioagents decreased root rot from 47.2% in *F. oxysporum* treatment to 29.4-35.4% in bioagents, with maximum reduction by *L. laccata,* followed by *P. tinctorius, T. harzianum* and *T. viridi*. The study revealed that, besides improving growth, bioagents especially mycorrhiza protected seedlings against root rot pathogens in blue pine. Inoculation of bioagents in presence of root rot pathogens proved superior over pathogen-inoculated control. Decrease in root rot disease index by antagonistic fungi including mycorrhiza may be attributed to the competition for nutrient base and space, cross protection of roots, alteration in host metabolism and improved nutrient supply. Chakravarty and Unestam (1987) reported that growth and development of mycorrhizal species was strongly suppressed by root rot pathogens *F. oxysporum* and *R. solani*. Various bioagents reportedly have greater rhizosphere competence and parasitizes the pathogenic fungi (Naik, 2003; Gao *et al*., 2010). The antagonists in rhizosphere likely compete with the pathogen for host surface and nutrients and inhibit pathogenic growth through antibiosis/mycoparasitism mechanisms (Howell, 2003; Sharma *et al*., 2010.). This seems to have reduced the seedling root decay. These speculations are substantiated by our *in vitro* bicontrol studies on *F. oxysporum* and *R. solani*. The principal mechanisms of *Trichoderma* spp. for disease control have been presumed to be those primarily acting upon the pathogens and include mycoparasitism, antibiosis and competition for resources and space (Harman, 2006). Thus reduction of root rot in blue pine seedlings incited by *F. oxysporum* and *R. solani* may be due to the biological control action of *T. harzianum* on these pathogens.

Pisolithus tinctorius alone showed 44.2% root colonization in comparison to 39.1% root colonization observed in *L. laccatta* after 180 days growth. In presence of *R. solani* the mycorrhizal root colonization was comparatively less, 24.2% in *P. tinctorius* and 19.5% in *L. laccata*. Almost similar effect of mycorrhiza was noticed in presence of *F. oxysporum*. Increased root colonization may be attributed to the competition for space and reduction in root biomass due to root decay by rot causing incitant, thereby leaving less surviving root area for symbiosis. 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. Antagonists or mycorrhizae inoculation in *Rhizoctonia* infected plants lessened the effect of root rot. Mycorrhizal root colonization was less in presence of *Rhizoctonia solani* and *F. oxysporum*. This indicates that the disease protection by ectomycorrhiza may involve multiple mechanisms including antibiosis, synthesis of fungistatic compounds by plant roots in response to mycorrhizal infection and physical barrier of fungal mantle around the plant root (Duchesne *et al*., 1987; Dar *et al*., 2007). The favourable effect of mycorrhizal fungus on plant growth and health may be attributed to the excretion of growth promoting substances by mycorrhizae or indirectly by alteration in root physiology, uptake of minerals and pattern of exudation into the mycorrhizosphere (Leyval and Berthelin, 1990).

CONCLUSION

The tested fungus biocontrol agents, including mycorrhiza, effectively mitigated root rot disease in blue pine. The bioagents once applied in field multiply in soil and when threshold population is

achieved they may reduce the disease incidence thereby ensure the successful establishment of pine seedlings at early stages. The fungal bioagents can be efficiently exploited in integrated disease management module.

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