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Research Article

Biomanagement of root rot of pine seedlings

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ABSTRACT: Studies were conducted on biomanagement of root rot of pine (*Pinus wallichiana*) seedlings during 2008-2009 at Shalimar campus, Srinagar, Kashmir. The pathogens associated with the disease were isolated, morphologically characterized and identified as *Fusarium oxysporum* f. sp. *pini* (Schlecht.) Synd. and Hans. and *Rhizoctonia solani* Kuhn, and *F. oxysporum* proved more pathogenic. In bioassays all the biocontrol agents tested inhibited the mycelial growth of *F. oxysporum* and *R. solani* in dual culture, however, *Trichoderma harzianum* and *T. viride* were more effective. *Pseudomonas fluorescens* showed strong antibiosis and developed zone of inhibition against both the pathogens *in vitro*. *Laccaria laccata* caused hyphal lysis of the pathogens in dual culture. *In vitro* evaluation of the culture filtrate of test antagonists revealed that culture filtrate of *P. fluorescens* had maximum inhibitory effect on mycelial growth and spore / sclerotial germination of test pathogens followed by *T. harzianum*, *T. viride* and *L. laccata.* Inoculation of *T. harzianum, P. fluorescens* and *L. laccata* individually or in combination significantly improved the growth and biomass of Kail pine seedlings *in vivo*. Combined inoculations of biocontrol agents showed synergistic growth promoting action. Seed germination was improved by the biocontrol agents with drastic reduction by pathogenic microorganisms. Biocontrol agents inoculated individually or in combination reduced the pathogenic effect (root rot) as compared to the control.

KEY WORDS: Biomanagement, *Pinus wallichiana,* root rot, *Fusarium oxysporum, Rhizoctonia solani*

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INTRODUCTION

Pinus wallichiana (A.B. Jack.), the blue pine or Bhutan pine, is an important conifer (*Pinaceae*: *Coniferales*) and is mainly propagated through nursery-raised seedlings. Pines like other forest trees face severe problems in successful regeneration under existing natural conditions and are often exposed to persistent pathogen attacks, particularly those causing root rot. *Fusarium* spp., *Rhizoctonia* spp., and *Cylindrocarpon* spp. are frequently isolated from the root of diseased conifer seedlings that cause root rot (Enebek *et al*., 1990). Despite attempts made by the nursery growers to inhibit the disease-causing organisms by applying fungicides and cultural methods, root rot continues to be serious problem. The demand for alternatives to chemical control of plant pathogens has become stronger owing to the concerns about the safety and environmental impacts of chemicals (Ooijkaas *et al.,* 1998). Biological control appears to be the only solution for long term sustainability and effective management of soil-borne diseases. Various strains of *Trichoderma* species have been found to be strong opportunistic invaders, fast growers and prolific producers of spores and antibiotics (Whipps and Lumsdon, 2001). Production of secondary metabolites like antibiotics, Fe-chelating siderophores and cyanide are most often associated with fungal suppression by fluorescent pseudomonads in the rhizosphere of several crops (Howell and Stipanovic, 1980). Ectomycorrhizae are essential for survival and early establishment of conifer seedlings, as they are mycotrophic in nature. They help in good growth and development of host plants in low nutrient soils and they also provide protection against various soil-borne pathogenic organisms. In view of the potential destructiveness of root rot of pine, three biocontrol agents, *viz*., *Trichoderma harzianum*, *Pseudomonas fluorescens* and *Laccaria laccata* were used separately and in combination in the rhizosphere of pine seedlings to work out their impact on the disease.

MATERIALS AND METHODS

In vitro *evaluation*

The causal organisms of root rot of *P. wallichiana* seedlings were isolated from the diseased roots of pine

seedlings collected from various forest nurseries in Kashmir Valley. The isolated pathogens were characterized and their pathogenicity was recognized by proving Koch's postulates. The fungal and bacterial antagonists were isolated from the soil collected from the rhizosphere of healthy *P. wallichiana* seedlings. The fungal antagonists were isolated on potato dextrose agar medium and the bacterial antagonists isolated on King's B medium (King *et al.*, 1954) by dilution plate method. The ectomycorrhizal fungi were isolated from the sporocarps collected from the canopy of *P. wallichiana* plantations on modified Melin-Norkran's medium (Marx, 1969). The cultures were purified, multiplied and maintained on their respective medium. The *in-vitro* antagonistic activity of isolated bioagents against root rot pathogens of blue pine seedlings was assessed by dual culture technique (Dennis and Webster, 1971). *In-vitro* antagonism in dual culture between ectomycorrhizal fungus and the root rot pathogens was studied on modified Melin Norkran's medium according to Marx (1969). Three replications were maintained per treatment. The radial growth of the pathogen was measured and the results were expressed as per cent growth reduction over control. The selected isolates were also tested for the production of inhibitory non-volatile metabolites in liquid medium by poisoned food technique at 60 per cent concentration as described by Mukherjee and Tripathi (2000). The culture filtrates of antagonists (15-day-old fungal, four-day-old bacterial and 30-day-old ecto-mycorrhizal cultural filtrates) were obtained by filtering the broth cultures through Whatman No. 1 filter paper. The filtrates were again passed through Seitz filter under vacuum to prevent contamination. The method adopted by Prasad *et al.* (1999) was followed. The antibiotic potential of antagonists against spore and sclerotial germination of *Fusarium oxysporum* and *Rhizoctonia solani*, respectively, were also tested. One ml each of culture filtrate and spore suspension of *F. oxysporum* was placed on each cavity of a sterile two-concavity glass slides and placed on glass tubings in moist chamber. The spore suspension in sterile water served as control. Observations spore germination were recorded with the help of a compound microscope (40x) and expressed as per cent reduction over control. Sclerotia of *R. solani* were placed on sterile filter paper impregnated with culture filtrates of antagonists and filter paper impregnated with sterile distilled water served as control. After incubation for five days, sclerotial germination was recorded and expressed as per cent reduction over control.

Greenhouse evaluation

The antagonists were evaluated against root rot pathogens of *P. wallichiana* seedlings in pot culture under greenhouse conditions. A soil-sand mixture (2:1) was sterilized at 1.4 kg cm^{-2} for one hour for three successive days. One kg of sterilized potting mixture was put in each plastic bag of 1.5 kg capacity. The pathogen inoculum multiplied on sand-maize medium (9:3) was incorporated @10 g kg^{-1} of mixture. Ectomycorrhizal fungal inoculum prepared in vermiculite based carrier according to the method of Marx and Bryan (1975) was added 15 days before sowing @15 ml kg^{-1}. A talc-based formulation of *Trichoderma harzianum* prepared as per Rudresh *et al.* (2005) with an inoculum load of 1 x 10^9 Cfu g^{-1}, was added @5 g kg^{-1} of potting mixture. *Pseudomonas fluorescens* multiplied on talc formulation according to Vidhyasekaran and Muthamilan (1995) with an inoculum load of 2.5 x 10^8 Cfu g^{-1}, was added @5 g kg^{-1} 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 48h at 40°C in dark. Five seeds were sown per bag, and after germination, the seedlings were thinned to one per bag. The germination percentage was recorded and shoot height, root length and dry weight of seedlings were measured 60 days after sowing. Root rot index was calculated on the basis of per cent root area affected on a scale of 1–5 as described by Ocamb *et al.* (2002).

RESULTS AND DISCUSSION

On the basis of morphological characteristics, pathogenicity and comparison with authentic descriptions, the pathogens responsible for disease development were identified as *Fusarium oxysporum* f. sp. *pini* and *Rhizoctonia solani.* These pathogens have been reported elsewhere as causal organisms of root rot of conifer seedlings from Wisconsin (USA), Uppsala (Sweden) and Ontario (Canada) forest nurseries (Landis, 1999; Martin *et al*., 2006). The isolated fungal antagonists were morphologically characterized and identified as *Trichoderma harzianum, Trichoderma viride* and *Trichoderma virens*. The bacterial antagonist *P. fluorescens* was identified on the basis of various morpho-physiological and biochemical tests. The ectomycorrhizal fungi *Laccaria laccata* and *Suillus granulates* isolated from sporocarps were identified on the basis of their morpho-cultural characteristics.

In vitro evaluation of isolated antagonists under dual culture revealed growth inhibition of root rot pathogens (*F. oxysporum* f. sp. *pini* and *R. solani*) by the test antagonists. Among the antagonists, *T. harzianum* caused maximum mycelial growth inhibition of 76.6 and 73.3 per cent in *F. oxysporum* and *R. solani*, respectively, in dual culture followed by *T. viride* (Table 1). The formation of hyphal coils by *T. harzianum* on pathogenic colonies was also noticed. *T. harzianum* exhibited strong mycoparasitic activity and completely overgrew the host mycelia once in contact with them. The present observations are in agreement with those reported by Pandey *et al.* (2005). *P. fluorescens* developed clear zones of inhibition against both the pathogens and caused 63-66 per cent mycelial growth inhibition. The formation of inhibition zone by

Table 1. ***In vitro*** **effect of various antagonists on mycelial growth and spore / sclerotial germination of** ***Fusarium oxysporum*** **f.sp.** ***pini*** **and** ***Rhizoctonia solani***

Treatment	% growth inhibition in dual culture (six dayinhibition after incubation)		Mycelial growth inhibition (%) by culture filtrate at 60% concentration		Spore / sclerotial germination (%) by culture filtrate at 60% concentration	
	F. oxysporum	*R. solani*	*F. oxysporum*	*R. solani*	*F. oxysporum*	*R. solani*
Trichoderma viride	69.4 (56.3)	70.1 (57.7)	59.6 (50.5)	53.6 (45.8)	47.5 (43.5)	41.8 (40.2)
Trchoderma harzianum	76.6 (61.6)	73.3 (58.9)	67.5 (55.2)	58.2 (49.3)	52.5 (46.4)	44.4 (41.7)
Trichoderma virens	48.5 (44.1)	47.6 (43.6)	50.0 (45.0)	44.8 (42.0)	41.6 (40.1)	35.3 (36.4)
Pseudomonas fluorescens	63.6 (51.9)	66.2 (53.0)	69.3 (56.3)	61.2 (51.7)	55.6 (48.2)	49.9 (44.9)
Laccaria laccata	46.4 (42.9)	45.4 (42.3)	48.4 (44.0)	39.2 (39.0)	41.8 (40.1)	36.3 (36.9)
Suillus granulates	40.8 (39.7)	43.7 (41.3)	34.2 (35.8)	28.7 (32.4)	32.3 (34.6)	29.2 (32.7)
Mean	58.4 (50.0)	57.9 (49.6)	54.8 (47.7)	47.6 (43.3)	45.2 (42.1)	39.4 (38.8)
CD (P = 0.05)	2.2	2.5	2.8	1.9	2.4	2.0

Figures in parentheses are arcsine transformed values

P. fluorescens suggests the involvement of strong antibiosis, possibly due to the production of volatile metabolites and diffusible chemicals produced by the antagonist. Development of inhibition zones by the antagonists with *F. oxysporum* and *R. solani* has been reported earlier by Rangeshwaran and Prasad (2000). The differential response of antagonists in inhibition of test pathogens may probably be due to the variation in the type, quantity and stability of metabolites produced including antibiotics.

Laccaria laccata in dual culture with *F. oxysporum* and *R. solani* caused hyphal lysis of both the pathogens. The interaction caused rupturing and twisting of the hyphae at initial stage, gradually the protoplasm showed desiccation and shrinkage. The mycoparasitic activity of ectomycrrhizal fungi against *R. solani* has earlier been reported by Zhao and Kuo (1988).

The culture filtrate of selected antagonists caused significant inhibition in mycelial growth of *F. oxysporum* and *R. solani*. However, the culture filtrate of *P. fluorescens* was more effective and caused 69.3 and 61.2 per cent hyphal inhibition of *F. oxysporum* and *R. solani*, respectively, followed by *T. harzianum* (67.5 and 58.2%, respectively). A similar trend was observed when the culture filtrates of biocontrol agents were assayed for inhibition of spore germination in *F. oxysporum* and sclerotial germination in *R. solani*. Maximum reduction in spore and sclerotial germination of the respective pathogens was observed by *P. fluorescens* followed by *T. harzianum*. The degree of inhibition observed in the present study is in agreement with Rudresh *et al.* (2005). The production of toxins, antibiotics and cell wall degrading enzymes by the bioagents in cell free culture filtrates may be the possible reason for the observed inhibition. *Trichoderma* spp. is known to produce chitinase and b-1-3, glucanase enzymes which may degrade the cell wall and lead to the lysis of hyphae of the pathogen (Wu *et al.*, 1986).

The mycorrhizal fungus *L. laccata* showed significant antagonistic activity against *F. oxysporum* and *R. solani* under *in vitro* conditions. The cell free culture extract of *L. laccata* not only inhibited the mycelial growth but also significantly reduced the spore / sclerotial germination of root rot pathogens. Sylvia and Sinchair (1983) observed that diffusible metabolites of *L. laccata* inhibited growth and caused distortion of hyphae of *F. oxysporum*. Hence the observed antagonistic action of *L. laccata* may be attributed to the release of substantial antibiotics and other antimicrobial metabolites against the pathogens.

Root rot index of Kail pine incited by *F. oxysporum* f. sp. *pini* and *R. solani* significantly decreased by the inoculation of biocontrol agents. The roots of Kail pine seedlings were found infected only when *F. oxysporum* or *R. solani* was inoculated in the medium which suggests that *F. oxysporum* and *R. solani* incite root rot in pine seedlings. *F. oxysporum* proved more pathogenic and

Table 2. Influence of biocontrol agents and *F. oxysporum* f. sp. *pini* on seed germination, growth and biomass of Kail pine seedlings

Treatment	Germination (%)	Seedling height (cm)	Root length(cm)	Dry weight(mg plant^{-1})
Laccaria laccata (LL)	74.7 (62.4)	9.6	11.6	206.7
Trichoderma harzianum (TH)	81.4 (64.2)	8.3	9.2	187.5
Pseudomonas fluorescens (PS)	77.6 (62.3)	8.8	10.0	192.4
Uninoculated control	72.3 (59.1)	7.2	8.0	151.2
LL+ FO	59.6 (50.5)	5.8	5.4	119.0
TH+FO	67.2 (55.0)	6.4	6.0	129.3
PF+FO	64.4 (53.5)	7.2	6.5	136.7
LL+TH+FO	73.3 (58.9)	8.0	8.0	159.0
LL+PF+FO	71.0 (57.3)	9.2	8.7	173.3
TH+PF+FO	77.4 (62.2)	8.2	7.5	154.2
TH+PF+LL+FO	83.2 (66.3)	9.8	10.7	197.8
Fusarium oxysporum	57.3 (49.2)	5.0	4.3	65.6
CD (P = 0.05)	2.5	0.9	1.0	6.0

Figures in parentheses are arcsine transformed values

Table 3. Influence of biocontrol agents on root rot index of Kail pine incited by *F. oxysporum* f. sp. *pini* and *Rhizoctonia solani*

Treatment	Root rot index (%)	
	Fusarium oxysporum f. sp. *pini*	*Rhizoctonia solani*
Laccaria laccata (LL)	27.7 (34.4)	25.5 (30.3)
Trichoderma harzianum (TH)	20.2 (26.6)	16.4 (23.8)
Pseudomonas fluorescens (PF)	23.1 (30.1)	18.5 (25.4)
LL+TH	14.0 (21.8)	11.5 (19.8)
LL+PF	19.7 (26.2)	14.8 (22.6)
TH+PF	10.3 (18.3)	8.1 (16.5)
LL+TH+PF	7.2 (15.7)	5.3 (12.8)
Control	48.0 (44.0)	31.7 (34.5)
CD (P = 0.05)	1.7	1.2

Figures in parentheses are arcsine transformed values

caused 16.3 per cent more root rot than *R. solani* (Table 3). Less plant growth and biomass was observed in pathogen infected Kail pine seedlings due to less healthy sites for colonization. In the present study, application of antagonists (*T. harzianum, P. fluorescens* and *L. laccata*) individually as well as in combination significantly reduced the root rot percentage of pine seedlings.

Trichoderma harzianum showed the highest antagonistic activity when inoculated individually and caused 58.3 and 48.2 per cent reduction over the control in root rot caused by *F. oxysporum* and *R. solani* respectively, followed by *P. fluorescens* and *L. laccata*. However, *T. harzianum* was reported to have greater rhizosphere competence and parasitizes the pathogenic fungi (Naik, 2003). The combined inoculations of biocontrol agents were most effective in reducing the root rot of Kail pine seedlings than the individual inoculation. Combined inoculation of *L. laccata* + *T. harzianum* + *P. fluorescens* caused maximum decrease (85.0 and 83.2%) in root rot over the control. The antagonists in the rhizosphere are likely to compete with the pathogen for the host surface and nutrients as well as the possibility of inhibiting pathogenic

growth through antibiosis/ mycoparasitism (Howell, 2003). Accordingly 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 assumed to be as those primarily acting upon the pathogens including mycoparasitism, antibiosis and competition for resources and space (Harman, 2006). Thus reduction of root rot in Kail pine seedlings incited by *F. oxysporum* and *R. solani* may be due to the biological control action of *T. harzianum* on these pathogens.

In the present study, reduction in root rot due to *L. laccata* may be attributed to the protection, induced resistance and release of antimicrobial compounds by the ectomycorrhiza fungus. Fungal sheath around roots seems to have restricted the advancement of pathogen into the mycorrhizal cortex (Zak, 1964). 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 the 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*.

Antagonistic activity of *P. fluorescens* demonstrated towards the root rot pathogen of Kail pine seedlings in the present study might be attributed to the production of lysing or toxic substances or antibiotics. Lim *et al.* (1991) reported that the *Pseudomonas stutzeri* YPL-1 released extracellular b1,3-glucanase and chitinase which are the key enzymes in the lysis of fungal cell walls. Fluorescent *Pseudomonas* spp. are reported to inhibit the plant pathogenic fungi in the rhizosphere by the production of siderophores (Laha *et al.,* 1992).

Biocontrol agents (*T. harzianum, P. fluorescens* and *L. laccata*) inoculated individually or in combination significantly improved the plant growth (seedling height and root length) and dry plant weight and improved the seed germination. *T harzianum* gave maximum seed germination (81.4%). The increase in plant growth and biomass was associated with growth promoting effect of biocontrol agents and protection against the pathogenic microorganisms. Among the bioagents inoculated individually, the ectomycorrhiza fungus *L. laccata* significantly showed higher plant growth with 33.7, 45.0 and 36.7 per cent increase in shoot height, root length and dry plant weight, respectively, as compared to uninoculated control (Table 2). The seedlings with considerable ecto-mycorrhizal colonization rapidly regenerate new lateral roots, create more new sites for ectomycorrhizae and thereby utilize available nutrients more efficiently than non-mycorrhizal seedlings (Marx and Hatchell, 1986). The favorable 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) or indirectly by alteration in root physiology, uptake of minerals and pattern of exudation into the mycorrhizosphere (Leyval and Berthelin, 1990). Combined inoculation of all the three bioagents (*L. laccata* + *T. harzianum* + *P. fluorescens*) in the presence of *F. oxysporum* represented the highest increase in growth and biomass of Kail pine seedlings. Seedling height, root length and dry weight of Kail pine seedlings were increased by 96.0, 148.8 and 201.5 per cent, respectively as compared to control (*F. oxysporum*). Steep increase in plant growth may be ascribed to the synergistic growth promoting action of biocontrol agents as well as more solubilization of mineral nutrients besides reducing the pathogenic effect of root rot fungus. *Pseudomonas fluorescens* seems to be a good candidate for optimising the efficiency of ecto-mycorrhizal mycelium inoculum. Garbay and Duponnois (1992) reported that *P. fluorescens* promoted mycorrhizal formation of *L. laccata* in Douglas fir and oak seedlings, thereby increased the growth of seedlings. Werner (2002) observed that mycorrhizal *Pinus sylvestris* seedlings inoculated with *T. virens* produced a significantly higher plant growth and biomass.

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