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In vitro study of fungicides and biocontrol agents against *Fusarium oxysporum* f.sp. *pini* causing root rot of Western Himalayan fir (*Abies pindrow*)

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Nine fungicides namely: carbendazim, hexaconazole, thiophonate methyl, triadimefan, metalaxyl, mancozeb, captan, copper oxychloride and chlorothalonil and seven bioagents namely: *Trichoderma harzianum*, *Trichoderma viride*, *Gliocladium virens, Lacaria laccata*, *Boletus edulis, Suillus placids and Russula lutea* were evaluated *in vitro* by using dual culture and cultural filtrate techniques for their effect on the inhibition of mycelial growth and spore germination of *Fusarium oxysporum* f.sp. *pini.* Among systemic fungicides maximum inhibition in mycelial growth and spore germination was observed in the carbendazim followed by other fungicides. While among non-systemic fungicides maximum mycelial growth inhibition and spore germination was observed in mancozeb followed by other fungicides. In dual culture maximum mycelial growth inhibition was recorded in *T. harzianum* followed by filtrates of bioagents.

Key words: Bioagents, cultural filtrate, dual culture, fungicides, *Fusarium oxysporum*

INTRODUCTION

Many fungi have been identified by various workers as causal organism of fungal rot diseases in all parts of the world. The plants are exposed to persistent pathogenic attacks, particularly those inciting root rot and wilt diseases at primary stages of plant. Root rot and wilt disease on conifers such as fir, pine and spruce at nursery stages is major problem since 1990's in Europe (Lilja et al., 2010). The principal root rot fungi which pose serious threat to fir and other conifers nurseries varying intensities include *Fusarium oxysporum*, *Rhizoctonia solani* and *Macrophomina phaseolina* (Wafaa and Haggag, 2002; Dar et al., 2011). The fungi penetrate into root epidermal cell wall, grow intercellulary, decompose cell wall constituents and persist by metabolizing cell contents. In view of highly devastating nature of root rot pathogens, effective disease management is essential to raise healthy 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 effective use of antagonistic bacteria, actinomycetes and fungi against several soil-borne pathogens has been demonstrated in several field and horticultural crops (Yobo et al., 2010). Symbionts, such

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as mycorrhizal fungi and free living organisms form integral components of pine rhizosphere and growth promoting interactions (Taylor and Alexander, 2005). The cultural filtrate of mycorrhiza, *Suillus cdlinius*, *Hebeloma mesophaeum* and *Paxillus* sp reportedly exhibit antagonistic effect on the mycelial growth and spore germination of *F. oxysporum* and *Pythium vexans* and the antifungal activity has been attributed to oxalic acid production (Quarraqi et al., 2005; Yamaji et al., 2006).

Chemical control measures have been tested and found effective in the control of diseases (Ogundana and Denis, 1981; Plumbley, 1985). Different protectants and systemic fungicides have been reported to be used *in vitro* against the fungus *Fusarium* sp. Fungicides may act on or interrupt the metabolic system of the pathogen (Bilgrami and Dube, 1976). The effectiveness of a fungicide depends on its innate toxicity and permeation. Certain protective fungicides although hazardous to environment are still used for the control of fungal diseases (Vaish and Sinha, 2003). Therefore, in the present investigation, inhibition of mycelial growth and spore germination of *F. oxysporum* exposed to different concentrations of some fungicides and bioagents were studied. The objectives of the study were to evaluate different fungicides and bioagents under lab conditions to find out the most effective one for final use. The results of these studies will be helpful to the growers to adopt the most suitable control strategy.

MATERIALS AND METHODS

The root rot pathogens were isolated from diseased roots, collected during the survey of fir (*Abies pindrow*) nurseries in years 2009 and 2010. Three to four surface-sterilized diseased root bits of 3 to 5 mm size were aseptically transferred to Potato Dextrose Agar (PDA) medium and plates incubated at 25 ± 2°C for seven days. 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). The fungal antagonists and ectomycorrhiza were isolated from the rhizosphere of fir trees by dilution plate method on PDA and Modified Melin Norkans 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 cultural-morphological characteristics and identified with the help of standard descriptions (Lakhanpal, 1988).

In vitro evaluation of various fungicides to check the colony growth and spore germination of the fungus *F. oxysporum* f.sp*. pini* was done through poisoned food technique described by Borum and Sinclair (1968) on PDA medium. The experiment was conducted in Completely Randomized Design (CRD) with 9 treatments and 3 replications. Five systemic fungicides namely: hexaconazole 5 EC, triadimefan 25 WP, carbendazim, 50WP, thiophonate methyl 70 WP, metalaxyl (Ridomil MZ) 68 WP, were tested each at 5 concentrations 10, 20, 30, 40, 50 ppm and four non-systemic fungicides viz, copper oxychloride 50 WP, mancozeb 75 WP, chlorothalonil 75 WP and captan 50 WP, were tested each at 5 concentrations 100, 200, 300, 400 and 500 ppm). After autoclaving, 25 ml of PDA medium amended with fungicides in 5 different concentrations in separate 100 ml flasks was poured in sterilized 90 mm petri plates. The PDA medium without fungicide was kept as control. 5 mm fungal disc of *F. oxysporum* f.sp*. pini* was picked from purified culture with the help of a sterilized cork borer and inoculated in the center of each petri plate. Three replicate plates were inoculated for each fungicidal concentration. The inoculated plates were incubated at 25 ± 2°C and mean colony diameter was measured after one week of inoculation. The percent inhibition in growth due to various fungicidal treatments at different concentrations was computed as follows:

Mycelial growth inhibition (%) = [(dc-dt)/dc] × 100

Where dc = average diameter of fungal colony in control, and
dt= average diameter of fungal colony in treatment group.

Spore suspensions of pathogen were taken from 7 day-old cultures on PDA containing at least 20 to 30 spores per microscopic field were made separately against five different concentrations of fungicides. One drop of about 0.1 ml of spore suspension was placed in a cavity glass slide containing a drop (about 0.1 ml) of fungicides. These slides were kept in moist chamber prepared by putting two folds of filter paper in both sides of petri-plates. These Petri plates were incubated at 25 ± 2°C for 24 h. Each treatment was replicated three times. The slides were finally examined under microscope for recording the percentage of conidial germination. The percent spore germination was recorded using formula given by Kiraly et al. (1974).

$$\text{Percent spore germination} = \frac{\text{Number of spores germinated}}{\text{Total number of spores examined}} \times 100$$

Dual culture

Antagonistic activity of fungal isolates (*Trichoderma harzianum*, *Trichoderma viride* and *Gliocladium virens*) against *F. oxysporum* f.sp*. pini* causing root rot of fir was assessed by dual culture technique (Utkhede and Rahe, 1983). Five mm mycelial discs each of one ten days old culture of test pathogen and biocontrol agent were taken with the help of a cork borer and placed on the fresh PDA plates about 60 mm apart in 90 mm petri-plate and incubated at 25 ± 2°C. The petri-plates with pathogen disc only served as control. Three replications for each treatment were laid out in a completely randomized design. Observations with respect to zone of inhibition on growth of the pathogen in dual culture as well as in control plates were recorded 7 days after incubation. The per cent inhibition in mycelial growth of the pathogen over control was calculated using formula given by Vincent (1947).

$$\text{Percent inhibition in mycelial growth/conidial germination} = \frac{C - T}{C} \times 100$$

Where, C = Mycelial growth/conidial germination in control
T = Mycelial growth/conidial germination in treatment

The *in vitro* antagonism in dual culture between ectomycorrhizal fungi (*Lacaria laccata*, *Boletus edulis, S. placids, Russula lutea*) and the root rot pathogen was studied on MMN agar according to Marx (1969). A 5 mm disc of 20 days old mycelial mat of the mycorrhizal fungus was placed about 2 cm away from the edge of 90 mm petri-plate containing 25 ml MMN agar medium. Fifteen days later 5 mm mycelial disc of pathogen was placed on the

opposite side of the plate, 4 cm away from the mycelial margin of the symbiont. Each fungus grown individually served as controls. The plates were incubated at 25 ± 2°C for six days in darkness. The observations on the radial growth of pathogen in treatment as well as in control plates were recorded and per cent mycelial inhibition calculated according to Vincent (1947). The morphology of the hyphae in the interaction zone was observed under light microscope.

Based on the growth and mycoparasitic nature; biocontrol agents were grouped in to various categories as per scale given by Bell et al. (1982) which is as follows:

(i) Strong antagonist: Growth of biocontrol agent very fast, it covered the entire medium surface and completely overgrew the pathogen.
(ii) Antagonistic: Growth of biocontrol agent very fast, it covered at least 2/3rd medium surface but without showing mycoparasitic action.
(iii) Moderate antagonist: Growth of biocontrol agent very slow but rapidly it overgrew the pathogen one in contact.
(iv) Slow antagonist: Growth of biocontrol agent and pathogen similar in magnitude, none appeared to be dominant to other.
(v) Poor antagonist: Growth of the pathogen fast, it colonized at least 2/3rd of the surface and appeared to withstand encroachment by the biocontrol agent.
(vi) Non antagonist: Growth of the pathogen very fast it completely overgrew the biocontrol agent and covered the entire medium surface.

Effect of cultural filtrates of antagonists on mycelia growth of test pathogen

The potato dextrose broth was prepared in 250 ml Erlenmeyer flasks. The standard cultural filtrate of antagonists @ 15 and 30 ml was added to 35 and 20 ml of sterilized broth, respectively in the flasks so as to make the final concentration of 30 and 60% of the cultural filtrate in broth. Each flask was inoculated with a 9 mm mycelial disc from the seven days old culture of the pathogen and inoculated at 25 ± 2°C for seven days. The resultant fungal growth was filtered through pre-weighed Watman No. 42 filter paper, dried at 60°C in hot air oven and weighed. The actual dry weight was obtained by subtracting the weight of the filter paper from the total filter paper mycelial weight.

RESULTS AND DISCUSSION

The non-systemic fungicides viz., Chlorothalonil 75 WP, Copper-oxychloride 50 WP, Mancozeb 75 WP and Captan 50 WP were evaluated at 100, 200, 300, 400 and 500 ppm concentrations on the basis of active ingredient whereas systemic fungicides namely: hexaconazole 5 EC, thiophenate methyl 70 WP, triadimefon 25 WP, metalayxl (Ridomil MZ) 68 WP and carbendazim 50 WP were evaluated at 10, 20, 30, 40 and 50 ppm concentrations also on the basis of active ingredient. The studies on *in vitro* evaluation of fungicides against *F. oxysporum* f sp. *pini* through poison food technique revealed that carbendazim at 50 ppm and mancozeb at 500 ppm concentration significantly inhibited the mycelial growth of test pathogen when compared to control. Tables 1, 2, 3 and 4 reveal that among the systemic fungicides carbendazim exhibited highest mycelial growth inhibition 100.0% and spore germination 84.5 at 50 ppm while among non-systemic fungicides mancozeb exhibited highest mycelial growth inhibition 100.0% and spore germination 80.0% at 500 ppm. These results are in accordance to the findings of Rana and Tripathi (1983), Prajapati et al. (2002), and Dubey and Kumar (2003). However, Gaur and Chakrabarti (2009) found captan and carbendazim to be most effective in arresting the mycelial growth of *F. mangiferae*. Nonsystemic fungicides prevent infection largely by inhibition of spore germination and germ tube elongation. The increased concentrations probably give a better effect (Iqbal et al., 2010).

In vitro evaluation of isolated antagonists under dual culture and culture filtrate conditions revealed growth inhibition of root rot pathogen (*F. oxysporum* f.sp. *pini*) by the test antagonists (Tables 5 and 6). Among the bioagents *T. harzianum* caused maximum mycelial growth inhibition of 92.5% in dual culture followed by *T. viride* (86.2%).These findings are in conformity with Rudresh et al., (2005) who noticed 77.0% inhibition of *F. oxysporum* by *T. viride* and completely overgrew the host mycelia once in contact with them. The formation of hyphal coils by *T. viride* on pathogenic colonies was also noticed.

L. laccata and *B. edulis* in dual culture with *F. oxysporum* f.sp. *pini* caused hyphal lysis of both the pathogens. Interaction caused rupturing and twisting of the hyphae at initial stage, gradually the protoplasm showed desiccation and shrinkage. Mycorrhizal fungi *L. laccata* and *B. edulis* showed maximum antagonistic activity against *F. oxysporum* f.sp. *pini* under *in vitro* conditions by 80.5 and 65.5% respectively. These findings are in conformity with Chakravarty and Unestam (1985) who reported similar findings with *L. laccata* on Dooglas fir root rot pathogen. The mycoparasitic activity of ectomycorrhizal fungi against *R. solani* has earlier been suggested by Zhao and Kuo (1988). Mycorrhizal fungus *L. laccata* showed significant antagonistic activity against *F. oxysporum* f.sp. *pini* under *in vitro* conditions. In the present study ectomycorrhizal fungi *L. laccata* and *Boletus edulis* under *in vitro* conditions showed variable response on fir root rot pathogen.

The culture filtrate of selected antagonists caused significant inhibition in mycelial growth of *F. oxysporum* f.sp. *pini*. However, the culture filtrate of *T. harzianum* was more effective and caused 77.9% hyphal inhibition of *F. oxysporum* f.sp. *pini*, followed by *T. viride* (74.6%). The production of toxins, antibiotics and cell wall degrading enzymes by the bioagents in cell free culture filtrates may be possible reasons for observed inhibition. *Trichoderma* spp. are known to produce chitinase and β-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 degree of inhibition observed in present study are in agreement with Rudresh et al. (2005) who observed 86.3 and 69.2% mycelial growth inhibition of *F.*

Table 1. *In vitro* effects of different concentrations of systemic fungicides on mycelial growth of *Fusarium oxysporum* f.sp. *pini.*

Fungicide/ Concentration	Mycelial growth inhibition (%)					
	10 ppm	20 ppm	30 ppm	40 ppm	50 ppm	Mean
Thiophenate Methyl (70 WP)	48.5 (46.6)	62.7 (54.3)	80.5 (65.5)	100.0 (90.0)	100.0 (90.0)	78.3 (69.2)
Hexaconazole (5 EC)	45.5 (43.7)	55.7 (49.5)	68.4 (57.4)	83.3 (67.3)	100.0 (90.0)	70.5 (61.5)
Triadimefan (25 WP)	40.2 (40.5)	50.5 (46.4)	62.0 (54.0)	77.5 (63.4)	92.4 (76.2)	64.5 (56.0)
Carbendazim (50 WP)	54.6 (48.3)	66.3 (55.7)	82.0 (66.2)	100.0 (90.0)	100.0 (90.0)	80.5 (74.8)
Metalaxyl (Ridomil MZ) (68 WP)	42.4 (41.0)	50.7 (46.4)	60.5 (51.4)	74.2 (62.7)	88.4 (67.4)	63.2 (53.7)
Mean	47.0 (44.2)	57.5 (50.4)	70.6 (59.0)	87.0 (74.6)	96.1 (82.7)	

CD (p= 0.05), Fungicide (A) 1.2, Concentration (B) 1.2, Factor (A X B) 2.0, *Figures in parenthesis are arc sine transformed values.

Table 2. *In vitro* effects of different concentrations of systemic fungicides on spore germination inhibition percentage of *Fusarium oxysporum* f.sp. *pini* after 24 h.

Fungicide/ Concentration	Spore germination inhibition (%)					
	10 ppm	20 ppm	30 ppm	40 ppm	50 ppm	Mean
Thiophenate Methyl (70 WP)	31.5 (33.8)	41.3 (39.9)	53.3 (46.8)	67.3 (55.1)	80.0 (63.8)	54.6 (47.8)
Hexaconazole (5 EC)	30.0 (33.6)	39.0 (38.6)	50.5 (45.3)	65.5 (53.7)	78.6 (63.4)	52.7 (46.9)
Triadimefan (25 WP)	26.5 (31.0)	37.0 (37.4)	49.3 (44.4)	62.5 (52.1)	75.5 (60.0)	49.5 (45.0)
Carbendazim (50 WP)	34.2 (35.6)	46.0 (42.6)	60.5 (50.7)	76.3 (60.8)	84.5 (66.5)	60.3 (51.3)
Metalaxyl (Ridomil MZ) (68 WP)	23.3 (29.0)	30.0 (33.1)	43.3 (40.9)	58.6 (49.5)	70.0 (56.9)	45.0 (41.9)
Mean	29.1 (32.6)	38.6 (38.3)	51.3 (45.6)	66.0 (54.2)	77.7 (62.1)	

CD (p= 0.05), Fungicide (A) 1.4, Concentration (B) 1.4, Factor (A X B) N.S., *Figures in parenthesis are arc sine transformed values.

Table 3. *In vitro* effects of different concentrations of non-systemic fungicides on mycelial growth of *Fusarium oxysporum* f.sp. *pini.*

Fungicide/ Concentration	Mycelial growth inhibition (%)					
	100 ppm	200 ppm	300 ppm	400 ppm	500 ppm	Mean
Captan (50 WP)	32.0 (34.5)	40.2 (40.5)	50.5 (46.4)	63.5 (55.5)	78.0 (64.0)	52.8 (48.1)
Mancozeb (75 WP)	53.3 (48.4)	60.5 (52.4)	70.4 (58.4)	82.2 (67.2)	100.0 (90.0)	73.2 (63.2)
Coperoxychloride (50 WP)	34.4 (35.6)	43.3 (42.2)	56.8 (50.4)	74.5 (62.9)	96.5 (79.6)	61.1 (54.1)
Chlorothalonil (75 WP)	45.5 (43.5)	53.5 (48.2)	63.5 (54.3)	75.5 (63.3)	100.0 (90.0)	67.6 (59.8)
Mean	40.3 (40.5)	48.8 (45.8)	60.3 (52.3)	74.9 (62.2)	93.3 (80.9)	

CD (p= 0.05), Fungicide (A) 1.01, Concentration (B) 1.01, Factor (A X B) 2.4, *Figures in parenthesis are arc sine transformed value.

Table 4. *In vitro* effects of various non-systemic fungicides on spore germination inhibition percentage of *Fusarium oxysporum* f.sp. *pini* after 24 h.

Fungicides/ Concentration	Spore germination inhibition (%)					
	100 ppm	200 ppm	300 ppm	400 ppm	500 ppm	Mean
Captan (50 WP)	26.5 (30.6)	33.3 (35.0)	42.5 (45.1)	55.0 (47.8)	70.0 (56.7)	45.4 (43.0)
Mancozeb (75 WP)	37.5 (37.4)	47.5 (43.2)	58.0 (49.7)	70.5 (56.9)	82.0 (63.1)	59.1 (50.0)
Coperoxychloride (50 WP)	30.5 (33.4)	38.3 (38.2)	48.5 (44.2)	60.0 (50.9)	74.2 (59.5)	50.3 (45.2)
Chlorothalonil (75 WP)	33.3 (35.2)	43.3 (41.1)	55.0 (47.8)	67.5 (55.1)	80.0 (61.6)	55.8 (48.1)
Mean	31.9 (34.1)	40.6 (39.3)	51.0 (46.7)	63.2 (52.6)	76.5 (60.2)	

CD (p= 0.05), Fungicide (A) 1.0, Concentration (B) 1.1, Factor (A X B) 2.3, *Figures in parenthesis are arc sine transformed values.

Table 5. *In vitro* effects of antagonists on mycelia growth of *Fusarium oxysporum* f.sp. *pini* in dual culture**.**

Antagonist	Growth inhibition (%)	Zone of inhibition	Anatgonism
Trichoderma viride	86.2 (65.0)	±	HA
Trichoderma harzianum	92.5 (74.7)	±	HA
Gliocladium virens	62.0 (50.5)	+	MA
Lacaria laccata	80.5 (63.7)	±	HA
Boletus edulis	70.5 (53.5)	+	HA
Russulla lutea	60.0 (50.0)	+	MA
Suillus placids	54.4 (47.4)	-	SL

CD (p= 0.05) 3.2, ± mycoparasitism, + zone of inhibition present: - zone of inhibition absent, HA = Highly antagonistic, MA =Moderately antagonistic, SL = Slow antagonistic.

Table 6. Effect of cultural filtrate of antagonists on the mycelial growth of *Fusarium oxysporum* f.sp. *pini.*

Treatments/ Concentration	30%	60%	Mean
T. harzianum	70.5 (56.9)	85.3 (55.1)	77.9 (56.0)
T. viride	67.3 (43.8)	82.0 (48.2)	74.6 (46.0)
G. virens	48.0 (46.1)	68.6 (40.5)	58.3 (43.3)
Lacaria laccata	56.0 (39.6)	75.5 (67.4)	65.7 (53.5)
Boletus edulis	52.5 (65.1)	71.3 (55.9)	61.9 (60.5)
Russulla lutea	42.3 (60.4)	63.6 (57.6)	52.9 (59.0)
Suillus placids	40.6 (52.9)	59.5 (50.3)	50.0 (51.6)
Mean	53.8 (52.1)	72.2 (53.6)	

CD (p= 0.05), Treatments (A) 0.97, Concentration (B) 0.52, Factor (A X B) 1.3, *Figures in parenthesis are arc sine transformed values.

oxysporum and *R. solani*, respectively, by culture filtrate of *T. harzianum*. Similarly, Maheshwari et al*.* (2002) observed 48.9 and 100% inhibition of sclerotial germination in *Sclerotium rolfsii* by the culture filtrate of *T. harzianum* and *P. fluorescens*, respectively.

The mycelial growth inhibition and the spore/sclerotial germination inhibition of test pathogens though varied with the antagonists generally showed significant increase with the increase in concentration of culture filtrate of bioagents. The quality and quantity of the inhibitory substances present in culture filtrate of antagonists could probably be responsible for observed differences in inhibition. These results are in conformation with Dubey and Patel (2001) and Bunker and Mathur (2001). *L. laccata* showed significant antagonistic activity against *F. oxysporum* f sp. *pini* under *in vitro* conditions. The cell free culture extract of *L. laccata* not only inhibited the mycelial growth but also significantly reduced the spore 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 (Duchesne et al*.*, 1987).

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