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Evaluation of Bioagents against the Infection of Green Mould (*Trichoderma* spp.) in *Pleurotus sajor-caju* Cultivation

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ABSTRACT

The aim of the present study was to check the efficacy of various bioagents against green mould (*Trichoderma* spp.) associated with the *Pleurotus* spp. cultivation both *in vitro* and *in vivo*. *Pleurotus* specie commonly known as oyster mushroom or dhingri mushroom is one of the most important mushrooms cultivated in india. Four bioagents namely *Pseudomonas fluorescence, Streptomyces* sp., *Bacillus cereus* and *Bacillus subtilis* (3 isolates., Bs-I, Bs-II and Bs-III) were screened against all *Trichoderma* spp. (*T. harzianum, T. viride* and *T. pseudokoninjii*) and *Pleurotus in vitro*. Antifungal efficiency was examined by streaking method. The percent inhibition produced by bioagents against *Trichoderma* spp. recorded was: *P. fluorescence* (35.2 to 41.8%), *Streptomyces* sp., (12.3 to 30.8%), *Bacillus cereus* (16.9 to 30.5%), Bs-I (35.1 to 40.9%), Bs-II (36.1 to 44.3%) and Bs-III (42.1 to 51.1%), respectively. Bacterial antagonists showing maximum inhibition against the pathogen and minimum inhibition against mushroom were further evaluated against the *Trichoderma* sp. in *in vivo* test in mushroom house. For *in vivo* test, sterilized lignite inoculated with the bacterial cultures of *P. fluorescence, B. subtilis* (Bs-I and Bs-II) was thoroughly mixed with the paddy straw substrate at 1, 2 and 3% concentrations, followed by the inoculation of 3 mL spore suspension of *Trichoderma* at the rate of 1×10^3 spores mL^{-1}. All tested antagonists produced more yield and reduced the disease intensity than control. The treatments which receive Bs-II as bioagent show maximum increase in yield (25.32 %) and minimum disease incidence (5.7%) at 3% concentration.

Key words: Efficacy, antagonists, mushroom, cultivation, inhibition, lignite

INTRODUCTION

Among commercially cultivated mushrooms *Pleurotus* species commonly known as oyster mushroom or dhingri mushroom is extensively cultivated throughout the world and contributed more than 24.1% of total world production (3.772 million metric tonnes in 1990) of mushroom (Munshi and Ghani, 2003). *Pleurotus* can be easily cultivated by simple method on number of base material which do not need composting. The mushroom (*Pleurotus sajor-caju*) is an edible basidiomycete having excellent flavour, taste and nutritional value. This mushroom has the highest protein content and has many other constituents such as Vitamin B_1 and Vitamin B_2 and low calorie levels. In addition, they are reported to be low in fat (2 to 3% by dry weight), a good source of essential aminoacids and contain 5 to 9% fiber (Yang *et al*., 2001). The culture of Oyster mushroom is becoming popular throughout the world because of its abilities to utilize a large variety of agricultural waste products (Stamets, 2000) and transform the ligno cellulosic biomass into food of

high quality, flavour and nutritive value. *Pleurotus* species have extensive enzyme systems capable of utilizing complex organic compounds that occur as agricultural wastes and industrial by-products (Baysal *et al*., 2003). *Pleurotus* spp. are having antiviral, anti-inflammatory, anticancer and immune modulation activities (Jose *et al*., 2002).

Pleurotus species are the most popular mushroom growing in India. In India, Jammu and Kashmir state comprises area of diverse climatic zones ranging from sub-tropical to temperate and alpine regions. Its cultivation in the Kashmir valley has increased manifold due to its easy cultivation on different agrowastes and also because of its medicinal importance. Unfortunately this mushroom is subject to many vagaries of nature viz., pests and diseases that adversely affect its production and productivity. Among the various moulds and competitors of *Pleurotus* spp. green moulds are reported to be devastating disease in the crop production of this mushroom. The main fungal species causing green mould have been identified as *Trichoderma viride* and *Trichoderma harzianum* (Sharma and Bahukhandi, 2003). *Trichoderma* spp. is most common antagonistic and mycoparasitic pathogen of *Pleurotus* crop causing green mould disease of *Pleurotus* crop (Oh *et al*., 2003). The main symptom of green mould disease is the appearance of greenish mycelium in the compost, bagging layer or fruiting bodies of *Pleurotus ostreatus*, 2-5 weeks after the beginning of production cycle. The pathogen inhibits the growth of mushrooms and in severe outbreaks, the fruiting bodies are not produced from contaminated beds (Park *et al*., 2005; Yu, 2001).

Green moulds are reported to be devastating disease in the crop production of mushrooms. It is also commonly occurring in mushroom houses in the Kashmir valley. Number of workers have recommended fungicidal treatment for management of this disease but growers are reluctant to use these chemicals as they also inhibit the growth of the *Pleurotus* mycelium and are non-economical and results in environmental pollution. This study was carried out to develop economically viable and eco-friendly fungistatic efficacy of bioagents against *Trichoderma* spp. both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Survey was conducted in 2007. The samples of pathogen were collected from M.R.T.C, SKAUST-K. The *in vitro* study was carried out in Division of Environmental Science, SKAUST-K and *in vivo* experiment in M.R.T.C, SKAUST-K, Srinagar.

***In vitro* evaluation:** Samples of *Pleurotus sajor-caju* substrate poly bags with green mould and green mould infected spawn bottles were collected from Mushroom Research and Training Center (M.R.T.C), SKAUST-K, Srinagar, during the period August 2007 to November 2007. In the laboratory, pieces of mycelium taken from green mould affected areas of infected paddy straw were placed on Potato Dextrose Agar (PDA) using a sterilized inoculating needle. The samples from infected bottles were obtained by dilution and plating of sampled material on rose benegal agar and also by dust sedimentation on the rose benegal agar medium. The plates were incubated at room temperature (27°C) until fungal growth was visible. A total of 14 isolates of *Trichoderma* spp. were collected. On the basis of cultural and morphological characteristics, *Trichoderma* isolates were classified into three species, namely *Trichoderma harzianum, Trichoderma viride* and *Trichoderma pseudokoninjii* (Park *et al*., 2004). The isolates were purified from dishes and maintained by periodic subculturing in PDA slants after every 15 days (Aneja, 2005).

The bacterial antagonists used during the study were obtained from Division of Environmental Science, SKAUST-K. Bioefficacy test of antagonists against *Trichoderma* spp. was done by streaking method. The respective bacterial bioagents were streaked on PDA plates. Four streaks were drawn on petriplates, in a square pattern. Each streak was 3 cm away from the center of the plate. After streaking, mycelial discs (5mm) each of Pathogen (*Trichoderma* spp.) and *Pleurotus sajor-caju* taken from the margin of 6 days old cultures, were inoculated in the center of PDA petriplates, separately. Each treatment was replicated thrice. The petri plates (no streaks) having Pathogen (*Trichoderma* spp.) and *Pleurotus sajor-caju* separately served as control. The petriplates were subsequently incubated at 26±1°C, till the complete growth was observed in control plates. Colony diameter of the *Trichoderma* spp. and *Pleurotus sajor-caju* in treated plates were recorded at two locations, right angle to each other and the average diameter was calculated. Percent growth inhibition over control was calculated according to the formula:

$$\text{Mycelial inhibition (\%)} = \frac{\text{Radial growth in control-Radial growth in treatment}}{\text{Radial growth in control}} \times 100$$

***In vivo* evaluation:** In this study, the bioagents which displayed least adverse effects on the growth of *Pleurotus sajor-caju* were evaluated to determine their inhibitory effect against the pathogen (*Trichoderma* spp.) during its cultivation. The cultivation trail was laid during 2009 in M.R.T.C, SKAUST-K.

Procurement of mushroom culture: The spawn of *Pleurotus sajor-caju* (Fr. Singer) used in present investigation was procured from Mushroom Research and Training Centre, Division of Plant Pathology, SKUAST-K. During the present investigation locally available cheap agricultural residue, paddy straw was used as base material for cultivation of *Pleurotus sajor-caju*.

Substrate pre-treatment: The chopping of paddy straw was done manually into bits of 3-5 cm in length and were cleaned thoroughly 2-3 times with tap water and then soaked in water for 12 h. These were then dipped in boiling water for 30 min, taken out, cooled in wooden basket and kept there till excess water was drained off. The desired moisture content of the straw was tested by squeezing the straw in between the palms and seeing that droplets of water do not trickle out from the straw.

***In vivo* test:** The most promising bacterial antagonists; *Bacillus subtillis* -I, *Bacillus subtilli*-II and *Pseudomonas fluorescence* were multiplied on Nutrient agar broth. Mass culture of bacterial antagonists was made on lignite. Each isolate was inoculated in the conical flasks (250 mL), containing 200 mL NA broth and incubated at 25±1°C for 48 h. After that, bacterial suspension was added in sterilized lignite powder at the rate of 1:2 v/w and mixed well under sterile conditions. This lignite powder was thoroughly mixed with the pre treated paddy straw substrate at the rate of 1, 2 and 3% (w/w) on dry weight basis just before spawning. The substrate was filled in polythene bags at the rate of 1 kg dry substrate per bag and the method of spawning was adopted using 1% spawn on dry weight basis of the substrate. Filling of substrate in the polythene bags was done in layers. Out of the three species of *Trichoderma*, *T. viride* was selected for the inoculation, as it shows the minimum inhibition by nearly all tested bioagents in *in vitro* test except *B. cereus* and Bs-II (Fig. 1). The straw amended with biofertilisers was inoculated with 3 mL ascospore suspension

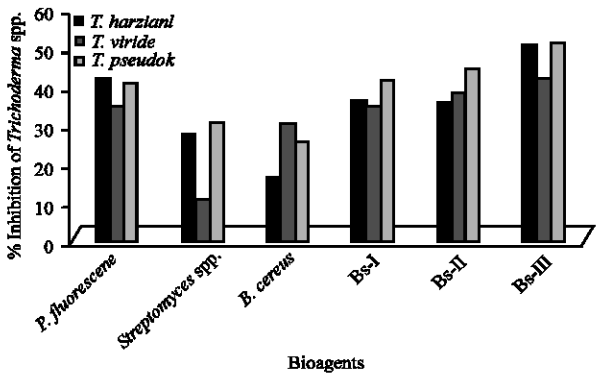

Fig. 1: Percentage of inhibition by bioagents on *Trichoderma* spp. mycelial growth

of *Trichoderma viride* with a spore load of (1×10^3 spores mL^{-1}) after spawning. Bags devoid of biofertilizers served as control. The filled bags were tied properly and 10-15 pinholes were made over the surface of the bag. Each treatment including check (no bioagent) was replicated 6 times in CRBD. The bags were then transferred to cropping room where temperature (28°C max. and 25°C min.) and relative humidity (80%) were maintained.

While carrying the above experiment *in vivo*, the observations on weight of 3 flushes (total yield) and disease incidence were recorded. In case of yield parameter, percent increase in yield in treated bags over control (un treated) was calculated using the formula:

$$\text{Percent increase in yield over control (\%)} = \frac{\text{Yield treatment-Yield control}}{\text{Yield treatment}} \times 100$$

Statistical analysis: In the *in vitro* experiments, R.B.D was applied. In *in vivo* trial, C.R.B.D was applied. All the experiments were analyzed statistically by the analysis of variance (ANOVA). The analyses of variance technique was applied for drawing conclusions from the data. The calculated value was compared with tabulated value at 5% level of probability for the appropriate degree of freedom.

RESULTS

Data presented in the Table 1 reveals that all bioagents were more or less inhibiting mycelial growth of *Trichoderma* spp. causing green mould in Dhingri (*Pleurotus* spp.) mushroom. *Bacillus sub*-III was highly effective against all *Trichoderma* spp., reduced the radial growth of *T. harzianum* by (50.4%), *T. viride* by (42.1%) and *T. pseudokoninjii* by (51.1%). *P. fluorescence*, Bs-I and Bs-II also show good antagonistic activity *in vitro* against *Trichoderma* spp. *Streptomyces* sp. and *Bacillus cereus* show the minimum inhibition in radial growth of *Trichoderma* spp. when compared to the other antagonists, reduced the radial growth of pathogen by (12.3 to 30.8%) (Table 1). None of the bioagents were able to cause complete inhibition of *Trichoderma* spp (Fig. 1). Amongst the bioagents evaluated *in vitro*, three most promising, Bs-I, Bs-II and *Pseudomonas fluoresence* were further evaluated *in vivo* against the disease.

In vivo evaluation of bioagents indicated that all the three bioagents at all concentrations of 1, 2 and 3% were significantly effective in reducing the Green mould of *P. sajor caju* as compared to the control (Table 3). The disease incidence in bioagent treatments varied from 5.7 to 55.5%. It

Table 1: Effect of various bioagents on the radial growth of *Trichoderma* spp. (T_1= *T. harzianum*, T_2= *T.viride* and T_3= *T. pseudokoninjii*) and *Pleurotus sajor-caju* (Pl)

	*Radial growth(mm)				% Inhibition over control			
Bacterial antagonist	Pl	T_1	T_2	T_3	Pl	T_1	T_2	T_3
P. fluorescence	70.0	47.0	56.6	48.6	17.6	44.6	33.3	42.7
	(56.7)	(43.2)	(48.7)	(44.1)	(24.7)	(41.8)	(35.2)	(40.7)
Streptomyces sp.	59.0	66.3	81.0	62.0	30.5	21.9	4.6	27.0
	(50.1)	(54.5)	(64.1)	(51.9)	(33.5)	(27.5)	(12.3)	(30.8)
Bacillus cereus	64.6	77.6	63.0	68.6	23.9	8.5	25.8	19.1
	(53.4)	(61.7)	(52.5)	(55.9)	(29.2)	(16.9)	(30.5)	(25.9)
B. subtilis-I	77.3	55.0	56.6	48.3	8.9	35.2	33.2	43.1
	(61.5)	(47.8)	(48.7)	(44.0)	(17.2)	(36.3)	(35.1)	(40.9)
B. subtilis-II	72.0	55.3	52.0	43.3	15.2	34.8	38.7	48.9
	(58.0)	(48.0)	(46.1)	(41.1)	(22.9)	(36.1)	(38.4)	(44.3)
B. subtilis-III	69.3	34.3	46.6	33.3	18.4	59.5	45.0	60.7
	(56.3)	(35.8)	(43.0)	(35.2)	(25.3)	(50.4)	(42.1)	(51.1)
Control	85.0	85.0	85.0	85.0	-	-	-	-
	(67.2)	(67.2)	(67.2)	(67.2)				
CD (0.05) =	1.27	9.42	2.95	7.20	1.39	7.91	10.22	5.80
SE =	2.23	5.35	1.68	4.09	2.48	4.44	5.14	3.26

Mean of three replications. Values in parenthesis are arc sine transformed values

Table 2: Influence of various bioagents on total yield of *Pleurotus sajor-caju* (Fr.) Singer during one month cropping period

		*Yield (g)		% increase in yield	Mean of 3 concentrations
Bioagent	Conc. (%)	Control value	Treatment	over control	(1.0, 2.0 and 3.0)
Bacillus subtilis-I	1.0	776.6	791.6	1.8	
	2.0	-	816.6	4.8	4.1
	3.0	-	825.0	5.8	
Bacillus subtilis-II	1.0	-	943.3	17.6	
	2.0	-	1016.6	23.6	22.1
	3.0	-	1040.0	25.3	
Pseudomonas fluorescence	1.0	-	915.0	15.1	
	2.0	-	971.6	20.0	19.4
	3.0	-	1010.0	23.1	

Mean of three flushes, SE = 45.9, CD at 5% = 76.6

is clear from the Table 3 that all the three bioagents were effective in reducing the disease incidence. However, *B. subtilis* (Bs-II) at higher test concentration of 3% proved significantly effective by exhibiting minimum mean disease incidence 5.7%. *P. fluorescence* and *B. subtilis* (Bs-I) each at 3% concentration exhibited 22.2 and 44.4% disease incidence, respectively. At 2% concentration, *B. subtilis* (Bs-II) proved to be effective in reducing the disease incidence upto 11.2% followed by *P. fluorescence* (22.3%) and *B. subtilis* (Bs-I) (44.4%). At the lowest concentration of 1%, *B. subtilis* (Bs-II) again proved to be effective in reducing the disease incidence by 33.3%, followed by *P. fluorescence* (44.4%) and *B. subtilis* (Bs-I) (55.5%). There was a direct correlation between the incidence of Green mould and yield of mushroom. All the bioagents significantly enhanced the yield of *P. sajor caju* as compared to control (Table 2). It was observed that the effect of concentration (1, 2 and 3%) on the yield was significant as the increase in concentration of

Table 3: Effect of bioagents on disease incidence of *Trichoderma* in Dhinghri mushroom crop

Bioagent	Conc. (%)	Disease incidence (%)	Mean bioagents
Bacillus subtilis-I	1.0	55.5 (48.1)*	
	2.0	44.4 (41.7)	48.1 (43.8)
	3.0	44.4 (41.7)	
Bacillus subtilis-II	1.0	33.3 (35.2)	
	2.0	11.2 (19.5)	16.7 (22.8)
	3.0	5.7 (13.8)	
P. fluorescence	1.0	44.4 (41.7)	
	2.0	22.3 (28.1)	29.6 (32.6)
	3.0	22.2 (28.1)	
Control (no bioagent)		88.8 (70.4)	

*Values in parenthesis are Arc sine transformed values, SE = 10.6, CD at 5% = 17.7

bioagents from 1 to 3%, the yield also increased. At higher concentration of 3%, maximum increase in yield over control (25.3%) was recorded in treatment which received *B. subtillis* (Bs-II) as bioagent. It was followed by *Pseudomonas fluorescence* (23.1%) and *Bacillus subtillis* (Bs-1) (5.8%) @ 3%. At the concentration of 2%, maximum increase in yield (23.6%) was shown by *Bacillus subtillis* (Bs-II), followed by *P. fluorescence* (20.0%) and *B. subtillis* (Bs-I) (4.8%). At 1% concentration, maximum increase in yield over control was recorded in *B. subtillis* (Bs-II) (17.6%) followed by *P. fluorescence* (15.1%) and *B. subtillis* (Bs-I) (1.8%).

DISCUSSION

All isolates of *Bacillus subtilis* (Bs-I, Bs-II and Bs-III), *Bacillus cereus, Streptomyces* sp. and *Pseudomonas flouresence* were effective against all *Trichoderma* spp. The bets bioagents showing inhibitory effect on *Trichoderma* spp. were isolates of *Bacillus subtilis* and *Pseudomonas florescence*. Constantinescu *et al.* (2004) also advocated that among 18 strains of *Bacillus subtilis* and 24 strains of *Pseudomonas* spp., 3 strains of *Pseudomonas* and 2 strains of *Bacillus subtilis* showed good antagonistic activity *in vitro* against *Trichoderma viridae*, the causal agent of green mould of *Pleurotus ostreatus*. Neither the bacteria cultures in *in vitro* tests nor their biopreparates inhibited the growth of *Pleurotus ostreatus* mycelium which is in contradictory with the present study as *Pleurotus* was also inhibited by bacterial biocides. Bs-II showed the maximum inhibition against *Trichoderma* spp . This might be due the effective antimicrobial substances produced by this bacteria (Wang and Ng, 2004). Chittihunsa *et al.* (2007) reported the antifungal efficiency of *Bacillus subtilis* against *Trichoderma* sp. and suppression of infection of *Trichoderma* spp. in oyster mushroom cultivation. Zhang *et al.* (1997) reported that out of 576 isolates of *streptomycetes*, 6 demonstrated the ability to inhibit the growth of *Trichoderma viride*. In the present study, yield was reduced in control inoculated with *Trichoderma* spore suspension with no bioagent. This finding was also advocated by Jayalal and Adikuram *et al.* (2007) who observed that green mould disease in Oyster mushroom (*Pleurotus ostreatus*) caused by *Trichoderma harzianum* results in considerable inhibition of growth of mycelium and fruit bodies of Oyster mushroom lowering the yield substantially. Mishra and Singh (2005) reported that the 10 Fluorescent *Pseudomonad* Isolates (FPIs) and an Actinomycete Isolate (AI), were evaluated *in vitro* against *Trichoderma viride* and *Agaricus bisporus*. In the case of bacterial biocides, Fluorescent *Pseudomonad* Isolate II (FPI-II) was effective and reduced the linear growth of *Trichoderma viridae* by approximately 73.68%. FPI-II resulted in highest yield of 13.23 and 16.62 kg compost/q in the first and second

crop, respectively, compared to 9.34 and 9.97 kg from their corresponding controls. Bhanwar and Thakur (2004) reported that wheat straw supplemented with *Bacillus polymixa* and *Pseudomonas straita* @ 2% increased the weight of sporophores of *Pleurotus* spp. Singh *et al.* (2000) studied that 2 isolates of fluorescent *pseudomonads*, designated as CIIb and CVa reduced time taken for pin head initiation and increased number of pin heads significantly. Their introduction in the casing mixture individually or in combinations significantly increased the yield of *Agaricus bisporus* and reduced time taken for primordial development by over 7 days.

CONCLUSION

Currently, the use of inhibitory microbial agent is one of the most possible methods for controlling some plant diseases. It is reasonable to collect and screen for more microbes with high activity to suppress the green mold infection. We found two most promising bioagents, *Bacillus subtilis*- II and *Pseudomonas* florescence that were able to minimize the infection of green mould both in *in vitro* and *in vivo* treatments. In *in vivo* trial, incorporation of these bioagents into the compost reduces the disease intensity and enhances the yield when compared to the check (no bioagent). However, there are still several further studies to be carried out. Moreover, breeding for mushroom that can resist *Trichoderma* sp. is one of the interesting aspects (Hatvani *et al.*, 2002) that will make mushroom cultivation a success.

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