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Research Article

In vitro efficacy of casing and compost isolated bacterial inoculants against *Verticillium fungicola* (Preuss) Hassebrauk and *Agaricus bisporus* (Lange) Imbach

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ABSTRACT: The aim of this study is to determine some effective antagonistic bacterial inoculants for the biological control of *Verticillium fungicola* causing dry bubble disease of white button mushroom (*Agaricus bisporus*). Out of the 32 bacterial inoculants isolated from the casing and compost materials and screened against *V. fungicola*, only 5 isolates were selected for further investigation. The cell free culture filtrates of these bacterial isolates were further evaluated against the pathogen and host fungus at different concentrations. The bacterial culture filtrates inhibited mycelial growth and conidial germination of *V. fungicola* to varied extents. The isolate PC-I showed maximum inhibition 71.50 per cent of mycelial growth and similar trends of inhibition were recorded in case of conidial germination (84.13 per cent of the pathogen) followed by PS-I, PS-II, *Bacillus thuringiensis*, BC-IX, BC-V and BC-IV, respectively. *Azotobacter* sp. proved less effective in inhibiting the mycelial growth of the pathogen. The culture filtrates of PS-I and PS-II stimulated the growth of *A. bisporus* at all the test concentrations, whereas the remaining culture filtrates inhibited its growth.

KEY WORDS: Bacterial inoculants, casing, compost, *in vitro*, *Verticillium fungicola*, white button mushroom

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INTRODUCTION

The white button mushroom, *Agaricus bisporus* derives its nutrition from specially prepared substrate known as compost, wherein microbial populations play an important role in converting and degrading the straw and form lignin-humus complex which is later on utilized by the mushroom mycelium (Flegg *et al.,* 1985). To induce sporophore formation in *A. bisporus* it is necessary to cover the mycelium colonized substrate with a layer of soil, neutralized peat moss or other similar materials. The application of these materials, termed the casing layer, is essential for initiation of the basidiomata (Eger, 1972; Flegg *et al.,* 1985). The microbes, especially the bacteria inhabiting casing soil have been known to act as the source of stimulus and nutrition for initiation and development of mushroom fruiting body (Curoto & Favelli, 1972; Eger, 1972; Peerally, 1978). The initiation and development of sporophore in *A. bisporus* are controlled by some specific groups of microflora, particularly pseudomonads, a component of bacterial flora invariably present in the casing soil (Hayes et al. 1969). Among the pseudomonads, siderophore producers constitute a sizable population. These siderophores act as growth promotion factors and some of them serve as potent antibiotics (Neilands, 1981). These are also known to reduce parasitic activities of various fungal pathogens (Byer and Sikora, 1990; Singh *et al.,* 2000). The present study was aimed to find an effective approach for the control of *Verticillium fungicola* causing dry bubble disease of *A. bisporus* using antagonistic bacterial inoculants.

MATERIALS AND METHODS

The pathogen *V. fungicola* was isolated following the standard procedure using Malt Extract Agar medium (Johnston and Booth, 1983). Mushroom sporophores showing typical symptoms of dry bubble disease (freshly collected) were repeatedly used for isolation of the pathogen. Morphological and cultural characteristics were studied under laboratory conditions and compared with standard description given by North and Wuest (1993) for identification of *V. fungicola*. The identity of the pathogen was also confirmed from National Bureau of Agriculturally Important Micro-organisms (NBAIMS), New Delhi. *Agaricus bisporus* culture used in the experiment was

obtained from Mushroom Research and Training Centre, SKUAST-K, Srinagar.

Samples from casing and compost were collected for isolation of bacterial inoculants employing serial dilution plate technique (Walksman, 1922). Selected bacterial colonies were streaked on to fresh nutrient agar, incubated at 25 ± 2°C and checked for purity. All the isolates were maintained on Nutrient agar prior to study of morphological and cultural characteristics, and biochemical tests were done by standard methods (Schaad, 1980). Pure cultures of the isolates were maintained on King's B media (King's *et al.,* 1954) and viability of the cultures were maintained through periodic transfer and storage at 2–5°C.

Out of 32 bacterial inoculants 5 isolates were selected for further investigation against the pathogen and the host fungus. The isolates of *Azotobacter sp.* and *Bacillus thuringiensis* were isolated from the commercial preparations. The bacterial culture filtrates were evaluated against *V. fungicola* (poisoned food and slide germination techniques) and against *A. bisporus* by poisoned food technique only (Kumar *et al.,* 1988). To prepare the cell free culture filtrates, these bacteria were grown on nutrient broth in 250ml Erlenmeyer flasks containing 100ml broth media and incubated at 28 ± 2°C for 15 days on a reciprocating shaker (120 strokes/minute). After incubation, the cultures were filtered through Whatman's filter paper No. 45 and centrifuged at 12000 rpm for 20 minutes and the supernatant was passed through 0.45µm millipore filters. This formed 100% stock solution of the cell free culture filtrates, which were evaluated at 10, 20, 40 and 60 per cent concentrations against both the pathogen and *A. bisporus* as per the technique of Nene and Thapliyal (1979). Culture filtrates at double the required concentrations were added to double strength Malt Extract Broth medium (MEB) in each of the 150 ml Erlenmeyer flasks in such a manner so as to get the final concentrations of the culture filtrates to the desired level and also to get the MEB stabilized to standard strength using sterilized distilled water. The flasks were inoculated with 5 mm diameter mycelial discs taken from ten-day old cultures of the pathogen and host with the help of a cork borer and incubated at 25 ± 2°C, maintaining three replications for each treatment. After the entire surface of medium was covered in control flasks, the mycelial mat was separated out by filtration, dried at 40°C for 72 hours and subsequently cooled. The mycelial mat was weighed and the actual dry weight of the mat was calculated by subtracting the weight of the filter paper. The per cent inhibition in mycelium growth was calculated as per the method of Vincent (1947). For studying the comparative efficacy of free culture filtrates at different concentrations slide conidial/spore germination technique as described by Montgomery and Moore (1938) was employed. The spore suspension of the test pathogen was prepared by dispensing a loopful of the fungal culture in sterilized distilled water and standardizing the spore concentration so as to give about 25 to 30 spores per low power microscopic field (10X). A volume of 0.5 ml of spore suspension was added to each 0.5 ml of the double strength bacterial cell free culture filtrates in test tubes, separately, shaken well and a drop of the mixture placed in cavity slide(s). The cavity slides were placed in moist chambers prepared by lining the 90 mm diameter Petri dishes from within with a double layer of moist filter paper, maintaining three replications for each treatment. The spore suspension diluted with equal volume of distilled water served as check. The slides were examined after 24 hrs of incubation at 25 ± 2°C for spore germination by taking counts at different microscopic fields on each cavity slide and the per cent inhibition in spore germination was computed. The analyses of the experiments were performed by using completely randomized design (CRD).

RESULTS AND DISCUSSION

The cell free culture filtrates inhibited the mycelial growth of *V. fungicola* significantly over check (Table 1). On overall mean basis, culture filtrate of PS-I exhibiting maximum inhibition (71.50%) followed by PS-II (57.00%) and *B. thuringiensis* (55.36%), with no significant difference between PS-II and *B. thuringiensis*. The culture filtrate of *Azotobacter* sp. exhibited the least (28.96%) inhibition of mycelial growth of the test pathogen. The culture filtrate concentrations were also found to exert statistically significant differences. On average, a minimum inhibition of 11.62 per cent was recorded at 10 per cent concentration, which gradually increased to 37.91, 66.48 and 94.34 per cent at 20, 40 and 60 per cent concentrations, respectively. There also existed a significant interaction between culture filtrates and their concentrations. At 10 per cent concentration, the culture filtrate of PS-I exhibited maximum (22.79%) inhibition in mycelial growth followed by PS-II (18.29%) and *B. thuringiensis* (10.38%). At 20 per cent concentration, PS-I was the best exhibiting 63.24 per cent inhibition of mycelial growth followed by *B. thuringiensis* (46.98%) and BC-IX (40.71%). At 40 per cent concentration, PS-I exhibited complete inhibition followed by PS-II (74.92%). At 60 per cent concentration, PS-I, PS-II, BC-V, BV-IX and *B. thuringiensis* showed complete inhibition of the test pathogen except BC-IV and *Azotobacter* sp. (91.61 and 68.78 per cent inhibition in mycelial growth of the pathogen). The effect of culture filtrates at 10, 20, 40 and 60 per cent concentrations on the conidial germination of *V. fungicola* is presented in Table 2. All the culture filtrates significantly inhibited the conidial germination of the pathogen, as compared to check. On average, maximum inhibition (84.13%) in conidial

Table 1. *In vitro* efficacy of various cell free culture filtrates in inhibiting the mycelial growth of *V. fungicola*

Culture filtrates	**Per cent inhibition mycelial growth at concentration (%)*				
	10	20	40	60	Means
PS-I	22.79 (28.48)	63.24 (52.67)	100.00 (89.96)	100.00 (89.96)	71.50 (65.27)
PS-II	18.29 (25.30)	34.82 (36.15)	74.92 (59.93)	100.00 (89.96)	57.00 (52.83)
BC-IV	8.86 (17.22)	32.48 (34.72)	60.01 (50.75)	91.61 (73.27)	48.24 (43.99)
BC-V	7.92 (16.32)	35.30 (36.43)	66.86 (54.83)	100.00 (89.96)	52.52 (49.39)
Azotobacter sp.	3.75 (10.96)	11.89 (20.15)	31.44 (34.09)	68.78 (56.01)	28.96 (30.30)
BC-IX	9.41 (17.78)	40.71 (39.62)	68.09 (55.59)	100.00 (89.96)	54.55 (50.73)
B. thuringiensis	10.38 (18.77)	46.98 (43.25)	64.09 (53.16)	100.00 (89.96)	55.36 (51.28)
Means	11.62 (19.26)	37.91 (37.57)	66.48 (56.90)	94.34 (82.72)	

	$SE_{(d)}$ ±	CD (P = 0.05)
Culture filtrates	(0.48)	(0.96)
Concentrations	(0.36)	(0.72)
Culture filtrates x Concentrations	(0.96)	(1.92)

*Mean of 3 replications; Figures within parentheses are arc sine angular transformed values

germination of the test pathogen was exhibited by PS-I followed by *B. thuringiensis* (80.51%) and PS-II (71.92%). Minimum inhibition (41.23 per cent) in conidial germination of the test pathogen was exhibited by *Azotobacter* sp. The concentrations and different culture filtrates were found to exert statistically significant differences. On average, the per cent inhibition in conidial germination was least (27.29%) at 10 per cent concentration, which increased to 56.89, 87.14 and 96.94 at 20, 40 and 60 per cent concentrations. At 10 per cent concentration, the culture filtrate of PS-I showed maximum (45.13%) inhibition in conidial germination followed by *B. thuringiensis* (37.71%) and PS-II (31.00 %) and least inhibition of 13.69 per cent by culture filtrate of *Azotobacter* sp., respectively. A similar trend in inhibition of conidial germination was exhibited by all the culture filtrates when assayed at 20 and 40 per cent concentrations. At 60 per cent concentration, all the culture filtrates exhibited complete conidial germination except *Azotobacter* sp. (78.63%).

The effect of culture filtrates on the mycelial growth of *A. bisporus* is presented in Table 3. All the culture filtrates significantly inhibited the mycelial growth compared to check, except PS-I and PS-II. The data revealed that on verage, the host mycelium growth was interestingly stimulated by PS-I (8.18%) and PS-II (4.44%). But maximum inhibition of host mycelium was exhibited by BC-V (56.16%) followed by *B. thuringiensis* (48.84%). The least inhibition (13.19 per cent) in mycelial growth of the host was exhibited by BC-IX. On average, the per cent inhibition in mycelial growth was least (3.36%) at 10 per cent concentration, which increased to 13.13, 30.75 and 49.32 per cent at 20, 40 and 60 per cent concentrations, respectively. At 10, 20, 40 and 60 per cent concen-trations, the culture filtrate of PS-I stimulated mycelial growth followed by PS-II as compared to check. At 10 per cent concentration, culture filtrate of BC-V exhibited maximum inhibition (6.48%) in mycelial growth followed by *B. thuringiensis* (6.34%). At 20, 40 and 60 per cent concentrations, the inhibition in host mycelial growth was maximum (44.10, 74.08 and 100%) in BC-V treated flasks followed by *B. thuringiensis* (15.77, 73.28 and 100%).

The existing scenario of mushroom production in Kashmir valley and other parts of the country is primarily in the hands of growers who have meager facilities at their mushroom farms and where conditions are impoverished and environmental controls minimum. Due to the prevalence of unhygienic conditions in and around the mushroom farms and lack of pasteurization facility dry bubble and other diseases continue to thrive and attack mushroom crops causing economic losses to the growers. Biological control is becoming as an alternative measure in controlling pathogens. In fact, closed growing systems such as mushroom growing houses are very suitable for the practice of biological control.

The results revealed clearly that among the microflora existing in the casing/compost the those having antagonistic potential could be used for management of different fungal diseases of *A. bisporus*. The earlier studies by Baker and Scher (1987), Jhune *et al.* (1990), Kloepper (1992), Ahlawat and Rai (1997), Bora *et al.,* 2000; Bhat *et al.,*

Table 2. *In vitro* efficacy of various cell free culture filtrates in inhibiting the conidial germination of *V. fungicola*

Culture filtrates	*Per cent inhibition mycelial growth at concentration (%)				
	10	20	40	60	Means
PS-I	45.13 (42.18)	91.41 (72.98)	100.00 (89.96)	100.00 (89.96)	84.13 (73.77)
PS-II	31.00 (33.81)	62.63 (52.30)	94.07 (76.15)	100.00 (89.96)	71.92 (63.05)
BC-IV	15.56 (23.20)	32.53 (34.74)	84.68 (67.00)	100.00 (89.96)	58.19 (53.72)
BC-V	25.41 (30.25)	60.33 (50.95)	92.65 (74.53)	100.00 (89.96)	69.59 (61.42)
Azotobacter sp.	13.69 (21.69)	22.37 (28.15)	50.26 (45.13)	78.63 (62.45)	41.23 (39.36)
BC-IX	22.55 (28.32)	44.63 (41.89)	88.33 (70.09)	100.00 (89.96)	63.87 (57.56)
B. thuringiensis	37.71 (37.86)	84.34 (66.72)	100.00 (89.96)	100.00 (89.96)	80.51 (71.13)
Means	27.29 (31.04)	56.89 (49.68)	87.14 (73.26)	96.94 (86.03)	
		$SE_{(d)}\pm$	CD (P = 0.05)		
Culture filtrates		(0.73)	(1.46)		
Concentrations		(0.55)	(1.10)		
Culture filtrates x Concentrations		(1.46)	(2.92)		

*Mean of 3 replications; Figures within parentheses are arcsine angular transformed values

Table 3. *In vitro* efficacy of cell free culture filtrates in inhibiting/stimulating the mycelial growth of *A. bisporus*

Culture filtrates	*Per cent inhibition mycelial growth at concentration (%)				
	10	20	40	60	Means
PS-I	2.90 (9.36)	4.24 (11.63)	8.92 (17.34)	16.69 (24.09)	8.18** (15.61)
PS-II	0.68 (4.45)	2.86 (9.72)	4.95 (12.83)	9.28 (17.71)	4.44** (11.18)
BC-IV	2.09 (8.29)	10.49 (18.84)	22.23 (28.11)	35.48 (36.54)	17.57 (22.94)
BC-V	6.48 (14.54)	44.10 (41.59)	74.08 (59.37)	100.00 (89.96)	56.16 (51.36)
Azotobacter sp.	2.31 (8.49)	8.42 (16.86)	16.95 (24.29)	54.72 (47.69)	20.60 (24.33)
BC-IX	2.74 (9.20)	6.08 (14.20)	14.85 (22.64)	29.11 (32.63)	13.19 (19.67)
B. thuringiensis	6.34 (14.29)	15.77 (23.36)	73.28 (58.86)	100.00 (89.96)	48.84 (46.61)
Means	3.36 (9.800)	13.13 (19.46)	30.75 (31.92)	49.32 (48.37)	
		$SE_{(d)}\pm$	CD (P = 0.05)		
Culture filtrates		(0.74)	(1.48)		
Concentrations		(0.56)	(1.12)		
Culture filtrates x Concentrations		(1.49)	(2.97)		

*Mean of 3 replications; Figures within parentheses are arc sine angular transformed values

(2000) and Mishra and Singh (2003) support the usefulness of bacterial flora in control of pathogens affecting the yield of *A. bisporus*.

Cell free culture filtrates used against *A. bisporus* revealed that the growth of mushroom mycelium was significantly higher in PS-I and PS-II as compared to other isolates. Bacterial biomass present in the mushroom substrate helps in mycelial growth (Stanek, 1972; Fermor and Wood, 1981; Sparling *et al.,* 1982), while the bacteria present in casing soil produce metabolites reported to trigger sporophore formation in *A. bisporus* (Park and Agnihotri, 1969; Ahlawat *et al.*, 2002).

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