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Antifungal Activity of Different Interspecies Combinations of Antagonistic *Bacillus Species* Against Phytopathogenic *Fusarium* and *Pythium Species*

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Abstract
Among the 140 isolates of *Bacillus* obtained from rhizosphere of healthy crop plants, twelve isolates were showed good antifungal activity against phytopathogenic *Fusarium* and *Pythium species.* Among these, the three most potent *Bacillus* isolates were selected and identified to species level on the basis of morphology, ability of endospore formation, cultural characters, biochemical characters as well as 16S-rRNA sequencing as- *B. thuringiensis*184, *B. cereus*220 and *B. subtilis* 252. Different random interspecies combinations were tested for their improvements in antifungal activity as compared to the individual isolates. Among the three interspecies combinations tested, *B. cereus*220 + *B. subtilis* 252 combination was found to be most effective against phytopathogenic *Fusarium* and *Pythium species.* The enhancement of P. I. values of two *Bacillus* combinations over the average P. I. values of individual species was found up to 12% in PDB and 16% on PDA. We conclude that, the combination of two antifungal *Bacillus* species was more effective against phytopathogenic fungi as compared to the individual species.

Keywords
Bacillus species, Antifungal activity, Phytopathogenic fungi.

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INTRODUCTION
The fungi contribute a major part of the phytopathogens in soil. The soil-borne fungi cause serious plant diseases such as root rot, crown or collar rot, damping-off, blights, fruit decay, wilts, etc. These diseases show necrosis type of symptoms indicated by decay or rotting of tissues due to derangement of cells. The soil-borne fungal pathogens mainly include the species of- *Phytopthora*, *Pythium*, *Fusarium*, *Colletotrichum, Macrophomina, Gauemannomyces*, *Corticium*, *Verticillium*, *Sclerotium*, etc.[1, 2] Among these soil-borne fungal pathogens, *Fusarium species* are considered to be very dynamic and taxonomically notorious.[3] The *Pythium* is relatively fast growing and

aggressive fungal pathogen, particularly at moist and low temperature conditions.[1, 2, 3]

Chemical control of crop diseases has created many problems related to environment and human health. Biological control of crop diseases using efficient strains of microorganisms is the powerful and safe remedy to avoid the crop-loss and harmful effects of chemical control. Among the bacteria, species of *Pseudomonas* and *Bacillus* are widely studied as biocontrol agents and some of them have been proved to be successful. The *Bacillus* species are characterized by ubiquitous presence, ability of endospore formation and the metabolic versatility.[4] The endospore formation ability of *Bacillus* species has been proved to be significant for prolong shelf-life of the biocontrol formulations without any cold storage.[5] Some of the important biocontrol species of *Bacillus* are- *B. cereus*UW85 for control of damping-off of alfalfa, *B. megaterium*B153-2-2 for control of Rhizoctonia root rot of soybean, *B. subtilis*GB03 for control of damping-off of cotton, *B. mycoides* for the control of wheat take-all, *B. subtilis* RB14 for biocontrol of *Rhizoctonia solani* causing damping-off of tomato plants.[4, 6, 7,8.] The *Bacillus* species also act as plant growth promoting rhizobacteria (PGPR), using different mechanisms. In China, success has been reported for the use of *Bacillus* species as 'yield increasing bacteria', when introduced as a seed inoculant on several crops, particularly wheat and rice.[9]

A specific mechanism or a combination of different mechanisms is involved in the biocontrol of plant pathogens by microorganisms. Some of the most commonly involved mechanisms are- Production of antibiotics and other toxic compounds, Competition for nutrients, Parasitism and Lysis, Detoxification and Degradation of virulence factors of phytopathogens and Induction of host resistance. [10,11,12, 13.]

Bacterial biocontrol products are available in the market in solid and liquid forms with antagonistic activity against phytopathogenic fungi e.g. 'ABTEC Pseudo' and 'ABTEC Bacillus' produced by Agro Biotech Research Centre, Ltd. *B. subtilis* GB03 sold today in the United States as 'Kodiak' for the control of damping-off of crop plants, mainly the cotton.[9]

The objective of the present work was to screen the efficient antifungal *Bacillus* isolates, to study the combined *in-vitro* antifungal activity of these isolates against phytopathogenic *Fusarium* and *Pythium* species for any enhancement, to prepare biocontrol formulations of successful combinations and to analyze for actual biocontrol efficiency in pot culture tests.

MATERIALS AND METHODS

Isolation of *Bacillus* species-

Rhizoplane soil samples of healthy cotton and tomato plants were collected from fields. One gram of each of these samples was separately added in 100ml sterile distilled water. The supernatant samples were decanted and heated at 65^{0}C for 20 min. in water bath so as to kill the heat sensitive microflora and to select the aerobic, heat resistant bacteria. The samples were cooled and a loopful of these were separately streaked on nutrient agar (NA) plates and incubated at 28^{0}C for 24h.[14, 15] Isolated colonies on NA were marked and subcultured on NA slants. Ability of the isolates to form endospore was tested by staining 48h nutrient agar cultures by Schaeffer-Fulton's method.[16] Gram nature and morphology was tested by Gram staining procedure and motility by hanging drop technique. Total 140 isolates of *Bacillus* were obtained and preserved.[15]

Isolation and Selection of phytopathogenic fungal cultures-

Fungal pathogens were isolated on PDA, from infected plant materials by tissue segment method and from disease conductive soils. They were identified on the basis of cultural characters on PDA (Fig.1.) and microscopic characters by mounting with cotton blue. Among the different phytopathogenic fungi isolated, *Pythium* and *Fusarium* species were selected for further study.[15]

Primary screening of antifungal *Bacillus* isolates-

The ability of the *Bacillus* species to inhibit the growth of phytopathogenic *Pythium* and *Fusarium* species was tested by 'Dual culture (co-culture) method' on PDA (Fig.2). 100µl of each bacterial culture was inoculated in wells (10mm diameter) at two sides of central fungal agar disc, equidistantly. [17,18,19.] Bacterial cultures were also applied with 6mm filter paper disc, instead of wells. Zones of fungal growth inhibition were observed after incubation at 28^{0}C for 72 hrs. Total twelve *Bacillus* isolates showed good antifungal activity against phytopathogenic *Pythium* and *Fusarium* species.

Fig.1 Phytopathogenic fungi isolated on PDA

Fig. 2 Primary screening of antifungal *Bacillus* isolates

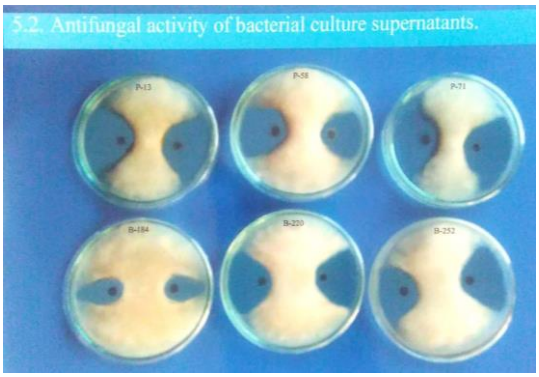
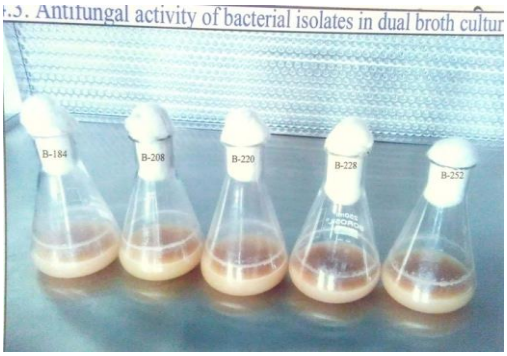


Fig. 3. Antifungal activity of *Bacillus* isolates in PDB by co-culture-



Secondary screening of efficient antifungal *Bacillus* isolates-

The efficiency of fungal growth inhibition of the antifungal isolates was tested by two methods: Dual culture method on PDA plates and Dual culture in PDB.

I) Co-culture on PDA plates- 100µl of 24hrs active broth culture of antagonist (10^6 CFU/ml) was added in 100ml sterile PDA cooled to 50^0C and poured in sterile Petriplates and allowed to solidify. 10mm PDA disc of *Fusarium* and *Pythium* culture was kept at the center of seeded PDA plates and incubated at 28^0C for 7days. A control plate without seeding of antagonist culture was kept for comparison. Diameters of fungal growth were measured and extent of antifungal activity of the isolates was measured in terms of percent inhibition (P I) of fungal growth using following formula and recorded in table 1.

$$\textbf{P. I.} = \frac{\textbf{(C-T) x 100}}{\textbf{C}}$$

Where 'C' is the diameter of fungal growth in control plate and 'T' is diameter of fungal growth in test plate. [20, 21.]

II) Co-culture in PDB flasks- 100µl of active NB culture of antagonist (10^6CFU/ml) and 100µl PDB culture of fungal pathogen was inoculated together in 100ml sterile PDB in 250ml Erlenmeyer flask (fig.3). One PDB

flask inoculated with fungal pathogen but without bacterial culture was labeled as 'control' and incubated with test flasks. After 7days incubation at 28°C and 120rpm in shaker-incubator, the mycelial growth of fungus was filtered using Whatman filter paper No.1 and weighed. The antifungal activity was measured in terms of percent inhibition of fungal growth using above formula and recorded in Table 2. In this case, 'C' is the wet weight of fungal growth in control and 'T' is the wet weight of fungal growth in tests [15, 22, 23.]

Identification of antifungal *Bacillus* isolates-

The efficient antifungal *Bacillus* isolates were further identified to species level on the basis of morphological characters, ability of endospore formation, cultural characters, important biochemical tests (oxidase, catalase, Voges-Proskauer test, nitrate reductase, manitol fermentation, lecithinase, and ability to grow at 50°C) and 16S rRNA sequencing performed at National Centre of Cell Science, Pune.[15]

Study of combined antifungal activity-

Three interspecies combinations were tested for improvement of antifungal activity- (*Bacillus thuringiensis*184 + *Bacillus cereus*220), (*Bacillus thuringiensis*184 + *Bacillus subtilis* 252) and (*B. cereus*220 + *B. subtilis* 252). 100µl of each culture in the pairs is inoculated in 100ml nutrient broth and incubated for 18 hrs. This fresh mixed culture is used to study combined antifungal activity against phytopathogenic *Pythium* and *Fusarium*, as described above.

RESULTS AND DISCUSSION

Table-1. Antifungal activity of *Bacillus* species and their interspecies combinations on PDA

Bacillus species	**Growth of *Pythium species* (mm)**	**P. I.* of *Pythium species***	**Growth of *Fusarium species* (mm)**	**P. I.* of *Fusarium species***
*B. thuringiensis*184	39	51.25	30	48.27
*B. cereus*220	35	56.25	27	53.44
B. subtilis 252	33	**58.75**	22	**62.06**
*B. thuringiensis*184 + *B. cereus*220	32	57.50 (+3.75)	28	51.72 (+0.87)
*B. thuringiensis*184 + *B. subtilis* 252	25	68.75 (13.75)	17	70.68 (15.43)
*B. cereus*220 + *B. subtilis* 252	22	**72.50** (15.00)	15	**74.13** (16.38)
Control	80	-	58	-

***P. I. – Percent Inhibition of fungal growth. All values are average of triplicates. Values in bracket indicate increase in P I values over the average P. I. of two individual species.**

Table-2. Antifungal activity of *Bacillus* species and their interspecies combinations in PDB

Bacillus species	**W. W.* of *Pythium species* (mg)**	**P. I. of *Pythium species***	**W. W.* of *Fusarium species* (mg)**	**P. I. of Fusarium *species***
*B. thuringiensis*184	2550	52.12	2905	54.75
*B. cereus*220	2390	54.23	2720	57.63
B. subtilis 252	2255	**56.81**	2700	**57.94**
*B. thuringiensis*184 + *B. cereus*220	2220	54.07 (+0.90)	2680	58.25 (+2.06)
*B. thuringiensis*184 + *B. subtilis* 252	1916	63.30 (+8.84)	2300	64.17 (7.83)
*B. cereus*220 + *B. subtilis* 252	1765	**66.20** (+10.68)	1940	**69.78** (+12.00)
Control	5222	-	6420	-

***W.W.- Wet Weight of fungal mycelium. All values are average of triplicates. Values in bracket indicate increase in P. I. values over the average P. I. of two individual species.**

Antifungal activity of individual *Bacillus* cultures-

Among the twelve antifungal *Bacillus* isolates, three isolates i.e. *B. thuringiensis*184, *B. cereus*220 and *B. subtilis*252 showed good antifungal activity (> 50%) in co-culture methods in PDA as well as PDB. Among these three isolates, *B. subtilis*252 showed maximum antifungal activity i.e. P. I. values 58.75% against *Pythium* and 62.06% against *Fusarium* (table-1) on PDA and 56.81% against *Pythium* and 57.94% against *Fusarium* in dual broth culture test in PDB (table-2). Similar *in-vitro* antagonistic activity of *Bacillus* species against phytopathogenic fungi has been observed by many workers. [7,8,9,19.]

Combined antifungal activity of *Bacillus* cultures-

Three interspecies combinations were tested for determination of any improvement of antifungal activity: *B. thuringiensis*184 + *B. cereus*220, *B. thuringiensis*184 + *B. subtilis* 252 and *B. cereus*220 + *B. subtilis* 252. Among these combinations, *B. thuringiensis*184 + *B. cereus*220 did not show considerable (<5%) improvement against *Pythium* as well as *Fusarium*, over the average P. I. value of the two individuals, in case of both the methods. The combination *B. cereus*220 + *B. subtilis* 252 showed maximum improvement in antifungal activity and proved to be the best, in case of both the phytopathogens on PDA as well as PDB. In general, the improvement in antifungal activity was relatively less in case of co-culture in PDB than that on PDA. On PDA, it showed PI value 72.50% (+15.00% over average PI) against *Pythium* and 74.13% (+16.38% over average PI) against *Fusarium*. In PDB, it showed PI value 66.20% (+10.68% over average PI) against *Pythium* and 69.78% (+12% over average PI) against *Fusarium*. Considerable improvement (> 5%) in antifungal activity was observed in case of two combinations (*B. thuringiensis*184 + *B. subtilis* 252) and (*B. cereus*220 + *B. subtilis* 252) by both methods in case of *Pythium* as well as *Fusarium*. These combinations were also proved effective to control *Pythium* and *Fusarium* infections in pot culture tests. Similar improvement in *in-vitro* antifungal activity and biocontrol efficiency by combinations of microbial cultures was observed by many workers. [24,25,26.27]

Mazzola *et al.*, (1995) observed improvement in biocontrol efficiency by combining the antagonist fluorescent *Pseudomonas* species than the individual.[28] De Boer *et al.*, (1999) observed that, specific combinations of fluorescent *Pseudomonas* RE8 and RS111 found to enhance suppression of Fusarium wilt of radish caused by *Fusarium oxysporum*.[29] De Boer *et al.*, (2003) observed that, soil application of combination of *Pseudomonas putida* WCS358 and RE8 with siderophore production and induced systemic resistance as mechanisms respectively, enhanced the protection of radish plants from Fusarium wilt to 50% as compared to 30% with single strain treatment.[30] Champawat and Sharma (2003) observed improvement in biocontrol efficiency with integrated management of diseases of brinjal, chili, cabbage and onion.[24] Schmidt *et al.*, (2004) observed improvement in biological control by combining *Pseudomonas fluorescens* and *Bacillus subtilis*.[31] Bohra and Mathur (2005), proposed the use of integration of bacterial biocontrol agents and neem formulations for ecofriendly disease management.[32] Howell C. R. observed that, *Trichoderma harzianum* can be used in combination with other species or with a chemical control agent for better results.[33,34] Rini and Sulochana (2006) observed that the combination *T. harzianum* (TR20) + *P. fluorescens* (P28) was most effective in reducing rhizoctonia root rot disease, i.e. 66.7% more efficient than the control, under greenhouse and field conditions.[34] Manjula *et al.*, 2004 observed improvement in biocontrol of stem-rot of groundnut by combined application of *Pseudomonas fluorescens* and *Trichoderma viride*.[35] These reports indicated appearance of specific interactions between the bacterial strains used for biological control. Biocontrol agents in combinations may persist longer in rhizosphere and utilize a wider array of biocontrol mechanisms.

CONCLUSION

We conclude that, the *in-vitro* antifungal activity of the two interspecies combinations i.e. *B. thuringiensis*184 + *B. subtilis* 252 and *B. cereus*220 + *B. subtilis* 252 was considerably greater than the average P. I. of individual species. The improvement in antifungal activity of the isolates in present study may be due to the synergistic antagonistic activity of *Bacillus* species, in respective combination.

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