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In-vitro evaluation of *Bacillus subtilis* strains for their antagonistic potential against different fungal Phytopathogens and their compatibility with commonly used fungicides

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Abstract
The aim of present study was to evaluate the antagonistic potential of 6 *Bacillus subtilis* strains (PBBSR1, PBBSR2, PBBSR3, PBBSR4, PBBSR5 and PBBSR6 against 5 fungal phytopathogens *viz*. *Drechslera oryzae*, *Fusarium monoliforme*, *Pythium* sp., *Sclerotium rolfsii*, *Curvularia* sp. and to check the compatability between the *B. subtilis* strains and 10 commercially used fungicides. Dual culture assay between the *B. subtilis* strains and the fungal pathogens showed all the strains to be effective in inhibiting mycelial growth of the studied pathogens with formation of inhibition zone. However, among the 6 strains, 2 strains (PBBSR1 and PBBSR2) were found to be most efficient with consistent results of efficacy under dual culture assay. Further, strains PBBSR1and PBBSR2 were checked for their compatibility with 10 commonly used fungicides that are usually recommended for disease management under *in-vitro* condition. A total of 8 fungicidal concentrations (25, 50, 100, 250, 500, 1000, 1500 and 2000 ppm) were considered for the test. The compatibility study showed 5 fungicides *viz*. Carbendazim 50% WP, Hexaconazole 5% SC, Thiophanate Methyl 70% WP, Azoxystrobin 18.2% + Difenconazole 11.4% SC and Azoxystrobin 23% SC to be compatible with both PBBSR1and PBBSR2 strains.

Keywords: *Bacillus*, antagonist, biological control, compatibility, fungicides

Introduction
Research over the last two decades has specified biocontrol of diseases as an alternative option for disease management. Emphasizing on the abilities of natural occurring biocontrol, in recent years, the interest in biological control of plant pathogens has been increased significantly which is an ecofriendly and cost effective approach that can substitute use of chemical fungicides. In nature, a wide variety of biocontrol agents exist, including bacterial and fungal antagonists that considerable enhance plant growth and health. Among them, bacterial antagonists are generally regarded as ideal due to ease of handling and their aggressive colonizing abilities in addition; the success of a biocontrol agent highly depends on how long it can survive under different adverse environmental conditions as well as it potential to control plant diseases. One of the bacterial candidates that perfectly fit the above mentioned criteria is the endospore-forming *Bacillus* species. Several studies have recognized the genus *Bacillus* being widely distributed in different ecological habitats and its ability to form endospore contributes to its extended survival and colonization in a wide range of stressful niches (Sharma *et al.*, 2019; Jamali *et al.*, 2020) [1, 2]. One of the most important characters of *Bacillus* species is its ability to produce diverse forms of secondary metabolites and a wide variety of structurally diverse antagonistic compounds. *Bacillus subtilis* strains commit about 4 - 5 per cent of their whole genomes to secondary metabolite production, with the ability to synthesize over two dozen structurally varied antimicrobial compounds (Stein, 2005) [3]. *Bacillus* strains can suppress and inhibit plant infections directly by releasing antimicrobial peptides (AMP), volatile compounds, and hydrolytic enzymes (Chitinases, Glucanases, and proteases), or indirectly by competing for a niche or nutrient requirements (Shafi *et al.*, 2017) [4]. Several *Bacillus* species have been proven to be effective against a broad range of phytopathogens (Shafi *et al.*, 2017; Fira *et al.*, 2018) [4, 5]. There have been many reports about *Bacillus* strains being antagonistic to multiple fungal and bacterial pathogens (Prasanna Kumar *et al.*, 2017; Myo *et al.*, 2019) [6, 7].

Though biocontrol agents can provide a good disease control with little or no environmental damage, its efficacy is often lower compared to fungicides (Jacobsen *et al*., 2004) [8]. Biological control in integrated disease management (IDM) using antagonistic microbes alone or as supplements to minimize the use of chemicals has become more prominent in recent years (Salman and Abuamsha, 2012; Boukaew *et al.*, 2013) [9, 10]. Combination of biological agents with chemical fungicides could be a practical alternative approach taking advantage of the strengths of both the methods while reducing their respective limitations associated with individual application of fungicides and biocontrol agents (Liu *et al.*, 2018) [11]. Integrated system could be associated with many advantages, like when conditions are temporarily unfavourable for the biocontrol activity, an associated fungicide could provide a reliable backup system (Salman and Abuamsha, 2012) [9]. However not only the fungicides will have deleterious effects on the pathogen but it may effect on the microbial antagonist as well. A proper understanding of the effect of fungicides on the pathogen and the antagonist would provide better information on selection of particular fungicides and fungicide tolerant antagonists (Devi and Prakasham, 2020) [12].

Considering the importance of *Bacillus* species as biocontrol agent, in the present study an attempt was made to evaluate antagonistic potential of 6 *Bacillus subtilis* strains *viz.* PBBSR1 (GenBank accession no. MZ995507), PBBSR2 (MZ995508), PBBSR3 (MZ995509), PBBSR4 (MZ995510), PBBSR5 (MZ995511) and PBBSR6 (MZ995512) against 5 fungal phytopathogens *viz. Drechslera oryzae, Fusarium monoliforme*, *Pythium* sp., *Sclerotium rolfsii*, *Curvularia* sp. (collected from Department of Plant Pathology, GBPUA&T, Pantnagar) through *in vitro* dual culture assay. Further, *in vitro* compatibility of *Bacillus subtilis* strains PBBSR1 and PBBSR2 with 10 commonly use fungicides was analyzed.

Materials and Methods

Dual culture assay of *Bacillus subtilis* strains against fungal Phytopathogens: The antagonism of *B. subtilis* strains against the fungal phytopathogens was tested *in vitro* by a dual culture technique followed by Kanjanamaneesathian *et al.* (1998) [13]. With the help of sterile cork borer fungal plug of 5 mm size was cut from fresh culture of respective pathogens and placed on one end of potato dextrose agar (PDA) plate at a distance of 2 cm from the edge. On the other end of the plate, at the same distance of 2 cm from the other edge 24 hr-old *B. subtilis* culture was streaked. Three replications were maintained for each *Bacillus* strain. The plates were kept for incubation at 28±1 °C for about 7 days. The zone of inhibition was observed when the pathogen growth stopped in dual culture plates. The efficacy of *B. subtilis* strains was based on their abilities to form wider inhibition zone.

Compatibility of *Bacillus subtilis* strains with fungicides

In the experiment two *B. subtilis* strains that were found most effective under *in-vitro* dual culture assay were chosen. The compatibility of 10 commonly used fungicides (Table 1) that are usually recommended for disease management was tested against these *Bacillus* strains by a poison food technique described by Krishnamoorthy *et al.* (2017) [14]. A total of eight concentrations (25, 50, 100, 250, 500, 1000, 1500 and 2000 ppm) of each fungicide were taken into study as described by Suneeta *et al.*, 2016 [15]. First, stock solutions of fungicides were prepared. Then different concentrations of each fungicide were prepared simply by pipetting the required volume from the stock and adding to the sterilized nutrient agar (NA) medium just before pouring to petri dishes. These fungicides amended NA plates were streaked with the freshly cultured *B. subtilis* strains and incubated at 30± 2 ^{0}C for 48 hr. Nutrient agar plates without any fungicide were kept as control plates. Three replications were maintained for every concentration of individual fungicide. For measuring the compatibility, bacterial growth on fungicide amended media were rated as +++ (Good); ++ (Moderate); + (Poor) and – (No growth) and compared with the growth on control plates. Good and moderate growth were considered as compatible reaction and poor and no growth as incompatible reaction

Table 1: Details of fungicides

Sl. No.	Trade name	Chemical name with active ingredient	Formulation
1.	Kvistin	Carbendazim 50% WP	Wettable powder
2.	Tilt 25 EC	Propiconazole 25% EC	Emulsifiable concentrate
3.	Folicur	Tebuconazole 25.9% EC	Emulsifiable concentrate
4.	CM-75	Carbendazim 12% + Mancozeb 63% WP	Wettable powder
5.	Nativo	Tebuconazole 50% + Trifloxystrobin 25% WG	Wettable granules
6.	Contaf	Hexaconazole 5% SC	Suspension concentrate
7.	Indofil M-45	Mancozeb 75% WP	Wettable powder
8.	Amistar	Azoxystrobin 23% SC	Suspension concentrate
9.	Hybritz	Azoxystrobin 18.2% + Difenconazole 11.4% SC	Suspension concentrate
10.	Control	Thiophanate Methyl 70% WP	Wettable powder

Statistical analysis

Experimental data were analyzed using standard analysis of variance (ANOVA) using the software OPSTAT (Sheoran *et al.*, 1998) [16]. Followed by Duncan's multiple range test (DMRT) at $p< 0.05$ using computer software package IBM SPSS Statistics v. 23 (Statistical Package for the Social Sciences)

Results and Discussions

Dual culture assay of *Bacillus subtilis* strains against fungal Phytopathogens

Dual culture study of the *B. subtilis* strains against *Drechslera oryzae* resulted in significant difference in their abilities to form inhibition zones. Maximum inhibition zone was observed in strain PBBSR2 (22.00 mm), followed PBBSR1

(21.67 mm) and were at par but significantly different from the other 4 *B. subtilis* strains. This was followed by the remaining 4 *B. subtilis* strains (ranging from 18.33 to 17.33 mm) which were at par to each other. Dual culture study between the *B. subtilis* strains and *Fusarium monoliforme* showed significant difference in inhibition zone formation. Maximum inhibition zone was observed in PBBSR1 and PBBSR2 (13.33 and 13.33 mm) and were at par but significantly different from the remaining strains. This was followed by strains PBBSR4 and PBBSR5 (10.00 and 9.00 mm) which were at par with each other. Least inhibition zone was observed in strains PBBSR3 and PBBSR6 (6.33 and 5.33 mm) which were at par. Dual culture study against *Pythium* sp. showed PBBSR2 and PBBSR1 as the most effective strains with maximum inhibition zone (20.00 mm) in PBBSR2 followed by PBBSR1 (18.33 mm) and were at par to each other but significantly different from the remaining 4 strains. This was followed by PBBSR4 (10.00 mm), PBBSR5 (7.67mm) and PBBSR3 (7.33 mm) which were at par with each other. Least inhibition zone was observed in PBBSR6 (6.00 mm). Dual culture study against *S. rolfsii* showed no significant difference in their abilities to form inhibition zones among 5 strains *viz*. PBBSR1 (10.67 mm), PBBSR2 (11.00 mm), PBBSR3 (12.00 mm), PBBSR4 (10.67 mm) and PBBSR5 (11.67) but they were significantly different from strain PBBSR6 which showed least inhibition zone (5.33 mm). Dual culture study between the *B. subtilis* strains and *Curvularia* sp. resulted in significant difference in formation of inhibition zones among the strains. The maximum inhibition zone was observed in PBBSR2 (16.00 mm) followed by PBBSR1 (15.33 mm) and were at par. This was followed by PBBSR3 (12.33 mm) and PBBSR5 (10.33 mm) which were at par. And the least inhibition zone was observed in PBBSR6 (4.00 mm) (Table 2 and Fig 1). The present experiment showed 2 *B. subtilis* strains (PBBSR2 and PBBSR1) as most efficient with consistent results of efficacy against all the 5 fungal phytopathogens. Similar work and findings have been reported by many authors, Grover *et al.* (2010) [17] analysed the antifungal activity of *B. subtilis* strain RP24 against variety of 12 phytopathogenic fungi. The result showed the inhibition zones ranging from a maximum value of 9.00 ± 0.82 mm to a minimum of 7.25 ± 0.71 against the different pathogens which is quite similar to the results observed in the present study. Ji *et al*. (2013) [18] evaluated the antifungal potential of *B. amyloliquefaciens strain* CNU114001 on 12 phytopathogenic fungi and reported that the bacterial strain exhibited strong inhibition of 50 to 70 per cent mycelial growth reduction over their respective controls**.** Jamal *et al.* (2015) [19] assessed the antifungal potential of *B. amyloliquefaciens* Y1 against 5 fungal and reported that the bacterial strain showed significant inhibition zones ranging from 18 to 6 mm over the pathogens. Similar studies and findings were reported by different authors (Prasanna Kumar *et al.*, 2017; Myo *et al.*, 2019) [6, 7].

Table 2: *In vitro* efficacy of *B. subtilis* strains against different fungal Phytopathogens in dual culture assay

***B. subtilis* strains**	**Inhibition zone (in mm)**				
	Drechslera oryzae	*Fusarium monoliforme*	*Pythium* sp.	*Scletorium rolfsii*	*Curvularia* sp.
PBBSR1	21.67 ± 0.88^{a}	13.33 ± 0.67^{a}	18.33 ± 0.88^{a}	10.67 ± 0.88^{a}	15.33 ± 0.33^{a}
PBBSR2	22.00 ± 0.58^{a}	13.33 ± 0.33^{a}	20.00 ± 0.58^{a}	11.00 ± 0.58^{a}	16.00 ± 0.58^{a}
PBBSR3	18.33 ± 0.88^{b}	6.33 ± 0.88^{cd}	7.33 ± 1.45^{bc}	12.00 ± 0.58^{a}	12.33 ± 0.67^{b}
PBBSR4	17.67 ± 1.33^{b}	10.00 ± 1.53^{b}	10.00 ± 1.00^{b}	10.67 ± 0.33^{a}	7.67 ± 0.33^{c}
PBBSR5	18.00 ± 0.58^{b}	9.00 ± 1.53^{bc}	7.67 ± 0.33^{bc}	11.67 ± 0.67^{a}	10.33 ± 1.20^{b}
PBBSR6	17.33 ± 0.67^{b}	5.33 ± 0.33^{d}	6.00 ± 2.00^{c}	5.33 ± 0.88^{b}	4.00 ± 1.00^{d}
C.D. (0.05)	2.68	3.14	3.67	2.12	2.36
C.V.	7.78	18.29	17.67	11.53	11.99

The mean values with standard errors in the same column followed by the same letter are not significantly different according to the Duncan test at P = 0.05.

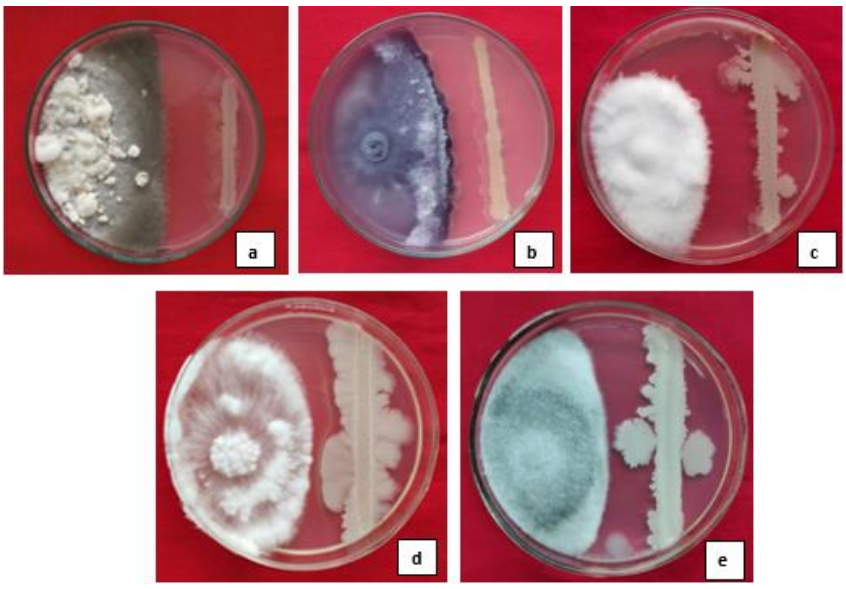

Fig 1: Dual culture assay showing inhibition zones by *Bacillus subtilis* strains (with PBBSR2 shown as reference strain) against different fungal Phytopathogens. (a) Dual culture assay of PBBSR2 against *D. oryzae*; (b) PBBSR2 against *F. monoliforme*; (c) PBBSR2 against *Pythium* sp; (d) PBBSR2 against *S. rolfsii*; (e) PBBSR2 against *Curvularia* sp.

Compatibility of *Bacillus subtilis* strains with fungicides

For this study, 2 *B. subtilis* strains (PBBSR2 and PBBSR1) that proved to be most efficient in *in vitro studies* were choosen. For measuring the compatibility, bacterial growth on fungicide amended media were rated as good (+++), moderate (++), poor (+) and no growth (-) and compared with the growth on control plates. The compatibility study revealed that both the *B. subtilis* strains had good growth (+++) at all the tested concentrations (25-2000ppm) of 3 chemical fungicides *viz*. Carbendazim 50% WP, Hexaconazole 5% SC and Thiophanate Methyl 70% WP, which showed its high tolerance and compatibility level up to 2000 ppm. With chemicals like Azoxystrobin 18.2% + Difenconazole 11.4% SC and Azoxystrobin 23% SC both the strains showed good growth (+++) at 250 to 500 ppm, moderate growth (++) to poor growth at 1000 to 1500 ppm and poor to no growth (-) at 2000 ppm, while these strains showed good growth (+++) at 25 ppm, moderate growth (++) at 50 ppm, poor growth (+) at 100 ppm and no growth (-) at 250 to 2000 ppm with Tebuconazole 50% + Trifloxystrobin 25% WG. With Propiconazole 25% EC, Tebuconazole 25.9% EC, Carbendazim 12% + Mancozeb 63% WP, Mancozeb 75% WP, both the strains showed poor (+) to no growth (-) even at lower concentration (25 and 50 ppm) which showed its incompatibility with these chemicals. The present study revealed that among the 10 fungicides studied, the *B. subtilis* strains showed high tolerance level with a total of 5 fungicides *viz*. Carbendazim 50% WP, Hexaconazole 5% SC, Thiophanate Methyl 70% WP, Azoxystrobin 18.2% + Difenconazole 11.4% SC and Azoxystrobin 23% SC which indicated their compatibility with these chemical fungicides (Table 3). Similar findings were reported by several workers (Krishnamoorthy *et al.,* 2017; Suneeta *et al.,* 2016) [14, 15]. Omar *et al.* (2006) [20] reported *B. megatherium* strain c96 to be highly tolerant to carbendazim under *in vitro* condition. Kumar *et al.* (2011) [21] studied compatibility of *B. subtilis* strain PSB5 with 10 chemical fungicides having concentrations of 50-2000 ppm. The strain showed compatible to kresoxim, carbendazim, difenconazole, azoxystrobin, tebuconazole and fosetyl Al. The strain showed incompatibility with tebuconazole + trifloxystrobin, propiconazole and propineb. Liu *et al.,* (2018) [11] reported that *B. subtilis* strain H158 tolerance with strobilurins even at higher concentrations which showed its compatibility with this fungicide.

Table 3: Compatibilty of *B. subtilis* strains PBBSR2 and PBBSR1 with different fungicides

Fungicides	Concentrations (ppm)							
	25	50	100	250	500	1000	1500	2000
Carbendazim 50% WP	+++	+++	+++	+++	+++	+++	+++	+++
Propiconazole 25% EC	+++	-	-	-	-	-	-	-
Tebuconazole 25.9% EC	-	-	-	-	-	-	-	-
Carbendazim 12% + Mancozeb 63% WP	+	-	-	-	-	-	-	-
Tebuconazole 50% + Trifloxystrobin 25% WG	+++	++	+	-	-	-	-	-
Hexaconazole 5% SC	+++	+++	+++	+++	+++	+++	+++	++
Mancozeb 75% WP	-	-	-	-	-	-	-	-
Azoxystrobin 23% SC	+++	+++	+++	+++	+++	++	+	+
Azoxystrobin 18.2% + Difenconazole 11.4% SC	+++	+++	+++	+++	+++	+++	++	+
Thiophanate Methyl 70% WP	+++	+++	+++	+++	+++	+++	+++	++

+++ (good); ++ (moderate); + (poor); – (no bacterial growth)

Conclusion

Among the 6 *B. subtilis* strains studied, 2 strains (PBBSR2 and PBBSR1) were found to be most efficient with consistent results of efficacy against all the pathogens tested. Further these strains were found compatible with 5 commonly used fungicides that are usually recommended for disease management. Future studies can be focused on *in-vivo* studies of these strains against the pathogens and integration between these strains and the fungicides can be explored under *in-vivo* condition.

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