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Efficacy of bacterial biocontrol agents and commercial fungicides against fusarium wilt of chrysanthemum

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Abstract
Fusarium wilt is one of the most devastating and destructive disease of chrysanthemum, resulting in significant yield loss. The main aim of this study is to identify the most effective bacterial biocontrol agents and fungicides for the management of Fusarium wilt under *in vitro* conditions. Among the different bacterial biocontrol agents, *Pseudomonas* isolate, *Ps* Ap and *Bacillus* isolate, *Bs* 6 inhibited the mycelial growth of *Fusarium* sp. to the extent of 57.77 per cent and 42.22 per cent over untreated control. Among the ten commercial fungicides, Carbendazim 50% WP and Tebuconazole 50% + Trifloxystrobin 25% WP were found to be most effective against *Fusarium* sp. in all the concentrations tested under *in vitro* conditions. Studies on the compatibility of bacterial biocontrol agents and chemicals revealed that, the fungicides Carbendazim, Tebuconazole + Trifloxystrobin and Propiconazole were found to be compatible at all the three concentrations *viz.*, 500, 1000 and 1500 ppm respectively.

Keywords: Fusarium wilt, biological control, fungicides, compatibility

Introduction
Chrysanthemums are commercial flowering plant belongs to the family *Asteraceae*. Chrysanthemum flowers are used for worshiping god, decorations and the blooms are used to make different cuisine because of their high medicinal properties. Despite the importance, the beauty of the chrysanthemum is spoiled by many fungal, bacterial and viral diseases. Among the major disease, wilt disease caused by *Fusarium* sp. is the most destructive disease and resulting in major yield loss. Management for the chrysanthemum wilt is difficult because of the persistence pathogen in the soil and the non-availability of resistant varieties (Garibaldi *et al*, 2009) [7]. Integrated management is considered as the effective method to control the Fusarium wilt of chrysanthemum. Hence, the present study is focused on the biological and chemical management of Fusarium wilt and to identify the compatibility of biocontrol agents with chemicals under *in vitro* conditions.

Materials and method
Collection and isolation of *Fusarium* sp.
The wilt pathogen, *Fusarium* sp. was isolated from the infected root portions of plants collected from major chrysanthemum growing areas of Krishnagiri, Dindigul, Dharmapuri and Theni districts of Tamil Nadu by tissue segment method. The infected root portion was washed with sterile water and cut into small pieces of about 1.5cm. The cut portions were surface sterilized with 1.0 % sodium hypochlorite (NaClO) solution for fifteen seconds and washed thoroughly thrice in the series of sterile distilled water to remove the traces of sodium hypochlorite. The sterilized pieces were dried on filter paper and placed on PDA medium, incubated at 28˚± 2˚c under dark condition for 5 to 7 days. After confirming the pathogen based on their cultural and morphological characters, the fungal growth was purified by hyphal tip method and transferred to fresh PDA plates for further maintenance.

Pathogenecity and virulence of *Fusarium* sp.
The pot culture experiment was conducted to assess the pathogenicity of *Fusarium* sp. Potting mixture comprising of laterite soil, sand and compost were mixed in the ratio of 3:1:1 and steam sterilized at 120 lb pressure in autoclave for 2 h on alternate days. The sterilized potting mixture was filled into the pots @ 5kg / pot. The *Fusarium* pathogen was mass multiplied separately in sorghum grains (Kamdi *et al*, 2012) [14]. Sorghum grains were soaked partially in a warm water at 45˚C, air dried by spreading it in blotting paper and filled in polypropylene bags (30/20 cm) up to 3/4th level by adding 10ml distilled water and steam sterilized at 120 lb

pressure for 2 h on alternate days. The pathogen was inoculated and incubated at room temperature (28±2 ^{0}C) for 14 days and used for testing the pathogenicity. The pathogen multiplied in the sorghum grain was incorporated into the sterile potting mix @ 5 per cent (w/w). Then three chrysanthemum seedlings were planted in each pot. Similarly, uninoculated control was also maintained. The pots were watered regularly up to saturation on alternate days. Observations were made regularly for the appearance of symptom development. After symptom development, re-isolation was done and compared with the original culture for confirmation of the Koch postulates.

Isolation of bacterial biocontrol agents

The bacterial biocontrol agents were isolated from rhizosphere soil collected from different regions of Tamil Nadu by serial dilution technique and dilutions were made upto 10^6. One ml of the suspension from each 10^3 to 10^6 dilutions were transferred to a sterile petri dishes containing King's B (KB) and Nutrient Agar (NA) medium for the isolation of *Pseudomonas* sp. and *Bacillus* sp. respectively. The plates were incubated at 28±2˚C for 48 hours. Colonies with characteristics of *Pseudomonas* sp. and *Bacillus* sp. were isolated individually and purified by streak plate method. The pure culture was maintained on an agar slant at 4˚C for further studies.

***In vitro* evaluation of bacterial biocontrol agents against *Fusarium* sp.**

The antagonistic activity of the bacterial biocontrol agents was tested against the most virulent strains of *Fusarium* sp. (FI3) by following the dual culture technique (Dennis and Webster, 1971) [6]. The bacterial isolates were streaked 1 cm away from the edge on one side of the sterilized petridish containing PDA medium and nine mm mycelial disc of virulent strain of the pathogen was placed on the opposite side of the petridish perpendicular to the bacterial streak. Three replications were maintained for each bacterial antagonists and a control was maintained by inoculating the pathogen alone at the end of the petridish containing PDA medium. The plates were incubated at room temperature (28±2˚C) for seven days. The effective antagonist was selected based on the per cent inhibition of mycelial growth of the pathogen. The per cent reduction over control was calculated by using the formula of Per cent inhibition over control = (C-T)/C × 100, where C is mycelial growth of pathogen in control and T is mycelial growth of pathogen in bacterial biocontrol agents inoculated plate.

***In vitro* evaluation of commercial fungicides against *Fusarium* sp.**

The efficacy of ten different commercial fungicides was tested against *Fusarium* sp. by poisoned food technique. Potato Dextrose Agar medium added with different concentrations of fungicides *viz.*, Copper-oxy-chloride 50% WP, Mancozeb 75% WP, Azoxystrobin 23% SC, Carbendazim 50% WP, Fosetyl Al 80% WP, Tricyclazole 75% WP, Propiconazole 25% WP, Tebuconazole 25% EC, Hexaconazole 45% + Zineb 68% WP, Tebuconazole 50% + Trifloxystrobin 25% WP at 500 ppm, 1000 ppm and 1500 ppm concentrations were poured separately in a sterilized petri dish and seven days old culture of most virulent *Fusarium* sp. (FI3) was placed in the centre of the petri dish. The inoculated plates were incubated at room temperature (28±2˚C) and appropriate control was maintained without adding fungicides. The per cent reduction over control was calculated by using the formula of Per cent inhibition over control = (C-T)/C × 100, where C is mycelial growth of pathogen in control and T is mycelial growth of pathogen in inoculated plate.

Compatibility test between bacterial biocontrol agents and effective fungicides

Disc diffusion method was used to test the compatibility of effective fungicides with bacterial bio-agents. The sterilized petri dish containing King's B and Nutrient Agar medium were inoculated with overnight cultures of effective isolates of *Pseudomonas* sp. and *Bacillus* sp. by spread plate method. Filter paper discs in the diameter of 5 mm were dipped in test tube containing different concentrations of fungicide solutions (500 ppm, 1000 ppm and 1500 ppm) and placed on the biocontrol agents inoculated plates. The plates were incubated at room temperature and three replications were maintained for each fungicide. The compatibility of the fungicide with bacterial biocontrol agents were analysed by measuring the diameter of the inhibition zone.

Statistical analysis

Analysis of variance (ANOVA) and the SPSS version 17.0 were used to analyse the experimental data. The treatment means were separated at 5% significance level using Duncan's Multiple Range Test (DMRT).

Results and discussion

Collection and isolation of *Fusarium* sp.

Six isolates of *Fusarium* sp. have been isolated from the infected root portions of the plants collected from various chrysanthemum growing areas of Tamil Nadu. The isolates of *Fusarium* sp. were named as FI1, FI2, FI3, FI4, FI5 and FI6. (Table 1).

Pathogenicity of *Fusarium* sp.

The wilt disease severity in the pathogen inoculated chrysanthemum plants ranged from 47.38 to 82.10 per cent. Among the six isolates tested in the present study, the *Fusarium* isolate FI3 collected from Milagaipatti of Dindigul exhibited the typical symptoms such as yellowing of leaves, wilting of plants, vascular discolouration on inoculated plant after 40 days and caused the maximum severity of 82.10 per cent wilt incidence and it was followed by 71.86 per cent incidence in isolate FI2. The remaining isolates of *Fusarium* collected from Sejjalapatti, Aandipatti, Samichettipatti and Gudalur recorded 47.38 to 68.62 per cent wilt incidence (Table 1).

Isolation of bacterial biocontrol agents

A total of ten isolates of *Pseudomonas* sp. and four isolates of *Bacillus* sp. were isolated from different rhizosphere soil samples. The isolates of *Pseudomonas* sp. were named as *Ps* Ad, *Ps* Rk, *Ps* Sp, *Ps* Mp, *Ps* Ap, *Ps* Mv, *Ps* Gd, *Ps* Sc, *Ps* Tp, *Ps* Om and *Bacillus* sp. were named as *Bs* Rk, *Bs* Sp, *Bs* Ap and *Bs* Gd based on its location. The *Bacillus* sp. *Bs* 6, *Bs* 8, *Bs* 9, *Bs* 3, *Bs* 17 and *Bs* 18 were obtained from the Department of Plant Pathology, AC & RI, Madurai.

***In vitro* evaluation of bacterial biocontrol agents against *Fusarium* sp.**

In vitro antagonism of ten isolates of *Pseudomonas* sp., against the mycelial growth of *Fusarium* sp. indicated that, the isolate *Ps* Ap was most effective in inhibiting the mycelial

growth of virulent isolate of *Fusarium* spp (FI3) to the maximum of 57.77 per cent. It was followed by *Ps* Ad with 55.55 per cent, *Ps* Sp with 48.88 per cent and *Ps* Mv with 45.55 per cent. The minimum mycelial growth reduction was recorded in the isolate *Ps* Tp with 21.11 per cent (Table 2, Plate 1). Among the ten *Bacillus* isolates screened, *Bs* 6 showed the maximum inhibition percentage to an extent of 42.22 per cent against the mycelial growth of *Fusarium* sp. It was followed by the isolate *Bs* 18, which exhibited the mycelial growth reduction of 37.77 per cent over control. *Bs* Gd exhibited the minimum mycelial growth reduction of *Fusarium* sp. (20.00 per cent) (Table 3, Plate 2). The results were in accordance with the work reported by other workers. Karthick *et al*, (2017) [16] tested thirteen isolates of *Bacillus* sp. against *Fusarium oxysporum* f. sp. *ciceri* by dual culture method. The isolate B was most effective with the per cent inhibition of 53.00 per cent. Shahzaman *et al*, (2016) [24] reported the bio efficacy of thirty isolates of *Pseudomonas fluorescens* against the *Fusarium* sp. The antagonist *Pf* 3 was found most effective with inhibition percentage of 93.33 per cent.

I*n vitro* evaluation of commercial fungicides against *Fusarium* spp

Ten commercial fungicides *viz.,* Copper-oxy-chloride, Mancozeb, Azoxystrobin, Carbendazim, Fostyl Al, Tricyclazole, Propiconazole, Tebuconazole, Hexaconazole + Zineb, Tebuconazole + Trifloxystrobin were screened for antifungal activity against *Fusarium* spp at 500, 1000 and 1500 ppm concentrations. Carbendazim and Tebuconazole + Trifloxystrobin recorded 100 per cent inhibition of pathogen tested under all the concentrations. Other fungicides like Copper-oxy-chloride, Mancozeb, Fosetyl Al, Propiconazole, Tebuconazole @ 1500 ppm level showed 100 per cent reduction of the pathogen and Azoxystrobin was found to be the least effective among all the chemicals (Table 4, Plate 3). The result was in accordance with the findings of Patra and Biwas (2016) [22]. They tested ten different fungicides and found the effectiveness of the combination product *i.e.* Tebuconazole + Trifloxystrobin, which had 100 per cent inhibition over control. Maitlo *et al.* (2014) [19], and Mahmood *et al.* (2015) [18] reported the effectiveness of carbendazim against *F. Oxysporum* f. sp. *ciceri*.

Compatibility test between bacterial biocontrol agents and effective fungicides

The effective fungicides such as Carbendazim, Tebuconazole + Trifloxystrobin and Propiconazole were tested for their compatability with effective *Pseudomoas* isolate (*Ps* Ap) and *Bacillus* isolate (*Bs* 6) at 500, 1000, 1500 and 2000 ppm concentrations. The result revealed that Carbendazim, Tebuconazole + Trifloxystrobin and Propiconazole were compatible with *Pseudomonas* sp. and *Bacillus* spp (Table 5, Plate 4). at all the concentrations. The result obtained was similar to the findings of Kiran *et al*. (2018) [17]. They tested four fungicides for the compatibility with *Bacillus subtilis* and found that Tebuconazole was compatible at all the concentrations tested. The experimental results concluded that, the biocontrol agents can be used effectively in combination with fungicides for the control of Fusarium wilt of Chrysanthemum.

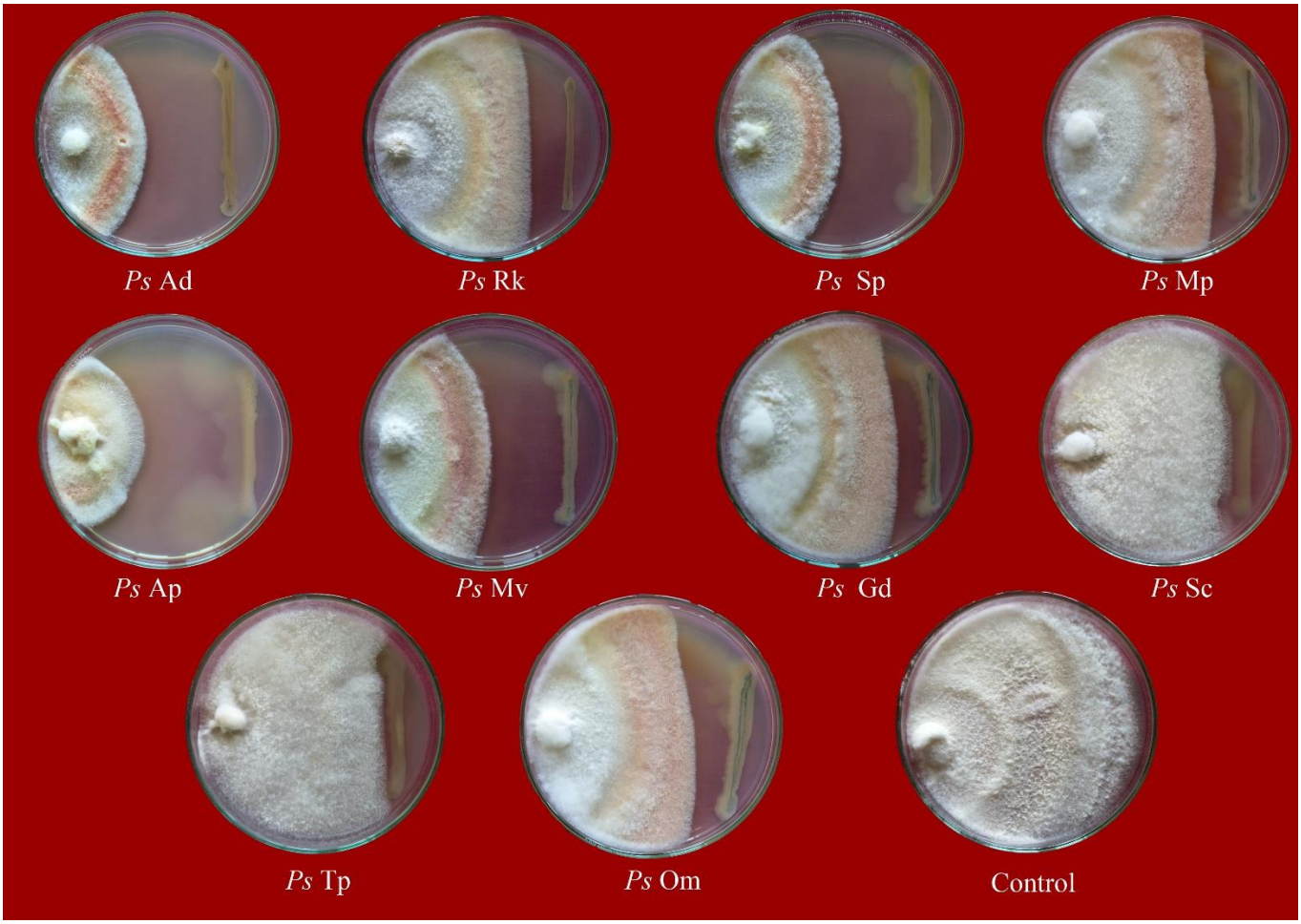

Plate 1: *In vitro* antagonistic efficacy of *Pseudomonas* sp. against *Fusarium* sp. (FI3)

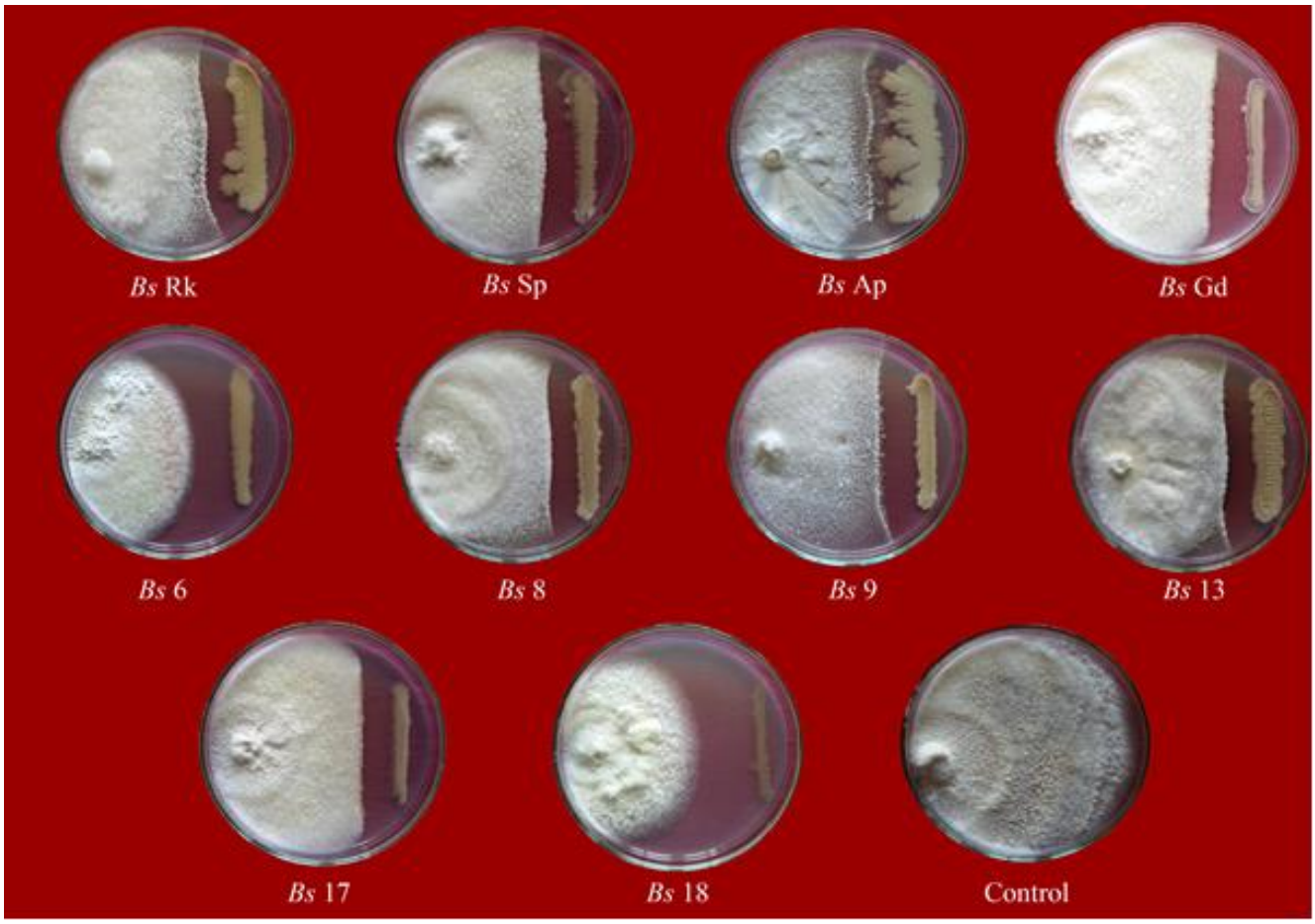

Plate 2: *In vitro* antagonistic efficacy of *Bacillus* sp. against *Fusarium* sp. (FI3)

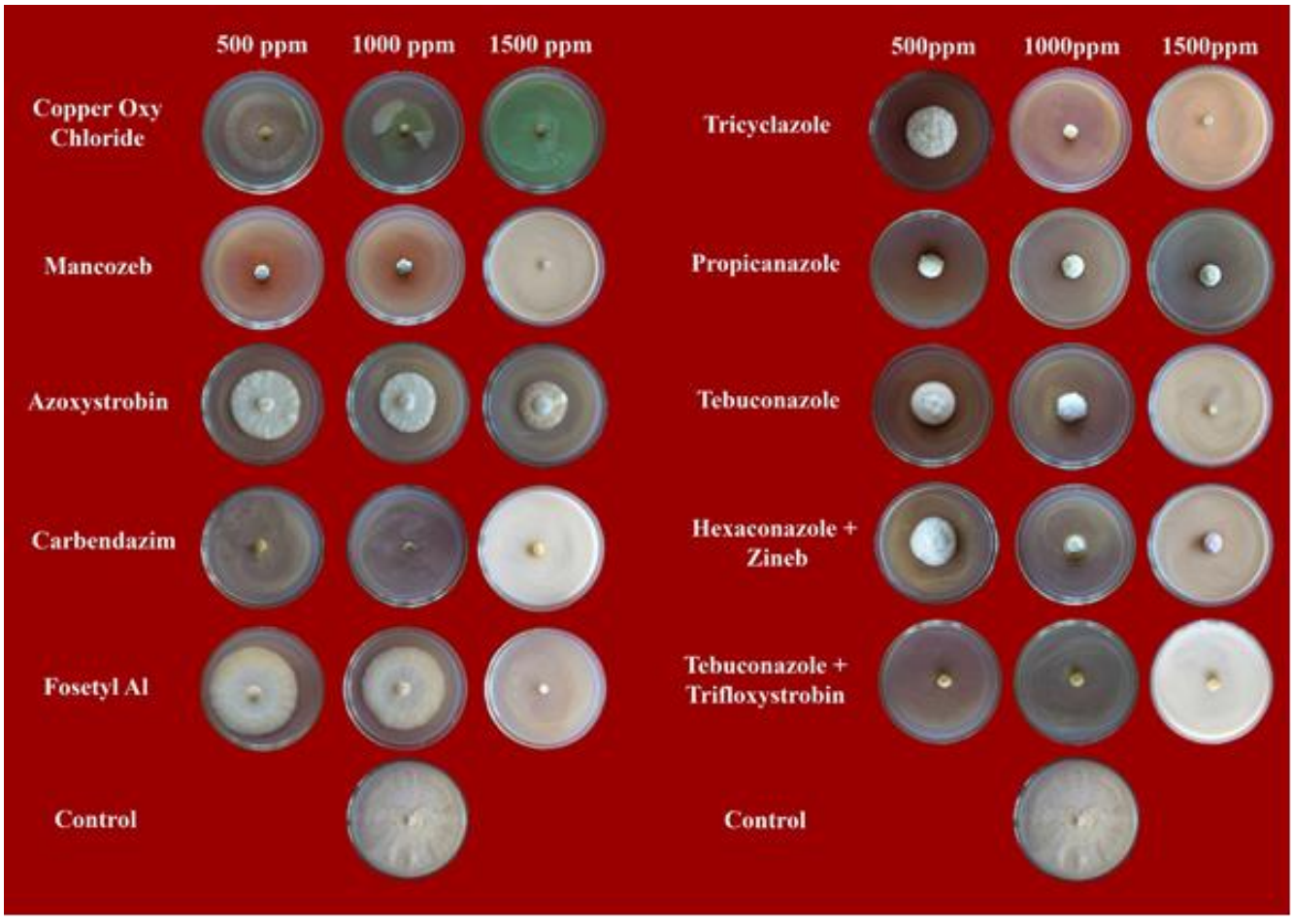

Plate 3: *In vitro* evaluation of commercial fungicides against *Fusarium* sp. (FI3)

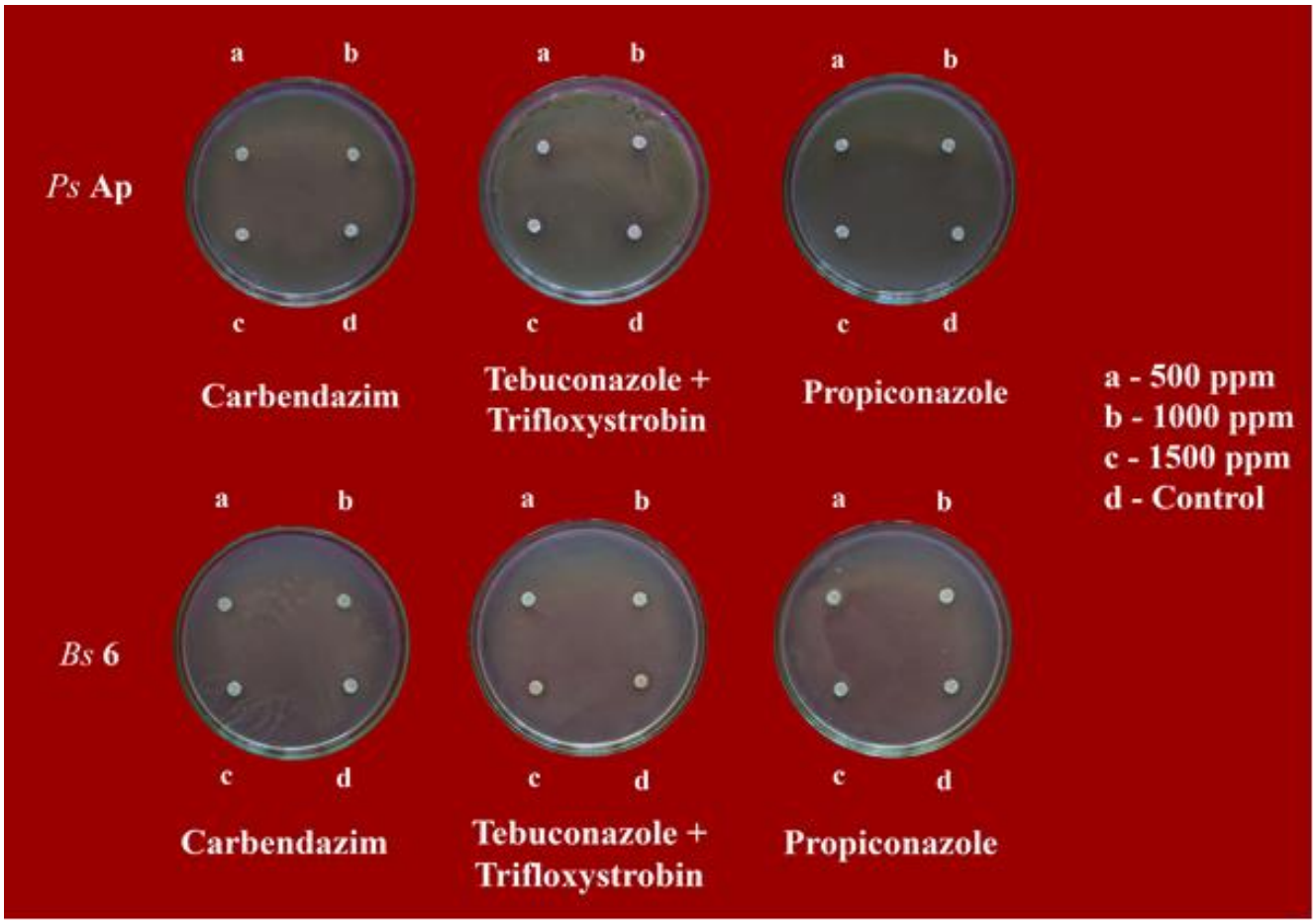

Plate 4: Compatibility test between bacterial biocontrol agents and effective fungicides

Table 1: Pathogenicity of *Fusarium* sp. in chrysanthemum

S. No.	Isolate No.	Place of isolate collection	District	Disease severity (%)	Days taken for symptom development
1	FI1	Sajjalapatti	Krishnagiri	68.62	45
2	FI2	Rayakottai	Krishnagiri	71.86	43
3	FI3	Milagaipatti	Dindigul	82.10	40
4	FI4	Andipatti	Dindigul	54.90	47
5	FI5	Samichettipatti	Dharmapuri	50.95	52
6	FI6	Gudalur	Theni	47.38	59
7	Control	-	-	0.00	0.00

Table 2: *In vitro* efficacy of *Pseudomonas* sp. against the mycelial growth of virulent isolate of *Fusarium* sp. (FI3)

S. No.	Isolates	Mycelial growth of *Fusarium* spp in (cm)*	Mycelial growth inhibition over control (%)
1	*Ps* Ad	4.0^{g}	55.55 (48.19)
2	*Ps* Rk	5.8^{e}	35.55 (36.60)
3	*Ps* Sp	4.6^{f}	48.88 (44.36)
4	*Ps* Mp	5.9^{e}	34.44 (35.93)
5	*Ps* Ap	3.8^{g}	57.77 (49.47)
6	*Ps* Mv	4.9^{f}	45.55 (42.45)
7	*Ps* Gd	6.2^{de}	31.11 (33.90)
8	*Ps* Sc	6.7^{bc}	25.55 (30.36)
9	*Ps* Tp	7.1^{b}	21.11 (27.35)
10	*Ps* Om	6.4^{cd}	28.88 (32.51)
11	Control	9.00^{a}	-
	CD (P=0.05)	0.45	

*Mean of three replications
Mean followed by a common letter are not significantly different at 5% level by DMRT.
Data in parenthesis are arc sine transformed values

Table 3: *In vitro* efficacy of *Bacillus* sp. against the mycelial growth of virulent isolate of *Fusarium* spp (FI3)

S. No.	Isolates	Mycelial growth of *Fusarium* spp in (cm)*	Mycelial growth inhibition over control (%)
1	*Bs* Rk	5.9^{e}	34.44 (35.93)
2	*Bs* Sp	6.2^{d}	31.11 (33.90)
3	*Bs* Ap	5.8^{ef}	35.55 (36.60)

4	*Bs* Gd	7.2 [b]	20.00 (26.57)
5	*Bs* 6	5.2 [g]	42.22 (40.52)
6	*Bs* 8	6.4 [cd]	28.88 (32.51)
7	*Bs* 9	6.2 [d]	31.11 (33.90)
8	*Bs* 13	6.5 [c]	27.77 (31.80)
9	*Bs* 17	6.6 [c]	26.66 (31.09)
10	*Bs* 18	5.6 [f]	37.77 (37.92)
11	Control	9.00 [a]	-
	CD (P=0.05)	0.44	

*Mean of three replications
Mean followed by a common letter are not significantly different at 5% level by DMRT.
Data in parenthesis are arc sine transformed values

Table 4: Effect of fungicides on mycelial growth of *Fusarium* sp. (FI3)

Treatment No.	Fungicides	Mycelial diameter (cm)*		
		500 ppm	1000 ppm	1500 ppm
T1	Copper-oxy-chloride 50% WP	7.6[b] (15.00)	5.8[c] (35.55)	0.0[e] (100)
T2	Mancozeb 75% WP	1.6[g] (82.22)	1.2[f] (86.66)	0.0[e] (100)
T3	Azoxystrobin 23% SC	6.7[c] (25.55)	5.5[c] (38.88)	4.7[b] (47.77)
T4	Carbendazim 50% WP	0.0[h] (100)	0.0[g] (100)	0.0[e] (100)
T5	Fostyl Al 80% WP	7.5[b] (16.66)	7.0[b] (22.22)	0.0[e] (100)
T6	Tricyclazole 75% WP	4.6[d] (48.88)	1.3[f] (85.55)	0.0[e] (100)
T7	Propiconazole 25% WP	2.3[f] (74.44)	2.1[d] (76.66)	1.8[c] (80.00)
T8	Tebuconazole 25% EC	3.8[e] (57.77)	2.0d[e] (77.77)	0.0[e] (100)
T9	Hexaconazole 45% + Zineb 68% WP	4.2[d] (53.33)	1.7[e] (81.11)	1.2[d] (86.66)
T10	Tebuconazole 50% + Trifloxystrobin 25% WP	0.0[h] (100)	0.0[g] (100)	0.0[e] (100)
T11	Control	9.0[a]	9.0[a]	9.0[a]
CD		0.58	0.45	0.26

*Mean of three replications
Mean followed by a common letter are not significantly different at 5% level by DMRT.
Figures in parentheses are per cent inhibition of mycelial growth over control

Table 5: Compatibility test between bacterial biocontrol agents and effective fungicides

S. No	Fungicides	Inhibition Zone (mm)*					
		Ps Ap			*Bs* 6		
		500 ppm	1000 ppm	1500 ppm	500 ppm	1000 ppm	1500 ppm
1	Carbendazim	5	5	5	5	5	5
2	Tebuconazole + Trifloxystrobin	5	5	5	5	5	5
3	Propiconazole	5	5	5	5	5	5

*****Mean of three replications
5 mm indicates 0% inhibition (Disc diameter)

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