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Evaluation of the biocontrol potential of *Bacillus subtilis, Pseudomonas aeruginosa* and *Trichoderma viride* against bacterial wilt of Tomato

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Abstract

Bacterial wilt, caused by the soil-borne pathogen *Ralstonia solanacearum* is an important disease of tomatoes, causing plant death and significant yield losses. The three known antagonistic organisms *Bacillus subtilis, Pseudomonas aeruginosa* (KUCd1) and *Trichoderma viride* were tested under *in vitro* and *in vivo* conditions against *Ralstonia solanacearum*. *B. subtilis* showed a significant antagonistic effect when pre-inoculated before the pathogen. Both under *in vitro* and green house conditions, a combination of the two bacteria *Bacillus subtilis* and *Pseudomonas aeruginosa* (KUCd1) in equal proportion also lowered the disease incidence by 51-55%.The studies indicate the efficacy of *B. subtilis* and the consortium of *Bacillus subtilis* and *Pseudomonas aeruginosa* (KUCd1) as potential bio-control agents against the bacterial wilt of tomato.

INTRODUCTION

Ralstonia solanacearum [1] is a known bacterial pathogen causing wilt in a wide range of host plants including a large number of economically important agricultural crops. Since it is a soil borne pathogen and host resistance is limited, the disease is difficult to control. Especially in the important crop plant tomato, bacterial wilt resistance has not been stable worldwide and yield losses of up to 90 percent have been recorded. Disease control using chemicals is another feasible option but there are several negative effects of use of chemicals starting from development of pathogen resistance to the chemical agents to irreversible environmental hazards.

In recent years PGPRs (Plant Growth Promoting Rhizobacteria) have been reported to be potential biological control agents as they are known for growth promotion as well as causing disease reduction in crops [2]. Among PGPRs, fluorescent pseudomonas has been reported to be effective against a broad spectrum of plant pathogens [3]. Similarly the sporulating Gram positive bacteria like *Bacillus* spp. have also been used successfully for plant disease control [4]. Amongst fungi, *Trichoderma* spp. are known to be effective biological means to control soil borne diseases [5].

However the relative competence of these genera as consortia or singly against bacterial wilt is yet to be compared. The objective of this work was therefore to comparatively evaluate the efficacy of the strains of *P. aeruginosa* KUCd1, *B. subtilis* and *T. viride* which are important representatives of the three genera as bio-control agents against bacterial wilt in tomato.

MATERIAL AND METHODS

Source of the pathogen

The causal organism of bacterial wilt, *Ralstonia solanacearum* was isolated from tomato (*Lycopersicon esculentum*) in Malda district of West Bengal. The isolate was grown on TTC medium, screened based on disease symptoms (in field and greenhouse) and molecular analysis (using OLI1/Y2primers) [6-8]. and was stored in sterilized distilled water in cork screwed bottles at 24°C.

Source of the antagonistic organisms

The bacterial isolates *Pseudomonas aeruginosa* (KUCd1), a strain reported to be cadmium tolerant and PGP (plant growth promoter) [9]; *Bacillus subtilis* and the fungal isolate *Trichoderma viride* were obtained from Prof. S.K Mukherjee, Department of Microbiology, University of Kalyani.

In vitro assay

Pseudomonas aeruginosa (KUCd1), *Bacillus subtilis* and *Trichoderma viride* were evaluated against the bacterial wilt pathogen *in vitro*. The experimental designs were complete randomized design (CRD) with six replications. Cross culture method, filter paper disk method [10] and perforated agar plate method [11] were used for the first and second antagonists and dual culture method was applied for *T.viride* respectively. PDA medium was used in the experiments in order to favor the growth of *R.solanacearum* and the potential antagonists. T test procedure was applied to test the significance differences in the distance of inhibition of pathogen by antagonists and DMRT was used to compare the mean performance among treatments.

Greenhouse assay

Tomato seeds (*Lycopersicon esculentum* c.v. *Pathorkuchi*) were sown in pots filled with sterilized soil and three sets of replica of two true leafed seedlings were used for each experiment. One set of control tomato plants were grown without inoculation and one set inoculated with *R.solanacearum* . The antagonistic potential was tested individually for *Pseudomonas aeruginosa* (KUCd1), *Bacillus subtilis* and *Trichoderma viride*, and also with a combination of *Pseudomonas aeruginosa* (KUCd1) and *Bacillus subtilis*. Soil pretreatment with antagonists were done one week prior to the pathogen inoculation by soil drenching method at 10^8cfu/ml. 20ml suspension of the pathogen also (10^8cfu/ml) was inoculated into the soil as a water suspension through soil drenching after seven days of application

of the antagonist .The disease incidence was calculated over a period of 40 days, beginning at 7 days after inoculation using the method reported by Yun Cao *et al* [12] with the following formula: Percent disease incidence = [(number of infected plants)/total number of plants] × 100. (Experiment 1).

In Experiment 2, the applications of antagonists were done one week after pathogen inoculation. The inoculum concentration and method of inoculation were kept same as in Experiment 1.Disease incidence was recorded for 40days from the day of pathogen inoculation.

Statistical Analysis

Experiments were designed as a completely randomized design (CRD) with six replications, and analyses were conducted using the R software (The R Foundation for Statistical Computing). The means were compared by Duncan multiple-ranges test (DMRT) at $P \leq 0.01$

RESULTS

In vitro Assay

Among the three antagonists, *Bacillus subtilis* had the highest distance of inhibition of the pathogen growth (Table 1a and b). The antagonistic organisms differed significantly ($P \leq 0.01$) on the basis of their mean inhibition zone and *T.viride* was found to be least effective in the *in vitro* experiments. Filter paper disc method showed smaller inhibition zones than cross-streak method and Perforated agar medium Method.

***In vivo* Assay -** In Experiment 1, the lowest disease incidence (51.12%) was found with 1:1 *Pseudomonas aeruginosa* (KUCd1) and *Bacillus subtilis* consortia after 42days from day of inoculation. Pots inoculated singly with *Pseudomonas aeruginosa* (KUCd1), *Bacillus subtilis* and *Trichoderma viride* had disease incidence of 65.43%, 83.1% and 92.01% respectively.

As evident from the Table.2a, application of any one of the treatments delayed the time of appearance of the first disease symptoms. On the 14th day from inoculation of pathogen, treated plants showed significant lowering in disease incidence as analysed through the Duncan's Multiple Range Test (DMRT) analysis except for the plants treated only with *T.viride* as bio-control agent. Use of *B. subtilis* singly and in combination with *P aeruginosa* showed almost similar result in controlling the disease till 28 days from the day of inoculation. The performance of the consortia of *B.subtilis* and *P.aeruginosa* were significantly better than *B.subtilis* alone from 35th days after application When the control plants were completely wilted by 42 days, plants treated with combination of *B. subtilis* + *P.aeruginosa* were only 50% infected; higher incidences were observed for treatment with *B. subtilis* (65.43).Thus from the greenhouse assay , treatment 2, (*B. subtilis*) and treatment 5, (*B subtilis* + *P.aeruginosa*) were found to lower the disease incidences significantly.

In Experiment 2, where the antagonists were added after one week of pathogen inoculation, the efficacy of the combination of *Pseudomonas aeruginosa* (KUCd1) and *Bacillus subtilis* was maximum with a disease incidence percent significantly different from the untreated plants, as against the antagonists used singly. Pots where *T. viride* was added were found to show highest disease severity almost equal to the control plants. However, the disease incidence was high when application of antagonists was done one week after pathogen inoculation. This may be due to the abundance growth of pathogen in the soil which outcompetes in number thus preventing the antagonistic organisms to reach a suitable population for the bio-control activity.

DISCUSSION

Recent studies have indicated that biological control of bacterial wilt disease could be achieved using antagonistic bacteria [13-14] . Toyota and Kimura [15]have reported the suppressive effect of some antagonistic bacteria on *R.solanacearum*. Some naturally occurring antagonistic rhizobacteria such as *Bacillus* spp. [16], *Pseudomonas* spp. [17] have been known to control this disease. In our study, a comparative analysis of the antagonistic potential of three known bio-control agents has been done to identify a stable antagonist against bacterial wilt of tomato. Lack of correlation between *in vitro* result and biological control *in vivo* has been documented in other studies on other pathogens [18-19]. To make a confirmative analysis and to compare the antagonistic behavior of these organisms at both cultural and field conditions, *in vitro* and *in vivo* assays have been designed.

Among the strains used in our experiment, *Bacillus subtilis* have been reported to be effective in the management of bacterial wilt disease in tomato [20]. Our study was in harmony with this where in both *in vitro* and in greenhouse *B. subtilis* emerged to be the best application in controlling bacterial wilt. In the greenhouse assay, we observed *B. subtilis* to be the most effective individually over the other two antagonists. In previous studies, inhibition had been observed when the antagonist was applied before or simultaneously with the pathogen, while antagonist was less effective when applied after pathogen inoculation. This indicated that the biocontrol effects of the *B. subtilis* strains are more likely to be a preventive effect rather than a therapeutic effect on the disease [21]. Our results (Table 2a and 2b) were in confirmation with these earlier findings. Thus, amount of suppression of disease incidence depends on the antagonist and the time of application. The bacterial strain *P. aeruginosa* KUCd1 used in our study is a cadmium tolerant strain reported to have PGP effect and it has also shown antagonistic effect towards several plant pathogens [22]. This strain proved to be an effective antagonist against *R. solanacearum* when applied before pathogen inoculation and lowered disease incidence by 15-20% till 35 days after pathogen inoculation. Use of antagonist consortium was restricted to *B. subtilis* and *P. aeruginosa* KUCd1, as mutual antagonism of the bacterial strain *B. subtilis* with *Trichoderma* spp has been reported earlier [23]. The consortium with an equal proportion of the two bacteria gave the lowest disease incidence in the 35-42 days of the study. This enhancement may be due to multiple mechanisms involved in the action of biological control of the disease [24].

T. viride was found to be the least potential antagonist against bacterial wilt of tomato. Though it seemed effective initially when appearance of first disease symptoms were delayed, our analysis showed that from 14 days after inoculation the disease incidence in *T.viride* treated plants were not significantly lower than the control plants. One of the features that make *Trichoderma* species effective as biocontrol agents against plant pathogenic fungi is their chitinase activity [25-26]. Probably because of this reason when treated against bacterial pathogens, the antagonistic activity of *Trichoderma viride* seems to be not as powerful as when used against pathogenic fungi.

Among the four methods of culture, cross streak method gives significantly ($P \leq 0.01$) highest inhibitory zone, suggesting it to be best suited method in dual culture tests. The filter paper disc method on the other hand produced the smallest inhibition zone

Table 1a. *In- vitro* antagonistic effect of the bacterial strains against *R. solanacearum*

Antagonist	*R. solanacearum*					
	Filter paper disc method		Cross streak method		Perforated agar plate method	
	Inhibition zone(mm)	% Inhibition	Inhibition zone(mm)	% Inhibition	Inhibition zone(mm)	% Inhibition
1.*Bacillus subtilis*	7.6	30.4	9.6	38.4	9.5	38
	8.1	32.4	11.2	44.8	7.9	31.6
	8.0	32	10.8	43.2	9	36
	8.0	32	10.1	40.4	8.5	34
	7.5	30	9.9	39.6	8.6	34.4
	7.8	31.2	11.8	47.2	8.5	34
Mean:	7.8b		10.6a		8.35b	
SD :	0.19		1		0.4	
2.*P.aeruginosa* KUCd1	3.7	14.8	6.6	26.4	6.0	24
	3.4	13.6	6.9	27.6	6.4	25.6
	3.0	12	8.0	32	6.5	26
	3.1	12.4	7.3	29.2	7.4	29.6
	3.2	12.8	7.0	28	5.5	22
	2.6	10.4	7.5	30	6.2	24.8
Mean:	3.17c		7.2a		6.1b	
SD: :	0.48		0.5		0.8	

Column1: P (T<=t) two-tail 2.545; Column2: P (T<=t) two-tail 0.0001; Column3: P (T<=t) two-tail 0.0015

*Means with the same letter in the same row are not significantly different. ($P \leq 0.01$)

Table 1b. *In- vitro* antagonistic effect of *T. viride* against *R. solanacearum*

Antagonist	Inhibition Zone (in mm) in dual culture	% Inhibition
Trichoderma viride	3.4	13.6
	2.6	10.4
	2.8	11.2
	3.1	12.4
	4.6	18.4
	3.7	14.8
Mean	3.8	
SD	0.8	

Table 2a. Mean values of Disease Incidence Percentage when pre-inoculated with antagonists.

Treatment	Antagonist used	Disease Incidence %					
		7Days	14Days	21Days	28Days	35Days	42Days
1. *R. solanacearum*	-	26.2	35.56a	46.53a	69.9a	84.28a	94.7a
2. *R. solanacearum*+ *B. subtilis*	*B. subtilis*	0	12.91c	22.60c	28.55c	49.27c	65.43c
3. *R.solanacearum* + *P.aeruginosa*	*P.aeruginosa*	0	22.74b	30.27b	49.55b	68.37b	83.1b
4. *R. solanacearum* + *T. viride*	*T. viride*	0	32.47a	43.47a	65.49a	81.73a	92.01a
5. *R. solanacearum*+ *B.subtilis*+ *P.aeruginosa*	*B. subtilis*+ *P.aeruginosa*	0	9.34c	19.68c	25.80c	37.08d	51.12d

Analysis of variance and Mean comparison done by Duncan's Multiple Range Test

*Means with the same letter in the same column are not significantly different. ($P \leq 0.01$)

Table 2b. Mean values of Disease Incidence Percentage in Tomato with post-inoculation of antagonists over 42 days.

Treatments	Antagonist used	Disease Incidence %					
		7Days	14Days	21Days	28Days	35Days	42Days
1.*R. solanacearum*	-	25.54a	35.56a	46.53a	69.9a	84.28a	94.7a
2. *R. solanacearum*+ *B. subtilis*	*B. subtilis*	20.57c	30.65b	43.93b	65.59c	82.60a	92.58ab
3. *R.solanacearum* + *P.aeruginosa*	*P.aeruginosa*	24.62a	31.08b	44.59ab	66.57bc	84.27a	92.47ab
4. *R. solanacearum* + *T. viride*	*T. viride*	25.63a	35.44a	45.74ab	67.29b	84.51a	94.47a
5. *R. solanacearum*+ *B. subtilis*+*P.aeruginosa*	*B.subtilis*+*P.aeruginosa*	21.97b	29.28b	40.15c	62.76d	81.31a	91.65b

Analysis of variance and Mean comparison done by Duncan's Multiple Range Test

*Means with the same letter in the same column are not significantly different. ($P \leq 0.01$)

A B

Fig. 1. Diseased tomato plants (Experiment 1) on 35th day from day of pathogen inoculation with (a) *R.solanacearum* treated plant against control plant, and (b) *R.solanacearum* treated plant against *R.solanacearum* + *B. subtilis* treated plant.

probably due to slower growth rate of the antagonistic bacteria as seen from the DMRT test in table 1a.

Thus, as seen from the results, consortium of two antagonists viz; *B subtilis* and *P. aeruginosa* KUCd1showed a significantly lower disease incidence till almost 45 days after pathogen inoculation. Field studies in different agro-climatic zones need be undertaken to confirm the effectiveness of these antagonistic strains under natural conditions and over a larger area.

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