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Comparative efficacy of diffusible and volatile compounds of tea rhizospheric isolates and their use in biocontrol

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ABSTRACT

An attempt was made to study *in vitro* production of antifungal substances by various strains of *Bacillus subtilis* and *Pseudomonas corrugata.* These strains were isolated from the rhizosphere of established tea *Camellia sinensis* L. located in North West Indian Himalayan regions *viz* Himachal Himalayas and Garhwal Himalayas. Selected strains were checked for their biocontrol potential against two phytopathogenic fungi *Fusarium udum* and *Alternaria solani* and the antagonists were found to cause inhibition in radial growth of the fungi. This diffusible effect was attributed to the production of diffusible and volatile compounds which were found to be potent antifungal in nature. The antifungal substances in bacterial strains were found to be extracellular, proteinacious in nature. The colony forming units (cfu) of pathogenic fungi were reduced by the diffusible and volatile compounds produced by antagonists. The inhibition in cfu was more by volatile compounds than by diffusible compounds. The volatile compounds included aldehydes, alcohols, ketones, sulphides and HCN.

Keywords: *Bacillus*, *Pseudomonas*, *Camellia sinensis*, fungi, biocontrol.

INTRODUCTION

Tea is attacked by a number of fungal and bacterial pathogens. An important step in disease control programmes involves selection of effective biocontrol agents. Various microorganisms like species of *Bacillus, Pseudomonas, Gliocladium* and *Trichoderma* have been recognized for their biocontrol potential against selected pathogens of tea (Agnihothrudu, 1999).

It has been reported that natural rhizosphere is often inimical to pathogens because antagonists form part of the rhizosphere community (Lynch, 1987). The overall interactions amongst tea roots, microbes and environmental conditions prevailing in the tea rhizosphere seem to favor the growth of microbes which are known to produce strong antibiotics with potential biocontrol agents. These rhizosphere bacteria with antagonistic properties improve plant growth by suppressing either major or minor pathogens (saprophytes) and providing first line of defense (Weller, 1988).

The established rhizosphere of tea has been proposed as an excellent site for the isolation of biocontrol agents (Pandey and Palini, 2002-2003). *Bacillus subtilis,* in particular, has been found to be the dominant bacterium associated with tea roots and these rhizospheric associated bacteria are considered ideal for biocontrol (Pandey and Palini, 1997, 2004). A siderophore producing strain of *Pseudomonas* isolated from the tea

rhizosphere also showed *in vitro* antibiosis against a number of plants pathogenic fungi (Dileep and Bezbaruah, 1996, 1999).

Keeping this in view, a study was planned to investigate (i) *in vitro* antibiosis aspects of *B. subtilis* and *P. corrugata* soil isolates from tea rhizosphere on pathogenic fungi *F. udum* and *A. solani,* (ii) comparative efficacy of diffusible and volatile compounds as antifungal agents.

MATERIALS AND METHODS

Bacterial strains

The antagonists used in the present study were isolated from the tea rhizosphere situated in the North West, the Himachal and the Garhwal Himalayas. In the Himachal Himalayas, three sites were selected viz Banuri tea experimental garden, Bundla tea estate and Rajpur tea estate, Palampur, Himachal Pradesh. In the Garhwal Himalayas, three sites were also selected, one from IIP tea gardens and two from Prem Nagar tea gardens, Dehradun, Uttaranchal. *Bacillus subtilis* soil isolates from Himachal Pradesh were designated as HPAB, while *B. subtilis* and *P. corrugata* from Uttaranchal were designated as UAAB and UAAP, respectively. Soil isolates of *Bacillus* and *Pseudomonas* obtained from established tea rhizospheric soil were purified and identified.

Pathogenic fungi

The pathogenic fungi *Alternaria solani* and *Fusarium udum* were collected from the culture collection of Dr. S. Sharma, Department of Microbiology, SBS PGI, Balawala, Dehradun. The test fungi are known to cause diseases (*Fusarium udum*: Fusarium wilt and rots, *Alternaria solani*: Leaf spot and leaf blight) (Bilgrami et. al., 1991).

Maintenance of microbial cultures

Fungal and bacterial isolates were maintained at 4 °C by repeated subculture on potato dextrose, nutrient agar (Hi-Media) (*Bacillus*) and King's B media (Hi-Media) (*Pseudomonas*) slants, respectively. The preliminary observations on antagonistic properties of these bacteria were recorded on the basis of inhibition zones developed around bacterial colonies by fungus by diffusion method. On the basis of this screening, the selected bacterial strains were tentatively classified as antagonistic agents.

Antagonism Assays

For examining antagonism due to diffusible compounds, a fungal lawn of test fungus was grown on potato dextrose agar (PDA) plates. Discs of 7 mm diameter from the fungal lawns were cut and inoculated on PDA plates. A sterilized Whatman filter paper disc of 5 mm diameter, dipped in nutrient broth containing the bacterial culture (10^8 cfu/ml), was inoculated about 1.5 to 2.0 cm away from the fungal disc. The plates were incubated in an inverted position at 28 °C. The observations were recorded after 24, 72 and 120 h of incubation by measuring the growth of the fungus towards and away from the bacterial colony. Inhibition of the fungal growth was calculated using the formula:

$$\frac{R1 - R2}{R1} \times 100$$

Where, R1 (a control value) represents a radial distance grown by the fungus in the direction of the antagonist and R2 represents the distance on a line between the inoculation positions of the fungus and the bacteria.

Antagonism due to volatile compounds was evaluated by preparing a bacterial lawn on Nutrient agar and Potato dextrose agar medium. After incubation for 24 h, the lid was replaced by a plate containing agar blocks of 7 mm diameter with a test fungus grown on potato agar medium. The two plates were sealed by the parafilm. Control sets were prepared without bacteria in the bottom plate. The Petri dishes were incubated at 28 °C and observations were recorded after 24, 72 and 120 h. The per cent growth inhibition of the test fungus was calculated using the formula:

$$\frac{r1 - r2}{r1} \times 100$$

Where r1 (a control value) represents the radial growth of the fungus in control sets without bacteria, and r2 represents the radial growth of the fungus in sets inoculated with the bacterium.

Enumeration of Antagonized Fungi

A 4 mm diameter agar block with fungal growth on it was taken from the antagonized (showing inhibition on plates in

both diffusible and volatile sets) and control plates after 120 h incubation. This agar block was serially diluted and plated on PDA plates. The plates were incubated at 28 °C and counts were recorded after 3 days. The per cent inhibition in colony forming units (cfu) was calculated using the formula:

$$\frac{C1 - C2}{C1} \times 100$$

Where C1 (a control value) represents the cfus of fungi from control plates and C2 represents the cfus of fungi from antagonized plates.

Location of diffusible antimicrobial substances

To determine the antimicrobial substances (extracellular or membrane bound), isolates of *B. subtilis* and *P. corrugata* were grown in nutrient broth and King's B broth, respectively, for 24-36 h at 30 °C and centrifuged at 6000 x g for 20 min to separate culture filtrate (CF) and cell pellet (CP). Cell free extract (CFE) was obtained after sonicating the cells three times for 15 s, respectively, with a gap of two min each using an ultrasonicator and recentrifuging the cells at 10,000 x g for 10 min under cold conditions at 4 °C. Each of the samples was tested for inhibitory activity against selected pathogenic fungi using the diffusion method as described earlier in section Antagonism Assays.

Determination of proteinacious nature of antimicrobial substances, HCN and siderophore production

To determine the nature of antimicrobial substances (for proteins only) produced by antagonistic bacteria, ammonium sulfate precipitation as well as proteolytic enzyme treatment methods were used (James and Gutterson, 1986). Culture filtration (supernatant after centrifugation at 10,000 x g for 20 min) of bacterial strains were treated with the proteolytic enzyme trypsin (200 µg trypsin was dissolved in 0.5 ml of phosphate buffer, pH 7.5) and was incubated at 37 °C for one hour. Subsequently, each sample was analyzed for residual antagonistic activity. HCN production was determined according to the method of Bakker and Schippers (1987). The universal chemical assay as described by Schwyn and Neilands (1987) was used for the detection of siderophore production by antagonistic bacteria.

Quantitative analysis of bacterial volatiles

The bacterial cultures were grown on nutrient agar medium (this medium supported good growth for tested pathogenic fungi, *Bacillus* and *Pseudomonas* sp.) sealed with Parafilm for two week at 25 °C. Aseptically, a hole was made in the plastic Petri plates before inoculation and from that gas was withdrawn with the help of a syringe. The gas phase of the bacterial cultures was analyzed using GC-MS separation as described by Strobel et al. (2001).

RESULTS

In the present investigation, all the *B. subtilis* and *P. corrugata* isolates from tea rhizosphere were screened for biocontrol agents against two phytopathogenic fungi viz *F. udum* and *A. solani*. Twelve of the *Bacillus* and four of the *Pseudomonas* soil isolates were found to inhibit the radial growth of the test fungi. This effect was attributed to the production of diffusible and volatile compounds, which were antifungal in nature. Volatiles compounds analyzed by GC-MS were found to fall into several classes of chemical substances viz alcohols, lipids, esters, acids and ketones; all were present in one to two weeks old cultures of bacteria. The effect of diffusible and volatile compounds produced by *B. subtilis* and *P. corrugata*, evaluated in terms of reduced radial growth of fungi is presented in Table 1. The values ranged from 13.3 to 50 % in *F. udum* and 12.1 to 37.8 % in *A. solani* due to the secretion of diffusible compounds and from 3.2 to 22.5 % in *F. udum* and 5.0 to 32.2 % in *A. solani* due to production of volatile compounds. Although a few bacterial strains could not show antagonism against the tested fungi, out of 16 isolates of *Bacillus* and *Pseudomonas*, five best isolates, namely HPAB1, HPAB4, HPAB5, UAAP1 and UAAB5, showed maximum inhibition test against fungi, and therefore, were selected for further antibiosis tests. The inhibitory effect was further confirmed by reduction in cfu of antagonized fungi. Experiments on cfu developed from antagonized fungal growth revealed that volatile compounds caused greater inhibition than the diffusibles (Table 2).

Table 1: Per cent inhibition in radial growth of pathogenic fungi caused by diffusible and volatile compounds produced by tea rhizospheric isolates 72 hrs after incubation

S. No.	Isolates	*F. udum.*		*A. solani*	
		Diffusible	Volatile	Diffusible	Volatile
1	$HPAB_1$	44.4 ± 7.5	22.5 ± 2.5	34.3 ± 5	32.2 ± 4
2	$HPAB_4$	37.5 ± 9	14.5 ± 2	32.3 ± 8	5.0 ± 1.5
3	$HPAB_5$	40.0 ± 5	4.8 ± 1	37.8 ± 4.5	13.5 ± 2
4	$HPAB_6$	13.3 ± 2	3.2 ± 1	37.5 ± 7	16.9 ± 4
5	$UAAB_9$	28.5 ± 5	12.9 ± 2.5	-	-
6	$UAAB_{10}$	22.8 ± 8	9.6 ± 3	19.1 ± 4	-
7	$UAAB_{11}$	26.6 ± 3.5	-	-	-
8	$UAAB_{12}$	-	-	19.0 ± 6.5	15.2 ± 4.5
9	$UAAB_{14}$	-	-	12.1 ± 3	-
10	$UAAB_{15}$	40.0 ± 6	6.4 ± 1.5	29.0 ± 6.5	10.1 ± 3
11	$UAAB_{17}$	16.6 ± 4	20.9 ± 2	32.3 ± 4	18.6 ± 2
12	$UAAB_{18}$	28.5 ± 7	8.0 ± 2	-	-
13	$UAAP_1$	45.9 ± 5	3.2 ± 1	23.3 ± 9	22.0 ± 9
14	$UAAP_3$	43.2 ± 3.5	6.4 ± 0	30.0 ± 0	25.0 ± 6
15	$UAAP_4$	24.2 ± 5.5	4.8 ± 1.5	18.7 ± 2	6.7 ± 2.5
16	$UAAP_5$	50.0 ± 0	22.5 ± 4	27.2 ± 7.5	20.0 ± 0

cfu at n x 10^4
Values are Mean ± SD of three determinations.

Table 2: Comparative account of cfu developed from the control and antagonized pathogenic fungi

S. No.	Isolates	*A. solani* (cfu control plate 96)		*F. udum* (cfu control plate 176)	
		Diffusible	Volatiles	Diffusible	Volatiles
1	$HPAB_1$	73 ± 9	64 ± 8.5	83 ± 10	72 ± 7
2	$HPAB_4$	62 ± 7	59 ± 9	77 ± 12	53 ± .5
3	$HPAB_5$	78 ± 13.5	63 ± 10	94 ± 9.5	72 ± 4
4	$UAAP_1$	59 ± 9	51 ± 9	38 ± 8	16 ± 8
5	$UAAP_5$	67 ± 8	53 ± 15	25 ± 10.5	21 ± 5.5

cfu at n x 10^4
Values are Mean ± SD of three determinations.

A perusal of Figures 1 and 2 shows the cfu inhibition by diffusible compounds ranged from 18.75 to 38.54 % in *A. solani* and 46.59 to 85.79 % in *F. udum* due to diffusible compounds. Inhibition due to volatile compounds ranged between 33.33 to 47.79 % in *A. solani* and 59.0 to 90.9 % in *F. udum.* Isolates $HPAB_1$ and $UAAP_5$ were found to be most effective antagonistic agents amongst the entire *B. subtilis* and *P. corrugata* isolates, respectively.

As shown in Table 3, a full and partial inhibition was obtained after ammonium sulfate precipitation and trypsin (proteolytic) tested culture filtrate against the plant pathogens. This confirms the proteinacious nature of the diffusible antifungal substances produced by selected antagonists. HCN production was also observed in selected bacterial soil isolates.

No inhibitory activity was exhibited by any of the cell pellets, but clear zones of inhibition were produced by culture filtrate and small zones by cell free extract, also showing clearly that most of the antimicrobial substances are extracellular and some part of it is bound to the cell wall, as small zones were observed with cell free extracts (Table 4).

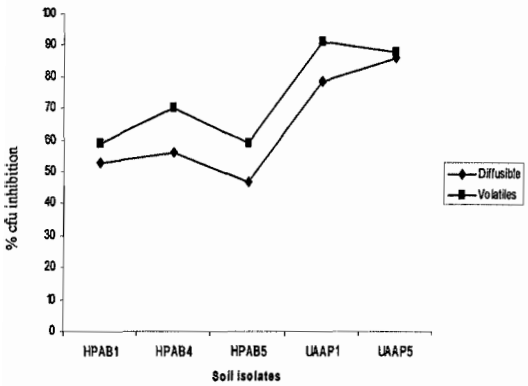

Figure 1: Comparative cfu % inhibition by diffusible and volatile compounds in *F. udum*.

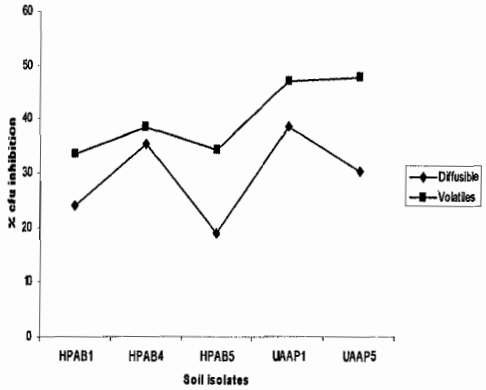

Figure 2: Comparative cfu % inhibition by diffusible and volatile compounds in *A. solani*.

Table 3: Effect of ammonium sulphate and trypsin treatment on antifungal substances produced by *Bacillus* and *Pseudomonas* isolates, HCN and siderophore production.

Isolates	HCNp	Sp	*F. udum*			*A. solani*		
			Cf	ASt	Tt	Cf	ASt	Tt
HPAB1	+	+	+	+	±	+	+	±
HPAB4	+	+	+	+	±	+	+	±
HPAB5	+	+	+	+	±	+	+	±
UAAP1	+	+	+	+	±	+	+	±
UAAP5	+	+	+	+	±	+	+	±

HCNp = HCN production; Sp = Siderophore production; Cf = Culture filtrate; ASt = Ammonium sulphate treatments; Tt = Trypsin treatment; + Good zone of inhibition; ± Partial zone of inhibition.

Table 4: Location of diffusible antimicrobial substances produced by *B. subtilis* and *P. corrugata* isolates.

Isolates	*A. solani*			*F. udum*		
	Cell pellets	Culture filtrate*	Cell free extract**	Cell pellets	Culture filtrate	Cell free extract
HPAB1	-	+	+	-	++	+
HPAB4	-	+	+	-	++	+
HPAB5	-	+	+	-	++	+
UAAP1	-	+	+	-	++	+
UAAP5	-	+	+	-	++	+

* Culture filtrate obtained after centrifugation; ** Cell free extract obtained after sonication and centrifugation of cells; - No zone of inhibition; + Presence of inhibition zone; ++ Large zone of inhibition

DISCUSSION

Sixteen soil isolates of *B. subtilis* and *P. corrugata* from scores of soil isolates were selected after screening their antagonistic properties against *A. solani* and *F. udum*. In this study, the antibiosis aspects of these bacterial strains especially the production and comparative efficacy of diffusible and volatile compounds against the selected plant pathogenic fungi have been investigated. Our studies led us to see the prevalence of these

properties among the tested antagonistic bacteria.

Bacillus and *Pseudomonas* soil isolates were used for biocontrol experiments on the basis of their occurrence pattern because each of these species dominated in Himachal Himalayan region and in Uttaranchal Himalayan region respectively. The established tea bushes as are present in Kangra region of Himachal Himalayas grow in close proximity to each other and in this way allows the root exudates to accumulate; hence, there is a greater inhibitory effect. Species of *Bacillus* were found to be best colonizers of these tea roots; because of their spore forming nature, they can survive under adverse conditions. *Pseudomonas* species were altogether absent (Pandey and Palni, 1997). On the other hand, in case of abandoned tea bushes as are present in Dehradun region of Uttaranchal Himalayas which are scattered, less canopy and foliage is present. So probably the root exudates in this case do not accumulate in the rhizosphere (Pandey and Palni, 2004). Therefore, *Pseudomonas* species, which is a common soil bacterium, were able to dominate this region and *Bacillus* species, although were isolated from this region, were not dominant.

The diffusible antimicrobial substances inhibited the radial growth of the fungi and there was reduction in cfu, compared to the control. Regarding the nature of the diffusible antifungal substances, it might be a protein (as seen by ammonium sulfate precipitation and the trypsin effect) linked to a complex material of unknown nature and having antifungal property (Verma et al., 2001), which requires further detailed studies or may be due to siderophore production or combination of both. To understand the location of antimicrobial substances in cells, culture filtrate (CF) and cell free extract (CFE) of soil bacterial isolates, the inhibitory effects were seen only with CF and CFE, whereas no zone of inhibition was found in case of cell pellets. It was presumed that antimicrobial substances are produced extracellularly and a fraction of it might be membrane bound as also seen by some inhibitory effects by CFE. It is also quite possible that there might exist two substances, out of which one is extracellular and the other remains attached to the cell membrane until lysis, as seen by Howell and Stipanovic (1980) in the case of *P. fluorescens* where one antibiotic is extracellular and the other comes out only after lysis.

There are reports that, under iron-limiting conditions, bacteria produce a range of iron chelating compounds or siderophores which have a very high affinity for ferric iron. These bacterial iron chelators are thought to sequester the limited supply of iron available in the rhizosphere making it unavailable to pathogenic fungi, thereby restricting their growth (O'Sullivan and O'Gara, 1992; Dileep and Bezbaruah, 1999; Loper and Henkels, 1999).

It is also worth noticing that the volatile organic compounds inhibited more mycelial growth as compared to the diffusible compounds. On an average (mean of test fungi), per cent inhibition in cfu of *A. solani* was 29.37 % by diffusible and 40.16 % by volatile compounds; also, in *F. udum,* similar results have been observed where 63.97 % inhibition was by diffusibles and 73.37 % by volatiles. Therefore, it is evident that volatile compounds like alcohols, ketones, acids, aldehydes and HCN are more potent fungicides, 36.73 % higher in *A. solani* and 14.69 % in *F. udum* over the diffusible antifungal substances like siderophores, proteins and enzymes. So, it was concluded that volatile compounds probably cause certain structural deformations in the fungal mycelium rather than mere reduction in radial growth as in case of diffusible compounds. Due to this structural deformation, the values obtained for cfu of antagonized fungi in case of antagonism due to volatile compounds are higher than the cfu values of antagonized fungi due to diffusible compounds. Similar findings have also been reported by Chaurasia et al. (2004) and Dilantha et al. (2005).

In the present study, potential biocontrol agents were isolated and selected from the tea rhizosphere of Indian Himalayan region. This study further proves the importance of established rhizosphere for the isolation, screening and selection of efficient biocontrol agents. These agents show antagonistic effects against pathogenic fungi, and thus, can be used for disease management programmes.

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