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Established and Abandoned Tea (*Camillia sinensis* L.) Rhizosphere: Dominant Bacteria and Their Antagonism

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Abstract

Some parts of the Indian Himalayan region are covered by established and abandoned tea bushes. Rhizospheric soils of these plants were studied for bacterial dominance and antagonism. Representatives of *Bacillus* and *Pseudomonas* genera were found to dominate the rhizosphere of established and abandoned tea bushes, respectively. Amongst the isolated species *Bacillus subtilis* and *Bacillus mycoides* appeared to be closely associated with roots of established tea bushes while the rhizosphere of abandoned tea bushes was dominated by *Pseudomonas putida*. Four isolates of both *B. subtilis* and *P. putida* were selected on the basis of maximum antibacterial activity. The bacteriocin-like activity of *B. subtilis* and *P. putida* strains was detected to be active over a range of temperature 0–50°C and was sensitive to proteolytic enzymes. Incubation of indicator strains with different concentrations of bacteriocin-like substances confirmed their bactericidal activity. Various species of *Bacillus* and *Pseudomonas* behaved antagonistically amongst themselves due to the production of bacteriocins under *in vitro* conditions.

Key words: *Bacillus*, *Pseudomonas*, *Camillia,* rhizosphere, antagonism, bacteriocin

Introduction

The rhizosphere of established tea bushes has some specific characteristics, which are associated with the long lived nature of tea plants, viz. negative rhizospheric effect, lowering of soil pH, antagonistic activities among microbial communities and dominance of certain species (Sood *et al*., 2007). The overall interactions between 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 use as biocontrol agents. It has been reported that natural rhizosphere is often inimical to pathogens, because antagonists form a part of the rhizosphere community (Lynch, 1987).

The specific microbial population of rhizosphere is found to be affected by soil pH, temperature and moisture. Bacterial populations associated with the tea rhizosphere are found to be strongly affected by the negative rhizosphere effect in contrast to fungi which are less affected (Pandey and Palni, 1999) whereas positive rhizosphere effect has been observed in case of bacteria from a newly developed tea garden (Palni *et al*., 1998).

Bacteria inhabit highly competitive environments, such as plant-soil root interface (rhizosphere). In such niches, bacteria are constantly competing for nutrients and ecological space. As a consequence, bacteria have devised various offensive tools for intra and interspecies competition, such as the production of some antibiotic substances, bacteriocins and bacteriolytic enzymes. In all likelihood, the bacteriocins constitute the most abundant and diverse class of antimicrobial agents.

The purpose of this study was to establish, whether or not tea rhizosphere *Bacillus* and *Pseudomonas* species produce bacteriocidal compounds and, if so, characterize them preliminary to confirm their bacteriocin-like character as well as to investigate their role in rhizosphere population dynamics of established and abandoned tea bushes.

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Experimental

Materials and Methods

Sampling area. The present study was carried out from 2005 to 2006 at two sites in the Shivalik hills of Western Himalayas, the Himachal Himalayas and the Garhwal Himalayas. In the Himachal Himalayas, three sites were selected viz. Banuri tea experimental garden, Bundla tea estate and Rajpur tea estate, all near Palampur, Himachal Pradesh. In the Garhwal Himalayas also three sites were selected, one from IIP tea gardens and two from Prem Nagar tea gardens both in Dehradun, Uttaranchal.

Collection of soil samples. Soil samples were collected with the help of a "Carpentor augor", placed into plastic bags, transported to the laboratory and kept at 4°C under field moist conditions until analysis. Only the soil which closely adhered to the tea roots was used for rhizosphere studies while for non-rhizosphere samples, soil was collected from the same garden but from areas well away from tea bushes.

Isolation, selection and enumeration of dominant bacteria. A number of soil samples were taken at 0–15 cm depth from different tea gardens to isolate and enumerate bacterial population. The number of bacteria was calculated on nutrient agar by spread plate using 1 g soil and appropriate dilution (Atlas, 1997). The bacterial colonies were isolated and purified by sub-culturing. The representative bacteria dominating the rhizosphere were grouped separately. These bacteria were identified up to genus level on the basis of their colony morphology and biochemical tests. *B. subtilis* and *P. putida* isolates due to their higher percentage of occurrence have been chosen for assay for bacteriocin production (Table I).

Table I
List of bacterial strains used in study isolated from Himachal Himalayas and Uttaranchal Himalayas

S. No.	*B. subtilis* (Himachal Himalayas)	S. No.	*P. putida* (Uttaranchal Himalayas)
1	HPAB7	1	UAAP1
2	HPAB12	2	UAAP4
3	HPAB13	3	UAAP7
4	HPAB41	4	UAAP22

Evaluation of R:S ratio and assessment of rhizosphere effect. For the estimation of rhizosphere (R) to soil (S) bacteria ratio (R:S ratio) appropriate soil dilutions from both rhizospheric as well as non rhizospheric region were plated on nutrient agar medium and the R:S ratio was determined using the formula:

$$\text{R:S ratio} = \frac{\text{CFUs of rhizosphere bacteria}}{\text{CFUs of non-rhizosphere bacteria}}$$

Detection of antimicrobial activity by the deferred agar spot test (DAS). This test was carried out on tryptic soya agar or broth (TS) (pH 7.3 ± 0.2) and Brain Heart Infusion Agar or broth with 0.1% glucose (BHIG) (pH 7.4 ± 0.2) (Atlas, 1997). Ten µl of culture of *Bacillus* and *Pseudomonas* test strains grown for 7 to 8 h in TS and BHIG broth were spot inoculated on the surface of TS and BHIG agar, respectively. After 18–24 h of incubation at 30°C the plates were overlaid with 5 ml of suitable soft media (0.75% agar) inoculated with 100 µl of indicator culture in the stationary phase (approximately 10^5 cells/ml). Inhibition zones were observed after 24–48 h of incubation under appropriate conditions. Clear zones of inhibition with sharp edges around spots were considered as possitive results.

Detection of antimicrobial activity by agar well diffusion assay (AWD). The soft agar inoculated with indicator microorganisms, as described in the DAS assay, was poured onto the BHIG agar plates. Wells of 5 mm diameter were cut in the agar and filled with 50 µl of bacteriocin preparation (protein concentration was about 1.5 mg/ml), prepared from overnight culture by centrifugation at 4000 × g for 20 min, neutralized with 5 mM NaOH to the final pH 7.0 and then filter sterilized (0.2 µm type, Millipore filter) (Cintas *et al.*, 1995). Plates were preincubated at 4°C for about 2 h to allow the diffusion of any inhibitory metabolites into the surrounding agar, and then incubated at the 30°C. The plates were examined for a clear zone in the agar surrounding the wells.

Sensitivity of bacteriocins to different temperatures. The neutralized sterilized supernatants of tested strains of *B. subtilis* and *P. putida* were heated at 45, 60, 75 and 90° C for 15 min and tested with AWD assay as described above.

Sensitivity of antimicrobials to enzymes. Enzymes indicated in Table IV (100 µg/ml) were added to 150 µl samples of culture supernatants. Samples were incubated for 1 h at 37°C (or 42°C in case of Proteinase K) before being tested for antimicrobials activity. Supernatants without added enzyme were used as control. After 1 h of incubation, the samples were heated at 95°C for 5 min to inactivate the enzymes and cooled on ice (Larsen and Jorgensen, 1999). Bacteriocin activity survay was tested by the AWD method

Effect of bacteriocin on indicator strain. An exponentially growing culture (250 µl) of the indicator strain of *B. mycoides* and *P. syringae* (10^7 cells/ml) was suspended in 50 mM phosphate buffer (pH 6.0) and exposed for a maximum of 140 min to various concentrations of bacteriocin (0, 25, 40 and 75 µg/ml of protein). At different times the survival count of bacteria (cfu/ml) was determined using the AWD method.

Results and Discussion

In the present study, a large number of bacteria was isolated from the rhizosphere of established and abandoned tea bushes from various tea growing areas of the Himalayan region. Various species belonging to the genus *Bacillus* were selected but *B. subtilis* and *B. mycoides* dominated the rhizosphere in established tea bushes. The other selected species were *Bacillus polymyxa* and *Bacillus cereus* (~15% each) – see Table II.

Table II
Dominance of bacterial isolates in tea rhizosphere of Himalayan regions as indicated by occurrence (%)

Isolate number	Occurrence (%)	Species
Himachal Himalayas		
1	*B. subtilis*	45.3
2	*B. mycoides*	17.3
3	*B. polymyxa*	15.1
4	*B. cereus*	14.8
Uttaranchal Himalayas		
1	*P. putida*	55.6
2	*P. fluorescens*	11.3
3	*P. cepacia*	6.8
4	*P. syringae*	3.9
5	*B. subtilis*	7.8
6	*B. mycoides*	5.0
7	*B. polymyxa*	4.5
8	*B. cereus*	3.3

Values are mean of three repetitions

It was interesting to note that contrary to the dominance pattern in the established tea rhizosphere of Himachal Himalayas, where *Bacillus* species (45.3 %) were dominant, *Pseudomonas* species were found to be dominant in the abandoned tea rhizosphere of Uttaranchal Himalayas with occurrence percentage of 55.6 % for *P. putida*, 11.3 % for *P. fluorescens*, 6.8 % for *P. cepacia* and 3.9 % for *P. syringae*. *Bacillus* species were also present in abandoned tea bushes and ranged from 3.3 to 7.8 % (Table II). Tea bushes exhibit several remarkable features, *e.g.* the negative rhizosphere effect, strong antagonistic activities amongst microbial communities in the rhizosphere (Pandey and Palni, 1996; Pandey *et al.*, 1997) and the long lived nature of tea plants. These factors collectively, may have helped in the development of a particular bacterial population which is well adapted to tea rhizosphere. Species of *Bacillus*, due to their spore forming nature, can survive under adverse conditions like low temperature in winter.

Table III
R:S ratio values (cfu) of bacteria from different sites indicating negative rhizosphere effect

Isolate No	Sites	Rhizospheric soil	Non-rhizospheric soil	R:S ratio
Himachal Himalayas				
1	Banuri tea estate	218.6±39.3	293.4±45.2	0.74
2	Bandala tea estate	178.4±25.5	242.3±47.5	0.73
3	Rajpur tea estate	204.2±30.1	277.8±39.0	0.73
Uttaranchal Himalayas				
1	IIP tea gardens	240.5±28.4	252.5±36.0	0.95
2	Prem Nagar 1	232.0±35.5	284.9±26.5	0.81
3	Prem Nagar 2	212.7±24.0	268.0±19.5	0.79

Values are means of three determinations of ± SD; cfu at n × 10^4

The bacterial population was found to be clearly suppressed in the rhizosphere of established tea bushes and the counts were low in the rhizospheric soil as compared to non rhizospheric soil (Table III). The values obtained from all sites were found below 1% and the values were lower in the tea bushes of the Himachal region (established tea bushes) as compared

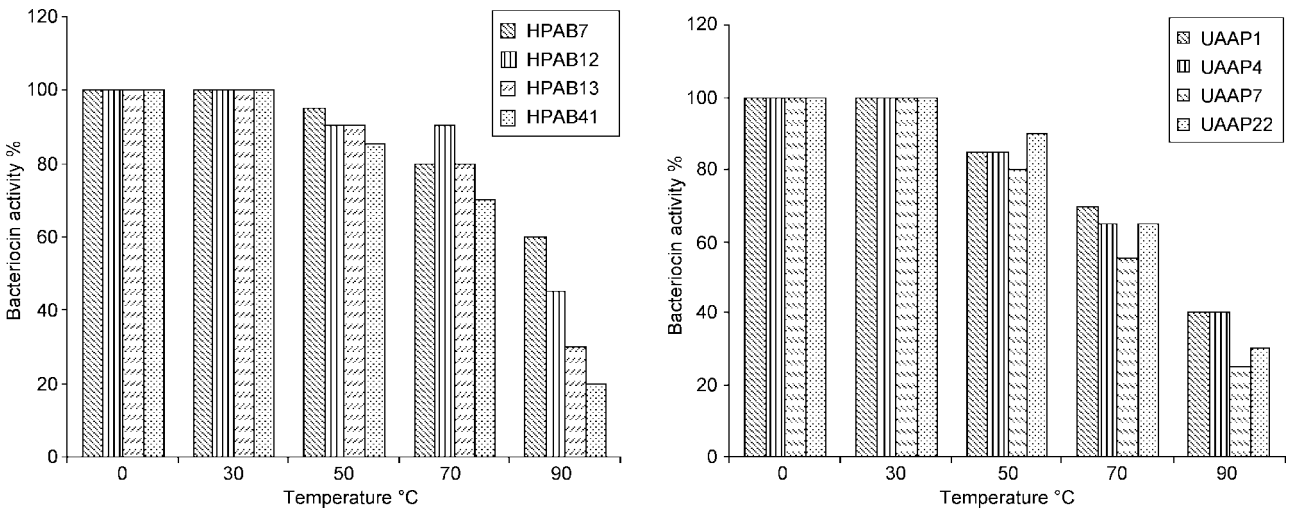

Fig. 1. Effect of temperature on the bacteriocin-like activity of *Bacillus subtilis* (A) and *Pseudomonas putida* strains (B) expresssed as % of basic activity.

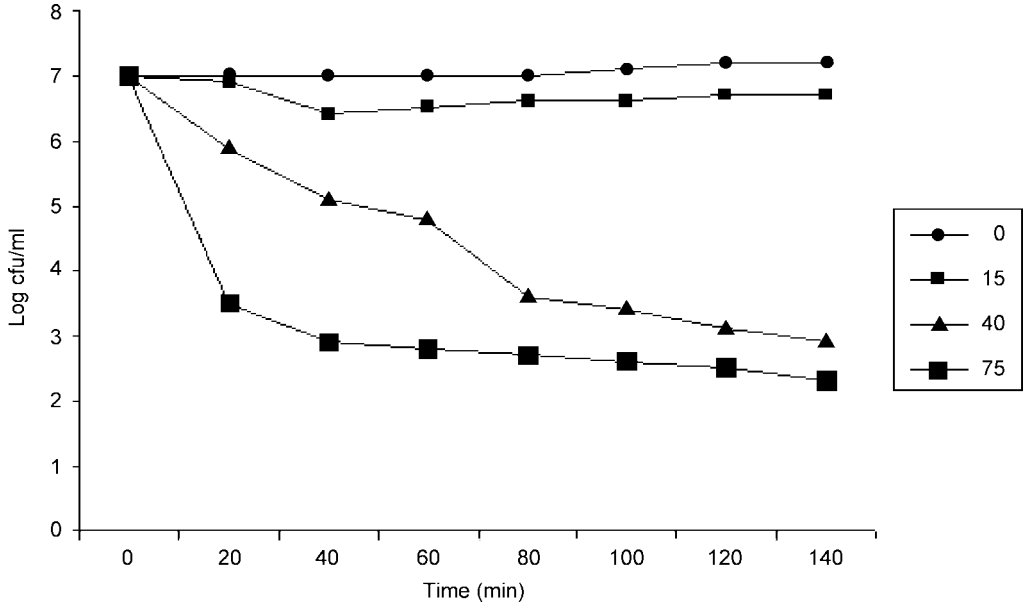

Fig. 2. Different bacteriocin concentrations (µg/ml) of *B.subtilis* strain HPAB12 affecting the growth of *B. mycoides*.

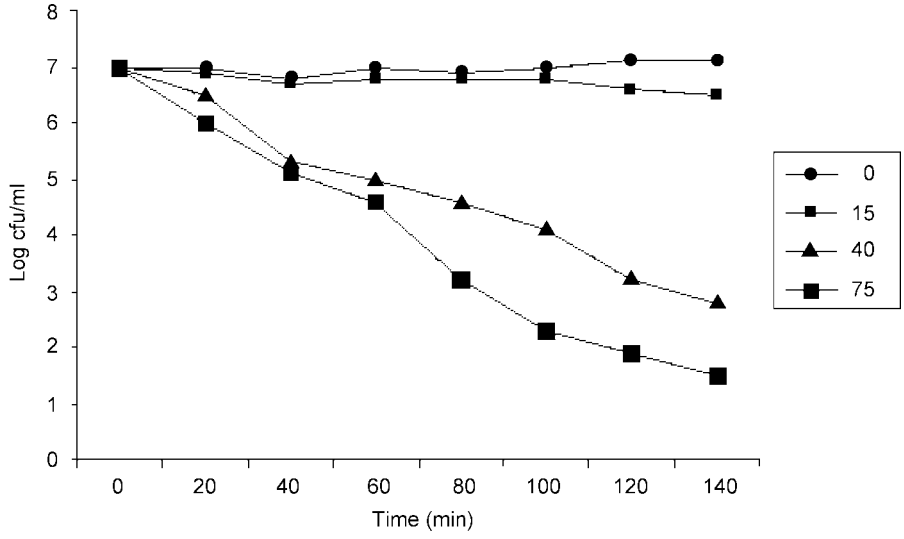

Fig. 3. Different bacteriocin concentrations (µg/mg) of *P. putida* strain UAAP1 affecting the growth of *P. syringe.*

to the Uttaranchal region (abandoned tea bushes) (Pandey and Palni, 1996). Tea plants prefer acidic soil and also the decomposed leaf debris tends to result in further lowering of pH, this seems to be a factor resulting in low microbial population in established tea rhizosphere. However, in the case of abandoned tea bushes, which are scattered, less foliage and canopy are present. Probably, in this case the root exudates do not accumulate in the rhizosphere, hence although below 1, a higher value for R:S ratio was observed. After heating for 15 min at 30°C, the activity of the tested bacteriocin was the same as that of the untreated control samples. Heating for 15 min at 50°C partially reduced the activity of bacteriocin while at 70°C the *Bacillus* strain HPPB12 showed partial loss, compared to others. Heating at 90°C for 15 min affected the activity of tested *Bacillus* and *Pseudomonas* strains (Fig. 1A and B respectively). The stability of bacteriocin over a wide range of heat treatments up to 70°C indicated that its bactericidal function is active in a variety of different conditions; this is an interesting feature in view of their potential use as antagonistic agents (Thomas and Wimpenny, 1996; Mataragas *et al.*, 2003). To determine whether bacteriocin had a bactericidal or bacteriostatic effect, various concentrations of the bacteriocin were added to the indicator *B. mycoides* and *P. syringae* strains. The survival of the indicator bacteria were determined using the AWD method at different time intervals of bacteriocin addition. The incubation of *B. mycoides* cells with bacte-

Table IV
Sensitivity to enzymes of bacteriocin-like substances in the supernatants of *B. subtilis* and *P. putida* strains

Enzymes	HPAB7	HPAB12	HPAB13	HPAB41	UAAP1	UAAP4	UAAP7	UAAP22
Catalase	+	+	+	+	+	+	+	+
Trypsin	–	–	–	–	–	–	–	–
Chymotrypsin	–	–	–	–	–	–	–	–
Protease	–	–	–	–	–	–	–	–
Lipase	+	+	–	+	–	–	+	–
Phospholipase C	+	+	+	+	+	+	+	+
Amyloglucosidase	–	–	+	–	–	–	+	+
Proteinase K	–	–	–	–	–	–	–	–
Lysozyme	+	+	+	+	+	+	+	+
DNase	+	+	+	+	+	+	+	+
Ribonuclease A	+	+	+	+	+	+	+	+

(+) resistance to treatment; (–) sensitivity to treatment

Table V
Antibacterial activity of *B. subtilis* and *P. putida* strains against other species of *Bacillus* and *Pseudomonas*

	Bacillus subtilis				*Pseudomonas putida*			
	HPAB7	HPAB12	HPAB13	HPAB41	UAAP1	UAAP4	UAAP7	UAAP22
B. mycoides	+	+	+	–	–	–	–	–
B. polymyxa	+	–	+	–	–	–	–	–
B. cereus	–	+	+	–	–	–	–	–
P. fluorescens	–	–	–	–	–	–	+	+
P. cepacia	–	–	–	–	+	–	+	–
P. syringae	–	–	–	–	+	+	–	+

+ Antibacterial activity present; – Antibacterial activity absent

riocin decreased the cfu count; therefore, it indicates its bactericidal activity (Fig. 2 and 3). With the increase in bacteriocin concentration and incubation time the bactericidal effect increased. The proteinaceous nature of substance presenting bacteriocidal activity may be concluded basing on the loss of activity after treating with proteolytic enzymes (Ward and Somkuti, 1995).

The antimicrobial activity of *B. subtilis* and *P. putida* strains was not affected by treatment with lysozyme, DNase, Ribonuclease A, phospholipase C and catalase, while it was completely lost after treatments with trypsin, chymotrypsin, protease and proteinase K (Table IV). Lipase and amyloglucosidase affected the bacteriocin activities in some strains. The treatment with catalase had no effect on the bacteriocidal activity, indicating that hydrogen peroxide was not involved in antimicrobial activity. The inactivation of activity by amyloglucosidase in five strains and by lipase by four strains might be an indication that beside proteinaceous subunit, some lipid or carbohydrate components are also involved in antibacterial activity.

Most strains of *B. subtilis* were antagonistic to *B. mycoides* and its growth was often inhibited. *B. subtilis*, which was the most dominant bacteria of the tea rhizosphere and which exhibited the best antagonistic activity against *B. mycoides* and inhibited its growth on dilution plates as well as after obtaining pure cultures. Strains of *B. subtilis* inhibited *B. mycoides*, *B. polymyxa* and *B. cereus*, *P. putida* also behaved antagonistically towards other species (Table V). This antagonism presented by *B. subtilis* and *P. putida* could be preasumably due to the production of bacteriocin (Riley and Gordon, 1999). Our result draws support from Singh *et al.* (2007), who studied the antagonism by dominant *Bacillus* species (bacteriocin producers) to tea rhizospheric fungal community. In this way, in established tea bushes, the best adapted species of *Bacillus* (due to spore formation) which are also the most dominant species (due to antagonistic activities) in tea rhizosphere compared to the other *Bacillus* species. In abandoned tea bushes, *P. putida* due to its antagonistic behavior side lined other *Pseudomonas* species. It is interesting to note that dominant microbial species were those, which are known to produce antimicrobial metabolites like bacteriocin.

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