

## COLONIZATION OF RHIZOSPHERE OF TEA BY GROWTH PROMOTING BACTERIA

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### ABSTRACT

Based on a detailed study conducted to isolate microbes from soil samples collected from various tea gardens located in the Indian Himalayan region, two bacteria namely *Bacillus subtilis* and *Pseudomonas corrugata* have been selected as promising inoculants for field application in tea gardens. Bioassays based on the inoculation of seed raised and tissue culture raised tea plants had earlier indicated the biocontrol and growth promotion properties of selected bacteria.

With a view to introduce these bacterial isolates eventually in the tea gardens, suspension cultures were raised and applied (inoculated) in the rhizosphere region of both seedling and cutting raised (four clones) young tea plants under net-house conditions. Monthly enumeration of bacterial (including free living nitrogen fixers), fungal and actinomycetes populations, up to a period of one year, indicated excellent rhizosphere colonization by the inoculated bacteria. The presence of "introduced" bacteria in the rhizosphere was confirmed by the use of antibiotic markers. The inoculated tea plants have been transferred to new plantation sites, near Kausani in District Bageshwar, Uttarakhand for further monitoring of growth and overall performance.

Key words: India; *Bacillus subtilis*; *Pseudomonas corrugata*; plant growth promotion; rhizosphere colonization; antibiotic resistance

### INTRODUCTION

Rhizosphere microorganisms, which are closely associated with plant roots, have been named as plant growth promoting rhizobacteria (PGPR; Glick, 1995). Their tolerance to adverse environmental conditions, capacity to produce antibiotics (including antifungals), hydrocyanic acid, indoleacetic acid, siderophores and 1-aminocyclopropane carboxylic acid (ACC) deaminase, and ability to solubilize phosphates and effectively colonize roots are responsible for plant growth promotion (Budzikiewicz, 1997; Duffy and Defago, 1999; Burd *et al.*, 2000; Ellis *et al.*, 2000; Meyer, 2000). The

potential of PGPR in relation to improved tea growth has also been recently recognized (Pandey *et al.*, 2000; Pandey and Palni, 2002-03). With a view to improve the overall health of tea bushes, two promising bacterial inoculants, namely *Bacillus subtilis* and *Pseudomonas corrugata*, were selected for introduction. These species were found to exhibit antifungal and phosphate-solubilizing activities and showed good survival at low temperature. For successful field application, the introduced PGPR should have, amongst other earlier said characteristics, the ability to compete against native microflora and maintain sufficient numbers (population) to bring about the desired effect over a time span sufficient for the plant to favorably respond (Weller, 1983; Bashan, 1998; Whipps, 2001).

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In the present investigation, the rhizospheric competitiveness of the two selected bacterial inoculants, initially isolated from rhizosphere of tea viz. *Bacillus subtilis* and *Pseudomonas corrugata*, was determined using identified antibiotic markers in young tea plants under net house conditions, prior to their field transfer. The effect of inoculations on the native microflora in rhizosphere and overall growth of tea plants was also determined.

## MATERIALS AND METHODS

### Tea Clones

Cutting raised 6 months old plants of T-78 (China hybrid, released by Tukdah Tea Estate, Darjeeling, West Bengal) and UPASI-9 (or Athrey, chemotaxonomically confirmed as China hybrid, selected at Brooklyn Tea Estate, Nilgiri Hills, South India) and one year old seedlings (raised from biconal seeds) of BSS- 379 (China hybrid) and BSS- 449 (small leaved Assam hybrid; both released by Tocklai Experimental Station, Jorhat) were obtained from Kumaun Mandal Vikas Nigam, Kausani, Uttaranchal. The plants were maintained in polybags (21 cm height, 7 cm diameter containing approx. 1.25 kg of soil, pH 5.4).

### Inoculation Experiments

*Bacillus subtilis* (NRRL B-30408) and *Pseudomonas corrugata* (NRRL B-30409), initially isolated from the rhizosphere of established tea bushes and examined for their biocontrol and growth promotion properties (Pandey and Palni, 1997; Pandey *et al.*, 1997; Pandey *et al.*, 2000), were used as inoculants. The bacterial cultures were maintained on slants of tryptone-yeast (TY) extract agar and *Pseudomonas* isolation agar

respectively, through regular subculturing and storage at 4°C.

A total of three treatments per clone were used: (1) control - rhizosphere supplied with TY broth, without bacteria, (2) rhizosphere inoculated with suspension culture of *Bacillus subtilis* and (3) rhizosphere inoculated with suspension culture of *Pseudomonas corrugata*. Bacterial suspensions were raised in 250 ml Erlenmeyer flasks containing 100 ml of TY extract broth, and 25 ml of the suspension containing approximately  $10^7$  cells ml<sup>-1</sup> was applied in the rhizosphere region of tea plants. Seventy five plants (cutting or seed raised) were taken for each treatment, including control. The plants were allowed to grow in shade under a net house in the Institute premises; watering was carried out at regular intervals.

### Rhizosphere Studies

These experiments were conducted with the soil samples collected from the rhizosphere of tea clone BSS- 449.

### Enumeration of Native Microflora

Soil samples from the control as well as inoculated plants were collected (in triplicate) at monthly intervals up to a period of 12 months. Three groups of microorganisms, viz. bacteria, actinomycetes and fungi, were enumerated to determine the rhizosphere colonization using the serial dilution technique (Johnson and Curl, 1972). Nutrient agar (for bacteria), Actinomycetes isolation agar (for Actinomycetes) and potato dextrose agar (for fungi) were used for these enumerations. Jensen's agar (Jensen, 1954), a nitrogen-free medium, was used for the enumeration of a specific group of bacteria. Following incubation at 28°C for 1 week, the plates were observed for colony forming units.

### Colonization of Rhizosphere by Bacterial Inoculants

For determining the rhizosphere colonization by the introduced bacteria, soil samples were collected from inoculated plants. The enumerations for *B. subtilis* and *P. corrugata* were carried out from respective treatments using the serial dilution technique. The antibiotic profile of *B. subtilis* was worked out on TY extract agar supplemented with different concentration(s) of 10 antibiotic(s) (data not presented); rifampicin and gentamycin ( $50 \mu\text{g ml}^{-1}$ ) were selected as resistance markers in the present study. *P. corrugata* was enumerated on *Pseudomonas* isolation agar supplemented with ampicillin ( $1500 \mu\text{g ml}^{-1}$ ), penicillin ( $2000 \mu\text{g ml}^{-1}$ ) and carbenicillin ( $2500 \mu\text{g ml}^{-1}$ ; Pandey *et al.*, 2001). Each experiment was conducted in triplicate.

### Growth Monitoring

Observations were recorded after 10 months of inoculation regarding increment in shoot length and stem girth over initial values in all treatments and all clones. Data were statistically analysed using the standard method of Snedecor and Cochran (1967).

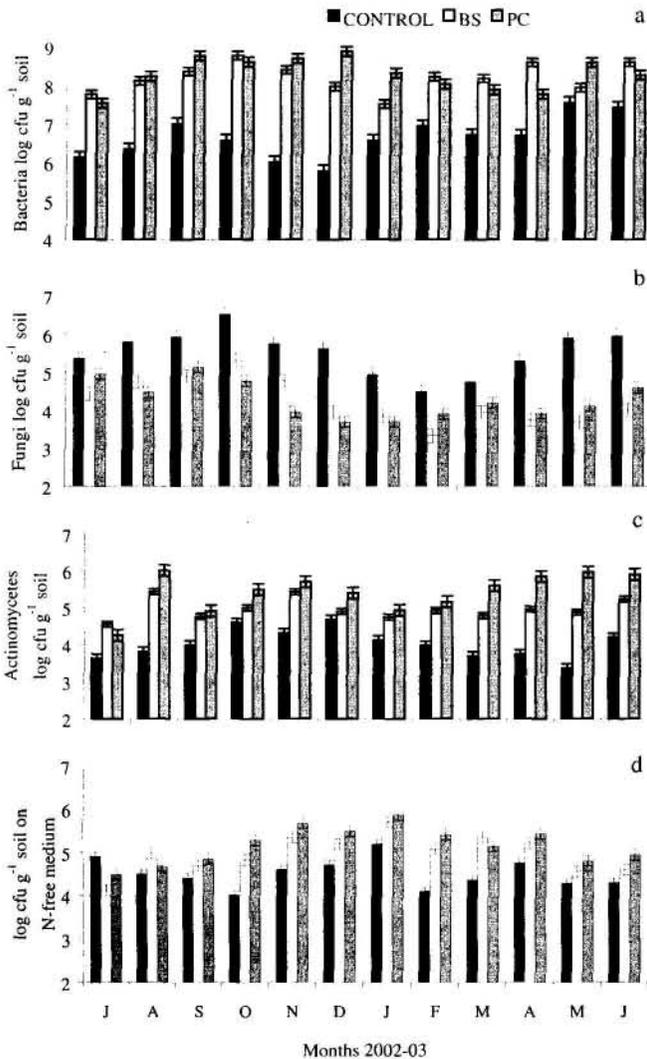
### RESULTS AND DISCUSSION

The effect of bacterial inoculations on the microbial communities in the rhizosphere of tea (clone BSS-449) can be seen in Fig. 1. Although the bacterial inoculation was found to stimulate the populations of native bacteria, actinomycetes and a group of bacteria capable of growing on a N-free medium, it suppressed the rhizospheric fungal population (statistically significant,  $p \leq 0.05$ ). The maximum population of native bacteria  $\text{g}^{-1}$  of rhizosphere soil was 8.781 (October) and 8.889 (December) log units

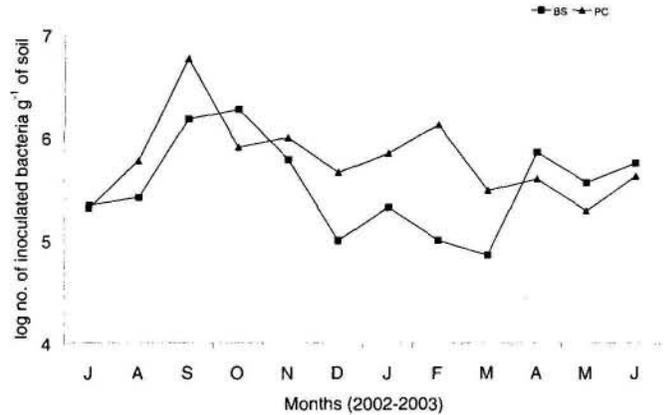
for *B. subtilis* and *P. corrugata* treatments, respectively; the corresponding values in control plants during the same months were 6.563 and 5.782 log units respectively. The population of actinomycetes in *B. subtilis* and *P. corrugata* treated plants was maximum in August; the values were 5.435 and 6.012 log units  $\text{g}^{-1}$  of soil, respectively, as against the control value of 3.819 log units  $\text{g}^{-1}$  of soil. The highest population of bacteria, capable of growth on N-free medium, was recorded highest in the month of January as 6.716 and 6.871 log units  $\text{g}^{-1}$  in case of *B. subtilis* and *P. corrugata* treated plants, respectively, against the control value of 4.614 log units  $\text{g}^{-1}$  of soil. Maximum suppression of the fungal population was found to occur in the month of February in *B. subtilis* treated plants (3.326 against the control value of 4.503 log units  $\text{g}^{-1}$  of soil). Similarly, in case of *P. corrugata* treatment, the maximum suppression of the fungal population was recorded in December; 3.7 and 5.632 log units  $\text{g}^{-1}$  of soil in treated and control rhizosphere, respectively. The stimulation of native microflora in the rhizosphere of various plant species by bacterial inoculations has been reported (Pandey *et al.*, 1998, 1999). The suppression of fungal population, probably through the production of antibiotics, is indicative of the antifungal property of introduced bacteria (Ellis *et al.*, 2000; O'Sullivan and O'Gara, 1992).

The use of antibiotic markers confirmed the ability of inoculated bacteria to colonize the tea rhizosphere (Fig. 2). The use of genetic markers, such as intrinsic levels of resistance to various antibiotics, is one of the simple and rapid methods for strain identification (Josey *et al.*, 1979). The *B. subtilis* counts in the rhizosphere of *B. subtilis*

**Fig. 1. Influence of bacterial inoculations on microbial populations in the rhizosphere of tea (clone BSS-449). Cfu = colony forming units. The data were analyzed using a single factor ANOVA ( $P \leq 0.05$ ) and the bars indicate standard error. Figs A, B, C and D represent microbial colonies belonging to bacteria, fungi, actinomycetes and a specific group of bacteria growing on N-free medium, respectively**



**Fig. 2. Colonization of tea rhizosphere by the bacterial inoculants. Data were analyzed using a single factor ANOVA ( $P \geq 0.05$ ) and the bars indicate standard error. The values are in respect of clone BSS-449**



treated tea plants varied from 4.854 (March) to 6.274 log units  $g^{-1}$  of soil (October), and in case of *P. corrugata* treated plants the population ranged from 5.285 (May) to 6.673 log units  $g^{-1}$  of soil (September). The individual populations of introduced bacteria were found to differ significantly (at  $p \leq 0.05$  level) during the complete growth period. *P. corrugata* maintained higher population during winter in comparison to *B. subtilis*, showing greater tolerance to low temperature. The individual populations of the two inoculants, however, became more or less similar 12 months after the inoculation. A similar pattern of root colonization in wheat rhizosphere by species of *Bacillus* sp. L324-92R<sub>12</sub> and *Pseudomonas* sp. 2-79RN<sub>10</sub> has been reported (Kim *et al.*, 1997). The use of antibiotic resistance as a marker is simple, rapid and sufficiently sensitive method for selection as well as enumeration of the introduced bacterium that exhibits resistance to selective antibiotics (Kluepfel, 1993).

The bacterial inoculations were seen to positively influence the measured growth parameters, viz. increment in shoot length and stem girth, in all four clones of tea (Table 1). While the treated plants, in general, appeared healthy and showed better growth, the differences were statistically significant in terms of enhancement of stem girth by both the bacteria in clone T-78. The beneficial effects of plant growth promoting rhizobacteria (PGPR), especially those belonging to genus *Bacillus* or *Pseudomonas*, in improving the overall health and growth of various plants including tea have been described (Weller, 1988; Glick, 1995; Pandey *et al.*, 2000; Sharma and Johri, 2003). Besides the plant-microbe interaction, ecotypic specificity and the role of environmental factors seem to be of considerable importance (Chanway and Holl, 1993). In the present study both the inoculants used, namely *B. subtilis* and *P. corrugata*, were originally isolated from the rhizosphere of tea bushes growing under temperate conditions. This is likely to have contributed towards better root colonization efficiency observed for either of the test inoculants. The inoculations resulted in improving the subsequent growth of all four clones of tea used in this study. The inoculated plants have been transferred to the plantation site for further monitoring. The study has implications, in terms of developing these bacteria as bioinoculants, for use in tea plantation programmes being undertaken in Uttaranchal.

**Table 1:** The effect of bacterial inoculations on the growth of tea clones.

Tea clones	Treatment	Increment in shoot length over initial (cm)±SE	Increment in stem girth over initial (mm)±SE
T-78	Control	27.77±1.07	1.51±0.06
	<i>Bacillus subtilis</i>	32.69±1.80	2.14±0.24*
	<i>Pseudomonas corrugata</i>	30.65±1.13	2.69±0.10*
	LSD (p=0.05)	6.59	0.53
UPASI-9	Control	31.82±1.62	1.78±0.08
	<i>Bacillus subtilis</i>	37.18±2.18	2.14±0.18
	<i>Pseudomonas corrugata</i>	36.12±1.89	2.16±0.10
	LSD (p=0.05)	7.18	0.50
BSS-449	Control	21.81±1.06	1.14±0.02
	<i>Bacillus subtilis</i>	24.51±1.54	1.22±0.10
	<i>Pseudomonas corrugata</i>	23.81±1.32	1.25±0.08
	LSD (p=0.05)	4.94	0.27
BSS-379	Control	11.01±0.79	1.13±0.05
	<i>Bacillus subtilis</i>	15.72±0.67*	1.34±0.03
	<i>Pseudomonas corrugata</i>	11.06±0.19	1.10±0.11
	LSD (p=0.05)	2.55	0.28

SE= Standard error; each treatment consisted of 25 plants, in triplicate, and thus the values are an average of 75 plants. Results were recorded 10 months following the bacterial inoculations.

\* Significantly different

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