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RHIZOSPHERE ASSOCIATED BACTERIA AND SOIL PHYSICO-CHEMICAL PROPERTIES OF TEA GARDEN

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Abstract

In the rhizosphere, plant-microbe associations play critical part in major ecosystem processes. The pHs of the collected rhizosphere soil were acidic (4.24 - 4.77) and favorable for the established tea orchards. Soil texture was either sandy clay loam or sandy loam in nature. Organic carbon of the samples ranged from 0.91 - 1.19%, depicting the soil falling in very low to low category due to monoculture of tea for a long time. Total nitrogen content of the soil samples ranged in between 0.10 and 0.13%. Moreover, Phosphorus and important metal ions *viz.* potassium, calcium and magnesium were measured. Mean heterotrophic bacterial load of the soil samples ranged from $1.59 \pm 0.22 \times 10^6$ to $10.88 \pm 2.31 \times 10^6$ cfu/g and from $1.58 \pm 0.29 \times 10^6$ to $6.93 \pm 0.79 \times 10^6$ cfu/g on NA and PYG, respectively. Maximum bacterial count on both media was observed in samples of Saloon Section Area plot while the lowest counts were found in samples of D1 Section plot. Sixteen bacterial isolates were selected and purified for identification, which was conducted by amplifying ~ 600 bp fragments of 16S rDNA. All the isolates were Gram positive and rod shaped. *Bacillus* was found to be the dominant genus (63%) in the tea rhizosphere soil. Other isolates were identified as varied species of *Lysinibacillus* (19%), *Paenibacillus* (12%) and *Brevibacterium* (6%). A phylogenetic tree was generated which showed only one major cluster comprising of two sub-clusters grouping *Paenibacillus* sp. in one and *Lysinibacillus* along with *Bacillus* sp. in another. Present observation indicated that *Bacillus* sp. is a major organism and has a strong role in evolution among the tea rhizospheric bacteria.

Introduction

The amount of microbial species in the rhizosphere may fluctuate from thousands to millions and accordingly, the interactions between roots and soil microbes are often specialized on the basis of co-evolutionary pressures (Duffy *et al.* 2004, Morrissey *et al.* 2004). In the rhizosphere, plant-microbe interactions play important roles in many vital ecosystem processes, including carbon sequestration and nutrient cycling (Singh *et al.* 2004). Positive plant-microbe interactions include plant-microbe symbioses, such as plant associations with plant-growth promoting rhizobacteria (PGPR), epiphytes and mycorrhizal fungi. These interactions have been proved to have many beneficial impacts on plants, including disease suppression (Mendes *et al.* 2011), increased nutrient availability and uptake and increased immunity to abiotic (Zolla *et al.* 2013) and biotic stresses (Zamioudis and Pieterse 2012), each leading to increases in plant productivity.

Many studies have revealed that soil microbial communities are affected by numerous factors i.e. plant species, soil types and agricultural management (Berg and Smalla 2009, Jangid *et al.* 2008). Other beneficial effects of soil microbes on plants include nutrient and water uptake enhancement, dinitrogen fixation, phytohormone production, symbiosis, disease and pest control and enhancement of soil quality. Thus, soil and its microbial communities are obligatory for

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biogeochemical cycles of soil nutrients, crop quality improvement and agroecosystem sustainability (Stark *et al.* 2008). Interactions between roots and microbes are common in rhizosphere and can be enhanced thus increasing plant growth and crop yield, also preventing deleterious effects of phytopathogenic organisms on the plants (Chakraborty *et al.* 2015).

Complex interactions between soil microbial communities and environmental factors in the tea orchard ecosystem have recently attracted the attentions of many researchers. Tea plant (*Camellia sinensis* (L.) O. Kuntze) of family Theaceae is one of the oldest organized plantation practices and one of the most popular beverages worldwide, produced from leaves and buds of the plant (Senthilkumar *et al.* 2015). It is a perennial woody plant grown as a major cash crop in many developing countries including Bangladesh. This crop is cultivated on 2.85 million ha worldwide and with a total production of 3.87 million tons per annum (FAO 2010). There are 163 tea estates in Bangladesh distributed in Moulvibazar, Habiganj, Chittagong, Sylhet and Panchagarh. Small areas in Brahmanbaria and Rangamati have also been planted with tea (Sana 1989).

One of the most attractive potential uses of 16S rRNA gene sequence informatics is to provide genus and species identification for isolates that fail to match with any recognized biochemical profiles. The cumulative results from studies to date suggest that 16S rRNA gene sequencing provides genus identification in most cases (90%) but less so regarding species (65 to 83%), with very limited number of the isolates remaining unidentified.

Hence, the present study was undertaken to enumerate and exploit the heterotrophic bacterial communities in tea rhizosphere that may have potential to make soil mineral nutrients available for plant usage, diminish the use of synthetic fertilizers, antagonize target pathogens and conserve the environment.

Materials and Methods

Bangladesh Tea Research Institute (BTRI) situated at Srimangal Upazila (24°08'-24°28' N, 91°36'-91°48' E) of Moulvibazar, Sylhet, Bangladesh was chosen as the sampling site. Soil samples were collected from four experimental plots of BTRI i.e. Ph.D. plot, D1 Section plot, Project Area plot and Saloon Section Area plot. Age of the tea plants of the sampling orchards were about 25 - 30 years.

Tea rhizosphere soil samples were collected from healthy plant-grown fields with the help of an auger from three separate locations of each of the mentioned plot areas during August 2016. Soil samples containing roots and root adhered soil were collected from 0 to 23 cm depth of randomly selected mature tea plants. Soil samples were carried to the laboratory in sterile plastic bags at 4°C and stored at the same temperature for further analysis.

Soil samples were used for physico-chemical analyses. Soil texture was determined by hydrometer method and soil pH was determined by using Jenway-3505 pH meter (soil : distilled water = 1 : 2.5). Soil organic carbon (in %) was determined following Walkley and Black wet oxidation method (1934). For determination of total nitrogen, Micro Kjeldahl steam distillation method was adopted and available phosphorus was determined colorimetrically by Bray-II ascorbic acid method (Huq and Alam 2005) using the UV spectrometer (PerkinElmer EZ301). For determination of available potassium, calcium and magnesium, samples were extracted with 77% ammonium acetate solution where potassium was determined by flame photometer (Buck Scientific PFP-7) and calcium and magnesium were determined by Atomic Absorption Spectrophotometer (Analytikjena nova 400P).

Bacterial enumeration and isolation from the samples were completed using nutrient agar (NA) (Eklund and Lankford 1967) and peptone yeast extract glucose (PYG) agar media (Atlas 1997). The pH of the media was adjusted to 6.0 before sterilization. One g of sample was

suspended in 100 ml of sterile water in a conical flask and then kept in shaking condition (200 rpm) at 37°C for 30 min. Enumeration and isolation of bacteria was then done by ten-fold serial dilution technique (Greenberg *et al.* 1998). The inoculated plates were inverted and incubated (aerobically) at 37°C for 24 hrs in an incubator (Memmert GmbH + Co Kg 8540 Sehwabach). After incubation, plates having well discrete colonies were selected for counting using a colony counter (Digital colony counter, DC-8 OSK 100086, Kayagaki, Japan). Discrete bacterial colonies were isolated immediately after counting. Based on distinct colony morphology, further selection was made. Selected isolates were purified by repeated streaking and stored in NA slants at 4°C for further analysis.

Molecular identification of the bacterial isolates was conducted by amplifying ~600bp fragments of 16S rDNA using 5'-16S rRNA: CCAGACTCCTACGGGAGGCAGC, 3'-16S rRNA: CTTGTGCGGGCCCCCGTCAATTC primers. Supernatant of heat lysed cell suspension was used as template for PCR amplification. Temperature and conditions maintained for PCR amplification were followed as our previous work (Saha *et al.* 2015). The amplified products were separated electrophoretically on 1.25% agarose gel. The amplified bands were sequenced (Macrogen, South Korea) and analyzed through NCBI-BLAST database (http://blast.ncbi.nlm.nih.gov/) and rRNA BLAST (http://bioinformatics.psb.ugent.be/cgi-bin/rRNA/blastform.cgi) programs to find out possible similar organisms in the databases.

A phylogenetic tree of the isolates was generated using neighbor joining (NJ) distance-based algorithm of phylogenetic analysis. Sequences obtained from BLASTN (nucleotide blast) were in FASTA format and relation between each sequence could be known by multiple sequence alignment using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/).

Statistical analysis was performed with the Statistical Package for the Social Sciences (SPSS) v.16.0 for windows (SPSS, SAS Institute Inc. Cary, USA). Effect of each parameter was studied in triplicate (n = 3) and the data were presented as the mean ± standard deviation (Sd). One-way ANOVA for the multiple comparisons were subjected using 5% level of significance.

Results and Discussion

Soil physico-chemical properties of different sampling sites are shown in Table 1. pHs of the samples were found to be acidic (4.24 to 4.77) having similar results to Gafur and Sultana (2013). Age of the tea bushes played a key role in lowering the pH of rhizosphere soil as age of the plants are inversely proportionate with soil pH (Wang *et al.* 2010). This is also held responsible as one of the crucial factors responsible for suppressing microbial communities in tea rhizosphere compared to other plant rhizospheres. However, Yu *et al.* (2004) found that generally not soil pH but tea orchard age had significant effect on microbial biomass, microbial community diversity and community composition and structure. Soil textural class was either sandy clay loam (SCL) or sandy loam (SL) in nature. Gafur and Sultana (2013) also reported related results of soil texture in soils of different tea valleys of Bangladesh. The organic carbon of the samples ranged from 0.91 - 1.19% making the collected soil samples very low to low category considering organic carbon percentage (FRG 2012). Total nitrogen ranged in between 0.10 and 0.13%. Amount of phosphorus and other available metal ions in the soil *viz.* potassium, calcium and magnesium are shown in Table 1.

Mean heterotrophic bacterial load of the rhizosphere soil samples from four plots ranged in between $1.59 \pm 0.22 \times 10^6$ and $10.88 \pm 2.31 \times 10^6$ cfu/g on NA and in between $1.58 \pm 0.29 \times 10^6$ and $6.93 \pm 0.79 \times 10^6$ cfu/g on PYG medium (Table 2). For the heterotrophic bacterial count, differences in mean among the four selected tea orchard plots were observed. Maximum heterotrophic rhizospheric bacterial counts on both media (14.06×10^6 cfu/g on NA and 7.65 ×

10^6 cfu/g on PYG) were observed in the sample collected from Saloon Section Area plot. While, the lowest bacterial counts (1.43×10^6 cfu/g on NA and 1.19×10^6 cfu/g on PYG) were found in samples of D1 Section plot. The suppression of the bacterial population in tea orchard soils may have been caused by the definite acid rhizosphere environment, long-term monoculture cropping system and the toxicity and accumulation of antimicrobial substances (Zhao *et al*. 2012).

Table 1. Soil properties of different sampling sites.

Sampling site	Soil		Organic C (%)	Total N (%)	Average available metal ions (ppm)			
	Texture	pH			P	K	Ca	Mg
Ph.D. plot	SCl	4.77	1.19	0.13	25.57	36.91	84.65	19.70
D1 Section plot	SCl	4.53	1.08	0.12	14.70	40.22	76.29	13.36
Project area plot	SL	4.47	1.02	0.11	10.95	43.10	75.62	11.90
Saloon section area plot	SL	4.24	0.91	0.10	6.26	29.38	64.85	9.70

Table 2. Heterotrophic bacterial load of the tea rhizosphere soil samples.

Sampling site	Replicate No.	Heterotrophic Bacterial Load ($\times 10^6$ cfu/g) on			
		NA	Mean ± Sd	PYG	Mean ± Sd
Ph.D. plot	01	4.89	4.08 ± 0.71a	3.77	3.87 ± 0.14a
	02	4.19		4.07	
	03	3.17		3.76	
D1 Section plot	01	1.90	1.59 ± 0.22b	1.68	1.58 ± 0.29b
	02	1.43		1.87	
	03	1.44		1.19	
Project area plot	01	3.22	2.55 ± 0.50c	2.03	2.51 ± 0.38c
	02	2.04		2.53	
	03	2.38		2.97	
Saloon section area plot	01	14.06	10.88 ± 2.31d	7.65	6.93 ± 0.79d
	02	8.63		7.31	
	03	9.94		5.84	

Means followed by different small letters in columns indicate significant differences ($p < 0.05$).

During this investigation, among the primarily isolated 38 bacteria, 16 were selected based on their distinctive colony morphology and purified for detailed study. Cakmakci *et al.* (2010) isolated total of 944 colonies from the acidic tea rhizosphere and majority (41%) of them were identified belonging to the genus *Bacillus*, *Pseudomonas*, *Pseudoalteromonas*, *Arthrobacter* and *Micrococcus*.

All the isolates were Gram positive and rod shaped. Out of 16 only one isolate was non-spore former (isolate No. 10) (data not shown). The present findings show a greater abundance of Gram positive bacteria in the tea rhizosphere, in agreement with previous studies of Cakmakci *et al*. (2010) and Xue *et al.* (2008). Similarly, Rau *et al*. (2009) and Rusznyak *et al*. (2008) showed higher level of Gram positive *Bacillus* and *Paenibacillus* species in the wild grass and reed rhizospheres compared to Gram negative species.

Identification of the bacterial isolates was conducted by amplifying 16S rRNA and size of the amplified DNA band in the gel was approximately 600 bp (Fig. 1). The obtained sequences were analyzed and identification of the selected isolates was completed (Table 3). Results indicated that among the tea rhizosphere bacteria, *Bacillus* was the dominant genus (63%) with 10 isolates belonging to this genus (Fig. 2). Other isolates were identified as different species of *Lysinibacillus* (19%), *Paenibacillus* (12%) and *Brevibacterium* (6%). Dutta *et al.* (2015) identified *Bacillus pseudomycoides* strain SN29 from tea estates of Assam, India which had excellent plant growth promoting (PGP) activity.

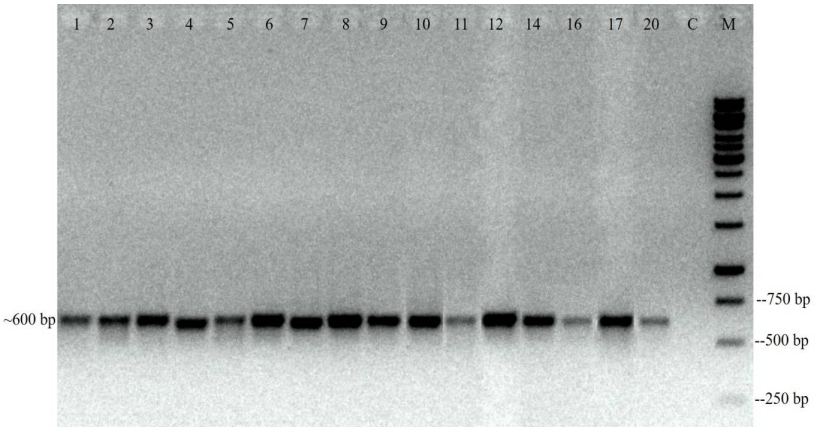

Fig. 1. PCR amplification of part of the 16S rRNA of 16 isolates. Lane M is the 1.0 kb ladder, Lane C is the negative control and lanes 1 - 20 are representing 16 different bacterial isolates.

Table 3. Identification of the isolates based on 16 sRNA sequencing.

Isolate No.	Scientific name	Strain	Max. coverage score	Identity match (%)
1	*Lysinibacillus macroides*	DSM 54	966	99
2	*Bacillus pseudomycoides*	JSM 05182085	983	99
3	*Paenibacillus glycanilyticus*	PZG A17	1022	99
4	*Bacillus subtilis*	THt3-1	1044	99
5	*Bacillus megaterium*	Y18-01	1016	99
6	*Lysinibacillus fusiformis*	BCH963	1018	99
7	*Paenibacillus* sp.	ML2-3	995	99
8	*Bacillus* sp.	JZDN22	1024	99
9	*Bacillus* sp.	MZ4	1024	99
10	*Brevibacterium samyangense*	SST-8	972	99
11	*Lysinibacillus sphaericus*	T12-16	1020	99
12	*Bacillus cereus*	03BB102	1022	99
14	*Bacillus* sp.	YACS15	1055	99
16	*Bacillus flexus*	T9-27	1027	99
17	*Bacillus* sp.	PR-2J	1007	99
20	*Bacillus cereus*	TYg2-1	1042	99

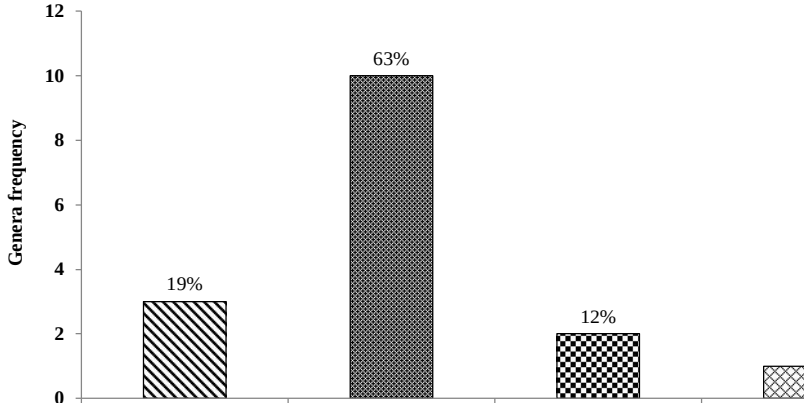

Fig. 2. Frequency of the identified bacterial isolates associated with tea rhizosphere soil.

A phylogenetic tree of the 16 isolates was generated using neighbor joining (NJ) distance based algorithm (Fig. 3). It was detected that there was only one major cluster comprising of two sub-clusters. These sub-clusters grouped *Paenibacillus* sp. in one cluster and *Lysinibacillus* along with *Bacillus* sp. This observation suggested the evolutionary divergence of these bacteria and indicated their evolutionary trend. On the other hand, there were also two small sub clusters completely comprised of *Bacillus* sp. This remark pointed out that *Bacillus* sp. is a major organism in the tea rhizosphere and holds a strong role in evolution.

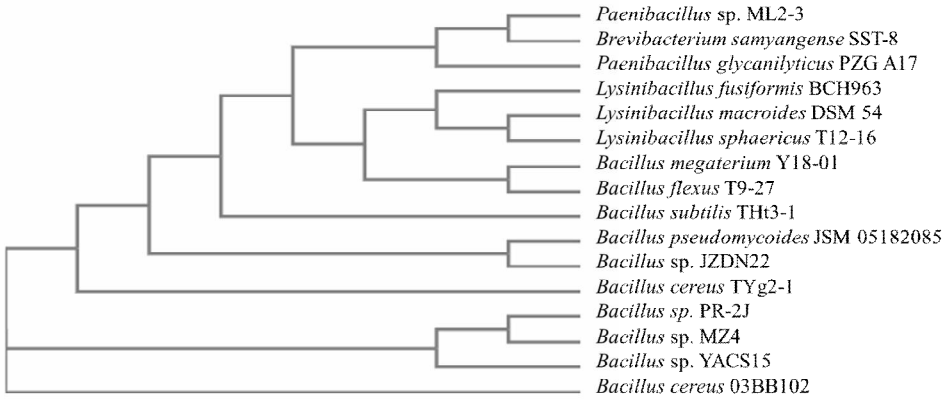

Fig. 3. Phylogram of the 16 different isolates based on their 16S rRNA sequences obtained from BLAST.

The isolated *Bacillus* spp. were identified up to five distinct species level *viz. B. pseudomycoides, B. subtilis, B. megaterium, B. cereus* and *B. flexus*. According to Cakmakci *et al.* (2010), soil pH was most important soil property for *Bacillus* and *Paenibacillus* diversity. The tea rhizosphere appeared to exhibit a fine example of natural selection where under a specific set of environmental conditions, over a prolonged period, growth of various species belonging to the genus *Bacillus* and related bacteria were favored and variants were found also within this same species. Sood *et al.* (2007) also demonstrated various dominant species of *Bacillus* in established tea bushes *viz. B. subtilis, B. mycoides, B. polymyxa* and *B. cereus*.

The present research showed that monoculture of tea in acidic soils may diminish soil nutrients gradually. Some bacteria were found to be associated with tea rhizospheric soil however their variety seemed to be limited may be due to negative selection pressure of the acidic soil environment and longtime monoculture of the crop. Further researches are needed to exploit the positive roles of the isolated bacteria such as nitrogen fixation, phytohormone creation, symbiosis, disease or pest control and soil quality upgradation therefore increase in the crop yield and quality improvement.

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