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RESEARCH ARTICLE

The Variability of the Predominant Culturable Plant Growth-Promoting Rhizobacterial Diversity in the Acidic Tea Rhizosphere Soils in the Eastern Black Sea Region

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ABSTRACT

The purpose of this study was to investigate the diversity of cultivable nitrogen fixing, phosphate solubilising and total bacteria originated from 580 rhizospheric acidic soils samples of tea plants grown at 62 locations. Based on FAME profiles of over 1428 rhizoplane bacteria, 63 bacterial genera were identified with a similarity index > 0.3, but 56.4% of the identified isolates belonged to six genera: *Bacillus* (37.02%), *Pseudomonas* (12.67%), *Stenotrophomonas* (5.71%), *Paenibacillus* (6.58%), *Arthrobacter* (4.35%) and *Brevibacillus* (3.98%). Most of the total, N_2-fixing and P-solubilizing bacteria isolated were Gram positive (59.9, 58.8 and 56.3%) and Gram negative constituted only 40.1, 41.2 and 43.7%. Among different groups, *Firmicutes*, *Gammaproteobacteria* and *Actinobacteria* comprised the largest groups contributing to about 50.3 and 46.6%, 30.8 and 32.5%, and 8.3 and 9.6% of the total N_2-fixing and P-solubilizing isolates, respectively. *B. cereus*, *P. fluorescens*, *B. megaterium*, *S. maltophilia*, *P. putida*, *B. licheniformis*, *B. pumilus*, *B. subtilis* and *P. polymyxa* were the most frequent N_2-fixing and P-solubilizing species in the acidic tea rhizosohere soils. In these studies were evaluated to represent the dominant culturable diversity of diazotrophs and phosphobacteria, and thus potentially beneficial to the growth and survival of tea plants in that specific acidic ecosystem of eastern Black Sea region.

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Introduction

The composition of the bacterial community associated with plant roots is influenced by a variety of plant and environmental factors (Poonguzhali et al. 2006). Soil and plant species affect the indigenous bacterial soil communities. Some studies conclude that plant species have the greatest effect on community structure (Costa et al. 2006) whereas others shown that soil type have the greatest effect (Fierer and Jackson 2006). Rhizosphere microorganisms in turn having a great impact on root biology, influence plant growth, nutrition and development. Microorganisms colonizing the rhizosphere can affect plant growth both positively and negatively, the term plant growth promoting rhizobacteria (PGPR) often describes beneficial rhizobacteria that stimulate plant growth (Asghar et al. 2002). Selection of an efficient PGPR requires an understanding of the composition and diversity of the root-associated bacteria, and characterization of its plant growth promotion-related properties. For this reason, there has been considerable interest in examining the effect of soil type, plant species and root zone location on bacterial community structure in the rhizosphere (Varmazyari and Çakmakçı, 2018). Furthermore, a good selection of PGPR strain requires an understanding the dynamic and composition of the bacterial

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communities colonizing the rhizosphere and characterization of its PGP related properties (Donate-Correa et al. 2004; Çakmakçı et al., 2010).

Tea (Camellia sinensis) the most important plant of Turkey is used in the traditional preparation of its national food and is planted widely on acidic soils. Turkey has the second largest tea market in the world. Turkish tea is produced on the eastern Black Sea coast, which has a mild climate with high precipitation and fertile soil. Tea gardens are usually grown as a monoculture and receive considerable amounts of fertilization, root exudates, and leaf litter (Çakmakçı et al., 2010). There is very little knowledge on the rhizosphere microbiology of the tea plants (Xue et al., 2006; Çakmakçı et al., 2010). Also, little information is available regarding the microbial community characteristics in tea garden soil ecosystems (Xue et al. 2008). Apart from our studies, the effect of the tea plants on the rhizospheric bacteria has not been studied so far in this area. These pioneering studies have been carried out on the diversity and functional importance of N_2-fixing bacteria (NFB) and P-solubilizing bacteria (PSB) in the acidic tea orchard soils in the eastern black sea region. A significant portion of the research results have already been published (Çakmakçı et al., 2010) and this article briefly summarizes some of the data on bacterial diversity. As a result, the objective of this present study was to isolate and identify plant growth promoting rhizobacteria from the rhizosphere of tea grown in eastern Black Sea region, and characterize them for phosphate solubilization and and nitrogen fixation.

Materials and Methods

Soil Samples, Isolation and Identification of Bacteria

The field of rhizosphere microbiology of tea garden soil ecosystems unexplored and in this work, we have isolated the bacterial population from the rhizosphere of tea plants production zones of various agro climatic regions of Rize and Trabzon during June–September, 2006-2016. The study area is located on the eastern Black Sea region, most of the country in Rize, between 40°50' and 41°20' N and 38°49' and 41°28' E. The tea gardens were established between 1938 -1944 years and now all of them are between 65-70 years old. It was found that the acidity of tea soils are too high depends on the quality and quantity of used nitrogen and in 80% of the soils the P content is low or too low. The tea plantations have been surveyed and 580 acidic soils samples from 62 locations were collected.

Rhizosphere soil samples were collected from healthy field-grown plant. Ten gram of the soil for each individual tea plant adhering to the roots, considered the rhizospheric soil was mixed and used for the bacterial isolation procedures (Çakmakçı et al., 2010; Karagöz et al., 2012). Uprooted plants along with a good amount of non-rhizosphere soil were brought immediately to the laboratory in polythene bags and air-dried. The non-rhizosphere soil was removed by gentle shaking whereas the soil adhering strongly to the root was referred to as rhizosphere soil. Rhizobacteria isolates were randomly selected from agar-solidified trypticase soy broth, and identified using fatty acid methyl ester (FAME) profiles. The method was carried out according to the described procedure already (Çakmakçı et al., 2010; Karagöz et al., 2012). Only strains with the similarity index (SIM) ≥0.3 were considered a good match (Oka et al. 2000). Fatty acid methyl ester (FAME) analysis is a well-established method for bacterial identification based on whole cellular fatty acids derivatized to methyl esters, analyzed by gas chromatography (Poonguzhali et al. 2006).

Nitrogen Fixation and Phosphate Solubilisation

Isolation and purification of N_2-fixing strains were carried out in an N-free solid malate-sucrose medium (NFMM) modified from Döbereiner (19899. Modified NFMM medium per liter distilled water (sucrose, 10.0 g; L-malic acid, 5.0 g; $MgSO_4$, H_2O, 0.2 g; $FeCl_3$, 0.01 g; NaCl, 0.1 g; $CaCl_2.2\ H_2O$, 0.02 g; K_2HPO_4, 0.1 g; KH_2PO_4, 0.4 g; $Na_2MoO_4.H_2O$, 0.002 g) with 18 g agar for solid mediumwas used for isolation. The medium adjusted to pH 7.2 with 1 N NaOH prior to agar addition and was then sterilized at 121 ºC for 20 min in an autoclave (Xie et al., 2003). N-free medium was used in order to obtain nitrogen fixing PGPR (Piromyou et al., 2011). Phosphate solubilization activity of the bacterial isolates was detected on Pikovskaya (1948) and National Botanical Research Institute's phosphate growth medium (NBRIP-BPB). NBRIP-BPB contained (per liter): glucose, 20 g; $Ca_3(PO_4)_2$, 10 g; $MgCl_2\ 6H_2O$, 5 g; $MgSO_4\ 7H2O$, 0.25 g; KCl, 0.2 g; $(NH_4)_2SO_4$, 0.1 g, and BPB, 0.025 g. To compare the reproducibility of the halo formation, pH indicator bromophenol blue was supplemented phosphate growth medium. Phosphate solubilization was was carried out according to the described procedure already (Çakmakçı et al., 2010).

Results

In our studies, a total of 1428 colonies were selected from the acidic tea rhizosphere. Over 1428 rhizoplane bacteria were randomly selected from agar-solidified trypticase soy broth, and identified using fatty acid methyl ester (FAME) profiles. The MIDI system identified (SIM > 0.3) 56.4% (805 out of 1428) of the bacteria isolated from the rhizosphere of tea and 38.9% (556 out of 1428) of the bacteria fixed nitrogen and 31.2% (446 out of 1428) solubilized P from insoluble calcium phosphate on NBRIP medium (Table 1). These isolates showed significant differences in their phosphate solubilizing potential, their extend of phosphate solubilization ranged between 22.9–172.8 mg L^{-1} liquid medium. Eight hundred five dominant, morphologically distinct rhizobacteria were purified, which belonged to 63 genera and 122 species. Furthermore, it assigned an additional 30.2% (431 out of 1428) of the tea isolates a taxonomic name based on a similarity index of less than 0.3. About 30.2% of the bacterial isolates could not be classified to genus since their similarity indices were <0.3 indicating no close matches. The majority of them were identified as belonging to the *Bacillus*, *Pseudomonas*, *Paenibacillus*, *Stenotrophomonas*, *Arthrobacter* and *Brevibacillus* genus. The analysis of FAME profiles for the

total bacteria isolated, facilitated their classification under four bacterial divisions: *Bacteroidetes* (1.1%), γ, β and α-subdivisions of *Proteobacteria* (29.6%, 6.2% and 3.2%, respectively), *Firmicutes* (50.1%), and *Actinobacteria* (9.8%). Also about 13.4% of the isolates were classified as flagged since there were no matches or the analysis was of unacceptable quality (Table 1).

Table 1. Diversity of culturable P-solubilizing and N_2-fixing bacteria in the acidic tea rhizosphere soils

Taxonomic identification	Order	Bacterial strain FAME identification	Number of isolates[a]	N_2-fixing isolates	P-solubilizing isolates
Alphaproteobacteria	*Rhizobiales*	*Rhizobium radiobacter*	8	6	4
		Rhizobium rubi	1	1	1
		Phyllobacterium rubiacearum	2	1	1
		Roseomonas fauriae	6	5	4
		Ochrobactrum anthropi	2	2	1
	Rhodobacterales	*Paracoccus denitrificans*	2	2	1
Betaproteobacteria	*Burkholderiales*	*Burkholderia cepacia*	11	8	7
		Burkholderia pyrrocinia	4	4	3
		Ralstonia eutropha	3	2	2
		Ralstonia pickettii	1	1	1
		Ac. xylosoxidans denitrificans	6	5	3
		Acidovorax facilis	3	1	3
		Acidovorax konjaci	1		1
		Alcaligenes faecalis	16	9	8
Gammaproteobacteria	*Xanthomonadales*	*Lysobacter enz. enzymogenes*	15	12	10
		Pseudoxanthomonas sp	5	4	3
		Stenotrophomonas acidaminiphila	10	6	6
		Stenotrophomonas maltophilia	36	28	21
	Pseudomonadales	*Pseudomonas alcaligenes*	7	5	4
		Pseudomonas agarici	5	3	2
		Pseudomonas aurantiaca	2		1
		Pseudomonas chlororaphis	3	2	1
		Pseudomonas fluorescens	39	28	26
		Pseudomonas mucidolens	1	1	
		Pseudomonas putida	28	22	19
		Pseudomonas pseudoalcaligenes	1		1
		Pseudomonas stutzeri	3	3	1
		Pseudomonas syringae maculicola	3	3	2
		Pseudomonas atrofaciens	3	2	1
		Pseudomonas sp.	7	4	3
		Acinetobacter calcoaceticus	15	9	7
		Acinetobacter lwoffii	5	3	2
	Alteromonadales	*Pseudoalteromonas tetraodonis*	1	1	1
		Aeromonas hydrophila	1	1	1
	Enterobacteriales	*Cedecea davisae*	1	1	1
		Enterobacter intermedius	2	1	1
		Citrobacter freundii	2	1	2
		Ewingella Americana	2	1	1
		Erwinia crysanthemi	3	1	3
		Hafnia alvei	4	3	4
		Photorhabdus luminescens	5	4	3
		Proteus vulgaris	5	4	3
		Rahnella aquatilis	3	2	2
		Providencia alcalifaciens	1		1
		Serratia fonticola	4	3	3
		Serratia marcescens	4	4	3
		Serratia plymuthica	2	2	1
		Pantoea agglomerans	3	3	3
Firmicutes	*Bacillales*	*Bacillus amyloliquefaciens*	2	1	
		Bacillus atrophaeus	10	9	8
		Bacillus badius	1	1	1
		Bacillus alcalophılus	2	1	1
		Bacillus cereus	78	55	28
		Bacillus coagulans	8	2	5

Taxonomic identification	Order	Bacterial strain FAME identification	Number of isolates[a]	N_2-fixing isolates	P-solubilizing isolates
		Bacillus globisporus	1	1	
		Bacillus laevolacticus	20	17	12
		Bacillus licheniformis	27	23	18
		Bacillus lentus	4	2	1
		Bacillus megaterium	37	28	25
		Bacillus mycoides	11	7	4
		Bacillus parabrevis	1	1	1
		Bacillus pumilus	26	20	16
		Bacillus simplex	6	3	2
		Bacillus sp	14	8	7
		Bacillus sphaericus	26	13	8
		Bacillus subtilis	24	20	16
		Bacillus thuringiensis	2	1	
		Paenibacillus alvei	1	1	1
		Paenibacillus azotofixans	2	2	1
		Paenibacillus larvae	3	2	2
		Paenibacillus lentimorbus	6	4	3
		Paenibacillus macquariensis	7	5	6
		Paenibacillus polymyxa	20	17	12
		Paenibacillus validus	14	11	8
		Brevibacillus choshinensis	12	8	5
		Brevibacillus centrosporus	8	4	3
		Brevibacillus parabrevis	3	2	1
		Brevibacillus reuszeri	9	3	4
		Geobacillus stearothermophilus	1	1	1
		Kurthia gibsonii	1	1	1
		Kurthia sibirica	11	3	5
Actinobacteria	*Actinomycetales*	*Arthrobacter pascens*	2	1	1
		Arthrobacter agilis	4	3	2
		Arthrobacter aurescens	3	1	1
		Arthrobacter crystallopoites	1	1	1
		Arthrobacter citreus	2	1	2
		Arthrobacter globiformis	6	5	4
		Arthrobacter mysorens	4	1	2
		Arthrobacter viscosus	13	6	5
		Kocuria rosea	6	4	4
		Micrococcus lylae	5	4	4
		Micrococcus luteus	11	6	7
		Brevibacterium liquefaciens	4	2	1
		Microbacterium chocolatum	1	1	1
		Rhodococcus erythropolis	11	8	6
Bacteroidetes	*Flavobacteriales*	*Chryseobacterium indologenes*	5	3	3
Others[a]			32	18	13
No library match			192		
Unidentified[b]			431		
Total			1428	556	446

[a]Others includes the genera: *Brevundimonas*, *Methylobacterium, Rhodobacter, Xanthobacter*, *Comamonas, Kingella*, *Variovorax, Xanthomonas, Raoultella*, *Yersinia, Photobacterium, Clostridium*, *Enterococcus*, *Sporosarcina*, *Staphylococcus*, *Cellulomonas, Curtobacterium*, *Nocardia*, *Bergeyella, Flavobacterium Sphingobacterium*, N_2-fixing and P-solubilizing bacteria in these genera were only detected once or twice.
[b]Isolates named with a similarity index < 0.3.

The bacterial community of tea rhizosphere was composed by 40.1% Gram-negative, 59.9% of Gram-positive bacteria. The identified Gram-positive groups, comprising the community, were classified into four orders; *Actinomycetales, Bacillales, Clostridiales* and *Lactobacillales*. The order *Bacillales* was the most diverse, and was composed of seven different genera; *Bacillus*, *Paenibacillus*, *Brevibacillus*, *Kurthia*, *Geobacillus*, *Staphylococcus* and *Sporosarcina*. The order *Actinomycetales* was represented by eight genera: *Arthrobacter*, *Rhodococcus*, *Kocuria*, *Brevibacterium*, *Micrococcus*, *Cellulomonas, Curtobacterium* and *Microbacterium*; while the order *Clostridiales* and *Lactobacillales* was represented by the genera *Clostridium and* Enterococcus (Table 1).

Among non-enteric Gram-negative *Pseudomonads* and *Xanthomonads* group were the most abundant with three species identified (*Pseudomonas fluorescens* 12.1%, *Pseudomonas putida* 8.7% and *Stenotrophomonas maltophilia* 11.1%). Among Gram-negative bacteria, sixteen strains of

Alcaligenes faecalis, fifteen strain each of *Lysobacter enzymogenes enzymogenes* and *Acinetobacter calcoaceticus*, eleven strains of *Burkholderia cepacia*, eight strains of *Rhizobium radiobacter*, seven strains each of *Pseudomonas alcaligenes* and *Pseudomonas* sp. and six strains each of *Roseomonas fauriae and Achromobacter xylosoxidans denitrificans* were identified. The genus *Bacillus* was the 61.8% of the Gram-positive population (298 out of 482), with a prevalence of *B. cereus* (26.1%), followed by *B. megaterium* (12.4%), *B. licheniformis* (9.1%), *B. pumilus* and *B. sphaericus* (8.7%) and *B. subtilis* (8.1%). Also, the *Bacillus* group was the most abundant with five other species identified (*B. laevolacticus*, *Bacillus* sp., *B. mycoides*, *B. atrophaeus*, *B. coagulans*). Gram-positive *Paenibacillus* genus was the second most abundant (11.0%) with seven species identified (*P. polymyxa*, *P. validus*, *P. lentimorbus*, *P. macquariensis P. larvae*, *P. azotofixans* and *P. alvei*). Among the other Gram-positive bacteria, twelve strain of *Brevibacillus choshinensis*, thirteen strain of *Arthrobacter viscosus*, eleven strain each of *Kurthia sibirica*, *Micrococcus luteus* and *Rhodococcus erythropolis*, nine strains of *Brevibacillus reuszeri* and seven strain of *Brevibacillus centrosporu* were identified. *Arthrobacter* (7.3%) included the species *A. viscosus*, *A. mysorens*, *A. globiformis*, *A. agilis*, *A. aurescens*, *A. citreus*, *A. Pascens* and *A. crystallopoites*.

We selected two hundred and fourteen different potential PSB from a pool of 805 rhizobacterial isolates obtained from the tea rhizosphere on the basis of their P-solubilizing and N_2-fixing ability on NBRIP and N-free solid malate sucrose medium (NFMM) medium. Table 1 shows that 446 and 556 out of the 805 tested isolates had potential for P-solubilization and N_2-fixation, which 52 different known bacterial genera represented by *Bacillus* (34.3 and 38.1%), *Pseudomonas* (13.7 and 13.1%), *Paenibacillus* (7.4 and 7.6%), *Stenotrophomonas* (6.1 and 6.1%), *Arthrobacter* (3.8 and 3.2%), *Brevibacillus* (2.9 and 3.1%) as the predominant genera. Among different groups, *Firmicutes*, *Gammaproteobacteria* and *Actinobacteria* comprised the largest groups contributing to about 46.6 and 50.5%, 32.5 and 30.7 % and 9.6 and 8.3% of the total P-solubilizing and N_2-fixing isolates, respectively.

Discussion

The taxonomic identities of 63 genera from approximately 1428 rhizospheric root-associated bacteria isolated from 580 rhizospheric soil samples of tea, grown at 62 sites were determined. Of these 1428 isolates, 13.4% (192 isolates) could not be identified by the MIDI system since there were no matches or the analysis was of unacceptable quality. Also about 30.2% of the isolates (431/1428) were identified with a SIM <0.3 which indicates a tentative identification, and were not included in further analysis. Identification of the bacterial isolates was more successful in the tea rhizosphere samples expressing an overall identification of about 56.4% of the total isolates. *Bacillus*, *Pseudomonas*, *Paenibacillus*, *Stenotrophomonas*, *Arthrobacter* and *Brevibacillus* genera were the most prominent N_2-fixing and P-solubilizing groups in the rhizosphere and soil populations analysed.

Characterization of the isolates on the basis of their FAME profiles revealed the presence of both Gram-positive and Gram-negative bacteria within the tea rhizosphere soils although larger number was that of Gram-positive. Most of the rhizospheric bacteria isolated were Gram-positive (59.9%) and Gram-negative constituted only 40.1%. Out of a total of 805 isolates, 323 belonged to Gram-negative, which included 238 γ-proteobacteria, 50 ß-proteobacteria, 26 α-proteobacteria,; 9 isolate belonged to the Bacteroidetes group. Major α-proteobacterial genera recovered from tea rhizospheres included several species of *Rhizobium*, *Roseomonas*, *Phyllobacterium* and *Paracoccus*. Major ß-proteobacterial included *Alcaligenes*, *Burkholderia* and *Achromobacter*, while *Pseudomonas*, *Stenotrophomonas*, *Acinetobacter*, and *Lysobacter* dominated the γ-proteobacterial genera. The 556 Gram-positive isolates included 403 *Firmicutes* and 79 *Actinobacteria*. The data obtained show a greater abundance of Gram-positive bacteria in the tea rhizosphere, in agreement with previous studies (Xue et al., 2008; Rau et al. 2009; Çakmakçı et al., 2010, Karagöz et al., 2012; Varmazyari and Çakmakçı 2018) that show a higher level of Gram-positive *Bacillus* and *Paenibacillus* species in the in the tea garden soils. Also the tea rhizoplane was dominated by *Bacillus* (37% or 298/805 identified isolates). The widely studied *Bacillus*, *Pseudomonas* and *Paenibacillus* genus represents one of the most diverse genera in the plant rhizosphere and soil populations (Beneduzi et al., 2008; Çakmakçı et al., 2010) and these species can be characterized with the ability to tolerate unfavourable conditions (Borsodi et al., 2007). Bacterial identification by the MIDI system indicated that *Bacillus*, *Pseudomonas* and *Paenibacillus* genera inhabit the rhizosphere of tea, and soil pH was the characteristic most closely related with their diversity.

We conducted a survey of dominant culturable N_2-fixing and P-solubilising bacteria naturally colonizing a mild climate with high precipitation and acidic soil in the eastern Black Sea biogeographical tea growing regions. The highest percentage of NFB and PSB was recorded *Firmicutes* in, followed by the *Gammaproteobacteria*. The results obtained indicated that *Bacillus*, *Pseudomonas*, *Paenibacillus*, *Stenotrophomonas*, *Brevibacillus* and *Arthrobacter* genera were the most prominent culturable groups in the rhizosphere and soil populations. Bacteria from these genera are generally regarded as good phosphate solubilisers, nitrogen fixers and plant growth promoters (Xie et al., 2003; Şahin et al., 2004; Çakmakçı et al., 2006, 2007; Chen et al. 2006; Poonguzhali et al. 2006; Beneduzi et al. 2008; Hariprasad and Niranjana 2009; Rau et al. 2009; Varmazyari and Çakmakçı 2018). *B. cereus* was the most dominant culturable NFB and PSB in the acidic tea rhizosphere, followed by *P. fluorescens*, *B. megaterium*, *S. maltophilia*, *P. putida*, *B. licheniformis*, *B. pumilus*, *B. subtilis* and *P. polymyxa*. The ability of a few soil microorganisms to convert insoluble forms of phosphorus to an accessible form is an important trait in plant growth-promoting bacteria for increasing plant yields. We demonstrate that the natural acidic soil supports a diverse group of potential PSB. These PSB could serve as efficient biofertilizer candidates for improving the P-nutrition of crop plants. The advantage of using natural soil isolates over the genetically manipulated or the one which has

been isolated from a different environmental set up is the easier adaptation and succession when inoculated into the plant rhizosphere (Chen et al. 2006). Use of these acid tolerant P-solubilizing and N_2-fixing bacteria as bio-inoculants will increase the available N and P in soil and the N and P uptake by plants, helps to minimize the mineral fertilizer application, reduces environmental pollution and promotes sustainable agriculture. This strain could be useful in the formulation of new inoculants, improving the cropping systems into which it can be most profitably applied. The identification and the isolation of PGPR from acidic and P-deficient soils, which combine the ability to fix nitrogen and solubilize phosphate, could also significantly increase the productivity of crops in acidic soil.

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