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The effect of bacteria-based formulations on tea (*Camellia sinensis* L.) growth, yield, and enzyme activities

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Abstract: *The effect of bacteria-based formulations on tea (*Camellia sinensis L.*) growth, yield, and enzyme activities.* There is an increasing need to use microorganisms for safe crop production for consumers, as well as to prevent environmental pollution and ensure the sustainability of agriculture and agricultural resources. The objective of this study was to evaluate possible effects of mineral fertilizer (NPK), one commercial liquid bio-fertilizer and ACC deaminase-containing, N_2-fixing, and P-solubilizing bacteria-based bio-fertilizers in triple strain combinations (BF1: *Bacillus subtilis* RC28 + *Paenibacillus polymyxa* RC05 + *Pseudomonas fluorescens* RC77; BF2: *Bacillus subtilis* RC63 + *Paenibacillus polymyxa* 24/3 + *Pseudomonas fluorescens* 48/3; BF3: *Bacillus atrophaeus* 36/10 + *Paenibacillus polymyxa* 28/3 + *Pseudomonas fluorescens* 51/2; BF4: *Bacillus subtilis* 39/3 + *Bacillus subtilis* RC63 + + *Pseudomonas fluorescens* 53/6; BF5: *Bacillus subtilis* RC521 + *Paenibacillus polymyxa* 66/6 + *Pseudomonas fluorescens* RC77; BF6: *Bacillus megaterium* 12/1 + *Paenibacillus polymyxa* RC35 + *Pseudomonas fluorescens* 48/3) on the growth and enzyme activities in tea under natural acidic conditions over three years. The bio-fertilizer formulations stimulated overall plant growth, including shoot development, plant height, trunk diameter, leaf area, leaf yield, chlorophyll and anthocyanin content**,** and activities of oxidative, catalytic, hydrolytic and anti-oxidative enzymes, in the Turkish registered tea clones Tuğlalı-10. In addition, inoculation with bacterial formulation affected the activities of enzymes such as glutathione reductase, glutathione S-transferase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, polyphenol oxidase, peroxidase, urease, 5-dehydroshikimate reductase, and alcohol dehydrogenases. However, plant growth responses were variable and dependent on the formulations and parameter evaluated. The selected effective bio-formulations could play an important role in understanding the plants' tolerance and adaptation to stress, and may contribute to improving the quality of tea products. Their ability to enhance plant growth will enable reductions in inputs of chemical fertilizer, and they have the potential to be used as a bio-fertilizer in sustainable and organic tea production. Our results indicate that a higher leaf yield potential in tea plants with bacterial inoculation can be expected on acidic soils in Turkey.

Key words: tea (*Camellia sinensis* L.), multi-trait rhizobacteria, bio-fertilizers, mixed inoculations, enzyme activity

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INTRODUCTION

The beneficial, freely occurring plant growth-promoting rhizobacteria (PGPR) are an alternative method of increasing

crop productivity that can reduce the use of chemical fertilizers. At present, the use of biological approaches is becoming more popular as a supplement to chemical fertilizers for improving crop yield. In this regard, PGPR have found a potential role in developing sustainable systems in crop production [Hayat et al. 2010]. Therefore, their use as biofertilizers for sustainable agriculture is important. In turn, having a great impact on root biology, plant growth, nutrition and development, PGPR are important for long-term sustainability. PGPR can promote the growth and productivity of plants through various mechanisms. They promote plant growth directly by providing the host plant with synthesized compounds, facilitating nutrient uptake, fixing atmospheric nitrogen, mineralizing and solubilizing phosphorus and other minerals, producing siderophores that solubilize and sequester iron, providing ACC-deaminase to hydrolyse endogenous ACC into ammonia and a-ketobutyrate instead of ethylene, secreting phytohormones (e.g. auxins, cytokinins, gibberellins, ethylene) that enhance various stages of plant growth, and synthesizing enzymes that modulate plant growth and development [Lucy et al. 2004, Gray and Smith 2005, Cappellari et al. 2013].

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. Tea is an economically important perennial leaf crop which requires more nitrogen than most other crops, and nitrogen application significantly increases both the yield and quality of tea [Han et al. 2008]. The response of tea leaf yield to nitrogen fertilizer application under suitable growing conditions with adequate rainfall is also significantly higher. Therefore, to improve the yield of tea leaves, fertilizer is applied to tea orchards, and its use has increased year after year. Many studies show that excess amounts of chemical fertilizer application can contribute to low N use efficiency and cause tea orchard soil acidification as well as serious water and environmental pollution [Han et al. 2008, Hirono et al. 2009, Liu et al. 2012]. Microorganisms are important in agriculture to promote the circulation of plant nutrients and minimize the need for chemical fertilizers. Plant-associated N_2-fixing and P-solubilizing bacteria are regarded as a possible alternative to inorganic nitrogen fertilizers, and PGPR strains have previously attracted the attention of agriculturists as soil inoculums to improve plant growth and yield [Şahin et al. 2004, Çakmakçı et al. 2006, 2007, Chen et al. 2006]. In the last few decades, it has been observed that plant growth-promoting rhizobacteria (PGPR) are valuable for agriculture as a tool for improving crop performance and environmental conditions.

Previous studies demonstrating the application of PGPR in soil have resulted in significant increase in growth of young tea bushes and contributed to the reduction of the use of chemicals in tea plantations [Chakraborty et al. 2006, Çakmakçı et al. 2013]. Recent studies have indicated that the development of stable formulations of PGPR is of great importance and is a promising approach to sustainable tea cultivation [Çakmakçı et al. 2014]. Even so, information on the use of bio-fertilizers on tea is very scarce, and the use of these kinds of bacteria in tea production is also lim-

ited because tea is grown in only a few countries in the world [Çakmakçı et al. 2010]. Enzymes play an important role in the antioxidant system, oxidation, formation of tea compounds, tea manufacturing and black tea production process, defence mechanism and quality in tea plants, and the biosynthesis of polyphenols, aromatic and flavonoid compounds. Little is known about the inoculation of PGPR and their effect on the activities of different oxidative, catalytic, hydrolytic and anti-oxidative enzymes in tea plants. The aim of this work was to evaluate the effects of co-inoculation with ACC deaminase-containing, N_2-fixing and/or P-solubilizing bacteria-based bio-fertilizers on growth promotion, yield, and enzyme activities in tea seedlings.

MATERIAL AND METHODS

The objective of this study was to evaluate possible effects of mineral NPK fertilizer (300 mg N + 60 mg P + 120 mg K per rooted cutting), one commercial liquid bio-fertilizer (CLBF: a commercial liquid bio-fertilizer containing *Bacillus megaterium*, *Pantoea agglomerans* and *Pseudomonas fluorescens*) and ACC deaminase-containing, N_2-fixing and P--solubilizing bacteria-based bio-formulations in triple strain combinations (BF1: *Bacillus subtilis* RC28 + *Paenibacillus polymyxa* RC05 + *Pseudomonas fluorescens* RC77; BF2; *Bacillus subtilis* RC63 + + *Paenibacillus polymyxa* 24/3 + *Pseudomonas fluorescens* 48/3; BF3: *Bacillus atrophaeus* 36/10 + *Paenibacillus polymyxa* 28/3 + *Pseudomonas fluorescens* 51/2; BF4; *Bacillus subtilis* 39/3 + *Bacillus subtilis* RC63 + *Pseudomonas fluorescens* 53/6; BF5: *Bacillus subtilis* RC521 + *Paenibacillus polymyxa* 66/6 + + *Pseudomonas fluorescens* RC77; BF6: *Bacillus megaterium* 12/1 + *Paenibacillus polymyxa* RC35+ *Pseudomonas fluorescens* 48/3) on growth and enzyme activities in tea under natural soil conditions, by conducting pot experiments over three years at the Ataturk Tea and Horticultural Research Institute in Rize. Sources and some biochemical characteristics of the bacterial strains used in the bio-formulations are given in Table 1. The experiment was arranged as a completely randomized design with nine treatments and four replicates (each having five rooted sapling cuttings). For this experiment, pure cultures were grown in 50% strength tryptic soy broth on a rotary shaker (120 rpm; 25°C) for 3 days. Bacteria were then harvested by centrifugation (ca. 3,000× g for 10 min), washed and re-suspended in 10 mM sterile phosphate buffer, pH 7.0 to a density of 10^9 cfu·ml^{-1} for the bacterial strains. For triple inoculation, equal volumes (10^9 cfu·ml^{-1} of each inoculant) of three cultures were mixed and then applied to tea saplings. The rooted cuttings were surface-sterilized prior to inoculation by soaking in 25% commercial-grade bleach for 5 min, followed by thorough washing under running tap water and air-drying aseptically overnight at room temperature. Young rooted cuttings of uniform height were inoculated with each of the bacteria-based bio-fertilizer formulations. Bacterial inoculation involved dipping the root system of the saplings into a suspension of each bio-formulation for 60 min, prior to planting. Fresh and dry leaf weight, shoot weight, average and total shoot length, shoot and trunk

TABLE 1. Sources and biochemical characteristics of the bacterial strains used in bio-formulations

Bio-formulation	Bacterial strain	Source	Oxidase	Catalase	Sucrose	N_2-fixation	P-solubilization	ACC deaminase activity
BF1	*Bacillus subtilis* RC28	grapevine	W+	+	+	+	+	S+
	Paenibacillus polymyxa RC05	wheat	-	+	-	S+	W+	S+
	Pseudomonas fluorescens RC77	raspberry	+	S+	W+	S+	+	S+
BF2	*Bacillus subtilis* RC63	raspberry	+	S+	-	S+	W+	S+
	Paenibacillus polymyxa 24/3	tea	+	+	-	+	-	ND
	Pseudomonas fluorescens 48/3	tea	+	+	-	W+	+	S+
BF3	*Bacillus atrophaeus* RC36	tea	-	S+	+	S+	+	+
	Paenibacillus polymyxa 28/3	tea	-	+	+	S+	W+	S+
	Pseudomonas fluorescens 51/2	tea	+	+	+	S+	+	+
BF4	*Bacillus subtilis* 39/3	tea	W+	S+	+	S+	W+	+
	Bacillus subtilis RC63	raspberry	+	S+	-	S+	W+	S+
	Pseudomonas fluorescens 53/6	tea	+	+	W+	S+	+	S+
BF5	*Bacillus subtilis* RC521	grapevine	-	S+	+	S+	-	S+
	Paenibacillus polymyxa RC66	raspberry	+	+	-	S+	-	+
	Pseudomonas fluorescens RC77	raspberry	+	S+	W+	S+	+	S+
BF6	*Bacillus megaterium* 12/1	tea	W+	+	-	+	S+	ND
	Paenibacillus polymyxa RC35	raspberry	W+	+	W+	S+	W+	ND
	Pseudomonas fluorescens 48/3	tea	+	+	-	W+	+	S+

S+ – strong positive reaction; + – positive reaction, - – negative reaction; W+ – weak positive reaction; BF – bio-formulation; ND – not determined.

diameter and plant height were collected for all rooted tea cuttings.

Tea leaf samples (apical bud and first two leaves) were washed three times with 50 mM Tris–HCl + 0.1 M Na_2SO_4 (pH 8.0), and each was homogenized by liquid nitrogen, transferred to 100 mM PVP + 10 mM NaN_3 + 50 mM Tris––HCl + 0.1 M Na_2SO_4 (pH 8.0) buffer, and centrifuged at 4°C, 15,000 g for 60 min [Çakmakçı et al. 2009]. Glucose--6-phosphate dehydrogenase (G6PD; EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (6PGD; EC 1.1.1.44) activities were determined according to the method of Beutler [1984]. The increase in A_{340} was monitored over 3 min. One unit of enzyme activity was defined as a reduction of 1 μmol $NADP^+$ min^{-1} under the assay conditions.

Protein content, glutathione reductase (GR; EC 1.8.1.7) and glutathione S-transferase (GST; EC 2.5.1.18) enzyme activities were determined according to methods described by Bradford [1976], Habig and Jacoby [1981], Carlberg and Mannervik [1985] respectively. One unit of GR enzyme activity was defined as the oxidation of 1 μmol NADPH per min under the assay conditions. One unit of GST activity was defined as the formation of 1.0 μmol product min^{-1} (extinction coefficients at 340 nm: 6.2 $mM^{-1}{\cdot}cm^{-1}$ for NADPH, and 9.6 $mM^{-1}{\cdot}cm^{-1}$ for the glutathione-2,4-dinitrobenzene conjugate).

The activities of polyphenol oxidase (PPO; EC 1.14.18.1), peroxidase (POD; EC: 1.11.1.7), 5-dehydroshikimate reductase (DHSK; EC: 1.1.1.25) and alcohol dehydrogenase (ADH; EC: 1.1.1.1) were assayed by the methods of Sanderson [1966], Hatanaka et al. [1974], Lee et al. [1991] and Mei et al. [2009], respectively. One unit of PPO and POD activity was defined respectively as an increase of 0.001 and 0.1 units of absorbance per min at 420 and 470 nm. One unit of DHSK reductase activity was defined as the formation of 1.0 μmol NADPH min^{-1}, while one unit of ADH activity was defined as the amount of ADH catalysing the reaction to produce 1 μM NADH per min in a spectrophotometer at 25°C (extinction coefficient at 340 nm: 6.22 $mM^{-1}{\cdot}cm^{-1}$). Results for PPO, POD, ADH and DHSK reductase enzyme activities were expressed as unit g^{-1} of dry weight (DW). Enzymatic activities were determined spectrophotometrically at 25°C using a Shimadzu 1208 UV spectrophotometer (Kyoto, Japan).

The experiments were performed in a completely randomized design with four replicates. Enzyme activities were determined on three samples from each replicate. The data were subjected to analysis of variance using SPSS13.0 (SPSS Inc. Waltham MA) and the means were separated according to Duncan's multiple range test.

RESULTS AND DISCUSSION

Three years of trials showed that treatments including bacterial formulations and fertilizer application significantly affected the parameters investigated compared with the control, depending on the years, bacterial formulations and growth parameter evaluated. Among the treatments tested, inoculation with BF4 and BF6 mixed bio-formulations and NPK fertilizer application increased trunk diameter, plant height, shoot and

leaf weight, fresh and dry leaf weight, second and third leaf area, chlorophyll (SPAD) and anthocyanin (ACI) contents of tea plants significantly compared with the control; the maximum yield and growth parameters in tea were found with the BF4 formulation (Table 2). In both years, all treatments significantly increased plant height. Except for the BF3 and BF5 formulations, all tested treatments significantly increased the shoot and leaf weight of tea saplings. In both years, trunk diameter, plant height, shoot and leaf weight and fresh and dry leaf weight were greatest with the inoculation of BF4, whereas the highest levels of second and third leaf area and chlorophyll (SPAD) and anthocyanin (ACI) contents were found with the application of NPK (Table 2).

The increases on inoculation as compared with the control plants ranged between –0.2 and 15.7% for trunk diameter, 13.9 and 22.0% for plant height, –0.7 and 41.2% for shoot and leaf fresh weight, –0.3 and 42.0% for leaf fresh weight, 1.4 and 45.1% for leaf dry weight, –1.4 and 13.0% for second leaf area, –3.1 and 13.0% for third leaf area, –1.4 and 12.4% for chlorophyll (SPAD) content, and –1.3 and 12.4% for anthocyanin (ACI) content. Mineral fertilizer application as compared with the control plants increased trunk diameter, plant height, shoot and leaf weight, fresh leaf weight, dry leaf weight, second leaf area, third leaf area, chlorophyll (SPAD) and anthocyanin (ACI) content by 14.0, 17.5, 35.3, 38.4, 41.0, 16.3, 16.3, 12.5 and 14.4%, respectively. It is particularly notable that in the third year, growth of tea (shoot and leaf weight, fresh and dry leaf weight) was significantly more

TABLE 2. The effect of different combinations of bacteria and fertilizer applications on the average trunk diameter, plant height, shoot and leaf weight, fresh and dry leaf weight, chlorophyll (SPAD) and anthocyanin (ACI) contents and leaf area of Turkish tea clone Tuğlalı-10

Treatments	GR	GST	G6PD	6PGD	PPO	POD	ADH	DHSK
	mg^{-1} protein				g^{-1} leaf DW			
First year (2013)								
Control	1.53 ±0.29 c	1.02 ±0.02 d	0.76 ±0.07 c	0.88 ±0.17 c	7.17 ±0.38 c	19.1 ±4.92 f	1.17 ±0.18 b-d	2.46 ±0.56 d
NPK	2.56 ±0.23 ab	2.61 ±0.11 b	0.80 ±0.23 c	1.12 ±0.23 c	8.99 ±1.03 a	25.6 ±4.13 ef	0.80 ±0.05 d	2.42 ±0.44 d
CLBF	2.47 ±0.20 b	1.70 ±0.23 c	1.62 ± 0.18 b	1.07 ±0.15 c	7.67 ±1.06 bc	19.9 ±3.66 f	0.87 ±0.22 cd	2.28 ±0.48 d
BF1	2.90 ±0.32 ab	1.81 ±0.14 c	2.91 ±0.24 a	2.77 ±0.31 a	7.40 ±1.32 a-c	30.2 ±4.19 de	0.80 ±0.24 d	3.52 ±0.47 c
BF2	1.35 ±0.23 cd	2.39 ±0.28 b	0.88 ±0.25 c	0.95 ±0.30 c	6.89 ±0.48 bc	51.7 ±3.41 a	1.73 ±0.31 a	4.32 ±1.56 ab
BF3	0.90 ±0.19 d	2.66 ±0.55 b	3.08 ±0.59 a	2.01 ±0.52 b	6.75 ±1.99 c	20.2 ±2.83 f	1.33 ±0.31 a-c	2.91 ±0.54 cd
BF4	2.52 ±0.27 b	2.59 ±0.45 b	0.99 ±0.11 c	1.46 ±0.16 bc	8.55 ±1.12 ab	51.3 ±7.86 ab	1.30 ±0.38 a-c	3.09 ±0.87 cd
BF5	3.09 ±0.22 a	4.31 ±0.27 a	1.17 ±0.41 bc	2.93 ±0.41 a	6.66 ±1.24 c	36.2 ±5.48 cd	1.07 ±0.29b-d	4.47 ±1.52 a

BF6	2.98 ±0.35 ab	1.73 ±0.04 c	0.71 ±0.18 c	0.94 ±0.16 c	8.63 ±1.12 a	43.7 ±7.91 b-c	1.52 ±0.42 ab	3.59 ±0.45 bc
Average	2.26	2.31	1.44	1.57	7.63	33.1	1.18	3.21
Second year (2014)								
Control	1.59 ±0.31 d	1.09 ±0.02 d	0.87 ±0.07 c	0.93 ±0.17 d	7.05 ±0.58 d	19.8 ±5.29 e	1.09 ±0.16 c	2.41 ±0.62 de
NPK	2.68 ±0.24 bc	2.78 ±0.12 b	0.86 ±0.24 c	1.18 ±0.24 cd	9.49 ±0.69 a	26.9 ±3.04 de	1.05 ±0.26 c	2.74 ±0.61 c-e
CLBF	2.59 ±0.20 c	1.81 ±0.24c	1.72 ±0.18 b	1.13 ±0.16 cd	8.30 ±1.03 a-d	20.1 ±3.55 e	1.01 ±0.07 c	2.13 ±0.50 e
BF1	3.03 ±0.34 ab	1.92 ±0.15 c	2.95 ±0.24a	2.98 ±0.33 a	7.81 ±0.99 b-d	30.7 ±3.39 cd	1.08 ±0.21 c	3.64 ±0.47 a-c
BF2	1.44 ±0.25 d	2.47 ±0.29 b	0.94 ±0.26c	1.00 ±0.32 d	7.23 ±0.55 cd	53.2 ±4.96 a	1.91 ±0.18 a	4.07 ±1.10 ab
BF3	0.94 ±0.19 e	2.81 ±0.58 b	3.16 ±0.60 a	2.13 ±0.53 b	7.00 ±1.63 d	20.9 ±2.73 e	1.44 ±0.45 bc	3.12 ±0.75 b-e
BF4	2.68 ±0.28 bc	2.76 ±0.47 b	1.04 ±0.11 c	1.58 ±0.17 c	8.67 ±0.98 a-c	53.5 ±8.95 a	1.58 ±0.26 ab	3.59 ±0.33 a-d
BF5	3.26 ±0.24 a	4.42 ±0.28 a	1.25 ±0.42 c	3.09 ±0.44 a	6.81 ±0.43 d	37.0 ±4.27 c	1.32 ±0.38 bc	4.72 ±1.32 a
BF6	3.15 ±0.37 a	1.84 ±0.04 c	0.83 ±0.12 c	0.99 ±0.17 d	9.13 ±1.13 ab	45.2 ±5.37 b	1.66 ±0.36 a	4.04 ±0.55 ab
Average	2.37	2.43	1.52	1.67	7.94	34.2	1.35	3.38
Third year (2015)								
Control	1.98 ±0.16 e	1.40 ±0.03 d	1.34 ±0.12 e	0.99 ±0.12 d	7.20 ±0.13 c	21.0 ±5.41 d	0.93 ±0.04 d	2.57 ±0.73 c
NPK	2.87 ±0.13 c	2.55 ±0.09 b	1.37 ±0.08 de	1.38 ±0.11 c	9.40 ±0.33 a	30.2 ±3.54 c	0.96 ±0.17 d	2.70 ±0.41 c
CLBF	2.47 ±0.11 d	2.44 ±0.21 bc	1.72 ±0.16 c	1.50 ±0.09 c	8.43 ±0.45 a-c	21.7 ±4.01 d	0.91 ±0.23 d	2.43 ±0.24 c
BF1	2.60 ±0.17 d	2.18 ±0.13 c	2.81 ±0.21 a	2.73 ±0.26 a	8.03 ±0.75 bc	31.9 ±4.31 bc	0.93 ±0.13 d	3.87 ±0.63 ab
BF2	1.97 ±0.25 e	2.40 ±0.12 bc	1.38 ±0.10 de	1.47 ±0.21 c	7.20 ±0.50 c	49.7 ±3.27 a	1.87 ±0.27 a	4.09 ±0.54 a
BF3	2.03 ±0.14 e	2.52 ±0.41 b	2.26 ±0.34 b	1.82 ±0.17 b	7.12 ±0.68 c	21.1 ±2.95 d	1.48 ±0.16 bc	3.03 ±0.57 bc
BF4	2.43 ±0.08 d	2.55 ±0.23 b	1.38 ±0.14 de	1.59 ±0.13 bc	8.57 ±0.92 ab	50.3 ±3.07 a	1.47 ±0.19 bc	3.87 ±0.55 ab
BF5	3.72 ±0.22 a	3.80 ±0.13 a	1.64 ±0.20 cd	2.68 ±0.17 a	7.15 ±0.52 c	37.7 ±5.71 b	1.19 ±0.32 cd	4.66 ±0.95 a
BF6	3.36 ±0.15 b	2.17 ±0.03 c	1.54 ±0.08 c-e	1.42 ±0.13 c	8.70 ±0.79 ab	45.6 ±4.64 a	1.64 ±0.28 ab	4.25 ±0.42 a
Average	2.60	2.45	1.72	1.73	7.98	34.3	1.26	3.50

GR – glutathione reductase; GST – glutathione S-transferase; G6PD – glucose-6-phosphate dehydrogenase; 6PGD – 6-phosphogluconate dehydrogenase; PPO – polyphenol oxidase; POD – peroxidase; ADH – alcohol dehydrogenase; DHSK – 5-dehydroshikimate reductase. All strains used in these bio-formulations are explained in Table 1.

Values are means ±SE; averages of the same column values (each section separately) followed by the same letter did not differ significantly in Duncan's multiple range tests at 0.05% significance.

enhanced by BF4 inoculation and NPK fertilizer application than by other treatments (Table 2).

Inoculation with rhizobacteria-based bio-formulations stimulated overall plant growth, including shoot development, plant height, trunk diameter, leaf yield, chlorophyll and anthocyanin content, leaf area, and activities of oxidative, catalytic, hydrolytic and anti-oxidative enzymes of the Turkish tea clones Tuğlalı--10. As reported previously, the effect of PGPR is a complex process, and depends on the bacterial strain and population, the plant-bacterial strain combination, the plant genotype, the growth parameters evaluated, and environmental conditions [Şahin et al. 2004, Çakmakçı et al. 2006]. Previous studies demonstrating the application of PGPR in soil have resulted in significant increase in growth of young tea bushes and contributed to the reduction of the use of chemicals on tea plantations [Chakraborty et al. 2006, 2012, Çakmakçı et al. 2013, 2014, Çakmakçı 2016, Zhan et al. 2016].

According to the three-year results, all bio-formulation inoculations and fertilizer applications significantly increased glutathione S-transferase (GST) activity in the leaves of tea plants. Except for the BF2 and BF3 formulations, all treatments increased glutathione reductase (GR) activity in each of the three years (Table 3). Glucose-6-phosphate dehydrogenase (G6PD) activity was greatest with the application of BF3 and BF1, whereas the highest level of 6-phosphogluconate dehydrogenase (6PGD) was observed after BF5 and BF1 inoculations, followed by BF3. Among the various treatments tested, BF2, BF4, and BF6 caused the maximum enhancement in peroxidase (POD)

TABLE 3. The effect of different combinations of bacteria and fertilizer applications on enzyme activities in tea leaves

Treatments	GR	GST	G6PD	6PGD	PPO	POD	ADH	DHSK
	mg^{-1} protein				g^{-1} leaf DW			
	First year (2013)							
Control	1.53 ±0.29 c	1.02 ±0.02 d	0.76 ±0.07 c	0.88 ±0.17 c	7.17 ±0.38 c	19.1 ±4.92 f	1.17 ±0.18 b-d	2.46 ±0.56 d
NPK	2.56 ±0.23 ab	2.61 ±0.11 b	0.80 ±0.23 c	1.12 ±0.23 c	8.99 ±1.03 a	25.6 ±4.13 ef	0.80 ±0.05 d	2.42 ±0.44 d
CLBF	2.47 ±0.20 b	1.70 ±0.23 c	1.62 ± 0.18 b	1.07 ±0.15 c	7.67 ±1.06 bc	19.9 ±3.66 f	0.87 ±0.22 cd	2.28 ±0.48 d
BF1	2.90 ±0.32 ab	1.81 ±0.14 c	2.91 ±0.24 a	2.77 ±0.31 a	7.40 ±1.32 a-c	30.2 ±4.19 de	0.80 ±0.24 d	3.52 ±0.47 c
BF2	1.35 ±0.23 cd	2.39 ±0.28 b	0.88 ±0.25 c	0.95 ±0.30 c	6.89 ±0.48 bc	51.7 ±3.41 a	1.73 ±0.31 a	4.32 ±1.56 ab
BF3	0.90 ±0.19 d	2.66 ±0.55 b	3.08 ±0.59 a	2.01 ±0.52 b	6.75 ±1.99 c	20.2 ±2.83 f	1.33 ±0.31 a-c	2.91 ±0.54 cd
BF4	2.52 ±0.27 b	2.59 ±0.45 b	0.99 ±0.11 c	1.46 ±0.16 bc	8.55 ±1.12 ab	51.3 ±7.86 ab	1.30 ±0.38 a-c	3.09 ±0.87 cd
BF5	3.09 ±0.22 a	4.31 ±0.27 a	1.17 ±0.41 bc	2.93 ±0.41 a	6.66 ±1.24 c	36.2 ±5.48 cd	1.07 ±0.29 b-d	4.47 ±1.52 a
BF6	2.98 ±0.35 ab	1.73 ±0.04 c	0.71 ±0.18 c	0.94 ±0.16 c	8.63 ±1.12 a	43.7 ±7.91 b-c	1.52 ±0.42 ab	3.59 ±0.45 bc

Average	2.26	2.31	1.44	1.57	7.63	33.1	1.18	3.21
Second year (2014)								
Control	1.59 ±0.31 d	1.09 ±0.02 d	0.87 ±0.07 c	0.93 ±0.17 d	7.05 ±0.58 d	19.8 ±5.29 e	1.09 ±0.16 c	2.41 ±0.62 de
NPK	2.68 ±0.24 bc	2.78 ±0.12 b	0.86 ±0.24 c	1.18 ±0.24 cd	9.49 ±0.69 a	26.9 ±3.04 de	1.05 ±0.26 c	2.74 ±0.61 c-e
CLBF	2.59 ±0.20 c	1.81 ±0.24c	1.72 ±0.18 b	1.13 ±0.16 cd	8.30 ±1.03 a-d	20.1 ±3.55 e	1.01 ±0.07 c	2.13 ±0.50 e
BF1	3.03 ±0.34 ab	1.92 ±0.15 c	2.95 ±0.24 a	2.98 ±0.33 a	7.81 ±0.99 b-d	30.7 ±3.39 cd	1.08 ±0.21 c	3.64 ±0.47 a-c
BF2	1.44 ±0.25 d	2.47 ±0.29 b	0.94 ±0.26 c	1.00 ±0.32 d	7.23 ±0.55 cd	53.2 ±4.96 a	1.91 ±0.18 a	4.07 ±1.10 ab
BF3	0.94 ±0.19 e	2.81 ±0.58 b	3.16 ±0.60 a	2.13 ±0.53 b	7.00 ±1.63 d	20.9 ±2.73 e	1.44 ±0.45 bc	3.12 ±0.75 b-e
BF4	2.68 ±0.28 bc	2.76 ±0.47 b	1.04 ±0.11 c	1.58 ±0.17 c	8.67 ±0.98 a-c	53.5 ±8.95 a	1.58 ±0.26 ab	3.59 ±0.33 a-d
BF5	3.26 ±0.24 a	4.42 ±0.28 a	1.25 ±0.42 c	3.09 ±0.44 a	6.81 ±0.43 d	37.0 ±4.27 c	1.32 ±0.38 bc	4.72 ±1.32 a
BF6	3.15 ±0.37 a	1.84 ±0.04 c	0.83 ±0.12 c	0.99 ±0.17 d	9.13 ±1.13 ab	45.2 ±5.37 b	1.66 ±0.36 a	4.04 ±0.55 ab
Average	2.37	2.43	1.52	1.67	7.94	34.2	1.35	3.38
Third year (2015)								
Control	1.98 ±0.16 e	1.40 ±0.03 d	1.34 ±0.12 e	0.99 ±0.12 d	7.20 ±0.13 c	21.0 ±5.41 d	0.93 ±0.04 d	2.57 ±0.73 c
NPK	2.87 ±0.13 c	2.55 ±0.09 b	1.37 ±0.08 de	1.38 ±0.11 c	9.40 ±0.33 a	30.2 ±3.54 c	0.96 ±0.17 d	2.70 ±0.41 c
CLBF	2.47 ±0.11 d	2.44 ±0.21 bc	1.72 ±0.16 c	1.50 ±0.09 c	8.43 ±0.45 a-c	21.7 ±4.01 d	0.91 ±0.23 d	2.43 ±0.24 c
BF1	2.60 ±0.17 d	2.18 ±0.13 c	2.81 ±0.21 a	2.73 ±0.26 a	8.03 ±0.75 bc	31.9 ±4.31 bc	0.93 ±0.13 d	3.87 ±0.63 ab
BF2	1.97 ±0.25 e	2.40 ±0.12 bc	1.38 ±0.10 de	1.47 ±0.21 c	7.20 ±0.50 c	49.7 ±3.27 a	1.87 ±0.27 a	4.09 ±0.54 a
BF3	2.03 ±0.14 e	2.52 ±0.41 b	2.26 ±0.34 b	1.82 ±0.17 b	7.12 ±0.68 c	21.1 ±2.95 d	1.48 ±0.16 bc	3.03 ±0.57 bc
BF4	2.43 ±0.08 d	2.55 ±0.23 b	1.38 ±0.14 de	1.59 ±0.13 bc	8.57 ±0.92 ab	50.3 ±3.07 a	1.47 ±0.19 bc	3.87 ±0.55 ab
BF5	3.72 ±0.22 a	3.80 ±0.13 a	1.64 ±0.20 cd	2.68 ±0.17 a	7.15 ±0.52 c	37.7 ±5.71 b	1.19 ±0.32 cd	4.66 ±0.95 a
BF6	3.36 ±0.15 b	2.17 ±0.03 c	1.54 ±0.08 c-e	1.42 ±0.13 c	8.70 ±0.79 ab	45.6 ±4.64 a	1.64 ±0.28 ab	4.25 ±0.42 a
Average	2.60	2.45	1.72	1.73	7.98	34.3	1.26	3.50

GR – glutathione reductase; GST – glutathione S-transferase; G6PD – glucose-6-phosphate dehydrogenase; 6PGD – 6-phosphogluconate dehydrogenase; PPO – polyphenol oxidase; POD – peroxidase; ADH – alcohol dehydrogenase; DHSK – 5-dehydroshikimate reductase. All strains used in these bio-formulations are explained in Table 1.

Values are means ±SE; averages of the same column values (each section separately) followed by the same letter did not differ significantly in Duncan's multiple range tests at 0.05% significance.

activity in tea, while BF2 and BF6 most effectively promoted alcohol dehydrogenase (ADH) activity. In the three years the highest 5-dehydroshikimate reductase (DHSK) activities were obtained from the BF5 formulation, followed by BF2 and BF6, while the lowest DHSK activity was recorded for the control and for the application of commercial liquid bio-fertilizer (Table 3). In general, GR, GST, 6PGD and DHSH activities were greatest with the application of BF5, whereas the highest levels of POD and ADH activities were found on treatment with BF2 (Table 2). BF4 inoculations and fertilizer application caused maximum enhancement in the growth and leaf yield of tea, while NPK application and BF6 inoculation were the most effective promoters of PPO activity.

Inoculation with some of the multitrait rhizobacterial formulations enhanced seedling growth and defence-related enzymes, such as GR, GST, POD and PPO, in tea leaves. The screening of rhizobacteria that show multiple plant growth-promoting (PGP) traits suggests that they have good potential for field testing and applications in improving the growth of tea [Çakmakçı 2016]. The beneficial role of PGPR formulations has been reported by some other researchers [Hafeez et al. 2006, Çakmakçı et al. 2007, Chakraborty et al. 2012, Çakmakçı 2016]; it might be attributed to IAA and siderophore production, N_2-fixation, P-solubilization, ACC deaminase activity, or even other non-evaluated PGPR traits that stimulate plant growth. Since inoculation caused a differential increase in leaf defence and quality-related enzymes like ADH and PPO activity, as well as activation of other plant enzymes, this may indicate that activation of these enzymes in tea leaves would be differentially affected by different formulations. To alleviate stress, plants have evolved an effective antioxidant system composed of antioxidant enzymes such as GR, GST and POD. Glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD) catalyse the biosynthesis of polyphenols [Magoma et al. 2003], while 5-dehydroshikimate reductase (DHSK) is important in the biosynthesis of flavonoid compounds [Sanderson 1966]. The synthesis of flavonoids in tea requires enzymes of the shikimate pathway, and DHSK reductase is a key regulatory enzyme in the process. Also, the oxidation of plant aldehydes to their corresponding alcohols is due to ADH activity.

Enzymes play an important role in the tea manufacturing process and in the antioxidant system of the plants. Polyphenol oxidase and peroxidase are thought to play a role in the fermentation process, oxidation and formation of black tea compounds [Balentine et al. 1997, Emdadi et al. 2009, Stodt et al. 2014]. Also, they lead to the formation of black tea polyphenols and aroma compounds characteristic of black tea and involved in the plants' defence mechanism against environmental stresses [Harbowy and Balentine 1997]. Polyphenols, which are antioxidant compounds, are the major category of secondary metabolites in tea plants [Lu et al. 2014]. Phenolic compounds are responsible for certain characteristics of black tea, such as colour and taste.

Of the bacterial formulations, BF4 and BF6 caused the greatest increase in PPO activity, while BF2, BF4 and BF6

most effectively promoted POD activity. Of the formulations tested, BF3 consistently gave PPO and POD activities equal to or lower than those of the control plants. During the manufacturing of green tea these enzymes are undesirable, because they can catalyse the oxidation of catechins, which can negatively affect the quality of green tea. On the other hand, these enzymes are desirable in the process of black tea production, and can catalyse the transformation of catechin to theaflavins (TF) and thearubigins (TR), responsible for the typical flavour and colour of black tea [Jiang 2008]. The roles of PPO and PO in transforming catechin compounds to TF and TR have also been highlighted, and this has direct bearing on the quality of black tea [Samanta et al. 2015]. Both of the oxidative enzymes PPO and POD are of considerable importance in black tea processing and for the quality and flavour of the tea. Enzyme PPO plays the main role in enzymatic browning of the leaves, and together with enzyme POD has a synergistic effect on the oxidation of phenolic compounds. Enzyme POD plays an important role in the formation of theaflavin-related compounds during black tea fermentation [Sang et al. 2004]. Our results may indicate that the activation of these enzymes in tea leaves would be differentially affected by different bio-formulations.

The application of NPK fertilizer and the BF4 and BF6 formulations increased the chlorophyll and anthocyanin content in tea leaves, and enhanced the growth and yield parameters of tea. Chlorophyll, the main component of the colour in green tea, may influence the net photosynthesis rate and the tea quality. Anthocyanins are responsible for leaf colour, antioxidant activity and physiological and biochemical processes. Chlorophyll is a highly important pigment as its quantity determines the final colour of non-fermented green tea infusion. The fermentation process transforms chlorophylls into pheophorbides and pheophytins, which give rise to the dark colour of black tea, as reported by Ošťádalová et al. [2014], who suggested that chlorophylls may be an important stable indicator of tea quality. Increasing contents of chlorophylls and anthocyanin would contribute to the production of quality teas. This is in agreement with the results of Joshi et al. [2015], who reported that tea from anthocyanin-rich cultivars could be used to make specialty teas with high antioxidant activity. Additional studies are required to explain the mechanism by which PGPR affects the tea quality, anthocyanin content, and responses of different oxidative, hydrolytic and antioxidant enzymes.

CONCLUSIONS

Inoculation with rhizobacteria-based bio-formulations had the potential to induce a greater amount of enzymes, leading to increased growth and yield. Bacterial formulations caused high growth, leaf weight and enzyme activity, but the effect was strongly dependent on the inoculant strain formulations and parameters evaluated. Among the various bio-formulations tested, BF4 (*B. subtilis* 39/3 + *B. subtilis* RC63 + *P. fluorescens* 53/6) and BF6 (*B. megaterium* 12/1 + *P. polymyxa* RC35 + *P. fluorescens* 48/3) were found to be most effective in

promoting the growth, yield and quality of tea. Our results may be of importance for further research on PGPR in relation to tea growth, yield and quality, processing technology, and stress tolerance. The application of such bio-formulations may result in the reduction of application of harmful chemicals, protect the environment and biological resources, and prevent the accumulation of nitrates and phosphates in agricultural soils. A biological fertilization strategy may change a tea plantation's soil fertility, physical and chemical environment, enzyme activity, stress tolerance and microorganism community, and can also affect the tea yield and quality.

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Streszczenie: *Wpływ preparatów zawierających bakterie na wzrost, plon i aktywność enzymatyczną herbaty* (Camellia sinensis *L.*). Istnieje rosnąca potrzeba stosowania mikroorganizmów w uprawach, w celu uzyskania żywności bezpiecznej dla konsumentów, przeciwdziałania zanieczyszczeniu środowiska oraz utrzymania zrównoważonego rolnictwa i zachowania rolniczych zasobów. Celem tej pracy była ocena możliwego wpływu nawozu mineralnego (NPK), komercyjnego bionawozu płynnego oraz biopreparatów zawierających bakterie na wzrost i aktywność enzymatyczną herbaty uprawianej przez trzy lata na naturalnie kwaśnych glebach. Do porównań użyto zawierające deaminazę ACC, wiążące azot i solubizujące fosfor bakteryjne biopreparaty, które zawierały potrójne kombinacje szczepów bakteryjnych (BF1: *Bacillus subtilis* RC28 + *Paenibacillus polymyxa* RC05 + *Pseudomonas fluorescens* RC77; BF2: *Bacillus subtilis* RC63 + *Paenibacillus polymyxa* 24/3 + *Pseudomonas fluorescens* 48/3; BF3: *Bacillus atrophaeus* 36/10 + *Paenibacillus polymyxa* 28/3 + *Pseudomonas fluorescens* 51/2; BF4: *Bacillus subtilis* 39/3 + *Bacillus subtilis* RC63 + *Pseudomonas fluorescens* 53/6; BF5: *Bacillus subtilis* RC521 + *Paenibacillus polymyxa* 66/6 + *Pseudomonas fluorescens* RC77; BF6: *Bacillus megaterium* 12/1 + *Paenibacillus polymyxa* RC35 + *Pseudomonas fluorescens* 48/3). W zarejestrowanym w Turcji klonie herbaty Tuğlalı-10 biopreparaty stymulowały ogólny wzrost roślin, w tym rozwój i przyrost elongacyjny pędów, średnicę pędu głównego, powierzchnię i plon liści, zawartość chlorofilu i antocyjanów, aktywności oksydacyjną, katalityczną, hydrolityczną i antyoksydacyjną herbaty. Inokulacja preparatami bakteryjnymi wpływała również na aktywność enzymów, takich jak: reduktaza glutationowa, s-transferaza glutationowa, dehydrogenaza glukozo-6P, dehydrogenaza 6-fosforoglukonianu, oksydaza polifenolowa, peroksydaza, ureaza, reduktaza 5-dehydroszikimianu oraz dehydrogenazy alkoholowe. Reakcja wzrostowa roślin była jednak różna i zależna od rodzaju formulacji oraz ocenianego parametru. Wyselekcjonowane, efektywne biopreparaty mogą pełnić istotną funkcję w zrozumieniu tolerancji roślin i ich adaptacji do stresu. Mogą one także przyczyniać się do polepszenia produktów uzyskiwanych z herbaty. Zdolność biopreparatów do stymulacji wzrostu roślin sprawia, że mogą być one wykorzystywane jako bionawozy w zrównoważonej i organicznej produkcji herbaty, co także może przyczynić się do ograniczenia stosowania chemicznych nawozów. Nasze wyniki wskazują, że inokulacje bakteryjne mogą zwiększać plon liści herbaty na kwaśnych glebach w Turcji.