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Research Article

# Çoklu Özellikli Bakteri Esaslı Biyo-Formüllerin Çayın Gelişim, Verim ve Enzim Aktiviteleri Üzerine Etkisi

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## Öz

Bu araştırma mineral gübre, ticari mikrobiyal gübre ve azot fikseri, fosfat çözücü ve ACC deaminaze aktivitesine sahip bakteri esaslı üçlü kombinasyonlar halinde uygulanan üç farklı mikrobiyal gübre formülasyonunun (BF9: *Bacillus megaterium* 47/9 + *Paenibacillus macquariensis* RC696 + *Pseudomonas fluorescens* 9/7; BF10: *Bacillus megaterium* RC665 + *Paenibacillus macquariensis* RC382 + *Pseudomonas fluorescens* 9/7; BF11: *Bacillus simplex* RC64 + *Pseudomonas putida* 3/10 + *Burkholderia pyrrocinia* RC134) asidik tarla koşullarında üç yıllık sürede çay gelişim ve enzim aktiviteleri üzerine etkisinin belirlenmesi amacıyla yürütülmüştür. Deneme tesadüf bloklarında altı uygulama ve dört tekerrürlü (her bir tekerrürde beş çay öbeği) olarak kurulmuştur. Uygulanan bakteri formülasyonları yaprak alanı, yeşil yaprak verimi, klorofil içeriği ve bitki gelişimini teşvik etmiştir. Ayrıca, bakteri formülasyonu aşılamaları yaprakta; glutatyon redüktaz (GR), glutatyon S-transferaz (GST), glukoz 6-fosfat dehidrogenaz (G6PD), 6-fosfoglukonat dehidrogenaz (6PGD), polifenol oksidaz (PPO), peroksidaz (POD), 5-dehidroksişikimat redüktaz (DHSK) ve alkol dehidrogenaz (ADH), enzim aktivitelerini değiştirebilmiştir. Seçilen etkin, aside toleranslı ve çoklu özelliklere sahip bakteri esaslı biyo-formülasyonlar, strese karşı bitki toleransı ve adaptasyonunu artırabilir, çay işleme teknolojisinde önemli bir rol oynayabilir ve çay ürünlerinin kalite konseptine katkıda bulunabilir. Bu çalışma, bu yerli faydalı rizobakteri izolatlarının, çay mahsulünün büyümesini teşvik etmek için mikrobiyal aşılama veya biyogübre olarak kullanılma potansiyeline sahip olduğunu ve sürdürülebilir çay yetiştiriciliği için umut verici olduğunu göstermektedir.

**Anahtar kelimeler:** *Camellia sinensis* L., birlikte aşılama, biyolojik gübre, verim ve kalite, bitki gelişmesini teşvik edici bakteri

# Effect of Co-Inoculation of Multi-Traits Bacteria Based Bio-Formulations on the Growth, Yield and Enzyme Activities of Tea

## Abstract

The aims of the present study were to investigate the effectiveness of mineral fertilizer (NPK), one commercial and three $N_2$-fixing, P-solubilizing and/or ACC deaminase-containing bacteria based bio-fertilizers in triple strains combinations (BF9: *Bacillus megaterium* 47/9 + *Paenibacillus macquariensis* RC696 + *Pseudomonas fluorescens* 9/7; BF10: *Bacillus megaterium* RC665 + *Paenibacillus macquariensis* RC382 + *Pseudomonas fluorescens* 9/7; BF11: *Bacillus simplex* RC64 + *Pseudomonas putida* 3/10 + *Burkholderia pyrrocinia* RC134) were evaluated for their growth and enzyme activities of tea under acidic soil conditions, in three years. The experiment was conducted in a completely randomized design with six treatments and four replicates. Bio-fertilizers formulations stimulated overall plant growth, including leaf area, green leaf yield, chlorophyll content and enzyme activities in tea leaf. In addition, inoculation with bacterial formulation, activities of the different enzymes like glutathione reductase, glutathione S-transferase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, polyphenol oxidase, peroxidase, urease, 5-dehydroshikimate reductase, and alcohol dehydrogenases also changed. The selected effective acid-tolerant multi-traits bacteria based bio-formulations could play an important role in understanding the plant tolerance and adaptation to stress, processing technology, and may contribute to the concept of the quality of tea

products. This study shows that these indigenous beneficial rhizobacteria isolates have the potential to be used as microbial inoculation or bio fertilizer to stimulate tea crop growth and are promising for sustainable tea cultivation.

**Key words:** *Camellia sinensis* L., co-inoculation, bio-fertilizers, yield and quality, plant growth promoting rhizobacteria

## Introduction

Tea is a woody perennial tree, which has remained in the field for several years, is a plant that is harvested several times a year, usually three times, and impoverishes the soil in terms of nutrients and microorganisms. Due to the frequent removal of two leaves and a bud for processing, tea as a leaf harvest crop is fed with abundant nutritional supplement fertilizers, especially N fertilizers. In particular, nitrogen and phosphorus, which are necessary for plant growth and development and additionally limit production, are applied in large quantities and continuously in tea orchards. While N application is constantly increasing in tea pods especially to increase leaf yield, even if it is applied continuously or the total amount in the soil is sufficient, phosphorus usage efficiency is low because it is constantly fixed to unavailable forms. In most cases, although acidic tea soils contain high amounts of total phosphorus, most of them are insoluble as Fe and Al phosphates.

While N increases yield and quality in tea cultivation, P deficiency inhibits photosynthesis in tea. On the other hand, excessive application of N fertilizer affects acidification of tea garden soil (Han et al., 2008), groundwater pollution (Liu et al., 2012), as well as nitrification rates (Xue et al., 2006). Some of the previous tea research has shown that repeated and excessive application of chemical fertilizers can lead to low N use efficiency and cause tea orchard soil acidification, deterioration of soil properties, potential nitrate leaching, gaseous N emissions, persistence of chemicals in plant products, and serious water and environmental pollution (Tokuda and Hayatsu, 2004; Han et al., 2008; Kamau et al., 2008; Hirono et al., 2009; Liu et al., 2012; Çakmakçı, 2016). With N fertilization, nitrate accumulation occurs in soil, plant, underground and surface waters. Today, agriculture relies on high input of agricultural chemicals and excessive application of chemical fertilizers directly endangers natural resources; causing degradation of water resources such as lakes, rivers and seawater resources, soil pollution and depletion of soil quality. In addition, increased use of fertilizers is associated with eutrophication and can lead to deterioration of aquatic organisms.

Tea leaves are constantly harvested, which requires continuous N and P fertilizers, and the rainfall regime of the region triggers this. On the other hand, most of the fertilizers applied in excessively rainy, high humidity and extreme sloping areas where tea is grown are lost by immobilization, evaporation and especially washing. High levels of N and P fertilization losses that is released, leaching and runoff in rainy and extreme sloping area mix with water, reduce drinking water quality and biodiversity, and threaten producers and consumer health. Undoubtedly, excessive use of chemical fertilizers is responsible for changing the soil quality and soil microbial population (Nath et al., 2013), and result in several problems, such as the persistence of chemicals in plant crops and adverse impact on the soil environment, which ultimately leads to inefficiency (Chakraborty et al., 2009). In addition, the intensive chemical fertilizer application creates a highly selective environment and adversely affects microbial diversity (Gulati et al., 2011). Despite the increase in input costs in existing agriculture, additional fertilizer does not increase the yield, because of soil productivity and biological activity decrease and acidity increases. On the other hand, due to the use of synthetic fertilizers for years in traditional tea production, soil health deteriorates due to the lack of micro elements and microbial activity, soil acidity increases, yield and product quality decrease. Effects of excessive fertilization and nutrient loss from tea plantations and orchards include water and nitrate pollution, loss of biodiversity and wildlife habitat, sedimentation of waterways, nutrient runoff, eutrophication and algal bloom. Due to environmental awareness, health concerns and the growing demand for organic tea in Turkey, an ecological, sustainable or organic production approach that can replace the traditional approach is becoming increasingly common.

Soil bacteria that are free-living, used as biological control and biological fertilizers in agriculture and benefit to plants are defined as plant growth-promoting rhizobacteria (PGPR) (Çakmakçı et al., 2011). PGPR are important in agriculture in order to promote the cycling and circulation of plant nutrients, stimulate plant growth, inhibit of plant ethylene synthesis, enhance stress resistance and reduce the need for chemical fertilizers as much as possible. PGPR can colonize near growing roots and use root exudates

as a carbon source, affecting plant growth both directly and indirectly through many important bacterial properties, such as nitrogen fixation, production of plant growth hormone and enzymes, solubilisation of inorganic phosphate and mineralization of organic phosphate, 1-aminocydopropane-1-carboxylic acid (ACC) deaminase ability, reduction in ethylene levels, nutrient uptake and production of siderophores (Hayat et al., 2010; Liu et al., 2015; Çakmakçı et al., 2017a). Since wrong and repeated overuse of chemical fertilizers has a detrimental effect on soil quality and health by destabilizing soil fertility and soil microbial community structure, interest in PGPR has increased in recent years and its use as biological fertilizers is increasingly common.

PGPR are considered as an alternative or complement for inorganic fertilizers to certain extent and has been used in low input agriculture to increase plant growth and productivity (Çakmakçı et al., 2006, 2007, 2011; Park et al., 2005; Sahin et al., 2004; Chen et al., 2006). Today, by researchers all over the world, these bacteria are isolated and identified from various sources, especially the plant rhizosphere, and then their potential effects are determined and then used single or in combination with other bacteria in agriculture. The use of microorganisms is needed for the production of reliable healthy foods, prevention of environmental pollution, sustainable agriculture and protection of agricultural resources.

The soils of tea land in Turkey are located at the eastern end of the Black Sea coast and are usually acidic condition. Tea plants are grown as a usually monoculture in Turkey, over and unbalanced fertilizer use increases production costs, reduces productivity and leads to water pollution (Çakmakçı et al., 2010). The use of PGPR is an important approach known to affect growth, yield and nutrient intake by a number of mechanisms. Because the leaves of the tea plant are used, beneficial bacteria can be important in organic and sustainable tea production. For the reason, the effect of new biological formulas with multiple properties, isolated from the rhizosphere, on growth promotion, yield and enzyme activities in tea plants was evaluated.

## Material and Methods

The present study was conducted to isolate and use PGPRs associated with tea (*Camellia sinensis* L.) from 413 soils samples from the rhizosphere of tea production zones of the rainy eastern Black Sea region, Turkey. To characterize the culturable bacteria isolated, the analysis of fatty acid methyl ester (FAME) profiles by the Sherlock Microbial Identification System and the microbial substrate use model by BIOLOG were used for bacterial identification. These bacterial ability to fix $N_2$ was determined with N free semi solid medium, as described by Döbereiner (1988), and phosphate-solubilizing ability was determined in National Botanical Research Institute's phosphate growth medium (NBRIP-BPB). Growth of bacterial isolates in nitrogen-free culture medium demonstrated their non-symbiotic nitrogen fixation ability (Rau et al., 2009; Han et al., 2005). The ACC deaminase abilities of all strains were determined by testing their growth ability to grow in DF (Dworkin and Foster, 1958) minimal salt medium supplemented with 3 mmol ACC as a single N source as defined by Penrose and Glick (2003). The measure of the ACC deaminase activity was determined by quantifying the amount of α-ketobutyrate produced, as described by Honma and Shimomura (1978). ACC deaminase activity of the isolates was measured using a spectrophotometer that measured the absorbance at 540 nm, the α-ketobutyrate produced from the enzymatic cleavage of ACC and was expressed as µmol mg protein$^{-1}$ h$^{-1}$.

In this study, we selected nine natural potential PGPR strains for their $N_2$-fixing, P-solubilizing properties, and/or ability to use ACC as a single nitrogen source. Triple combinations of these strains were prepared and then, field experiments were carried out in Rize Atatürk Tea and Horticulture Research Institute in three years and tested for their effects on tea plant growth and yield increasing potential (Table, 1). In the study conducted in tea orchards, treatments were as follows: a) three bacterial consortia inoculation; b) commercial liquid bio-fertilizer inoculation; c) mineral NPK fertilizer (800 kg ha$^{-1}$ year$^{-1}$ in compound NPK; 25-5-10 fertilizer); and d) control without bacterial inoculation and NPK fertilizer application. The experiment was arranged as a completely randomized block design with six treatments, with each treatment repeated four times and each repeat consisted of five tea saplings.

For this experiment, pure cultures of strains were grown in 0.5-strength Tryptic Soy Broth (TSB) for 3 days at 25 °C on a rotary shaker at 120 rpm. Bacteria were harvested by centrifugation at 3000 x g for 10 min. After centrifugation, bacteria were washed in 10 mM phosphate buffer solution (pH: 7.0) and re-suspended in the same buffer solution at a density of $10^9$ cfu ml$^{-1}$ for the bacterial strains.

Table 1. Biochemical characteristics of the bacterial strains used in bio-formulations

| Biofor-mulation | Bacterial strain | Oxi-dase | Cata-lase | Suc-rose | $N_2$-fixation | P-solubili-zation | ACC deaminase activity |
|---|---|---|---|---|---|---|---|
| BF9 | *Bacillus megaterium* 47/9 | - | + | - | S+ | S+ | S+ |
| | *Paenibacillus macquariensis* RC696 | - | + | - | + | + | ND |
| | *Pseudomonas fluorescens* 9/7 | + | + | + | S+ | + | S+ |
| BF10 | *Bacillus megaterium* RC665 | - | + | W+ | S+ | S+ | + |
| | *Paenibacillus macquariensis* RC382 | - | S+ | - | S+ | W+ | S+ |
| | *Pseudomonas fluorescens* 9/7 | + | + | + | S+ | + | S+ |
| BF11 | *Bacillus simplex* RC64 | + | + | - | + | + | ND |
| | *Pseudomonas putida* 3/10 | + | + | + | S+ | S+ | ND |
| | *Burkholderia pyrrocinia* RC134 | W+ | + | - | S+ | + | 4 |

"S+": strong positive reaction, "+": positive reaction, "W+": weak positive reaction, BF: bioformulations; ND: not determined.

For the three microorganisms based liquid bio-fertilizers, frozen bacterial culture seeded in petri dish Nutrient Agar (NA) containing medium, incubated for 24 hours at 27 °C. Pure colonies were taken from fresh culture and transferred to Nutrient Broth (NB) culture media. Horizontal shaker incubator developed a 24-hour culture, inoculated in NB containing the liquid culture media, previously prepared by fermenters and sterilized by autoclaving at 121 °C for 20 min. Bacteria were developed 24 h optimum pH, oxygen, and temperature values. The microbial consortium consisting of the three strains was prepared by mixing an equal volume of each bacterial strain and then blended with carrier (Çakmakçı et al., 2014). Bacteria inoculated organic liquid carrier, the optimum growth conditions were incubated in the bioreactor. Counts of viable bacteria per millilitre as Colony Forming Unit (CFU) made in bacterial concentration was $1x10^8$ cells/ml at the end of 48 hours, during which time exceeds, packaging made completely sterile conditions, the product has been kept in a room temperature at 24 °C. The bio-fertilizer had $10^8$ bacterial cells $g^{-1}$ carrier at the time of application to soil. Appropriate consortia cultures were injected into the rhizosphere area around the root of six-year-old seedlings at the time of fertilizer application.

For the enzymes analysis, initially tissue samples 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 + 10mM $NaN_3$ + 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. Glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD) activities were determined according to the method of Beutler (1984). Protein contents were determined according to methods described by Bradford (1976), using bovine serum albumin as the standard protein. Glutathione reductase (GR), glutathione S-transferase (GST), peroxidase (POD), polyphenol oxidase (PPO), 5-dehydroshikimate reductase (DHSK) and alcohol dehydrogenase (ADH) enzyme activities were measured according to methods of Carlberg and Mannervik (1985); Habig and Jacoby (1981); Mei et al. (2009); Lee et al. (1991); Sanderson (1966) and Hatanaka et al. (1974), respectively. Enzyme activities were measured with a Shimadzu UV-1208 spectrophotometer (Kyoto, Japan) at 25 °C. Chlorophyll contents of the top fourth and fifth leaves were measured using a SPAD-502 chlorophyll meter (Konica-Minolta, Japan) to measure leaf greenness of the plants. Mean of five readings from each leaf was recorded as SPAD value. Enzyme activities were determined by measuring three times in each replicate. All experimental data were subjected to analysis of variance (ANOVA) using the SPSS 13.0 software package for Windows. Means comparison was performed with the use Duncan's Multiple Range Test.

## Results and Discussion

Three years of field trials showed that treatments with microbial consortia and NPK fertilizer significantly affected the parameters investigated compared to the control in tea. However, the effects and success of the applications have varied depending on the years, microbial consortia and the parameters tested. All applications, especially mineral fertilizers, tested bacterial formulas and commercial liquid microbial fertilizers increased fresh and dry leaf weight, and G6PD and 6PGD activities compared to the control; the maximum yield and growth parameters in tea were found in NPK application and bacterial consortium BF11 (*B. simplex* RC64 + *P. putida* 3/10 + *B. pyrrocinia* RC134) inoculation (Table, 2).

Table 2. The effect of bacterial consortia and NPK fertilizer on the fresh and dry leaf weight, chlorophyll contents, leaf area and enzymes activities in leaves of tea.

| Treatments* | Sum of three distinct harvests period** | | Chlo-rophyll contents (SPAD) | Second leaf area ($cm^2$) | Third leaf area ($cm^2$) | Enzymes activities in tea leaves (Units $mg^{-1}$ protein) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Fresh leaf weight (g/tea bushes) | Dry leaf weight (g/tea bushes) | | | | GR | GST | G6PD | 6PGD |
| **First year (2017)** | | | | | | | | | |
| Control | 775.7 d | 371.9 d | 75.5 d | 12.2 c | 21.3 c | 1.47 d | 1.36 c | 1.07 b | 1.08 d |
| NPK | 980.5 a | 466.3 a | 85.2 a | 15.3 a | 26.9 a | 2.72 a | 2.23 a | 1.56 a | 1.59 bc |
| CLBF | 846.0 c | 412.6 c | 74.5 d | 13.4 bc | 23.6 bc | 2.13 c | 2.54 a | 1.72 a | 1.77 b |
| BF9 | 933.3 b | 447.1 b | 81.2 bc | 14.6 ab | 26.0 ab | 2.45 b | 1.58 bc | 1.53 a | 1.54c |
| BF10 | 902.6 b | 445.5 b | 77.9 c | 14.0 ab | 25.7 ab | 1.46 d | 1.65 bc | 1.58 a | 2.14 a |
| BF11 | 941.2 b | 460.7 ab | 82.3 b | 14.8 ab | 26.5 a | 2.65 a | 1.82 b | 1.57 a | 1.69 bc |
| Average | **896.6** | **434.0** | **79.4** | **14.1** | **25.0** | **2.14** | **1.86** | **1.50** | **1.63** |
| **Second year (2018)** | | | | | | | | | |
| Control | 705.2 d | 324.6 d | 69.2 c | 11.29 c | 19.91 c | 1.59 d | 1.49 c | 1.16 c | 1.10 d |
| NPK | 918.5 a | 412.3 a | 84.6 a | 14.22 a | 25.07 a | 3.01 ab | 2.33 a | 1.74 b | 1.76 bc |
| CLBF | 812.7 c | 369.4 c | 75.2 b | 12.58 bc | 22.18 bc | 2.35 c | 2.34 a | 1.85 ab | 1.95 ab |
| BF9 | 864.6 b | 389.1 b | 82.8 a | 13.77 ab | 24.28 ab | 2.86 b | 2.11 a | 1.80 b | 1.65 c |
| BF10 | 852.7 b | 385.5 b | 81.1 a | 13.21 ab | 23.28 ab | 1.52 d | 1.80 b | 1.75 b | 2.04 a |
| BF11 | 896.3 a | 410.2 a | 84.3 a | 14.00 ab | 24.68 ab | 3.07 a | 2.22 a | 2.02 a | 2.00 a |
| Average | **841.7** | **381.9** | **79.5** | **13.18** | **23.23** | **2.40** | **2.06** | **1.71** | **1.75** |
| **Third Year (2019)** | | | | | | | | | |
| Control | 793.8 d | 375.9 c | 74.7 c | 12.96 b | 22.37 c | 1.69 d | 1.52 c | 1.18 c | 1.26 c |
| NPK | 964.6 a | 442.8 a | 87.1 a | 15.78 a | 28.90 a | 3.12 a | 2.48 a | 1.80 b | 1.84 ab |
| CLBF | 862.3 c | 399.8 b | 78.2 c | 14.07 ab | 24.92 bc | 2.44 c | 2.64 a | 1.95 b | 2.04 a |
| BF9 | 951.9ab | 438.9 a | 85.4 ab | 15.41 a | 27.70 ab | 2.88 b | 2.02 b | 1.80 b | 1.73 b |
| BF10 | 935.5 b | 433.3 a | 83.1 b | 14.78 a | 26.16 ab | 1.72 d | 1.86 b | 1.83 b | 2.06 a |
| BF11 | 954.8ab | 450.4 a | 86.9 a | 15.67 a | 28.41 a | 3.13 a | 2.43 a | 2.15 a | 2.04 a |
| **Average** | **910.4** | **423.5** | **82.6** | **14.78** | **26.41** | **2.50** | **2.16** | **1.78** | **1.83** |

*Control: without bacteria inoculation or mineral fertilizers; NPK fertilizer (800 kg $ha^{-1}$ $year^{-1}$ in compound NPK; 25-5-10 fertilizer); CLBF, commercial liquid bio-fertilizer; GR, glutathione reductase; GST, glutathione S-transferase; G6PD, glucose-6-phosphate dehydrogenase; 6PGD, 6-phosphogluconate dehydrogenase; BF9-BF11, all strains used in these bioformulations were explained in Table 1

**Values followed by different letters in a column (each section separately) were significantly different ($p < 0.05$), using Duncan's multiple range test

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In three years highest fresh and dry leaf weight and chlorophyll content were obtained from NPK fertilizer application followed by BF11 and BF9 inoculations while the lowest fresh and dry leaf weight as recorded for control and commercial bio-fertilizer applied plots (Table, 2). As an average of three years and sum of three distinct harvests period inoculations of tea plants with CLBF, BF9, BF10 and BF11 gave increases over control

respectively of by 10.8, 20.9, 18.3, and 22.7 % in fresh leaf weight, by 10.2, 18.9, 17.8, and 23.2 % in dry leaf weight, by 3.9, 13.7, 10.3, and 15.5 % in chlorophyll contents, by 9.8, 20.1, 15.2, and 22.0 in second leaf area and by 11.2, 22.6, 18.2 and 25.2 third leaf area. NPK applications, however, increased fresh leaf weight up to 25.9 %, dry leaf weight by 23.2, chlorophyll contents by 17.1, 24.9 %, second leaf area by 24.3 and third leaf area by 27.2 %.

Our results clearly indicate the beneficial effect of mixed formulations the $N_2$-fixer and P-solubilizer in inoculants production. Our previous experiments show that triple inoculation with multi-trait PGPR resulted in higher plant height, shoot and leaf weight than uninoculated control (Çakmakçı et al., 2013). Application of the bacterial formulations resulted in significant increase in growth of young tea, measured in terms of growth and yield. Similarly, previous studies have shown that PGPR application causes a significant increase in the growth and yield of young tea bushes (Sharma et al., 2002; Chakraborty et al., 2006; Saravanakumar et al., 2007; Çakmakçı et al., 2014), and help in the reduction of the use of chemicals, N and P fertilizers in tea plantations (Chakraborty et al., 2009; Saikia et al., 2011; Princy et al., 2015). Although beneficial bacteria can reduce excessive use of chemical fertilizers without compromising plant growth and productivity, scientific knowledge on regarding the further development, improvement and use of such biofertilizer technology for a perennial crop such as tea is limited to a few studies (Nepolean et al., 2012; Chakraborty et al., 2012; Tennakoon et al., 2019). However, in processes combining indigenous beneficial PGPR with ¾ of the recommended N and P fertilizers (Saikia et al., 2011), or with 50% of synthetic fertilizers (Princy et al., 2015), a significantly sustainable higher yield was observed in tea compared to the recommended chemical fertilizer alone. Previous studies have indicated that development of stable formulation of PGPR is an important and promising approach for sustainable tea cultivation (Çakmakçı et al., 2014). In this regard, it was emphasized that especially rhizobacteria with natural multiple plant growth-promoting (PGP) traits have a better potential for field-testing and applications in improving tea growth (Çakmakçı, 2016). Similarly, the cultivable tea rhizobacterial isolates were found to have PGP activity such as phosphate solubilization, indol acetic acid and siderophore production, and effectively stimulate the growth of rice and corn seedlings (Bhattacharyya et al., 2020). The technology of the use of mixed inoculants aimed at stimulating plant growth by combining different mechanisms of different microorganisms provides a better nutritional balance for plants and an improvement in nutrient intake. The result of the study suggest that co-inoculations with newly native multi-traits bacteria stimulated parameters such as shoot growth, leaf yield, leaf area, chlorophyll content and enzyme activities. Previous studies demonstrated the co-inoculations with multi-trait bacteria is more effective than single inoculations in promoting plant growth and providing a more balanced nutrition for various crops, and has the potential to use more and provide excellent results (Şahin et al., 2004; Madhaiyan et al., 2010; Valverde et al., 2006; Yu et al., 2012) and tea crops (Çakmakçı et al., 2012; 2013; 2014; 2017b).

Except for first year, all treatments significantly increased chlorophyll content and glutathione S-transferase activities of tea plants. Except for commercial liquid bio-fertilizer (CLBF), all treatments increased second and third leaf area of tea plants in last two years. Also, except for BF10 mixed bioformulations, all treatments tested significantly increased glutathione reductase activity in the leaves of tea plants. The application of NPK fertilisers, effective formulations increased the chlorophyll content in tea-leaves, and enhanced the growth parameters of tea. Chlorophyll, the main component of the colour in green tea, is an important pigment that can affect the net photosynthesis rate and tea quality. Among the treatments tested, while polyphenol oxidase increased the most with NPK application, BF11 was the most effective promoter in terms of the activity of peroxidase, alcohol dehydrogenase and 5-dehydroshikimate reductase. In the first year, BF11 inoculation gave the most appropriate results in terms of POD, ADH and DHSK activities, while NPK application provided the highest chlorophyll content and PPO activity (Table, 2; 3).

Co-inoculation with multi-traits bacteria enhanced tea orchard growth, leaf yield and activities of oxidative, catalytic, hydrolytic and anti-oxidative defence related enzymes of tea plants, such as, GR, GST, POD, G6PD, 6PGD, ADH and DHSK in tea leaves. Similarly, accumulation of POD and PPO enzymes was observed in tea plants treated with *P. fluorescens* (Saravanakumar et al., 2007).

Table 3. The effect of co-inoculation of bacteria and NPK fertilizer applications on the enzymes activities in tea leaves (First year)

| Treat-ments | Polyphenol oxidase (PPO)* | | Peroxidase (POD) | | Alcohol dehydrogenases (ADH) | | 5-dehydroshikimate reductase (DHSK) | |
|---|---|---|---|---|---|---|---|---|
| | Units $g^{-1}$ leaf DW | Units $mg^{-1}$ protein | Units $g^{-1}$ leaf DW | Units $mg^{-1}$ protein | Units $g^{-1}$ leaf DW | Units $mg^{-1}$ protein | Units $g^{-1}$ leaf DW | Units $mg^{-1}$ protein |
| Control | 7.25 c | 0.072 c | 14.86 c | 0.18 bc | 1.49 bc | 0.050 b | 2.41 b | 0.09 c |
| NPK | 9.03 a | 0.097 b | 18.85 c | 0.13 c | 0.68 e | 0.021 c | 2.32 b | 0.08 c |
| CLBF | 8.45 ab | 0.104 b | 16.09 c | 0.13 c | 1.02 d | 0.032 c | 1.86 c | 0.06 d |
| BF9 | 7.34 c | 0.050 d | 31.40 b | 0.21 b | 1.64 ab | 0.058 ab | 3.85 a | 0.14 a |
| BF10 | 5.98 d | 0.042 d | 37.79 a | 0.27 a | 1.46 c | 0.050 b | 3.53 a | 0.12 b |
| BF11 | 8.09 b | 0.179 a | 40.91 a | 0.30 a | 1.77 a | 0.069 a | 3.87 a | 0.14 a |
| **Average** | **7.69** | **0.091** | **26.65** | **0.20** | **1.34** | **0.047** | **2.98** | **0.10** |

*Averages of the same column values (each section separately) followed by same letter did not differ significantly from Duncan's multiple range tests at ($p < 0.05$) significance.

However, the changes in quality and defense-related enzyme activity in tea-leaves varied depending on the formulations applied. G6PD and G6PD enzymes, both of which belong to the pentose phosphate pathway, play a very important role in N assimilation and the plant's resistance and adaptation to environmental stresses, while antioxidant enzymes such as GR, GST and POD, which are important determinants for establishing plant defense, act as regulators that play a role in protecting plants from stress (Liu et al., 2007; Sarkar et al., 2009; Gill and Tuteja, 2010; Nikolaeva et al., 2010; Lin et al., 2013; Çakmakçı et al., 2017b). Both the oxidative key enzymes PPO and POD, which are naturally found in fresh tea leaves, are very important in the processing of black tea and the formation of black tea compounds, oxidation of catechins accumulation of theaflavins, quality, flavor and color of black tea (Stodt et al., 2014; Samanta et al., 2015; Takemoto and Takemoto, 2018). Plants activate an effective antioxidant defense system consisting of antioxidant enzymes such as GR, GST and POD to reduce stress, alleviate the harmful effects of oxidative stress, and develop tolerance to various environmental stress conditions. G6PD and 6PGD enzymes are involved in the biosynthesis of polyphenols (Magoma et al., 2003), DHSK is one of the key regulatory enzymes of the shikimat pathway, plays an important role in the biosynthesis of flavonoid compounds (Sanderson, 1966), while ADH is responsible for the oxidation of aldehydes to alcohols.

PPO and POD play a role in the fermentation process, oxidation and formation of tea compounds (Emdadi et al., 2009; Stodt et al., 2014), thereby forming polyphenols and flavour compounds unique to black tea, and also play a role in the defense mechanisms of plants against environmental stresses (Harbowy and Balentine, 1997). Enzymes play a role in the plant's antioxidant defense system, as well as in the tea production process and in phenolic compounds that form some black tea properties such as color and taste (Çakmakçı et al., 2017c).

The application of NPK fertilisers, BF9 and BF14 formulation increased the chlorophyll content and second and third leaf area, and enhanced the growth and yield parameters of tea. Chlorophyll is a highly important pigment as its amount determines the final colour of non-fermented green tea infusion. Additional studies are required to explain the mechanism by which PGPR affects the tea quality, and different oxidative, catalytic, hydrolytic and antioxidant enzymes responses. Among the various bio-formulations tested, BF11 (*B. simplex* RC64 + *P. putida* 3/10 + *B. pyrrocinia* RC134) and BF9 (*B. megaterium* 47/9 + *P. macquariensis* RC696 + *P. fluorescens* 9/7) were found most effective in promoting growth, yield and quality of tea**.** On the other hand, this study clearly demonstrates the importance of using locally adapted microbial inoculants from the same site, plant and soil series in the field level application of PGPR. In previous research, the poor performance of bacteria due to poor adaptation in a different soil type than those from which they were isolated has been clearly demonstrated in field studies. The importance of preparing biological fertilizers with local microorganisms is emphasized (Das et al., 1997; Akbari et al., 2007; Vikram et al., 2007; Sangeeth et al., 2008), and it has been repeatedly proven that a pool of endemic bacteria of a region may contain highly efficient genotypes and is likely to perform better than the exotic strains (Çakmakçı et al., 2010; Devi et al., 2011; Dutta et al., 2015; Dutta and Thakur, 2017). Indigenous rhizosphere associated soil microbial inhabitants with wide array of plant growth promoting activity could be beneficial for tea cultivation

## Conclusions

Inoculation with beneficial plant-related multi-trait rizobacteria-based bio-formulations that exhibit plant growth promoting properties can play an important role in promoting and/or enhancing plant growth and enzyme activities. Multi-strain bacterial formulations can change on the tea plantation of soil fertility, physical and chemical environment, enzyme activity, stress tolerance, microorganism's community. And they can also affect the tea yield and quality, but inoculant's efficacy was strongly dependent on the inoculant strain formulations and plant parameters evaluated. Microbial consortium inoculations associated with native microbiota with more than one plant beneficial function are used to increase tea growth, yield and quality, while in addition can also protect plants from biotic and abiotic stress.

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