Yilmaz H (2024)
Notulae Botanicae Horti Agrobotanici Cluj-Napoca
Volume 52, Issue 4, Article number 14067
DOI:10.15835/nbha52414067
Research Article

Biofortification of black chickpea (*Cicer arietinum* L.) through plant growth-promoting rhizobacteria: enhancing nutritional and bioactive compounds

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Abstract

The chickpea (*Cicer arietinum* L.), often called the “poor man’s meat”, is a legume with remarkable nutritional value. Recently, its recognition as a functional food has grown, owing to its ability to improve human nutrition and reduce disease risk. This study explores the biofortification potential of black chickpea seeds through the targeted application of *Bacillus subtilis, B. megaterium*, and *Rhizobium cicer*. By inoculating the seeds, these rhizobacterial treatments aim to naturally enhance the bioactive compound profile by manipulating the plant root systems. The study comprehensively assesses key parameters including total antioxidant activity, protein content, total phenolic content, and macro- and micronutrient composition of bacterial inoculated black chickpeas grown under the ecological conditions of Bolu, Türkiye. Results showed that rhizobacterial inoculation significantly improved all measured traits compared to the control group. *B. subtilis* treatment increased total antioxidant activity by 10.6% and total phenolic content by 19.5%. Protein content exhibited by approximately 11% across all treatments. *R. cicer* treatment led to the most pronounced increases in macro- and micronutrients, particularly in calcium (38.5%), potassium (82.7%), magnesium (26.35%), phosphorus (15.23%), iron (155.3%), and zinc (44.21%). These results demonstrate that rhizobacterial treatments can significantly enhance black chickpeas’ biochemical and nutritional quality. Thus, biofortified black chickpeas offer a promising, sustainable strategy for addressing global micronutrient deficiencies and combating hidden hunger, providing a valuable tool for improving food security.

Keywords: biostimulants; food composition; legume; micronutrient malnutrition; phenolics; total antioxidant capacity

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Introduction

Chickpea (*Cicer arietinum* L.), a member of the Fabaceae family, is the third most cultivated legume globally. In 2022, global chickpea production reached 18.09 million tons, primarily concentrated in Asian countries (FAO, 2024). Chickpeas are exceptionally nutrient-dense, containing 18-23% protein, 40-50% carbohydrates, various essential vitamins and minerals, 17.4% dietary fiber, and 6-7% fatty acids (Tosh and Yada 2010; Sofi *et al*., 2020; Kumari, 2023). Beyond their basic nutritional profile, chickpeas contain bioactive

Received: 11 Aug 2024. Received in revised form: 22 Oct 2024. Accepted: 14 Nov 2024. Published online: 25 Nov 2024.
From Volume 49, Issue 1, 2021, Notulae Botanicae Horti Agrobotanici Cluj-Napoca journal uses article numbers in place of the traditional method of continuous pagination through the volume. The journal will continue to appear quarterly, as before, with four annual numbers.

compounds with antioxidants, antifungal, antibacterial, anticancer, and anti-inflammatory properties, significantly enhancing their health benefits (Ferreira *et al*., 2019), essential for the nutrition of millions in impoverished regions (Grewal *et al*., 2020). Chickpeas are separated into two groups as "Kabuli" and "Desi" in terms of grain size and plant traits (van der Maesen, 1972). The Desi type is a small seed size (0.1-0.3 g), dark, and has a ridged surface, while the Kabuli type is a larger seed size (>0.3 g) than the Desi type and has a thin bright cover surface. Notably, black chickpeas surpass cream-beige varieties in their content of antioxidants, flavonoids, and polyphenols, establishing them as functional foods with enhanced health benefits (Segev *et al*., 2010; Kaur and Prasad, 2021).

The Green Revolution was an agricultural movement in the mid-20th century that increased food production through high-yielding crop varieties, chemical fertilizers, and modern farming techniques, especially benefiting developing nations (Paul *et al*., 2024), emphasis on high-yield grain crops, particularly wheat and rice, has inadvertently reduced the cultivation of legumes such as chickpeas. This shift has resulted in diets heavily dependent on carbohydrates, particularly in low-income countries, contributing to widespread micronutrient malnutrition, commonly known as hidden hunger (Çakmak *et al*., 2010; Burchi *et al*., 2011). Hidden hunger is characterized by deficiencies in essential vitamins and minerals, such as iron and zinc, which are vital for overall health and development (Khush *et al*., 2012). To address this issue, biofortification, or biological enrichment of crops, has emerged as a promising strategy. This approach aims to enhance the nutritional quality and bioavailability of agricultural products through modern biotechnology, traditional plant breeding, and optimized agricultural practices (Parihar *et al*., 2021). However, the extensive use of chemical fertilizers to enhance crop yields has led to significant environmental degradation and soil health deterioration (Singh *et al*., 2020). Therefore, it is imperative to develop sustainable fertilization strategies that are both effective and environmentally friendly.

Plant growth-promoting rhizobacteria (PGPR) present an innovative and eco-friendly solution for biofortification. These beneficial soil bacteria enhance plant growth by colonizing roots and increasing nutrient availability in the rhizosphere (Prasad *et al*., 2015). PGPRs play a crucial role in nutrient uptake, such as zinc, phosphate, potassium, and calcium, while also promoting the production of phytohormones that stimulate plant growth (Cakmakcı *et al*., 2007; Khan *et al*., 2019). In addition to nutrient enhancement, several studies have shown that PGPR applications can significantly boost the accumulation of bioactive compounds like antioxidants and phenolic compounds. For instance, *Bacillus subtilis* has been demonstrated to increase total phenolic content and antioxidant activity in tomatoes (Chandrasekaran *et al*., 2019), while co-inoculation of *Bacillus megaterium* and *B. subtilis* in wheat resulted in significant increases in antioxidant enzyme activities, microbial biomass, and nutrient content, ultimately enhancing grain yield and quality (Iqbal *et al*., 2022). Furthermore, *Rhizobium* spp. treatments in crops like chickpeas, common beans, and lentils have been shown to enhance mineral uptake, particularly iron and zinc, thereby improving both plant health and nutritional quality (Yadav and Verma, 2014; Tena *et al*., 2016; Massa *et al*., 2020). By interacting with plant roots, these rhizobacteria improve the rhizosphere environment, influencing plant growth directly and indirectly (Yilmaz and Karik, 2022). As a result, the application of PGPR not only contributes to improved plant growth but also offers a natural means of increasing the levels of bioactive molecules in plants, such as antioxidants and phenolic compounds, along with essential minerals (de la Osa *et al*., 2021). This makes PGPR an invaluable tool in sustainable biofortification strategies for various crops.

Given their rich micronutrient content, chickpeas offer a viable solution to alleviate hidden hunger by addressing iron and zinc deficiencies while simultaneously enhancing soil fertility through symbiotic nitrogen fixation (Grewal *et al*., 2020; Basu and Parida, 2023). Recent studies have demonstrated that these biostimulants enhance the chemical composition of various plants, including *Phaseolus vulgaris* (Radegani and Rahmani, 2010), *Cicer arietinum* (Sing *et al*., 2013; Khan *et al*., 2018), *Oryza sativa* (Mishra *et al*., 2006), *Glycine max* (Couto *et al*., 2011), and *Pisum sativum* (Ejaz *et al*., 2020). In this context, the present study investigates the effects of different PGPRs (*Bacillus subtilis, Bacillus megaterium, and Rhizobium cicer*) on the total antioxidant activity, total phenolic content, protein levels, and macro- and micronutrient (especially Fe and Zn) profiles of black chickpeas. This research addresses a significant gap in the existing literature by focusing on the relatively understudied black chickpea variety and seeks to advance our understanding of sustainable agricultural practices.

Materials and Methods

Plant material and field experiment

The Turkish chickpea variety "KATRAN" was used as the plant material, obtained from Olgunlar Seed Company (*https://olgunlar.com.tr/tohum/katran/*). Field experiments were conducted in the Bolu province (40°41'15.5"N - 31°35'28.9"E) with three replications and four treatments (*B. subtilis, B. megaterium, Rhizobium cicer* and control (non-inoculated). The research was conducted using a randomized block design with three biological replications. Each experimental plot measured 3.6 m^2 (2 m × 1.8 m), with chickpeas sown at a row spacing of 30 cm and a planting density of 60 seeds per m^2. The spacing between plots and blocks was set at 1 m and 2 m, respectively. The seeds were sowed in April 2022 and harvested in September. Data were collected from 20 plants randomly selected from each plot.

The climate data for the 2022 growing season and the soil characteristics of the experimental site are presented in Tables 1 and 2, respectively. Throughout the season, temperatures remained consistent with long-term averages, while rainfall and relative humidity levels were noticeably lower.

Table 1. The climate data of the Bolu in 2022

Parameter	April	May	June	July	August	September
RH in 2022 (%)	65.0	66.0	79.0	70.0	75.0	69.0
Long-term RH (%)	70.0	71.7	72.1	69.5	69.2	71.1
MT in 2022 (°C)	10.0	13.0	17.0	19.0	21.0	16.0
Long-term MT (°C)	10.3	13.2	17.1	18.5	20.5	16
TR in 2022 (kg/m^2)	29.0	39.0	163.0	20.0	54.0	10.0
Long-term TR (kg/m^2)	51.1	62.5	63.2	28.9	27.5	27.5

Notes: RH: Relative humidity, MT: Mean temperature, TR: Total rainfall

Soil quality analyses of the experimental area were conducted at the Central Research Institute of Soil, Fertilizer, and Water Resources, under the Ministry of Agriculture and Forestry in Ankara, Türkiye. The results are presented in Table 2.

Table 2. Physical and chemical properties of soil

Physical Properties		Exchangeable cations (mg kg^{-1})	
Texture	Clay Loam	K	1037
Organic carbon (%)	2.71	Ca	6305
pH (1/2.5)	7.24	Mg	761.5
Salt (dS/m)	0.75	Cu	3.59
Lime (%)	1.60	Fe	5.00
P_2O_5 (kg da^{-1})	44.04	Zn	2.43
N (%)	0.14	Mn	6.20

Bacterial application

The bacterial strains *Bacillus megaterium* and *B. subtilis* were obtained from the culture collection unit of the Department of Genetics and Bioengineering, Faculty of Engineering, Yeditepe University, Turkey. *R. cicer* was obtained from the Central Research Institute of Soil, Fertilizer, and Water Resources, affiliated with the Ministry of Agriculture and Forestry. The bacterial cultures, grown on nutrient agar, were stored at +4 °C prior to inoculation. The bacterial application protocol followed the methods of Yılmaz and Kulaz (2019) with minor modifications (including the use of CMC to coat the seeds). Prior to planting, 100 seeds were inoculated with 10 mL of bacterial solution at a concentration of 10^8 CFU/mL.

Determination of total phenolic and DPPH scavenging activity

The total phenolic content (TPC) was determined using a modified version of the Waterhouse (2002) method. 1.6 mL of distilled water, 50 µL of methanolic extract, and 50 µL of Folin-Ciocâlteu reagent were mixed and stirred. Following this, 300 µL of a 7% (w/v) calcium carbonate solution was added and vortexed. The mixture was then allowed to stand for 2 hours at room temperature in the dark. Absorbance was measured at 760 nm using a UV-Vis spectrophotometer (SP-UV1100, DLAB, Beijing, China). The absorbance values were subsequently converted to phenolic content using a standard curve ($R^2 = 0.99$) derived from gallic acid concentrations ranging from 0.5 to 6 mM.

For the measurement of DPPH scavenging activity, 2,2-Diphenyl-1-picrylhydrazyl (Sigma-Aldrich, Darmstadt, Germany) was utilized, targeting a final absorbance range of 0.7–0.8. A volume of 2 mL of methanol extract was determined, followed by the addition 50 µL of the sample, 1.45 mL of ethanol, and 0.5 mL of DPPH solution were sequentially added and vortexed. The mixture was then left to react for 15 minutes at room temperature. The absorbance was measured at 520 nm using a UV-Vis spectrophotometer. The scavenging capacity of DPPH was calculated from the absorbance values (A) obtained; DPPH (%) = $(A_{blank} - A_{sample})/A_{blank}.100$

Determination of total protein and mineral composition

The crude protein content of the seeds was determined using the Kjeldahl method. The resultant values were multiplied by 6.25 (N × 6.25) and expressed as a percentage, following the AACC (1999) method. For mineral analysis, chickpea seeds were ground into a fine powder. One gram of the ground samples was weighed and soaked for 12 hours in 3 mL of nitric acid. After this time, the samples were pre-digested at 200 °C and then subsequently cooled. After cooling, 1 mL of perchloric acid was added, and the wet digestion process continued until the solution became clear. Once digestion, was complete the solutions were filtered using filter paper and brought to a final volume of 25 mL. Phosphorus (P) analysis in the solution was conducted using an ICP-OES (Thermo Scientific, USA) device. Potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), and zinc (Zn) analyses were performed using atomic absorption spectrophotometry (Thermo Scientific, ICE 3000).

Statistical analysis

The research was carried out in a randomized block design. Results could include ANOVA that can provide the effect of blocking. Treatment means were compared using the Least Significant Difference (LSD) test at a significance level of $p \leq 0.001$. Correlation analysis was performed by eliminating endogenous correlations among the studied traits, ensuring that correlations were calculated exclusively between characteristics. Pearson's coefficient was employed for the correlation analyses, and data visualization was carried out using the 'corrplot' package in R (Wei et al., 2017). The relationships among the studied traits were analyzed using principal component analysis (PCA) with the 'ggplot2' package in R Studio (Wickham, 2016).

Results

Effects of rhizobacterial applications on antioxidant activity, total phenol, protein, and nutrient accumulation in black chickpeas

The ANOVA results for rhizobacterial applications on black chickpeas showed significant differences among treatments for several variables. Total phenolic content (TPC), calcium (Ca), magnesium (Mg), phosphorus (P), potassium (K), and zinc (Zn) accumulation were all significantly affected ($p \leq 0.001$), with phosphorus showing the highest F-value (F = 358.13), followed by zinc (F = 262.75). The %CV values ranged from 4.93% for protein content to 24.65% for potassium (K), indicating the least variation in protein content and the highest variation in potassium accumulation across treatments (Table 3).

Table 3. ANOVA table showing the minimum, maximum, mean, standard error, F-value, and %CV for rhizobacterial applications in black chickpeas

Variables	Min.	Max.	Mean	Std. Error	F value $_{App}$	F value $_{Block}$	%CV
DPPH	66.28	73.29	70.21	0.856	12.06**	4.16*	5.33
TPC	0.82	0.98	0.88	0.006	124.41***	26.52**	7.75
Protein	25.21	28.31	27.25	0.181	60.33***	0.81^{ns}	4.93
Ca	195.66	271.11	240.38	3.013	149.91***	2.41^{ns}	13.99
Mg	109.33	138.11	121.69	1.345	79.94***	7.49**	9.46
P	513.55	591.77	557.11	1.773	358.13***	14.45**	5.43
K	337.66	616.77	484.66	20.94	30.05***	3.22^{ns}	24.65
Fe	4.63	11.81	7.16	0.352	85.65***	2.67^{ns}	22.59
Zn	1.89	2.74	2.42	0.022	262.75***	0.39^{ns}	13.48

Significant at * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

Total antioxidant capacity, total phenolic and protein content

Significant differences between treatments were observed at $p \leq 0.05$ for total antioxidant capacity. The control group's DPPH value was 66.3%. The highest DPPH level was noted in the *B. subtilis* inoculation (73.3%), followed by the *B. megaterium* inoculation (71.4%). This indicates a 10.6% increase in DPPH levels relative to the control group (Figure 1). For TPC, significant differences were observed between treatments at $p \leq 0.05$, and concentrations ranged from 0.82 to 0.98 mg mL^{-1} GAE. The TPC in the control application was 0.82 mg mL^{-1} GAE, while the highest value of 0.98 mg mL^{-1} GAE was observed in the *B. subtilis* inoculation. Bacterial inoculation increased protein accumulation in the seeds. The highest protein contents were observed in the *R. cicer* (28.31%) and *B. subtilis* (28.07%) treatments, while the lowest was in the control group (25.21%). Protein content increased by 11% in *R. cicer* inoculation compared with the control treatment. However, no statistically significant difference was observed between the *B. subtilis* and *R. cicer* treatments (Figure 1; Table 3).

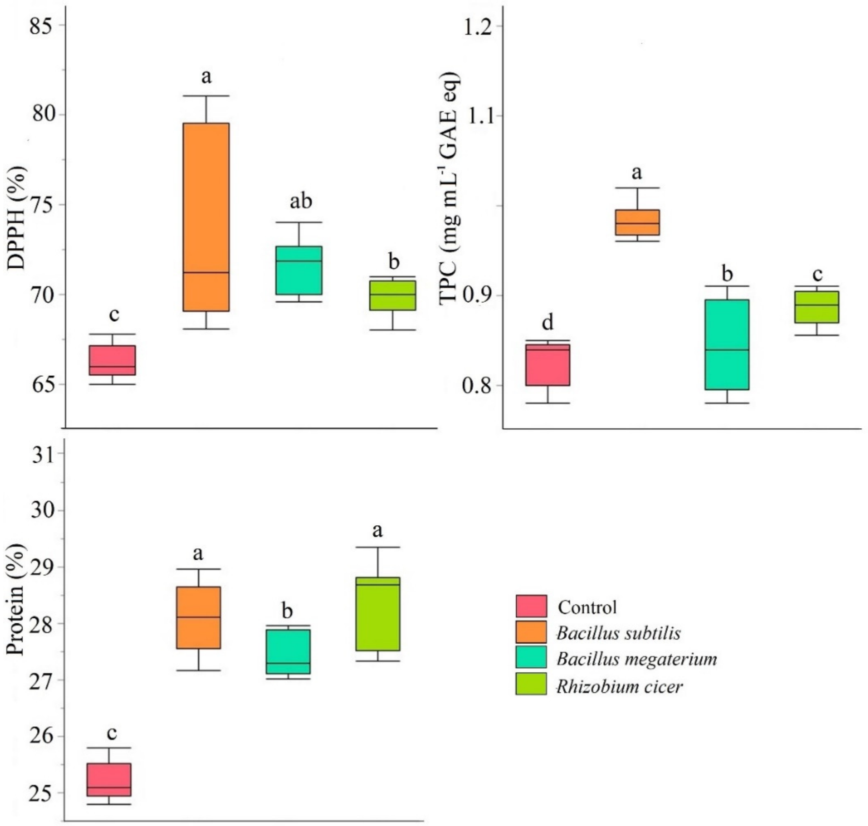

Figure 1. Effect of different PGPR (*B. subtilis* (BS), *B. megaterium* (BM), and *R. cicer* (RC)) on total antioxidant capacity by DPPH, TPC (total phenol content), and protein content of black chickpea.
Different letters indicate significant differences according to LSD test

Mineral content

Calcium (Ca) accumulation in seeds ranged from 195.7 to 271.1 mg 100 g^{-1}, with significant differences observed between treatments ($p \leq 0.001$). The highest Ca accumulation in black chickpeas was recorded in the *B. subtilis* (270.1 mg 100 g^{-1}) and *R. cicer* (271.1 mg 100 g^{-1}) treatments (Figure 2, Table 3). Notably, *R. cicer* increased calcium accumulation in seeds by 38.5%.

Magnesium (Mg) accumulation in seeds varied between 109.3 and 138.1 mg 100 g^{-1}, with significant differences between treatments ($p \leq 0.001$). The highest magnesium content, 138.1 mg 100 g^{-1}, was observed in the *R. cicer* inoculation, representing a 26.35% increase compared to the control group.

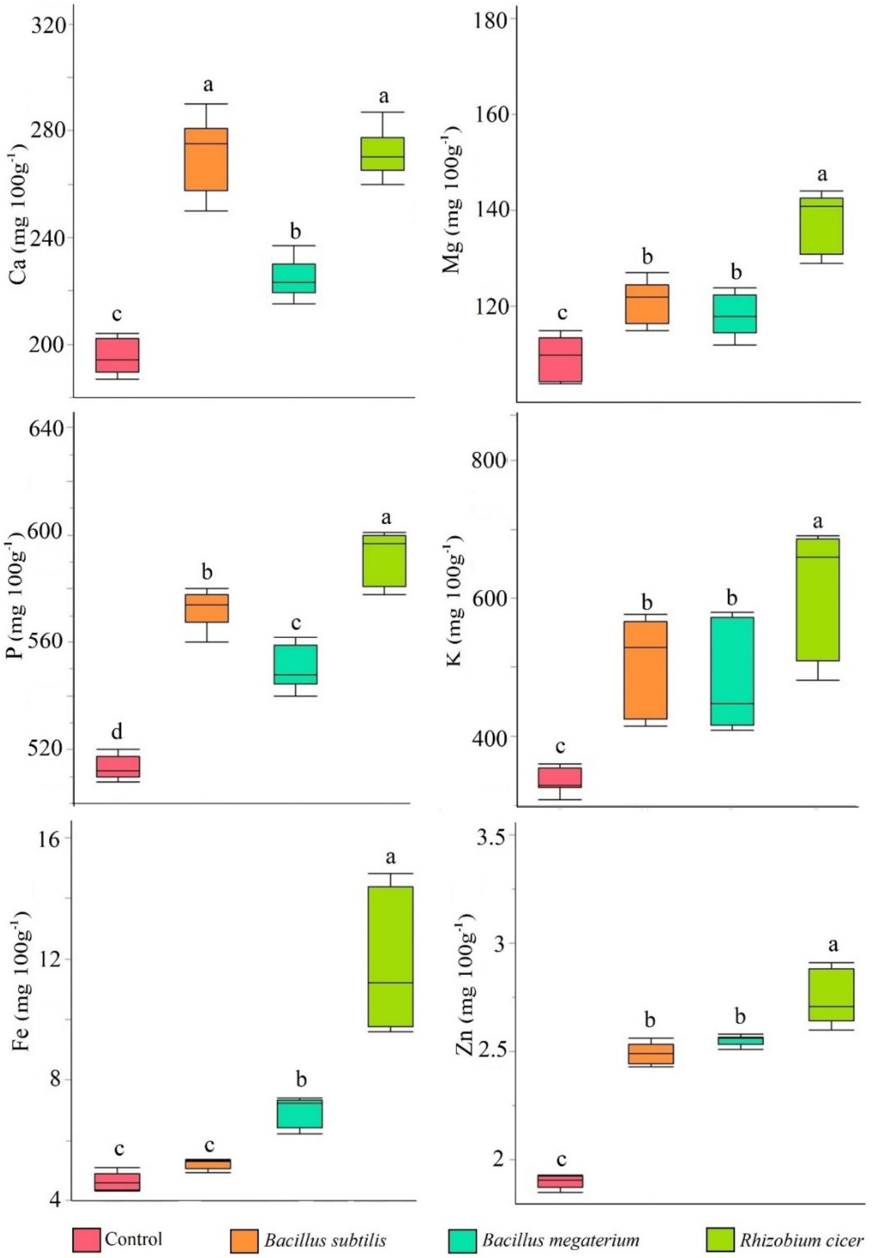

Figure 2. Effect of bacterial inoculation (*B. subtilis* (BS), *B. megaterium* (BM), and *R. cicer* (RC)) on the mineral contents of black chickpea

According to the LSD test, different letters represent significant differences: ($p \leq 0.001$).

Potassium (K) was the most abundant element in black chickpeas, with its content exceeding the control group in all treatments. K accumulation in seeds ranged between 337.7 and 616.8 mg 100 g^{-1}, with significant differences between treatments ($p \leq 0.001$). The control group contained 337.7 mg 100 g^{-1} of potassium, while the highest concentration, 616.8 mg 100 g^{-1}, was achieved with *R. cicer*, marking an 82.7% increase compared to the control.

After potassium, phosphorus (P) was identified as the second most abundant element in black chickpeas. P accumulation in seeds varied from 513.5 to 591.8 mg 100 g^{-1}, with significant differences between treatments

($p \leq 0.001$). The highest phosphorus content, 591.8 mg 100 g^{-1}, was found in the *R. cicer* inoculation, which led to a 15.2% increase compared to the control.

Iron (Fe) accumulation in seeds varied between 4.63 and 11.82 mg 100 g^{-1}, showing significant differences between treatments ($p \leq 0.001$). The highest Fe content was observed in the *R. cicer* treatment (11.82 mg 100 g^{-1}). The control group contained 4.63 mg 100 g^{-1}, and *R. cicer* increased iron levels by 155.3%, representing the highest relative increase among the mineral nutrients studied.

Zinc (Zn) accumulation in seeds ranged from 1.89 to 2.74 mg 100 g^{-1}, with significant differences between treatments ($p \leq 0.001$). The control group showed a zinc content of 1.89 mg 100 g^{-1}, while *R. cicer* raised the zinc level by 44.21%. The highest Zn content, 2.74 mg 100 g^{-1}, was recorded in the *R. cicer* inoculation (Figure 2, Table 3).

Correlations among studied characteristics

The correlation matrix highlighted the relationships between antioxidant activity, total phenolic content, protein, calcium, potassium, magnesium, phosphorus, iron, and zinc (Figure 3). Notably, a strong positive and statistically significant correlation was observed between Ca and P (r=0.94), indicating a robust relationship between the increase in P content and Ca levels. Similarly, the high positive correlations between calcium and magnesium (r=0.78) and zinc and iron (r=0.73) suggest that these minerals are closely interrelated and potentially affect each other's bioavailability and uptake in plants.

The strong positive correlation between magnesium and iron (r=0.84) suggests a linked uptake, likely due to their roles in similar metabolic processes, such as chlorophyll synthesis. Similarly, the positive correlation between magnesium and zinc (r=0.82) indicates that these elements may be taken up together, reflecting their joint involvement in key enzymatic functions. The correlation analysis between protein, phosphorus, and zinc revealed significant positive relationships. A very high positive correlation was observed between protein and P (r=0.89), indicating that as protein content increases, phosphorus levels also tend to increase. Similarly, a strong positive correlation was identified between protein and zinc (r=0.89), suggesting a concurrent rise in zinc content with increasing protein levels. Furthermore, the correlation between phosphorus and zinc was also positive and substantial (r=0.91), highlighting a significant association where higher phosphorus levels correspond to higher zinc content. The positive correlation observed between zinc and phosphorus may be attributed to the phosphate solubilizing abilities of *B. subtilis, B. megaterium*, and the nutrient-enhancing effects of *R. cicer*, which likely improved the bioavailability of both elements in the soil. In the study, a weak negative correlation was observed between total phenol and iron (r=-0.05). This suggests that as total phenol content increases, iron levels might slightly decrease; however, this relationship is weak and was not statistically significant.

Principal Component Analysis interrelations among the studied characteristics

Principal component analysis (PCA) shows that PC1 and PC2 accounted for 82.8% of the total variation, with PC1 explaining 66.6% and PC2 accounting for 16.2% of the variance (Figure 4). *B. subtilis* inoculation was strongly associated with increased total phenolic content and antioxidant capacity, indicating its superior role in enhancing these traits. *R. cicer* was closely linked to improvements in several macro- and micronutrients, including iron, magnesium, potassium, zinc, phosphorus, and calcium. In contrast, *B. megaterium* did not show a strong association with any specific variables. The PCA further confirmed that *B. subtilis* and *R. cicer* treatments were most effective in improving the bioactive and nutrient profiles of black chickpeas compared to the control group, which showed no significant relationships with any of the analyzed variables.

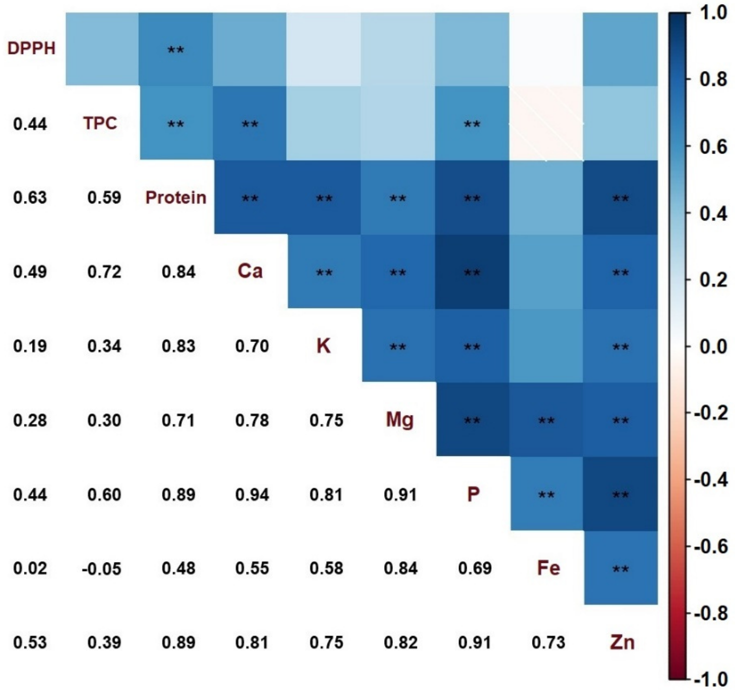

Figure 3. Correlations between the studied characteristics in black chickpea
* and ** indicates significance at $p \leq 0.05$ and $p \leq 0.01$ respectively. TPC: total phenol content, DPPH: total antioxidant capacity, Ca: calcium, K: potassium, Mg: magnesium, P: phosphorus, Fe: iron, Zn: zinc.

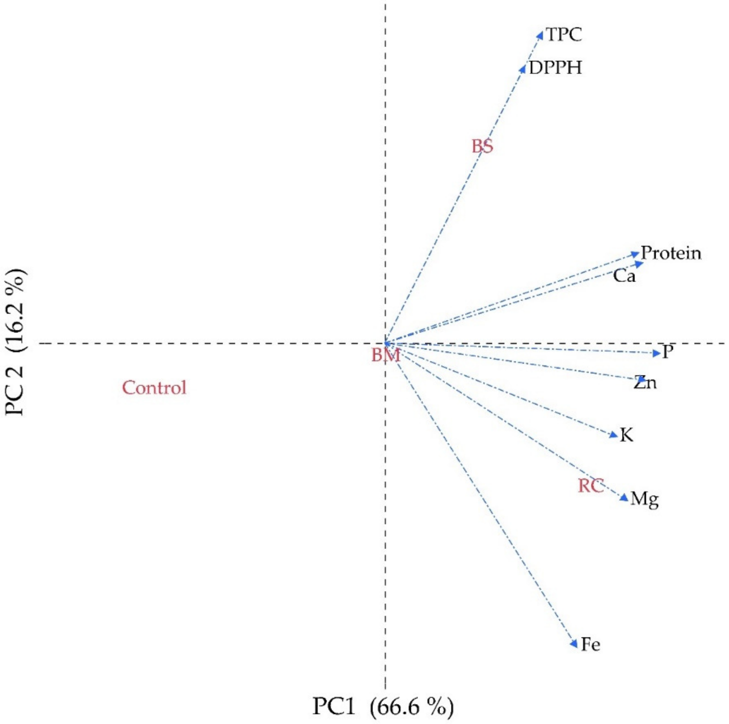

Figure 4. The biplot PCA analysis illustrates the distribution of PGPR treatment
RC: *R. cicer*, BS: *B. subtilis*, BM: *B. megaterium*, TPC: total phenol content, DPPH: total antioxidant capacity, Ca: calcium, K: potassium, Mg: magnesium, P: phosphorus, Fe: iron, Zn: zinc.

Discussion

Plants colonized by beneficial bioinoculants, both internally and externally, exhibit enhanced development and productivity (Jha and Saraf, 2015). Plant growth-promoting rhizobacteria and *Rhizobium*, key bacterial biostimulants, significantly improve nutrient and mineral uptake in plants (Hamid *et al*., 2021). Consistent with these findings, our study revealed that inoculation with PGPR and *Rhizobium* significantly enhanced the total antioxidant capacity and total phenolic content of black chickpea varieties compared to non-inoculated plants.

The interaction between plants and beneficial microorganisms, particularly PGPR, plays a pivotal role in modulating the phenylpropanoid pathway, which is crucial for plant defense and growth (Guo *et al*., 2021). PGPR, including *B. subtilis*, can induce systemic acquired resistance (Ramos *et al*., 2022) and regulate the expression of phenylpropanoid biosynthetic genes, leading to the accumulation of flavonoids and other phenylpropanoid metabolites that contribute to enhanced plant defense mechanisms (Stringlis *et al*., 2018; Jeon *et al*., 2022). Moreover, PGPR are associated with the increased production of reactive oxygen species (ROS)-scavenging enzymes such as superoxide dismutase and catalase, thereby improving the plant's antioxidant capacity (Desoky *et al*., 2020).

In this study, *B. subtilis* likely upregulated these pathways, resulting in elevated phenolic compound production and antioxidant activity. *Rhizobium* spp. further contributed by enhancing phenolic biosynthesis and stress tolerance through nitrogen fixation and the secretion of phytohormones like indole-3-acetic acid (Velázquez *et al*., 2019; Menéndez *et al*., 2020). These mechanisms underline the role of rhizobacteria in promoting both plant health and bioactive compound profiles, making them crucial in improving plant resilience and nutritional value (Mitra *et al*., 2024).

Specifically, *Bacillus* species have been shown to induce the production of antioxidant enzymes and phenolic compounds, bolstering plant resistance to environmental stress (Radhakrishnan *et al*., 2017; Padró *et al*., 2021). In this study, the highest DPPH and total phenolic content levels were observed in plants inoculated with *B. subtilis*, corroborating previous studies that highlight the positive impact of bacterial inoculation on enhancing antioxidant capacity and phenolic content. Similar enhancements were observed in barley plants inoculated with rhizobacteria, where increased antioxidant and phenolic compounds contributed to improved ROS scavenging (Slimani *et al*., 2023). Additionally, *Bacillus lentimorbus* inoculation in several vegetable species—*Lactuca sativa, Trigonella foenum-graecum, Daucus carota*, and *Spinacia oleracea*—resulted in increased DPPH and TPC levels, indicating the efficacy of PGPR in improving radical scavenging capacity (Nautiyal *et al*., 2008). *Rhizobium* inoculation has similarly been shown to enhance phenolic compound production and increase resistance to *Rhizoctonia solani* infection in rice (Mishra *et al*., 2006). Moreover, *Bradyrhizobium japonicum* inoculation in soybeans activated DPPH activity and enhanced phenolic content, further supporting the beneficial effects of these inoculants on plant antioxidant systems (Couto *et al*., 2011). These findings underscore the pivotal role of rhizobacteria in enhancing the antioxidant defenses of various plant species, thereby improving their resilience to environmental stressors.

In addition to their role in enhancing antioxidant activity, PGPR and *Rhizobium* also play a crucial role in nitrogen metabolism, leading to increased protein content in plants. Studies have demonstrated that bacterial inoculation significantly increases protein content in crops like *Phaseolus vulgaris* L., *Vigna radiata* L., *Lens culinaris, Pisum sativum* L., *Glycine max,* and *Cicer arietinum* L. (Yadegari *et al*., 2010; Aamir *et al*., 2013; Singh *et al*., 2018; Yousaf *et al*., 2019; Khaitov *et al*., 2020; Ibrahim and Sawah, 2022). The increased protein content observed in this study is linked to enhanced nitrogen uptake and assimilation, driven by key enzymes such as nitrate reductase and glutamine synthetase (Shi *et al*., 2014). *R. cicer*, as a symbiotic nitrogen-fixing bacterium, forms root nodules where atmospheric nitrogen is converted into ammonium, a readily usable form of nitrogen for plants (Signorelli *et al*., 2020; Shahid *et al*., 2021). This ammonium is assimilated via the glutamine synthetase-glutamate synthase (GS-GOGAT) pathway, enabling its incorporation into amino acids and proteins (Nouwen *et al.*, 2021). Moreover, *B. subtilis* and *B. megaterium*, though non-symbiotic, indirectly support nitrogen fixation by secreting phytohormones like indole-3-acetic acid (IAA) and gibberellins, which enhance root growth and nutrient absorption (Yousuf *et al.*, 2017; Sansinenea *et al.*, 2019; Nafisah *et al.*, 2022). These bacteria also produce extracellular enzymes and organic acids that solubilize soil-bound nutrients, further boosting nitrogen uptake and protein synthesis (Radhakrishnan *et al.*, 2017). *Bacillus* and *Rhizobium* species not only enhance iron uptake through siderophore production but also utilize their nifH genes to optimize nitrogen fixation, a process that relies heavily on iron availability (Liu *et al.*, 2023). Siderophores—low-molecular-weight compounds with a high affinity for iron—bind to iron in the soil, and specific bacterial proteins and transporters recognize these iron-siderophore complexes, enabling the uptake of iron into bacterial cells (Grandchamp *et al.*, 2017). Once inside the cells, bacteria release iron from the siderophore complexes, ensuring its availability for critical processes such as nitrogen fixation and photosynthesis (Khan *et al.*, 2016). This siderophore-mediated iron uptake plays a crucial role in improving iron bioavailability, leading to the 155.3% increase in iron content observed in *R. cicer*-inoculated plants.

Additionally, *Bacillus* and *Rhizobium* species contribute to the uptake of essential macronutrients such as potassium, calcium, and magnesium. *Bacillus* species secrete organic acids and phytohormones that improve root growth, increasing the surface area for nutrient absorption. Potassium uptake is enhanced by the activation of specific potassium transporter proteins in the root cells, which helps maintain osmotic balance and supports enzyme activation in the plant (Yousuf *et al.*, 2017). Calcium uptake is facilitated by the acidification of the rhizosphere, which releases calcium bound to soil particles, and *Rhizobium* species also promote calcium availability through their role in nodulation (Bonilla and Bolanos, 2010). Magnesium, essential for chlorophyll synthesis, is absorbed more efficiently through the expansion of root surface area induced by the secretion of phytohormones and organic acids by these bacteria (Vessey, 2003; Shen *et al.*, 2011). This enhanced uptake of K, Ca, and Mg not only supports critical biochemical processes but also improves overall plant health and productivity. These phytohormones mainly support the development of plant root tissue, increase root elongation, and promote the development of lateral roots and root hairs (Bottini *et al.*, 2004; Riefler *et al.*, 2006; Zaidi *et al.*, 2009). These findings not only reveal the key mechanisms involved in enhancing plant growth and nutrient accumulation but also support the fundamental relationships observed in our correlation analysis between mineral contents and biochemical parameters.

The correlation results from our study highlight significant relationships between mineral contents and biochemical parameters, aligning with existing literature on plant nutrition and crop quality enhancement. Wang *et al.* (2013) emphasized potassium's crucial role in plant stress response, and our findings support this with strong correlations between potassium, protein, and phenolic content, indicating a synergistic effect on plant health. Potassium's importance likely stems from its role in maintaining osmotic balance and activating stress-related enzymes. Marschner (2011) described how phosphorus and zinc contribute to protein synthesis, a relationship confirmed by our data, showing positive correlations with protein content. However, excessive phosphorus can reduce zinc uptake, a well-documented antagonistic interaction in nutrient management. White and Broadley (2003) highlighted calcium's role in plant development, which our results confirm through positive correlations between calcium and other nutrients like potassium and phosphorus. However, high calcium levels can interfere with magnesium uptake, negatively affecting processes like photosynthesis (Cakmak, 2008). Our results also emphasize zinc's role in biofortification, supported by strong correlations with iron and phosphorus. However, managing phosphorus-zinc balance is crucial to avoid antagonistic effects that could limit zinc absorption. In summary, while synergistic interactions between potassium, calcium, and phosphorus enhance plant growth, antagonistic relationships, such as those between phosphorus-zinc and calcium-magnesium, highlight the need for balanced nutrient management. Effective nutrient optimization through tailored fertilization is key to improving plant health and crop quality. The PCA analysis further confirms these interrelations, with protein, Ca, P, and Zn co-moving in plant metabolism. Phenolic content and antioxidant capacity also align, reinforcing the role of phenolics in stress response. Studies demonstrate the critical interaction between phosphorus, zinc, and iron for nutrient homeostasis and crop quality (Xie *et al.*, 2019), supporting our findings. Additionally, research on zinc-efficient genotypes mirrors our observations of zinc's synergistic effects with other nutrients, emphasizing the importance of targeted nutrient management for optimizing plant growth and protein content (Peleg *et al.*, 2008; Jalal *et al.*, 2024).

Conclusions

The findings of this study underscore the significant benefits of inoculating black chickpeas with plant growth-promoting rhizobacteria and *Rhizobium*. The study demonstrated that the seeds were biologically fortified, exhibiting high protein levels, essential minerals such as calcium, potassium, and magnesium, and notably increased iron content. The role of *R. cicer* in enhancing the iron content of black chickpea seeds, which is vital for addressing iron deficiency anaemia, was elucidated for the first time. Additionally, the marked improvement in total antioxidant capacity and phenolic content reflects the increased resilience of black chickpeas to environmental stressors through the production of antioxidant enzymes and phytohormones. These results highlight the dual benefits of PGPR and Rhizobium inoculations: not only do they enhance agricultural productivity and plant health, but they also offer a sustainable solution to nutrient deficiencies. By integrating these biofertilizers into agricultural practices, the study suggests a substantial contribution to improving public health outcomes and advancing sustainable agricultural practices. Thus, this approach can be a pivotal strategy in combating global malnutrition and food insecurity.

Authors' Contributions

HY: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Resources; Software; Validation; Visualization; Writing - original draft; Writing - review and editing.

All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

Acknowledgements

The author would like to thank Olgunlar Seed Company for providing the Turkish chickpea variety "KATRAN" used as the plant material in this study

Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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