

Growth, Nutrient Uptake, and Yield Promotion of Broccoli by Plant Growth Promoting Rhizobacteria with Manure

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Abstract. This study was conducted to investigate the effects of root inoculations with *Bacillus cereus* (N₂-fixing), *Brevibacillus reuszeri* (P-solubilizing), and *Rhizobium rubi* (both N₂-fixing and P-solubilizing) on plant growth, nutrient uptake, and yield of broccoli in comparison with manure (control) and mineral fertilizer application under field conditions in 2009 and 2010. Bacterial inoculations with manure compared with control significantly increased yield, plant weight, head diameter, chlorophyll content, nitrogen (N), potassium (K), calcium (Ca), sulfur (S), phosphorus (P), magnesium (Mg), iron (Fe), manganese (Mn), zinc (Zn), and copper (Cu) content of broccoli. The lowest yield per plant, plant weight, stem diameter, and chlorophyll content were recorded in the control, but the manure with *Bacillus cereus* (BC), *Rhizobium rubi* (RR), and *Brevibacillus reuszeri* (BR) inoculations increased yield 17.0%, 20.2%, and 24.3% and chlorophyll content by 14.7%, 14.0%, and 13.7% over control, respectively. Bacterial inoculations with manure significantly increased uptake of macronutrients and micronutrients by broccoli. In conclusion, seedling inoculation with BR and especially RR may partially substitute costly synthetic fertilizers in broccoli.

Many agricultural soils of the world are deficient in one or more of the essential nutrients needed to support healthy plants. Acidity, alkalinity, salinity, anthropogenic processes, and erosion can lead to soil degradation. Addition of fertilizers and/or amendments is essential for a proper nutrient supply and maximum yields. Chemical fertilizers often have low use efficiency, meaning that only a portion of

the applied nutrients is taken up by plants (Adesemoye et al., 2009). Estimates of overall efficiency of applied fertilizer have been reported to be about or lower than 50% for N, less than 10% for P, and ≈40% for K in mineral fertilizer and are lower in manure. Plants that efficiently absorb and use nutrients greatly enhance the overall efficiency of applied fertilizers, reducing cost of inputs and preventing loss of nutrients to ecosystems (Baligar et al., 2001).

Fertilizer use efficiency can be optimized by fertilizer management practices that apply nutrients at the right rate, time, and place in both conventional and organic farming. There must be a balance between optimal nutrient use efficiency and optimal crop productivity (Roberts, 2008). Intensive farming practices, which demand high yield and quality, require extensive use of chemical fertilizers, which

are costly and can create environmental problems. The use of chemical fertilizers to enhance soil fertility and crop productivity has often negatively affected the complex system of biogeochemical cycles. For example, fertilizer use has caused leaching and runoff of nutrients, especially N and P, leading to environmental degradation. Therefore, interest has grown in environmentally sustainable and organic agricultural practices (Esitken et al., 2005).

One potential way to decrease negative environmental impacts resulting from inefficient use of chemical fertilizers is inoculation with plant growth promoting rhizobacteria (PGPR). These bacteria exert beneficial effects on plant growth and development and therefore may be used as biofertilizers for agriculture (Adesemoye et al., 2009). So far numerous bacterial species, mostly associated with the plant rhizosphere, have been tested and found to be beneficial for plant growth, yield, and crop quality. They have been called PGPR including the strains in the genera *Acinetobacter*, *Alcaligenes*, *Arthrobacter*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Beijerinckia*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Rhizobium*, and *Serratia* (Bashan and de-Bashan, 2005; Han and Lee, 2005). Capabilities of PGPR range from enhancing yields of organic systems to pollution control. Success of PGPR in organic systems is attributed to more rapid breakdown of organic matter, enhanced availability of nutrients, and improved soil properties, and these effects are mostly explained by the release of metabolites directly stimulating growth. All the mechanisms by which PGPR promote plant growth are not fully understood but may include the ability to produce plant hormones such as auxins, cytokinins, gibberellins, and inhibit ethylene production; a symbiotic N₂ fixation; solubilization of inorganic phosphate and mineralization of organic phosphate and/or other nutrients; antagonism against phytopathogenic microorganisms by production of siderophores; the synthesis of antibiotics, enzymes, and/or fungicidal compounds; and competition with detrimental microorganisms (Caballero-Mellado et al., 2007).

A principal problem of organic farming is the low nutrient status of most organic fertilizers (Mengel and Kirkby, 1987). Nutrient content of organic fertilizers varies widely depending on the source and moisture content. This problem is further compounded by the difficulties in assessing the value of organic fertilizers through direct analysis of total quantities of plant nutrients. Thus, field experiments are needed to determine the nutrient availability and efficiency of most organic fertilizers. This is the result of the slow and variable release rates of nutrients during decomposition of organic materials (Hsieh and Hsieh, 1990). Biofertilizers with PGPR can be used to increase soil productivity and plant growth in sustainable agriculture through increasing mineral and organic fertilizer use efficiency (Adesemoye et al., 2009).

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Most studies with PGPR were carried out to determine direct beneficial effects on plant growth but lack information about the effect of PGPR on organic fertilizer use efficiency in plant growth. Therefore, a study was conducted to investigate the effects of PGPR inoculations with manure on plant growth parameters, nutrient uptake, and yield of broccoli in comparison with mineral fertilizer application under field conditions.

Materials and Methods

Plant material. Broccoli 'Monet F1' (*Brassica oleracea* L., var. *italica*) plants, middle season variety, as plant material was used.

Field experiments. Field trials were conducted in Experimental Farm of Ataturk University in Erzurum in Eastern Anatolia (lat. 29°55' N; long. 41°16' E; altitude 1850 m), Turkey, in 2009 and 2010. The soil at the experimental area had 28.3% sand, 32.2% silt, and 39.5% clay. Relevant soil chemical characteristics were as follows: soil pH 7.3; organic matter 3.5%; P: 0.9 mg P₂O₅ per 100 g soil and K 123 mg K₂O per 100 g soil. Approximately 30 t·ha⁻¹ manure, which contained 2.4% N, 1.8% K₂O, 2.1% P₂O₅, and electrical conductivity (EC) of 6.3 dS·m⁻¹, was applied to the beds before planting. With an average temperature of 5.7 °C and total rainfall of 402.5 mm (Table 1), plant growth in the region is restricted to the period between May and October. The second year of the experiment received higher and more even distribution of rainfall in the growing months of May, July, and August, whereas average monthly air and soil temperatures were generally higher in the first year of the experiment (Table 1). Average daily air temperature ranged between 8.6 and 28.3 °C in the growing season of 2009 and between 11.2 and 22.6 °C in the growing season of 2010. Average daily soil temperature ranged between 14.4 and 24.8 °C in the first growing season and between 13.7 and 24.3 °C in the second growing season (Table 1).

Broccoli seeds were sown into plastic trays filled with peat (pH: 5.5, EC: 250 dS·m⁻¹, N: 300 mg·L⁻¹, P: 131 mg·L⁻¹, K: 333.33 mg·L⁻¹, organic matter: 2%). Seedlings were initially grown in a greenhouse and then transplanted after \approx 1 month in rows (0.50 m apart with intrarow spacing of 0.45 m) in late June in both years. Each plot consisted of 25 plants. Treatments consisted of 1) control (manure + 100 kg·ha⁻¹ rock phosphate including 18% P₂O₅—organic); 2) mineral fertilizer (N 120 kg·ha⁻¹ as urea and P 180 kg P₂O₅/ha as triple superphosphate 48% P₂O₅); 3) BC (manure + rock

phosphate + *Bacillus cereus*); 4) BR (manure + rock phosphate + *Brevibacillus reuszeri*); and 5) RR (manure + rock phosphate + *Rhizobium rubi*). Marketable broccoli heads were harvested in September in both years. Inner rows were used for sampling and harvest. Entire plants were harvested at ground level and weighed from each plot when the terminal buds were swollen but not opened. Stem diameter was measured from the ground level. Plants were then cut 20 cm below the top of head, which was trimmed to obtain a marketable product. Head diameter was measured across the widest part of the head. The plant materials were weighed for leaf and head dry matter ratio and then dried in an oven at 70 °C until a constant mass was reached.

Chlorophyll readings. A portable chlorophyll meter (SPAD-502; Konica Minolta Sensing, Inc., Japan) was used to measure leaf greenness. SPAD-502 chlorophyll meter estimates total chlorophyll amounts in leaves in a non-destructive method (Neufeld et al., 2006). For each plant, measurements were taken at four locations on each leaf, two on each side of the midrib on all fully expanded leaves (Khan et al., 2003), and the same leaves were used for chemical analyses (Alcantar et al., 2002).

Bacterial strains, culture conditions, media, and treatment. Bacterial strains were originally isolated from the rhizosphere of tea plants naturally grown in Rize, Turkey, and identified as *Bacillus cereus*, *Brevibacillus reuszeri*, and *Rhizobium rubi* based on fatty acid methyl ester analysis using the MIDI system (Sherlock Microbial Identification System Version 4.5; MIDI, Inc., Newark, DE). For this experiment, the bacterial strains were grown on nutrient agar. A single colony was transferred to 250-mL flasks containing NB and grown aerobically in flasks on a rotating shaker (95 rpm) for 24 h at 27 °C. The bacterial suspension was then diluted in sterile distilled water to a final concentration of 10⁸ colony-forming units (cfu)/mL. Bacterial suspensions were measured and concentration of bacterial population arranged to the desired level (10⁸ cfu/mL) using a spectrophotometer at 630 nm (a ultraviolet/VIS Aqumat spectrophotometer; Thermo Electron Spectroscopy Ltd., Cambridge, U.K.) (AOAC, 2005). Bacterial applications of *Bacillus cereus*, *Brevibacillus reuszeri*, and *Rhizobium rubi* were performed using a dipping method in which seedling roots were inoculated with the bacterial suspensions at the concentration of 10⁸ cfu/mL in sterile water \approx 60 min before transplanting. Control plants were dipped into sterile water. The

bacterial strains *Bacillus cereus* and *Rhizobium rubi* were able to grow in N-free basal medium indicating its N-fixing potential. In the present study, P-solubilizing activities of *Brevibacillus reuszeri* and *Rhizobium rubi* were measured according to the qualitative methods (Mehta and Nautiyal, 2001).

Mineral analysis. At heading is a good time to assess the relationship between broccoli nutrient content and soil nutrient pools (Jones et al., 1991; Mengel and Kirkby, 2001). Therefore, tissue subsamples were taken during heading (five youngest leaves), then oven-dried at 68 °C for 48 h and ground, and passed a 1-mm sieve size. The Kjeldahl method and a Vapodest 10 Rapid Kjeldahl Distillation Unit (Gerhardt, Konigswinter, Germany) were used to determine total N (Bremner, 1996). Macro- [P, K, S, Ca, Mg, and sodium (Na)] and microelements (Fe, Mn, Zn, and Cu) were determined after wet digestion of dried and ground subsamples using a HNO₃-H₂O₂ acid mixture (2:3 v/v) with three steps [first step; 145 °C, 75% radio-freqeucy power (RF), 5 min; second step; 180 °C, 90% RF, 10 min; and third step; 100 °C, 40% RF, 10 min] in microwave digestion (Bergof Speedwave Microwave Digestion Equipment MWS-2; Berghof Products and Instruments, Eningen, Germany) (Mertens, 2005a). Tissue P, K, S, Ca, Mg, Na, Fe, Mn, Zn, and Cu were determined using an inductively coupled plasma spectrophotometer (Optima 2100 DV, ICP/OES; Perkin-Elmer, Shelton, CT) (Mertens, 2005b).

Data analysis. Experiments were repeated in 2 years. Each experiment consisted of a randomized complete block experimental design with three blocks. All data in the present study were subjected to analysis of variance using SPSS 18 (PASW Statistics 18) (SPSS Inc., 2010) statistical program. Means were separated by Duncan's multiple range tests. There were no significant interactions by year; therefore, the data were pooled.

Results

Effects of bacterial treatments on broccoli yield and growth parameters. There were no significant interactions for effects across years for the characteristics investigated. Across 2 years, bacterial treatments increased head yield per plant, plant weight, stem diameter, head diameter, and chlorophyll reading values but not head dry matter ratio or leaf dry matter ratio of broccoli in this study compared with control (Table 2).

Lowest head yield per plant, plant weight, stem diameter, head diameter, and chlorophyll reading values were recorded in the control. Highest head yield per plant, plant weight, stem diameter, head diameter, and chlorophyll reading values were obtained from mineral fertilizer and RR inoculations with manure application. Relative yield, which is the broccoli yield expressed as percentage of the highest yield obtained from mineral fertilizer application, was the 76.5% for control, 89.5% for BC, 92.4% for BR, and 95.1% for RR treatments (Fig. 1). On the other hand, BC,

Table 1. Climatic data on the experimental site.

Climatic factors	Years	May	June	July	August	September	Total/avg	
							Growth season	Annual
Total rainfall (mm)	1985–2010	70.5	47.9	27.4	17.1	24.4	187.3	402.5
	2009	41.6	72.2	20.7	3.5	29.2	167.2	367.4
	2010	58.3	61.8	41.9	30.4	0.1	192.5	488.8
Average air temperature (°C)	1985–2010	10.6	14.9	19.3	19.4	14.7	15.8	5.7
	2009	11.4	18.4	20.3	22.6	14.1	17.4	6.4
	2010	12.8	14.4	18.1	18.6	15.2	15.8	5.5

Table 2. Effects of plant growth promoting rhizobacteria applications on plant yield, yield parameters, and total chlorophyll content of broccoli plant.

	Mineral	Organic	Organic + BC	Organic + BR	Organic + RR
Yield per plant (g)	801 a ²	613 c	717 b	740 ab	762 ab
Plant weight per plant (g)	2324 a	1877 b	2208 a	2202 a	2416 a
Stem diameter (cm)	2.71 a	2.41 b	2.38 b	2.60 ab	2.59 ab
Head diameter (cm)	13.86 a	12.02 b	12.75 ab	12.54 ab	13.98 a
Chlorophyll content	74.16 ab	69.58 b	79.71 a	78.59 a	77.96 a
Head dry matter ratio (%)	11.80 NS	13.04	13.22	12.55	11.84
Leaf dry matter ratio (%)	15.48 b	16.03 a	15.25 bc	14.93 c	15.54 b

²Mean values in lines followed by a different letters differ significantly at $P < 0.05$.

NS = Non-significant.

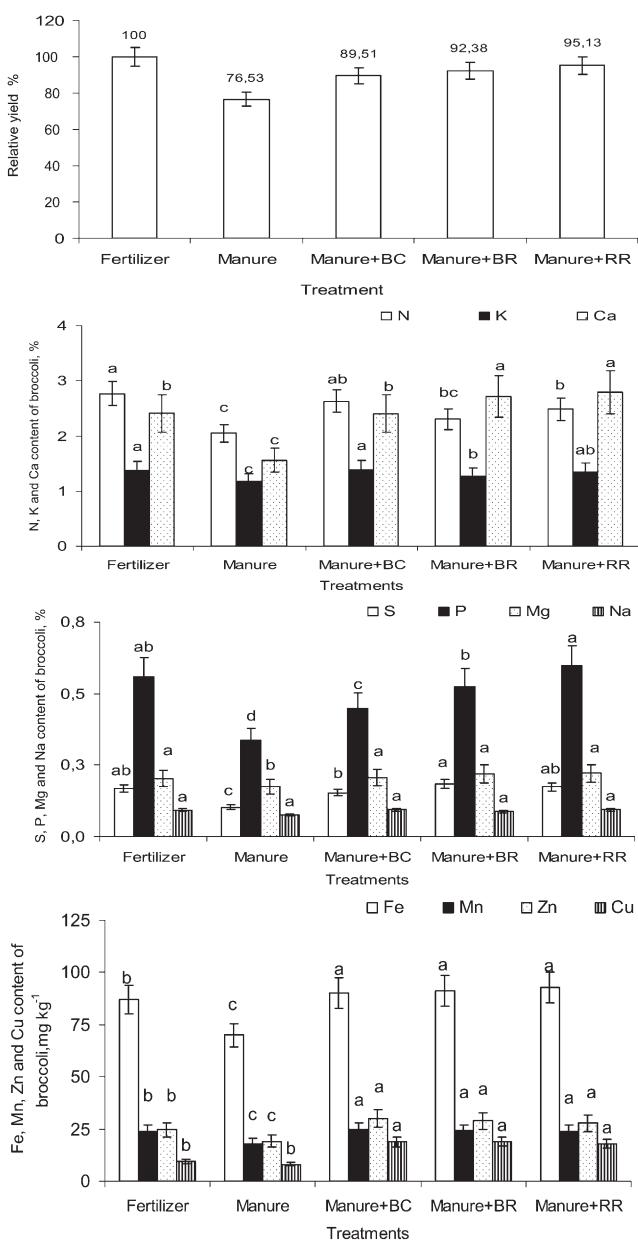


Fig. 1. Relative yield; nitrogen (N), potassium (K), calcium (Ca), sulfur (S), phosphorus (P), magnesium (Mg), sodium (Na), iron (Fe), manganese (Mn), zinc (Zn), and copper (Cu) content of broccoli in response to plant growth promoting rhizobacteria (PGPR) applications. Different letters on top of bars indicate differences ($P < 0.05$). Vertical bars indicate the mean \pm se.

BR, and RR inoculations with manure application increased 17%, 20%, and 24% in yield per plant and 17%, 17%, and 29% in plant weight compared with control (Table 2), respectively. The positive effect recorded for the

inoculation of PGPR clearly indicated that BC, BR, and RR were able to increase the relative yield of broccoli.

SPAD chlorophyll reading values increased with PGPR treatments compared

with the control. BC, BR, and RR inoculations with manure application increased 14%, 14%, and 13% in chlorophyll compared with control, respectively (Table 2). SPAD chlorophyll values of broccoli leaves were correlated with tissue total N concentrations from the laboratory analysis, which means that chlorophyll values increased values with PGPR treatments (Table 2; Fig. 1).

Effects of biofertilizer on plant nutrient element content. Results showed bacterial inoculations significantly affected ionic compositions of plant leaves (Fig. 1). In this study, the data demonstrated that bacterial applications increased N, P, K, Ca, Mg, S, Fe, Mn, Zn, and Cu contents of leaves compared with the control.

N_2 -fixing and P-solubilizing PGPR strains promoted plant nutrient element (PNE) contents of leaves of the plant. Although mineral fertilizer had the highest N content of leaves, BC with manure had the highest K, Mn, and Zn; BR with manure had the highest S and Cu; and RR with manure had the highest Ca, P, Mg, and Fe (Fig. 1). Leaf nutrient content for K, Mn, and Zn increased 18.6%, 36.4%, and 56.7% for BC with manure; S and Cu contents of leaves increased 78.5% and 134% for BR with manure; and Ca, P, Mg, and Fe contents of leaves increased 78.8%, 76.7%, 26.5%, and 22.9% for RR inoculations with manure application compared with control, respectively (Fig. 1).

Discussion

This is the first study to demonstrate that PGPR root inoculation with manure can increase yield, growth, and PNE contents of broccoli plants. Two years of trials under field conditions showed that inoculations with *Bacillus cereus*, *Brevibacillus reuszeri*, and *Rhizobium rubi* significantly increased yield, yield parameters, and PNE uptake of broccoli. However, the strain *B. cereus* is a member of potential pathogens in risk Group 2, so we could not suggest using it as a biofertilizer in every condition. Bacterial applications with manure positively affected head yield per plant, plant weight, stem diameter, head diameter, and chlorophyll reading compared with the control; and RR was the most effective PGPR in the treatment. The yield and plant growth enhancement effects of bacteria used in this study on broccoli could be explained with the N_2 -fixing and P-solubilizing capacity of bacteria. The effects of a combined treatment of PGPR (mixture of *Bacillus* sp., *B. subtilis*, *B. erythropolis*, *B. pumilus*, and *P. rubiacearum*) plus 50% chemical fertilizer (% CF + biofertilizer) on the growth of lettuce were compared by Young et al. (2003). Results showed there was a 25% increase of lettuce yield for the treatment of 1/2 CF + biofertilizer compared with that of the CF treatment, indicating that at least 50% of chemical fertilizer can be saved as a multifunctional biofertilizer when used along with a chemical fertilizer.

Young et al. (2004) evaluated the effects of PGPR (mixture of *Bacillus* sp. *B. subtilis*, *B. erythropolis*, *B. pumilus*, and *P. rubiacearum*)

on rhizosphere microbial activity and the growth of water celery in a field experiment. Results showed that the dry weight of water celery in the treatment with 50% organic compound fertilizer (OCF) with PGPR (PGPR + 1/2 OCF) was increased by 34% compared with the treatment with 100% organic compound fertilizer added.

Numerous studies have shown the improvement in plant growth and development in response to seed or root inoculation with various microbial inoculants capable of producing plant growth regulators (Zahir et al., 2004). Trials with rhizosphere-associated plant growth-promoting N₂-fixing and P-solubilizing bacteria indicated yield increases in many crops such as wheat (Ozturk et al., 2003), barley (Cakmakci et al., 2001; Ozturk et al., 2003), sugar beet (Cakmakci et al., 2001), canola (de Freitas et al., 1997), and maize (Pal, 1998). Because of their spore-forming ability, plant growth-promoting strains are readily adaptable to commercial formulation and field application (Liu and Sinclair, 1993). The positive effects of *Bacillus* and *Rhizobium* on the yield and growth of crops such as chickpea, apple, strawberry, spinach, tomatoes, sugar beet, barley, and wheat were explained by N₂-fixation ability, phosphate-solubilizing capacity, indole acetic acid, and antimicrobial substance production (Cakmakci et al., 2001, 2007; Elkoca et al., 2008; Esitken et al., 2010).

The effect of PGPR applications on chlorophyll readings in the study is in good agreement with the results of Yildirim et al. (2008a, 2008b) in radish grown in saline conditions. PGPR treatments gave elevated reading values when compared with the non-inoculated controls in these studies. They proved that SPAD chlorophyll meter readings in leaves were correlated with tissue total N concentrations. This phenomenon might be caused by PGPR treatments with N₂-fixing capabilities, promoting N uptake by plant roots.

In plants treated by PGPR with manure, the PNE concentration of leaves may provide important information about the effect of bacterial inoculation in PNE uptake. In this study, it was found that bacterial treatments increased PNE contents of broccoli plants compared with the control. Generally, the enhancements in macro-/micronutrient contents were more pronounced in PGPR treatments with manure, whereas mineral fertilizer also resulted in significant nutrient increases in plant leaf. Enhancement of mineral uptake by plants should result in an increased accumulation of minerals in leaves of the plant. The use of the N₂-fixing and P-solubilizing PGPR in chickpea (Elkoca et al., 2008), barley (Cakmakci et al., 2007), tomato (Adesemoye et al., 2010), lettuce (Lai et al., 2008), and strawberry (Gunes et al., 2009) stimulated macro- and micronutrient uptake such as N, P, K, Ca, Mg, Fe, Mn, Zn, and Cu, which were consistent with our results. On the other hand, the study results could suggest that PGPR application with manure could reduce over 50% the required N and P fertilizer to

get the same yield with mineral fertilizer application. Bacterial inoculations with manure significantly also increased uptake of macronutrients and micronutrients by broccoli. Study results indicated that at least 50% of chemical fertilizer could be saved when a PGPR application was used along with manure fertilizer.

The enhancement of N uptake by plants inoculated with the PGPR strains used in this study could be through associative N fixation based on the tests with JNFB medium. On the other hand, the PGPR have been reported to promote the growth of the plant and increase the root surface area or the general root architecture. Plants growing better in turn release higher amounts of carbon (C) in root exudates. The release of more C prompts an increase in microbial activity, and this process continues in a cycle. The whole process makes more nutrients available from the soil pool, influencing nutrient flux into plant roots, and the plant is able to take up more available nutrients (Adesemoye et al., 2009).

Conclusions

The present study demonstrates that PGPR applications in addition to manure can be used to increase broccoli growth and yield without using mineral fertilizer. Efficient plant nutrition management should ensure both enhanced and sustainable agricultural production and safeguard the environment. Obviously, the use of chemical fertilizers and manures cannot be eliminated at this time without drastically decreasing food production. At the same time, the harmful environmental side effects of fertilizer use such as leaching, runoff, emission, and eutrophication of aquatic ecosystems worldwide cannot go unabated. Hence, there is an urgent need for integrated nutrient management that targets agricultural inputs and lowers the adverse environmental impacts of agricultural fertilizers and practices. Chemical, organic, or microbial fertilizer has its advantages and disadvantages in terms of nutrient supply, soil quality, and crop growth. So, developing a suitable nutrient management system that integrates use of these three kinds of fertilizers may be a challenge to reach the goal of sustainable agriculture. In view of environmental pollution in case of excessive use of mineral fertilizers and as a result of the high costs in the production of N and P fertilizers, bacteria tested in our study may well be suited to achieve sustainable and ecological agricultural broccoli production without decreasing yield compared with mineral fertilization application. An important nutritional problem of developing countries is micronutrient malnutrition, also called hidden hunger. Data may suggest that inoculations with PGPR have some potential to increase use efficiency of organic fertilizer in both organic and conventional farming.

In conclusion, *Brevibacillus reuszeri* and *Rhizobium rubi* were found to have great potential for use as PGPR to increase production in broccoli like in many other

crops previously tested and to be used in development of biofertilizer needed for organic agricultural production because of rendering these insoluble phosphates into a soluble form and increasing organic matter mineralization through the process of acidification, chelation, and exchange reactions in plant growth media. Considering environmental pollution with excessive use of synthetic fertilizers and high costs in the production of N and P fertilizers, the bacteria tested in our study may be a promising alternative as a biofertilizer for vegetable production in sustainable and organic agricultural systems; however, much research is still needed.

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