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Synergist effects of some PGPR bacteria and sodium nitroprusside in pepper plant

Zeliha Kayaaslan[1] **Servet Aras**[2] **Gökçe Aydöner Çoban**[3]

[1]Department of Plant Protection, Faculty of Agriculture, Yozgat Bozok University, Yozgat, Turkey
[2]Department of Horticulture, Faculty of Agriculture, Yozgat Bozok University, Yozgat, Turkey
[3]Department of Horticulture, Faculty of Agriculture, Yozgat Bozok University, Yozgat, Turkey

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Corresponding Author
Servet Aras
servet.aras@yobu.edu.tr

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Abstract

Plant growth promoting rhizobacteria (PGPR) represent promotes plant growth by increasing the supply or availability of nutrients to the host plant. These bacterial applications are environmentally friendly techniques and their use has become widespread recently. Some PGPRs can increase nitrogen (N) fixation and have phosphate (P) solubilizing property. In the current study, we evaluated the synergistic effects of some useful bacteria and sodium nitroprusside (SNP, a nitric oxide donor) in pepper plant. Nitric oxide (NO) acts as a signal molecule in plants and has important role in plant-bacteria symbiosis interaction. Three PGPR strains namely, *Enterobacter cloacae* (ZE-2), *Pseudomonas putida* (ZE-12) and *Acinetobacter calcoaceticus* (ZE-13) were used and the bacteria possess phosphorous-solubilizing and nitrogen-fixing properties. The applications of PGPRs alone and with combination of SNP (0.1 mM) were performed to the plant rhizosphere (the roots) through irrigation two times with two weeks interval starting with seedling planting. End of the study, many morphological parameters including stem diameter, plant height and biomass were improved by all applications compared to control. Root:shoot dry weight ratio decreased by the applications. Stem diameter, plant height and biomass were significantly increased with all treatments compared to control. The yield was found higher in all applications compared to control and the highest increase in the yield was provided by *Enterobacter cloacae* (ZE-2) application. Dry matter allocation in upper part of the plants provided higher plant yield. The applications significantly affected cell expansion and division. SNP increased the effect of *Acinetobacter calcoaceticus* (ZE-13) bacteria on cell division in leaf cells and midrib size. Furthermore, *Pseudomonas putida* (ZE-12) increased the yield combining with SNP compared to alone use. The increase in the plant growth is related with the midrib size. The application of PGPR with SNP could be a promising approach in plant growing.

Keywords: Biofertilizer, *Capsicum annuum*, Nitric oxide, PGPR, Yield

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INTRODUCTION

Plant growth promoting rhizobacteria (PGPR) represent a wide variety of soil bacteria which colonize the roots of plants that enhance plant growth (Vessey, 2003). PGPR often called as biofertilizers when applied to seed, plant surfaces, or soil, colonizes the rhizosphere and promotes growth by increasing the supply or availability of nutrients to the host plant (Zaidi et al., 2015). Useful bacteria applications are eco-friendly techniques that conserve the environment against pollution caused by agrochemical products (Tahiri et al., 2022). Many PGPRs have useful effects on plant growth, nutrient availability, and synthesis of hormones (İpek et al., 2017). Some PGPRs can increase nitrogen (N) fixation (Dixon and Kahn, 2004). Phosphate (P) solubilizing property was also reported in many PGPRs (Ergin and Gülser, 2016). Furthermore, some PGPRs are

used against environmental stress factors including salinity (Vaishnav et al., 2016), drought (Admassie et al., 2022) and limey soil (Aras et al., 2018).

A wide variety of bacteria including species of Bacillus, Alcaligenes, Pantoea, Enterobacter have been used for the beneficial effects in many plants (Collavino et al., 2010; Arıkan et al., 2018). The useful effects of Alcaligenes, Agrobacterium, Staphylococcus, Bacillus and Pantoea strains were reported in pear trees under high limey soil conditions (İpek et al., 2017). García et al. (2003) reported that Bacillus and Pseudomonas strains improved tomato and pepper growth. Islam et al. (2013) stated that some N-fixing bacteria increased tomato and pepper growth.

Another way to improve plant growth is nitric oxide (NO) in plants. Among the NO donors, sodium nitroprusside (SNP) is one of the most utilized one (Filippou et al., 2012). Many studies showed that SNP controls stomatal movements, photosynthesis and flowering (Takahashi and Yamasaki 2002; He et al., 2004). SNP triggered early xylem formation in peach bud (Aras, 2022c). SNP application improved cortical cells and xylem vessels in peach leaves (Aras, 2022b). Furthermore, many studies showed that SNP application improved the tolerance against environmental stress factors in many plants (Hayat et al., 2012; Esringu et al., 2016; Kaya et al., 2019; Aras et al., 2020).

NO acts as a signal molecule in plants (Siddiqui et al., 2011). NO possesses important role in plant-bacteria symbiosis interaction (Wang and Ruby, 2011). Vaishnav et al. (2016) reported that NO improved the effects of PGPRs on salinity stress in soybean. A few study were conducted on the interaction between NO and bacteria (Sharma et al., 2021). Thus, in the current experiment we studied the relation between SNP and some useful bacteria including *Enterobacter cloacae*, *Pseudomonas putida* and *Acinetobacter calcoaceticus* in pepper plant. The plant growth, yield and leaf cellular physiology were evaluated in the study with application of PGPRs alone and with combination of SNP.

MATERIALS AND METHODS

Bacterial strains

Within the scope of PGPR acquisition studies, pepper plant growing areas in Tokat province and its districts were visited and samples were taken from the root zone soil of the plant. During sampling, pepper plants that showed better development and appeared healthier than other plants were selected. Bacteria obtained using various isolation methods were purified according to different colony structures. Purified isolates were identified by proteomics at Mustafa Kemal University Plant Health Clinic Application and Research Center using MALDI-TOF MS method. As a result of identification, it was determined that there were three PGPR strains: *Enterobacter cloacae* (ZE-2), *Pseudomonas putida* (ZE-12) and *Acinetobacter calcoaceticus* (ZE-13). In order to determine that the isolates obtained were not plant pathogens, they were subjected to hypersensitivity tests on tobacco plants (*Nicotiana tabacum* cv. Samsun) and softening tests on potato slices. Positive result was not observed in hypersensitive test in tobacco (Belgüzar et al., 2021). None of the isolates was able to cause the death of local cell of tissue in tobacco leaves and rotting of potato slices. Therefore, the bacteria are non-pathogenic.

Bacterial isolates are stored as stock cultures in the Phytopathology laboratory of Yozgat Bozok University, Faculty of Agriculture, Department of Plant Protection. King B medium was used for the growth of bacteria. Bacterial suspensions colony densities of bacteria were prepared by measuring the absorbance value of $2x10^{-8}$ (A 600:0.3) using a spectrophotometer with sterile water.

In vitro screening of bacteria properties

The property of the bacteria to solubilize phosphorus (P) was evaluated using National Botanical Research Institute's phosphate growth medium (NBRIP) (Nautiyal, 1999). The assay was carried out using a randomized design with three replicates. Three drops of cell suspension were inoculated onto the surface of NBRIP agar plate media, followed by incubation for a week at 25 °C. One colony with the largest clear zone was selected (Johri et al., 1999; Mirik et al., 2008). Nitrogen fixation property of the bacteria was evaluated N-free Jensen agar media (Ahmad et al., 2005).

Pot experiment and bacteria and SNP applications

This study was conducted in a semi-controlled greenhouse (average 25°C temperature, 65% relative humidity) at Yozgat Bozok University, in Turkey. Seedlings of pepper (*Capsicum annuum* L. cv. Çetinel) were planted in 4 L plastic pots filled with substrate and agricultural perlite (4:1). The applications were performed to the plant rhizosphere (the roots) through irrigation two times with two weeks interval starting with seedling planting. Bacterial suspensions were diluted to a final concentration of 10^9 cfu mL^{-1} and applied to the pepper roots. 0.1 mM SNP dose was chosen according to the previous experiments (Kaya et al., 2019; Shams et al., 2019). The applications were performed at the same time. Control plants were not treated with any bacteria or SNP. The study was carried out in a completely randomized design with 3 replicates per treatment and 4 seedlings were used per replicate. The plants were evaluated two months after applications initiation. The yield was determined during the season.

Morphological Responses and Yield

Stem diameter (mm) was measured with a digital caliper. Plant biomass (root+shoot) was determined with a precision balance. Plant height (mm) and root length (mm) were measured with a ruler. Root and shoot dry weights were measured after drying the plant material at 70ºC for 48-72 hours. The value of root:shoot in dry weight was calculated as the dry weights of root/shoot. Yield (g) was determined per plant.

Histological Responses

The samples of the leaves were stored in FAA (formaldehyde, alcohol, acetic acid) solution. Freehand cross sections of leaf midrib were utilized for microscopic evaluation. The midrib sections were subjected to toluidine blue O for cortical cell observation (O'Brien et al., 1964) and visualized with a light microscope (Olympus CX21; Olympus, Tokyo, Japan) coupled to a digital camera.

Statistical Analyses

The statistical analyses were performed with the statistical software package SPSS, version 20.0. Data were subjected to Duncan's test at a significance level of $P < 0.05$. Heatmap and principal component analysis (PCA) was performed using XLSTAT Software.

RESULTS

In the present study, all bacteria had the ability of phosphorous-solubilizing and nitrogen-fixing. The PGPRs alone or combining with SNP improved plant growth and yield in pepper. Stem diameter, plant height and biomass were significantly increased with all treatments compared to control (Table 1). Figure 1 shows the growth increment in pepper provided by the applications.

Figure 1. Pepper plant growth increment provided by PGPRs and SNP

Table 1. Effect of PGPRs and SNP on stem diameter, plant height and biomass in pepper

Treatments	Stem diameter (mm)	Plant height (mm)	Plant biomass (g)
Control	3.97 b	37.6 e	30.0 cd
SNP	4.39 ab	47.0 d	40.3 b
ZE-2	4.60 a	55.1 bc	40.7 b
ZE-12	4.72 a	66.6 a	49.1 a
ZE-13	4.87 a	67.3 a	40.9 b
ZE-2+SNP	4.60 a	60.6 ab	24.7 d
ZE-12+SNP	4.65 a	56.8 bc	35.3 bc
ZE13+SNP	4.63 a	48.6 cd	39.5 b

Root:shoot dry weight ratio decreased by the applications (Table 2). Root length differently affected by the applications. SNP, ZE-2 and ZE-12+SNP increased root length compared to control, when the other applications decreased the value. The yield was remarkably affected by the applications. The highest yield was obtained in ZE-2 application.

Table 2. Effect of PGPRs and SNP on root:shoot in dry weight, root length and yield in pepper

Treatments	Root:shoot in dry weight	Root length (mm)	Yield (g/plant)
Control	0.9056 a	28.5 ab	230 g
SNP	0.5317 b	29.0 a	495 c
ZE-2	0.5526 b	29.5 a	608 a
ZE-12	0.5595 b	25.2 bc	285 f
ZE-13	0.7761 ab	24.0 c	430 e
ZE-2+SNP	0.5654 b	22.0 c	575 b
ZE-12+SNP	0.5666 b	30.5 a	471 d
ZE-13+SNP	0.7854 ab	22.0 c	305 f

We evaluated pepper leaf midrib with histological analyses (Figure 2). The applications significantly affected cell expansion and division (Table 3). The highest cortical cell diameter was found in ZE-2 application that demonstrates the highest increase in cell expansion was found in ZE-2 application. ZE-13+SNP application had the highest number of cortex cell layer among the treatments. In general, the results showed that the treatments did not have a significant effect in cortex cell layer. Moreover, we evaluated midrib diameter in the study. ZE-13+SNP had the highest midrib diameter among the treatments followed by ZE-12+SNP.

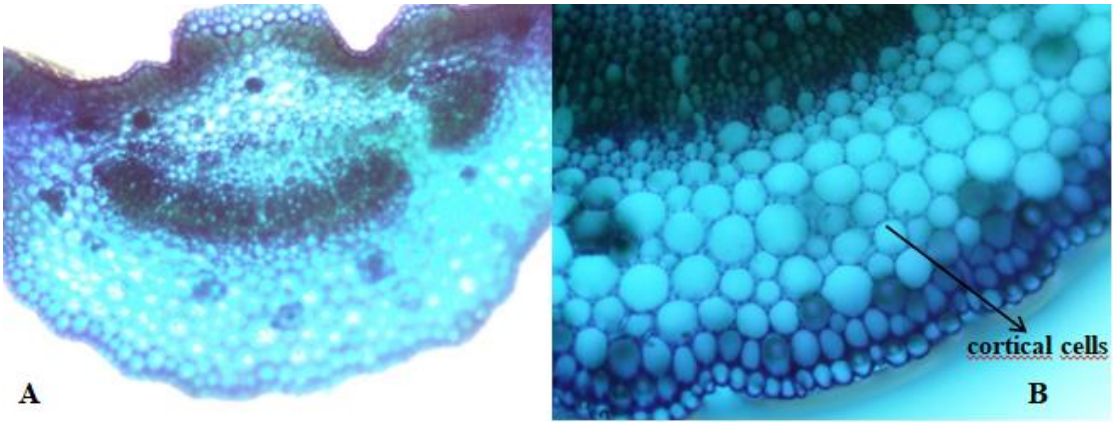

Figure 2. General view of cortical cells of leaf midrib in pepper, A: cross section of leaf midrib, B: cortex of midrib

The normalized heatmap approach was applied to assess all results provided by PGPRs and SNP in pepper (Figure 3). Two main clusters were detected: the first cluster is closely linked to number of cortex cell layer and root:shoot DW, and the second cluster is linked to root length, yield, plant biomass, midrib diameter, cortical cell diameter, stem diameter and plant height. The applications were categorized into two: first is control; and second is SNP, ZE-2+SNP, ZE-2, ZE-12+SNP, ZE-13+SNP, ZE-12 and ZE-13. We also performed PCA to examine the overall distribution trend among applications (Figure 4). Principal Component 1 (PC1) and Principal Component 2 (PC2) were the first and second largest dimensions of data difference and explained the results by 44.13 and 21.79%, respectively. PCA has scattered the applications in four quarters of the biplot. Biplot indicates that PGPRs alone or combining with SNP improved plant growth, histological response and yield compared to control.

Table 3. Effect of PGPRs and SNP on cortical cell diameter, number of cortex cell layer and midrib diameter in pepper

Treatments	Cortical cell diameter (µm)	Number of cortex cell layer	Midrib diameter (µm)
Control	41.3 c	5.54 ab	872 d
SNP	48.2 bc	6.39 ab	927 cd
ZE-2	58.3 a	5.46 ab	1015 bc
ZE-12	54.1 ab	5.22 b	1043 ac
ZE-13	54.6 ab	5.92 ab	1049 ac
ZE-2+SNP	54.1 ab	5.65 ab	990 bd
ZE-12+SNP	56.1 ab	5.45 ab	1106 ab
ZE-13+SNP	52.3 ab	6.61 a	1163 a

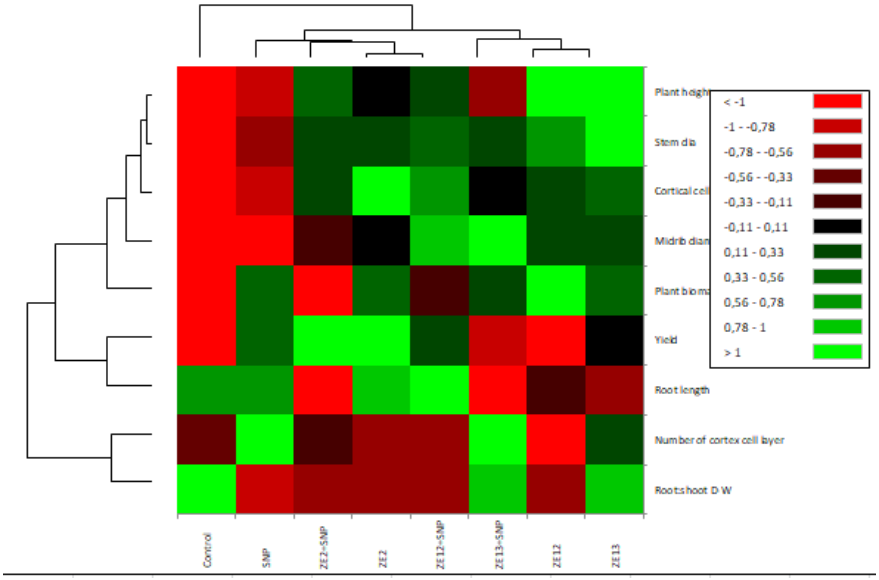

Figure 3. Normalised heatmap responses of the morphological and histological attributes and yield of pepper.

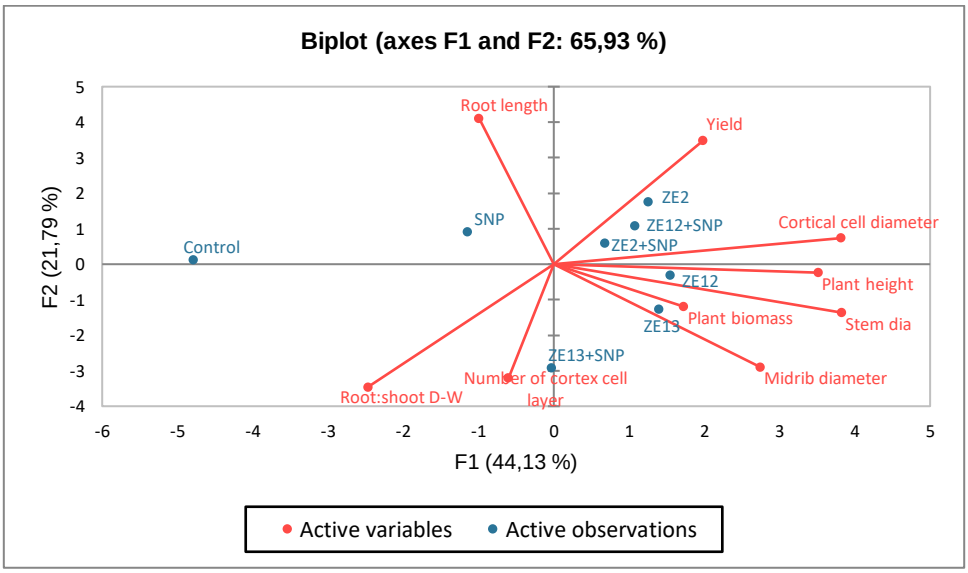

Figure 4. Principal component analysis of the morphological and histological responses and yield of pepper. The mean values of the replicates are shown.

DISCUSSION

The present study demonstrated the effects of some PGPRs alone or combining with SNP in pepper plant. PGPR application possessed a remarkable effect on plant growth and yield.

In this study, which was conducted within the scope of determining the effects of some plant growth promoting rhizobacteria and mycorrhizae on the growth and development of *Dahlia variabilis* (star flower), which is widely produced as cut flower and outdoor ornamental plant in the world and is increasingly widespread in our country, *Enterobacter cloacae* (ZE-2), *Bacillus cereus* (ZE-7), *Pseudomonas putida* (ZE-12), *Acinetobacter calcoaceticus* (ZE-13), *Burkholderia cepecia* (7-a-2) bacterial species and commercially available mycorrhiza (5000 ppm) were applied to the seeds of *D. variabilis* variety 'Violet'. Germination rate (%), seedling height (cm), stem diameter (mm), number of leaves (number), plant fresh weight (g), plant dry weight (g), root length (cm), root fresh weight (g), root dry weight (g) and SPAD value and chlorophyll content were measured. As a result, it was determined that the applications had different effects on D. variabilis. It was determined that *P. putida* (ZE-12) application increased germination by 12% compared to the control, and *A. calcoaceticus* (ZE-13) application increased seedling height by 32.9% (Alkaç et al., 2022a).

In another study, the effects of *Zinnia elegans* L. Zesty and *Dahlia variabilis* L. 'Figaro Violte' on the development of varieties were investigated. *Dahlia variabilis* L. seedlings were planted in suspensions prepared from ZE-12, ZE-13 and ZE-12+ZE13, while *Z.elegans* seedlings were planted in suspensions prepared from ZE-2, ZE-7, ZE-12, ZE-13, ZE-12+ZE-13. Seedlings to which no rhizobacteria and mycorrhiza applications were made were used as the control group. As a result, all parameters except root dry weight of the applications applied in the *D. variabilis* trial remained at the same values as the control. ZE-13 application was effective in root dry weight. It was determined that the applications applied in *Z.elegans* seedlings increased the flower stalk thickness and number of leaves, and especially ZE-13 application was the most effective application. As a result, it was determined that there is a potential for applications that will not negatively affect environmental and human health in ornamental plant cultivation (Alkaç et al.,2022b).

PGPR applications improved plant growth compared to control. The highest increment in stem diameter, plant height and plant biomass was found in PGPR applied plants. PGPR combining with SNP also increased the parameters compared to control. However, the increments were found higher in the use of PGPR alone. Many experiments showed that PGPR application can enhance plant growth in many plants (Mia et al., 2010; Wang et al., 2022). The increase in plant growth is frequently associated with a higher N fixation (Olivera et al., 2004). Aminifard et al. (2012) stated that nitrogenous fertilizer promoted plant growth in pepper. In the current study, we evaluated the effect of phosphorus-solubilizing and N-fixing bacteria on plant growth. The increase in plant growth may be a result of N-fixing property of the bacteria. Bulut (2013) reported that some phosphorus-solubilizing and N-fixing bacteria increased wheat plant growth. Moreover, many N-fixing bacteria enhanced plant growth in many plants (Mohammed et al., 2012; Devi et al., 2022). SNP also increased plant growth both the use of alone and combining with the bacteria. Exogenous SNP application improves plant growth by mediating the level of endogenous NO (Li et al., 2022) and endogenous NO plays important role in photosynthesis (Fatma et al., 2016). In a previous study, we reported that SNP improved chlorophyll biosynthesis and xylem vessel in peach leaves (Aras, 2022b). Xylem is responsible for water and mineral uptake (Brodersen et al., 2019) that may play pivotal role in plant growth.

Moreover, we evaluated root:shoot ratio in dry weight in order to dry matter distribution between shoot and root. All applications decreased the ratio compared to control (Table 2). The decline in root length and increment in plant height were also found in SNP and PGPR applications that provides that SNP, PGPR alone and combining with SNP applications favoured an investment in plant shoot compared to root. Thus, the applications altered the pattern of dry matter distribution favouring the shoot growth and allocated the dry matter in the shoots. In perennial (woody) plants, dry matter allocation in shoots may be a problem and reported in cherry trees under stress conditions (Aras et al., 2019), because the roots are storage organ and must be improved for the next years. However, in annual (herbaceous) plants the shoot growth is important due to dry matter distribution into fruits. In the current experiments, the pepper yield was found higher in all applications compared to control and the highest increase in the yield was provided by ZE-2 application. We consider that dry matter allocation in upper part of the plants provided higher plant yield.

Plant growth increment relies on the coordinated progression of cell division and expansion. Expansion of a tissue consists of two phases. In the first phase, cell division occurs. In the second phase, cell division has ceased and cell expansion initiates (Gonzalez et al., 2012; Aras, 2022a). In the current experiment, we evaluated pepper leaf midrib cell expansion and division. All treatments improved plant growth compared to control, parallel with that the cell expansion increased. The increase in the cell expansion may be related with the ability of nitrogen fixation of the bacteria. N influences cell expansion, growth and development (Scheible et al., 2004). Moreover, the effect of P also studied in cell division and expansion (Kavanová et al., 2006) and the authors stated that P slightly affected cell division and expansion. Therefore, the growth increment may be attributed to the N-fixing ability rather than P solubilizing. The cell division was not affected by the treatments except ZE-13+SNP. The results showed that the plant growth is a result of cell expansion rather than cell division provided by the PGPRs

and SNP. As far as we know, it is the first report on the effects of useful bacteria on plant cell physiology. SNP also affected the cell division and expansion. Many studies showed that NO promoted cell division and expansion in several plants (Han et al., 2009; Arun et al., 2017) could thus contribute to plant growth increment. NO might regulate the cell division and expansion interacting with auxin and cytokinin (Otvos et al., 2005). Midrib diameter was also assessed in the present study. Midrib consists of xylem, phloem and cortical cells has pivotal roles in water and mineral distribution (Aras et al., 2021, Aras and Endes, 2023). All treatments increased midrib diameter compared to control. The increase in the plant growth is related with the midrib size. Because, water and mineral uptake through midrib influences the plant growth and yield (Aras et al., 2021).

Principal component analysis and heatmap analysis suggest that PGPRs alone or combining with SNP improved the plant growth, cell division and expansion and yield compared to control and SNP alone application. The interpretive usefulness of PCA has been reported in many studies (Carillo et al., 2019; Kılıç, 2023).

CONCLUSION

Our results demonstrated that the PGPRs alone or combining with SNP improved the pepper plant growth and yield. SNP increased the effect of ZE-13 bacteria on cell division in leaf cells and midrib size. Furthermore, ZE-12 increased the yield combining with SNP compared to alone use. The application of PGPR with SNP could be a promising approach in plant growing.

Compliance with Ethical Standards

Peer-review

Externally peer-reviewed.

Declaration of Interests

The authors declared that for this research article, they have no actual, potential or perceived conflict of interest.

Author contribution

The contribution of the authors to the present study is equal. All the authors read and approved the final manuscript. All the authors verify that the Text, Figures, and Tables are original and that they have not been published before.

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