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Comparative Assessment of Gamma-Polyglutamic Acid and *Bacillus subtilis* cells as Biostimulants to Improve Rice Growth and Soil Quality

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ABSTRACT
Chemical fertilizers have been widely used to improve rice production; however, their excessive use can have harmful environmental effects. Therefore, biostimulants are a sustainable option to promote rice yield and improve soil quality. This research focusses on the use of gamma-polyglutamic acid (γ-PGA) and *Bacillus subtilis* cells as biostimulants to improve rice growth and soil quality. The sand culture technique was performed to determine germination and growth of rice seedlings, and greenhouse experiments were conducted to evaluate the performance of rice yields. The soil quality was investigated by measuring physical and chemical characteristics. The results demonstrated that γ-PGA and *B. subtilis* cells were efficient biostimulants for germination by significantly increasing the seedling vigor index. γ-PGA considerably improved the growth parameters of 21-day-old rice seedlings by significantly increasing dry weight, total sugar, total free amino acids and total chlorophyll content compared to the control. In greenhouse experiments, γ-PGA had a positive influence on all physical characteristics and rice grain yield indicators compared to *B. subtilis* cells and controls. Furthermore, γ-PGA and *B. subtilis* cells had a stronger impact than controls on improving soil quality, and γ-PGA had a notable effect on soil physical properties rather than on their chemical properties. Based on these findings, γ-PGA outperformed *B. subtilis* cells as a natural biostimulant to increase rice productivity and improve the quality of paddy soil.

Keywords: microbial products, paddy soil, plant growth regulator, rice productivity, soil quality improvement, soil amendment

INTRODUCTION

In conventional agriculture, chemical fertilizers have been commonly applied to improve soil fertility, particularly those that involve rice (*Oryza sativa* L.), which is the main staple food consumed by almost all Asian countries. One of the main rice producers in the world is Thailand, where synthetic fertilizer use has increased more than tenfold, from approximately 20 kg/ha in 1980 to about 250 kg/ha in 2008. More than 95 percent of agricultural synthetic fertilizers are imported (Suebpongsang et al., 2020). Global consumption of nitrogen (N), phosphorus (P_2O_5) and potassium (potash) increased from 184,017 in 2015 to 201,667 tons in 2020 (Food and Agriculture Organization of the United Nations, 2017). Depending on the types of soil, fertilizers and plants, the amount of fertilizer taken up by plants is typically low, leading to a loss of 60% to 90% of the fertilizer to the environment (Adesemoye and Kloepper, 2009). As a result, crop productivity does not always increase linearly with the use of chemical fertilizer (Iqbal et al., 2020) and excessive fertilizer generates serious environmental problems, including greenhouse gas emissions (Li et al., 2020), surface water eutrophication, soil acidity, soil organic matter and soil fertility degradation (Lou et al., 2022; Wang et al., 2020).

As the demand for organic products increases, organic rice cultivation is becoming more popular, thereby increasing the need for organic fertilizers. Organic fertilizers can be produced from animal, plant or microbial sources, and their use can influence soil physicochemical properties due to the organic matter content and nutritional balance (Shang et al., 2020). Numerous studies have recently been carried out on the impact of organic fertilizers and other alternatives on rice grain yield. The application of a cow manure-based organic fertilizer led to a significant increase in rice yield. (Aziz et al., 2014; Atman et al., 2018). Vermicompost is another organic agricultural fertilizer that is nutritionally and microbially active and includes growth hormones (Joshi et al., 2015). Although the application of organic manure can aid in soil rehabilitation, it is insufficient to provide the essential nutrients required for optimal growth. This leads to the use of chemical and organic fertilizers in combination, which has been observed in many studies focusing on improving productivity and crop yield. Anisuzzaman et al. (2021) demonstrated that the combination of organic and inorganic fertilization improves not only crop yield but also soil fertility. Vermicompost, on the other hand, significantly increases rice productivity when combined with other traditional inputs (Rahman and Barmon, 2019). Since a single application of organic fertilizer may be insufficient to meet the nutritional requirements of the crop, the development of an alternative organic fertilizer rich in minerals such as nitrogen or phosphorus may be a viable alternative.

One of the most effective strategies for sustainable crop management techniques to maintain soil fertility and quality is the use of beneficial microorganisms and/or their secreted compounds. Among various genera, *Bacillus* species are gram-positive, aerobic, endospore forming, and plant growth-promoting rhizobacteria (PGPR) that exist predominantly in the environment (Kashyap et al., 2019). The growth-promoting characteristics of *Bacillus* sp. include the formation of phytohormones, siderophores, and phosphate solubilization, together with 1-aminocyclopropane-1-carboxylate deaminase inputs (Backer et al., 2018; Poveda and González-Andrés, 2021). Some *Bacillus* spp. also exhibit antagonist properties and induce systemic resistance of plants against pathogens (Chowdhury et al., 2015). Ding et al. (2005) revealed that the *nifH* gene in *Bacillus* spp. was responsible for the production of nitrogenase, an enzyme that can fix atmospheric nitrogen and deliver it to plants to promote plant growth. γ-Polyglutamic acid (γ-PGA), an anionic biodegradable biopolymer of high molecular mass made up of D- and L-glutamic acid polymerized by glutamyl linkages, is one of the compounds produced by various species of *Bacillus* (Wang et al, 2022). It is secreted as an extracellular viscous substance that promotes plant growth and provides protection against harmful environmental factors. (Chunhachart et al., 2014; Kotabin et al., 2017). γ-PGA demonstrates tremendous potential and promise for agricultural applications. Several studies demonstrated that γ-PGA improved *Brassica rapa* subsp. *chinensis* productivity and its uptake of nutrients N, P and K (Zhang et al., 2017), as well as the number of tillers, the number of seeds per spike and the yield of winter wheat (Xu et al., 2013b). Additionally, γ-PGA increased the content of soluble amino acids, soluble protein and total nitrogen in Chinese cabbage leaves (Xu et al., 2014). However, few studies have been conducted on rice, which requires a different type of cultivation and weed management in which fields are flooded when young seedlings are planted. Therefore, this research aimed to determine the effect of γ-PGA and *Bacillus subtilis* cells as biostimulants on rice growth and soil quality characteristics. The findings of this study could be useful for future applications of γ-PGA or *Bacillus* cells as an alternative to chemical fertilizers to accelerate rice growth and improve some soil quality.

MATERIAL AND METHODS

Preparation of γ-PGA and B. subtilis cells

γ-PGA was prepared according to the method described by Kotabin et al. (2017). *Bacillus subtilis* NBRC16449 was obtained from the Division of Microbiology, Department of Science and Bio-innovation, Faculty of Liberal Arts and Sciences, Kasetsart University, Thailand, cultured in Luria Bertani broth (LB) broth and incubated at 37±2 °C with 150 rpm for 18 h. Then, 5 ml of inoculum was added to 100 ml of γ-PGA before incubation at 37±2 °C for 48 h with shaking at 150 rpm. Bacterial cells were centrifuged at 8,000 rpm at 4 °C for 15 min. γ-PGA in the supernatant was separated with cold ethanol and desalted by dialysis at 4 °C for 24 h. The γ-PGA solution was freeze dried and kept in a desiccator prior to use. For cell preparation, the cell pellet was washed once with 0.2 M phosphate buffer (pH 7) and centrifuged at 8,000 rpm at 4 °C for 15 min. The pellet was resuspended in a 0.1% peptone physiological salt (PPS) solution after the supernatant was removed. The OD_{650} value of the cell suspension was then adjusted to 1.0 with a PPS solution to achieve approximately 10^8 cfu/ml. Subsequently, concentrations of 10^2, 10^4, 10^6, and 10^8 cfu/ml were prepared using a 10-fold dilution method.

Germination of rice seedlings

Oryza sativa L. cv. KDML 105 rice seeds were acquired from the Phayao Rice Seed Center in Phayao Province, Thailand. Seeds were surface sterilized with 10% sodium hypochlorite for 10 min before being washed with distilled water (Kotabin et al., 2017). The germination of rice seedlings was carried out using a modified sand culture test (Othman et al., 2017). Briefly, the sand was sieved, washed seven times with tap water, and then dried at 180 ± 2 °C for 3 h. Sand was mixed with γ-PGA and *B. subtilis* cells to achieve final concentrations of 50, 100, 300, and 500 mg/kg of sand and 10^2, 10^4, 10^6, and 10^8 cfu/kg of sand. Twenty-five milliliters of deionized water and 0.1% PPS solution were used as controls. Ten seeds were sown in a plastic pot and watered with 20 ml of distilled water every two days. The seeds were grown in a growth chamber at 28±2 °C with a 12:12 h light-dark cycle (15,000 lux) for 7 days. Germination was monitored at 24-h intervals until 7 days after sowing. The percentage of germination (GP), the germination energy (GE), the speed of germination (SG), and the seedling vigor index (SVI) were obtained (Vibhuti et al., 2015). Physical characteristics, that is, the lengths of the roots and shoots and the fresh and dry weights, were also determined.

Growth and development of 21-day-old rice seedlings

The experiment was carried out using a sand culture test with *Oryza sativa* L. cv. KDML 105 rice seeds and the same concentrations of γ-PGA and *B. subtilis* cells as described above. Twenty-five milliliters of deionized water, 0.1% PPS solution, Hoagland's half- and full-strength nutrient solution were used as controls. During the rice seed growing period, 20 ml of distilled water was used to water once a week in each treatment. The physical characteristics of the rice seeds were investigated after 21 days of growth. Total sugar content was determined using the phenol–sulfuric acid method (Kurzyna-Szklarek et al., 2022) and the total free amino acid content was determined using the ninhydrin carbon dioxide method (Anantharaman et al., 2017) with leucine serving as a standard. The total chlorophyll content was determined using the method described Wintermans and De Mots (1965).

Physical properties and performance of rice yield in the greenhouse

A greenhouse experiment was carried out at the Kamphaeng Saen Campus of Kasetsart University in Nakon Pathom, Thailand. The experiment was carried out from December 2021 to February 2022. The experimental soil was taken from the surface (30 cm depth) of a rice paddy field in Nakhon Pathom Province, Thailand. The soil was then air dried, ground and sieved through a 6 mm mesh to remove rock and plant debris. Then 5 kg of air-dried sieved topsoil was placed in 12-inch plastic pots. Twelve treatments consisting of soil mixed with γ-PGA (50, 100, 300, and 500 mg/kg of soil), soil mixed with *B. subtilis* cells (10^2, 10^4, 10^6, and 10^8 cfu/kg of soil) and four different controls, including deionized water (DI), 0.1% peptone, full-strength dose and half-strength dose of chemical fertilizers were carried out. All treatments were tested in three replicates, totaling 36 pots. Distilled water was added to the plastic pots and stirred with the soil until the soil became sludge. Rice seeds were surface sterilized with 10% sodium hypochlorite and rinsed three times with sterile DI prior to planting (Kotabin et al., 2017). Different N: P: K fertilizer formulas were applied as a positive control: a full-strength dose of 16: 10: 0

fertilizer (0.4 g/pot) on day 20 and a 46: 0: 0 fertilizer (0.1 g/pot) on days 40 and 90 after planting. Half-strength fertilizer doses were applied similarly (0.2 g/pot N: P: K ratio 16: 10: 0; 0.05 g/pot N: P: K ratio 46:0:0). Seven days after planting, each pot was seeded with ten seeds and then trimmed to five healthy seedlings. A two-centimeter depth of water layering was maintained throughout the ripening period. At maturity, 120 days after planting, the plants and rice grains were harvested in each pot for analysis of physical properties. The physical properties of rice, such as the length of the roots and shoots, as well as the fresh and dry weights, were determined. The number of tillers, the number of panicles, the size of the panicle and the fresh and dry weights of the panicle were determined. The parameters of rice yield were determined, such as the number of grains, the grain weight, the 100-grain weight, and the number of unfilled grains.

Soil quality analysis

After harvesting rice seedlings, soil samples were air-dried, ground and sieved through a 2-mm mesh for further analysis. Soil bulk density was determined according to Black and Hartge (1986), while soil moisture and porosity were determined using the method of Carter and Ball (1993). The organic matter (OM) in the soil was measured as described by Nelson and Sommers (1996). Standard soil analytical protocols were used to determine pH, electrical conductivity (EC), cation exchange capacity (CEC), total nitrogen, total iron, available phosphorus and exchangeable cations (K^+, Ca^{2+}, Mg^{2+}, Na^+) (van Reeuwijk, 2002).

Statistical analysis

Each experiment was carried out three times. The results are presented as averages and standard deviations (SD). All data were statistically evaluated using analysis of variance, then Duncan's multiple range test (DMRT) to confirm significant differences between treatments at $p \leq 0.05$. The principal component analysis (PCA) was calculated using the multivariate statistical package (MVSP) for Windows.

RESULTS AND DISCUSSION

Effect of γ-PGA and *Bacillus* cells on the germination, growth, and development of rice seedlings

All germination measures, such as germination percentage, germination energy, and germination speed, were found to follow the same trend, with 100% of these values observed in plants treated with 300 and 500 mg/kg γ-PGA and 1×10^6 and 1×10^8 cfu/kg *Bacillus* cells (Figure 1). Furthermore, SVI clearly increased as the concentrations of γ-PGA and *Bacillus*

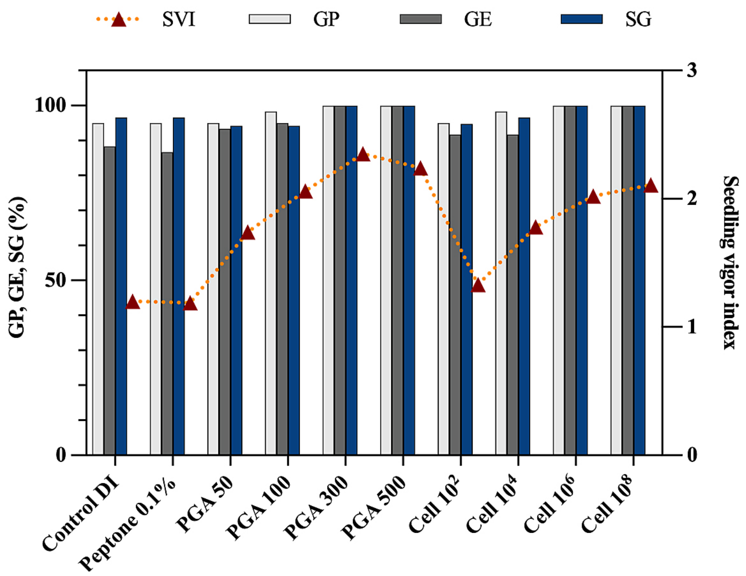

Figure 1. Effect of γ-PGA and *B. subtilis* cells on 7-day-old germination parameters (GP = germination percentage, GE = germination energy and SG = speed of germination)

cells increased. Here, 500 mg/kg γ-PGA had a higher SVI value (2.24) than 10^8 cfu/kg *Bacillus* cells (2.11) (Figure 1). It is likely that γ-PGA significantly increased the ability of plants to absorb nutrients by increasing both root biomass and activity (Zhang et al., 2017). Glutamic acid, the structural unit of γ-PGA, can affect plant development by serving as a signaling molecule, particularly by influencing root development (Forde, 2014) and considerably accelerating callus induction and embryogenic callus development (Sun and Hong, 2010).

Furthermore, all concentrations of γ-PGA and *Bacillus* cells tended to increase the length of the rice seedling shoot compared to controls. Rice seedlings treated with 300 and 500 mg/kg γ-PGA had the highest dry weight, with values of 23.5 and 22.3 mg, respectively. It should be noted that the root length of these treatments was shorter than that of the controls (Table A1); however, according to observations, the γ-PGA treatments showed a stimulating effect on the development of the lateral roots (Figure A1). This could be due to the positive effect of the L-Glu structural unit in γ-PGA. Walch-Liu et al. (2006) reported that external L-glutamate at concentrations ranging from 50 µM to 50 mM could limit primary root growth while stimulating lateral root branching in *Arabidopsis*, leading to increased plant root density inside glutamate rich regions of the soil. Other studies have reported the effective use of nano γ-PGA as a carrier system for the plant growth regulator gibberellic acid (GA_3), resulting in 74.1% more lateral roots for *Phaseolus vulgaris* compared to seeds treated with GA_3 alone (Pereira et al., 2017)

Table A1. Effect of γ-PGA and *B. subtilis* cells on the physical parameters of the 7-day-old germination test

Treatment	Shoot length (cm)	Root length (cm)	Fresh weight (mg)	Dry weight (mg)
Control DI	$5.09±0.03^{b}$	$10.29±0.07^{a}$	$66.2±1.4^{b}$	$12.6±0.2^{ab}$
Peptone 0.1%	$5.11±0.11^{b}$	$9.80±0.13^{ab}$	$90.1±6.7^{a}$	$12.5±1.4^{b}$
γ-PGA 50 mg/kg	$5.30±0.08^{ab}$	$8.99±0.10^{b}$	$83.6±3.8^{a}$	$18.3±2.0^{ab}$
γ-PGA 100 mg/kg	$5.58±0.02^{ab}$	$7.80±0.60^{cd}$	$85.1±1.5^{a}$	$20.9±4.7^{ab}$
γ-PGA 300 mg/kg	$5.51±0.14^{ab}$	$7.63±0.42^{cd}$	$84.2±3.4^{a}$	$23.5±6.2^{a}$
γ-PGA 500 mg/kg	$6.04±0.15^{a}$	$7.15±0.18^{cde}$	$84.0±1.7^{a}$	$22.3±0.9^{ab}$
10^2 cfu/kg	$5.33±0.20^{ab}$	$9.36±0.14^{ab}$	$92.4±0.4^{a}$	$14.0±0.6^{ab}$
10^4 cfu/kg	$5.74±0.07^{ab}$	$8.09±0.33^{c}$	$93.1±0.3^{a}$	$18.1±3.0^{ab}$
10^6 cfu/kg	$5.70±0.07^{ab}$	$6.97±0.25^{de}$	$86.1±7.4^{a}$	$20.2±4.3^{ab}$
10^8 cfu/kg	$5.83±0.61^{ab}$	$6.51±0.06^{e}$	$87.7±6.6^{a}$	$21.1±0.7^{ab}$

Note: Mean ± SD values with different lowercase superscripts are significantly ($p < 0.05$) different; DI = Deionized water; cfu = Colony forming unit.

Figure A1. Effect of γ-PGA on the root of 7-day-old rice seedlings: A = Deionized water, B = γ-PGA 50 mg/kg, C = γ-PGA 100 mg/kg, D = γ-PGA 300 mg/kg, E = γ-PGA 500 mg/kg

The growth of rice seedlings was also observed for a 21-day period using a sand culture test to determine the physical and biochemical parameters of the rice seedlings. Seedlings treated with γ-PGA exhibited a significant increase in shoot length and dry weight, similar to the results of the 7-day germination test (Table 1; Figure 2). This could be because γ-PGA increased the activity of the key enzymes involved in nitrogen assimilation in leaves and roots such as nitrate reductase, glutamine synthetase and glutamate dehydrogenase, resulting in a significant growth increase (Xu et al., 2014). The length of the roots was reduced in several of cases, particularly when a high concentration of γ-PGA (300 and 500 mg/kg) was applied (Table 1; Figure 2), resulting in a lower seedling dry weight when compared to those low concentrations of γ-PGA (50 and 100 mg/kg). The addition of *Bacillus* cells (10^6 and 10^8 cfu/kg) exhibits a similar trend with an increase in γ-PGA concentration, indicating that γ-PGA may be produced during bacterial growth in the seedling rhizosphere. Therefore, the growth of a larger lateral root system in seedlings stimulated by γ-PGA supplied and produced by *Bacillus* cells, as described above, could account for the maximum dry weight of seedlings. This increased lateral root growth is beneficial for agriculture because it increases soil water and nutrient uptake, thus reducing fertilizer use in the field (Lynch and Brown, 2012). This

Table 1. Effect of γ-PGA and *B. subtilis* cells on the physical and biochemical parameters of 21-day-old rice seedlings

Treatment	Shoot length (cm)	Root length (cm)	Seedling dry weight (g)	Total sugar (mg/g FW)	Total free amino acid (mg/g FW)	Total chlorophyll (μg/g FW)
Control DI	$8.14{\pm}0.25^{f}$	$11.95{\pm}0.17^{ab}$	$3.69{\pm}0.16^{d}$	$3.10{\pm}0.05^{d}$	$0.63{\pm}0.14^{c}$	$28.90{\pm}0.22^{d}$
Peptone 0.1%	$9.06{\pm}0.15^{cd}$	$11.26{\pm}0.05^{bc}$	$5.55{\pm}0.23^{bc}$	$3.22{\pm}0.11^{d}$	$0.42{\pm}0.05^{c}$	$31.21{\pm}0.22^{cd}$
½ HS	$9.63{\pm}0.23^{bc}$	$11.91{\pm}0.08^{ab}$	$5.39{\pm}0.15^{bc}$	$4.03{\pm}0.25^{cd}$	$0.81{\pm}0.36^{c}$	$33.01{\pm}0.47^{bcd}$
FHS	$9.84{\pm}0.05^{b}$	$12.42{\pm}0.03^{a}$	$5.25{\pm}0.28^{c}$	$4.88{\pm}0.34^{bc}$	$1.29{\pm}0.18^{bc}$	$38.10{\pm}1.09^{ab}$
γ-PGA 50 mg/kg	$11.51{\pm}0.16^{a}$	$10.83{\pm}0.60^{cd}$	$6.75{\pm}0.14^{a}$	$5.72{\pm}0.39^{ab}$	$1.39{\pm}0.16^{bc}$	$38.66{\pm}0.13^{ab}$
γ-PGA 100 mg/kg	$10.94{\pm}0.16^{a}$	$9.46{\pm}0.10^{e}$	$6.29{\pm}0.48^{ab}$	$6.37{\pm}1.08^{a}$	$2.48{\pm}0.14^{b}$	$37.09{\pm}2.23^{abc}$
γ-PGA 300 mg/kg	$11.15{\pm}0.17^{a}$	$8.97{\pm}0.13^{ef}$	$6.08{\pm}0.44^{abc}$	$6.24{\pm}5.57^{ab}$	$3.89{\pm}0.67^{a}$	$37.97{\pm}2.68^{ab}$
γ-PGA 500 mg/kg	$11.40{\pm}0.04^{a}$	$8.83{\pm}0.06^{ef}$	$6.04{\pm}0.23^{abc}$	$6.47{\pm}0.09^{a}$	$3.94{\pm}1.27^{a}$	$42.77{\pm}1.32^{a}$
10^2 cfu/kg	$8.51{\pm}0.40^{def}$	$11.11{\pm}0.10^{c}$	$6.12{\pm}0.33^{abc}$	$5.39{\pm}0.05^{ab}$	$0.59{\pm}0.03^{c}$	$38.98{\pm}0.34^{ab}$
10^4 cfu/kg	$8.78{\pm}0.23^{def}$	$10.28{\pm}0.06^{d}$	$6.03{\pm}0.21^{abc}$	$5.57{\pm}0.10^{ab}$	$0.74{\pm}0.03^{c}$	$39.42{\pm}2.67^{ab}$
10^6 cfu/kg	$8.84{\pm}0.25^{de}$	$9.46{\pm}0.35^{e}$	$5.91{\pm}0.46^{abc}$	$5.83{\pm}0.13^{ab}$	$1.11{\pm}0.08^{bc}$	$39.38{\pm}4.83^{ab}$
10^8 cfu/kg	$8.16{\pm}0.05^{ef}$	$8.50{\pm}0.09^{f}$	$6.02{\pm}0.05^{abc}$	$5.68{\pm}0.30^{ab}$	$1.28{\pm}0.16^{bc}$	$42.43{\pm}1.00^{a}$

Note: Mean ± SD values with different lowercase superscripts are significantly ($p < 0.05$) different; DI = Deionized water; ½ HS = Half strength of the Hoagland solution; FHS = Full strength of the Hoagland solution; cfu = Colony-forming unit.

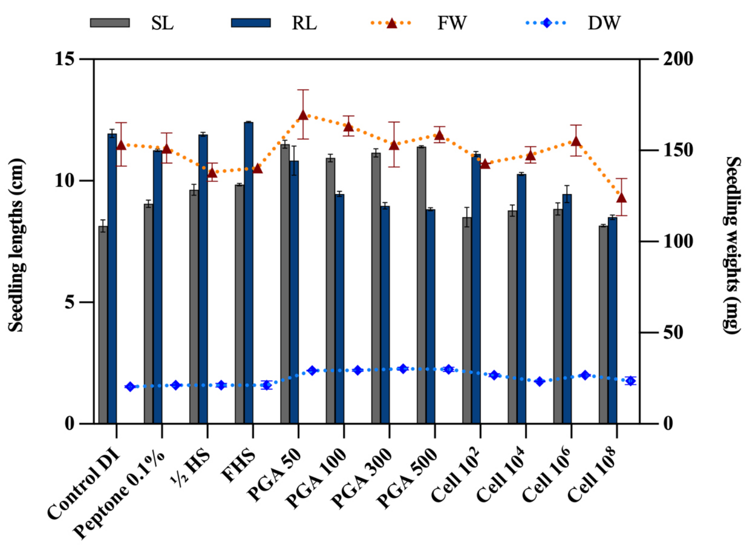

Figure 2. Effect of γ-PGA and *B. subtilis* cells on the physical parameters of 21-day-old seedlings. (SL = seedling length, RL = root length, FW = fresh weight, and DW = dry weight)

could be an advantage of using γ-PGA as a plant growth biostimulant.

High concentrations of γ-PGA and *Bacillus* cells generally resulted in a substantial increase in all biochemical parameters of rice seedlings compared to all controls (Table 1). High total sugar content was observed in seedlings treated with 100, 300 and 500 mg γ-PGA/kg sand. As biostimulant concentrations increased, total sugar levels of rice seedlings increased significantly by 17.21–32.58% and 10.45–19.47% in γ-PGA and *Bacillus* cells, respectively, compared to FHS treatment. A similar response was observed in *Brassica rapa* subsp. chinensis treated with γ-PGA, where the concentration of soluble sugar was 20.8–37.8% higher than that of the positive control during the study period (Zhang et al., 2017). The highest levels of total free amino acids were found in seedlings treated with 300 and 500 mg γ-PGA/kg sand, which showed 3.89 and 3.94 mg/g FW, respectively. On the contrary, no significant differences were observed between *Bacillus* cell-treated seedlings and those treated with FHS (Table 1). The results are consistent with those of Xu et al. (2014), who discovered that γ-PGA significantly increased the content of soluble protein and free amino acids in Chinese cabbage leaves. In contrast, Zhang et al. (2017) observed a decrease in the free amino acid content of *Brassica rapa* subsp. *chinensis* grown in soil treated with γ-PGA. This could be due to differences in the types of plants and growth conditions used. The highest amount of total chlorophyll was produced when biostimulants were used at the highest concentrations (500 mg γ-PGA/kg sand and 108 cfu/kg sand). This result was comparable to that of Xu et al. (2013a), who discovered that γ-PGA increased the chlorophyll content of rapeseed. Furthermore, *B. subtilis* can increase plant photosynthetic activity by modifying leaf photosynthetic performance and chlorophyll concentration in *Vicia faba*.

Improvement of the physical properties and performance of rice production in the greenhouse

A greenhouse experiment was carried out to evaluate the influence of γ-PGA and *Bacillus* cells on rice production, growth and development. γ-PGA and *Bacillus* cells, as well as positive controls, dramatically improved the length of the rice shoot compared to the negative control (DI). Although a similar trend was observed with 21-day-old rice seedlings, the root lengths tended to be shorter than those in the control group. However, our observations indicate that the lateral roots of rice treated with γ-PGA appear to be considerably larger. All γ-PGA and *Bacillus* cell concentrations increased fresh weight, varying from 14.12 to 16.79 g. Additionally, γ-PGA at 50 mg/kg significantly improved the dry weight of rice (Table A2). All applied concentrations of γ-PGA resulted in a high number of tillers and panicles, especially the lowest concentration of γ-PGA at 50 mg/kg. The maximum concentration of *Bacillus* cells (10^8 cfu/kg) tended to promote these parameters as well. Furthermore, 500 mg/kg γ-PGA produced the highest panicle sizes and the highest fresh and dry weights (Table 2). This could be

Table A2. Effect of γ-PGA and *B. subtilis* cells on some physical parameters of rice in greenhouse experiment

Treatment	Shoot length (cm)	Root length (cm)	Shoot + Root fresh weight (g)	Shoot + Root dry weight (g)
Control DI	121.23±4.14^{b}	28.04±0.90^{ab}	8.54±0.05^{b}	3.69±0.19^{d}
Peptone 0.1%	135.18±2.64^{a}	28.02±0.54^{ab}	14.72±0.69^{a}	5.55±0.23^{bc}
½ FCF	130.38±0.97^{a}	28.70±0.97^{a}	13.83±0.19^{a}	5.39±0.15^{bc}
FCF	131.18±1.26^{a}	28.05±0.64^{ab}	11.83±2.37^{ab}	5.25±0.28^{c}
γ-PGA 50 mg/kg	136.27±1.37^{a}	27.34±0.68^{abc}	14.12±0.42^{a}	6.75±0.14^{a}
γ-PGA 100 mg/kg	135.28±1.50^{a}	28.03±0.54^{ab}	16.79±3.52^{a}	6.29±0.48^{ab}
γ-PGA 300 mg/kg	129.43±4.78^{a}	27.06±0.76^{abc}	15.97±0.50^{a}	6.08±0.44^{abc}
γ-PGA 500 mg/kg	135.96±0.83^{a}	26.00±0.16^{c}	15.16±1.33^{a}	6.04±0.23^{abc}
10^2 cfu/kg	129.29±1.15^{a}	26.77±0.23^{abc}	16.67±0.83^{a}	6.12±0.33^{abc}
10^4 cfu/kg	131.31±1.95^{a}	27.54±0.05^{abc}	15.56±1.01^{a}	6.03±0.21^{abc}
10^6 cfu/kg	128.97±1.73^{a}	27.26±0.42^{abc}	16.65±0.39^{a}	5.91±0.46^{abc}
10^8 cfu/kg	130.55±3.34^{a}	26.38±0.32^{bc}	16.50±1.74^{a}	6.02±0.05^{abc}

Note: Mean ± SD values with different lowercase superscripts are significantly ($p < 0.05$) different; DI = Deionized water; ½ FCF = Half strength of chemical fertilizer; FCF = Full strength of chemical fertilizer; cfu = Colony forming unit

Table 2. Effect of γ-PGA and *B. subtilis* cells on the physical parameters of rice in the greenhouse experiment

Treatment	Number of tillers (per plant)	Number of panicles (per pot)	Size of panicle (cm)	Panicle fresh weight (g)	Panicle dry weight (g)
Control DI	2.06±0.06^{e}	10.00±0.57^{c}	17.68±0.69^{e}	2.180±0.058^{d}	1.510±0.026^{b}
Peptone 0.1%	2.80±0.11^{d}	14.66±2.02^{b}	18.94±0.35^{de}	2.926±0.099^{abc}	2.076±0.043^{a}
½ FCF	2.93±0.13^{cd}	15.00±1.00^{b}	18.40±0.13^{e}	2.681±0.168^{abc}	1.917±0.159^{ab}
FCF	2.93±0.13^{cd}	15.33±0.88^{b}	20.27±0.39^{cd}	2.874±0.053^{ab}	2.194±0.039^{a}
γ-PGA 50 mg/kg	3.80±0.20^{a}	19.66±2.33^{a}	22.66±0.66^{a}	2.812±0.152^{abc}	2.213±0.022^{a}
γ-PGA 100 mg/kg	3.26±0.17^{bc}	16.00±0.00^{ab}	22.82±0.81^{a}	2.692±0.059^{abc}	2.136±0.162^{a}
γ-PGA 300 mg/kg	3.46±0.13^{ab}	17.33±0.33^{ab}	22.80±0.51^{a}	3.010±0.071^{ab}	2.198±0.050^{a}
γ-PGA 500 mg/kg	3.46±0.06^{ab}	17.00±0.57^{ab}	23.24±0.46^{a}	3.032±0.188^{a}	2.263±0.219^{a}
10^{2} cfu/kg	2.93±0.06^{cd}	18.00±2.51^{ab}	20.62±0.18^{bc}	2.553±0.111^{c}	2.145±0.234^{a}
10^{4} cfu/kg	3.00±0.00^{cd}	15.00±0.00^{b}	21.22±0.14^{bc}	2.674±0.010^{abc}	2.047±0.303^{a}
10^{6} cfu/kg	3.00±0.11^{cd}	15.00±0.57^{b}	21.99±0.37^{ab}	2.613±0.169^{bc}	2.072±0.167^{a}
10^{8} cfu/kg	3.06±0.06^{cd}	18.33±0.88^{ab}	20.72±0.20^{bc}	2.818±0.131^{abc}	2.028±0.187^{a}

Note: Mean ± SD values with different lowercase superscripts are significantly ($p < 0.05$) different; DI = Deionized water; FCF = Full strength of chemical fertilizer; ½ FCF = Half strengths of chemical fertilizer; cfu = Colony-forming unit.

because, as previously stated, γ-PGA had a direct impact on the NO_3^-–N level in the soil, which is a key factor in promoting these growth characteristics. Xu et al. (2016) demonstrated that up-regulation of genes involved in nitrogen assimilation and synthesis of brassinosteroids, jasmonic acid, and lignins by γ-PGA provides a better explanation for why γ-PGA increases plant growth and improves stress tolerance. Another explanation, based on soil quality experiments, is that γ-PGA affects the bulk and porosity of the soil, which also had a related effect on the community of rhizosphere bacteria, particularly plant growth promoting bacteria, in the soil. γ-PGA improves the yield of Chinese cabbage by increasing the relative abundances of potential plant growth-promoting bacteria in the soil in a concentration-dependent manner (Bai et al., 2020) and plays a role in colonization of bacterial roots, indicating a potential role for γ-PGA in *B. subtilis*–plant interactions (Yu et al., 2016).

In this experiment, *B. subtilis* NBRC16449 is capable of producing γ-PGA, which benefits plants, and *B. subtilis* NBRC16449 itself is capable of secreting enzymes that degrade PGA to hydrolyze PGA to glutamate, which can be metabolized to ammonia or nitrate through soil microbes (Su et al., 2020). *Bacillus* is one of the most studied rhizobacteria that promotes plant growth and development. The colonization of plant roots by *B. subtilis* is advantageous for both the bacterium and the host plant. In *B. subtilis*, chemotaxis plays an important role in the location and colonization of young roots (Allard et al., 2016). The bacterial genome contains several chemoreceptor genes that govern cell development and the connection between bacteria and other living species. The chemoreceptors found in *B. subtilis* allow it to locate a specific habitat, plant roots (Su et al., 2020; Yang et al., 2015). Previous research demonstrated that rice plant exudates attract *Bacillus* species (Bacilio et al., 2003), and *Arabidopsis* root exudates play a crucial role in attracting *B. subtilis* while promoting root colonization (Allard et al., 2016). Some species of *Bacillus* release ammonia from nitrogenous organic matter (Hayat et al., 2010). *Bacillus* spp. secretes phosphatases and organic acids that acidify the surrounding environment to accelerate the conversion of complex inorganic phosphate to a simple available phosphate that is taken up by plant roots (Kang et al., 2014)

γ-PGA demonstrated the potential to improve all indicators of rice grain yield performance. γ-PGA at 50 mg/kg resulted in a considerable increase in the number of grains/pot. γ-PGA also had a positive effect on grain weight and 100 grain weight compared to controls. Additionally, all γ-PGA concentrations had the lowest value for the number of unfilled grains. Compared to the negative control, the increase in grain number clearly shows that 50 mg/kg of γ-PGA produced the maximum percentage of 192 followed by 10^8 cfu/kg *Bacillus* cells with PIC of 161% (Table 3).

Data analysis using PCA showed that axes 1 and 2 represented 80.7% and 9.67% of total variance, respectively (Figure 3). All γ-PGA concentrations had a beneficial effect on both the

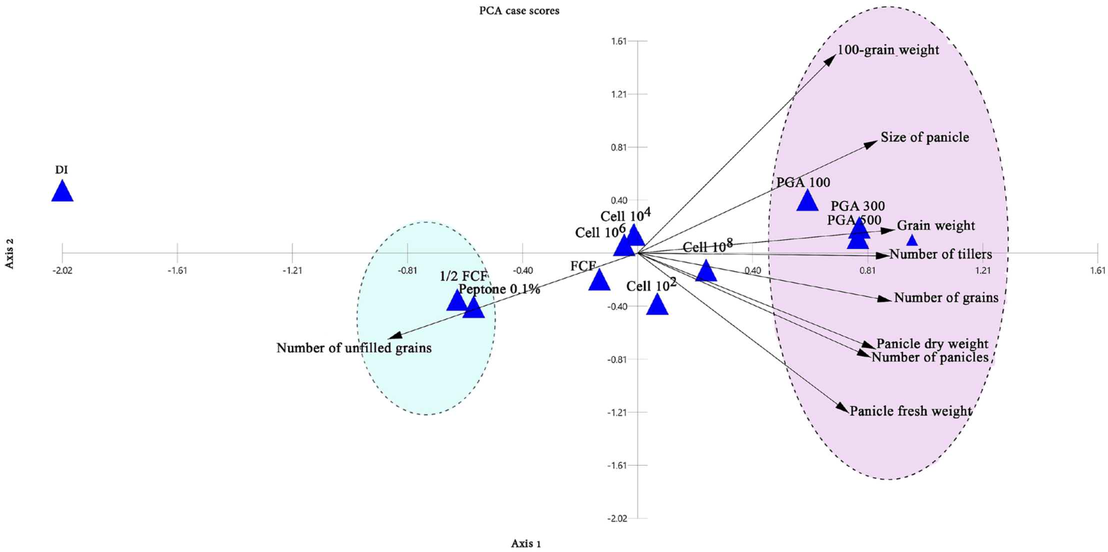

Figure 3. Principal component analysis (PCA) of rice yield parameters under different treatments (DI = Deionized water; FCF = Full strength of chemical fertilizer; ½ FCF = Half strength of chemical fertilizer)

Table 3. Effect of γ-PGA and *B. subtilis* cells on rice yield performance

Treatment	Number of grains (grain/pot)	Grain weight (g/pot)	100-grain weight (g)	Number of unfilled grains (grain/plant)	PIC (%)
Control DI	$695.73±31.64^{e}$	$194.73±10.17^{e}$	$2.33±0.013^{bc}$	$18.30±1.53^{a}$	-
Peptone 0.1%	$1,316.79±221.41^{d}$	$314.65±28.24^{d}$	$2.33±0.012^{bc}$	$15.12±1.64^{b}$	89
½ FCF	$1,367.63±147.62^{cd}$	$357.23±12.36^{cd}$	$2.33±0.03^{bc}$	$16.21±0.72^{ab}$	96
FCF	$1,441.91±61.17^{bcd}$	$380.74±8.34^{bcd}$	$2.38±0.03^{abc}$	$11.50±0.61^{c}$	107
γ-PGA 50 mg/kg	$2,032.38±184.30^{a}$	$517.83±41.20^{a}$	$2.47±0.05^{abc}$	$6.06±0.36^{d}$	192
γ-PGA 100 mg/kg	$1,732.64±35.30^{abc}$	$521.77±12.40^{a}$	$2.51±0.09^{a}$	$6.41±0.48^{d}$	149
γ-PGA 300 mg/kg	$1,812.09±34.86^{ab}$	$527.40±23.92^{a}$	$2.48±0.08^{ab}$	$5.54±0.60^{d}$	160
γ-PGA 500 mg/kg	$1,820.03±105.55^{ab}$	$517.00±22.58^{a}$	$2.45±0.04^{abc}$	$5.62±0.43^{d}$	161
10^2 cfu/kg	$1,735.50±219.46^{abc}$	$460.26±55.01^{ab}$	$2.31±0.04^{c}$	$9.72±0.20^{c}$	149
10^4 cfu/kg	$1,465.35±30.19^{bcd}$	$419.33±32.29^{bc}$	$2.43±0.07^{abc}$	$9.54±0.49^{c}$	110
10^6 cfu/kg	$1,457.85±97.49^{bcd}$	$408.39±26.50^{bc}$	$2.39±0.01^{abc}$	$10.24±0.55^{c}$	109
10^8 cfu/kg	$1,819.06±90.49^{ab}$	$463.94±25.33^{ab}$	$2.41±0.02^{abc}$	$9.81±0.64^{c}$	161

Note: Mean ± SD values with different lowercase superscripts are significantly ($p < 0.05$) different; PIC = Percentage increase in the treatment compared to the control based on number of grains/pot; DI = Deionized water; FCF = Full strength of chemical fertilizer; ½ FCF = Half strength of chemical fertilizer; cfu = Colony-forming unit.

physical and the yield performance indicators of rice. As demonstrated on the right-hand side of axis 1, all concentrations of γ-PGA had a positive impact on the weight of 100 grains, the size of the panicle, the weight of the grains/pot, the number of tillers, the number of grains, the number of panicles and the fresh and dry weights of the panicle. Furthermore, the effect of *Bacillus* cells on these parameters is unclear, except for their maximum concentration (10^8 cfu/kg), which revealed a slight increase in these parameters. To summarize, γ-PGA outperformed *Bacillus* cells in terms of physical properties and rice yield performance and showed a considerable increase in practically all indicators compared to controls. This suggests that γ-PGA could be a viable alternative growth promoter for rice production.

Soil quality

In addition to rice growth and development, soil characteristics are another highly significant aspect to consider. Therefore, an experiment was designed to determine the effect of γ-PGA and Bacillus cells on the physical and chemical qualities of the soil. Both γ-PGA and *Bacillus* cells improved several physical parameters of the soil compared to other positive controls, such as peptone, half- and full-strength chemical fertilizer. Although the difference was not statistically significant, OM amounts increased by 13.73% with the γ-PGA treatment (500 mg/kg) and by 26.67% with *Bacillus* cells (10^8 cfu/kg). Total nitrogen also increased by 25% and 33.3%, respectively, in the γ-PGA and *Bacillus* cells compared to treatment with FHC (Table 4). This finding was supported by Zhang et al. (2017), who found that on Day 1, the γ-PGA treatment had a lower NO_3^-–N level in the soil than the chemical fertilizer treatment. However, from day 7 through the completion of the trial (60 days), the γ-PGA treatment had higher NO_3^-–N concentrations than the chemical fertilizer treatment. This result could imply that γ-PGA will gradually release nitrogen to provide N nutrition to the crop at a later stage of plant growth (Xu et al., 2013a). Treatments with FCF and ½ FHC significantly reduced total Fe, but γ-PGA did not. On the contrary, these treatments resulted in a significantly higher available P content than γ-PGA and *Bacillus* cells. In both γ-PGA and *Bacillus* cells, exchangeable K was shown to provide the largest amount. However, exchangeable Ca and Mg remained consistent across all treatments. Furthermore, when γ-PGA and *Bacillus* cells were added, the amount of exchangeable Na increased significantly compared to controls (Table 4). In terms of physical characteristics of the soil, 500 mg/kg γ-PGA resulted in a significantly increased soil moisture content and bulk density, which were also significantly reduced to 1.12 g/cm^3. Furthermore, soil treated with 500 mg/kg γ-PGA exhibited a significant increase in total porosity (Table 5), which is consistent with Chen et al. (2018), who revealed that the small condensed aggregates formed by γ-PGA reduced the bulk density of the

Table 4 Effect of γ-PGA and *B. subtilis* cells on the chemical properties of soil

Treatment	pH	EC (ds/m)	CEC (me/100g)	OM (%)	Total N (%)	Total Fe (%)	Available P (mg/kg)	Exchangeable cation (mg/kg)			
								K	Ca	Mg	Na
Control DI	7.80^{a}	3.11^{b}	22.14^{ab}	2.01^{a}	0.10^{a}	24.23^{ab}	39.05^{cd}	156.01^{a}	5,607.50^{a}	594.75^{a}	88.75^{ef}
Peptone 0.1%	7.78^{ab}	3.22^{ab}	24.53^{a}	2.19^{a}	0.11^{a}	18.25^{c}	39.31^{bcd}	131.40^{a}	5,520.00^{a}	589.12^{a}	110.95^{d}
½ FCF	7.80^{a}	3.01^{b}	25.82^{a}	2.47^{a}	0.12^{a}	19.12^{c}	40.40^{b}	126.10^{a}	5,252.50^{a}	550.50^{a}	103.15^{e}
FCF	7.80^{a}	3.33^{ab}	26.12^{a}	2.55^{a}	0.12^{a}	20.72^{bc}	43.39^{a}	133.30^{a}	4,435.00^{a}	594.25^{a}	76.37^{f}
γ-PGA 50 mg/kg	7.77^{ab}	3.54^{a}	21.55^{ab}	2.82^{a}	0.14^{a}	25.42^{a}	35.23^{e}	140.18^{a}	5,645.00^{a}	621.12^{a}	137.95^{b}
γ-PGA 500 mg/kg	7.79^{ab}	3.28^{ab}	21.59^{ab}	2.90^{a}	0.15^{a}	24.20^{ab}	38.14^{d}	197.98^{a}	5,942.50^{a}	619.62^{a}	162.00^{a}
10^2 cfu/kg	7 .72^{b}	3.14^{b}	17.92^{b}	3.10^{a}	0.16^{a}	25.28^{a}	39.82^{bc}	182.50^{a}	5,922.50^{a}	628.62^{a}	131.05^{bc}
10^8 cfu/kg	7 .77^{ab}	3.40^{ab}	22.83^{ab}	3.23^{a}	0.16^{a}	21.25^{abc}	39.01^{cd}	319.89^{a}	5,288.75^{a}	626.75^{a}	118.50^{cd}

Note: Mean ± SD values with different lowercase superscripts are significantly ($p < 0.05$) different; DI = Deionized water; FCF = Full strength of chemical fertilizer; ½ FCF = Half strength of chemical fertilizer; cfu = Colony-forming unit; EC = Electrical conductivity; CEC = cation exchange capacity; Total N = total nitrogen; Total Fe = Total iron.

Table 5. Effect of γ-PGA and *B. subtilis* cells on the physical properties of the soil

Treatment	Soil physical properties		
	Soil moisture (%)	Bulk density (g/cm^3)	Total porosity (%)
Control DI	34.47±0.81^{b}	1.79±0.01^{a}	36.01±0.90^{c}
Peptone 0.1%	34.11±1.00^{b}	1.82±0.06^{a}	36.81±0.50^{bc}
½ FCF	34.77±1.55^{b}	1.80±0.03^{a}	37.60±0.61^{bc}
FCF	35.10±0.92^{b}	1.83±0.10^{a}	35.13±0.02^{c}
γ-PGA 50 mg/kg	40.07±1.04b	1.49±0.03^{b}	43.73±0.52^{b}
γ-PGA 500 mg/kg	52.18±6.07^{a}	1.12±0.12^{c}	62.76±5.35^{a}
10^2 cfu/kg	35.33±0.77^{b}	1.75±0.06^{a}	38.00±0.17^{bc}
10^8 cfu/kg	38.33±3.34^{b}	1.76±0.03^{a}	40.12±0.88^{bc}

Note: Mean ± SD values with different lowercase superscripts are significantly ($p < 0.05$) different; DI = Deionized water; FCF = Full strength of chemical fertilizer; ½ FCF = Half strength of chemical fertilizer; cfu = Colony-forming unit.

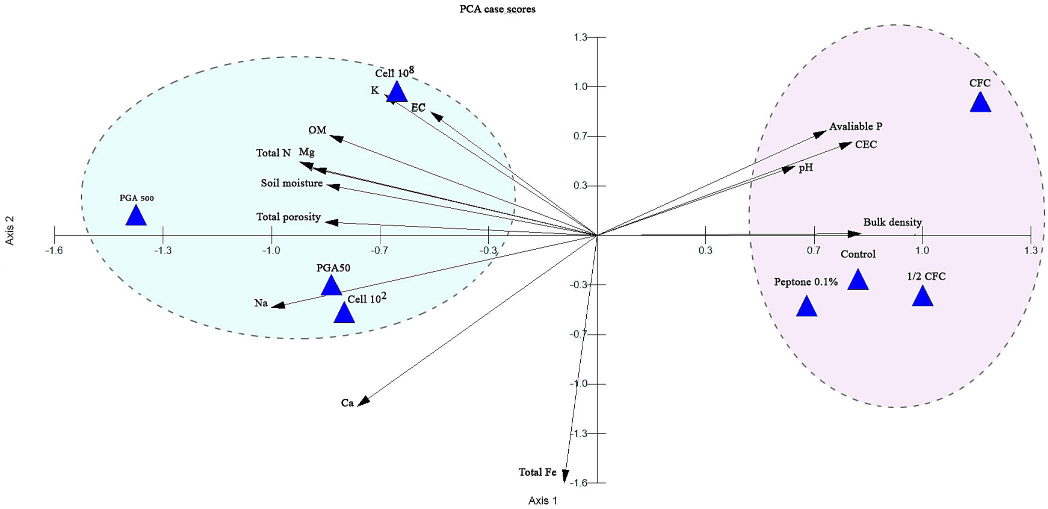

Figure 4. Principal component analysis (PCA) of soil quality parameters under different treatments (DI = Deionized water; FCF = Full strength of chemical fertilizer; ½ FCF = Half strength of chemical fertilizer; EC = Electrical conductivity; CEC = cation exchange capacity; Total N = total nitrogen; Total Fe = Total iron, available P = Available phosphorus)

soil, leading to higher soil porosity and improved soil structure.

The principal component analysis (PCA) demonstrated that axes 1 and 2 of PCA represented 48.2% and 18.40% of the total variance in the dataset, respectively (Figure 4). The correlation between different substances incorporated into the soil and soil quality parameters helped separate the two scenarios, as indicated by PCA axis 1. In Scenario 1, a correlation of available P, CEC, pH, and bulk density was found with all controls on the right-hand side of axis 1. On the contrary, both γ-PGA (50 and 500 mg/kg) and *Bacillus* cells (10^2 and 10^8 cfu/kg) affected EC, OM, total N, exchangeable K, Mg, Na, and Ca, moisture content, and total porosity of the soil, as illustrated on the left-hand side of axis 1 (Scenario 2). Therefore, treatment with γ-PGA and *Bacillus* cells had a greater impact on soil quality than the controls. Additionally, γ-PGA had a noticeable effect on the physical qualities of the soil, rather than their chemical characteristics.

CONCLUSIONS

The rice growth and production study showed that the addition of γ-PGA had a positive effect on all physical parameters of rice and indicators of grain production. *B. subtilis* cells appeared to perform worse than γ-PGA. However, compared to the full dose of chemical fertilizer, the highest concentration of *B. subtilis* cells demonstrated results similar to those of the control. In terms of soil quality, γ-PGA and *B. subtilis* cells increased various soil chemical indicators, including EC, OM quantities, total nitrogen content, and all exchangeable cations. On the other hand, a high concentration of γ-PGA clearly had a favorable influence on the physical qualities of the soil. Based on these findings, γ-PGA clearly demonstrated the potential of its rice growth stimulating effects and improvement of soil quality. However, in *B. subtilis* cells, the results were inconclusive. This could be due to the inappropriate concentration applied in this experiment. Because the cost of γ-PGA is still expensive and may not be suitable for agricultural applications, the production of γ-PGA-containing compost by solid-state fermentation utilizing food waste such as soybean residue or wastewater from the soybean extraction process may be an alternative, although further research is needed.

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