



Enhancing Soybean Growth with Phytohormone-enriched PGPR Bioinoculants and Biofertilizers in Plant Stress Physiology

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ABSTRACT

Background: The present investigation aimed to evaluate the effects of plant growth-promoting rhizobacteria (PGPR) bioinoculants and their carrier-based biofertilizers on the physiology of soybean.

Methods: Seeds of two soybean varieties (cv. NARC-1 and cv. William-82) were either soaked in broth cultures of two PGPR strains, *Planomicrobium chinense* (MF616408) and *Pseudomonas putida* (KX574857), containing 10⁶ cells/mL, or coated with biofertilizers. Three biofertilizers, namely biostimulant, biozote and biofert, were used.

Result: Significant increases in root and shoot weight were observed with both liquid bioinoculants and carrier-based biofertilizers compared to the control. The consortium of *P. chinense* and *P. putida* resulted in the highest proline content and superoxide dismutase (SOD) activity. Biostimulant-treated plants exhibited higher catalase (CAT) activity and phenolics content. Indole-3-acetic acid (IAA) and gibberellic acid (GA) levels were enhanced in plants treated with PGPR and biofertilizers. Specifically, *P. chinense* and the biostimulant significantly promoted IAA content in cv. William-82, while biozote and *P. chinense* treatments increased GA content in both varieties. The consortium of *P. chinense* and *P. putida* showed the lowest ABA levels. The highest IAA/ABA and GA/ABA ratios were observed in plants treated with *P. chinense*. Liquid bioinoculants had a more pronounced effect on plant growth than carrier-based biofertilizers, though biofertilizers had stronger residual effects on rhizosphere soil organic matter. The combined effects of phytohormones, sugar, protein and proline production and antioxidant enzyme activity influenced plant growth and development.

Key words: Biofertilizer, Bioinoculants, PGPR, Phytohormones.

INTRODUCTION

Sustainable agriculture is vital for a growing population. Rhizobacteria, used as inocula and biofertilizers, enhance plant growth by solubilizing nutrients and producing phytohormones like auxins and cytokinins, which lower ethylene levels (Etesami *et al.*, 2015; Mondal *et al.*, 2024). Biofertilizers, containing live PGPR strains, are applied to seeds or soil to improve plant growth and nutrient mobilization (El-Ghamry *et al.*, 2018). Their shelf life is about two to three months (Maheswari and Elakkiya, 2014) and they can supplement chemical fertilizers (Ahmad *et al.*, 2013; Tejesh *et al.*, 2024).

Soybean, rich in oil and protein, benefits from biofertilizers, enhancing sustainable crop production and soil quality (Bhowmik and Das, 2018; Kumari, 2019). Phytohormones like auxins, gibberellins and abscisic acid regulate growth and stress responses. Rhizobacteria produce these hormones, improving plant resilience (Kozłowski and Pallardy, 1996; Liu *et al.*, 2016; Ullah *et al.*, 2018). For example, *Pseudomonas* and *Bacillus* species enhance nutrient content and yield in wheat (Khan *et al.*, 2021; Mutumba *et al.*, 2018).

This study focuses on the effects of PGPR and biofertilizers on soybean growth, emphasizing phytohormone production to optimize biofertilizer use.

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MATERIALS AND METHODS

Seed procurement and setup

Soybean seeds (NARC-1 and William-82) from NARC Islamabad were planted in 24×30 cm pots with a soil-sand mix (3:1), 10 seeds per pot, in a greenhouse at the University of Wah during the 2019-2020 season (25-30°C, 71-75% humidity).

Biofertilizer and bioinoculant preparation

Biozote biofertilizer (NARC) and a biostimulant made from 500 g autoclaved pressed mud and 85 mL *Pseudomonas putida* and *Planomicrobium chinense* broth (OD 0.98±0.01 at 660 nm) were used. Biofert 1 (*Azotobacter chroococcum*), Biofert 2 (*Azospirillum lipoferum*) and Biofert 3 (*Pseudomonas putida*) were also prepared with pressed mud.

Seed sterilization and inoculation

seeds were sterilized with 5% sodium hypochlorite for 2-3 minutes, rinsed and coated with 100 g Biozote and biostimulant using 0.5% sucrose solution. Another set was soaked in broth cultures of *P. chinense*, *P. putida* and their consortium for 72 hours at 28°C (OD 0.99±0.01 at 660 nm). Plant growth and biochemical parameters were assessed 15 days after sowing (Samasegaran *et al.*, 1982; Asad *et al.*, 2004).

Biochemical analyses

Protein content

Protein content of fresh leaves was determined following the method of Waterborg and Matthews (1984).

Proline content

The proline content of leaves was determined following the method of Bates *et al.* (1973).

Phenolics content

Phenolics content of leaves was measured following the method of Singleton and Jones (1999).

Sugar content

The sugar content in plant leaves was determined following the method of Dubois *et al.* (1956) as modified by Johnson *et al.* (1966).

Antioxidant enzyme assays

Superoxide dismutase (SOD) activity

Superoxide dismutase activity was determined following the method of Beauchamp and Fridovich (1976).

Catalase (CAT) activity

Catalase activity was determined following the method of Kumar *et al.* (2010).

Phytohormones content of leaves

IAA, GA and ABA content were determined using Kettner and Dörffling (1995) method. ABA retention time was 6.2 min.

IAA, GA, ABA and SA content of biofertilizers

After two months, biofertilizer (100 g) was suspended in 100 mL water, centrifuged and the supernatant was adjusted to pH 2.3-3.0 for phytohormone extraction (Seskar *et al.*, 1998).

Soil sampling and analysis

Rhizosphere soil samples of soybean were collected after 15 days of seed sowing below 7-10 cm from the surface.

Soil samples were homogenized and sieved through a 2 mm sieve and processed for physicochemical analyses. Soil organic matter was determined following the method of Walkley and Black (1934).

Phosphorus and potassium content of soil:

The phosphorus (P) content of soil was determined using a spectrophotometer following the method of Olsen *et al.* (1954). Potassium (K) content of soil was determined using a flame photometer following the method of Knudsen *et al.* (1983).

Statistical analysis

Data were analyzed using Statistix 8.1 software. ANOVA and Tukey HSD test at a 5% significance level were used for treatment comparisons.

RESULTS AND DISCUSSION

Shoot length

All treatments showed significant increases in shoot length compared to the control (Fig 1). The maximum increase (40% in V1 and 35% in V2) in shoot length was observed in plants treated with a consortium of *Pseudomonas putida* and *Planomicrobium chinense* compared to the control. Both biofertilizers exhibited significant enhancement in shoot length, with no significant differences between them.

Shoot weight

All treatments showed significant increases in shoot weight (Fig 1) compared to the control in both varieties, with the cv. NARC-1 variety being more responsive. The maximum increase (46.8% and 53.4%) was observed in plants of cv. NARC-1 and cv. William-82, respectively, treated with *P. chinense* compared to the control. Biozote and Biostimulant showed similar increases over control, 28% and 35%, respectively.

Root fresh and dry weight

The maximum increase in root fresh weight was observed in *P. chinense* inoculated plants: 87% for cv. NARC-1 and 76% for cv. William-82. *Pseudomonas putida* also significantly increased root dry weight. Biostimulant treatment resulted in a 71% increase in V1 and 73% in V2. Biozote biofertilizers showed similar effects on fresh root weight in both varieties, with increases of 39% in V1 and 37% in V2. Biozote biofertilizer also increased root dry weight by 39.4% in cv. NARC-1 and 31.3% in cv. William-82. The consortium of *P. putida* and *P. chinense* further increased fresh root weight by 52% in V1 and 55% in V2.

Phytohormone content of leaves

IAA content

All treatments significantly increased IAA content compared to the control, with the highest increase in cv. William-82 treated with *P. chinense*, showing a 3.8-fold increase (Fig 2).

GA content

All treatments significantly increased GA content. *P. chinense* treatment led to an 8.2-fold increase in cv. William-82.

Biozote biofertilizers also increased GA content but to a lesser extent (3.7- and 4.2-fold in cv. NARC-1 and cv. William-82, respectively) (Fig 2).

ABA content

All treatments, except the PGPR consortium, significantly decreased ABA content. *P. chinense* and *P. putida* showed notable reductions, particularly in cv. William-82.

Biofertilizers decreased ABA content by 42% in V1 and V2, while cv. William-82 showed a 30% decrease (Fig 2).

Phytohormones ratio

The IAA/ABA ratio reflects the balance between the growth-promoting hormone IAA and the stress-responsive hormone abscisic acid (ABA). IAA/ABA ratio was highest in *P. chinense*-treated plants, indicating growth promotion.

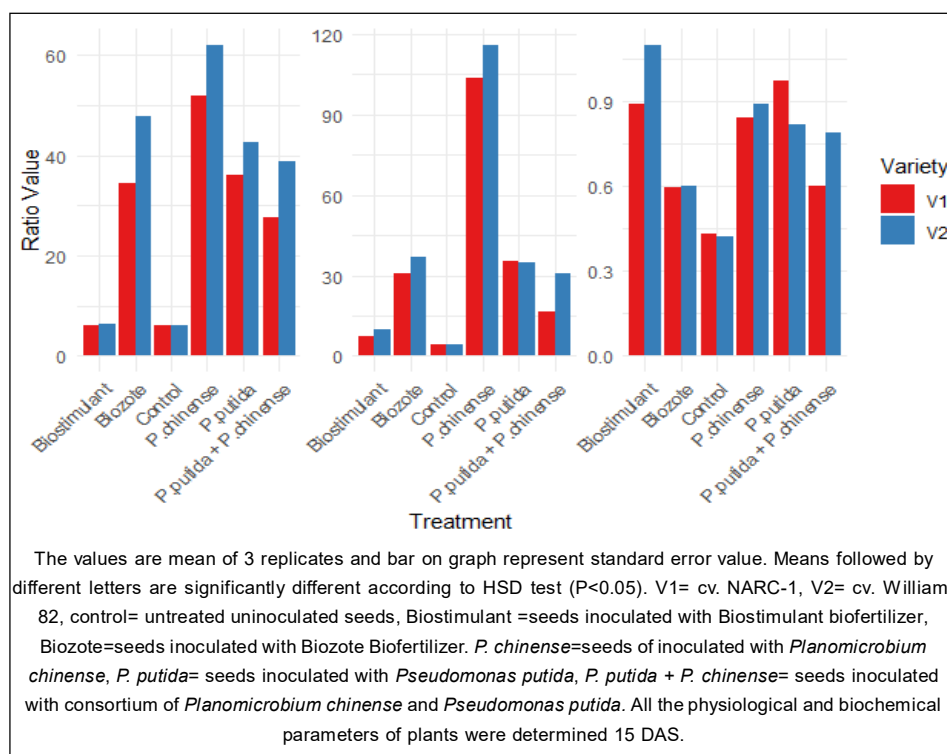


Fig 1: Effect of biofertilizers/bioinoculants on shoot length (cm), shoot and root fresh weight (g) of soybean plants.

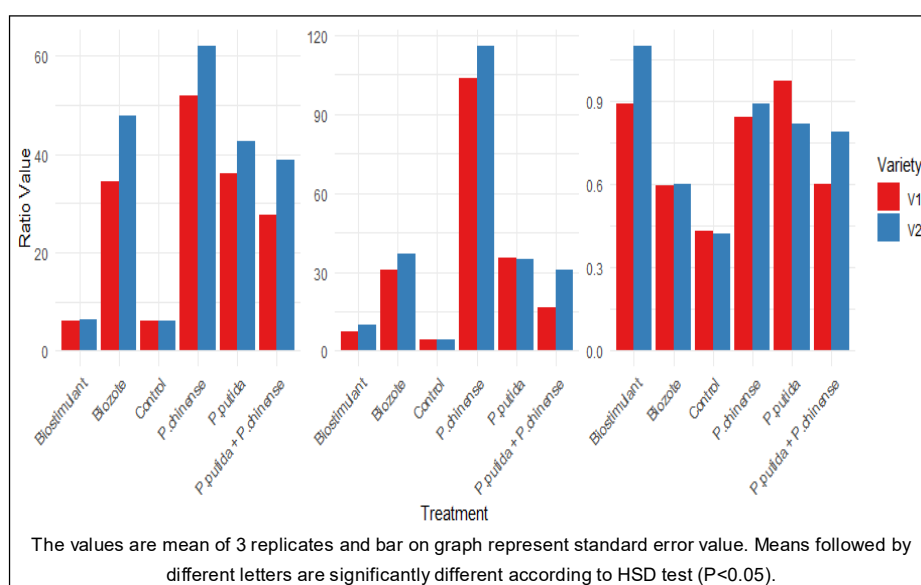


Fig 2: Effect of biofertilizers/bioinoculants on IAA, GA and ABA content of leaves (ug/100 g) respectively.

Lowest in biostimulant-treated plants, suggesting a balanced stress response. Whereas the GA/ABA ratio highest in *P. chinense*-treated plants, indicating growth promotion. Lowest in biostimulant-treated plants, indicating a higher stress response (Table 1). Overall, the data suggest that different treatments influence the phytohormonal balance differently in soybean plants, with *P. chinense* treatment generally resulting in a hormonal profile skewed towards growth promotion, while biostimulant treatment tends to induce a more balanced hormonal response, potentially enhancing stress tolerance.

Correlation analysis of phytohormones and growth parameters

In cv. NARC-1 (V1), significant positive correlations were found between fresh root weight and IAA and GA content and between GA content and shoot weight. ABA content negatively correlated with IAA, GA and growth parameters. In cv. William-82 (V2), significant positive correlations were found between shoot length, shoot weight, fresh root weight

and IAA and GA content. Notably, shoot length had a highly significant positive correlation with GA content (Table 2).

Phytohormone content of biofertilizers

IAA and GA content of biofertilizers (after 60 days of preparation)

Fig 3 illustrates the variations in indole acetic acid (IAA) and gibberellic acid (GA) content among different biofertilizers after 60 days of preparation. Among the tested biofertilizers, Biostimulant exhibited the highest level of IAA, followed by Biofert 3. Regarding GA content, Biozote demonstrated the highest concentration, followed by Biofert 1 and Biofert 2, which showed similar levels to Biostimulant. Notably, Biofert 3 exhibited the lowest GA content among all the tested biofertilizers (Fig 3).

ABA and SA content of biofertilizers

The abscisic acid (ABA) and salicylic acid (SA) content in the biofertilizers, Biostimulant exhibited the highest ABA content, followed by Biofert 1, with Biozote and Biofert 3

Table 1: Ratios between IAA, GA and ABA of cv. NARC-1 (V1) and cv. William-82 (V2).

Treatments	IAA/GA (V1)	IAA/GA (V2)	IAA/ABA (V1)	IAA/ABA (V2)	GA/ABA (V1)	GA/ABA (V2)
Biostimulant	0.89	1.1	7.25	9.66	6.2	6.375
Biozote	0.597	0.6	30.89	37	34.47	47.8
<i>P. chinense</i>	0.84	0.89	103.57	116	52	62.1
<i>P. putida</i>	0.97	0.82	35.4	34.96	36.1	42.6
<i>P. putida</i> + <i>P. chinense</i>	0.6	0.787	16.7	30.58	27.62	38.81
Control	0.43	0.42	4.5	4.2	6.1	6.21

Table 2: Correlation between phytohormones and growth parameters of V1 and V2.

Parameters	IAA	GA	ABA	Shoot length	Shoot weight	Fresh root weight	Dry root weight
V1							
IAA	1						
GA	**0.95845	1					
ABA	-0.709	-0.51569	1				
Shoot length	0.486501	0.610262	-0.13761	1			
Shoot weight	*0.816962	**0.9087	-0.39878	*0.865827	1		
Fresh root weight	***0.9526	***0.976	-0.58765	0.718087	***0.9529	1	
Dry root weight	0.381133	0.349163	-0.47131	0.658015	0.609373	0.521734	1
V2							
IAA	1						
GA	**0.94525	1					
ABA	-0.76171	-0.10404	1				
Shoot length	*0.879312	**0.9977	-0.10404	1			
Shoot weight	**0.903134	**0.9989	-0.03812	-0.88873	1		
Fresh root weight	***0.9789	*0.75286	-0.06407	-0.90082	0.999586	1	
Dry root weight	0.408894	0.32	-0.44344	0.242843	0.029389	0.017255	1

*Significant at $P < 0.05$; **Significant at $P < 0.01$; ***Significant at $P < 0.001$.

showing similar levels. In contrast, Biofert 2 displayed the lowest ABA content among the tested biofertilizers. Both biofertilizers showed the production of SA during storage, with Biozote producing approximately three times more SA compared to Biostimulant (Fig 4).

Proline and protein contents

Fig 5 revealed that all treatments showed increases in proline content of leaves compared to the control. The maximum increase in proline content was observed in plants inoculated with a consortium of *Planomicrobium chinense* and *Pseudomonas putida* (1.22-fold and 1.3-fold in cv. NARC-1 and cv. William-82, respectively) compared to the uninoculated control. Biozote biofertilizers increased proline content by 72% and 68.5% in V1 and

V2, respectively, compared to the control. Similarly, the consortium of *Planomicrobium chinense* and *Pseudomonas putida* showed a 1.56-fold and 2.71-fold increase in protein content in leaves of V1 and V2, respectively, compared to the control. Biostimulant exhibited a 2.95-fold increase in V1 and a 2.85-fold increase in V2 over the control.

Sugar and phenolic contents

All treatments increased leaf sugar content, with the highest increase (1.77-fold) in V2 treated with *Pseudomonas putida* (Fig 5). Biostimulant and Biozote treatments showed similar increases (1.5-fold and 1.45-fold, respectively). Phenolic content also increased in all treated plants, with *P. putida* showing the highest increase (84% in V1 and 87% in V2).

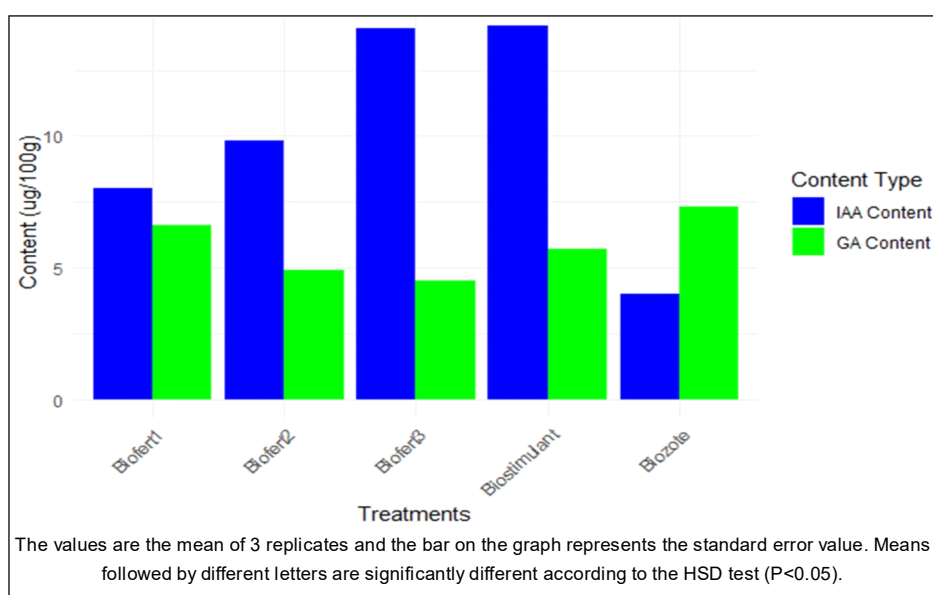


Fig 3: IAA and GA content of Biofertilizers.

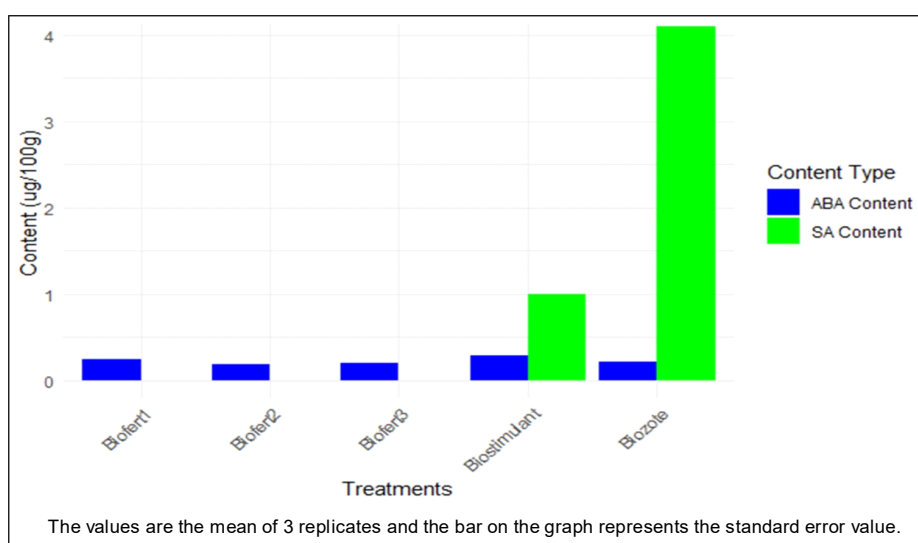


Fig 4: ABA and SA content of Biofertilizers in storage.

The *P. putida* and *Planomicrobium chinense* consortium also significantly boosted phenolic content (73% in V1 and 80% in V2). Biozote showed smaller increases (35% in V1 and 40% in V2).

Superoxide dismutase (SOD) and catalase (CAT) activities

Pseudomonas putida treatment led to the highest increase in SOD activity (4.56-fold in V1 and 4.31-fold in V2). Biostimulant increased SOD by 1.11-fold in V1 and 0.7-fold

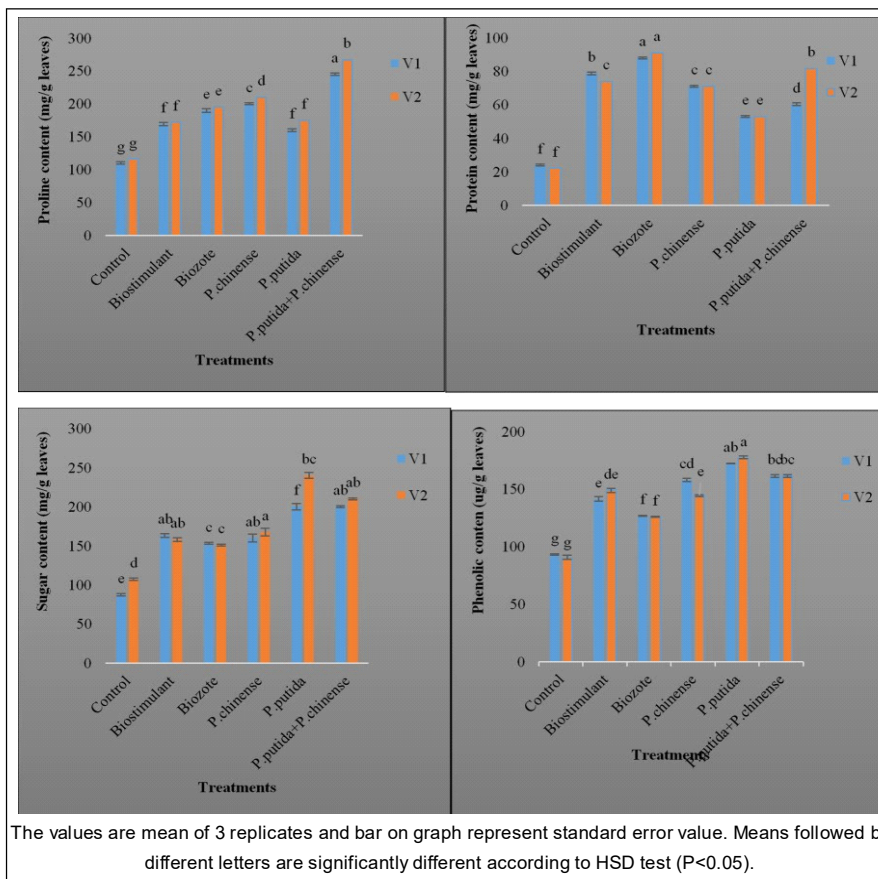


Fig 5: Effects of biofertilizers/bioinoculant on proline, protein, sugar and phenolic contents of *Glycine max* (L.).

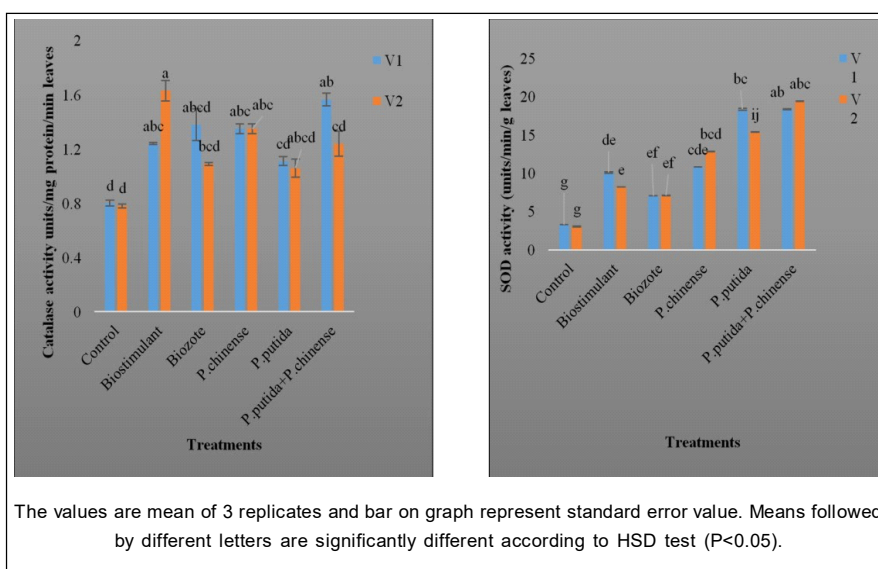


Fig 6: Effect of biofertilizers/bioinoculant on catalase (CAT) and SOD activities of *Glycine max* (L.).

in V2, like Biozote. The highest catalase activity was seen in V2 treated with Biostimulant (1.28-fold) and V1 treated with the consortium (1-fold). Biozote increased catalase activity by 72.5% in V1 and 40.3% in V2. Biostimulants had a more pronounced effect on V2, while the PGPR consortium was more effective in V1.

Soil parameters

Biostimulant-treated plants had the highest nutrient (phosphorus and potassium) and organic matter content in the soil (Fig 7). Biozote increased phosphorus by 61.4% in V1 and 75.8% in V2. The PGPR consortium increased phosphorus by 65.5% in V1 and 67.1% in V2. *P. chinense* increased potassium by 1.02-fold in V1 and 1.10-fold in V2, while *P. putida* increased it by 82% in V1 and 91.5% in V2. Biozote also led to the highest increase in organic matter (93.3% in V1 and 83.1% in V2). Biofertilizers generally had a more pronounced effect on soil content than bioinoculants, with cv. William-82 being more responsive to all treatments (Fig 7a and b).

The application of PGPR (Plant Growth-Promoting Rhizobacteria) in sustainable agriculture has seen a global rise due to their eco-friendly nature, sustainability and production of phytohormones (Prasad *et al.*, 2020). Our study revealed significant differences between carrier-based inocula (biofertilizers) and PGPR inocula without carrier (bioinoculant) on plant physiology. The local variety NARC (V1) showed greater responsiveness in growth parameters, while the exotic variety Williams 82 (V2) exhibited a stronger response to biochemical parameters, such as the production of osmoregulators (proline and sugar) and antioxidant enzyme activities (SOD and catalase). Proline, a beneficial solute, enhances cellular osmolarity during water limitation and serves as a potent nonenzymatic antioxidant (Eesha *et al.*, 2024; Mirzae *et al.*, 2020).

The liquid inocula without carrier (bioinoculant) were more effective for growth parameters, likely due to the higher cell density per ml compared to biofertilizers. Plants treated with *P. chinense* showed higher biomass (shoot and root weight), attributed to a significant increase in IAA content and a high IAA/GA ratio (0.84 and 0.89 in V1 and V2, respectively). This finding aligns with previous reports on *Planomicrobium chinense*, which enhances root and shoot weight through exopolysaccharides and indole acetic acid production (Khan, 2018).

Phytohormone content was correlated with growth parameters. Hormones act in concert for correlative control and the ratio of promoters to inhibitors determines their physiological effects. The correlation values showed that root and shoot weight and length were significantly correlated with IAA and GA content and negatively correlated with ABA content. The highest IAA/ABA ratio was observed in *P. chinense*-treated plants, while the minimum ratio was found in biostimulant-treated plants in both varieties. Similarly, the GA/ABA ratio was highest in *P. chinense*-treated plants. *Pseudomonas plecoglossicida*, a novel species from soybean rhizosphere, also produces IAA and GA (Astiani *et al.*, 2020). *Pseudomonas aeruginosa* and *Bacillus endophyticus* enhance IAA content and biomass in *Arabidopsis thaliana*, aiding osmotic stress tolerance (Shanthi *et al.*, 2024). Biostimulants increased ABA in V1, while Biozote enhanced ABA and SA production in V2. The high SA content and low ABA in Biozote suggest its potential for inducing pathogen resistance.

Enhanced protein production was more pronounced in biofertilizer-inoculated plants compared to bioinoculants, with *P. chinense* being particularly effective and V2 being more responsive. Younis *et al.* (2019) reported that Biozote contains *Rhizobium* strains capable of nitrogen fixation, resulting in higher protein content. A consortium of *Rhizobium*, *Pseudomonas* and *Bacillus* species increases protein content, aiding in the biofortification of food (Yasmeen *et al.*, 2019).

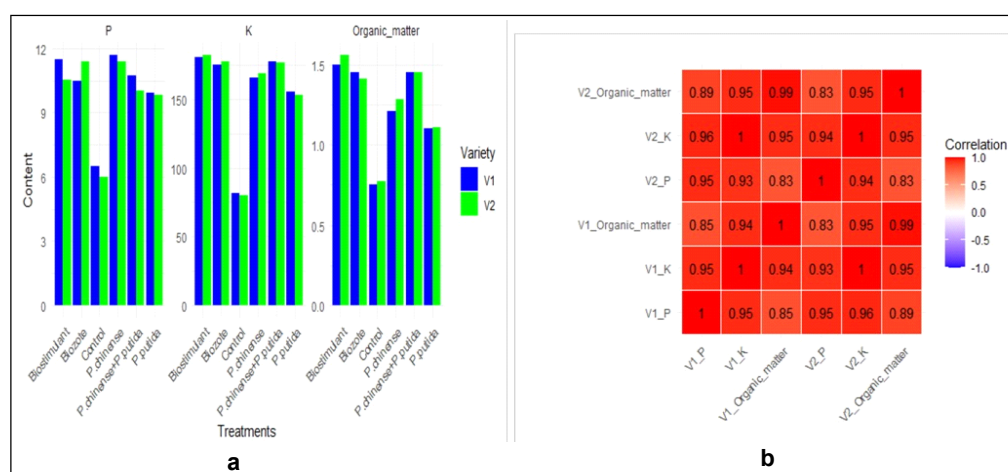


Fig 7: a) Effects of biofertilizers/bioinoculants on organic matter (%) and nutrients contents (mg/kg) of rhizosphere soil of soybean. b) show the correlation matrix of P, K and organic matter.

Biofertilizers were less effective than liquid PGPR inocula (bioinoculants) in osmoregulator production, as evidenced by lower proline and sugar levels in leaves. *P. chinense*, alone or in consortium, enhanced proline production in leaves, with the exotic variety Williams 82 being more responsive. *Pseudomonas putida* treatment increased sugar content but resulted in lower proline levels than *P. chinense*, suggesting *P. putida* may use sugar predominantly as an energy source and osmoregulator. *Pseudomonas aeruginosa* and *Burkholderia gladioli* increase sugar and carbohydrate content in *S. lycopersicum* under heavy metal stress (Khanna *et al.*, 2019).

Phenols or polyphenols, essential for plant defense and pigmentation, were higher in bioinoculant-treated plants, particularly with *P. putida*. This increase is similar to findings in tomato plants, where *P. putida* acted as a biocontrol agent against pests (Bano and Muqarrab, 2017). *P. chinense* had lower SOD but higher catalase activity than *P. putida*, suggesting better ROS detoxification. The V2 variety showed a stronger response to biostimulants, consistent with findings that *Bacillus firmus* boosts antioxidant enzyme activity in soybeans (Eesha *et al.*, 2024).

Soil organic matter, crucial for plant growth and environmental health (Brady and Weil, 2016), was significantly enhanced by biostimulants, which also increased K content more than Biozote. The residual benefits of biofertilizers/bioinoculants on soil fertility can aid subsequent crops. Macronutrients (N, P, K) are vital for crop health, preventing chlorosis and necrosis, supporting seedling development and ensuring robust growth (Bessa *et al.*, 2019).

Our results confirm the effectiveness of PGPR, especially bioinoculants, in enhancing plant growth and stress tolerance via phytohormone modulation and improved biochemical responses. Future research should investigate the long-term impacts of PGPR on soil health and crop productivity to bolster sustainable agriculture.

CONCLUSION

PGPR bioinoculants and biofertilizers enhance soybean growth, biochemical content and soil fertility, with carrier-free bioinoculants proving more effective than carrier-based biofertilizers. Future research should optimize formulations and application methods for biofertilizers to maximize their benefits in sustainable agriculture. This study's greenhouse setting limits its applicability, necessitating further field trials to confirm the results in diverse agricultural conditions.

Statements and declarations

The authors declare that they have no known competing interests.

Author contribution

Asghari Bano: Designed and supervised the project. Haleema Tariq and Naeem Khan: Carried out experiments and wrote the initial draft. Asghari Bano, Mahmoud F. Seleiman and Naeem Khan: Revised the MS.

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Conflict of interest

The author declares d no conflicts of interest.

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