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ENHANCING *SPINACIA OLERACEA* GROWTH UNDER ARSENIC STRESS BY BIOCHAR COATED-PHOSPHORUS AND RHIZOBACTERIA

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Abstract

Arsenic (As), a naturally occurring contaminant poses a significant threat to plant growth and agriculture worldwide. It can disturb the nutrients uptake, decrease the chlorophyll contents, and induce oxidative stress in plants. To overcome this issue, use of biochar-coated phosphorus (BCP) and rhizobacteria (RB) can be effective amendments. Biochar, a carbon-rich material, can be coated with phosphorus to improve P availability for minimization of As uptake. It can also proliferate RB which secretes growth hormones and improve root hairs that facilitates water and nutrients uptake. Therefore, current study investigates the effects of RB+BCP as amendments for mitigation of As toxicity and improvement of spinach plant growth. Four treatments (control, BCP, RB, and RB+BCP) were applied using a completely randomized design in four replications. Results showed that treatment RB+BCP under As toxicity significantly enhanced spinach plant height (22.79%), plant fresh weight (30.40%), and plant dry weight (36.76%) compared to the control. A significant improvement in chlorophyll a (24.36%), chlorophyll b (65.08%), and total chlorophyll (31.99%) content over control under As toxicity, validated the effectiveness of RB+BCP. In conclusion, RB+BCP has potential to improve chlorophyll b by enhancement in P uptake and modulation of antioxidants which in turn can improve spinach growth under As toxicity. Further field-level investigations are recommended to establish RB + BCP as best amendment in terms of cost-benefit ratio, considering its effectiveness in toxicity alleviation and soil fertility enhancement across various crops and under diverse climatic conditions.

Key words: Antioxidants; Arsenic stress; Biochar-coated phosphorus; Chlorophyll content; Rhizobacteria

Introduction

Arsenic (As) is a highly toxic heavy metal that can harm plants (Ganie *et al.*, 2024). It is a non-essential metalloid (Ghosh *et al.*, 2023) that can make plant morphology and development irregular, thus decreasing their growth (Sinha *et al.*, 2023). High As uptake in plants disturbs biomass production, photosynthesis, and nutrient uptake. It also causes metabolic disturbances and cellular damage (Nabi *et al.*, 2021). Furthermore, generating reactive oxygen species (ROS) by As toxicity is another allied negative effect that induces oxidative stress in plants (Zaidi *et al.*, 2024). Inoculating rhizobacteria and biochar application as amendments can play a vital role in overcoming this critical issue.

Plant growth-promoting rhizobacteria (PGPR can minimize As uptake in plants, improve plant growth, minimize oxidative stress, and is an environmentally friendly approach (Joshi *et al.*, 2023). They employ different mechanisms to mitigate As toxicity, i.e., As immobilization, modulation, and transformation of plant antioxidant-based defense system (Mondal *et al.*, 2021). Rhizobacteria can also regulate phytohormones and enhance nutrient acquisition by improving root growth (Zafar-ul-Hye *et al.*, 2021a, Zafar-ul-hye *et al.*, 2021b, Muhammad *et al.*, 2022, Danish *et al.*, 2024). In addition to the above, inoculating As-resistant PGPR has become popular because of its eco-friendly nature and cost-effective approach (Zubair *et al.*, 2016).

On the other hand, biochar is a carbon-rich material produced by pyrolyzing natural materials at high temperatures and low oxygen content. It decreases the release of carbon in the air, thus minimizing air pollution via carbon sequestration (Tomczyk *et al.*, 2020). When treated with nutrients, it improves soil fertility, promoting plant development (Shi *et al.*, 2023). At the same time, its porous nature upgrades soil aeration and water maintenance, ensuring a consistent nutrient supply for root development and plant health (Khan *et al.*, 2024). Biochar

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upgrades microbial action and reduces nutrient draining, advancing vigorous plant development and increasing yields (Majumder *et al.*, 2024). It can also minimize the uptake of heavy metals due to high sorption ability (Sharma *et al.*, 2022).

Spinach (*Spinacia oleracea* L.) is a crucial vegetable in the diet because of its high dietary benefit, containing fundamental minerals and nutrients (Sarma & Bhavya, 2024). It is a rich source of essential minerals like calcium, iron, potassium, vitamin C, phosphorus, and salt (Rashid *et al.*, 2022). Due to enrichment with flavonoids, it is an important part of water-solvent polyphenols, known for their potent antioxidant properties (Montenegro-Landívar *et al.*, 2021). However, As toxicity in spinach plants negatively impacts germination, growth, and photosynthesis, increasing oxidative stress, cell damage, necrosis, chlorosis, and possibly plant death (Sun *et al.*, 2023). That's why the selection of spinach crop in the current experiment was done as it not only facilitates the study of As stress mechanisms but also addresses a significant environmental health concern in As-contaminated areas.

The study aimed to examine the impact of biochar-coated phosphorus (BCP) and rhizobacteria RB on spinach cultivated under arsenic stress. The study covers the knowledge gap regarding investigating the most representative attributes of spinach plants that become positively affected by the combined use of BCP and RB and play an imperative role in improving spinach growth. The current study's novelty lies in using BCP and RB as a combined amendment for spinach plants under As toxicity. It is hypothesized that BCP application with RB might potentially improve the plant growth of spinach plants, chlorophyll, and nutrient uptake under As toxicity.

Material and Methods

Experimental site: In 2022, a pot experiment was conducted in the experimental area of Research Solution (30°09'41.6"N 71°36'38.0" E). Random soil sampling was done from the research area to characterize soil physicochemical properties. The soil characteristics were as follows: Total nitrogen (%), 0.02 (Bremner, 1996); clay loam in texture (Donald & Hanson, 1998); Available phosphorus (µg/g), 6.21 (Kuo, 2018); pH, 8.09 (Page *et al.*, 1983); EC*e* (dS/m), 2.25 (Rhoades, 1996); Extractable Na (µg/g), 111 (Donald & Hanson, 1998); soil organic matter (%), 0.49 (Nelson & Sommers, 1982); Extractable potassium (µg/g), 122 (Pratt, 2016). The irrigation characteristics are as follows: EC (µS/cm), 383; Carbonates (meq./L), 0.01; pH, 7.17 (Estefan *et al.*, 2013); Sodium (mg/L), 102; Bicarbonates (meq./L), 4.58; Ca+Mg (meq./L), 2.62; Chloride (meq./L), 0.01. Following a meticulous thinning process post-germination, four seedlings were kept per pot.

Synthesis of biochar: At first, fruit and vegetable waste were collected from local markets (30°11'30.1"N 71°28'48.3" E). After sun drying, pyrolysis was performed at 525 ± 5°C for 94 min. The attributes of the biochar incorporate TN (%): 0.52, pHs: 8.12, TP (%): 0.2, ECe (dS/m): 3.65, Unpredictable Matter (%): 32, Fixed carbon (%): 51, Debris Content (%): 18, and TK (%): 0.68.

Synthesis of biochar coated phosphorus: To prepare biochar-coated phosphorus, single superphosphate, and biochar were added to a steel container. A binder solution, i.e., 10% sugar solution (100ml/kg), was gradually added to the mixture while stirring was done to ensure an even coating. Once uniformly coating was completed, the mixture was spread on a tray and air-dried for 2h.

Rhizobacteria: The BLAST method was utilized to distinguish heat-tolerant rhizobacteria *Bacillus altitudinis* from the cotton rhizosphere by contrasting 16S rRNA fractional sequencing and GenBank information (Hall *et al.*, 2011, Yu *et al.*, 2011). Rhizobacteria (RB) are distinguished utilizing promotion number MW287246, 16S rRNA comparability (%) 100, and the nearest related organic entity in Genbank, *B. altitudinis* (41KF2b) promotion number NR042337.1. Gram staining (+ve), Catalase (+ve), Oxidase (+ve), and ACC-deaminase action (+ve) are among the attributes of RB (Glick *et al.*, 1995), IAA creation (+ve) (Sarwar *et al.*, 1992), Zn solubilization (- ve) (Kumar *et al.*, 2012), siderophore creation (+ve) (Schwyn & Neilands, 1987), chitinase creation (+ve)) (Dunne *et al.*, 1997) and HCN creation (+ve) (Bakker & Schippers, 1987).

Arsenic toxicity: The salt of $NaAsO_2$ was used for the development of 75 mg As/kg toxicity. The soil spiking was done for 21 days at 25±˚C at 65% field capacity with a humidity of 60%. The continuous mixing of soil was performed manually after each 5 days.

Collecting and sowing of plant material: The study used spinach seeds that were surface disinfected with 5% sodium hypochlorite and 95% ethanol. After sterilization, contamination was washed 3 times with deionized sterilized water. Ten sterilized seeds were sown in each pot containing 5 kg of soil (as per the treatment plan), and two seedlings per pot were maintained by thinning after germination.

Experimental design, treatment plan, and environmental conditions: The treatments include control, BCP (biochar-coated phosphorus), RB (*B. altitudinis*), and RB+BCP. All the treatments were applied under no AS and with AS stress. A completely randomized design (CRD) was used in the trial, with four replications for each treatment. The controlled environmental conditions, i.e., 5-6 hours of light, 20±5°C temperature, and 60±5% humidity, were maintained throughout the experiment.

Fertilizer: To meet their dietary requirements, the plants were given a preset measure of nitrogen (N), phosphorous (P), and potassium (K) at a proportion of 60:40:25 kg/section of land (N = 1.110g, P = 0.7425g, and K = 0.1875g each pot). During different development stages, like planting, watering, and flowering, artificial fertilizers were utilized to create the vital parts of plants. Sulfur (S), zinc (Zn) at 33%, and borax (B) at 11% were likewise sprinkled on the harvests as micronutrients. As micronutrients, every section of land got 3 kilograms of borax, 6 kilograms of zinc, and 5 kilograms of sulfur.

Harvesting and data collection: After 55 days from sowing, the plant's morphological attributes data were calculated using an analytical grade balance. To determine dry weight, samples were subjected to 70°C heating for 48 hours in an oven.

Chlorophyll contents and carotenoids: The chlorophyll in freshly harvested leaves was measured using Arnon's methods (Arnon, 1949). The grinding of samples was done with 5 ml of 80% acetone. The absorbance was subsequently measured at 645, 663 nm, and 480nm wavelength to calculate chlorophyll contents (Arnon, 1949) and carotenoids (Kirk & Allen, 1965).

Antioxidants: Superoxide dismutase (SOD) activity was calculated by the reduction inhibition of nitro blue tetrazolium (NBT) at 560 nm (Dhindsa *et al.*, 1982). For peroxidase (POD) activity was measured at 420 nm by using the method of (Hori *et al.*, 1997). Catalase (CAT) activity was evaluated by the breakdown of H_2O_2 and reduction in absorbance at 240 nm, due to H_2O_2 decomposition (Aebi, 1984). Thiobarbituric acid (TBA) was introduced to a malondialdehyde (MDA) sample extract, resulting in the formation of a colored complex, and the absorbance was measured at 532 nm (Hernández & Almansa, 2002). For APX activity, the reaction between ascorbic acid and H_2O_2 was observed at 290nm wavelength (Nakano & Asada, 1981).

N, P, and K leaves: Initially, nitrogen sulfuric acid (Mills & Jones, 1991) and phosphorus and potassium di-acid mixture (Miller, 1997) were used for digestion. To measure nitrogen content using a modified micro-Kjeldahl method (Mills & Jones, 1991). The phosphorus content was determined at 420 nm using the strategy for (Mills & Jones, 1991). Potassium content was estimated by utilizing a flame photometer.

Statistical analysis: The data was examined utilizing the standard statistical protocol of (Steel *et al.*, 1997). Using OriginPro software and Excel 365, MS Office, paired comparisons were carried out using the Tukey test, with a significant level set at $p<0.05$. OriginPro software was utilized for convex hull and hierarchical cluster plots (Anon., 2021).

Results

Growth attributes: Under no AS stress, adding BCP, RB, and RB+BCP treatments resulted in a significant increase in plant height 13.09%, 6.21%, and 19.45%, plant fresh weight 15.20%, 7.46%, and 28.40%, and plant dry weight 25.68%, 9.75%, and 42.10% than the control. In AS stress, applying BCP, RB, and RB+BCP treatment showed a significant increase in plant height 16.48%, 7.16%, and 22.79%, plant fresh weight 20.95%, 10.50%, and 30.40%, and plant dry weight 24.26%, 12.13%, and 36.76% from the control (Fig. 1A-C).

Chlorophyll contents and carotenoids: In comparison to the control, chlorophyll content increased by 12.17%, 6.09%, and 18.26%; with BCP, RB, and RB+BCP treatments, chlorophyll b rise by 28.51%, 14.89%, and 43.40%, total chlorophyll increased by 16.18%, 8.25%, and 24.43%, and carotenoid content increased by 12.50%, 4.81%, and 20.67% under no AS stress. Under AS stress, a substantial 16.30%, 8.24%, and 24.36% increases in chlorophyll a, 42.86%, 17.46%, and 65.08% increase in chlorophyll b, 21.28%, 9.97%, and 31.99% in total chlorophyll, and 24.09%, 12.41%, and 35.77% increase in carotenoid content by adding BCP, RB, and RB+BCP compared to the control (Fig. 2 A-D).

Gass exchange attributes: In no AS stress, the photosynthetic rate increased by 19.63%, 10.21%, and 32.12% with BCP, RB, and RB+BCP; transpiration rate increased by 15.48%, 9.95%, and 21.94%, and stomatal conductance increased by 10.11%, 5.74%, and 16.19% from the control. In comparison to the control, adding BCP, RB, and RB+BCP treatments under AS stress showed 24.18%, 12.43%, and 37.72% increases in photosynthetic rate, 31.69%, 16.44%, and 49.33% increase in transpiration rate, and 7.30%, 2.80%, and 10.32% in stomatal conductance (Fig. 3A-C).

Antioxidant activity: POD level decreased by 39.84%, 14.29%, and 94.35% under no AS stress, SOD level decreased by 41.28%, 18.46%, and 60.42%, CAT level decreased by 28.96%, 14.70%, and 37.36%, APx showed 99.31%, 31.96%, and 99.75% decrease and 54.92%, 24.17%, and 99.28% decrease in MDA was observed with BCP, RB, and RB+BCP treatments compared to the control. Adding BCP, RB, and RB+BCP treatments under AS stress, resulting a 24.49%, 13.99%, and 36.79% decrease in POD, SOD levels decreased by 19.30%, 9.68%, 28.65%, CAT levels decreased by 11.78%, 8.31%, and 17.99%, APx decreased by 44.34%, 17.45%, and 74.40% and 23.77%, 10.13%, and 24.14% decrease in MDA over the control (Fig. 4).

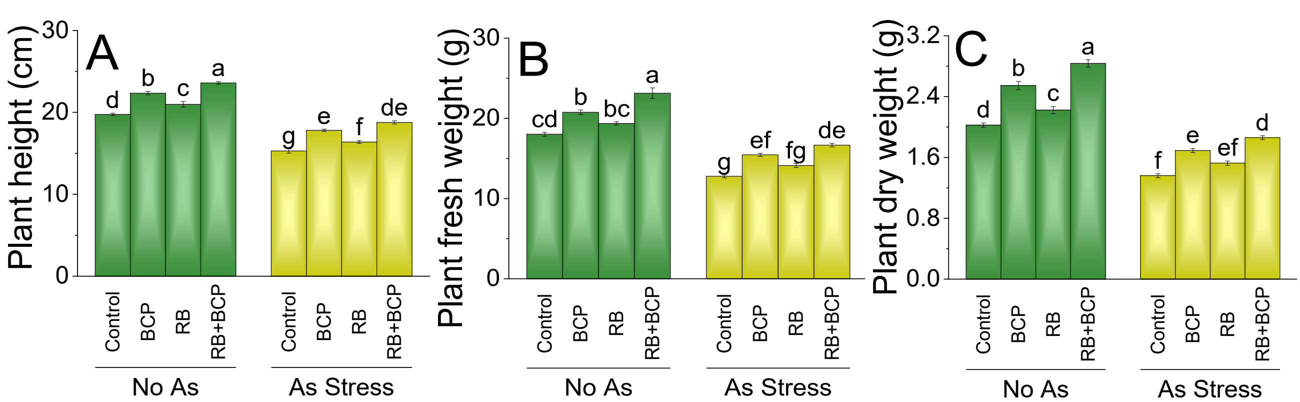

Fig. 1. The impact of various treatments on the height of plants (A), fresh weight (B), and dry weight (C) of spinach cultivated without arsenic and under arsenic stress. The Tukey test indicated significant differences at ($p<0.05$); different letters on the bars represent the average of four replicates.

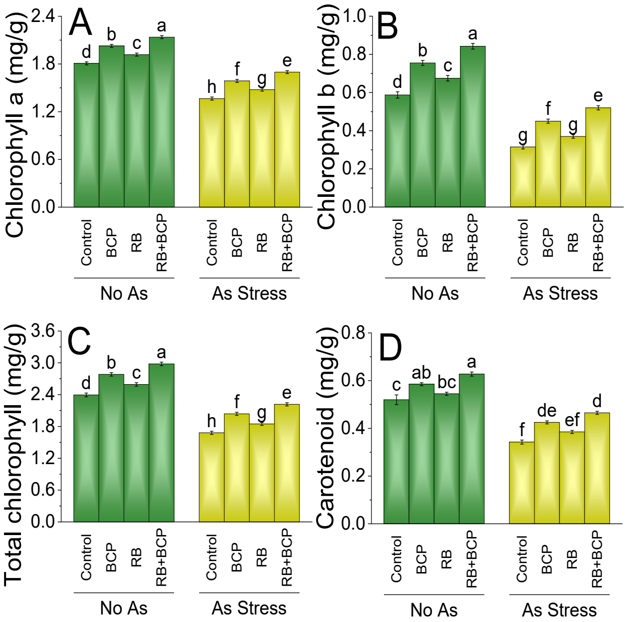

Fig. 2. The impact of various treatments on chlorophyll a (A), b (B), total (C), and carotenoid (D) levels in spinach cultivated without arsenic and under arsenic stress. The Tukey test indicated significant differences at ($p<0.05$); different letters on the bars represent the average of four replicates.

Plant N, P, and K: Applying BCP, RB, and RB+BCP treatment under no AS stress resulted in a significant increase in plant N 8.18%, 4.53%, and 12.08%, plant P 8.24%, 4.61%, and 11.98%, and plant K increased by 9.04%, 5.05%, and 13.30% than the control. In AS stress, applying BCP, RB, and RB+BCP treatment caused an increase in plant N by 4.71%, 1.66%, 7.94%, plant P increased by 15.14%, 7.50%, and 24.74%, and plant K increased by 11.44%, 5.23%, and 19.28% from the control (Fig. 5).

Convex hull and hierarchical cluster analysis: The Control group showed a broad distribution with scores ranging from -6.71028 to 1.63084 on the PC1 axis and from -0.4974 to 0.53982 on the PC2 axis. This group occupied a wide area on the negative side of PC1 and varied considerably along PC2. The BCP group, characterized by scores ranging from -3.34978 to 4.7776 on PC1 and from -0.16627 to 0.04062 on PC2, formed a more compact cluster primarily on the positive side of PC1, with some overlap in the negative region. The RB group had scores ranging from -5.04156 to 3.50812 on PC1 and from -0.40256 to 0.25162 on PC2. This group also formed a distinct cluster, with its points largely situated in the negative region of PC1 but extending into the positive side, suggesting a more diverse spread than the BCP group. The RB+BCP group exhibited scores ranging from -1.69906 to 7.24722 on PC1 and from -0.35313 to 1.09025 on PC2. This group spanned a significant range on both axes, indicating a diverse distribution that included both negative and positive sides of PC1 (Fig. 6A).

The no As group had scores ranging from 0.08342 to 7.24722 on PC 1 and from -0.4974 to 1.09025 on PC 2. These scores formed a cluster predominantly on the positive side of PC 1, with slight variations along PC 2. The distribution of these points indicates a relatively high degree of similarity within the no As group. In contrast, the As Stress group showed scores ranging from -6.71028 to -0.74642 on PC 1 and from -0.35313 to 0.53982 on PC 2. This group occupied the negative side of PC 1, demonstrating a clear separation from the No As group. The spread of the As Stress points was more varied along PC 2, suggesting a broader range of responses under stress (Fig. 6B).

Initially, chlorophyll a and total chlorophyll formed a cluster with a high similarity (0.01796), which further clustered with chlorophyll b (similarity index of 0.07305), creating a larger cluster indicative of related pigment parameters. Transpiration rate and plant phosphorus (P) showed a similarity index of 0.09076, clustering together and suggesting a link between these physiological processes. These clustered with plant height at a similarity index of 0.13751, indicating that plant height is moderately related to these physiological parameters. Chlorophyll-related clusters (chlorophyll a, total chlorophyll, and chlorophyll b) combined with plant height at a similarity index of 0.06446, forming a larger cluster that signifies the interrelationship between plant height and chlorophyll content. Photosynthetic rate and stomatal conductance also formed a cluster with a similarity index of 0.28502, reflecting their close functional relationship. Plant nitrogen (N) showed a relatively high similarity index (0.40357) when clustered with the combined physiological parameters, indicating its significant role in overall plant function. Plant potassium (K) clustered with transpiration rate and plant P at a similarity index of 0.46762, linking these nutrient and physiological parameters. Plant fresh and dry weights showed high similarity (0.51806) and clustered together, emphasizing the correlation between these biomass parameters. These further clustered with carotenoid content (similarity index of 0.52235), indicating a relationship between biomass and carotenoid levels. SOD and MDA, both stress-related parameters, showed a high similarity (0.52364) and formed a distinct cluster, which later integrated with other physiological parameters. The antioxidant enzymes POD and CAT also clustered at a high similarity index (0.81446), forming a distinct group. As the analysis progressed, larger clusters formed, integrating various physiological and biochemical parameters. APX, another stress-related enzyme, clustered with other antioxidant enzymes at a similarity index of 0.95859, indicating a strong relationship among this stress (Fig. 6C).

Discussion

Arsenic toxicity: Arsenic is a toxic heavy metal that disrupts cellular homeostasis when it becomes part of a plant body (Zhang *et al.*, 2021). It interacts with thiol-containing enzymes and proteins, resulting in metabolic imbalance and plant dysfunction (Sharma, 2012). Overgeneration of ROS and cellular damage are major adverse effects of As in plants. Higher synthesis of ROS causes severe damage to cells via triggering lipid peroxidation, DNA fragmentation, and protein oxidation (Nahar *et al.*, 2022). It also causes chlorosis in plants by interfering with the synthesis of chlorophyll precursors, which also decreases photosynthetic efficiency (Sil & Biswas, 2022). Furthermore, chemical similarities of As with P established a competition for P uptake via phosphate transporters (PHTs) (Geng *et al.*, 2005). Impaired root hairs, a decrease in the development of lateral roots, and a change in the pattern of root exudation secretion also played an imperative role in minimizing nutrient uptake under As toxicity (Zaidi *et al.*, 2024).

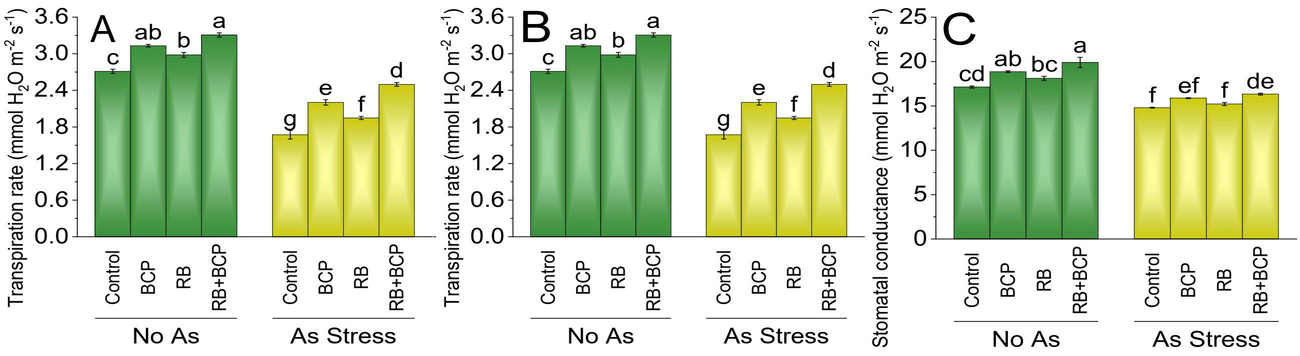

Fig. 3. The impact of various treatments on the photosynthetic rate (A), transpiration rate (B), and stomatal conductance (C) of spinach cultivated without arsenic and under arsenic stress. The Tukey test indicated significant differences at ($p<0.05$); different letters on the bars represent the average of four replicates.

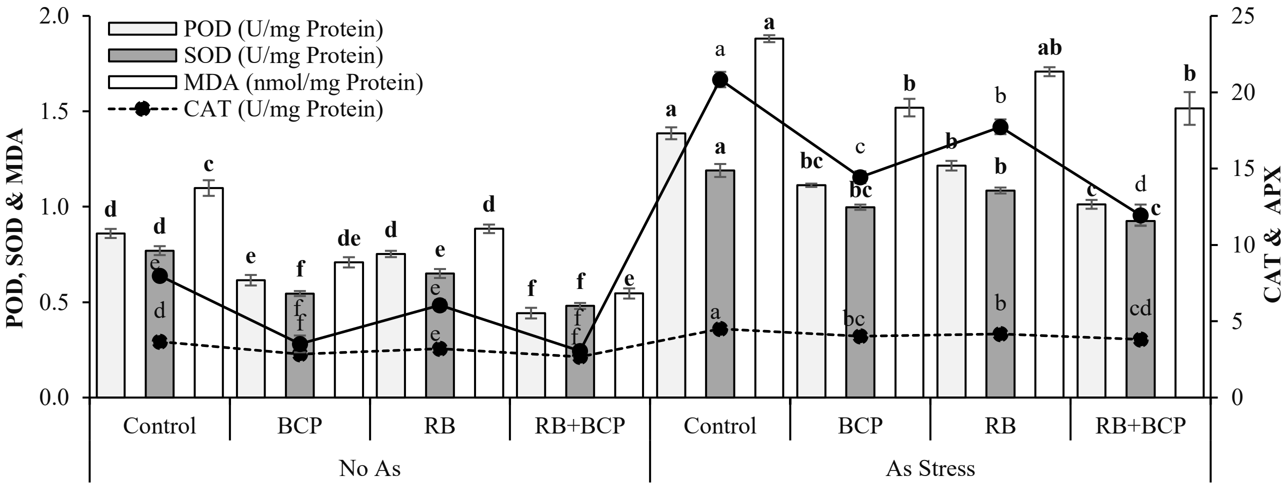

Fig. 4. The effect of different treatments on POD, SOD, CAT, APx, and MDA of spinach grown under no arsenic and arsenic stress. The Tukey test measured significant differences at ($p<0.05$); distinct letters on the bars are the mean of four replicates.

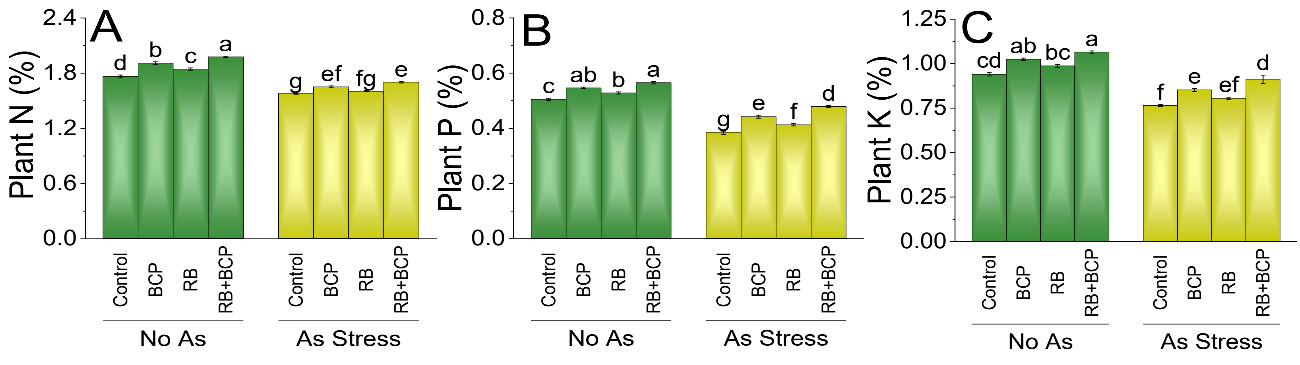

Fig. 5. The effect of different treatments on plant N (A), P (B), and K (C) of spinach grown under no arsenic and arsenic stress. The Tukey test measured significant differences at ($p<0.05$); distinct letters on the bars are the mean of four replicates.

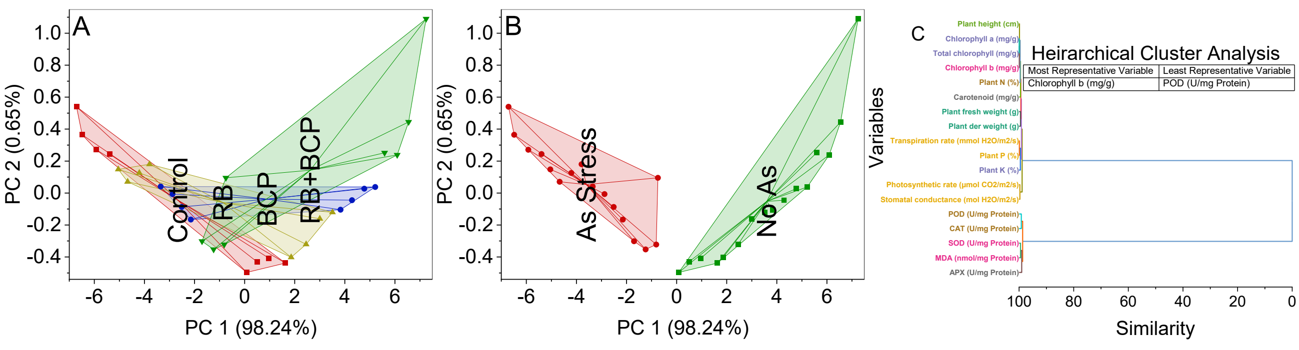

Fig. 6. Convex hull cluster plot for treatments (A), arsenic (As) stress levels (B), and hierarchical cluster analysis (C) concerning the attributes examined.

Rhizobacteria: Rhizobacteria can enhance plant stress resilience by inducing systemic resistance and producing antioxidants to decrease arsenic-induced oxidative damage (Mondal *et al.*, 2021). They play a fundamental part in root improvement (branching, elongation) by releasing phytohormones like auxins and gibberellins, improving nutrient consumption productivity (Khan *et al.*, 2021). Rhizobacteria produce growth-promoting chemicals and enzymes that improve the effectiveness of the root surface region, nutrients, and water retention (Chauhan *et al.*, 2021). Oxidase-positive rhizobacteria have the potential to change arsenite (As^{3+}, more toxic and mobile form) into arsenate (As^{5+}, less harmful and readily absorbed by plant roots form) (Biswas & Sarkar, 2019). These rhizobacteria can prevent cellular damage caused by As toxicity via their catalase activity (converting H_2O_2 into water and oxygen) (Ullah *et al.*, 2024). As rhizobacteria used in the current study also possess all such characteristics, the improvement in spinach plant growth under As toxicity becomes validated.

Biochar: Adding biochar improves soil cation exchange capacity and nutrient availability (Liu *et al.*, 2012, Schulz & Glaser, 2012, Lopes *et al.*, 2022). One key mechanism by which biochar reduces arsenic toxicity is surface complexation and adsorption (Sharma *et al.*, 2022, Zhang *et al.*, 2022). The oxygen-containing functional groups, i.e., carboxyl, hydroxyl, and carbonyl present on the surface of biochar, have the potential to bind particularly arsenate (As^{5+}), thus decreasing its mobility in soil (Kumar *et al.*, 2022). Additionally, biochar often has iron, calcium, and aluminum metal oxides, which facilitate immobilization through precipitation (Wang *et al.*, 2021). Better proliferation of rhizobacteria in the presence of biochar is another allied factor that facilitate the plants to improve their growth under As toxicity (Malik *et al.*, 2022, Liao *et al.*, 2024). Similar results were also noted in the current study; addition of BCP improves the spinach plants nutrient uptake and chlorophyll contents.

Rhizobacteria × Biochar coated phosphorus: Phosphorus deficiency minimizes chlorophyll synthesis and electron transport by restricting ATP synthase activity and changing thylakoid lumen pH (Carstensen *et al.*, 2018). Chlorophyll b, is an important part of plant's photosynthetic machinery. Its synthesis also requires energy and precursors (Voitsekhovskaja & Tyutereva, 2015). Adenosine triphosphate (ATP) energy provides this essential energy to plants, and phosphorus is vital for their formation (Mullen, 2019). In the current study, similar results were noted, such as the application of BCP and rhizobacteria improving the P concentration in plants. This improvement was directly associated with the enhancement in the chlorophyll b synthesis, which is the most representable attribute for improving spinach growth under As toxicity. Literature showed that enhancement in chlorophyll b due to chlorophyllide a oxygenase (CAO) improves the photosynthetic capacity, leading to higher carbohydrate synthesis (Tanaka *et al.*, 2001, Biswal *et al.*, 2012). This, in turn, gives more energy for root development, which helps in better nutrient uptake under arsenic toxicity (Tanaka *et al.*, 2001, Biswal *et al.*, 2012).

Conclusion

In conclusion, RB+BCP as an amendment can increase spinach plants chlorophyll b contents (most representative attribute) under arsenic stress. This improvement in chlorophyll b contents was associated with better uptake of P, which is an important element in making ATP. Besides that, applying RB+BCP also regulates antioxidant activities and growth regulators (IAA produced by RB) that might have played an imperative role in decreasing oxidative stress in spinach, resulting in better root elongation and growth. Further field-level investigations are recommended to establish RB + BCP as the optimal amendment in terms of cost-benefit ratio, considering its effectiveness in toxicity alleviation and soil fertility enhancement across various crops and under diverse climatic conditions.

Authors Contributions: Conceptualization, U.Y. and S.D.; methodology, M.J. and R.K.I.; investigation, S.S., U.E.H., and G.F.; formal analysis, A.A.K. and R.A.R.; data curation, S.S. and R.A.R.; writing, original draft preparation, R.A.R. and S.D.; writing, review and editing, M.I.A., A.E.A.S., N.M.A.M., U.Y., and S.D.; visualization, A.A.K. and M.I.A.; supervision, U.Y. and S.D.; project administration, S.D.; funding acquisition, A.E.A.S. and N.M.A.M. All authors have read and agreed to the published version of the manuscript.

Data Availability Statement: All data generated or analyzed during this study are included in this published article.

Competing Interests: The authors declare no competing interests.

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