

Synergistic effect of co-application of biochar and a PGPR, *Bacillus cereus*, on spinach yield under reduced chemical fertilizer regime

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Excessive use of chemical fertilizers in agriculture contributes substantially to greenhouse gas emissions and underground water pollution. Therefore, there is an urgent need to reduce the use of chemical fertilizers without compromising agricultural productivity. Biochar and plant growth-promoting bacteria (PGPR) could be an environment-friendly and cheaper solution in this regard. We evaluated the effect of a sugarcane-bagasse derived biochar on *Spinacia oleracea* under 75% or 50% of the recommended fertilizers, in presence or absence of a PGPR i.e., *Bacillus cereus*. Spinach was grown on a sandy clay loam soil in pots for 60 days. Biochar was mixed at 1 or 2% (w: w basis) with soil in all possible combinations of 75% or 50% of the recommended fertilizers. Plants receiving 100% of recommended fertilizers served as control. Biochar significantly increased fresh leaf biomass, with the maximum increase of 152% found where 2% biochar + 75% fertilizer were added. Moreover, co-application of biochar with PGPR further increased the leaf biomass, with the maximum increase of 208% found when 2% biochar and PGPR were co-applied under 50% fertilizer regime. Biochar significantly increased the soil nitrate content, with the maximum increase of 529% found in 2% biochar addition under 50% fertilizer application. Co-application of PGPR and biochar further increased soil nitrate content with the maximum increase of 750% found in 1% biochar application under 75% fertilizer regime. Phosphorus availability was only increased by 1% biochar application with maximum increase of 250% under 75% fertilizer regime. Co-application of PGPR with 1% biochar increased the microbial biomass by 91% and 75% under 75% and 50% fertilizer application respectively. Principal component analysis showed that the soil nutrients i.e., nitrate, and available phosphorus and microbial biomass, which were overwhelmingly improved under co-application of biochar and PGPR, were closely related to spinach leaf and root biomass. We concluded that the co-application of biochar and the PGPR acted synergistically in increasing the nutrient availability for plants thereby leading to higher productivity. Therefore, we recommend that this technique can be a viable solution for reducing the input costs on fertilizer in addition to boosting plant productivity.

Keywords: Antioxidant enzymes, Available phosphorus, Microbial biomass, Mineral nitrogen, Reactive oxygen species, Root: shoot ratio.

INTRODUCTION

The excessive application of synthetic fertilizers since the green revolution has cast dire environmental effects in terms of greenhouse gas emissions, nitrate leaching to groundwater, and eutrophication (Tilman *et al.*, 2002, 2011; Balasuriya *et al.*, 2022). If the current trajectory of use of the mineral fertilizers continues till 2050, the global nitrogen (N) use would climb to 250 Mt year⁻¹ from the current 100 Mt year⁻¹, possibly leading to major environmental effects like elevated

greenhouse gas levels and severe water pollution (Vitousek *et al.*, 1997; Burney *et al.*, 2010). Therefore, there is a constant search for alternative technology improvement, adaptation and transfer to minimize the use of synthetic fertilizers.

Biochar (BC), a carbon rich product made by pyrolyzing organic materials under limited oxygen, is being considered an emerging technology (Lehmann *et al.*, 2006; Woolf *et al.*, 2010). Mostly, it is made from organic wastes. It has gained a widespread attention due to its multiple potential uses in waste management, carbon sequestration, improving soil

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quality and health, remediation of contaminated soils and water etc. (Haider *et al.*, 2022). Its application in agriculture has been shown to improve nutrient availability, enhance water holding capacity, boost plant growth, and improve soil health indicators in addition to mitigating greenhouse gas emissions (Jeffery *et al.*, 2011; El-Naggar *et al.*, 2015; Riaz *et al.*, 2017). Their use has particularly been recommended in low fertility soils.

Biochar has the capacity to retain and provide macronutrients depending on nutrient status of the precursor feedstock (Gul and Whalen, 2016). The BC has more advantages than adding its feedstock directly because the former increases nutrient retention and use efficiency and reduces nutrient leaching compared to the latter (Haider *et al.*, 2014; Iqbal *et al.*, 2015). However, adding biochar alone does not replace the nutrients from synthetic fertilizers (Alburquerque *et al.*, 2013). A few studies have mixed biochar with lower levels of synthetic fertilizers than recommended and found mixed results. For instance, Alburquerque *et al.*, (201) found 42% lower plant growth and 57% lower wheat grain yield when 40% of recommended fertilizer (RF) and 2.5% BC were used, although the maximum growth and yield was found with maximum fertilizer application (100% recommended fertilizer) and 2.5% BC (Alburquerque *et al.*, 2013). In a field study, 500 kg BC ha⁻¹ combined with 50% of the recommended fertilizer was found to match the grain yield of wheat that was produced by adding 100% of recommended fertilizer only (Ijaz *et al.*, 2019). Further studies must be performed to assess the potential of biochar to reduce the need of synthetic fertilizers for different crops.

Plant growth promoting rhizobacteria (PGPR) are known since long for improving plant growth under normal and stressed conditions (Rashid *et al.*, 2016; Saeed *et al.*, 2021). Different PGPR carrying the potential of producing phytohormone indole acetic acid, ethylene lowering ACC-deaminase, solubilizing rock phosphate or fixing atmospheric nitrogen have been proven in pot and field studies. Owing to these traits, they are considered environmentally safe biofertilizers for crop production. Previously, they have been added in combination with organic amendments to boost plant growth and yields (Mengual *et al.*, 2014; Arif *et al.*, 2017). Recently, they were co-applied with biochar, particularly under abiotic stresses like drought, salinity and heavy metals for improving plant growth (Akhtar *et al.*, 2015; Shanta *et al.*, 2016; Zafar-ul-Hye *et al.*, 2020; Çiğ *et al.*, 2021), managing plant disease (Rasool *et al.*, 2021) and improving soil properties (Sarfraiz *et al.*, 2017; Kari *et al.*, 2021). However, the possibility of producing similar or higher plant yields while co-applying the PGPR and biochar under reduced fertilizer regimes has rarely been studied.

The objectives of the study were: 1) to assess the potential of sugarcane bagasse-derived biochar to maintain or increase the spinach growth under reduced addition of synthetic fertilizers, and 2) to evaluate the combined potential of a PGPR, *Bacillus*

cereus and the biochar to increase or maintain spinach production, with respect to application of hundred percent of the recommended fertilizers. Our first hypothesis was that the biochar addition along with reduced synthetic fertilizer will at least maintain the same plant growth as the 100% recommended fertilizer application without biochar. Secondly, we hypothesized that the PGPR would enhance the plant growth compared to 100% recommended fertilizer application by interacting with biochar and releasing mineral nutrients from it.

MATERIALS AND METHODS

Experimental set up: The experiment was set up in the botanical garden of Government College University Faisalabad (31.4161° N, 73.0700° E). A sandy clay loam was sampled from a riverine near Faisalabad, Pakistan for this pot experiment. Sugarcane bagasse, collected from Ayub Agricultural Research Institute, was pyrolyzed at 450 °C to make biochar for the experiment (Lehmann *et al.*, 2006). The main physico-chemical characteristics of the soil and the biochar (BC) are given in the Table 1.

Table 1. Physico-chemical properties of the soil and sugarcane bagasse derived biochar (BC) used in the experiment

Parameter	Soil	Sugarcane bagasse derived Biochar (BC)
Sand (%)	65.67 ± 1.76	
Silt (%)	8.33 ± 2.33	
Clay (%)	25.47 ± 0.33	
Soil Texture	Sandy clay loam	
pH	8.05 ± 0.02	8.97 ± 0.01
Electrical conductivity (d S m ⁻¹)	0.16 ± 0.02	1.55 ± 0.11
Organic C (g kg ⁻¹ dry matter)	8.89 ± 0.33	295.46 ± 80
Total N (g kg ⁻¹)	4.4 ± 1.01	7.41 ± 0.97
Total P (g kg ⁻¹)	2.24 ± 0.55	19.55 ± 0.67

The biochar was mixed with soil at the rate of 1 or 2% on weight basis before filling the pots. Synthetic fertilizer was also mixed with the soil before pot filling. Hundred percent of recommended nitrogen and phosphorus i.e., 90 kg ha⁻¹ and 30 kg ha⁻¹ was mixed in the form of ammonium nitrate and di-ammonium phosphate respectively, for control treatment. Each pot received about 3.6 kg of soil. For inoculating seeds with a plant growth promoting rhizobacterium (PGPR), a *Bacillus cereus*, bearing indole acetic acid producing and phosphate solubilization traits, was used. This strain has already been isolated and tested by one of the authors of this study (Shahid *et al.*, 2020). Seeds of a local cultivar, Lahori Spinach VI, of *Spinacia oleracea* were purchased from Vegetable Research Station, Ayub Agriculture Research Institute Faisalabad, Pakistan. Eight to ten seeds were sown

per pot, whereby six plants were kept alive for the experiment after germination. The treatments where PGPR inoculation was done, seeds were soaked in *Bacillus cereus* inoculum for 15 mins under laminar flow before sowing. The details and the acronyms of the treatments of the experiment are given in the Table 2. A completely randomized design (CRD) was followed. All the treatments were replicated three times whereas the experiment was replicated twice for taking two harvests. Optimum moisture conditions were provided for all the pots.

Table 2. Details and acronyms of the treatments of the experiment.

Treatment details	Acronym used in the paper
100 % recommended synthetic fertilizer	Control
1%BC + 75% of the recommended fertilizer	1BC+75RF
1%BC + 50% of the recommended fertilizer	1BC+50RF
2%BC + 75% of the recommended fertilizer	2BC+75RF
2%BC + 50% of the recommended fertilizer	2BC+50RF
1%BC + 75% of the recommended fertilizer + inoculation with PGPR	1BC+75RF+PGPR
1%BC + 50% of the recommended fertilizer + inoculation with PGPR	1BC+50RF+PGPR
2%BC + 75% of the recommended fertilizer + inoculation with PGPR	2BC+75RF+PGPR
2%BC + 50% of the recommended fertilizer + inoculation with PGPR	2BC+50RF+PGPR

Measurement of plant growth: Plants were allowed to grow for 30 days before first harvest was done. The aboveground biomass was neatly cut at the base before destroying the pots for recovering roots and soil for further analyses. Roots were removed from the moist soils by gentle taping followed by washing with distilled water. Plant parts were weighed fresh (fresh biomass) and left on the shelf for air drying followed by drying in the oven at 70°C till the constant weight to measure the dry weight of leaves and roots. A fresh leaf from each treatment was used to determine the photosynthetic pigments, antioxidant enzymes and reactive oxygen species. Twenty-five days after the first harvest, plants were again harvested for measuring fresh leaf and root biomass (second harvest).

Chlorophyll content and carotenoids: Half gram of a fresh leaf was grounded in 10 ml of 80% methanol followed by the centrifuging of the extract at 10,000 rpm for 10mins. The absorbance was read at spectrophotometer (Analytik Jena SPECORD 200 PLUS) and chlorophyll a and b contents were calculated following Arnon, (1949). Carotenoids were calculated according to Taylor and Davies, (1976).

Determination of H₂O₂ contents & lipid peroxidation: In order to determine hydrogen peroxide (H₂O₂) content, finely

grounded 0.5g of fresh leaf was homogenized in ten ml of trichloro-acetic acid (0.1% TCA) on an ice bath followed by the centrifuging the extract at 12,000rpm for 10 mins. The absorbance of the supernatant and standards was read at 390nm after mixing with potassium phosphate buffer (0.5 ml) and potassium iodide (1ml, 1 M).

Measurement of superoxide dismutase, catalase, peroxidase and ascorbate peroxidase activity: Superoxide dismutase activity in fresh leaves was measured by using photo-reducing nitroblue tetrazolium (NBT) (Beauchamp and Fridovich, 1971). In a 650ul phosphate buffer, 100ul of methionine was added. Then, 50ul of riboflavin was also added in addition to 100ul of enzyme extract. NBT was then added and the mixture was well shaken. Then, the mixture was placed under 15 W fluorescent lamps for 10 minutes to start the reaction. The absorbance was read on a spectrophotometer at 560nm. For measuring catalase activity (CAT), reaction mixture was prepared by adding H₂O₂ (0.1ml) and enzyme extract (0.1ml) in a phosphate buffer (2.8ml). The reduction in absorbance was read on spectrophotometer at 240nm for 120s with intervals of 20s (Maehly and Chance, 1954). For measuring peroxidase activity (POD), 0.1ml of guaiacol and 0.1ml of enzyme extract were added to a 2.7ml of phosphate buffer and mixed well. The absorbance was taken at six intervals over 120 seconds at 470nm. One unit of POD activity was considered equivalent to a change of 0.01 unit per minute in absorbance. Specific enzyme activity was expressed as enzyme per mg protein (Maehly and Chance, 1954). Half ml of the supernatant was mixed with 0.5 mM AsA, sodium phosphate buffer (pH 7), 0.1mM H₂O₂, and 0.25ml of EDTA for ascorbate peroxidase (APX). The absorbance of this mixture was read at 290nm on a UV-visible spectrophotometer.

Mineral nitrogen, available phosphorus and microbial biomass: Given a very low amount of ammonium ions in the studied soil, only nitrate content was measured and termed as mineral N. Salicylic acid nitration method was employed to determine soil nitrate (Cataldo *et al.*, 1975). Briefly, twenty ml of 0.5M K₂SO₄ was used to extract ten gram of fresh soil after shaking at 60 rpm for 30 minutes. N-free filter paper was used to filter the extracts. For developing the color, half ml of the filtered extracts or standards was taken in labelled glass tubes. One ml of acidified salicylic acid (5%) was added to each test tube, vortexed followed by resting for 30 mins. Ten ml of sodium hydroxide (4M) were added to each tube before keeping it still for 1hr for development of color. The absorbance of the mixtures was determined by using spectrophotometer at 410nm.

Available phosphorus was determined as a blue phosphate molybdic acid complex at 882 nm on a visible spectrophotometer (Olsen and Sommers, 1982). Briefly, moist soil samples were extracted in 0.5M sodium bicarbonate solution (1:20 soil solution ratio) following 30 mins of end over end shaking. Five ml of the extracts were

poured in a flask and sulfuric acid was used to reach at pH 5 resulting into a total volume of 20ml. Sixteen ml of ammonium heptamolybdate (0.001 M) working solution and 2ml ascorbic acid were added one after the other. The prepared solutions were read at 882nm for absorption on a UV-Visible spectrophotometer.

Microbial biomass carbon was determined by using the fumigation-extraction method (Vance *et al.*, 1987). Five grams of moist soil were fumigated with ethanol free chloroform in a vacuum desiccator for 48 h at 25°C. After removing the fumigants, the soils were extracted with 0.5 M K₂SO₄ (1:5 soil to solution ratio) following reciprocal shaking for 30 mins. The extracts were filtered with a Whatman filter paper. Similarly, another set of soils was extracted but without fumigation. A modified Walkley-Black method was used to determine the dissolved organic carbon in the extracts (Walkley and Black, 1934; Jackson, 1962). Microbial biomass carbon (MBC) is the dissolved organic C in fumigated soils minus that in non-fumigated ones corrected with an extraction factor of 0.45 (Joergensen, 1996).

Statistical analyses: One way analysis of variance (ANOVA) was applied to determine the effect of biochar and co-application of biochar and PGPR under reduced fertilizer regimes in comparison to hundred percent fertilizer application (control) on plant growth parameters i.e., fresh and dry leaf and root weights, root:shoot ratio; chlorophyll pigments i.e., total chlorophyll, chlorophyll a & b, chlorophyll a: chlorophyll b, carotenoids and total chlorophyll: carotenoids; antioxidant enzymes i.e., SOD, CAT, POD, APX; reactive oxygen species i.e., and H₂O₂; soil variables i.e., soil nitrate and available phosphorus content and microbial biomass carbon. The ANOVA was followed by a least significant difference (LSD) test, where 95% confidence interval among means (i.e., $P < 0.05$) was considered as a significant difference among means. The relationship of plant growth, physiological, and chemical parameters among each other and with soil parameters was tested for principal components analysis (PCA) on correlation metrics. All the statistical analyses, including the PCA, were performed in Statgraphics Centurion.

RESULTS

Plant growth: All the combinations of biochar and reduced fertilizers (BC+RF) significantly increased the fresh leaf biomass (g pot⁻¹) of *Spinacia oleracea* for the first harvest (Figure 1a). Across all the BC+RF combinations, this increase was on average 95% higher (22.9 ± 3.2 g pot⁻¹) than that in the control treatment (11.7 ± 0.8 g pot⁻¹). Inoculation with *Bacillus cereus* PGPR further increased the fresh leaf biomass raising it to 148% higher (i.e., 29.0 ± 2.48 g pot⁻¹) than the control treatment. The maximum fresh leaf biomass in an individual treatment (36.2 ± 1.6 g pot⁻¹, 208% higher than control) was shown by the 2BC+50RF+PGPR treatment

followed by the 1BC+75RF treatment (29.6 ± 1.2 g pot⁻¹, 152% higher than control).

Adding biochar with reduced fertilizer also significantly increased the fresh root biomass except in the 1BC+50RF and 2BC+75RF treatments where it remained similar to control (Figure 1b). Overall, across all combinations of biochar and reduced fertilizer, without PGPR inoculation, fresh root biomass was 92% higher (i.e., 7.5 ± 1.2 g pot⁻¹) than the control (i.e., 3.9 ± 0.06 g pot⁻¹). Adding PGPR on top of biochar further increased the fresh root biomass up to 168% (i.e., 10.5 ± 0.9 g pot⁻¹) than the control. Similar to fresh leaf biomass, the maximum fresh root biomass in an individual treatment was found in 2BC+50RF+PGPR treatment (13.0 ± 1.1 g pot⁻¹, 233% higher than control).

The response of dry leaf and root weights to various treatments followed a trend almost similar to that shown by the fresh leaf and root biomass respectively (Figure 1c & d). Briefly, all the PGPR treatments showed significantly higher increase in dry leaf matter than BC+RF treatments, which was 338% higher than the control treatment. Whereas, all the BC+RF treatments showed 88% higher dry leaf weight than the control treatment. This distinction between PGPR inoculated and not-inoculated was not as clear in dry root biomass as it was in dry leaf biomass. Only 1BC+75RF+PGPR, 2BC+75RF+PGPR and 2BC+50RF+PGPR showed higher dry root biomass than the control treatment.

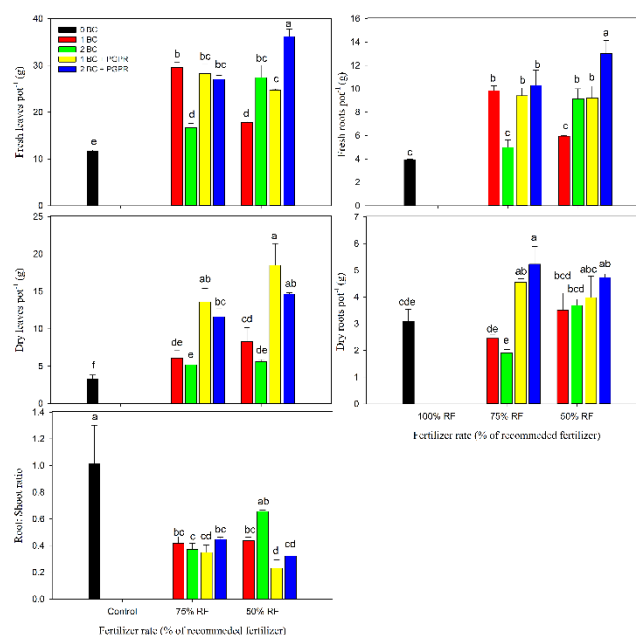


Figure 1. Fresh leaf biomass (A), fresh roots biomass (B), dry leaf weight (C), dry root weight (D) per pot and root: shoot ratio, obtained after first harvest, in response to different combinations of biochar (BC) and recommended fertilizers (RF) as well as BC+RF plus plant growth promoting

bacteria. Each bar represents mean of three replicates whereas standard errors of means are shown as error bars. Letters on top of bars show a significant difference based on a one-way ANOVA followed by LSD test ($P < 0.05$).

The highest root: shoot ratio, calculated based on the dry weights of root and shoot biomass, was shown by the control treatment followed by 2BC+50RF treatment (Figure 1e). All the remaining treatments significantly reduced the root:shoot ratio. Across the BC+RF combinations, an average decrease of 47% compared to the control treatment was observed. Adding PGPR in addition to BC+RF decreased the root:shoot ratio by 51% across all the combinations.

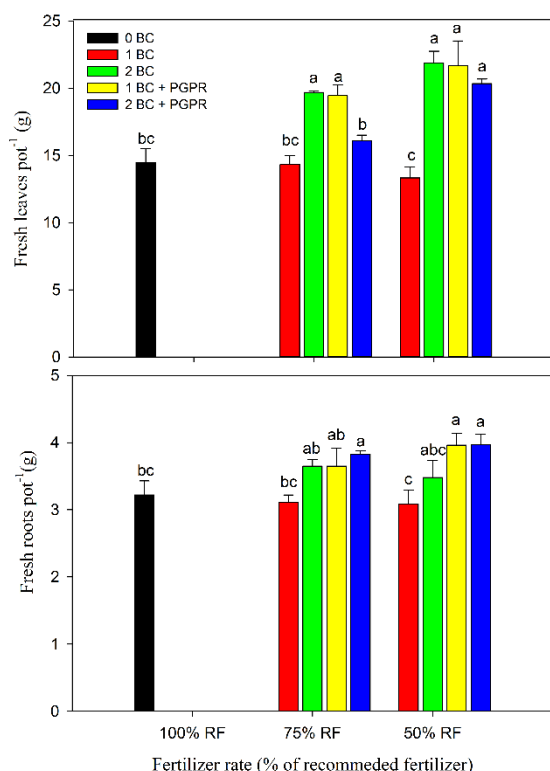


Figure 2. Fresh leaf biomass (A) and fresh root biomass (B), obtained after second harvest, in response to different combinations of biochar (BC) and recommended fertilizers (RF) as well BC+RF plus plant growth promoting bacteria. Each bar represents mean of three replicates whereas standard errors of means are shown as error bars. Letters on top of bars show a significant difference based on a one-way ANOVA followed by LSD test ($P < 0.05$).

During the second harvest, only those BC+RF combinations significantly increased the fresh leaf biomass where either BC addition rate was 2% or the biochar was co-applied with the

PGPR (Fig. 2a). It is interesting to note that the 1% biochar addition did not sustain the increase in the leaf biomass in the second harvest. However, inoculating it with PGPR significantly increased the leaf biomass in 75% RF as well as 50% RF treatments. Another interesting result was that the inoculation with PGPR along with 2BC either reduced the leaf biomass to the level found in control (in 75%RF) or did not cause any further increase than 2BC only (in case of 50%RF). The increase in fresh leaf biomass (34-50%) was less as compared to that observed in the first harvest (95-148%). None of the BC+RF combinations significantly increased fresh root biomass. Three PGPR inoculated BC+RF combinations, i.e., 1BC+50RF+PGPR, 2BC+75RF+PGPR and 2BC+50RF+PGPR treatments significantly increased the fresh root biomass (Fig. 2b). However, this increase was very small i.e., ranging between 26 to 31%, as compared to that in the first harvest that ranged from 92 to 168%.

Photosynthetic pigments: There was no consistent effects of biochar or co-application of biochar and the PGPR on all the photosynthetic pigments (mg g⁻¹ fresh weight) (Fig. 3).

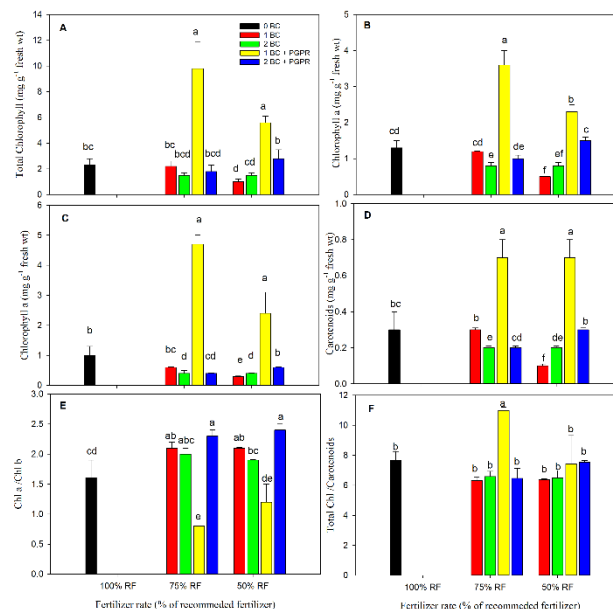


Figure 3. Effect of biochar and biochar plus PGPR inoculation under reduced synthetic fertilizers in comparison to 100% recommended fertilizers on total chlorophyll (A), Chlorophyll a (B), Chlorophyll b (C), carotenoids (D), ratio of Chl a/Chl b (E) and ratio of Total Chl/Car (F). Values are means of three replicates whereas the small letters within a column shows significant difference ($P < 0.05$) among means based on least significant difference test following One-way ANOVA.

The 1BC+50RF lowered total chlorophyll by 56% and PGPR inoculated 1BC+50RF and 1BC+75RF treatments increased the total chlorophyll content by 148% and 334% respectively, with respect to control. All the other treatments were similar to the control. Similarly, the Chlorophyll a was significantly reduced by 58% in 1BC+50RF treatment, and by 39% and 50% in 2BC+75RF and 2BC+50RF respectively, with respect to control. It was significantly increased in response to co-application of 1% BC and PGPR by 181% and 81% in 1BC+75RF+PGPR and 1BC+50RF+PGPR treatments respectively and by 18% in 2BC+50RF+PGPR, with respect to control (Figure 3b). Similarly, co-application of 1% BC and PGPR significantly increased the chlorophyll b by 382% and 143% under 75% RF (1BC+75RF+PGPR) and 50% RF (1BC+50RF+PGPR) regimes respectively, with respect to controls (Figure 3c). The same treatments significantly increased the carotenoids by 164% and 129% with respect to control treatment (Figure 3d). All the remaining treatments significantly reduced the Chlorophyll b and carotenoids compared to the control. Moreover, the ratio of chlorophyll a: chlorophyll b was significantly lower in the PGPR inoculated 1BC+50RF and 1BC+75RF treatments while all the others showed similar or higher value of this ratio when compared to the control treatment. No treatment effect was found on total chlorophyll: carotenoids ratio.

Effect of biochar and PGPR on antioxidant defense system:

The production of oxidant burden i.e., hydrogen peroxide (H_2O_2 , $\mu\text{mol g}^{-1}$ fresh weight) in leaves was significantly lowered by PGPR+ BC+RF combinations by 56-87% except the 2BC+75RF+PGPR treatment (Fig. 4). All the BC+RF combinations without PGPR inoculation did not induce any significant change in H_2O_2 .

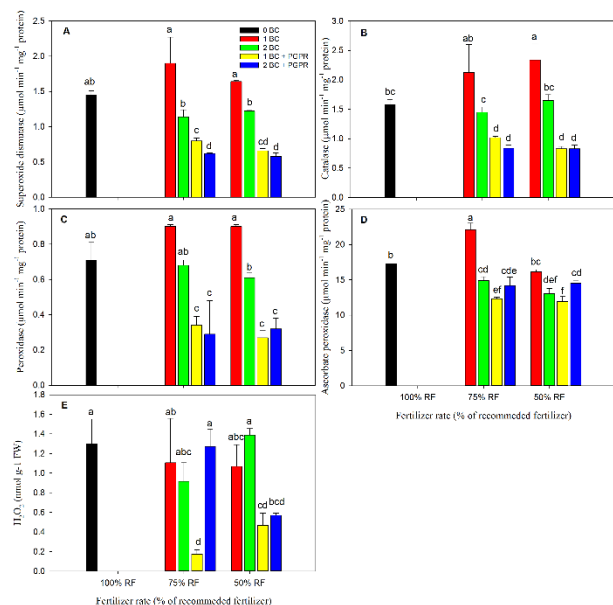


Figure 4. Effect of biochar and biochar plus PGPR inoculation under reduced synthetic fertilizers

in comparison to 100% recommended fertilizers on activities of superoxide dismutase (SOD) (A), catalase (CAT) (B), peroxidase (POD) (C), ascorbate peroxidase (APX) (D) and hydrogen peroxide (H_2O_2) (E). Values are means of three replicates whereas the small letters on top of bars show significant difference ($P < 0.05$) among means based on least significant difference test following one-way ANOVA.

Among the antioxidant enzymes, activity of the superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) was significantly lowered by 45-60%, 35-48%, and 52-62% respectively in all the PGPR inoculated BC+RF combinations (Figure 4A-C). However, all the PGPR-less BC+RF combinations showed similar SOD, CAT and POD activity as that in the control treatment (Figure 4). However, the activity of ascorbate peroxidase (APX) was significantly increased by 1BC+75RF while it was similar in 1BC+50RF treatment compared to control treatment. All the PGPR inoculated BC+RF combinations significantly reduced it in the range 16-31%. Moreover, 2BC+75RF and 2BC+50RF showed 14% and 24% lower APX activity than the control treatment.

Effect of biochar and PGPR on nitrate, available phosphorus and microbial biomass:

In general, biochar addition at both rates significantly increased the soil nitrate content under both the fertilizer regimes with respect to control, except the 1BC addition under 75%RF regime (Figure. 5A). This increase ranged from 327% to 529%, increasing with the rate of addition of biochar from 1% to 2%. Inoculation with the PGPR on top of biochar further increased the soil nitrate content with respect to BC only (60-343%), as well as control treatments (584-750%) but only under 75% RF fertilizer regime. Under 50% RF regime, inoculation with PGPR on top of 1BC didn't induce any change, whereas it significantly reduced it to control levels in 2BC treatment. Adding 1% BC significantly increased the available P by 256% and 130% with respect to controls under 75% and 50% RF regimes respectively (Figure 5B). However, co-applying PGPR with 1% BC under 75% RF significantly reduced the available P to the control level, whereas the same under 50% RF didn't cause any additional change. The addition of 2% BC with or without PGPR did not influence the available P. Only co-application of 2% BC and PGPR significantly increased the microbial biomass by 91% and 72% under 75% RF and 50% RF regime respectively, with respect to control (Figure 5C). None of the 1% BC, with or without co-application of the PGPR, induced any change in the microbial biomass.

Principal components analysis: The PCA revealed that most of the data (89.27%) could be explained by first two canonical axes (Figure 6). It is evident from the analysis that the root and shoot weights (fresh as well as dry) were closely related to microbial biomass carbon (MBC), soil nitrate, as well as

soil phosphorus. Moreover, co-application of biochar (2%) and PGPR under 50% RF regime (2BC+50RF+PGPR) is very closely related with MBC and consequently with shoot and root fresh weights. Whereas, the shoot and root weights were in negative relationship with biochar only application (2%) under 75% RF regime (2BC+75RF).

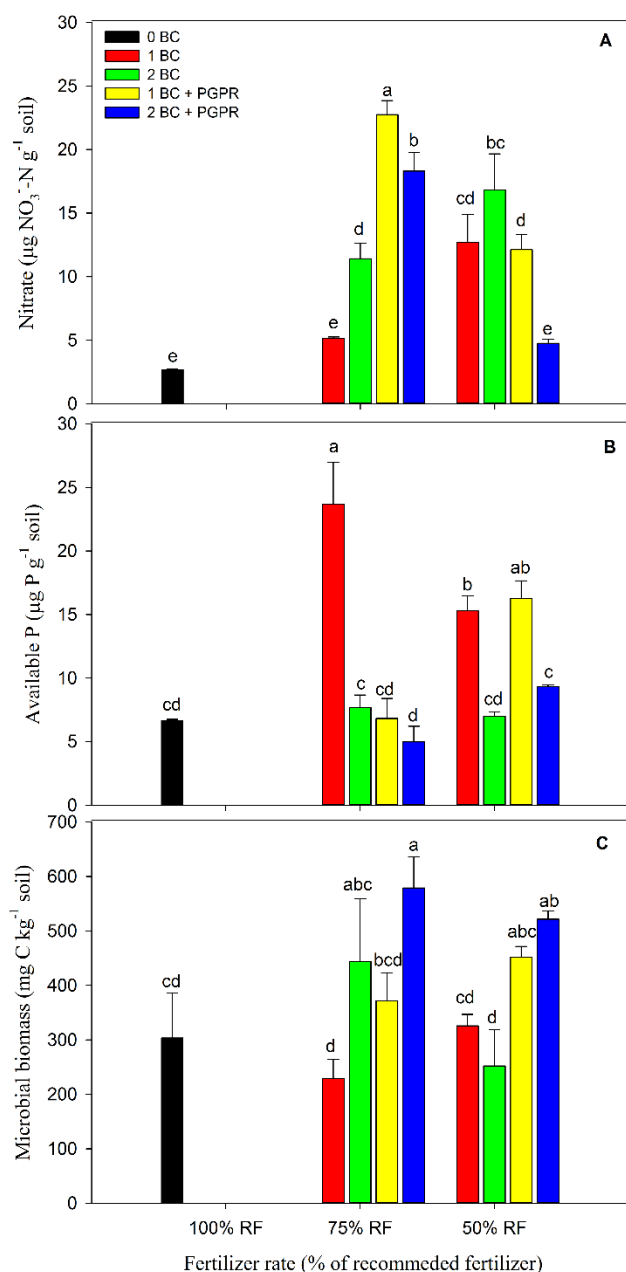


Figure 5. Soil nitrate content (A), available phosphorus (B) and microbial biomass (C) in response to different combinations of biochar (BC) and recommended fertilizers (RF) as well BC+RF plus plant growth promoting bacteria. Each bar

represents mean of three replicates whereas standard errors of means are shown as error bars. Letters on top of bars show a significant difference based on a one-way ANOVA followed by LSD test ($P < 0.05$).

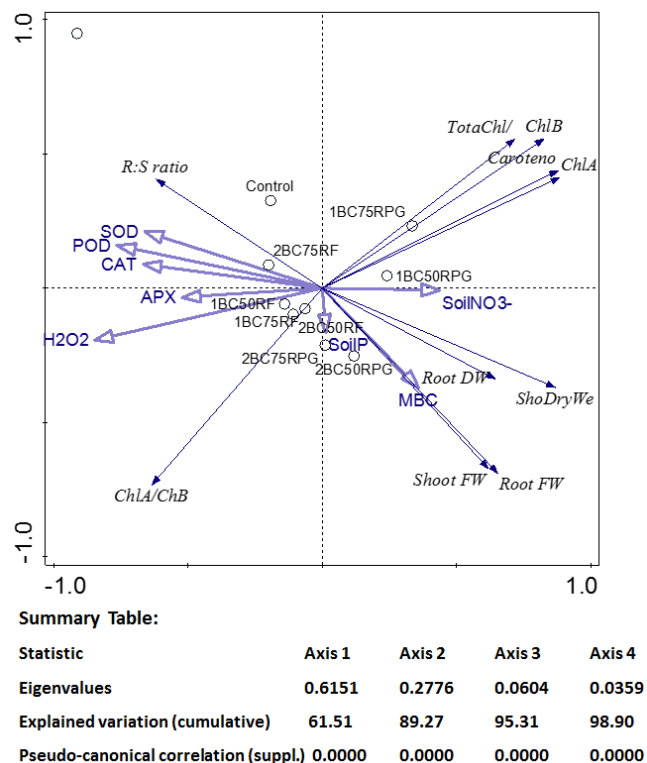


Figure 6. Principal component analysis plant growth, physiological, and chemical variables as well as soil variables; root shoot ration (R: S ratio), Total chlorophyll (Total Chl), Chlorophyll A (Chl A), Chlorophyll B (Chl B), Carotenoids (caroteno), Root dry weight (Root DW), Shoot dry weight (ShoDryWe), Root fresh weight (Root FW), Shoot fresh weight (Shoot FW), Microbial biomass carbon (MBC), Ascorbate peroxidase (APX), Hydrogen peroxide (H_2O_2), Catalase (CAT), Peroxidase (POD), Superoxide dismutase (SOD). The table under the figure shows statistical values of the analysis where the first two components explained the most of the variation (89.3%) in the data.

DISCUSSION

Plant growth: Co-application of biochar and a PGPR, *Bacillus cereus*, induced a positive synergistic effect on spinach leaf biomass compared to sole application of biochar (Figure 1). The co-application under reduced fertilizer regimes across different treatments increased the leaf biomass by 148% compared to control (where only 100%

recommended fertilizers were applied), while the sole application of biochar increased it by 95% only. Co-application of biochar and PGPRs has been found to increase the economic yield of various crops (Malik *et al.*, 2022). For instance, co-application of a timber waste biochar and the PGPR *B. amyloliquefaciens* enhanced wheat grain weight by 59%-77% under drought conditions (Danish and Zafar-ul-Hye, 2019; Zafar-Ul-Hye *et al.*, 2019). Similarly, in a greenhouse study, a PGPR *Pseudomonas fluorescens* in combination with biochar was found to improve cucumber growth by 77% under drought stress (Nadeem *et al.*, 2017). In another study, PGPR consortia along with 500kg ha⁻¹ biochar were found to be the best option to obtain optimum yield (40% higher than without co-application) of wheat at half the dose of recommended synthetic fertilizer (Ijaz *et al.*, 2019). However, none of the cited studies showed as high synergistic effect of co-application of PGPR and biochar as our study. The synergistic effect of the PGPR and biochar on leaf biomass continued till the second harvest, though it was mitigated compared to first harvest (Figure 2). Moreover, biochar only did not induce any increase in the second harvest. Plausibly, the easily accruable mineral nutrients from biochar had expired by the second harvest. However, the PGPR could further decompose the biochar and release the mineral nutrients leading to higher biomass production in co-applied treatments.

Although the biochar addition enhanced fresh root biomass, the root dry matter was significantly higher only in *B. cereus* inoculated BC+RF treatments (Figure 1d). The PGPR strain used in our study bears the trait of indole acetic acid production, which is a phytohormone known for lateral development of roots thereby explaining the increased root dry matter of spinach (Ijaz *et al.*, 2019; Shahid *et al.*, 2020). Moreover, the lower root: shoot ratios in co-applied treatments imply that the plants were having easy availability of nutrients and moisture saving them from investing more in root growth (Henry *et al.*, 2005).

Photosynthetic pigments: Almost all the treatments significantly enhanced total chlorophyll, chlorophyll a & b and carotenoids thereby explaining the higher aboveground growth of plants (Figure 3). Moreover, PGPR induced much higher increase in the contents of these pigments as compared to BC+RF only treatments. This, as well as the other PGPRs are known to improve the chlorophyll and carotenoid contents (Zafar-Ul-Hye *et al.*, 2019). These pigments are directly related to leaf nutritional status, especially N (Asai *et al.*, 2009). The soil nitrate content in soils of these treatments was significantly higher than control indicating that plants in these treatments were not N deficient as is also attested by higher growth.

Antioxidant enzymes and reactive oxygen species: Usually antioxidants and reactive oxygen species (ROS) in plant parts are measured in those studies where plants are subjected to some kind of stress like drought, salinity or presence of heavy

metals in soils (Haider *et al.*, 2019). Although no such stress was faced by plants in our study, the measurement of these variables corroborate the other findings of our study. For instance, the inoculation with PGPR substantially lowered the H₂O₂ as compared to BC+RF and control treatments (Figure 4). This indicates that the plants were facing minimum stress in growing under PGPR inoculation (Shahid *et al.*, 2020). Corresponding decrease in antioxidants activity further confirms that the plants did not need to produce antioxidants for scavenging the ROS which was already very low (Sharma *et al.*, 2012).

Soil available P, nitrate and microbial biomass: Biochar has been shown to contribute to soil mineral nutrients thereby leading to improved plant growth (Prommer *et al.*, 2014; Zhai *et al.*, 2015). Most of the treatments of our study increased the availability of phosphorus and nitrogen in solution (Figure 5). However, the PGPR, although known for its phosphate solubilizing trait (Shahid *et al.*, 2020), did not show any additional increase in phosphorus availability. It is not uncommon for PGPR traits to not translate in the real soil plant systems (Herrmann and Lesueur, 2013). We also determined the soil microbial biomass and found that most of the treatments tended to increase it, although it was significantly higher than control in only two treatments having 2BC with PGPR inoculation. Contradictory responses of microbial biomass to biochar have been reported from positive to neutral (Jeffery *et al.*, 2011; Shemawar *et al.*, 2021). However, the higher biomass, if not always significantly higher than control, in response to various BC+RF and BC+RF PGPR+ treatments in our study are in line with the plant growth, photosynthetic pigments and plant antioxidant defense results.

Mechanistic explanation of synergistic effect of biochar and PGPR on plant growth: The principal components analysis provides mechanistic understanding of the effect of application of biochar alone or co-application of biochar and *Bacillus cereus* on soil and plant attributes (Figure 6). The close link of soils variables i.e., microbial biomass, plant available phosphorus, and soil nitrate with plant attributes i.e., shoot and root biomass show that the increased nutrient availability and healthy microbial biomass underpinned the enhanced spinach production. It has been postulated that biochar and PGPR work in tandem to maximize the accrual of nutrients from biochar's decomposition. The biochar provides safe places for PGPR to thrive on due to its porous structure, whereby the PGPR as well as the extant microbial communities enhance release of nutrients from biochar (Malik *et al.*, 2022).

Conclusion: Potential of co-application of a PGPR i.e., *Bacillus cereus* and biochar to maintain or enhance productivity of spinach under reduced application of mineral fertilizers was tested in this study. Our results show that the co-application increased the spinach production up to two

times over two harvests when compared to the treatment where hundred percent recommended fertilizer was applied. Apparently, co-application of biochar and *Bacillus cereus* works in synergism. Biochar brings in nutrients, some of which become easily available to plants. The PGPR releases the other portion of nutrients from the biochar that do not accrue themselves. Consequently, the plant growth is boosted. These results indicate that the combined application of biochar and PGPR can be an effective technique to reduce the need of application of chemical fertilizers while enhancing plant production. This will serve to save the environment on the one hand and reduce the input cost on the other hand. However, we recommend that this technique should be assessed field trials over multiple growing seasons.

Conflict of interest: The authors declare that there is no conflict of interest.

Authors' contribution: MA & M conceptualized and conducted the experiment and wrote the original draft; SH, FM, AM, MI, MS, devised and performed methods, processed data and reviewed the manuscript; TS conceptualized research question, devised methodology, acquired funding and reviewed the manuscript. All authors have read and agreed to the published version of the manuscript.

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