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Alleviation of Salinity Induced Oxidative Stress in *Chenopodium quinoa* by Fe Biofortification and Biochar—Endophyte Interaction

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Abstract: Iron-biofortification is a sustainable food-based approach to combat iron deficiency by increasing iron content and bioavailability in agronomic crops. Siderophore producing microbes offer a sustainable and low-cost way to increase iron supply in crops. Also, certain substances released from organic amendments act as iron-chelators which increase the solubility as well as the availability of iron to plants. Present study investigated the role of siderophore-producing endophytic bacteria and biochar on iron-fortification of a novel crop quinoa in iron-limited saline conditions. The surface-disinfected seeds of quinoa were inoculated with *Burkholderia phytofirmans* PsJN (CFU = 10^9) and sown in saline soil (EC 20 dS m^{-1}) amended with biochar (1% *w*/*w*). Results revealed that biochar and PsJN particularly when applied together significantly enhanced plant growth, grain yield, and grain nutrient contents of quinoa. Strikingly, iron concentration in quinoa grains was increased up to 71% by the combined application of biochar and PsJN. Moreover, plant physiological parameters were also improved significantly by the integrated application. However, enzymatic/non-enzymatic antioxidants activities were decreased by integrated treatment thus ameliorated salinity stress. Our study suggests that integrated application of siderophore-producing bacteria and biochar could be a promising, sustainable and cost-effective strategy which is easily integratable into the existing farming practices to achieve food fortification with micronutrients in developing countries.

Keywords: plant-microbe interaction; biofortification; salinity; biochar; quinoa; nutrient homeostasis

1. Introduction

Iron deficiency in plant-based foods continues to pose significant public health problems in resource-limited settings. Hidden hunger for iron is significant nutritional disorder in the world, which is considered as a prominent cause of anemia [1]. In developing countries, about 40% of young children

and 50% of pregnant women are anemic [2]. A recent investigation showed that iron deficiency in the newborn babies is responsible for irretrievable influence on the structure, function and the development of the brain [1,3]. Iron deficiency can be cured either through pharmacological iron supplementation or through agriculture-based iron biofortification. Iron-biofortification is a sustainable food-based technique to combat iron deficiencies in humans that can be achieved through three main strategies: (i) increase of iron content in grains or edible parts of plants (ii) increase of the prebiotics concentration in the plant edible parts that favor iron absorption (iii) decrease of antinutrients like phytic acid that reduce iron absorption in the human gut by iron chelating [4].

Agriculture-based iron biofortification can be done by crop fertilization with iron chelates. However, in developing countries, this approach is not sustainable because it requires the long-term supplementation of iron fertilizers and is costly accompanying potential threats to the environment. Conversely, siderophore-producing microbes offer a sustainable and low-cost way to supply iron to the crops. Plant growth promoting bacteria (PGPB) are known to cause improvement in iron uptake in important food crops [5]. PGPB release siderophores into the surrounding environment, which scavenge iron by making iron-chelate complexes and enhance its uptake through growing roots [6]. In saline soils, oxidation states of iron fluctuate between soluble forms to relatively insoluble forms leading to the reduced iron availability in plants [7].

PGPB-based iron supply appeared to be more effective in iron-limited soils, where high pH and salinity reduce Fe availability to crop plants [8]. Under stress conditions, PGPB releases an enzyme 1-aminocyclopropane-1-carboxylic acid (ACC)-deaminase, which can mitigate the negative effect of salinity on root growth by lowering the ethylene concentration in the plants [9–12]. PGPB strain *Burkholderia phytofirmans* PsJN is well known to promote plant growth by improving essential nutrient uptakes like phosphorus, iron, and zinc or by releasing other plant growth regulators like ACC-deaminase and indole acetic acid [13]. Moreover, organic amendments when applied to soils secret certain chemical compounds which acts as chelators and sequester nutrients thus increase their bioavailability to crops [14]. Among the different forms of organic amendments, biochar is well known for improving soil health and nutrient mobilization [15,16]. It has been reported that biochar application enhances both the extent and rate of bacterial ferrihydrite reduction by mediating electron transfer processes [17].

Chenopodium quinoa (quinoa) has recently gained worldwide attention due to its high nutritious and gluten-free edible grains. It has been recognized as a key crop to improve world food security because of its potential to grow on salt-affected soils which are not suitable for other major food crops [18–20]. Until now, food-based iron biofortification is mostly studied either by Fe fertilization or through genetic modification of important crops. However, microbial-based and biochar-based iron biofortification offers more sustainable and cost-effective strategies to provide micronutrients (iron, zinc, etc.,) in developing countries. Moreover, iron biofortification of important agronomic crops like rice, wheat, and maize has been focused [21–24] but Fe biofortification of emerging food crop quinoa is hardly investigated. Here, we investigated the potential of plant growth-promoting bacterium *B. phytofirmans* PsJN and organic amendment (biochar) on the growth characters, yield parameters and iron biofortification potential of quinoa growing in iron-limited saline soil.

2. Materials and Methods

2.1. Preparation of Endophytic Inoculum

The plant growth supporting endophytic bacterium *Burkholderia phytofirmans* PsJN was donated by culture group of Bioresource Unit, Austrian Institute of Technology, Vienna, Austria. The inoculum of *B. phytofirmans* PsJN was prepared in 500 mL Erlenmeyer flask comprising 200 mL Luria-Bertani (LB) broth. The flask was incubated in an orbital shaking incubator (Firstek Scientific, Tokyo, Japan) at 180 rpm for 48 h at 28 ± 2 °C. The optical density of culture was measured at wavelength 600 nm via

spectrophotometer (Nicolet Evolution 300 LC, England, UK) and adjusted to $OD_{0.5}$ to attain an even cell density of bacteria (10^9 CFU mL^{-1}) for inoculation.

The soil was prepared by sieving via a 2 mm mesh to remove plant debris, clods, etc., and analyzed for various characters. The soil texture as measured through hydrometer method reported by Gee and Bauder [25] was found to be clay loam comprising of 39.0% sand, 29.0% silt and 32.0% clay. The pH of the soil paste was 7.9, and the electrical conductivity of soil was 1.98 dS m^{-1}. Plant available Fe was 3.9 mg kg^{-1} as extracted using 0.005 M DTPA [26]. Available phosphorus was measured by the method described by Watanabe and Olsen [27], nitrogen by Bremner and Mulvaney [28] and extractable potassium following the method of Richard [29]. Soil salinity level was maintained up to (20 dS m^{-1}) using sodium chloride salt. Tree twigs feedstock was pyrolyzed at 400 °C for production of biochar in a laboratory setup muffle furnace with 10 °C min^{-1} increase in temperature and 40 min residence time was maintained as described by Sanchez et al. [30]. The biochar was analyzed for physicochemical properties as pH 7.24, EC 1.62, cation exchange capacity 88.52 $cmol_c$ kg^{-1}, organic carbon 57.20%, nitrogen 1.29%, phosphorus 2.94%, potassium 2.26%, zinc 82.53 mg kg^{-1} and iron 89.36 mg kg^{-1}. The prepared biochar was then mixed at a rate of 1% (*w*/*w*) in the soil. The seeds of quinoa (cv. UAF-Q7) were obtained from the Laboratory of Alternative Crops, Department of Crop Physiology, University of Agriculture, Faisalabad, Pakistan. Surface-disinfected seeds of quinoa were soaked in liquid suspension of PsJN (10^9 CFU mL^{-1}) for one hour while un-inoculated seeds were dipped in broth without bacterial cells [31]. Six seeds were initially sown in polyethene lined pots containing 8 kg soil and after germination, two seedlings per pot (each representing one replicate) were maintained. The seeds were sown in mid of November 2016, and the mean maximum temperature was 22 ± 2 °C while mean minimum temperature was 11 ± 2 °C during the crop season. Pots were placed in rain protected wire-house of Institute of Soil and Environmental Sciences, University of Agriculture, Faisalabad, Pakistan under natural conditions. Iron was applied @ of 63 mg kg^{-1} using ($FeSO_4.7H_2O$) as Fe source. Recommended rates of nitrogen, phosphorus and potassium were applied at a proportion of 75, 60 and 30 kg ha^{-1}, respectively by means of urea, diammonium phosphate, and sulfate of potash. There were four treatments (i) control, (ii) biochar, (iii) PsJN and (iv) PsJN + biochar which were replicated thrice under completely randomized design (CRD).

2.2. Plant Growth Parameters

Plant height and dry mass of roots and shoots were recorded on maturity following standard procedures. Grains were threshed manually after harvesting the plants and air-dried under the shadow after washing. Samples of root and shoot were dried in the oven at 80 °C for 48 h.

2.3. Physiological Attributes

After 55 days of sowing, various gas exchange traits, from upper canopy entirely stretched leaves (two fully matured leaves per plant and four leaves per treatment) between 11:00 and 14:00 h were determined by using CIRAS-3 (PP System, Amesbury, MN, USA). Measurements were done on 1.7 cm^2 area of the leaf at 400 µmol mL^{-1} of carbon dioxide as well as 1000 µmol m^{-2} s^{-1} of photosynthetically active radiations (PAR). Leaf chlorophyll contents were assessed by using SPAD meter. Each sample of the leaf was measured at least six diverse spaces for SPAD measurement.

2.4. Extraction and Enzymatic/Non-Enzymatic Antioxidant Assays

Extraction of various enzymatic and non-enzymatic antioxidant was done by homogenizing solid fresh leaf material in an ice-cold solution comprising of 0.2 M potassium phosphate buffer (pH 7) having 0.1 mM EDTA. Glutathione reductase was estimated according to the method reported by Smith et al. [32]. The activities of oxidized glutathione (GSSG) and reduced glutathione (GSH) were calculated following [33] method. Whereas, Ascorbate peroxidase (APX) assay was performed by Nakano and Asada [34]. Roth and Gilbert, [35] classical method was adapted to monitor the activity

of superoxide dismutase (SOD) and the activity of glutathione-s-transferase (GST) was measured spectrophotometerically by the method given by Habig et al. [36].

2.5. Measurement of Stress Related Metabolites

The ROS (super oxide anion $O_2^{\bullet -}$) was measured according to the method described by Elstner and Heupel [37]. Whereas, to measure lipid peroxidation or malondialdehyde (MDA) concentration in leaves the original method given by Jambunathan [38] was applied.

2.6. PsJN colonization of Rhizosphere, Root, and Shoot

Rhizosphere and endophytic colonization of plant tissues by the inoculant strain PsJN were determined by dilution and plate counting technique. After harvesting, rhizospheric soil was sampled by roots agitation and collecting closely adhered soil. For colonization assay, soil slurry was prepared at a ratio of 1:3 (soil: NaCl) mixing 5 g rhizosphere soil with 15 mL of 0.9% (*w*/*v*) NaCl solution following agitation at (180 rpm) for 30 min at 28 °C. After complete soil particles sedimentation, serial dilutions up to 10^{-6} were plated onto tryptic soy agar (TSA) medium. Colonies were counted after incubating the plates at (28 ± 2 °C) for 48 h, and the colonization value was determined afterwards. For root/shoot colonization, 2 g of surface-sterilized samples of each were homogenized in 10 mL 0.9% NaCl solution by using a sterile mortar and pestle. The material was placed in a shaking incubator for 30 min at 28 °C. After settling the solid fraction, serial dilutions up to 10^{-5} were spread on TSA medium. 20 visible colonies were selected per treatment randomly, and their identity with that of inoculant strain was authenticated by restriction fragment length polymorphism (RFLP) analysis of the 16S–23S rRNA intergenic spacer (IGS) region [39].

2.7. Water Relations and Grain Quality Parameters

Relative water contents (RWC), relative membrane permeability (RMP) and membrane stability index (MSI) were measured from fully matured flag leaves. Relative water contents were assessed by the method given by Mayak et al. [40].

$$RWC = \frac{fresh\ weight - dry\ weight}{fully\ tugid\ weight - dry\ weight} \times 100 \quad (1)$$

For measuring RMP, leaves were cut and placed into test tubes consisting of 20 mL deionized water, and EC_0 was measured after vortexing samples for ten seconds. EC_1 of this solution was measured after 24 h of incubation at 4 °C. The tubes were autoclaved at 121 °C for 20 min to measure EC_2. Following formula was used to calculate RMP as defined by Yang et al. [41].

$$RMP(\%) = \frac{EC_1 - EC_0}{EC_2 - EC_0} \times 10 \quad (2)$$

For measuring MSI, leaf cuttings were weighed and transferred into test tubes containing 10 mL deionized water. These test tubes were set aside in water bath at 40 °C for 30 min, and EC_1 was noted. Then, tubes were set aside in water bath at 100 °C for 10 min to find out EC_2. The formula to calculate MSI as defined by Sairam et al. [42] is given below.

$$MSI = \left[1 - \frac{EC_1}{EC_2}\right] \times 100 \quad (3)$$

The concentration of protein in total grain samples was evaluated by Bradford method [43]. Phytate was examined through a process defined by Haug and Lantzsch [44]. After grinding, 60 mg of each grain sample was extracted with 10 mL (0.2 N HCl) 25 °C for 2 h and following measurement of respective concentration spectrophotometerically. Ash analysis of grain was done via methods of

AOAC [45]. All devices utilized for chemical and biochemical examination were soaked in diluted HNO_3 (pro analysis quality, Merck, Kenilworth, NJ, USA) and washed with deionized water.

2.8. Nutrient Analysis

Plant samples (roots and shoots) were digested following Wolf [46] method by using sulphuric acid (H_2SO_4) and hydrogen perchloric acid ($HClO_4$). The digested samples (grains) were crushed in a mill and passed through 0.5 mm sieve to perform chemical and biochemical analysis. For quantifying iron metal, known weight of ground sub-samples of total grains was placed on digestion in a di-acid mixture having ratio 2:1 (HNO_3: $HClO_4$) [47]. Concentrations of sodium and potassium in the root, shoot, and grains of quinoa were determined by a flame photometer (Jenway, PFP-7, Staffordshire, UK) and Na^+/K^+ ratio was calculated afterwards. The concentration of nitrogen in the roots, shoots and grains of quinoa was determined using Kjeldahl apparatus. Plant available phosphorus was determined using Barton reagents through Ashraf et al. [48] method. Iron concentration was measured on atomic absorption spectrophotometer (Perkin Elmer Aanalyst-100, PerkinElmer Inc, Waltham, MA, USA).

2.9. Statistical Analysis

Data recorded for growth, physiology, nutrients, and biochemical quality were subjected to one-way (ANOVA) analysis of variance using statistix 8.1® software (Statistix, Tallahassee, FL, USA). Significant differences among treatment means were computed by post hoc Tuckey's test ($P < 0.05$).

3. Results

3.1. Plant Growth Parameters

Plant growth parameters varied considerably upon amendment of plant growth-promoting endophyte PsJN alone and in a combination with biochar. Overall, both the sole bacterial inoculation and in combination with BC increased the growth, development and nutrient uptake of quinoa at both levels of iron fertilizer but the influence was more noticeable at 63 mg kg^{-1} iron level especially in the integrated application (PsJN + BC) (Table 1). In the sole application of PsJN and BC, plant height was increased up to 12 and 39%, respectively relative to the untreated control (EC, 20 dS m^{-1}). Whereas, the integrated use of PsJN and biochar resulted in a more striking effect on plant height that was recorded up to 94% more over untreated control. Similarly, integrated application significantly increased shoots and roots dry weights up to 38% and 46%, respectively at higher Fe level over control. Sole application of BC and PsJN increased shoots dry weight up to 13% and 10% at Fe 3.9 mg kg^{-1} while 27% and 24% increase was observed at Fe 63.9 mg kg^{-1}. Similarly, same treatments showed increase in roots dry weight (15%, 8%) and (38%, 31%) at both Fe levels, respectively as compared to control. Relatively little increase (7% and 10%) in grain yield was observed by separate application of BC and PsJN at low Fe level while up to 22 and 28% increase was observed when same treatments were integrated with Fe at higher level as compared to control. The maximum increase in grain yield (35%) was recorded through combined use of BC, PsJN and Fe (63.9 mg kg^{-1}) over control.

3.2. Physiological Attributes

Plant physiological characters showed the greatest improvement with the integrated application of endophyte PsJN and tree-twigs biochar under saline environment. The integrated application significantly increased photosynthetic and transpiration rate up to 41% and 138%, respectively compared to control. However, solely the biochar and PsJN boosted rate of photosynthesis up to 9 and 20%, respectively relative to control when both treatments were combined with Fe, relatively higher increase 26% and 34% were observed compared to control. Similarly, in transpiration rate, BC and PsJN showed 115 and 100% increase at Fe 63.9 mg kg^{-1} relative to control. The other physiological parameters such as stomatal and sub-stomatal conductance, integrated use performed better as compared to the sole application of bacteria and BC at both Fe levels compared to control (Table 1).

Table 1. The effect of biochar-*Burkholderia phytofirmans* PsJN integration on growth and physiological parameters of *Chenopodium quinoa* under salinity stress.

Treatment	Fe (mg kg^{-1})	Plant Height (cm)	Root Dry Weight (g pot^{-1})	Shoot Dry Weight (g pot^{-1})	Grain Yield (g pot^{-1})	Photosynthetic Rate (µmol CO_2 m^{-2} s^{-1})	Transpiration Rate (µmol H_2O m^{-2} s^{-1})	Stomatal Conductance (µmol m^{-2} s^{-1})	Internal CO_2 Concentration (µmol mol^{-1})
C	3.9	35.3 ± 1.45 g	1.3 ± 0.086 d	3.7 ± 0.165 c	6.8 ± 0.332 e	11.6 ± 0.185 e	1.3 ± 0.88 e	75.3 ± 2.02 e	274.7 ± 5.54 a
	63.9	45 ± 1.94 ef	1.4 ± 0.086 bc	4.4 ± 0.258 ab	7.8 ± 0.265 c	13.4 ± 0.317 cd	1.9 ± 0.56 cd	83.7 ± 2.18 de	254.3 ± 4.91 bc
BC	3.9	48.9 ± 1.67 de	1.5 ± 0.106 bc	4.2 ± 0.248 bc	7.3 ± 0.270 d	12.6 ± 0.523 de	2.1 ± 0.91 c	86.7 ± 2.60 cd	246.3 ± 4.33 cd
	63.9	61.2 ± 2.03 b	1.8 ± 0.128 ab	4.7 ± 0.355 ab	8.3 ± 0.292 b	14.6 ± 0.425 bc	2.8 ± 0.44 b	109.3 ± 3.52 a	231.7 ± 3.92 de
PsJN	3.9	39.5 ± 2.04 fg	1.4 ± 0.076 cd	4.1 ± 0.230 bc	7.5 ± 0.384 cd	13.9 ± 0.642 cd	1.7 ± 1.11 d	81.3 ± 2.02 de	264 ± 6.08 ab
	63.9	56.9 ± 1.76bc	1.7 ± 0.113 ab	4.6 ± 0.302 ac	8.7 ± 0.352 ab	15.5 ± 0.550 ab	2.6 ± 0.64 b	99.3 ± 3.17 b	241 ± 4.16 cd
BC+PsJN	3.9	51.7 ± 1.90 cd	1.5 ± 0.104 ab	4.1 ± 0.261 bc	7.7 ± 0.243 cd	14.4 ± 0.545 bc	2 ± 1.04 cd	94.3 ± 2.40 bc	239.7 ± 3.48 cd
	63.9	68.5 ± 1.96 a	1.9 ± 0.153 a	5.1 ± 0.346 a	9.2 ± 0.298 a	16.4 ± 0.497 a	3.1 ± 0.26 a	113.3 ± 3.84 a	220.7 ± 3.52 e

Quantities sharing similar letters are not different with each other. C: Control; BC: Biochar (1% *w*/*w*); PsJN: *Burkholderia phytofirmans*; Fe: Ferrous sulphate (15 kg ha^{-1}). Values are mean of three repeats ± SD.

3.3. Water Relations, Chlorophyll Content and Grain Quality Parameters

Data indicated that the integrated application of BC and PsJN also significantly increased the water relations of *Chenopodium quinoa* in terms of RWC, MSI and RMP up to 55%, 127%, and 64% respectively relative to their control treatments at Fe 63.9 mg kg^{-1} (Table 2). The sole application of BC and PsJN showed increase in RWC (20% and 14%), MSI (45% and 30%) and RMP (23% and 9%) at low Fe relative to their controls. However, BC and PsJN when combined with Fe showed increase 42% and 30% in RWC, 98% and 110% in MSI and 58% and 43% in RMP, respectively as compared to control. Likewise, the grain protein and ash levels were improved up to 61% and 57% over control by the simultaneous use of biochar and PsJN at higher Fe level. However, alone application of biochar and PsJN enhanced protein levels up to 9% and 18% at low Fe while up to 42% and 52%, respectively at higher Fe than control. Likewise, sole use of biochar and PsJN increased grain ash contents by 9% and 17% at low Fe, while 38% and 49% at high Fe level, respectively than control. In case of chlorophyll content, sole use of BC and PsJN enhanced 15 and 23% chlorophyll content as compared to control at higher Fe level. Maximum increase (32%) in chlorophyll content was recorded through combined use of BC and PsJN at Fe 63.9 mg kg^{-1} (Table 2).

Table 2. The effect of biochar-*Burkholderia phytofirmans* PsJN integration on water relations, chlorophyll contents and grain quality parameters of *Chenopodium quinoa* under salinity stress.

Treatment	Fe (mg kg^{-1})	RWC (%)	RMP (%)	MSI (%)	Protein (%)	Ash (%)	Chlorophyll (SPAD)
C	3.9	47.33 ± 1.53 d	42.43 ± 1.54 d	18.60 ± 0.66 e	9 ± 0.38 e	2.3 ± 0.15 e	47.5 ± 1.47 d
	63.9	54.16 ± 1.84 cd	55.90 ± 2.08 b	28.80 ± 1.10 cd	12.1 ± 0.35 c	3 ± 0.23 ad	51.6 ± 1.73 cd
BC	3.9	56.60 ± 1.59 c	52.20 ± 1.99 bc	26.90 ± 0.81 cd	9.8 ± 0.23 de	2.5 ± 0.15 de	50.9 ± 1.51 d
	63.9	67.13 ± 2.08 ab	66.80 ± 2.03 a	36.90 ± 1.10 b	12.8 ± 0.35 bc	3.2 ± 0.23 ab	54.7 ± 1.77 bc
PsJN	3.9	53.80 ± 1.59 cd	46.16 ± 1.48 cd	24.10 ± 0.64 d	10.6 ± 0.36 d	2.7 ± 0.15 ce	52.3 ± 1.33 cd
	63.9	61.53 ± 1.79 bc	60.73 ± 1.85 ab	39.20 ± 1.18 ab	13.7 ± 0.27 ab	3.4 ± 0.26 ab	58.5 ± 1.83 ab
BC+PsJN	3.9	59.00 ± 1.91 bc	54.43 ± 1.51 bc	29.80 ± 0.63 c	12.4 ± 0.32 c	2.8 ± 0.17 be	53.2 ± 1.87 c
	63.9	73.30 ± 1.84 a	69.70 ± 2.15 a	42.23 ± 1.39 a	14.5 ± 0.37 a	3.6 ± 0.21 a	62.6 ± 2.01 a

Quantities sharing similar letters are not different with each other. Values are mean of three repeats ± SD. C: Control; BC: Biochar (1% *w*/*w*); PsJN: *Burkholderia phytofirmans*; Fe: Ferrous sulphate (15 kg ha^{-1}). RWC = Relative water contents, RMP = Relative membrane permeability and MSI = Membrane stability index.

3.4. Nutrient Analysis

Data regarding Fe concentration in roots revealed that integrated application significantly increased root Fe concentration relative to control at both iron levels. However, the maximum increment 71% in root iron content was noticed when Fe was applied at 63.9 mg kg^{-1} along with BC and PsJN. The sole application of BC and PsJN showed 40% and 48% in shoot and 43% and 53% increase in grain Fe contents, respectively as compared to control whereas integrated application strikingly enhanced Fe concentration up to 70 and 71%, respectively in both parts over control (Figure 1).

Bacterial inoculation, especially in combination with BC enhanced major nutrients like nitrogen (N), phosphorus (P) and potash (K) concentration both in below and aboveground parts of the plant (Tables 3 and 4). Sole application of BC and PsJN showed increase (17% and 13%), (13% and 8%) and (31% and 19%) in root, shoot and grain N, respectively over control. Fe application along with BC and PsJN showed more promising increase in plant upper tissue compared to control. Maximum increase 66%, 58% and 90% in root, shoot and grain was observed through integrated application of BC and PsJN at 63.9 mg Fe/kg compared to control. Similar trend was observed regarding P and K concentration in upper plant tissue of quinoa by BC and PsJN application at 3.9 and 63.9 mg Fe/kg compared to control. Application of BC and PsJN inoculum decreased sodium (Na) concentration in roots as well as in shoots. The integrated application of biochar and PsJN showed highest decrease in Na level particularly at higher Fe dose that was 40%, 58%, and 66% less than control in the roots, shoots and grains, respectively (Table 4). Contrarily, plant potassium concentration was increased significantly (107%) over control especially with the integrated application of biochar and PsJN at 63.9 mg kg^{-1}. However, irrespective to the applied treatments, the Na^+/K^+ ratio was considerably

decreased as compared to control (Figure 2). Maximum decrease in Na^+/K^+ ratio of root, shoot and grain by 64%, 87% and 89%, respectively through combined use of BC and PsJN at 63.9 mg kg^{-1} as compared to control.

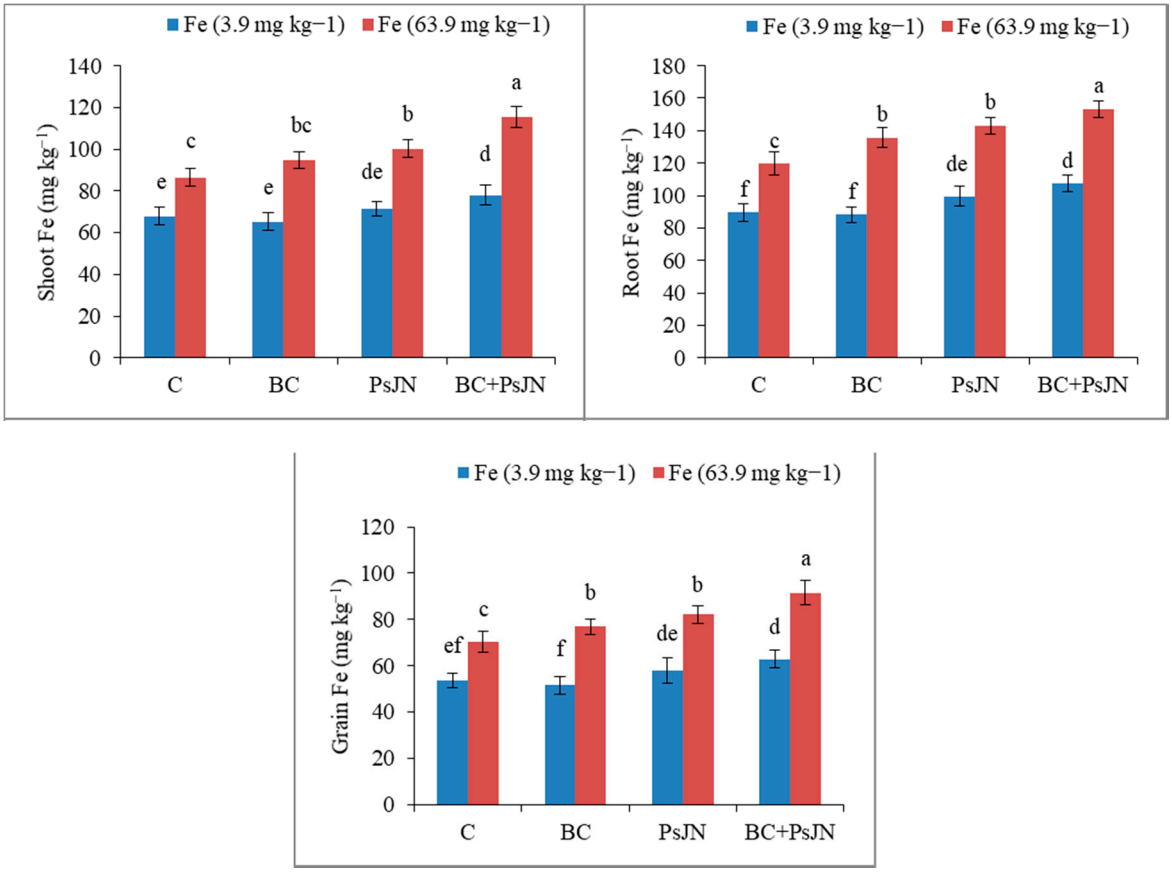

Figure 1. The effect of biochar-*Burkholderia phytofirmans* PsJN integration on Fe concentration of *Chenopodium quinoa* under salinity stress. Quantities sharing similar letters are not different with each other. C: Control; BC: Biochar (1% *w*/*w*); PsJN: *Burkholderia phytofirmans*; Fe: Ferrous sulphate (15 kg ha^{-1}). Values are mean of three repeats ± SD.

Table 3. The effect of biochar-*Burkholderia phytofirmans* PsJN integration on chemical parameters (N and *P*) of *Chenopodium quinoa* under salinity stress.

Treatment	Fe (mg kg^{-1})	N in Root (mg kg^{-1})	N in Shoot (mg kg^{-1})	N in Grain (mg kg^{-1})	*P* in Root (mg kg^{-1})	P in Shoot (mg kg^{-1})	*P* in Grain (mg kg^{-1})
C	3.9	21.6 ± 0.72 d	15.8 ± 0.47 d	9.1 ± 0.23 f	3.1 ± 0.15 e	2.1 ± 0.10 e	1.2 ± 0.06 f
	63.9	26.9 ± 0.98 c	18.8 ± 0.51 c	12.6 ± 0.55 d	4.8 ± 0.28 cd	3.1 ± 0.17 cd	2.1 ± 0.06 de
BC	3.9	25.2 ± 0.97 c	17.9 ± 0.53 c	11.9 ± 0.32 de	4.9 ± 0.26 cd	3.5 ± 0.21 bd	2.2 ± 0.09 de
	63.9	31.8 ± 1.18 b	21.9 ± 0.58 b	14.4 ± 0.41 c	6.1 ± 0.32 ab	4.8 ± 0.32 a	3.7 ± 0.0 7 b
PsJN	3.9	24.4 ± 0.75 cd	17.1 ± 0.43 cd	10.8 ± 0.32 e	4.6 ± 0.21 d	3.0 ± 0.15 d	2.1 ± 0.07 de
	63.9	33.3 ± 1.38 ab	23.8 ± 0.66 a	15.9 ± 0.72 b	5.6 ± 0.31 bc	4.0 ± 0.26 b	3.2 ± 0.08 c
BC+PsJN	3.9	26.1 ± 0.64 c	18.3 ± 0.55 c	12.1 ± 0.34 de	5.1 ± 0.30 cd	3.8 ± 0.21 bc	2.4 ± 0.07 d
	63.9	35.9 ± 1.21 a	25.0 ± 0.78 a	17.3 ± 0.42 a	6.8 ± 0.32 a	5.0 ± 0.32 a	4.0 ± 0.12 a

Quantities sharing similar letters are not different with each other. C: Control; BC: Biochar (1% *w*/*w*); PsJN: *Burkholderia phytofirmans*; Fe: Ferrous sulphate (15 kg ha^{-1}). Values are mean of three repeats ± SD.

Table 4. The effect of biochar-*Burkholderia phytofirmans* PsJN integration on chemical parameters (Na and K) of *Chenopodium quinoa* under salinity stress.

Treatment	Fe (mg kg^{-1})	Na in Root (mg kg^{-1})	Na in Shoot (mg kg^{-1})	Na in Grain (mg kg^{-1})	K in Root (mg kg^{-1})	K in Shoot (mg kg^{-1})	K in Grain (mg kg^{-1})
C	3.9	2.2 ± 0.06 a	1.7 ± 0.05 a	0.6 ± 0.06 a	19.2 ± 0.64 d	11.2 ± 0.63 e	7.2 ± 0.14 f
	63.9	1.9 ± 0.06 cd	0.8 ± 0.03 d	0.4 ± 0.03 b	25.7 ± 0.99 bc	18.2 ± 0.63 c	11.8 ± 0.50 cd
BC	3.9	1.9 ± 0.05 bc	0.9 ± 0.03 c	0.4 ± 0.03 b	26.2 ± 1.18 bc	19.2 ± 0.83 bc	12.5 ± 0.34 c
	63.9	1.6 ± 0.05 e	0.6 ± 0.03 e	0.25 ± 0.02 cd	29.3 ± 1.30 ab	21.1 ± 0.86 ab	13.6 ± 0.45 b
PsJN	3.9	2.1 ± 0.07 ab	1.1 ± 0.07 b	0.5 ± 0.04 a	23.2 ± 1.03 c	16 ± 0.55 d	11 ± 0.34 de
	63.9	1.8 ± 0.05 cd	0.6 ± 0.03 e	0.3 ± 0.02 bc	27.9 ± 1.27 ab	20.1 ± 0.57 bc	12.4 ± 0.30 c
BC+PsJN	3.9	1.8 ± 0.05 cd	0.7 ± 0.04 de	0.3 ± 0.03 bc	22.6 ± 0.92 cd	15.1 ± 0.40 d	10.1 ± 0.28 e
	63.9	1.3 ± 0.03 f	0.4 ± 0.02 f	0.2 ± 0.02 d	31.3 ± 1.52 a	22.9 ± 0.98 a	14.9 ± 0.36 a

Quantities sharing similar letters are not different with each other. C: Control; BC: Biochar (1% *w/w*); PsJN: *Burkholderia phytofirmans*; Fe: Ferrous sulphate (15 kg ha^{-1}). Values are mean of three repeats ± SD.

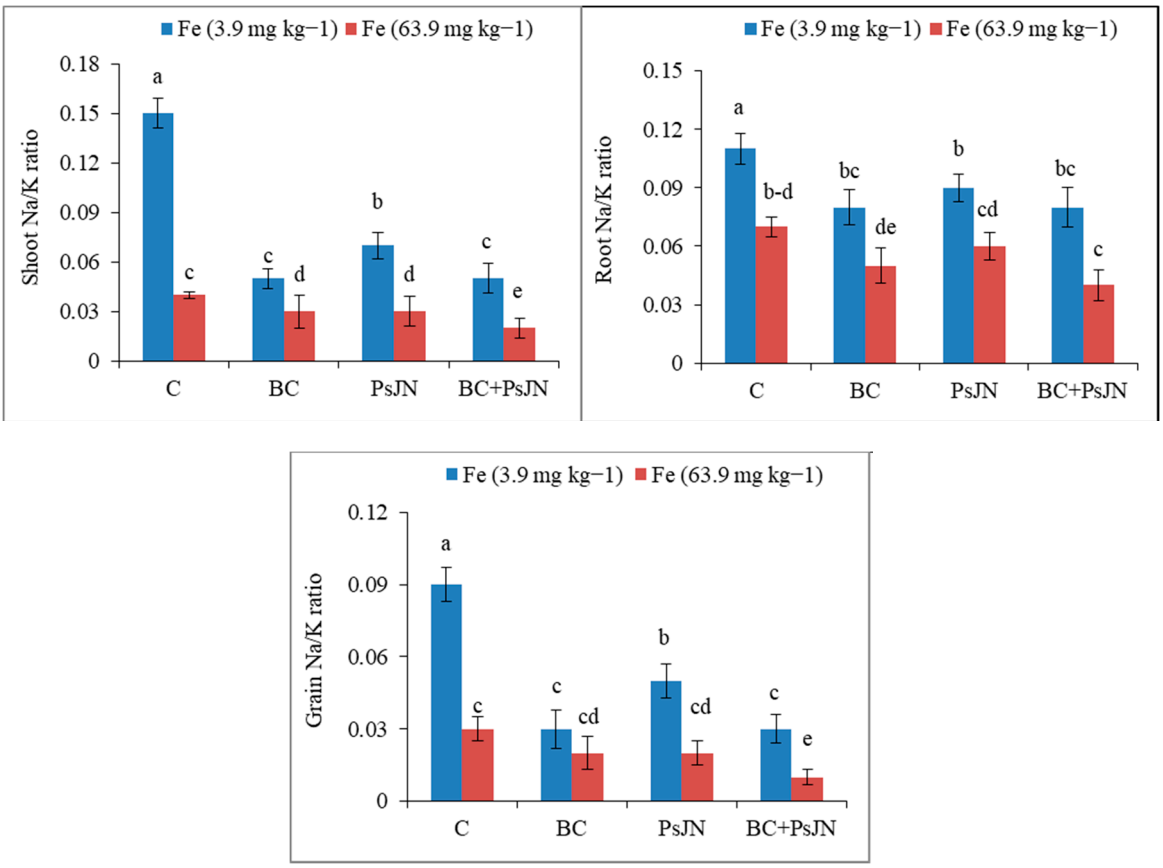

Figure 2. The effect of biochar-*Burkholderia phytofirmans* PsJN integration on Na/K ratio of *Chenopodium quinoa* tissues under salinity stress. Quantities sharing similar letters are not different with each other. C: Control; BC: Biochar (1% *w/w*); PsJN: *Burkholderia phytofirmans*; Fe: Ferrous sulphate (15 kg ha^{-1}). Values are mean of three repeats ± SD.

3.5. Enzymatic and/Non-Enzymatic Antioxidants and Stress Related Metabolites

Various assays for enzymatic or non-enzymatic antioxidant activities showed that treatment with biochar and PsJN especially when they were applied together significantly decreased glutathione reductase (GR) activity (61%), oxidized glutathione (GSSG) activity (27%), reduced glutathione (GSH) activity (54%), GSH-GSSG ratio (38%), ascorbate peroxidase (APX) activity (45%), superoxide dismutase (SOD) activity (53%), glutathione-s-transferase (GST) (56%), glutathione peroxidase (GPX) (47%), super-oxide anion (52%) and malondialdehyde activity (47%), as compared to control where plants were supplemented with 63.9 mg kg^{-1} Fe (Figures 3–5). Similarly, sole use of BC showed

decrease in GR (30% and 35%), GSSG (17% and 20%), GSH (22% and 33%), APX (14% and 20%), GPX (7% and 23%), SOD (15% and 34%), GST (25% and 34%), super-oxide anion (28% and 32%) and MDA (5% and 29%), respectively at low and high Fe levels relative to their controls. Similarly, single application of PsJN inoculum showed decrease in GR (44% and 46%), GSSG (20% and 22%), GSH (37% and 43%), APX (31% and 33%), GPX (25% and 34%), SOD (39% and 43%), GST (39% and 44%), super-oxide anion (42% and 40%) and MDA (13% and 38%), respectively at both Fe levels as compared to their controls.

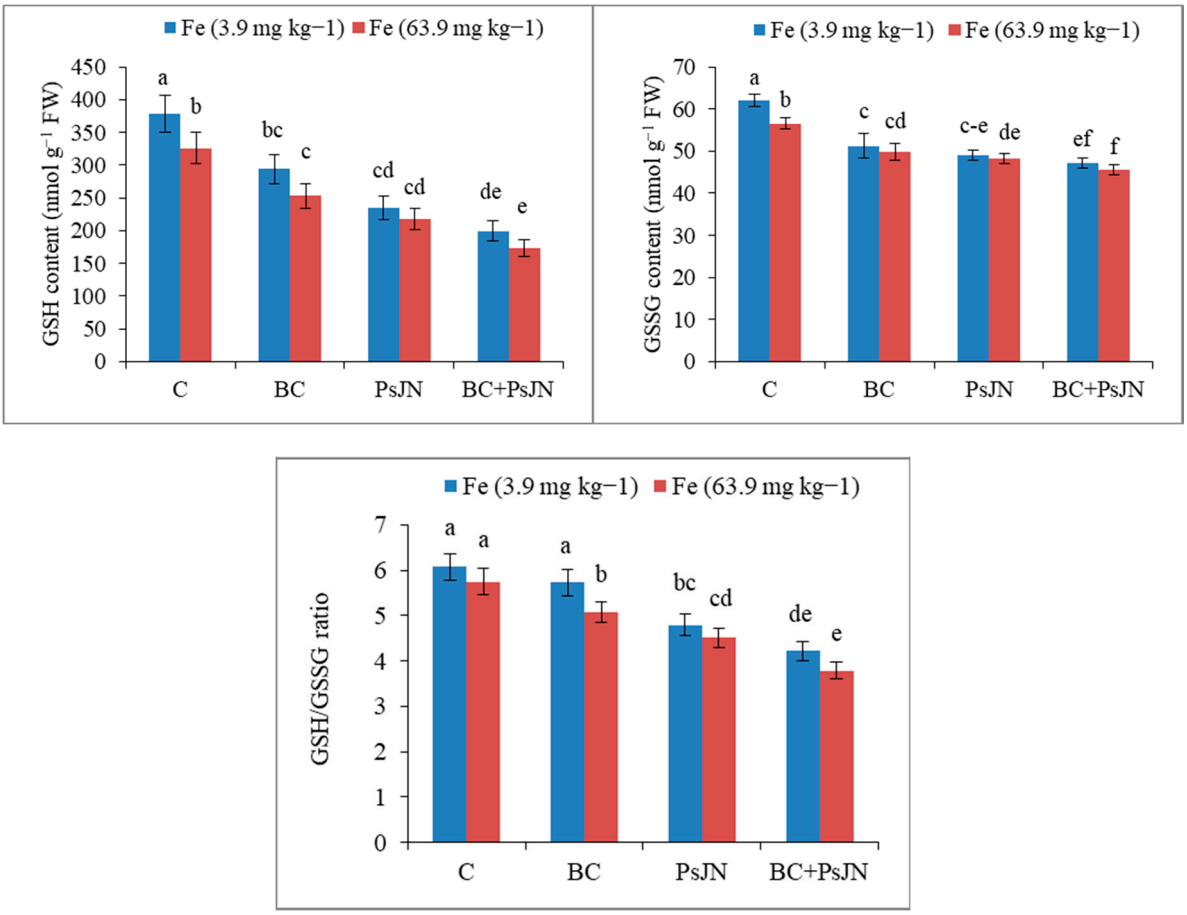

Figure 3. The effect of biochar-*Burkholderia phytofirmans* PsJN integration on Glutathione homeostasis of *Chenopodium quinoa* under salinity stress. GSH = Reduced glutathione, GSSG = Oxidized glutathione, GSH/GSS ratio. Quantities sharing similar letters are not different with each other. C: Control; BC: Biochar (1% *w*/*w*); PsJN: *Burkholderia phytofirmans*; Fe: Ferrous sulphate (15 kg ha^{-1}). Values are mean of three repeats ± SD.

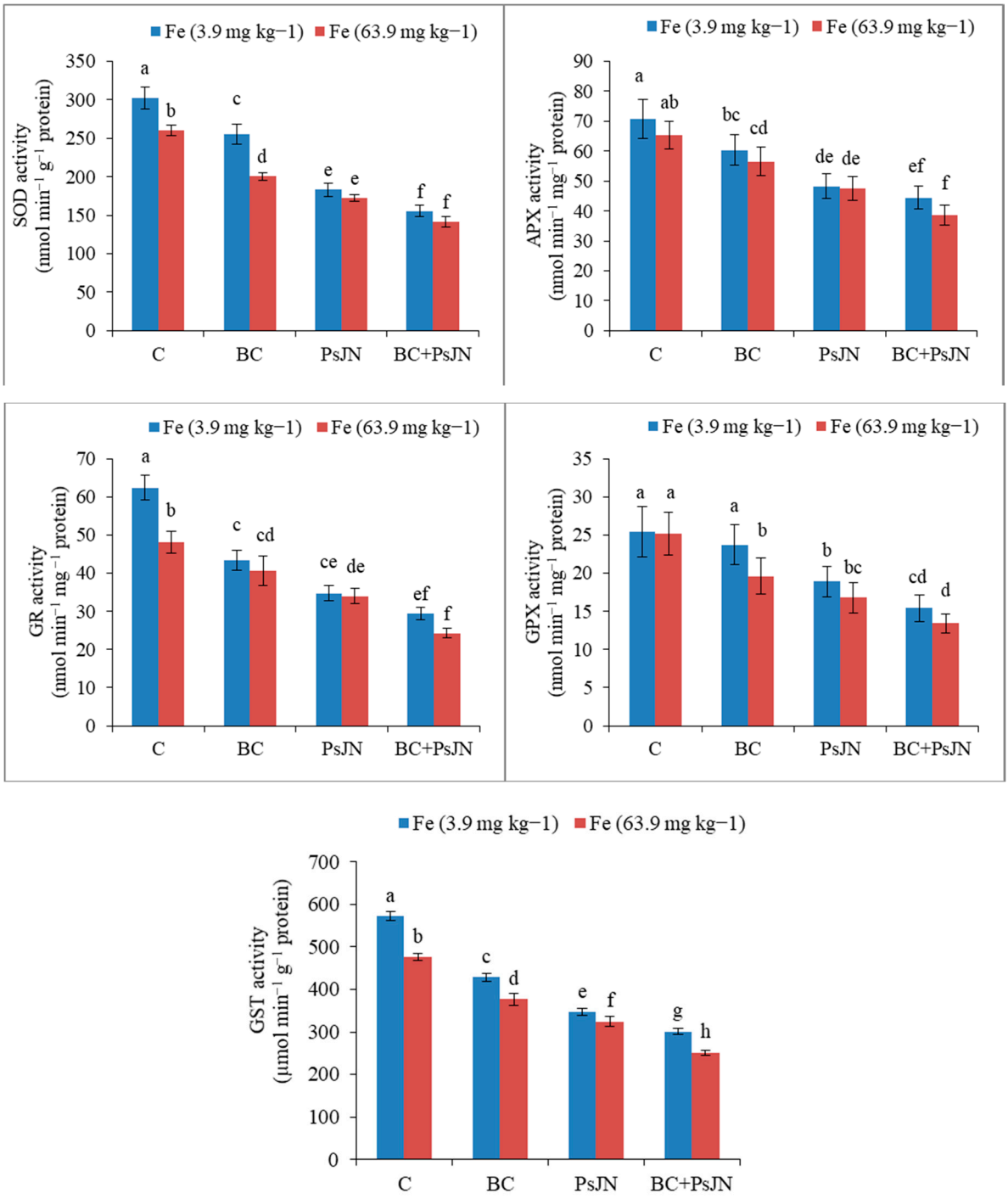

Figure 4. The effect of biochar-*Burkholderia phytofirmans* PsJN integration on antioxidant activity of *Chenopodium quinoa* under salinity stress. SOD = Super-oxide dismutase, APX = Ascorbate peroxidase, GR = Glutathione reductase, GPX = Glutathione peroxidase, GST = Glutathione S-transferase. Quantities sharing similar letters are not different with each other. C: Control; BC: Biochar (1% *w*/*w*); PsJN: *Burkholderia phytofirmans*; Fe: Ferrous sulphate (15 kg ha^{-1}). Values are mean of three repeats ± SD.

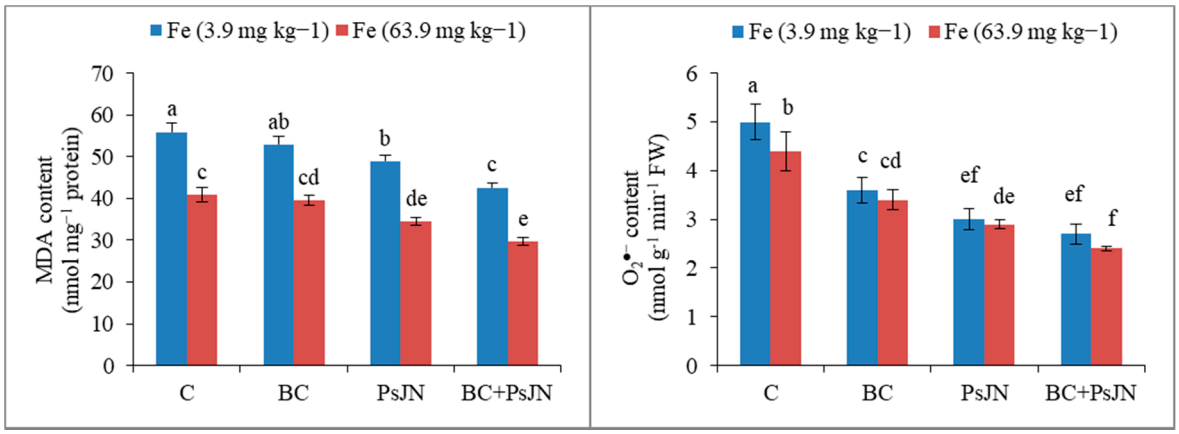

Figure 5. The effect of biochar-*Burkholderia phytofirmans* PsJN integration on stress related metabolites of *Chenopodium quinoa* under salinity stress. MDA; Malondialdehyde, O_2^-; Superoxide anion. Quantities sharing similar letters are not different with each other. C: Control; BC: Biochar (1% *w*/*w*); PsJN: *Burkholderia phytofirmans*; Fe: Ferrous sulphate (15 kg ha^{-1}). Values are mean of three repeats ± SD.

3.6. PsJN Colonization of Rhizosphere, Root, and Shoot

Efficient colonization of the applied strain was observed in root/shoot interior of quinoa under saline conditions (Figure 6). However, when integrated with biochar and Fe, the persistence of PsJN was more enhanced relative to sole inoculation in the rhizosphere and tissues of quinoa plants. Inoculation combined with biochar showed 2.73×10^5 CFU g^{-1} rhizosphere, 9.92×10^4 CFU g^{-1} root interior and 1.95×10^4 CFU g^{-1} shoot interior bacterial population. However, most CFU g^{-1} dry weight of the inoculant strain was recovered from the rhizosphere (5.73×10^5), root interior (4.53×10^5), and shoot interior (9.92×10^4) in the presence of biochar and Fe.

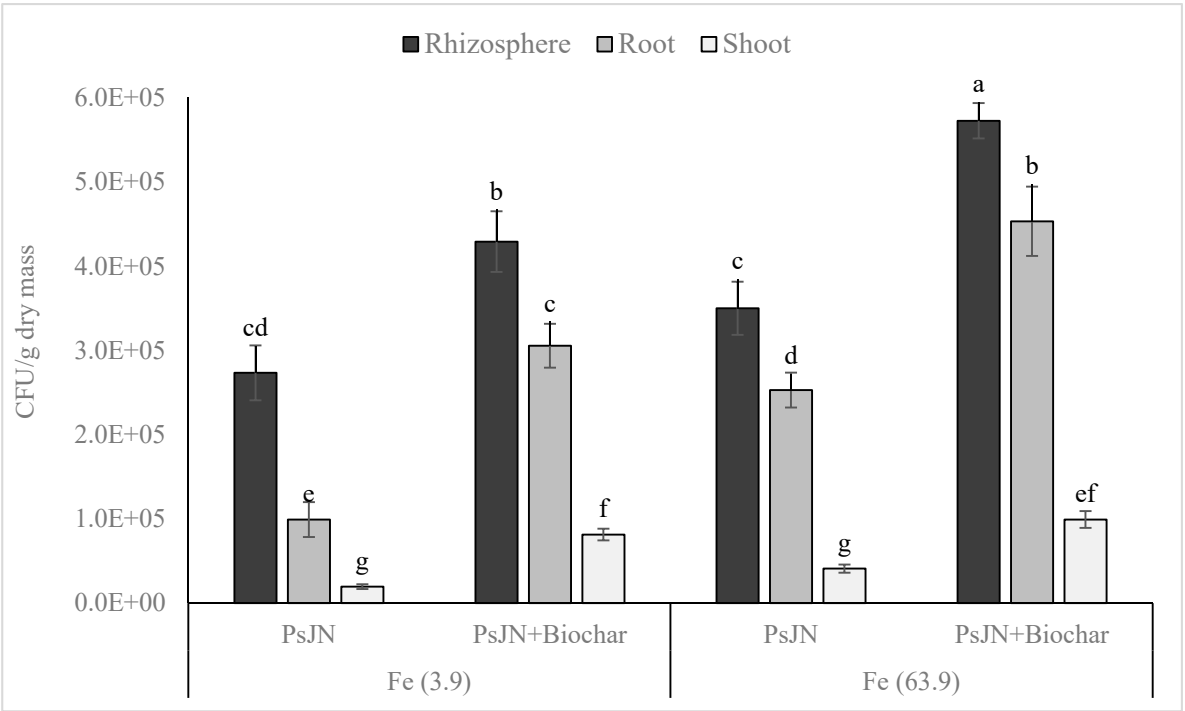

Figure 6. Persistence of selected endophytic strain in the shoot interior of *Chenopodium quinoa* under salinity stress. C: Control; BC: Biochar (1% *w*/*w*); PsJN: *Burkholderia phytofirmans*; Fe: Ferrous sulphate (15 kg ha^{-1}). Values are mean of three repeats ± SD.

4. Discussion

4.1. Plant Growth Parameters

Iron is a significant element for normal plant growth and development because it is a cofactor of numerous metabolic reactions, and its deficiency can adversely affect plant growth, photosynthesis, and respiration. Plants growing in saline soils often show typical symptoms of iron deficiency (chlorosis), as the solubility of iron (Fe) in the soil solution decreases with high pH [49]. In current experiment, we examined the effect of endophytic bacterium *Burkholderia phytofirmans* PsJN and organic amendment (biochar) on the growth, physiology, and iron-fortification of an emerging crop quinoa in saline soil. BC and PsJN application alone and in combination improved the plant growth, nutrient uptake, and antioxidant homeostasis apart from conferring salinity tolerance in plants. However, these effects were more significant with the combined application of biochar and PsJN. Salinity stress has been known to limit plant growth and yield through disturbing various physiological processes of crops [50]. We found significant reduction in growth attributes (plant height, shoot and root dry weights) of *Chenopodium quinoa* under saline conditions. However, application of biochar and *Burkholderia phytofirmans* PsJN significantly improved plant growth in the present study. This improvement in growth of plants might be associated with solubilization of nutrients from biochar [51]. This might also be due to the role of biochar and applied bacteria that have resulted in rhizosphere acidification leading to desorption of nutrients from biochar surface and /or soil colloids and thus have stimulated the growth attributes [31,51–53]. These observations are supported by previous studies, where siderophore producing bacteria including PsJN (which secrete hydroxamate-containing siderophore such as pyoverdin) often reported enhanced plant growth by facilitating Fe uptake and mediating plant tolerance against various abiotic stresses [13,54,55]. Moreover, biochar is known to play a critical role in iron biofortification of cereals in pH affected calcareous [22] as well as saline soils [56]. Biochar addition enhances nutrients availability to crops, increase water holding capacity of the soil and enhance microbial activity which might increase plant biomass accumulation under stressed conditions [57].

4.2. Plant Physiological and Biochemical Attributes

While it is well established that, water stressed plants close their stomata and ultimately lower the gaseous exchange attributes [58] and hence reduced photosynthetic activity. The influence on photosynthesis can be assessed from the influence on photosynthetic pigments. It has been described in certain studies that salinity stress causes decline in photosynthetic pigments of plants [50,59,60]. We found decreased physiological attributes in stressed plants. This reduction in physiological parameters might be attributable to disturbed metabolic machinery of the plants under stress [61]. It may also be due to increased osmotic stress in plants that causes shortage of water for plants and reduced rate of transpiration [62]. However, integrated application of biochar and PsJN improved physiological parameters such as stomatal conductance, photosynthetic rate, chlorophyll contents and transpiration rate in present study. By virtue of its high cation exchange capacity, biochar might have increased the availability of essential nutrients to stressed plants and hence enhanced physiological adaptations of quinoa plants under stress [63]. Moreover, bacterial inoculation can have a positive effect on plant physiology through uplifting chlorophyll contents, stomatal conductance, internal CO_2 concentration and relative water contents [31,64–67]. Certain PGPB have been recognized in imparting salinity resistance through direct stimulation of crops by providing fixed N, Fe sequestered by bacterial cells, phytohormones and soluble phosphates [11,68,69].

Enhanced crop productivity and improved nutritional quality grown on degraded soils are proposed to be the best solutions to combat malnutrition and hidden hunger [70,71]. Quinoa seeds are distinguished from cereals probably due to significant source of essential amino acids, vitamins and macro- and micro- nutrients [72,73]. Previously, several reports demonstrated reduced nutritional quality of quinoa grown on salt-affected soils [61,74,75]. In current study, plants grown on saline soil

showed significant reduction in nutritional parameters, however, the application of biochar and PsJN significantly improved nutritional attributes. This enhancement of nutritional status of amended plants might be due to the better provision of nutrients through biochar and endophytic bacteria [31,64] which might have helped plants to better adjust under unfavorable growth conditions [76].

4.3. Nutrients Homeostasis

Plant salinity tolerance capacity has been correlated with increased K^+ and decreased Na^+ uptake, which ultimately affects the Na^+/K^+ ratio in plants [77]. PGPB are well known to produce exopolysaccharides which can bind Na^+ and thereby reduce its uptake by the plants [10]. Because the strain PsJN has the potential to produce exopolysaccharides, therefore, its inoculation in present study decreased the contents of Na^+ available for plant uptake. These results are substantiated by Nadeem et al. [68], where inoculation of wheat seedlings with exopolysaccharide producing bacteria restricted Na^+ uptake and stimulated plant growth under saline condition. This may also be attributed to the higher Na^+ adsorption on surface of applied biochar. These results are in excellent agreement with [78], they reported enhanced growth of rice plants grown under salt-affected soil conditions due to synergistic application of biochar and PGPR.

Moreover, it is well known fact that Na^+ in higher levels hinders the uptake of other essential nutrients [79]. We found higher concentrations of macro nutrients especially nitrogen, phosphorous and potassium in plants amended with biochar and PSJN alone or in combination as compared to un-amended plants. These findings are in line with other studies describing increased availability of nutrients under the application of organic amendments [60,80]. Several other studies have confirmed the involvement of organic amendments (biochar and composts) in improved nutritional status of crops under saline soils due to increased soil aggregate stability, enhanced CEC, improved water retention, improved aeration and organic matter content [81,82]. In addition, we found increased contents of Fe in plants amended with biochar and PSJN relative to control. Previously, it was reported that inoculation of PGPR can facilitate uptake of micronutrients and increase plant tolerance against stress [83–85]. Moreover, Plant growth-promoting bacteria (PGPB) can mobilize the nutrients through various mechanism such as production of organic acids, rhizospheric acidification, nutrients chelation and by multiple exchange reactions [11,86]. It has been reported that PsJN is well-known PGPB, having many functional traits for plant growth promotion, nutrient uptake, and stress tolerance. PsJN found to be producing siderophores, indole acetic acid (IAA) and synthesis of ACC deaminase and its genome sequencing revealed that this strain harbors numerous genes for these traits and equipped with other important PGPB functions [13,87]. The synthesized siderophores release into the soil, bind iron and facilitate its uptake through TonB-dependent Fe-siderophore complex receptors [88]. Very recently, Shahid et al. [12] reported enhanced growth of rice through up-regulation of stress responsive genes due to inoculation of PGPR (*Achromobacter* sp. FB-14) showing ACC deaminase activity. Moreover, the production or modulation of plant hormones such as indole acetic acid and ethylene as well as nutrient solubilizing ability explain the possible mechanism of plant growth promotion of quinoa by PsJN.

4.4. Antioxidant Homeostasis

Under abiotic stress conditions, plant produce certain ROS such as hydrogen peroxide, superoxide, and hydroxyl radicals [89]. To avoid oxidative stress by scavenging ROS, plants are equipped with antioxidant defense characterized by various metabolites and enzymes. Among various enzymes, APX plays a critical role as powerful antioxidant H_2O_2-scavenger [90,91]. The antioxidants are produced under the stimuli of various stresses (such as salinity and other abiotic stress), and their concentration is frequently characterized as an assessment of oxidative stress. PsJN harbor numerous genes for ROS tolerance such as glutathione-S-transferases (GST), catalases, hydroperoxide reductases, superoxide, dismutases, and peroxidases [13]. In the present study, enzymatic antioxidants activities decreased especially by combined utilization of biochar and PsJN under saline condition. Our results are supported by the findings of Kanwal et al. [92] where biochar reduced the negative effects of salinity by

decreasing superoxide dismutase in wheat. Very recently, Saeed et al. [31] reported that combined use of endophytic bacteria along with amendment reverted the harmful effects of abiotic stress on *Brassica napus* growth by decreasing the activities of certain studied antioxidants.

4.5. Persistence of Endophyte in Shoot, Root and Rhizosphere

The persistence and activity of bacterial strain are governed by multifarious environmental factors (e.g., salinity). In this study, PsJN was found to be able to compete with the indigenous microbiota and effectively persisted in the plant environment (Figure 6) apart from promoting plant growth and nutrient uptake under saline environment. However, higher endophytic populations were observed in the soil treated with biochar, the viability of endophytic bacteria was further increased in the Fe enriched saline environment. The reason for the greater colonization of PsJN strain in BC amended soil might be due to availability of plentiful nutrients for normal microbial growth, provision of niches and shelter from predators. Moreover, microbial inoculation may have helped plants to meet their nutrient requirements under diverse environmental conditions [76]. Furthermore, Naveed et al. [93] demonstrated that under stressed conditions, plants undertake multiple metabolic and osmotic adjustments resulting in altered root exudation, which could affect the colonization and efficacy of bacterial inoculum. Moreover, PsJN strain harbor a wide range of genes for motility, chemotaxis, surface adhesion and quorum sensing which explain its ability of successful colonization in a variety of stressed environments [13]. The positive impact of biochar on soil microbial population and nutrient supply might explain the reason why the integrated application of biochar and PsJN perform best in our study.

5. Conclusions

The research findings suggested that the integrated application of *Burkholderia phytofirmans* PsJN and biochar have great ability to shed beneficial impacts on Fe bioavailability, growth, yield and nutritional value of quinoa grains grown in iron-limited saline conditions. The combined use of PsJN, BC and Fe enhanced growth, physiology, and mineral nutrition by alleviating salinity-induced oxidative stress as depicted by reduced antioxidants activity and Na^+/K^+ ratio of *C. quinoa*. The use of siderophore producing microbes with organic amendments might be a promising, sustainable and low-cost strategy for tackling micronutrient deficiencies in the low-and middle-income countries. Moreover, this approach can easily be integrated into the existing farming system to achieve food fortification with micronutrients.

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