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Influence of the Rhizobacterium *Rhodobacter sphaeroides* KE149 and Biochar on Waterlogging Stress Tolerance in *Glycine max* L.

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Abstract: In the context of the current climate change and increasing population scenarios, waterlogging stress in plants represents a global threat to sustainable agriculture production. Plant-growth-promoting rhizobacteria and biochar have been widely reported to mitigate the effects of several abiotic stresses. Hence, in the present study, we examined the effect of the rhizobacterium *Rhodobacter sphaeroides* KE149 and biochar on soybean plants subjected to sufficient water supply and waterlogging stress conditions. Our results revealed that KE149 and biochar inoculation significantly improved plant morphological attributes, such as root length, shoot length, and fresh biomass. The biochemical analysis results showed that the two treatments determined a significant drop in the levels of endogenous phytohormones (such as abscisic acid) under normal conditions, which were considerably enhanced under waterlogging stress. However, the jasmonic acid content increased with the application of biochar and KE149 under normal conditions, and it considerably decreased under waterlogging stress. Moreover, proline, methionine, and aspartic acid were significantly increased, whereas the phenolic and flavonoid contents were reduced with the application of the two treatments under waterlogging stress. These results suggest that the application of KE149 and biochar can be a safe biological tool with which to improve the physiology and productivity of soybean plants exposed to waterlogging stress.

Keywords: biochar; soybean; *Rhodobacter sphaeroides* KE149; waterlogging; abiotic stress

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1. Introduction

Exposure to abiotic stress is one of the major causes of low productivity of agricultural crops, and [1] waterlogging stress, in particular, is prevalent globally in agriculture sites [2]. Climate change, influenced by massive anthropogenic human activities, has further triggered water stress in lands used for agricultural production. In general, water stress is categorized as (i) water deficiency and (ii) excess water exposure, which includes waterlogging and complete submergence [3]. Plants face several physiological abnormalities when subjected to water stress. The main processes induced are the generation of reactive oxygen species (ROS) in cells, ionic imbalance, electrolytic leakage, and osmotic stress, which all ultimately result in cell death. To counteract these stress reactions, the plant undergoes several functional changes that include molecule signaling, antioxidant production, phytohormone modulation, and amino acid biosynthesis [3–5].

Phytohormones such as abscisic acid (ABA), jasmonic acid (JA), and salicylic acid (SA) are widely reported as being associated with stress tolerance [6]. ABA plays a key role in the regulation of stomata to confer water stress resistance [7,8], SA is a natural phenolic compound that activates the defense system against pathogens and abiotic stresses [9], and JA induces defense mechanisms, in addition to activating antioxidants and participating in amino acid accumulation and cross-signaling [10].

Antioxidants play a key role in minimizing the detrimental effects of oxygen radicals generated in plant cells in response to abiotic stress [11]. Among the antioxidants, flavonoids are known to improve yield quality, while phenolic compounds are known to scavenge ions to neutralize ROS [12]. Similarly, the amino-acid-like proline, methionine, and aspartic acid are actively involved in metal sequestration, radical scavengers, stabilizers of macromolecules, electron sinks, and manufacturing cell wall components [13]. Therefore, it is vital to improve plants' physiological aspects under stress in order to achieve optimal crop production.

Plant-growth-promoting rhizobacteria (PGPR) and biochar are emerging biostimulants that have been reported to improve metabolism and confer tolerance in crops subjected to several biotic and abiotic stresses [14,15]. Biochar and PGPR are biologically safe tools, and they work symbiotically to improve plant metabolism. The functional aspects of PGPR include the production of secondary metabolites, ion regulation, the improvement of ion absorption through roots, and translocation to shoots [16,17]. Similarly, biochar replenishes the soil, enhances carbon sequestration, and facilitates microbial growth [18,19]. Numerous studies have reported the beneficial role of PGPR application for plant growth and development [20]. In addition, Abideen et al. [18] showed that biochar application improved the growth of *Phragmites karka* under water stress conditions, and Kammann et al. [21] reported that appropriate doses of biochar improved the growth of *Chenopodium quinoa,* also subjected to water stress. Moreover, PGPR and biochar are widely known to improve the growth of several leguminous crops [22,23].

Leguminous plants are highly prone to waterlogging stress [24,25]. The photosynthetic activity of *Rhodobacter* spp. has been widely reported to mitigate stress in plants [26–28], and biochar is recognized as a stress reliever and growth promoter [29]. Considering these facts, the aim of the present study is to understand the metabolomic perspectives of soybean plants treated with biochar and the plant-growth-promoting bacterium *Rhodobacter sphaeroides* KE149, during exposure to waterlogging stress. The tested hypothesis is that PGPR and biochar have a potential role in regulating ion absorption through roots, regulating phytohormones, and amino acid synthesis in soybean plants exposed to waterlogging stress.

2. Materials and Methods

2.1. Selection of Microbes

The bacterium *Rhodobacter sphaeroides* KE149 was selected for the current experiment based on the results obtained from our previous study, where KE149 was reported to have various plant-growth-promoting traits, including the ability to confer resistance against water stress in adzuki bean plants [30].

2.2. Experiment Location, Method, and Design

The experiment was conducted at the greenhouse of Kyungpook National University, Daegu, Korea, located at longitude 128.587655° E and latitude 35.857655° N. Soybean (*Glycine max* L. cv. Taekwang) seeds were surface-sterilized with 1% Tween solution and 2% perchloric acid, and were washed thoroughly with sterile distilled water. The seeds were then sown into pots filled with autoclaved soil containing peat moss (13–18%), perlite (7–11%), coco-peat (63–68%), and zeolite (6–8%), with the following macro-nutrients: NH_4^+~90 mg/kg, NO_3^-~205 mg/kg, P_2O_5~350 mg/kg, and K_2O~100 mg/kg. Three-week-old soybean plants (50 per treatment) were treated with 10 mL of bacterial culture (10^8 CFU·mL^{-1}) and oak wood biochar (0.1% per pot). The biochar consisted of dolomite 10%, zeolite 5%, molasses 10%, peat 5%, and diameter 3–4 mm. Seven days later, soybean plants, with and without bacterial treatment, were subjected to waterlogging stress. The pots were kept in a tray and water was applied on daily basis to maintain the water level up to 5 cm above the soil surface. The experiment was designed as no stress (NS) and waterlogging stress (WLS). The length and fresh weight of shoots and roots in addition to chlorophyll content were measured after 7 days of waterlogging treatment, and plant samples were collected for further biochemical analyses.

2.3. Measurement of Realtive Water Content and Chlorophyll Content

The relative water content (RWC) was measured according to the method described by Wattoo [31]. The chlorophyll content was measured by using a CCM-300 chlorophyll content meter (Opti-Sciences, Hudson, NH). This instrument uses a fiber optic probe that detects the emission rate of red to far-red fluorescence (700 nm to 735 nm) [30].

2.4. Quantification of Endogenous Phytohormones

ABA: The method described by Bhusal et al. [32] was used to quantify the ABA content in plant shoots. In brief, one gram of freeze-dried sample was extracted with isopropanol and acetic acid (95:5 v/v). The obtained solution was filtered and an internal standard [(±)-3,5,5,7,7,7-d6)-ABA] was added. The extract was washed with 1 N NaOH and chlorophyll was separated by CH_2CL_2. Subsequently, polyvinylpolypyrrolidone was added and the solution was stirred for an hour; then it was filtered, evaporated, and finally extracted through ethyl acetate/diethyl ether. The ABA extracts were analyzed by GC-MS/SIM (6890 N Network GC System and 5973 Network Mass Selective Detector; Agilent Technologies, Santa Clara, CA, USA).

JA: The protocol described in Adhikari et al. [33] was used to obtain the JA extract. In brief, one gram of freeze-dried ground sample was extracted using acetone/citric acid (70:30 v/v) and filtered. The (9,10–$^2H_2$)-9,10- Dihydro-JA (100 ng) was added as an internal standard. Methylation was performed using diethyl ether followed by dichloromethane. The peaks were obtained using GC-MS/SIM technology (6890 N Network GC System and 5973 Network Mass Selective Detector; Agilent Technologies, Santa Clara, CA, USA).

SA: SA was quantified based on the method used by Adhikari et al. [34]. In brief, 0.5 g of lyophilized sample was extracted with 100% methanol, was centrifuged, and the solution obtained was vacuum-dried and suspended in trichloroacetic acid (5%). The solution was then partitioned by cyclopentane, ethyl acetate, and isopropanol, and subsequently it was dried using N_2. The excitation and emission peaks at 305 nm and 355 nm were recorded respectively using HPLC (Shimadzu RF-10AXL fluorescence detector).

2.5. Amino Acid and Antioxidant Analyses

The method described by Shahzad et al. [35] was used to quantify the amino acid content in soybean shoots. In brief, 0.05 g of lyophilized sample was suspended with 6 N HCl and charged with N_2. The sample was kept airtight at 110 °C on a hot plate for 24 h and was dried at 80 °C for the next 24 h. The extract was obtained with 0.02 N hydrochloric acid and injected into an amino acid auto-analyzer (Hitachi, Japan). For the analysis of antioxidants, phenolic and flavonoid contents were measured based on the methods described by Adhikari et al. [36] and Adhikari et al. [37], respectively.

2.6. Statistical Analysis

The present study was conducted using a completely randomized design with six replicates for each treatment. The data were analyzed in SAS 9.4 (SAS Institute, Cary, NC, USA) and evaluated using Duncan's multiple range test (DMRT) at $p \leq 0.05$.

3. Results

3.1. Effect on Morphological Attributes and Relative Water Content

The inoculation of KE149 and biochar in soybean improved morphological characteristics such as root and shoot length as well as total biomass. Under normal conditions, the plants treated with KE149 and biochar showed a 52% and 58% increase, respectively, in shoot weight. A similar trend was observed under waterlogging conditions, where the shoot weight increased by 26% and 29%, respectively in the two treatments. Minor differences were observed in shoot and root lengths under normal conditions. However, under waterlogging conditions, shoot length increased by 15% and 25% in the two treatments, respectively, and root length increased by 26% and 27%, also respectively (Table 1 and Figure 1).

Table 1. Effect of waterlogging stress and absence of stress ('no stress') in soybean plants.

	Shoot Length (cm/plant)	**Root Length (cm/plant)**	**Shoot Fresh Weight (g/plant)**	**Root Fresh Weight (g/plant)**	**RWC (%)**
			No Stress		
NT	22.88 ± 0.88 b	18.07 ± 1.06 a	4.22 ± 0.14 b	2.19 ± 0.09 b	71.7 ± 1.5 a
RS	24.08 ± 0.54 a	19.0 ± 1.67 a	6.43 ± 0.52 a	4.60 ± 0.06 a	72.0 ± 1.0 a
BC	25.60 ± 0.76 a	19.32 ± 1.21 a	6.69 ± 0.47 a	4.70 ± 0.04 a	72.3 ± 1.2 a
			Waterlogging Stress		
NT	19.70 ± 0.73 b	15.04 ± 0.44 b	3.86 ± 0.08 c	1.69 ± 0.24 b	68.3 ± 1.5 b
RS	22.80 ± 1.79 a	19.00 ± 0.86 a	4.87 ± 0.18 b	2.45 ± 0.32 a	74.0 ± 1.0 a
BC	24.72 ± 0.68 a	19.20 ± 1.10 a	5.00 ± 0.09 a	2.79 ± 0.24 a	76.0 ± 1.0 a

Each value represents the mean ± SD. Each data point represents the mean of at least three replicates. Different letters in the column after mean values represent the least significant differences at $p \leq 0.05$. (NT: no treatment; RS: *Rhodobacter spaheroides* KE149; BC: biochar.)

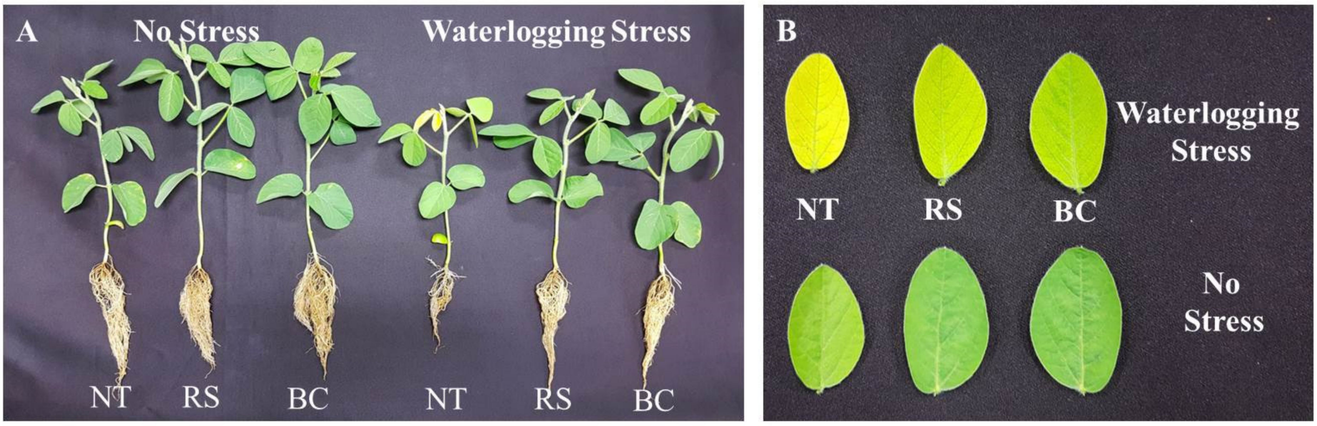


Figure 1. Effect of biochar and *R. sphaeroides* KE149 on (**A**) morphological characteristics and (**B**) color bleaching of soybean plants grown under sufficient water conditions and waterlogging stress. (NT: no treatment; RS: *Rhodobacter spaheroides* KE149; BC: biochar.)

3.2. Chlorophyll Content

The chlorophyll content of the first-, second-, and third-trifoliate leaves was measured. The results showed that KE149 and biochar increased the chlorophyll content by 57% in first-trifoliate leaves, and by similar amounts—namely 142% and 154%, respectively—in second-trifoliate leaves. In third-trifoliate leaves, a vast increase in chlorophyll content was observed for both treatments (Figure 2).

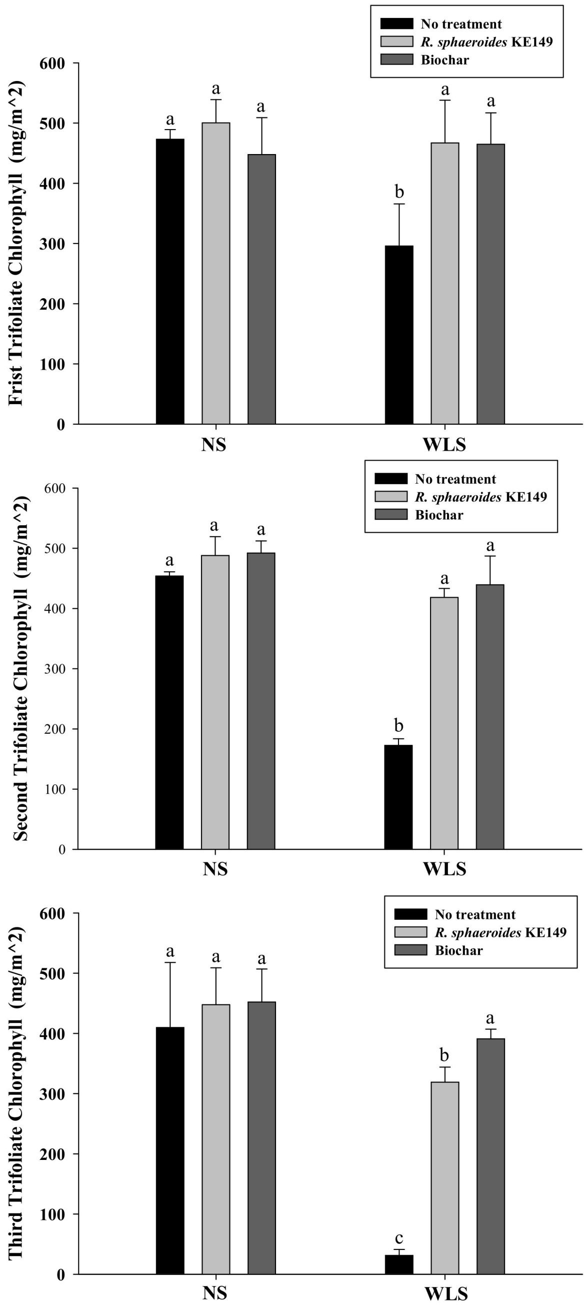


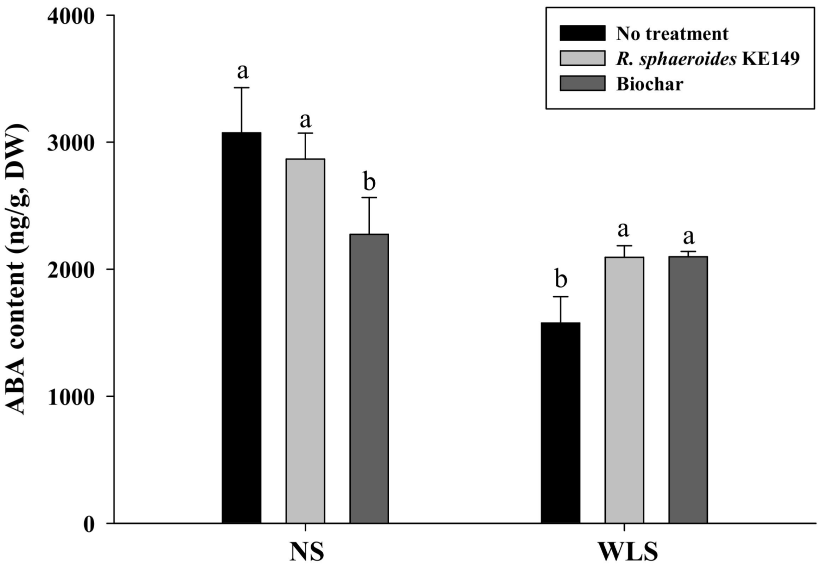
Figure 2. Effect of biochar and *R. sphaeroides* KE149 inoculation on chlorophyll content in the first-, second-, and third-trifoliate leaves of soybean plants. Each data point represents the mean of at least three replicates. Error bars represent standard deviations. Bars with different letters are significantly different from each other at $p \leq 0.05$. (NS: no stress; WLS: waterlogging stress).

3.3. Hormone Quantification

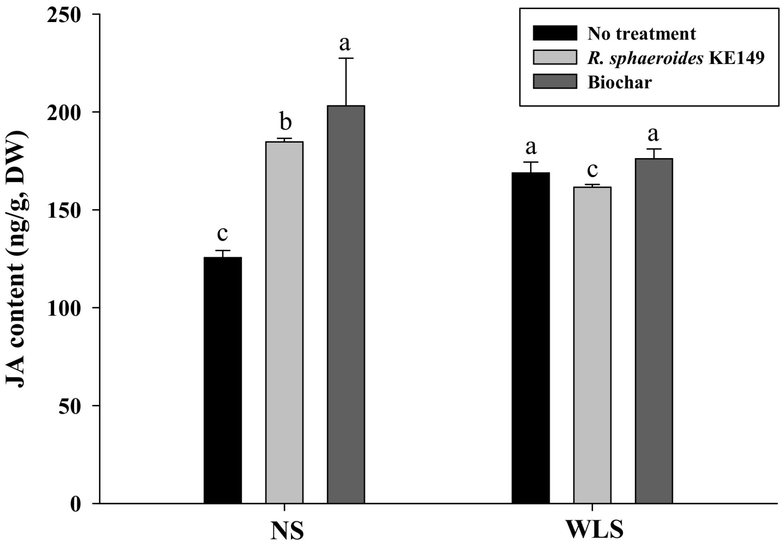
ABA: The ABA content significantly dropped by 6% and 25% in plants treated with KE149 and biochar, respectively, under normal stress conditions when compared to no treatment (NT). In contrast, under waterlogging stress, ABA considerably increased by 32% and 33% in plants treated with KE149 and biochar, respectively, compared to NT (Figure 3A).

JA: The JA content was significantly increased by 47% and 61% in plants treated with KE149 and biochar, respectively, under normal conditions. However, under stress conditions it dropped in KE149-treated plants, but it increased in biochar-treated plants (Figure 3B).

SA: The SA content was significantly enhanced under normal conditions in plants inoculated with KE149 and biochar. However, under waterlogging stress, biochar application increased the SA content, whereas no significant differences were observed in the KE149 treatment (Figure 3C).

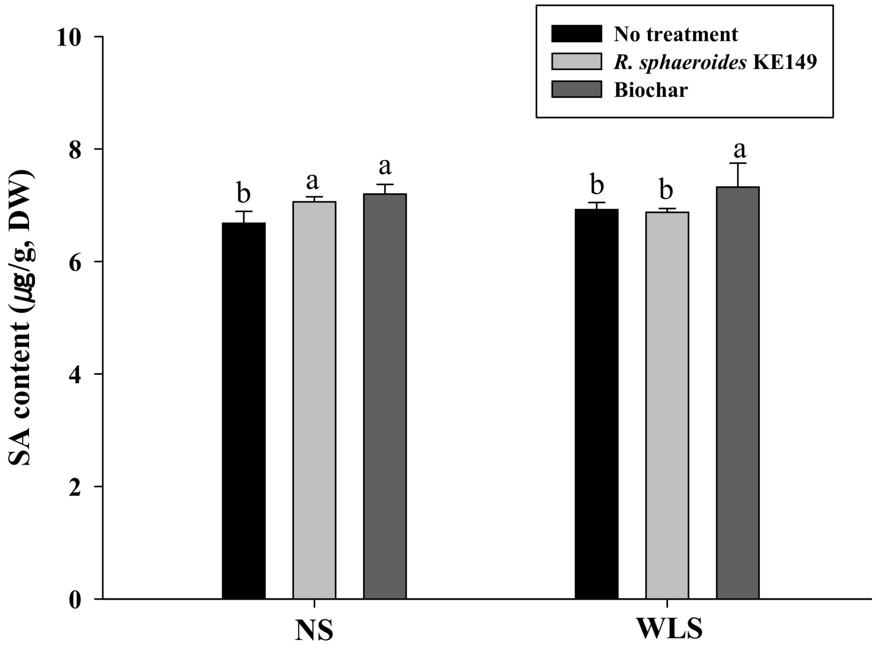


(**A**)



(**B**)

Figure 3. *Cont.*

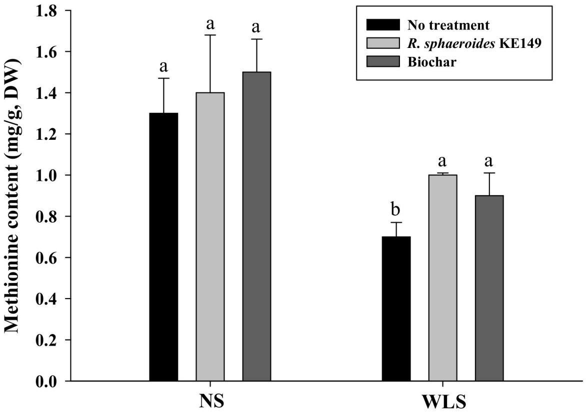


(**C**)

Figure 3. Quantification of endogenous phytohormones (**A**) abscisic acid, (**B**) jasmonic acid, and (**C**) salicylic acid content in soybean shoots following inoculation with *R. spharoides* KE149 and biochar under waterlogging and normal conditions. Each data point represents the mean of at least three replicates. Error bars represent standard deviations. Bars with different letters are significantly different from each other at $p \leq 0.05$. (NS: no stress; WLS: waterlogging stress.)

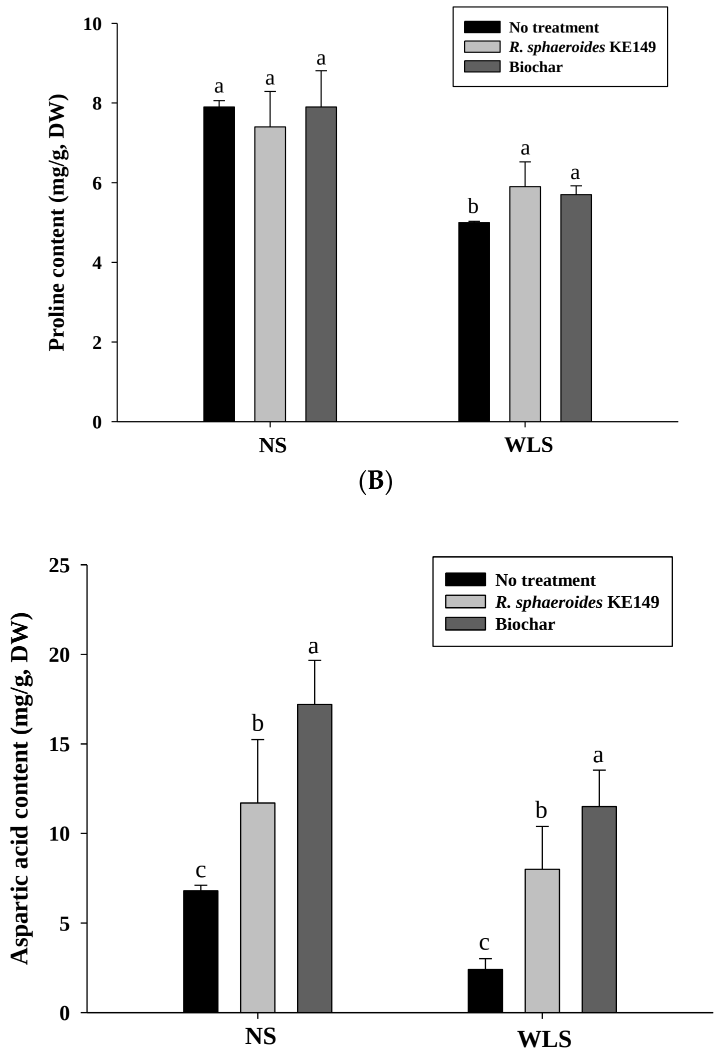
3.4. Amino Acid Analysis

Proline content was significantly enhanced by 18% and 14% in plants inoculated with KE149 and biochar, respectively, under waterlogging conditions. However, under normal conditions no significant differences were observed. Methionine content increased by 42% and 28% after KE149 and biochar application, respectively, under waterlogging stress. Aspartic acid increased by 233% and 379% in the two treatments, respectively, under stress conditions and, under normal conditions, its contents were enhanced by 72% and 152%, respectively, compared to the control (Figure 4).



(**A**)

Figure 4. *Cont.*



(**B**)

(**C**)

Figure 4. Quantification of (**A**) methionine, (**B**) proline, and (**C**) aspartic acid content in soybean shoots following inoculation with *R. spharoides* KE149 and biochar under waterlogging and normal conditions. Each data point represents the mean of at least three replicates. Error bars represent standard deviations. Bars with different letters are significantly different from each other at $p \leq 0.05$. (NS: no stress; WLS: waterlogging stress.)

3.5. Antioxidant Analysis

In the present study, the inoculation of KE149 and biochar significantly enhanced the phenolic content by 34% and 15%, respectively, under normal conditions. However, under waterlogging stress conditions, the content of this antioxidant significantly dropped by 24% and 44% in the two treatments, respectively. A similar trend was observed in flavonoid content, which increased by 8% in KE149-treated plants, while no significant differences were observed in biochar-treated plants, under normal conditions. However, under waterlogging stress, the flavonoid content significantly dropped by 10% and 16% in KE149- and biochar-treated plants, respectively (Figures 5 and 6).

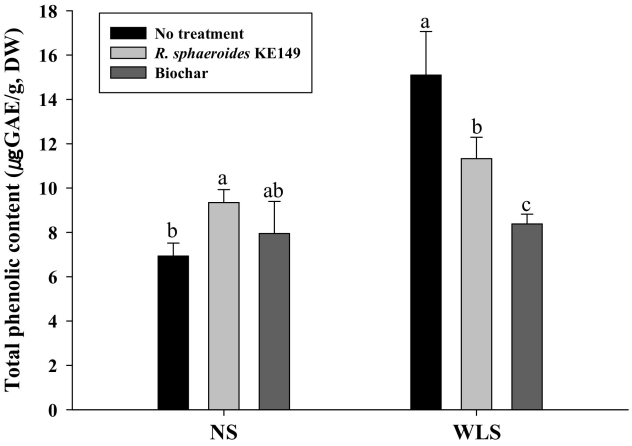


Figure 5. Effect of biochar and *R. sphaeroides* inoculation on the phenolic content of soybean shoots. Each data point represents the mean of at least three replicates. Error bars represent standard deviations. Bars with different letters are significantly different from each other at $p \leq 0.05$. (NS: no stress; WLS: waterlogging stress.)

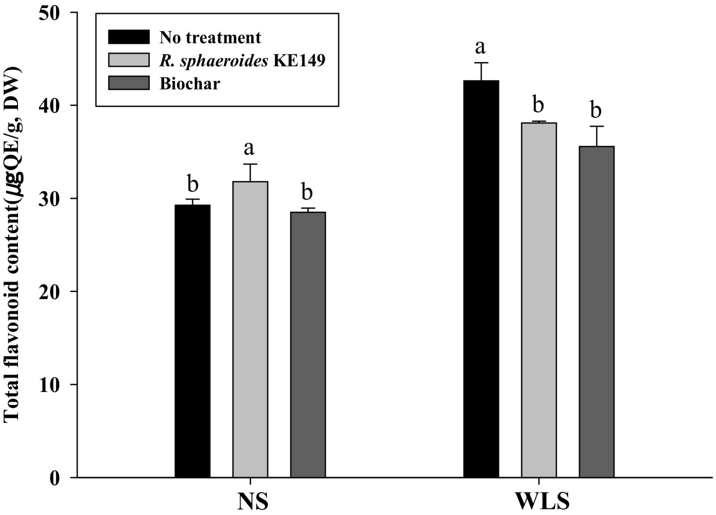


Figure 6. Analysis of flavonoid content in soybean shoots following inoculation with *R. spharoides* KE149 and biochar under waterlogging and normal conditions. Each data point represents the mean of at least three replicates. Error bars represent standard deviations. Bars with different letters are significantly different from each other at $p \leq 0.05$. (NS: no stress; WLS: waterlogging stress.)

4. Discussion

Biofertilizers are emerging biological tools used for promoting sustainable agriculture and combating threats to the environment. In the present study, the application of the rhizobacterum KE149 and biochar significantly improved plant-growth-promoting traits in soybean plants exposed to waterlogging stress. Here we discuss how KE149 and biochar application regulates various metabolic aspects, such as the functioning of endogenous phytohormones, amino acids, and the antioxidant system of soybean plants subjected to waterlogging stress.

A large number of studies reported that ABA maintains water levels in the cell as it controls stomatal activity [38]. In general, low water retention allows guard cells around stomata to close and, at higher moisture levels, it activates the stomatal conductance and stimulates the transpiration rate [39]. In our study, the ABA level was significantly enhanced by the inoculation of biochar and KE149, which might have protected cells from excess osmotic stress. Our results are in line with those by De Ollas et al. [40], which showed that the ABA level decreased under waterlogging conditions in tomato plants. Similar results were also shown by Arbona et al. [41], who observed a significant drop in the ABA level of *Carrizo citrange* plant roots exposed to flooding stress.

JA is reported to stimulate resistance in plants subjected to stress and is involved in crosstalk for the synthesis of other endogenous phytohormones, as well as amino acids. In the present study, the JA level was significantly enhanced by the application of KE149 and biochar under normal conditions. In contrast, under waterlogging stress, the level dropped after KE149 application, while no significant difference was observed when compared to the control. These results are in line with the findings by Shan et al. [42], who reported that JA application led to the improvement of *Agropyron cristatum*. Similarly, Yosefi et al. [43] reported that JA was involved in crosstalk with SA to improve water stress resistance in tomatoes. Maladaptive overproduction of JA may reduce plant growth and resistance against stress. Waqas et al. [44] recommended the application of appropriate doses of biochar, after reporting that excessive JA production in rice—following the application of biochar—resulted in physiological abnormalities. Hence, the lower level of of JA in our results indicates the significance of biochar doses on waterlogging stress in soybean plants.

SA is reported to induce systemic resistance in plants facing attacks by external pathogens or exposure to abiotic stress [45,46]. Our study showed that SA significantly increased after the application of biochar and KE149, indicating the higher resistance level conferred to the plants (Figure 5). These results are supported by the findings presented in Hayat et al. [47], who reported that the external application of SA improved the physiological status of tomatoes. Similar results have been shown by Sing et al. [48] in wheat. Moreover, our results are validated by various previous studies [49–51] that described the significance of SA in plant toxicity.

Amino acids are key components of protein structures, and precursors of hormonal pathways. The crosstalk between endogenous phytohormones is highly influenced by amino acid biosynthesis [52]. Under water stress, proline, methionine, and aspartic acid concentrations play a key role in adjusting the moisture level in plants, and in regulating phytohormones and antioxidants. In general, an increase in proline enhances ABA biosynthesis, which in turn controls the transpiration rate. Our study showed that the proline level significantly increased with biochar and KE149 treatments. A similar trend was observed for aspartic acid and methionine. Proline serves as an osmoregulant and it maintains the redox balance [53]. Similarly, aspartic acid enhances the mobility of amino acids in the phloem [54]. Methionine undergoes a chemical reaction that produces S-adenosylmethionine under the influence of ACC synthase, to finally synthesize ethylene [55]. As ethylene enhances tolerance to prolonged waterlogging stress [56], the observed effect of KE149 and biochar, in terms of amino acid accumulation, possibly triggered ethylene synthesis, which might have improved the resistance of plants against waterlogging stress.

Water stress generates ROS and imposes osmotic stress on plant cells [57]. To counteract or neutralize the negative effects of ROS, plants scavenge radicals [58]. In general, polyphenolic compounds and flavonoids are stimulated in response to biotic/abiotic stresses [57,59]. Their contents have been reported to counterbalance the effects of harmful oxygen radicals [59]. In the present study, the inoculation of KE149 and biochar significantly enhanced the polyphenol and flavonoid contents under normal conditions, while these decreased considerably under waterlogging stress. The drop in polyphenolic and flavonoid levels indicates the reduced effort made by the plants to combat the negative effects of oxygen radicals.

5. Conclusions

The present study shows that the inoculation of biochar and KE149 regulated phytohormones and amino acids, and strengthened the antioxidant systems of soybean plants.

Therefore, KE149 and appropriate doses of biochar can be considered as safe, eco-friendly biofertilizers to be used to mitigate waterlogging stress.

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