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ORIGINAL ARTICLE

Mitigating Combined Stresses Motivated by Salinity and *Rhizoctonia solani* using Brassinolide, and *Azotobacter chrocoocum* Enriched Biochar in Faba Bean (*Vicia faba*)

Gad A.G. *[1], Xu D.[2], Zhuo Z.[2], Zeyadah S.M.A.[1], Diab M.Y.[1], Gouda H.A.*[3]

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ABSTRACT

Faba bean (*Vicia faba* L.) is one of the major legume vegetable cultivated in Egypt. Naturally occurring biotic and abiotic stressors significantly reduce the growth and yields of crop plants. This study explored the potential of exogenous brassinolide (BL) application (10^{-7}), biochar, *Azotobacter chroococcum*, and *A. chroococcum* enriched biochar to alleviate the negative impacts of the combined stresses of salinity and *Rhizoctonia solani* on faba beans. Brassinolide and *Azotobacter* sp. enriched biochar treatments were effective in mitigating the negative impacts of the combined stresses by reducing *R. solani* incidence by 28 and 20%, respectively, and disease severity up to 35 and 30%, respectively. The growth parameters include plant height, as well as the fresh and dry weight of both the roots and shoots were promoted. Total chlorophyll (TCC) and carotenoids contents (CC) of faba bean salinity and *R. solani* combined stress treated plants significantly increased after BL (18.1 and 22.9 mg g^{-1} FW, respectively) and *Azotobacter* sp. enriched biochar (13.4 and 17.9 mg g^{-1} FW, respectively) treatments. Peroxidase (POD), polyphenol oxidase (PPO) and ascorbate peroxidase (APX) enzymes activities were upregulated after *Azotobacter* sp. co-culturing and BL treatments. Meanwhile, superoxide dismutase (SOD) significantly decreased compared to treated controls. Additionally, *Azotobacter* sp. enriched biochar treatment highly significant increased SOD activity compared to controls groups. These results suggest that these compounds tested can contribute to more sustainable management practices for faba bean cultivation when exposed to saline stresses and *R. solani* infections.

Keywords: Faba bean, *Rhizoctonia solani*, salt stresses, combined stress, *Azotobacter chrocoocum*, biochar, brassinolide.

***Correspondence : Ahmed, G. Gad**
E-mail: ahmed.gad@alexu.edu.eg
Hassan A., Gouda
E-mail: mycologist2010@yahoo.com

Ahmed, G. Gad
https://orcid.org/0009-0000-5300-2171
Mona, Y. Diab
Sabreen, M. A. Zeyadah
1-Department of Plant Pathology, Faculty of Agriculture-Elshatby, Alexandria University, Egypt.
Danping Xu
https://orcid.org/0000-0001-6131-2591
Zhihang Zhuo
2-College of Life Science, China West Normal University, Nanchong 637002, China.
Hassan A., Gouda
https://orcid.org/0000-0003-2255-7611
3-Plant Pathology Research Institute, Agriculture Research Centre, 12619 Giza, Egypt.

INTRODUCTION

Faba bean (*Vicia faba* L.) is a significant leguminous vegetable cultivated in Egypt. Egypt is the third largest producer of cultivated faba beans in Africa, following Ethiopia and Sudan (Merga *et al.*, 2019). The cultivated area in Egypt is approximately 89.815 thousand feddans and the production was approximately 410,000 tons in 2018 (Zohir, 2022). It is considered an important source of high-quality of protein and also contains vitamins, minerals, carbohydrates and beneficial compounds (Alghamdi, 2009).

Faba bean is globally challenged by various biotic and abiotic stressors. In Egypt, *Rhizoctonia solani* root-rot is considered a major limiting factor affecting yield and productivity (Abou-Zeid *et al.*, 1997; Eisa *et al.*, 2006, Abdel-Razik *et al.*,2012 and Abdel-Monaim, 2013). Salinity causes a serious problem in agriculture, it can limit crop growth and production (Safdar *et al.*, 2019). Soil salinization has intensified the aggressiveness of phytopathogenic fungi affecting cultivated crops. For example, faba beans diseases caused by *Fusarium equiseti*,

F. graminearum, and *Rutstroemia* sp., as well as potato wilting and rotting caused by *Fusarium solani*, have shown increased severity under salt stress conditions (Haddoudi *et al.,* 2021 and Tiwari *et al.*, 2024). The pathogenic *R. solani* aggressiveness was stronger in the presence of salinity, particularly affecting faba bean, tomatoes and strawberries (Abd El-Hai and El-Saidy2016; Mustafa *et al.,* 2021; Sekmen Cetinel *et al.,* 2021 and Mustapha *et al.,* 2022).

Azotobacter spp. are free-living, biological nitrogen-fixing aerobic bacteria that protected plants against *R. solani* infection on several crops such as faba bean, potato, cucumber, and eggplant (Meshram 1984; Hassouna *et al.,* 1998; Dashadi *et al.,* 2011; Matloob and Al-Baldawy, 2020, and Zian and Aly, 2020). Furthermore, they proved ameliorative effects on salinity-stressed plants such as faba bean and tomato. For instance, both the culture filtrate of *Rhizobial* spp. and *Azotobacter vinelandii* have been found to enhance faba bean and tomato seed germination, growth, and chlorophyll content (Nour El-Din *et al.*, 2011, Alghamdi, 2022). The combined stresses of drought and salinity were ameliorated in exposed eggplant plants after *A. vinelandii* and *A. chroococum* inoculation, whereas oxidative stress was reduced and osmoprotectants increased (Kiran *et al.,* 2025).

Biochar is a porous, carbon-rich product created through thermochemical conversion of various lignocellulosic feedstocks. These feedstocks include agricultural waste such as straw and corncobs, forestry waste like sawdust and wood chips, as well as organic and industrial waste, including manure and sludges (Singh *et al.,* 2022). The application of biochar increased plant biomass primarily by boosting root volume through enhanced soil organic carbon, enriched rhizosphere Firmicutes, and reduced oxidative damage to leaves (Li *et al.,* 2023). Biochar notably diminished EC and the loss of organic matter, while enhancing nutrient contents throughout the procedure of composting pig manure combined with rice straw (Chang *et al.,* 2017). The positive effects of biochar on soil microbial communities and plant development have become a focal point of research (Hossain *et al.,* 2020). With its large surface area, diverse functional groups, and high porosity, biochar serves as an exceptional medium for microbial colonization and activity (Zhao *et al.,* 2022). Biochar produced from rice husks has been adduced as an effective carrier for functional microbial applications in environmental remediation (Feng *et al.,* 2021). The combination of biochar with PGPR strains such as *Bacillus amyloliquefaciens* and *Paenibacillus polymyxa* contribute to improved nitrogen utilization efficiency and promote a healthier soil microbial community in tomato cultivation, proving to be more beneficial than the use of biochar or PGPR independently (Guan *et al.,* 2023).

Brassinolides are classified as steroid hormones stimulate the rate of cell division, shoot elongation, gravitropism, and differentiation of xylem (Anwar *et al.,* 2025). The use of exogenous BR notably increased chlorophyll content and enhanced photosynthetic traits of plants under various stress conditions (Niu *et al.,* 2016). BR enhanced the levels of essential inorganic ions while reducing toxic ions, effectively supporting ion balance particularly in the leaves, roots, and epicotyls of canola when subjected to salt stress (Liu *et al.,* 2014). Because of the significant impact of *Rhizoctonia* root-rot and salinity on the faba bean crop growth and productivity, this study was conducted with the following objectives: 1) to isolate the pathogen associated with the rotting of roots and stems of faba bean plants, and 2) to evaluate the use of *Azotobacter chroococcum*, biochar, and brassinolide in order to mitigate the negative effects of both the disease and salinity stress, ultimately aiming to enhance the growth and yield of the crop.

Materials and Methods

Fungal isolation and identification

Samples of plants showing symptoms of *root-rot* infection were brought to the

laboratory from different areas from and the infected plant parts in 2023 from Alexandria and Assiut governorates. They were cut into small pieces and then washed with distilled water and sterilized with (0.1%) sodium hypochlorite solution for 3 minutes. All the pieces dried with filter paper, 3-4 plant pieces placed using sterile forceps on petri dish with a diameter of 9 cm containing PDA (Gamliel *et al.*, 1996). After cultivation for 4 days at 25±2°C, the fungal isolates were identified morphologically depending macroscopic characteristics of the fungal colony according to Gilman (1957). Genomic DNA was extracted from the cultured fungus and PCR amplification of the internal transcribed spacer (ITS) region of the ribosomal DNA was performed with ITS1 (5'- TCCGTAGGTGAACCTGCGG-3') and ITS-4 (5'-TCCTCCGCTTATTGATATGC-3') primers pairs (White *et al.*, 1990). Amplicons were sequenced are compared to databases using BLAST (NCBI) to identify the specific *Rhizoctonia* species and phylogenetic analysis was conducted with MegAlign software (DNAStar, version 5.05).

Pathogenicity test

The isolated isolates of *Rhizoctonia solani* were evaluated for pathogenicity under greenhouse conditions. The isolates of *R.solani* were cultured on autoclaved barely seeds in 250 ml flask and incubated on 25 °C for two weeks for inoculum preparation. Plastic pots of 25 cm in diameter were filled with autoclaved soil (1:1, sand: clay) and inoculated with the fungal inoculum at rate of 3% (w/w). Pots inoculated soil was thoroughly mixed with the inoculum, and left for one week. Un-inoculated pots were used as a control. The seeds of faba bean (cv. Giza 716) underwent surface disinfection through immersion in a 1% sodium hypochlorite solution for three minutes, followed by multiple washes with sterilized water to remove residual hypochlorite. Seeds were allowed to dry on a filter paper and sown at a rate of five seeds per pot. Three pots were used for each treatment. The incidence of pre- and post-emergence damping-off was documented 15, and 30 days after sowing and calculated (Atwa, 2016).

Pre-emergence % = Total No. of un-germinated seeds / Total No. of planted seeds x 100

Post-emergence % = Total No. of rotted seedlings / Total No. of planted seeds x 100

Survived seedlings % = Total No. of survived seedlings / Total No. of planted seeds x100

Disease severity index

R. solani disease severity of faba bean were conducted on 60 days old seedlings, plants were uprooted thoroughly washed under running tap water, and were examined for lesions presence and size. Observations were recorded, described and scaled 0-4 scale according to (Muyolo *et al.*, 1993). Disease incidence and severity were calculated according formula mentioned below in Table 1 (El-Gazzar *et al.*, 2023).

%Disease Incidence = (Number of infected plants / Total number of plants) x 100%

% Disease Severity Index= [Σ (SD x BS)/ (ESD x TB)] x100

Where SD is the disease index on each plant, BS is total plant examined of that scale, ESD indicates the maximum disease index possible and TB is the total number of plants examined in each replicate.

Table 1. Disease severity of *R. solani* root rot on faba bean seedlings

Scale	Description
0	Healthy seedling
1	Root small brown lesions
2	Deep lesions on the roots or stem
3	Lesions surrounding the main root and reduced root length
4	Dead plant

In *vivo*, the activity of *A. chroococum*, biocahr and brassinolide on faba bean treated with 50 mM NaCl and *R. solani* combined stress

Treatments preparation

1. *A. chrocoocum* was cultured in a 250 ml conical flask containing 100 ml of nutrient broth medium at 28 °C and 180 rpm agitation for 48 h. The suspension was

centrifuged at 8000 rpm for 15 minutes. The supernatant concentration was adjusted to 1.2×10^7 CFU ml^{-1} (OD_{600}=1) and used for soil inoculation at 10 ml/kg soil (Prakongkep *et al.*, 2013).

2. Biochar was purchased from North Africa for Chemicals Company, Alexandria, Egypt. It was produced by paralyzing rice husks at 450 °C for 6 hours in an environment with limited oxygen (Prakongkep *et al.*, 2013).

3. *A. chrococum* enriched biochar was prepared by mixing fresh bacterial suspension and autoclaved biochar in ratio 20:1 (v: w) and incubated at 25 °C for 3 days (Chaudhary *et al.*, 2023). Rice husk biochar and *A. chrococum* enriched biochar treatments were applied at 1% of soil weight.

4. Salinity stress in 50 mM NaCl, plants were regularly fertilized and irrigated.

The sterilized seeds were treated with eight treatments as described in Table (2). Three replicate were specified for each treatment. Five seeds of faba bean (cv Giza 716) were sown for each pot filled with 3 kg sterilized soil (1:1, sand: clay). After 60 days from the planting, when the initial symptoms appeared, the disease incidence and severity were calculated according formula mentioned below in Table (1) (El-Gazzar *et al.*, 2023).

Table 2. *Azotobacter chroococum*, biocahr and brassinolide designated treatments on 50 mM NaCl and *R.solani* combined stress faba bean

Treatments	Description
T1	Healthy plants
T2	*R. solani*
T3	50 mM NaCl
T4	R.s + 50 mM NaCl
T5	*R.s* + 50 mM NaCl + Biochar
T6	*R.s* + 50 mM NaCl + Azotobacter enriched biochar
T7	R.s + 50 mM NaCl+ *A. chroococum*
T8	R.s + 50 mM NaCl+ brassinolide

Estimation the growth of plants

Fresh faba bean plant samples were collected after 60 days from each treatment, washed thoroughly under water to remove soil residues, and morphological characteristics such as root and shoot length, as well as fresh and dry weight, were measured.

Estimation of photosynthetic plant pigments

A leaf sample was collected 60 days after sowing. The leaves were then separated and ground down to extract photosynthetic pigments using 85% aqueous acetone. The concentrations of chlorophyll a, chlorophyll b, and carotenoids were calculated in mg g^{-1} FW following the method outlined by Lichtenthaler and Wellburn (Lichtenthaler and Wellburn, 1983).

Estimation of antioxidant enzymes activity

Plant root tissues were sampled and pulverized utilizing a 0.2 M TrisHCl buffer (pH 7.8) that included 14 mM-mercaptoethanol at a ratio of 1/3 w/v. The extracts underwent centrifugation at 10,000 rpm for 20 minutes at a temperature of 4°C. Bradford method was employed for total protein assay, where 40-µl crude extract was added to 940 µl of Bradford solution, and measuring absorbance at 595 nm (Bradford, 1976). The standard curve was constructed using bovine serum albumin. The estimated protein concentration was employed to determine the activity of antioxidants enzymes activity POD, PPO, SOD, and APX.

Polyphenol oxidase (PPO) activity

Polyphenol oxidase activity was determined according to the method described by Cheema and Sommerhalter (2015). The reaction mixture was composed of 200 µl of the enzyme extract mixed with 1.5 ml of 0.1 M sodium phosphate buffer (pH 6.5) and then 0.5 mL of catechol was placed and mixed together and finally completed the total volume to be 3 mL using sterile distilled water (Cheema and Sommerhalter 2015).

Peroxidase (POD) activity

Peroxidase activity was estimated according to Cakmak and Marschner (1992). The guaiacol peroxidase assay involved a solution of 50 mM phosphate buffer at pH 7, combined with 8.26 mM hydrogen peroxide

and 100 µl of enzyme extract. Absorption variations were observed at 470 nm (Cakmak and Marschner, 1992).

Superoxide dismutase assay (SOD)

Superoxide dismutase activity was estimated according to Beauchamp and Fridovich 1971. The reaction mixture (5 mL) comprised 50 mM potassium phosphate buffer, 0.1 µM EDTA, 0.013 mM methionine, two µM riboflavin, and 50 µL of protein extract, and was subjected to moderate light intensity (300 µmol m-2 s-1). In this study, a 50% reduction in NBT optical absorption at 560 nm compared to the control was deemed equivalent to one enzyme unit.

APX activity assay

Ascorbate peroxidase activity was estimated by measuring the reduction in absorption of 50 mM phosphate buffer (pH=7) containing 0.5 mM ascorbic acid and 0.15 mM hydrogen peroxide for 2 min at 290 nm. The reaction mixture consists of 100 µL of enzyme extract combined with 1.5 mL of buffer. The activity was calculated using the extinction coefficient (ε = 2.8 mMol-1 cm-1) and expressed as a unit per minute per milligram of protein (Nakano and Asada 1987).

Statistical analysis

The experiments statistical analyses were conducted using the SPSS statistical package (SPSS Version 16.0). Analysis of variance (ANOVA) was performed using one-way ANOVA followed by a post hoc Tuky test to analyse the significant difference among treatments. A p-value of≤0.05 was considered statistically significant.

Results

Identification of faba bean root-rot causal agent

Colonies are tan light brown colour in appearance after 7 days on PD, conidiophore characteristically branch out at a 90° or right angle and septate. A distinctive constriction is present at the point of origin for the branch. The ITS gene sequence alignment showed 96.76-98.42% identity to *R. solani* isolates and 99-100% coverage with several strains of *R. solani* isolates. Thus, isolate R (accession number: PV833208.1) was identified as *R. solani*. A phylogenetic tree based on the ITS rDNA sequences was constructed using MEGA 12 (Fig.1).

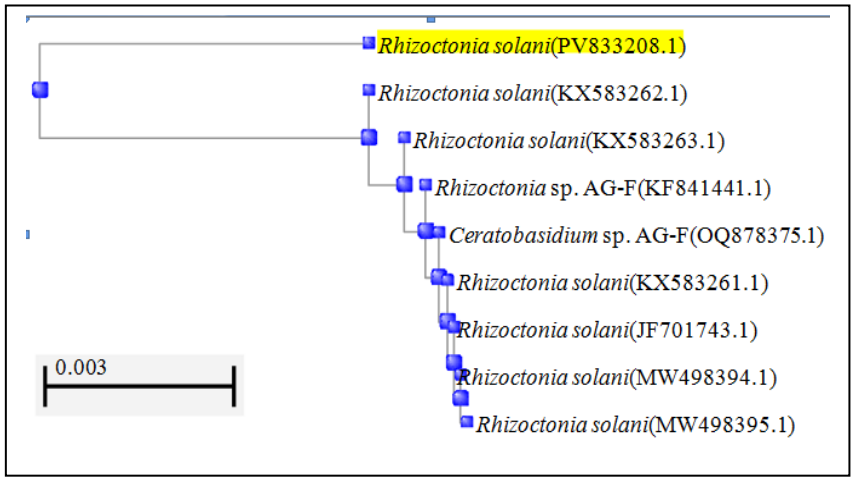

Fig. 1. Phylogenetic clustering based on the ITS rDNA gene sequence of *R. solani* constructed by the neighbour-joining method.

Pathogenicity test

According to the pathogenicity test, all tested *R. solani* isolates were capable of inducing the typical damping-off and root-rot symptoms, resulting in a reduced survival rate of the plants. The most virulent of rotten bean seeds was (No. 2) which was obtained from Alexandria governorate. Conversely, symptoms of seedling mortality induced by isolate (No. 2) recorded the highest infection

(Table 3). The disease symptoms were characterized by reddish lesions on the roots, which caused them to sink and girdle the stems, ultimately leading to plant death. These results demonstrate a high level of virulence in *R. solani* affecting faba beans.

Table 3. Pathogenicity test of *R. solani* isolates causal pathogen of root-rot of faba bean

Isolates	No.	localities	Pre-emergence %	Post-emergence %	Survival %
R. solani	1	Alexandria	30.5	12.3	57.2^{ef}
R. solani	2	Alexandria	32.7	18.3	49.0^{g}
R. solani	3	Alexandria	25.3	14.0	60.7^{cd}
R. solani	4	Assiut	20.0	13.0	67.0^{b}
R. solani	5	Assiut	22.3	15.0	62.7^{c}
R. solani	5	Assiut	25.3	15.3	59.4^{de}
Control					100^{a}

The effect of salinity and treatments on disease severity assessment

Disease severity obtained data showed that presence of salinity has significantly increase *R. solani* (No. 2) aggressiveness (T4) by infecting all plants (100%) exhibiting 69% disease severity compared to T2 representing *R. solani* treated plants (Table 4 and Fig. 2D). BL exogenous application (T8) significantly reduced DI and DSI up to 20 and 30%, respectively followed by T6 (28 and 35%, respectively) Table (4) and Fig.(2 h&F). Although T7 plants group (*A. chrococum* treated plants) significantly reduced DSI up to 30%, moderately reduced DI up to 48% and enhanced root structure and development (Table 4 and Fig. 2G). While individual applied biochar significantly increased DSI up to 65% compared T2 (60%) and DI was 60%.

Table 4. Disease incidence (DI) and disease severity of 50 mM NaCl and *R. solani* combined stress treated faba bean plants

Treatments	DSI%	DI%
T1 (control)	00 ±0.00 ^{f}	00 ±0.00 ^{e}
T2 (*R. solani*)	60 ±0.24 ^{c}	100 ±0.45^{a}
T3 (50 mM NaCl)	00 ±0.00 ^{f}	00 ±0.00 ^{e}
T4 (*R. solani*+50 mM NaCl)	69 ±0.25 ^{a}	100 ±0.4 ^{a}
T5 (*R. solani*+50 mM NaCl+Biochar)	65 ±0.20 ^{b}	60 ±0.35 ^{b}
T6 (*R. solani*+50 mM NaCl+ *Azotobacter* enriched biochar)	35 ±0.32 ^{d}	28 ±4.90 ^{d}
T7 (*R. solani*+50 mM NaCl+ *A. chrococum*)	30 ±0.30 ^{e}	48 ±4.90 ^{c}
T8 (*R. solani*+50 mM NaCl+BL)	30 ±0.30 ^{e}	20 ±0.25 ^{d}

Values are the mean ± Standard Error of three replicates. Data with the same letter (s) are not significantly different at $P \leq 0.05$ using the Tukey's test

The effect of treatments on faba bean plants growth parameters

The results in Table 5 show that the grown of faba bean plants under salinity and *R. solani* combined stress (T4) has the lowest a significant negative impact on lengths of shoot and root, fresh and dry weights of shoot, fresh and dry weights of root compared to all treatments T1,2,3..

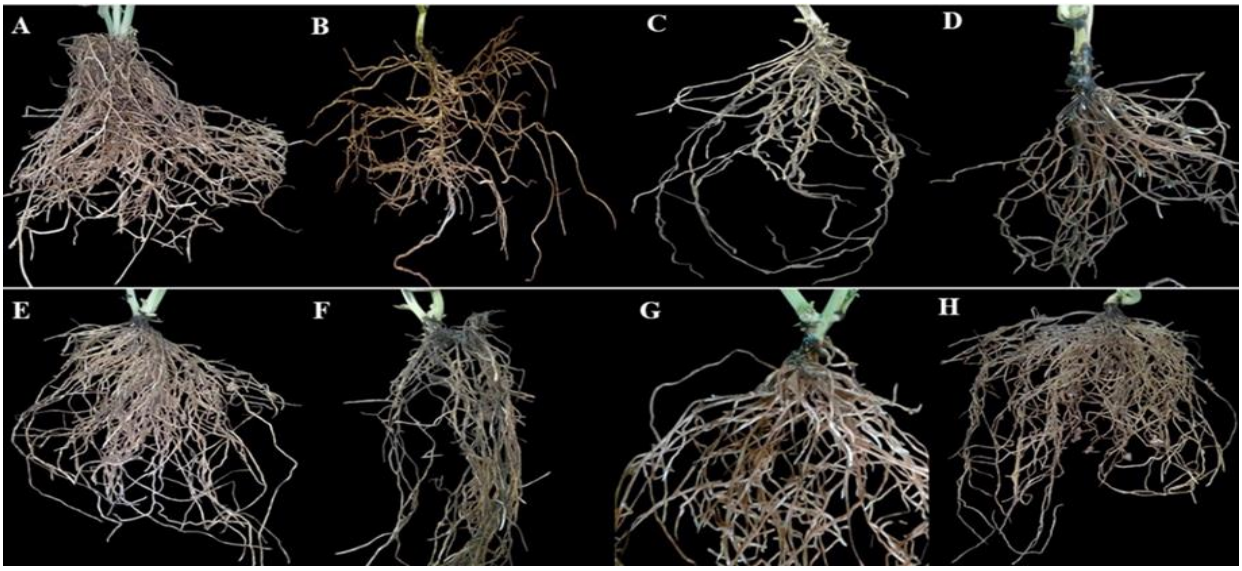

Fig. 2. Root-rot and lesion development of 60 days old faba bean plants exposed to combined stress of 50 mM NaCl and *R. solani*. A: healthy plants, B: *R. solani* inoculated plants, C:50 mM NaCl treated plants, D: 50 mM NaCl + *R. solani*, E: combined stress+biochar, F: combined stress Enriched, G: combined stress +*AZ. chrococum* and H: brassinolide treated plants.

On the other hand, all of the study's parameters; lengths of shoot and root, fresh and dry weights of shoot, fresh and dry weights of root were increased significantly in all treated plants. Furthermore, the best treatment when treated study's parameters with brassinolide 10^{-7} , which significant increases were observed in root length (28 cm), shoot length (60.8 cm), root fresh weight (17.1 g), shoot fresh weight (52.2 g), root dry weight (5.6 g) and shoot dry weight (10.2) followed by Azotobacter enriched biochar treatment) in root length (26.8 cm), shoot length (58.4 cm), root fresh weight (13.4 g), shoot fresh weight (50.6 g), root dry weight (4.6 g) and shoot dry weight (20 g) in comparison to T4, while individual applied biochar was the lowest one.

Table 5: Effect of treatments on growth attributes of *R. solani* and 50 mM NaCl combined stress treated faba bean plants

Treatment	Length (cm) of		Root weight (g)		Shoot weight (g)	
	Root	Shoot	RFW	RDW	SFW	SDW
T1	25.4±3^{a}	54.6±2.9 ^{bc}	12.6±3.4^{bc}	3.5±0.3^{c}	46.6±3^{bc}	15.1±1.1^{b}
T2	18.8±3^{c}	34.0±1.6 ^{e}	4.4±0.7^{ef}	1.1±0.1^{ef}	21.0±1.5^{e}	5.9±0.3^{d}
T3	20.2±3.3^{bc}	40.0±3.1 ^{d}	5.8±0.3^{ef}	1.7±0.2^{e}	24.3±2^{e}	6.9±0.3^{d}
T4	16.6±1.3^{c}	25.8±1.9 ^{e}	2.9±0.3^{f}	0.6±0.1^{f}	15.6±2.8^{f}	2.4±0.6^{e}
T5	24.8±1.3^{ab}	50.2±1.5 ^{c}	7.2±0.7^{de}	2.5±0.5^{d}	35.5±2.1^{d}	11.9±0.6^{c}
T6	26.8±2.1^{a}	58.4±1.8 ^{ab}	13.4±2.4^{b}	4.6±0.9^{b}	50.6±3.6^{ab}	20.0±0.7^{a}
T7	25.4±1.1^{a}	54.8±3.3 ^{bc}	9.4±0.7^{cd}	3.0±0.2^{cd}	41.9±2.4^{c}	14.8±0.6^{b}
T8	28±2.3 ^{a}	60.8±3.8 ^{a}	17.1±1.1^{a}	5.6±0.6^{a}	52.2±2^{a}	10.17±1.6^{c}

Values are the mean ± Standard Error of three replicates. Data with the same letter (s) are not significantly different at $P \leq 0.05$ using the Tukey's test. T1 (control), T2 (*R. solani* inoculated plants), T3 (50 mM NaCl exposed plants), T4 (*R. solani*+50 mM NaCl), T5 (*R. solani*+50 mM NaCl+Biochar), T6 (*R. solani*+50 mM NaCl+ Azotobacter enriched biochar), T7 (*R. solani*+50 mM NaCl+*A. chrococum*) and T8 (*R. solani*+50 mM NaCl+BL).

Physiochemical attributes

The effect treatments on photosynthetic pigments contents

Investigating effect of treatments on salinity and *R. solani* combined stress treated faba bean indicated that T4 (50 mM NaCl+*R. solani*) treatment was the lowest significant in TCC (2.1 mg g^{-1} FW) and CC (4.4 mg g^{-1} FW) among all treatments (Fig.3). Meanwhile, *A. chrococum* enriched biochar treatment (T6) was the most significant in TCC (18.1 mg g^{-1} FW) and CC (22.9 mg g^{-1} FW), followed by brassinolide T8 (13.4 and 17.9 mg g^{-1} FW, respectively). Although other treatments T5 (biochar) and T7 (*A. chrococum*) significantly ameliorated combined compared to T4, still significantly less than T1 (healthy control).

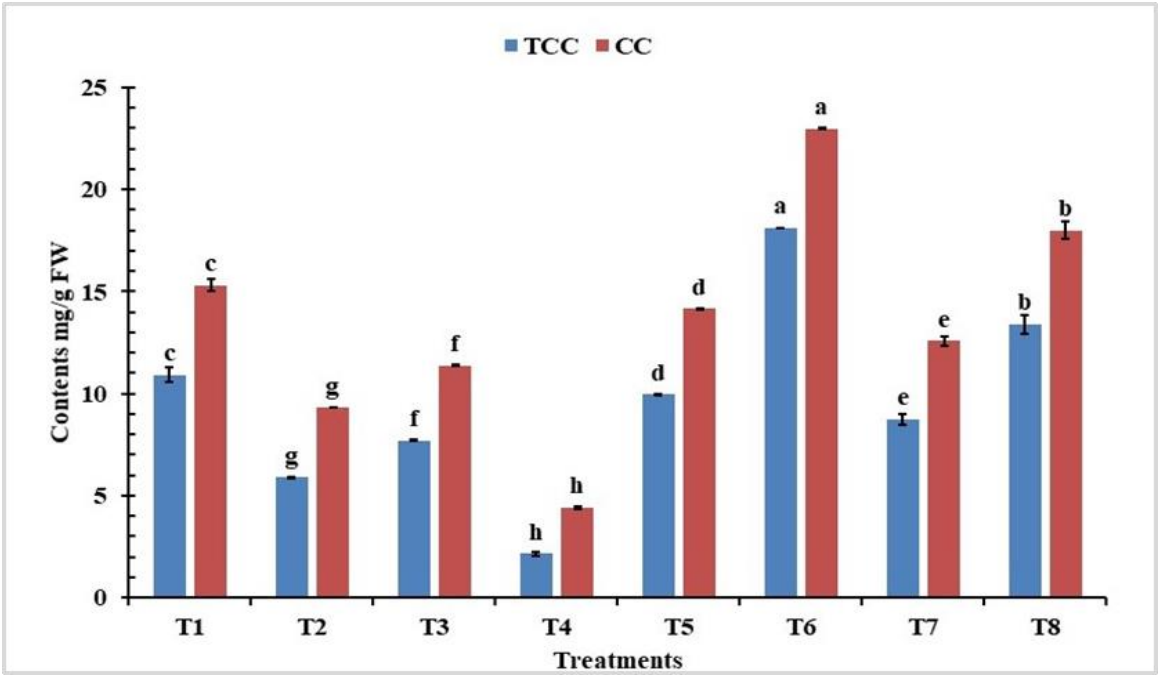

Fig 3. Total chlorophyll (TCC) and carotenoids contents of 60 days old faba bean plants exposed to combined stress of 50 mM NaCl and *R. solani*.

Values are the mean ± Standard Error of three replicates. Data with the same letter (s) are not significantly different at $P \leq 0.05$ using the Tukey's testT1 (control), T2 (*R. solani* inoculated plants), T3 (50 mM NaCl exposed plants), T4 (*R. solani*+50 mM NaCl), T5 (*R. solani*+50 mM NaCl+Biochar), T6 (*R. solani*+50 mM NaCl+ *Azotobacter* enriched biochar), T7 (*R. solani*+50 mM NaCl+*A. chrococum*) and T8 (*R. solani*+50 mM NaCl+BL). Columns with same colour and letters are not significantly different.

Oxidative enzymes response

POD activity was significantly unregulated in salinity and *R. solani* combined stress treatment (T4), compared to individual stressed plants in T2 (*R. solani* only) and T3 (salinity stressed plants), reaching levels of up to 11.66 U mg^{-1}protein min^{-1}; meanwhile treatments (T5, T6, T7 and T8) significantly increased POD compared to control groups (T1, T2, T3 and T4) with the highest activity observed in T7 (*A. chrococum* inoculated plants) by 13.92 U mg^{-1} protein/min (Fig. 4A). The activities of PPO and APX were maximized following T7 and T8 treatments with PPO reaching 4.8 and 5.04 U mg^{-1} protein/min, respectively, and APX achieving 32.79 and 30.86 U mg^{-1} protein/min, respectively (Fig. 4B&D). Conversely, the activity of the SOD enzyme significantly decreased after T7 (0.162 U mg^{-1} protein/min) and T8 (0.226 U mg^{-1} protein/min), while T6 showed the highest activity at 0.546 U mg^{-1} protein min^{-1} compared to all other treatments (Fig. 4C).

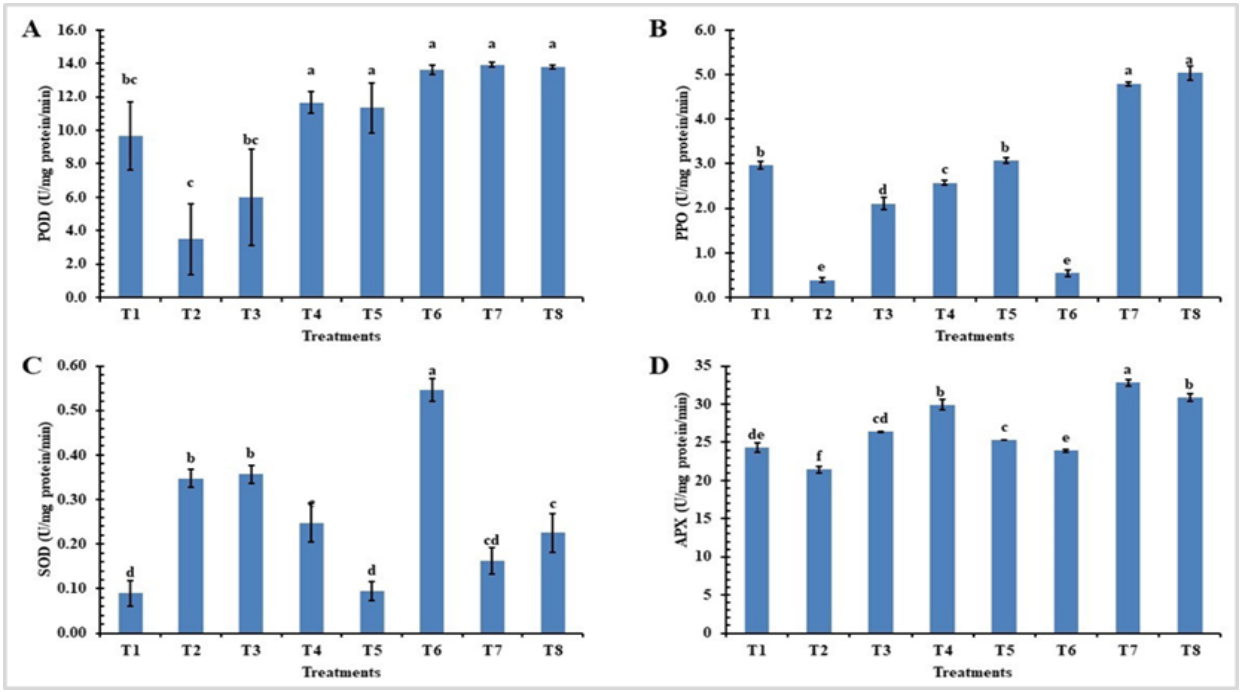

Fig.4. Activities of POD, PPO, SOD and APX antioxidant enzymes of salinity and *R. solani* combined stress 35 days old treated faba bean plants.

Values are the mean ± Standard Error of three replicates. Data with the same letter (s) are not significantly different at $P \leq 0.05$ using the Tukey's test. T1 (control), T2 (*R. solani* inoculated plants), T3 (50 mM NaCl exposed plants), T4 (*R. solani*+50 mM NaCl), T5 (*R. solani*+50 mM NaCl+Biochar), T6 (*R. solani*+50 mM NaCl+ Azotobacter enriched biochar), T7 (*R. solani*+50 mM NaCl+*A. chrococum*) and T8 (*R. solani*+50 mM NaCl+BL).

DISCUSSION

Salinity and *R. solani* root-rot among the most destructive abiotic and biotic stresses affecting faba bean plants, leading to significant reductions in crop quality and productivity. Therefore, it is crucial to find effective solutions that allow plants to thrive under combined stresses. In this study, we utilized *A. chrococum* (a biofertilizer), biochar (a soil amendment), and BL (a plant growth regulator) to enhance the tolerance of faba beans and mitigate the negative effects of these stressors.

The present study found that all treatments (*A. chrococum*, biochar, and brassinolide (10^{-7}) were more effective in reducing disease incidence of *R. solani* root-rot on faba bean and also increased the growth rate of faba bean plants. These results align with previous reports demonstrating antagonistic potential of *Azotobacter* spp. against *R. solani* and growth promotion (Dey *et al.*,, 2017, Abdel Latef *et al.*, 2021). Further studies indicated that Azotobacter enhances a plant's ability to absorb nutrients from the soil, particularly under salinity-stress conditions and enhances plant growth through various direct mechanisms, such as synthesizing phytohormones (including indole-3-acetic acid (IAA), gibberellic acid (GA), abscisic acid (ABA), and cytokinins) as well as exo-polysaccharides and volatile organic compounds. Indirect mechanisms include disease prevention, nutrient solubilization, and biological nitrogen fixation (Płaza *et al.*, 2022 and Ouf *et al.*, 2023). Brassinolide (BL) enhanced genes expression of both downstream and upstream of the systematic acquired resistance (SAR) pathways and wound-inducible disease resistance (Furio *et al.*, 2019b; Furio *et al.*, 2019a and Ding *et al.*, 2021). BRs can increase resistance to various stresses, such as salinity, which leads to improved yield and quality. This effect is attributed to Osmolytes like proline, glycine betaine, and total free amino acids (Zeng *et al.*, 2010 and Shahid *et al.*, 2014). Biochar

also promotes plant growth by enhancing microbial populations and significantly increasing soil nitrogen, phosphorus, and potassium content (Rondon *et al.*, 2007).
Photosynthesis is a crucial process for generating energy and providing organic materials to plants. In this study, we observed faba bean plants treated with *A. chrococum*-enriched biochar and BL exhibited the highest levels of total chlorophylls (TCC) and carotenoids (CC) compared to T4 (*R. solani* + 50 mM NaCl). Several previous studies have shown that Azotobacter's role in enhancing the production of proteins and enzymes linked to pigment stability, as well as the activity of electron transporters involved in photosynthesis (Pinnola *et al.*, 2016 and Enebe and Babalola, 2018). Brassinolide (BL) also significantly positively impacts the amounts of chlorophyll a, chlorophyll b, and total chlorophyll (Sedaghathoor *et al.*, 2017). BL plays a regulatory role in photosynthesis under both normal and stressful conditions, improving the efficiency of photosynthetic carbon fixation (Siddiqui *et al.*, 2018 and Al-Kanani and Al-Tamimi, 2025).

In the current study, the application of *A. chrococcum* and BL (10^{-7}) notably increased the levels of ascorbate peroxidase (APX), catalase, and peroxidase (POD) activities compared to the control group (T4: *R. solani* + 50 mM NaCl). These results are in agreement with previous reports demonstrating activities induction of various antioxidant enzymes, including catalase, peroxidase (POD), superoxide dismutase (SOD), ascorbate peroxidase (APX), and guaiacol peroxidase, in rhizobacteria and biochar co-treatments under drought stress in faba bean (Nafees *et al.*, 2024). The positive impacts observed on plant growth parameters, physicochemical properties, and enzymatic activities suggest that all treatments tested could be effectively applied in saline soils planted with faba bean and infected with *R. solani.*

CONCLUSION

The results suggest that the use of *A. chrococum*, biochar, and brassinolide (at a concentration of 10^{-7}) plays a beneficial role in enhancing plant disease resistance against *R. solani* root-rot in faba beans. Additionally, these treatments lead to significant improvements in various morphological parameters, as well as an increase in photosynthetic pigments, including total chlorophyll concentration, chlorophyll a, chlorophyll b, carotenoids, and antioxidant activity. The application of *A. chrococum*, biochar, and brassinolide is considered an important and environmentally friendly approach for agriculture, particularly in areas affected by fungal infections and saline stress.

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AUTHOR CONTRIBUTIONS

Majority contribution for the whole article belongs to the author(s). The authors read and approved the final manuscript.

COMPETING INTERESTS

The authors declare that they have no competing interests. The contents of the manuscript have neither been published nor under consideration for publication elsewhere.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

The author (s) hereby declare that NO generative AI technologies such as Large Language Models (Chat GPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

STATEMENT AND ETHICS DECLARATIONS

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the

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