



Biochar and plant growth-promoting rhizobacteria enhance physio-biochemical traits, secondary metabolites, oil, and grain yield of rapeseed under salinity stress

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Abstract

Salinity stress poses a significant constraint on global productivity. This study was designed to investigate the combined effects of inoculation with *Azotobacter chroococcum* (Az) and soil amendment with biochar on the performance of rapeseed (*Brassica napus* L.) under varying levels of NaCl-induced salinity. A factorial experiment evaluated key physio-biochemical responses, oxidative stress indicators, defense compound accumulation, and yield components. Salinity stress negatively impacted rapeseed growth, significantly reducing photosynthetic efficiency (Fv/Fm), chlorophyll content, relative water content (RWC), ATP levels, and ultimately grain and oil yields. Concurrently, salinity (120 mM and control treatments) increased indicators of oxidative damage — electrolyte leakage by 54%, malondialdehyde by 37%— and elevated levels of reactive oxygen species ($O_2^{\bullet -}$ at $97 \mu\text{mol g}^{-1} \text{FW h}^{-1}$ and H_2O_2 at $86 \mu\text{mol g}^{-1} \text{FW}$). Additionally, it induced defense responses such as increased antioxidant enzyme activity and osmolyte accumulation. However, the co-application of Az and biochar significantly mitigated these adverse effects of salinity. Notably, the combined treatment, particularly Az inoculation with an elevated biochar application rate (20%), demonstrated synergistic effects. This combination significantly enhanced photosynthetic efficiency, improved plant water status, and augmented antioxidant defense systems as well as osmotic adjustment mechanisms (including soluble sugars, proline, etc.) relative to untreated plants under saline conditions. Under these conditions, grain and oil yields increased substantially by 16.8% and 12.6%, respectively, compared to untreated saline-stressed plants. These findings highlight the potential of combining Az inoculation with biochar application as a promising and sustainable strategy to improve salinity tolerance and enhance the productivity of rapeseed in salt-affected agricultural environments.

Keywords Antioxidant enzymes · *Azotobacter Chroococcum* · Biochar application · Canola · Oil content · Grain production · Salinity stress

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Introduction

To sustainably meet the food and agricultural demands for a global population projected to exceed 9 billion by 2050, overall agricultural productivity is now estimated to require an average annual increase of 1.73%. However, recent trends show a significant productivity slowdown, with actual growth averaging only 1.12% per year between 2011 and 2020 (Steensland 2022; USDA ERS 2022). Rapeseed (*Brassica napus* L.), also known as canola, belongs to the Brassicaceae family. It is the second most important oilseed crop worldwide, containing approximately 40% oil. Rapeseed can produce up to one ton of oil per hectare, making it potentially the highest-yielding oil crop (Tanner et al. 2023).

Rapeseed is primarily cultivated in Eastern Europe and Asia (Nazari et al. 2022; Bhuiyan et al. 2019; Jing et al. 2020). Rapeseed is rich in functional compounds, including oils, phenolic compounds, dietary fiber, and proteins, associated with various health benefits (Matthaus et al. 2016; Jing et al. 2020). As a major oil-producing crop, it provides biodiesel, edible oil, and various industrial chemicals (Li et al. 2016; Tanner et al. 2023). Its oil ranks as the third most common cooking oil globally due to a high content of unsaturated fatty acids, which offer significant nutritional advantages. Increasing evidence indicates that diets high in unsaturated fatty acids confer numerous health benefits (Shen et al. 2023). Rapeseed oil contains essential fatty acids, including oleic acid (56.80–64.92%), linoleic acid (17.11–20.92%), and palmitic acid (4.18–5.01%) Matthaus et al. (2016). Salinity stress alters these fatty acid profiles, which are crucial for maintaining membrane integrity and fluidity under stress conditions. Understanding how salinity impacts fatty acid composition is essential for improving rapeseed's resilience and nutritional quality.

Globally, and particularly in semi-arid and arid countries such as Iran, salinity stress poses a significant constraint to crop cultivation, development, and productivity (Yaghoubian et al. 2022a; Khademian et al. 2019). Salinity and drought are among the primary abiotic stresses threatening global crop productivity, with over half of the world's arable land projected to face severe salinization by 2050 (Guo et al. 2014; Wang et al. 2003). This crisis underscores the urgent need to mitigate salinity's biochemical, molecular, and physiological impacts on crops.

Salinity stress induces ionic toxicity, oxidative stress, and osmotic imbalance, impairing water absorption, nutrient assimilation, and cellular stability (Carillo et al. 2011; Ashraf et al. 2018). These disruptions manifest as reduced leaf relative water content, membrane damage, chlorophyll degradation, and excessive reactive oxygen species (ROS) accumulation. ROS oxidize proteins, lipids, and nucleic acids, exacerbating cellular dysfunction (Foyer and Noctor 2005). To counteract this, plants activate antioxidative enzymes like superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX), which neutralize ROS and maintain redox homeostasis (Yaghoubian et al. 2021; Challaabathula et al. 2022). For instance, SOD converts superoxide radicals ($O_2^{\cdot-}$) into H_2O_2 , while CAT and APX further detoxify H_2O_2 into water and oxygen (Garratt et al. 2002). Enhanced antioxidant activity correlates with improved stress resilience, as seen in crops like rapeseed, where salinity severely impacts growth and oil quality (Sabagh et al. 2019; Wang et al. 2020).

Conventional strategies such as genetic engineering and breeding face significant economic and time limitations, driving research toward sustainable alternatives (Padikasan

et al. 2018) like microbial biotechnology. This approach has emerged as a feasible and cost-effective solution to address agricultural challenges in arid and saline environments (Yang and Fang 2015; Abdel Latef et al. 2020). Plant growth-promoting rhizobacteria (PGPR), including Az, enhance stress tolerance through mechanisms such as nitrogen fixation, siderophore production, and nutrient solubilization (Kumar et al. 2016; Abadi and Sepehri 2016). Additionally, PGPRs have been shown to significantly improve plant metabolism, development, and yield by mitigating salinity effects through a combination of direct and indirect pathways (Arora et al. 2020; Yaghoubian et al. 2022c; Monjezi et al. 2023). Studies demonstrate *A. chroococcum*'s efficacy in alleviating salinity stress in maize, sesame, wheat, and soybean by modulating osmolyte levels and antioxidant systems (Abd El-Ghany et al. 2015; Khademian et al. 2019; Chaudhary et al. 2013; Yaghoubian et al. 2022b).

Application of fertilizer amendments (especially organic fertilization systems) has been shown to enhance plant productivity and quality over time, particularly in dry sub-humid regions (Salehi et al. 2025a, b). Biochar, a pyrolyzed biomass, complements PGPR by adsorbing toxic ions (Na^+) and improving soil fertility (Mehmood et al. 2020; Verheijen et al. 2014). It mitigates oxidative stress in saline soils, as shown in common bean seedlings (Farhangi-Abriza and Torabian 2017). Numerous studies have shown that biochar has positive impacts on plant growth through both direct and indirect processes. These findings emphasize the potential of biochar as an effective tool for enhancing soil health and stimulating plant growth (Enders et al. 2012). However, the synergistic effects of Az and biochar on rapeseed under salinity stress remain insufficiently explored. Therefore, this study was designed to comprehensively evaluate the synergistic potential of Az and biochar in enhancing rapeseed's tolerance to salinity. The primary objectives were to: (1) quantify the effects of this co-application on key physiological parameters, including photosynthetic efficiency (F_v/F_m) and plant water status; (2) assess its role in mitigating oxidative damage by measuring indicators like electrolyte leakage and reactive oxygen species; (3) examine the enhancement of plant defense systems, such as antioxidant enzyme activity and osmolyte accumulation; and (4) determine the ultimate impact on critical grain and oil yield components under saline conditions.

Materials and methods

Experimental set-up

A factorial experiment was conducted using a randomized complete block design (RCBD) with three replications

in a greenhouse in Iran during 2019. Rapeseed (*Brassica napus* L.) plants were exposed to varying levels of salinity (0 [non-saline], 60, and 120 mM of NaCl), two levels of bacterial treatment involving non-inoculated and inoculated with Az (inoculated-Az), and biochar treatments (control, 10%, and 20% biochar). The selected biochar application rates (10% and 20%) were chosen based on published Literature to establish a dose-response relationship, while avoiding the potential negative impacts on plant growth and nutrient availability that have been reported at excessive concentrations. Biochar and inoculation with Az were selected as organic amendments to assess the biochemical and physiological responses of rapeseed under different salinity levels. The rapeseeds were surface-sterilized with a 2% solution of sodium hypochlorite prior to sowing. A schematic diagram of the experimental setup is provided in Fig. S1 (Supplementary Material) to facilitate understanding of the methodology.

Preparation of biochar and azotobacter

The preparation of biochar followed the method outlined by Qian and Chen (2013), with modifications. Maple residues (*Acer pseudoplatanus* L.) were converted into biochar through a pyrolysis process at 560 °C for 6 h in an anaerobic environment. The key physicochemical properties of the biochar were determined using an elemental analyzer (Elementar, Germany) and are detailed in Table 1. The bacterium used in this experiment was Az, a well-known free-living nitrogen-fixing bacterium. Key characteristics of Az include its ability to fix atmospheric nitrogen under aerobic conditions, produce phytohormones and other bioactive compounds that promote plant growth and enhance stress tolerance. These traits make Az an effective plant growth-promoting rhizobacterium (PGPR), particularly under abiotic stress conditions such as salinity.

Table 1 Physical and chemical properties of soil and Biochar used in the greenhouse experiment

Property	Unit	Soil	Biochar
Texture	—	Sandy loam	—
pH	—	7.1	7.9
EC	dS m ⁻¹	0.21	—
Organic carbon	%	0.42	—
Total nitrogen (N)	%	0.07	0.71
Carbon (C)	%	—	32.82
Hydrogen (H)	%	—	1.6
Oxygen (O)	%	—	29.01
Potassium (K)	mg/kg	166	3309
Magnesium (Mg)	mg/kg	—	983
Sodium (Na)	mg/kg	—	8.2
Calcium (Ca)	mg/kg	—	3386
Phosphorus (P)	mg/kg	34	—
Cation exchange capacity (CEC)	cmol/kg	—	19.9

Azotobacter chroococcum (strain designation and accession number CCSM-B.00440) was obtained from the Culture Collection of Soil Microorganisms (CCSM) at the Soil and Water Research Institute (SWRI), Karaj, Iran (CCSM 2021). For bacterial cultivation, Az was initially grown in Luria-Bertani (LB) Liquid medium, then transferred to 250 ml flasks containing 100 ml of the same medium. The flasks were placed in a shaking incubator at 28 °C and 120 rpm for 48 h to facilitate bacterial growth.

The bacterial culture was centrifuged at 5000 rpm for 5 min at a temperature of 4 °C. The resulting pellet was resuspended in autoclaved distilled water. The bacterial population concentration was adjusted to 8×10^8 colony-forming units (CFU) per milliliter. After sterilization, the seeds were inoculated with the bacterial suspensions and incubated for 6 h, following the method of Yaghoubian et al. (2022b).

Experimental condition and management

Soil samples were analyzed for chemical properties before sowing. The initial soil characteristics are presented in Table 1. To ensure thorough integration, the soil and biochar were mixed by passing the components through a 2 mm mesh screen before filling the pots. Each plastic pot (30 × 30 cm) was subsequently filled with 15 kg of this prepared soil mixture. Ten rapeseed seeds were sown in each pot. The pots were then placed in a greenhouse where temperatures were maintained at 20 to 25 °C (day/night) and relative humidity was kept between 50 and 60%.

Plants were irrigated daily during emergence and seedling development. Salt stress treatments commenced 28 days after planting; NaCl solutions were gradually added via irrigation to achieve final concentrations of 60 mM and 120 mM. Control plants received regular irrigation without added NaCl throughout the experiment. Plant samples for physiological and biochemical analyses were collected during the flowering stage. At physiological maturity, seeds were harvested from all plants to determine seed yield and quality characteristics.

Measurements of chlorophyll content, relative water content (RWC), and maximum photosystem II efficiency (Fv/Fm)

Chlorophyll content was measured following the method of Ghassemi et al. (2016). Rapeseed leaf samples (500 mg) were homogenized in 4 mL of 80% acetone and centrifuged at 12,000 g for 15 min at 4 °C. The absorbance of the resulting supernatant was measured at 645 nm and 663 nm. Fv/Fm was measured using a portable chlorophyll fluorometer. To determine the RWC, the most recently matured rapeseed

leaves were harvested, and their fresh weight (FW) was recorded. Subsequently, the leaves were used to determine turgid weight (TW) and dry weight (DW) according to the method described by Smart and Bingham (1974). RWC was calculated using Eq. 1:

$$\text{RWC (\%)} = \left[\frac{\text{Fw} - \text{Dw}}{\text{Tw} - \text{Dw}} \right] \times 100 \quad (1)$$

Measurement of adenosine triphosphate (ATP) content

Total ATP content was measured using the luciferin-luciferase method with a Luminometer (Novalum, Charm Sciences, Lawrence, MA, USA). Cellular ATP was extracted using a solution of 4% trichloroacetic acid (TCA) containing 2 mM ethylenediamine tetraacetic acid (EDTA), following the protocol described by Larsson and Olsson (1979).

Measurement of lipid peroxidation (as malondialdehyde [MDA] content) and membrane stability (via electrolyte leakage [EL])

Lipid peroxidation was quantified by measuring MDA levels in fresh rapeseed leaves using the method described by Dhindsa et al. (1981). Absorbance was measured at 532 nm, and the measurement was corrected for nonspecific absorbance by subtracting the absorbance measured at 600 nm. The MDA content was calculated using an extinction coefficient of $155 \text{ mM}^{-1} \text{ cm}^{-1}$ and expressed as $\text{nmol g}^{-1} \text{ FW}$.

Membrane stability was assessed by measuring electrolyte leakage (EL) according to the protocol of Shi et al. (2006). Fresh rapeseed leaves were placed in tubes containing 30 mL of deionized water and incubated at room temperature for 24 h. The initial electrical conductivity of the solution (EC1) was measured. Subsequently, the tubes containing the leaves and solution were heated to 95°C for 20 min, cooled to room temperature, and the final conductivity (EC2) was measured. Electrolyte leakage (EL) was calculated using Eq. 2:

$$\text{EL (\%)} = (\text{EC1}/\text{EC2}) \times 100 \quad (2)$$

Measurements of hydrogen peroxide (H_2O_2) and the oxygen radicals ($\text{O}_2^{\cdot-}$) content

Hydrogen peroxide (H_2O_2) in rapeseed leaves was quantified based on its reaction with titanium tetrachloride, measuring the absorbance of the resulting complex at 490 nm, following the method of Hsu and Kao (2007). The production rate

of $\text{O}_2^{\cdot-}$ was measured using the method described by Wang and Jiao (2000).

Antioxidant enzyme assays

Peroxidase (POX) activity was measured by monitoring the Change in absorbance at 490 nm, following the method of Gueta-Dahan et al. (1997). Ascorbate peroxidase (APX) activity was quantified by monitoring the Change in absorbance at 290 nm according to Nakano and Asada (1981). Superoxide dismutase (SOD) activity was determined based on its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT). One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of NBT reduction under the assay conditions. Catalase (CAT) activity was measured according to Singh et al. (2010) by monitoring the decrease in absorption at 240 nm. All enzyme activities were expressed as units per gram of fresh weight ($\text{U g}^{-1} \text{ FW}$).

Measurement of osmolytes and soluble proteins

Proline content was determined according to the method described by Bates et al. (1973). Rapeseed leaf samples (250 mg) were homogenized in 1.5 mL of 3% aqueous sulfosalicylic acid. An aliquot (1.5 mL) of the extract was reacted with ninhydrin (1 mL) and glacial acetic acid (1 mL) in vials. Proline concentration was quantified spectrophotometrically by comparison with a standard curve prepared using pure proline, and results were expressed as $\text{mg g}^{-1} \text{ FW}$. Soluble carbohydrate content was measured using the phenol-sulphuric acid method, following the protocol described by Kochert (1978). Glycine betaine (GB) concentration was determined following the procedure described by Grieve and Grattan (1983).

For soluble protein analysis, leaf samples (1000 mg) were homogenized in 4 mL Na-phosphate buffer and centrifuged at $12,000 \text{ g}$ for 10 min. The protein concentration in the supernatant was determined by measuring absorbance at 595 nm with a UV-Vis spectrophotometer.

Determination of DPPH radical scavenging activity

The scavenging activity against the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was determined using a modified procedure described by Asghari et al. (2018). Aliquots (20 μL) of the plant extract (prepared at a concentration of 2 mg/mL) were mixed with 180 μL of a 0.1 mM methanolic DPPH solution. After incubation in the dark for 30 min at room temperature, the absorbance of the mixture was measured at 517 nm. The percentage of DPPH radical scavenging activity was calculated using Eq. 3:

$$\text{DPPH (\%)} = (\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}) / \text{A}_{\text{control}} \times 100 \quad (3)$$

Here, $\text{Abs}_{\text{control}}$ represents the absorbance of the control reaction (DPPH solution mixed with the sample solvent), and $\text{Abs}_{\text{sample}}$ represents the absorbance of the reaction mixture containing the DPPH solution and the sample extract.

Measurement of soluble sugar content

Frozen leaf samples (0.2 g) were extracted with 3 mL of distilled water. The homogenate was filtered through filter paper. For sugar determination, an aliquot of the filtrate (0.5 mL) was mixed with 2.5 mL of concentrated sulfuric acid (98%) and 0.5 mL of 5% phenol solution. A standard curve was prepared using known concentrations (ranging from 0 to 8 mg mL⁻¹) of mannose, xylose, and glucose. Absorbance of the standards and samples was measured using a spectrophotometer. Total soluble sugar concentration was expressed as mg g⁻¹ FW.

Determination of oil content, fatty acid composition, and unsaturation index (UI)

Seed oil content was determined using the Soxhlet extraction method with petroleum ether for six hours. The content was calculated based on the mass difference of seed samples before and after extraction, following Ghassemi-Golezani et al. (2017). Fatty acid composition was analyzed as fatty acid methyl esters (FAMES) by gas chromatography (GC), using a modified method based on Hama (2017). The UI was calculated using Eq. 4, following Ghassemi-Golezani and Farhangi-Abriz (2018):

$$\text{UI} = (\Sigma \%C_{n:1} + 2\Sigma \%C_{n:2} + 3\Sigma \%C_{n:3}) / 100 \quad (4)$$

where $C_{n:1}$, $C_{n:2}$, and $C_{n:3}$ represent the percentages of oleic acid, linoleic acid and linolenic acids, respectively.

Statistical analysis

Experimental data were subjected to a two-way analysis of variance (ANOVA). Prior to ANOVA, data normality was checked using the Kolmogorov–Smirnov test, and homogeneity of variances was also assessed. Means for each trait were compared using Fisher's protected Least Significant Difference (LSD) test at $p \leq 0.05$ significance level. Statistical analyses were performed using GenStat (Version 12) and SAS (Version 9.4) software. Figures were generated using Microsoft Excel. Error bars shown represent the standard deviation (SD). In figures, different letters indicate statistically significant differences between means ($p \leq 0.05$), where applicable, as described in the legends.

Results

Chlorophyll content (Chl a and Chl b), relative water content (RWC), and maximum photosystem II efficiency (Fv/Fm)

Increasing salinity level significantly reduced chlorophyll a (Chl a), chlorophyll b (Chl b), and RWC in rapeseed leaves (Table 2). The lowest Chl a content (0.89 mg g⁻¹ DW), Chl b content (0.35 mg g⁻¹ DW), and RWC (65.47%) were observed in non-inoculated plants exposed to the highest salinity level (120 mM NaCl). Conversely, inoculation with Az significantly increased Chl a, Chl b, and RWC under both non-saline and saline conditions compared to non-inoculated controls (Table 2). Similarly, biochar application significantly enhanced these parameters, with higher values observed at the 20% application rate compared to the 10% rate under all conditions. Consequently, the combination of Az inoculation and 20% biochar application yielded the highest Chl a, Chl b, and RWC values observed in this study (Table 2).

Fv/Fm in rapeseeds leaves ranged from 0.52 to 0.78 across all treatments (Table 2). Salinity exposure significantly reduced Fv/Fm compared to the non-saline condition; the lowest value of 0.52 was observed in non-inoculated plants without biochar under 120 mM NaCl. However, under saline stress, Az inoculation combined with biochar application (both 10% and 20%) significantly increased Fv/Fm values. The highest Fv/Fm (0.78) was recorded in inoculated-Az plants treated with 20% biochar under 60 mM NaCl stress (Table 2).

Adenosine triphosphate (ATP) content

Increasing salinity significantly reduced ATP content in rapeseed leaves, particularly at the 120 mM NaCl level (Fig. 1e–f). Temporally, ATP content generally increased during early growth, reaching peak values at 9 weeks after sowing (WAS) under non-saline and 60 mM NaCl conditions. Under 120 mM NaCl, the peak was also observed at 9 WAS, but the subsequent decline commenced earlier than in the other treatments. The lowest overall ATP content across treatments was typically observed at 14 WAS (Fig. 1e–f). Both inoculation with Az and application of biochar (10% and 20%) significantly increased leaf ATP content compared to their respective controls under both non-saline and saline conditions. The positive effect of biochar was observed regardless of Az inoculation (Fig. 1e–f). The highest ATP content (2.37 µg ATP µg protein⁻¹) was recorded at 9 WAS in non-saline conditions for plants inoculated with Az and treated with 20% biochar (Fig. 1e–f).

Table 2 Effect of Az inoculation and Biochar application on chlorophyll a (Chl a), chlorophyll b (Chl b), relative water content (RWC), maximum photosystem II efficiency (Fv/Fm), electrolyte leakage (EL), and malondialdehyde content (MDA) in rapeseed leaves under varying salinity levels

Salt (mM)	Az	Biochar	Chl a (mg g ⁻¹ DW)	Chl b (mg g ⁻¹ DW)	RWC (%)	Fv/Fm	EL (%)	MDA (mmol g ⁻¹ FW)
0	Non-inoculated	Control	1.74±0.07 gh	0.84±0.06 fg	81.38±1.74 cd	0.74±0.01abc	25.08±3.07 f	10.37±1.24 hi
		10% biochar	1.89±0.13 ef	0.93±0.02 ef	82.49±1.43 cd	0.75±0.02ab	24.67±1.15 f	9.79±0.55 hi
		20% biochar	2.03±0.09 cd	1.07±0.04 bc	84.08±2.73 bc	0.76±0.04ab	23.94±0.81 f	8.75±0.49 i
	Inoculated-Az	Control	2.01±0.17 de	1.01±0.12 cd	84.05±2.95 bc	0.75±0.01ab	24.03±2.07 f	9.55±0.43 hi
		10% biochar	2.29±0.22 ab	1.18±0.09 ab	85.12±3.88 ab	0.77±0.01a	23.20±1.99 fg	8.23±0.22 i
		20% biochar	2.43±0.07 a	1.22±0.16 a	86.21±1.59 a	0.78±0.02a	21.83±1.64 g	7.64±1.68 i
60	Non-inoculated	Control	1.45±0.03 kl	0.69±0.08 hi	75.27±0.58 ef	0.62±0.02 ef	37.13±2.94 c	18.05±2.24 d
		10% biochar	1.66±0.16 ij	0.79±0.07 gh	78.31±1.61 de	0.70±0.03 cd	32.95±2.84 de	16.03±1.04 de
		20% biochar	1.74±0.08 gh	0.91±0.06 ef	80.09±0.94 cd	0.72±0.02bc	30.54±2.80 e	13.47±2.43 fg
	Inoculated-Az	Control	1.79±0.08 fg	0.86±0.06 fg	81.50±3.82 cd	0.67±0.04de	32.73±2.07 de	15.67±1.76 de
		10% biochar	1.88±0.14 fg	0.96±0.04 de	83.42±2.76 bc	0.74±0.01abc	30.58±0.53 e	12.11±1.86 gh
		20% biochar	2.22±0.15 bc	1.21±0.16 ab	85.91±1.54 ab	0.77±0.03a	25.61±3.41 f	10.18±2.95 hi
120	Non-inoculated	Control	0.89±0.02 m	0.35±0.04 j	65.47±1.35 h	0.52±0.02 g	54.28±1.77 a	36.78±2.31 a
		10% biochar	1.28±0.09 l	0.53±0.03 i	69.84±1.39 g	0.59±0.03f	47.59±2.47 b	31.14±3.01 b
		20% biochar	1.39±0.06 kl	0.71±0.08 gh	73.18±2.54 fg	0.65±0.02e	37.01±2.20 c	24.89±0.14 c
	Inoculated-Az	Control	1.33±0.08 kl	0.68±0.03 hi	70.33±2.91 g	0.58±0.03f	47.43±1.40 b	30.23±2.44 b
		10% biochar	1.53±0.13 jk	0.83±0.08 gh	78.14±3.55 de	0.67±0.01de	35.61±2.98 cd	23.28±0.77 c
		20% biochar	1.91±0.06 ef	1.05±0.09 bc	82.16±3.31 cd	0.74±0.02abc	25.16±1.70 f	14.34±0.83 ef

Treatments included three NaCl concentrations (0, 60, and 120 mM), two inoculation statuses (non-inoculated and inoculated-Az), and three biochar rates (control, 10%, and 20%). Values represent the mean of three replicates±standard deviation (SD). Means within each column followed by the same letter are not significantly different according to LSD test ($p \leq 0.05$).

Electrolyte leakage (EL) and malondialdehyde (MDA) content

EL and MDA content are indicators of oxidative stress and membrane damage in plants. The interaction between Az inoculation, biochar application, and salinity significantly influenced EL and MDA content in rapeseed leaves (Table 2). Specifically, the highest EL (54.28%) and MDA content (36.78 mmol g⁻¹ FW) were observed in non-inoculated plants without biochar exposed to 120 mM NaCl (Table 2). Inoculation with Az and application of biochar significantly reduced both EL and MDA content under non-saline and saline conditions compared to untreated controls (Table 2). For example, under non-saline conditions, the combination of inoculated-Az and 20% biochar reduced EL by 13% and MDA content by 26.3% compared to the control treatment.

Oxygen radicals (O₂^{•-}) and hydrogen peroxide (H₂O₂) levels

Salinity stress (60 and 120 mM NaCl) significantly increased O₂^{•-} and H₂O₂ levels in rapeseed leaves compared to non-saline conditions, regardless of inoculation or biochar treatments (Fig. 2a, b). Under non-saline conditions, inoculation-Az did not significantly alter O₂^{•-} or H₂O₂ levels compared to non-inoculated controls (Fig. 2a, b). However, under saline conditions, biochar application

significantly reduced the levels of both O₂^{•-} and H₂O₂ (Fig. 2a, b). The highest O₂^{•-} level (0.97 μmol g⁻¹ FW h⁻¹) and H₂O₂ level (0.86 μmol g⁻¹ FW) were recorded under 120 mM NaCl in non-inoculated plants grown without biochar (control) (Fig. 2a, b). Conversely, the combination of Az inoculation and 20% biochar application resulted in the lowest O₂^{•-} (0.35 μmol g⁻¹ FW) and H₂O₂ (0.22 μmol g⁻¹ FW) levels observed within each respective treatment condition (non-saline, 60 mM, and 120 mM NaCl), demonstrating the strongest mitigation effect (Fig. 2a, b).

Antioxidant enzymes activities (CAT, SOD, POX, APX)

Salinity stress significantly increased the activities of catalase (CAT), superoxide dismutase (SOD), peroxidase (POX), and ascorbate peroxidase (APX) in rapeseed leaves compared to non-saline conditions (Fig. 3a–d). Under non-saline conditions, Az inoculation did not significantly affect CAT or POX activity (Fig. 3a, c). However, under non-saline conditions, the application of 20% biochar significantly increased APX and SOD activities compared to the no-biochar control (Fig. 3b, d). The highest activities for all four enzymes under stress conditions (60 and 120 mM NaCl) were observed in plants inoculated with Az and treated with 20% biochar (Fig. 3a–d). For example, at 120 mM NaCl, the combination of Az inoculation and 20% biochar application increased by 96.6%, 113%, 103%, and 50% in CAT, SOD, POX, and APX activity, respectively,

compared to the non-inoculated, control treatment under the same salinity level (Fig. 3a–d).

Osmolytes content (soluble proteins, Glycine betaine, soluble carbohydrates, proline) and DPPH scavenging activity

Az inoculation, biochar application, salinity, and their interactions significantly affected the content of soluble proteins (SP), glycine betaine (GB), soluble carbohydrates (SC), proline, and DPPH scavenging activity in rapeseed leaves (Table 3). Increasing salinity stress resulted in higher levels of SP, GB, SC, proline, and DPPH activity compared to non-saline conditions, with the highest concentrations generally observed at 120 mM NaCl across all treatments (Table 3). Furthermore, under saline conditions, Az inoculation and biochar application (both 10% and 20%) led to significantly higher accumulation of these osmolytes and greater DPPH activity compared to the non-inoculated, no-biochar controls subjected to the same salinity level (Table 3). The maximum recorded values for SP ($5.87 \text{ mg g}^{-1} \text{ DW}$), GB ($24.41 \text{ mg g}^{-1} \text{ FW}$), SC ($27.82 \text{ mg g}^{-1} \text{ FW}$), proline ($51.63 \text{ mg g}^{-1} \text{ FW}$), and DPPH activity (57.74%) were observed in plants inoculated with Az and treated with 20% biochar under 120 mM NaCl stress. These values were significantly higher than those in the non-inoculated, no-biochar control plants under the same 120 mM NaCl stress level (Table 3).

Specific soluble sugar content (glucose, mannose, xylose)

The content of specific soluble sugars (glucose, mannose, and xylose) significantly increased in response to salinity stress, with the highest levels generally observed at 120 mM NaCl (Fig. 4a–c). Across all treatments and conditions, glucose content ranged from 5.40 to $13.44 \text{ mg g}^{-1} \text{ FW}$, mannose content ranged from 5.10 to $9.96 \text{ mg g}^{-1} \text{ FW}$, and xylose content ranged from 3.01 to $8.65 \text{ mg g}^{-1} \text{ FW}$ (Fig. 4a–c). Under salinity stress, inoculation with Az combined with biochar application resulted in significantly higher contents of glucose, mannose, and xylose compared to the non-inoculated plants without biochar (control) subjected to the same salinity level (Fig. 4a–c). Specifically, at 120 mM NaCl, plants inoculated with Az and treated with 20% biochar showed increases of 33.59% in glucose, 27.04% in mannose, and 35.80% in xylose content, compared to the control treatment (Fig. 4a–c).

Fatty acids composition and unsaturation index (UI)

Az inoculation, biochar application, and salinity significantly influenced the fatty acid composition and UI of rapeseed oil

(Table 4). Under non-saline conditions, the combination of Az inoculation and 20% biochar application significantly reduced the content of oleic acid (by 1.44%), stearic acid (by 10.11%), and palmitic acid (by 12.0%) compared to the non-inoculated, no-biochar control. Conversely, this same treatment resulted in the highest recorded levels of linoleic acid (19.43%) and linolenic acid (8.87%) under non-saline conditions (Table 4).

At 60 mM NaCl, while no significant differences were observed in the UI between treatments, some significant differences were present for major fatty acid contents between inoculated-Az and non-inoculated plants, and also due to biochar application compared to the no-biochar control (Table 4). At the highest salinity level (120 mM NaCl), the non-inoculated plants without biochar (control) exhibited the highest content of oleic acid (67.63%), stearic acid (2.46%), and palmitic acid (5.07%). The highest UI value (1.24) was recorded in non-saline conditions, observed in several treatments including those receiving Az inoculation and 20% biochar treatment (Table 4).

Oil content, grain yield, and oil yield

Salinity levels alone did not cause significant differences in seed oil content (Fig. 5a). However, oil content ranged from 39.13 to 47.47% across all treatments, with the highest content (47.47%) observed in plants inoculated with Az and treated with 20% biochar under 120 mM NaCl stress (Fig. 5a). Salt stress significantly reduced both grain yield and oil yield, with the greatest reductions observed at 120 mM NaCl (Fig. 5b, c). Biochar application and inoculated-Az significantly increased grain and oil yields under both non-saline and saline conditions compared to untreated controls (Fig. 5b, c). The relative yield increase due to these treatments was greater under non-saline and moderate (60 mM NaCl) stress conditions compared to high (120 mM NaCl) stress conditions. Overall, the highest grain yield ($1.55 \text{ g plant}^{-1}$) and oil yield ($3.66 \text{ g plant}^{-1}$) were obtained from inoculated-Az plants treated with 20% biochar under non-saline conditions (Fig. 5b, c).

Discussion

Azotobacter and Biochar mitigate salinity-induced reductions in photosynthetic parameters and ATP levels

Consistent with established knowledge, this study demonstrated that increasing salinity stress significantly impaired key physiological parameters in rapeseed. The present study observed marked reductions in chlorophyll a (Chl a),

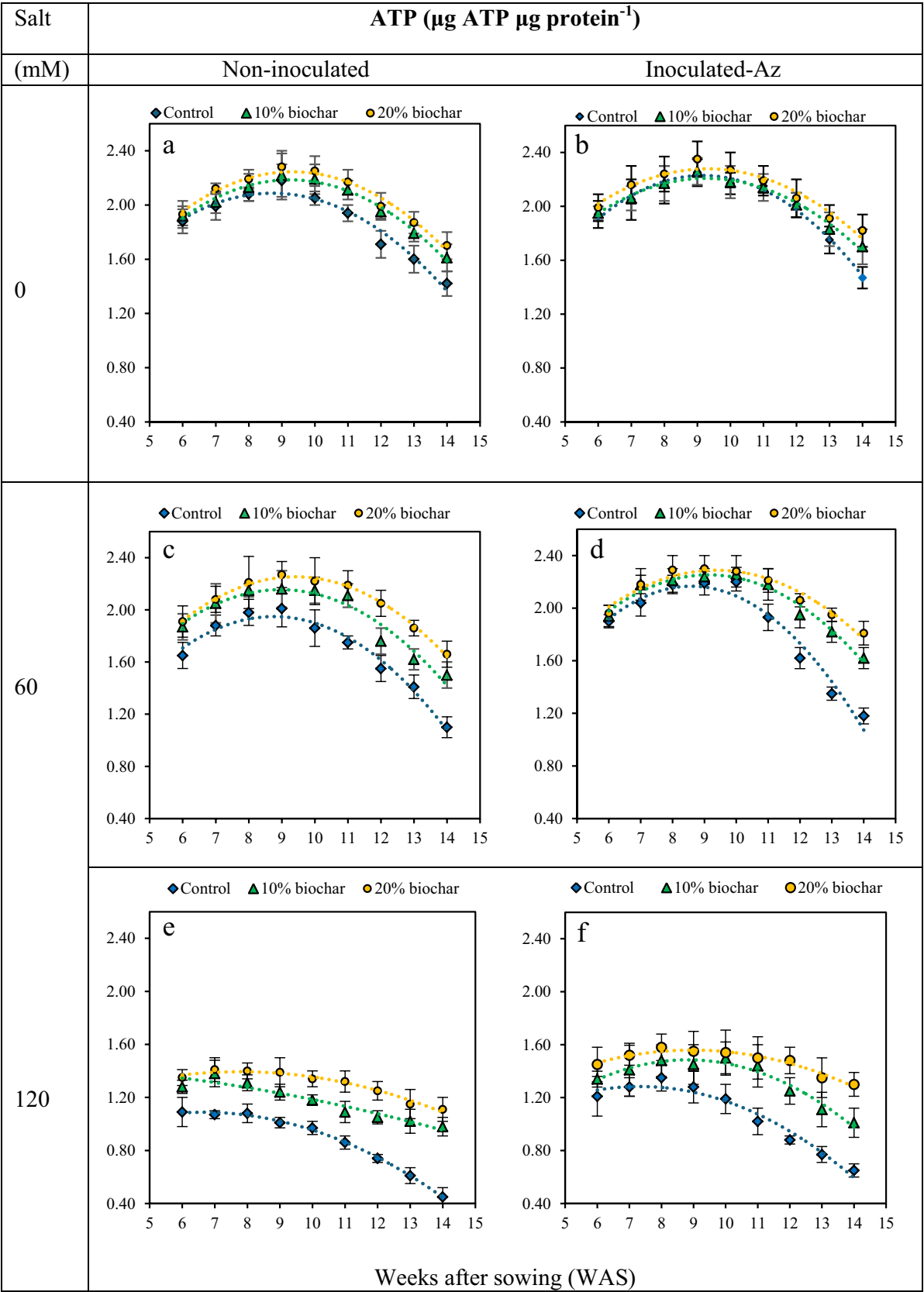


Fig. 1 Effect of Az and biochar application on changes on adenosine triphosphate (ATP) content in rapeseed leaves under varying salinity stress levels (NaCl concentrations). Panels (a, c, e) show the effect of biochar application (control, 10%, and 20%) in non-inoculated plants under 0, 60, and 120 mM NaCl, respectively, while panels (b, d, f) show the corresponding effect in inoculated-Az plants. Values represent the mean of three replicates $\pm$ standard deviation (SD). Error bars denote statistically significant differences (LSD test, $p \leq 0.05$). See Fig. 2 for treatments

chlorophyll b (Chl b), RWC, Fv/Fm (Table 2), and adenosine ATP content (Fig. 1e–f), particularly at 120 mM NaCl. Such chlorophyll degradation under salt stress is often attributed to oxidative damage and impaired synthesis pathways (Yaghoubian et al. 2021). The observed decline in RWC, a critical indicator of plant water status (Soltys-Kalina et al. 2016), reflects the osmotic challenge imposed by salinity and aligns with previous reports across various plant species subjected to salt stress (Kumar et al. 2010; Chang et al. 2014; Khademian et al. 2019; Yaghoubian et al. 2022a). Furthermore, the reduction in Fv/Fm likely indicates damage to the PSII reaction centers, possibly mediated by reactive oxygen species (Radić et al. 2013; Apostolova et al. 2006), while the concurrent decrease in ATP levels points towards disrupted energy metabolism stemming from impaired photosynthesis (Chatzigianni et al. 2023; Hannachi et al. 2022). The temporal pattern observed for ATP, peaking around 9 weeks after sowing before declining, suggests a developmental shift in energy dynamics potentially exacerbated by stress.

Crucially, inoculation with Az and the application of biochar, especially at the 20% rate, effectively counteracted these detrimental effects of salinity. Both treatments, individually and particularly in combination, significantly improved Chl content, RWC, Fv/Fm, and ATP levels compared to untreated controls under both non-saline and saline conditions. The enhancement of RWC by plant growth-promoting rhizobacteria (PGPR) like Az under salinity is well-supported, potentially linked to improved water uptake or hormonal signaling (García et al. 2017; Hosseini et al. 2017; Yaghoubian et al. 2021). Biochar likely contributed to improving soil moisture retention and nutrient availability, thus reducing overall stress perception by the plant. The restoration of higher Fv/Fm and ATP levels in Az- and biochar-treated plants suggests enhanced protection or repair of the photosynthetic machinery, particularly PSII, under salinity. This functional improvement parallels findings where other beneficial microbes, like arbuscular mycorrhizal fungi, mitigated photooxidative stress on PSII (Aroca et al. 2013). The observation that the Az+20% biochar combination often yielded the highest values for these parameters points towards a synergistic interaction, potentially stemming from complementary mechanisms: biochar conditioning the soil environment while Az directly promoting plant growth and stress resilience.

Additionally, extensive literature supports the potential for biochar to serve as a beneficial microbial carrier and to synergistically enhance the performance of plant growth-promoting bacteria such as Az (Sharma et al. 2025; Bolan et al. 2023). Biochar's porous structure and large surface area provide favorable microhabitats for beneficial microbes, supporting their establishment, survival, and activity in the soil (Bolan et al. 2023; Xiang et al. 2022). Recent meta-analyses and reviews also indicate that biochar application generally increases soil microbial diversity and biomass, which can facilitate improved nutrient cycling and plant growth under stress conditions (Xu et al. 2023). These findings, together with the observed plant responses, suggest that the combined application of Az and biochar may create a more supportive rhizosphere environment, further enhancing rapeseed resilience to salinity stress.

Azotobacter and biochar mitigate salinity-induced oxidative markers

This study observed that high salinity (120 mM NaCl) in the absence of ameliorating treatments led to elevated EL and MDA content in rapeseed leaves (Table 2). These results signify substantial oxidative stress leading to lipid peroxidation and membrane damage, a common consequence of salinity stress reported in previous research (Yaghoubian et al. 2021; Coppa et al. 2024). Such damage is often mechanistically linked to the overproduction of reactive oxygen species (ROS), including superoxide radicals ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) (Evelin et al. 2019; Farhangi-Abriz and Torabian 2017).

Conversely, the application of biochar and inoculation with Az significantly reduced EL and MDA levels under both non-saline and saline conditions (Table 2), indicating a protective role against salinity-induced oxidative damage. The finding that Az reduced EL and MDA under salinity aligns specifically with the results reported by Yaghoubian et al. (2021). Plant growth-promoting rhizobacteria (PGPR) like Az are known to enhance cell membrane integrity, potentially by mitigating oxidative damage (Abadi and Sepehri 2016). Therefore, the improved membrane integrity observed in Az- and biochar-treated plants likely resulted from reduced oxidative damage and the promotion of protective mechanisms against ROS.

Consistent with the observed reduction in membrane damage, Az inoculation combined with biochar application also significantly reduced the accumulation of $O_2^{\bullet-}$ and H_2O_2 under salinity stress (Fig. 2a, b). This finding is supported by previous studies demonstrating the capacity of biochar and/or microbial inoculants to decrease ROS levels in plants exposed to salt stress (Hashem et al. 2018; Arora et al. 2020). The observed reduction in $O_2^{\bullet-}$ and H_2O_2 likely

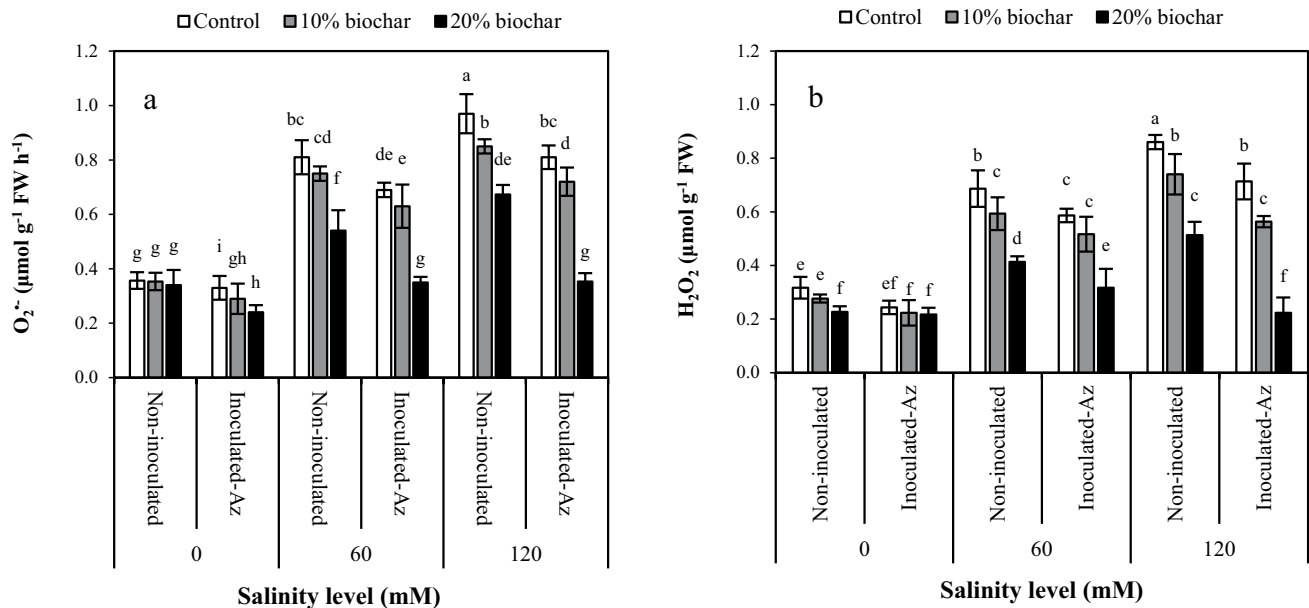


Fig. 2 Effect of Az and biochar application on oxygen radicals ($O_2^{\bullet-}$) content (a) and hydrogen peroxide (H_2O_2) content (b) in rapeseed leaves under varying salinity stress levels. Treatments included three NaCl concentrations (0, 60, and 120 mM), two inoculation statuses

(non-inoculated and inoculated-Az), and three biochar rates (control, 10%, and 20%). Values represent the mean of three replicates $\pm$ standard deviation (SD). Means within each panel sharing the same letter are not significantly different according to the LSD test ($p \leq 0.05$)

resulted from several contributing factors identified in this study, including the significantly enhanced activities of antioxidant enzymes (CAT, SOD, POX, APX) responsible for ROS detoxification (Fig. 3a–d). Furthermore, alterations in osmolyte levels (Table 3) and improved physiological status (higher RWC and Fv/Fm, Table 2) may have contributed to a better-balanced redox state and reduced ROS production. Collectively, these responses highlight a multi-faceted strategy by which combined Az and biochar application enhances rapeseed resilience to salinity-induced oxidative damage, offering potential practical applications in agriculture.

Modulation of antioxidant enzyme, osmolytes, and DPPH radical scavenging activity by azotobacter and biochar under salinity

Salinity stress triggered significant increases in the activities of key antioxidant enzymes—CAT, SOD, POX, and APX—in rapeseed leaves (Fig. 3a–d). This induction of enzymatic defenses represents a common adaptive plant response to combat oxidative stress under saline conditions, as documented across various species including soybean, safflower, and sesame (Gengmao et al. 2015; Khademian et al. 2019; Yaghoubian et al. 2021; Farhangi-Abriz and Ghassemi-Golezani 2018; Sharaya et al. 2023; Kanwal et al. 2024). While Az inoculation alone did not significantly alter CAT and POX activities under non-saline conditions, the application of 20% biochar did increase non-saline APX and SOD activities (Fig. 3b, d). This suggests biochar might improve

plant antioxidant status even without overt stress, possibly through enhanced nutrient availability or improved soil health (Jin et al. 2024).

Critically, the combination of Az inoculation and 20% biochar application yielded the highest activities for all four enzymes under salinity stress (60 and 120 mM NaCl) (Fig. 3a–d). This indicates a synergistic interaction enhancing the plant's enzymatic defense capacity beyond the levels induced by salinity alone. Such microbially-mediated enhancement of antioxidant systems under stress has been observed with other beneficial microbes, for instance, *Piriformospora indica* in mustard (Sharaya et al. 2023). It is proposed that the substantially elevated enzyme activities in the Az + biochar-treated plants played a key role in scavenging excess ROS ($O_2^{\bullet-}$ and H_2O_2) observed under salinity (Fig. 2a, b), thereby directly contributing to reduced MDA and EL (Table 2) and ultimately improving salt tolerance (Sarker and Oba 2020).

In response to increasing salinity, significant accumulation of compatible solutes—including soluble proteins (SP), glycine betaine (GB), soluble carbohydrates (SC), and proline—was observed, along with increased DPPH radical scavenging activity (Table 3). The accumulation of such osmolytes is a fundamental strategy plants employ to maintain osmotic balance, protect cellular structures and proteins, and potentially scavenge ROS under stress conditions (Sarkar et al. 2023; Wu et al. 2014). Proline, in particular, functions as a key osmoprotectant rapidly accumulated under osmotic stress (Verbruggen and Hermans 2008;

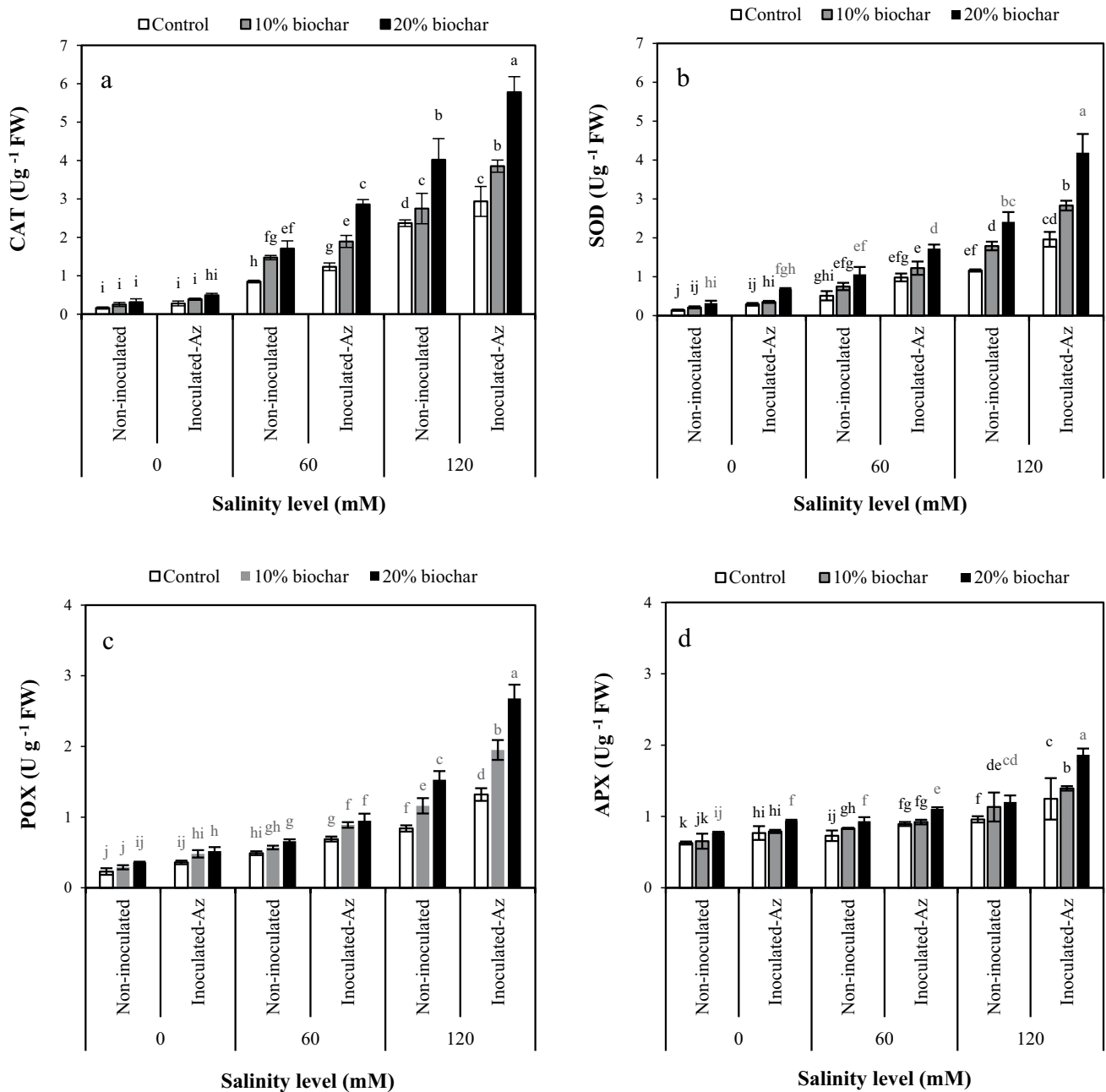


Fig. 3 Effect of Az and biochar application on catalase (CAT) (a), superoxide dismutase (SOD) (b), peroxidase (POX) (c), and ascorbate peroxidase (APX) (d) activity in rapeseed leaves under varying levels of salinity stress. Treatments included three NaCl concentrations (0, 60, and 120 mM), two inoculation statuses (non-inoculated and inocu-

lated-Az), and three biochar rates (control, 10%, and 20%). Values represent the mean of three replicates $\pm$ standard deviation (SD). Means within each panel sharing the same letter are not significantly different, according to the LSD test ($p \leq 0.05$)

Anwar Hossain et al. 2014). The observed increase in osmolyte levels under salinity is consistent with reports in other species, such as bean (Farhangi-Abriz and Torabian 2017).

Importantly, Az inoculation and biochar application further augmented the accumulation of these osmolytes and enhanced DPPH scavenging activity, especially under saline conditions, compared to untreated stressed plants (Table 3). The most substantial increases were consistently found

with the combined Az+20% biochar treatment, particularly under high salinity (120 mM NaCl). This synergistic effect suggests that Az and biochar together bolster the plant's capacity for both osmotic adjustment and non-enzymatic antioxidant defense. The enhancement of osmolyte accumulation, such as proline, by microbial inoculation under salinity has been previously reported (Zarea et al. 2012). This likely arises from a combination of factors, including

Table 3 Effect of Az inoculation and Biochar application on soluble proteins (SP), Glycine betaine (GB), soluble carbohydrates (SC), proline, and DPPH radical scavenging activity in rapeseeds under varying salinity levels

Salt (mM)	Az	Biochar	SP (mg g ⁻¹ DW)	GB (mg g ⁻¹ FW)	SC (mg g ⁻¹ FW)	Proline (mg g ⁻¹ FW)	DPPH (%)
0	Non-inoculated	Control	2.08±0.03 h	4.31±0.18 j	2.74±0.18 j	9.47±1.06 j	28.06±1.99 i
		10% biochar	2.15±0.09 gh	4.96±0.36 ij	3.69±0.23 ij	13.58±0.91 i	29.58±1.01 hi
		20% biochar	2.31±0.04 gh	6.28±0.21 h	5.73±0.09 gh	15.64±0.21 i	31.43±1.43 gh
	Inoculated-Az	Control	2.15±0.08 gh	5.89±0.20 hi	4.49±0.15 hi	14.57±0.85 i	30.11±1.07 hi
		10% biochar	2.34±0.07 gh	6.71±0.49 h	6.18±0.11 g	25.62±1.42 f	32.82±0.49 gh
		20% biochar	2.47±0.26 gh	8.83±0.68 fg	8.81±0.013ed	22.45±1.65 g	34.76±1.19 ef
60	Non-inoculated	Control	2.36±0.26 gh	6.93±0.34 h	5.31±0.24gh	18.08±1.93 h	33.12±0.56 gh
		10% biochar	2.48±0.03 gh	8.37±0.36 g	6.38±0.20 fg	24.75±1.81 fg	36.09±1.78 ef
		20% biochar	2.65±0.12 ef	10.73±0.17 e	8.03±0.41e	28.37±1.22 e	40.36±0.98 d
	Inoculated-Az	Control	2.59±0.11 fg	9.24±0.99 fg	7.73±0.17ef	24.51±0.55 fg	36.24±1.37 e
		10% biochar	2.76±0.17 ef	11.15±0.92 e	9.68±0.29d	33.06±1.38 d	39.42±4.19 d
		20% biochar	2.94±0.09 de	14.61±0.72 c	13.11±0.45c	37.66±2.05 c	45.24±0.79 c
120	Non-inoculated	Control	3.21±0.09 d	10.08±1.13 e	8.66±1.38ed	23.18±0.54 g	41.71±1.16 d
		10% biochar	3.75±0.31 c	13.27±0.22 d	12.94±0.64c	34.48±0.55 d	45.68±0.68 c
		20% biochar	4.49±0.54 b	17.25±0.74 b	15.63±0.52b	35.06±2.91 d	48.35±2.24 c
	Inoculated-Az	Control	4.08±0.56 c	13.04±0.44 d	14.39±1.72 bc	32.85±3.31 d	45.83±3.18 c
		10% biochar	4.66±0.24 b	17.43±0.97 b	15.82±1.17b	45.64±3.43 d	52.74±1.42 b
		20% biochar	5.87±0.17 a	24.41±1.77 a	27.82±1.36a	51.63±1.53 a	57.74±1.69 a

Treatments included three NaCl concentrations (0, 60, and 120 mM), two inoculation statuses (non-inoculated and inoculated-Az), and three biochar rates (control, 10%, and 20%). Values represent the mean of three replicates±standard deviation (SD). Means within each column followed by the same letter are not significantly different according to LSD test ($p \leq 0.05$)

improved nutrient assimilation, hormonal signaling influenced by Az, and the stress-alleviating effects of biochar on soil properties, allowing greater metabolic investment in these protective compounds. Collectively, the enhanced osmolyte accumulation and radical scavenging capacity contribute significantly to the overall resilience of Az- and biochar-treated rapeseed plants under saline conditions.

Modulation of soluble sugar, fatty acid composition, and unsaturation index (UI) by azotobacter and biochar salinity

The significant increase observed in this study in specific soluble sugars – glucose, mannose, and xylose – particularly under high salinity (120 mM NaCl) (Fig. 4a–c), represents an adaptive response by rapeseed. The accumulation of soluble sugars is a well-known mechanism for osmotic adjustment, helping plants maintain cellular turgor and protect structures from dehydration under osmotic stress. The observed increase in soluble sugar content under salinity is consistent with previous reports documenting similar responses in rapeseed (Khattab 2007) and other species such as bean (Farhangi-Abriz and Torabian 2017). Furthermore, the observed enhancement of glucose, mannose, and xylose accumulation in plants treated with Az and biochar, especially under stress (Fig. 4a–c), suggests these treatments improve the plant's capacity for osmotic adjustment. This improvement could potentially stem from multiple factors

induced by the treatments, such as enhanced photosynthetic efficiency providing more carbon precursors, altered carbon partitioning towards soluble sugar synthesis, or improved nutrient uptake facilitating metabolic processes.

Salinity stress significantly altered the fatty acid profile of rapeseed oil (Table 4). Under high salinity (120 mM NaCl), particularly in control plants lacking Az or biochar, a shift towards higher proportions of saturated (palmitic and stearic) and monounsaturated (oleic) fatty acids was observed, concurrent with a decrease in polyunsaturated fatty acids (linoleic and linolenic). This resulted in a lower UI under high stress. Such changes may reflect an adaptive mechanism to maintain membrane integrity under salinity, although a decrease in polyunsaturated fatty acids is often considered detrimental to the nutritional quality of the oil. The observed increase in oleic acid and decrease in linoleic and linolenic acids under salinity partially align with findings reported for soybean (Ghassemi-Golezani and Farhangi-Abriz 2018). These shifts are potentially linked to altered activity of fatty acid desaturase enzymes, which can be inhibited by salinity-induced ion toxicity or metabolic disruptions (Hernández et al. 2011).

In contrast, under non-saline conditions, the combination of Az inoculation and 20% biochar promoted a favorable shift in the fatty acid profile, significantly increasing the relative content of linoleic and linolenic acids while decreasing oleic, stearic, and palmitic acids (Table 4). Consequently, the highest UI value (1.24) was recorded under

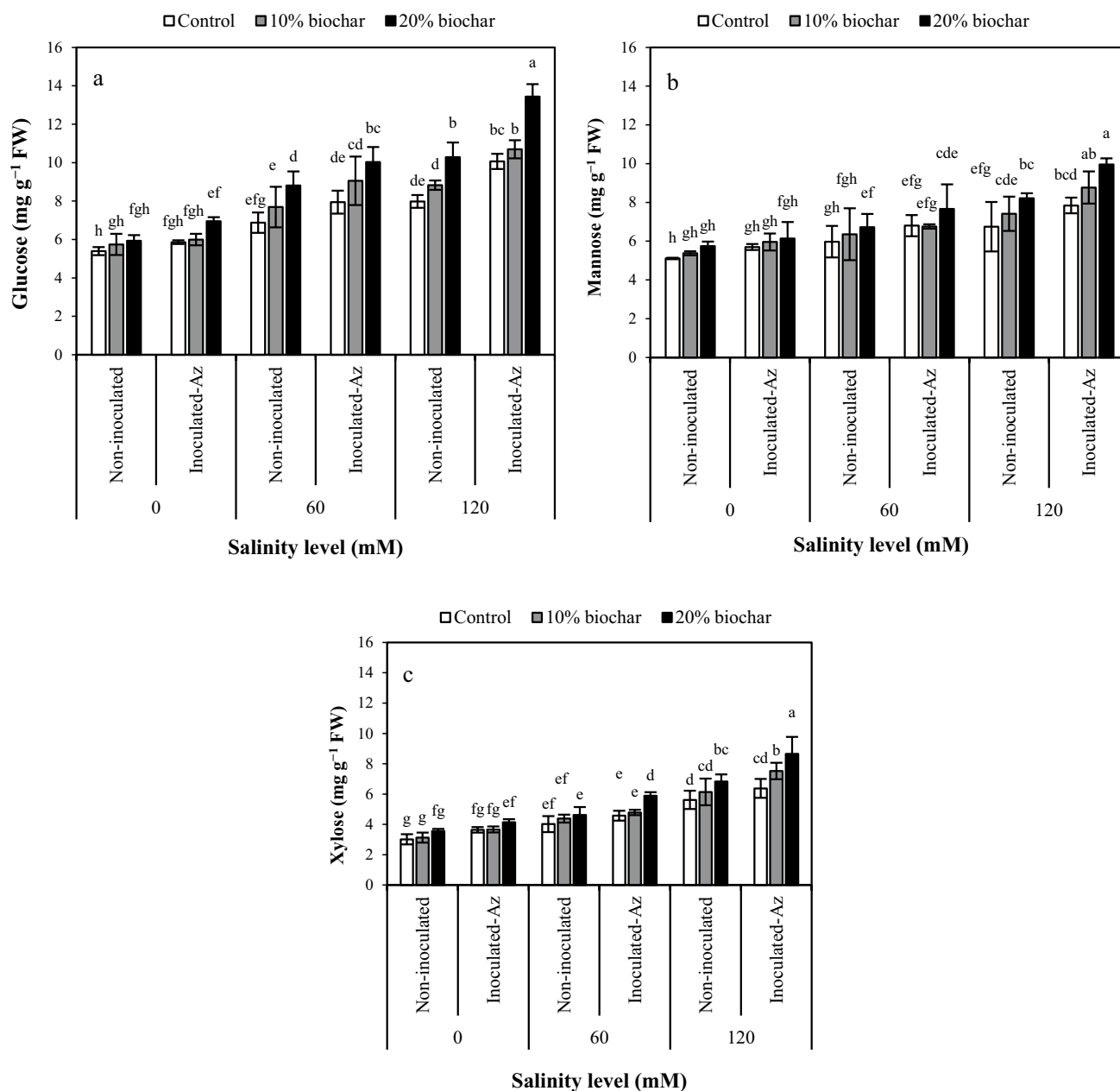


Fig. 4 Effect of Az and biochar application on glucose content (a), mannose content (b), and xylose content (c) in rapeseed leaves under varying levels of salinity stress. Treatments included three NaCl concentrations (0, 60, and 120 mM), two inoculation statuses (non-inoc-

ulated and inoculated-Az), and three biochar rates (control, 10%, and 20%). Values represent the mean of three replicates $\pm$ standard deviation (SD). Means within each panel sharing the same letter are not significantly different, according to the LSD test ($p \leq 0.05$)

these optimal conditions (observed in several treatments including those receiving Az and 20% biochar treatment). This suggests that Az and biochar can enhance pathways leading to unsaturated fatty acid synthesis under non-stress conditions, potentially improving oil quality. Enhanced fatty acid desaturation mediated by beneficial microbes has been reported previously (*P. indica* in barley; Baltruschat et al. 2008). However, while significant differences in major fatty acid contents were observed, the effects of Az or

biochar on the overall fatty acid profile and UI were not significant at moderate salinity (60 mM NaCl). These results highlight that the efficacy of these treatments in modulating lipid metabolism can be context-dependent and may interact complexly with the level of abiotic stress. These results underscore the potential of Az inoculation and biochar application to influence rapeseed oil quality, particularly by enhancing unsaturation under non-saline conditions, while

Table 4 Effect of Az and Biochar application on palmitic acid, stearic acid, oleic acid, Linoleic acid, linolenic acid, and unsaturation index (UI) in rapeseeds under varying salinity levels

Salt (mM)	Az	Biochar	Palmitic Acid	Stearic Acid	Oleic Acid %	Linoleic Acid	Linolenic Acid	UI
0	Non-inoculated	Control	4.17±0.19 cde	1.78±0.03 cde	59.65±0.40 de	18.18±0.69 cd	8.39±0.27 bcd	1.21±0.02 ab
		10% biochar	4.12±0.31 cde	1.75±0.15 cde	59.33±0.43 de	18.85±0.55 bc	8.47±0.85 bc	1.24±0.03 a
		20% biochar	3.99±0.30 cde	1.69±0.04 de	59.07±1.72 e	19.42±0.63 bc	8.65±0.35 bc	1.24±0.03 a
	Inoculated-Az	Control	4.03±0.18 cde	1.72±0.20 cde	58.83±2.01 f	19.01±1.96 bc	8.51±0.34 bc	1.22±0.04 ab
		10% biochar	3.98±0.06 cde	1.65±0.08 e	59.05±0.85 ef	19.71±0.31 b	8.89±0.11 b	1.23±0.01 ab
		20% biochar	3.67±0.23 e	1.60±0.06 e	58.79±0.36 f	19.43±0.35 a	8.87±0.59±a	1.24±0.01 a
60	Non-inoculated	Control	4.15±0.7 cde	1.99±0.11 bcd	62.77±1.11 b	17.12±1.72 cd	8.01±0.12 bcd	1.21±0.03 ab
		10% biochar	4.27±0.22 cd	1.9±0.05 cde	61.45±1.45 bc	17.79±0.76 bcd	8.21±0.42 bcd	1.21±0.04 ab
		20% biochar	4.03±0.25 cde	1.75±0.15 cde	59.9±2.21 cde	18.99±0.23 bc	8.56±0.20 bc	1.23±0.03 ab
	Inoculated-Az	Control	4.28±0.07 cd	1.78±0.17 cde	59.24±0.58 bc	18.66±1.36 bc	8.41±0.23 bc	1.21±0.02 ab
		10% biochar	4.01±0.12 cde	1.71±0.27 cde	58.96±0.83 cd	18.92±0.32 bc	8.50±0.82 bc	1.22±0.04 ab
		20% biochar	3.72±0.17 e	1.65±0.3 e	58.87±1.09 cde	19.21±1.55 bc	8.77±0.10 bc	1.23±0.02 ab
120	Non-inoculated	Control	5.07±0.17 a	2.46±0.26 a	67.63±1.04 a	14.22±0.50 e	7.19±0.42 de	1.17±0.03 b
		10% biochar	4.85±0.09 ab	2.16±0.17 b	63.94±1.81 bc	16.08±1.39 d	7.51±0.28 cde	1.19±0.02 ab
		20% biochar	4.39±0.21 bcd	2.02±0.21 bc	60.68±2.97 cde	17.25±0.62 bcd	7.87±0.14 cde	1.19±0.03 ad
	Inoculated-Az	Control	4.51±0.13 bc	1.89±0.21 cde	62.82±0.82 cd	17.14±0.45 cd	8.12±0.13 bcd	1.21±0.02 ab
		10% biochar	4.42±0.64 bc	1.83±0.07 cde	59.86±2.17 de	18.26±0.88 bcd	8.19±0.24 bcd	1.21±0.01 ab
		20% biochar	3.87±0.18 de	1.66±0.2 e	59.69±0.86 de	19.24±1.70 bc	8.64±0.42 bc	1.24±0.05 a

Treatments included three NaCl concentrations (0, 60, and 120 mM), two inoculation statuses (non-inoculated and inoculated-Az), and three biochar rates (control, 10%, and 20%). Values represent the mean of three replicates±standard deviation (SD). Means within each column followed by the same letter are not significantly different according to LSD test ($p \leq 0.05$)

also revealing the complex lipid metabolic responses under varying salinity levels.

Influence of azotobacter and biochar on rapeseed oil content, grain yield, and oil yield under salinity

While salinity level alone did not significantly affect seed oil content (Fig. 5a), the combination of Az inoculation and 20% biochar resulted in the highest oil content (47.47%), which was observed under high salinity stress (120 mM NaCl). This suggests that these treatments can enhance oil accumulation processes even under adverse conditions, complementing the potential modifications to oil quality (fatty acid composition) discussed previously. As expected, salinity stress significantly reduced both grain and oil yields (Fig. 5b, c). This reduction is likely attributable to the detrimental effects of high ion concentrations on plant metabolism, enzyme function, and overall growth, which can disrupt ion homeostasis and critical metabolic processes (Medhat 2002). Importantly, biochar application and Az inoculation significantly mitigated these yield losses under salinity and also enhanced yields under non-saline conditions compared to untreated controls (Fig. 5b, c). The yield improvements conferred by biochar can be linked to enhanced soil physical and chemical properties, potential reduction in ROS exposure, and overall stress alleviation, contributing to increased crop productivity even under

salinity, as supported by previous work (Graber et al. 2010; Thomas et al. 2013; Phuong et al. 2020).

Crucially, the higher grain and oil yields observed in treated plants strongly correlated with the improved Fv/Fm (higher Fv/Fm values), as documented earlier (Table 2). This internal consistency demonstrates that the enhanced physiological resilience fostered by Az and biochar, reflected in better photosynthetic performance, directly translated into improved yield outcomes. The relative yield increase due to these treatments was most substantial under non-saline and moderate (60 mM NaCl) stress conditions.

Collectively, these findings demonstrate that the co-application of Az and biochar provides a multi-faceted defense strategy against salinity stress in rapeseed. The individual benefits of each amendment are likely amplified through a synergistic interaction, which may be driven by biochar improving the soil micro-environment and potentially acting as a protective carrier for the microbial inoculant, thereby enhancing its survival and efficacy (Bolan et al. 2023; Sharma et al. 2025). While we did not directly measure soil structure, nitrogen fixation, or phytohormone production, these mechanisms are supported by previous studies and may contribute to the observed improvements in plant performance (Graber et al. 2010; García et al. 2017; Xu et al. 2023). The combined effects of these amendments led to improved physiological status, enhanced antioxidant capacity, and better osmotic adjustment, which ultimately translated to higher grain and oil yields under stress. A

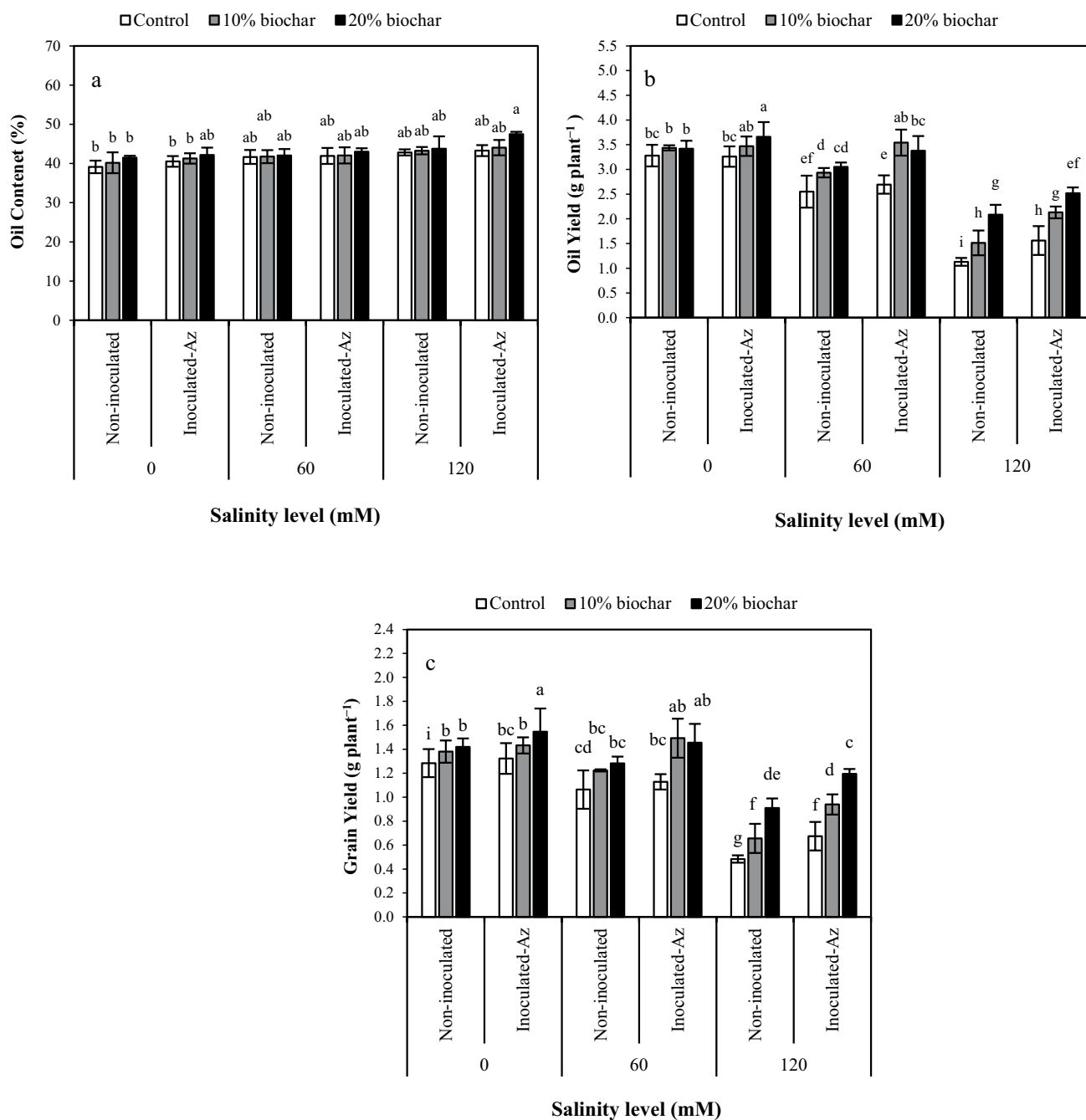


Fig. 5 Effect of Az and biochar application on oil content (a), oil yield (b), and grain yield (c) in rapeseed under varying levels of salinity stress. Treatments included three NaCl concentrations (0, 60, and 120 mM), two inoculation statuses (non-inoculated and inoculated-Az),

and three biochar rates (control, 10%, and 20%). Values represent the mean of three replicates $\pm$ standard deviation (SD). Means within each panel sharing the same letter are not significantly different, according to the LSD test ($p \leq 0.05$)

summary of these proposed, literature-supported mechanistic pathways is illustrated in Fig. 6. Further research focusing on the precise physiological and molecular mechanisms underpinning the observed synergistic effects on stress tolerance could yield valuable insights for developing integrated management practices and more resilient crop varieties for challenging agricultural settings.

Conclusions

This study demonstrated that while salinity stress severely impaired the physiological performance, yield components, and oil content of rapeseed, the combined application of the plant growth-promoting rhizobacterium Az and biochar served as an effective mitigation strategy. The novelty of

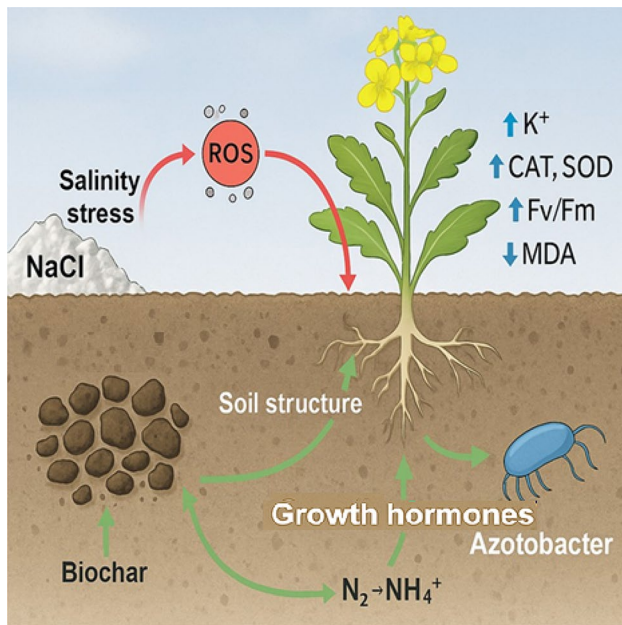


Fig. 6 Schematic representation of the proposed mechanisms by which biochar and *Azotobacter chroococcum* (Az) mitigate salinity stress and enhance rapeseed growth, yield, and oil content. Salinity stress induces both ionic and osmotic stress, leading to increased reactive oxygen species (ROS) production and cellular damage. Biochar application improves soil structure, water retention, and nutrient availability, while Az inoculation promotes plant growth via phytohormone production and nitrogen fixation. The synergistic interaction between biochar and Az—where biochar acts as a protective carrier—further enhances microbial survival and activity. Together, these treatments improve plant physiological resilience by enhancing ion homeostasis, increasing antioxidant enzyme activity, and promoting osmolyte accumulation, which collectively reduce oxidative damage and result in improved rapeseed growth, yield, and oil content under saline conditions (Sharma et al. 2025; Coppa et al. 2024; Jin et al. 2024; Xu et al. 2023; Yaghoubian et al. 2021; García et al. 2017; Farhangi-Abraz and Torabian 2017)

this work lies in demonstrating the synergistic potential of this specific microbe-soil amendment combination to bolster rapeseed resilience against salinity, primarily through enhanced photosynthetic efficiency (Fv/Fm, ATP), improved water status (RWC), activation of antioxidant defense systems (CAT, SOD, POX, APX), and beneficial modulation of osmolyte accumulation and fatty acid profiles. Notably, the 20% biochar rate combined with Az inoculation consistently provided the most substantial improvements. From a practical standpoint, these findings suggest that co-applying Az inoculants with biochar could represent a viable and sustainable agronomic practice for enhancing rapeseed cultivation and productivity in salt-affected agricultural regions, potentially reducing reliance on conventional inputs and contributing to soil health.

However, several limitations should be acknowledged. One key limitation is that this study did not directly assess microbial colonization or the specific interactions between

biochar and Az in the rhizosphere. While the results suggest a synergistic effect based on plant growth responses, direct evidence of colonization and mechanistic interactions is lacking. This limitation should be considered when interpreting the observed benefits and underscores the need for future research to include microbial colonization assays and detailed interaction studies. Additionally, this research was conducted under controlled greenhouse conditions with a specific soil type, and field validation is crucial to confirm the efficacy of this approach under variable environmental conditions and diverse agricultural systems. Future research should therefore prioritize field trials across different rapeseed cultivars and soil types to assess the long-term benefits and consistency of the combined treatment. Further investigations into the underlying molecular mechanisms driving the observed synergistic effects, along with economic analyses evaluating the cost-effectiveness of biochar and inoculant application, will be essential for facilitating the practical adoption of this promising strategy for sustainable agriculture in challenging environments.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Conflict of interest The authors have no conflict of interest.

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