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Research Article

Keywords: Antioxidant enzymes, Brassica juncea, Chlorophyll fluorescence, Photosynthesis, Reactive oxygen species, Stress tolerance

Posted Date: November 25th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11765644/v1

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SALICYLIC ACID- MEDIATED REGULATION OF GROWTH, PHYSIOLOGY, AND STRESS RESPONSES IN INDIAN MUSTARD UNDER ARSENIC STRESS

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Key message: The foliar spray of salicylic acid mitigates the stress generated by arsenic in mustard by modulating the photosynthetic characteristics and antioxidant enzymes.

Abstract

Arsenic (As) contamination is a major environmental problem that adversely affects plant growth, physiological processes, and crop productivity by inducing oxidative stress. The present study investigated the role of salicylic acid (SA) in mitigating As-induced toxicity in Indian mustard (*Brassica juncea* L.). Arsenic stress caused a dose-dependent decline in shoot and root growth, biomass accumulation, leaf area, SPAD chlorophyll value, photosynthetic rate, stomatal conductance, transpiration rate, intercellular CO_2 concentration, chlorophyll fluorescence parameters, and the activities of carbonic anhydrase and nitrate reductase. Exposure to As also increased the accumulation of superoxide radicals ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2), resulting in oxidative stress, stomatal abnormalities, nutrient imbalance, and cellular damage, as confirmed by histochemical staining, scanning electron microscopy, energy-dispersive X-ray analysis, and confocal microscopy. Although antioxidant enzyme activities and proline content increased under As stress as adaptive responses, these were insufficient to prevent oxidative injury. Foliar application of SA significantly alleviated the detrimental effects of As by improving growth, photosynthetic performance, chlorophyll fluorescence, nutrient status, and enzyme activities while reducing ROS accumulation and cellular damage. SA further enhanced antioxidant defense and proline accumulation, thereby strengthening stress tolerance. The ameliorative effects of SA were most pronounced under moderate As stress (25 and 50 mg kg^{-1} soil). Overall, SA improved arsenic tolerance in *B. juncea* by maintaining photosynthetic efficiency, enhancing antioxidant and osmoprotective responses, and reducing ROS-mediated cellular injury.

Keywords: Antioxidant enzymes; *Brassica juncea*; Chlorophyll fluorescence; Photosynthesis; Reactive oxygen species; Stress tolerance.

1. Introduction

Indian mustard (*Brassica juncea* L. Czern. & Coss.) is an important oilseed and leafy vegetable crop widely cultivated for its nutritional and economic value. It contributes significantly to

food security and agricultural income, particularly in South Asian countries. However, its productivity is adversely affected by various environmental stresses, including contamination of agricultural soils with toxic metals and metalloids. Among these contaminants, arsenic (As) is considered one of the most hazardous environmental pollutants due to its high toxicity, persistence, and widespread occurrence in soil and water systems (Ahmad et al., 2021; Darani et al., 2022). Arsenic contamination originates from both natural and anthropogenic activities and poses serious risks to agricultural sustainability, food safety, and human health. The problem is particularly severe in several regions of India where elevated arsenic levels have been reported in groundwater and agricultural soils (Marghade et al., 2022).

In the environment, arsenic occurs mainly as arsenate [As(V)] and arsenite [As(III)], with toxicity largely dependent on its chemical form and bioavailability. Plants absorb As(V) via phosphate transporters due to its structural similarity to phosphate, whereas As(III) is taken up through aquaporin channels (Geng et al., 2024). Once absorbed, arsenic interferes with numerous physiological and biochemical processes, resulting in impaired growth and development.

Arsenic toxicity suppresses seed germination, inhibits root and shoot growth, reduces leaf area and biomass accumulation, and ultimately decreases crop productivity (Sinha et al., 2023). At the cellular level, arsenic induces excessive production of reactive oxygen species (ROS), including superoxide radicals and hydrogen peroxide, leading to oxidative stress. The resulting oxidative damage affects membrane integrity, proteins, nucleic acids, and photosynthetic machinery, thereby disrupting normal plant metabolism (Hasanuzzaman et al., 2020; Nahar et al., 2022). Photosynthesis is particularly sensitive to arsenic stress, with reductions in chlorophyll content, photosystem II efficiency, and carbon assimilation frequently reported in arsenic-exposed plants.

Plants possess antioxidant defense systems to detoxify ROS and maintain cellular redox balance. However, under severe arsenic stress, these endogenous defense mechanisms are often insufficient to prevent oxidative damage. Therefore, the use of stress-mitigating compounds has emerged as an effective strategy for improving plant tolerance to heavy-metal and metalloid toxicity.

Salicylic acid (SA) is an important plant signaling molecule involved in the regulation of growth, development, and stress responses. In addition to its role in plant defense, SA has been shown to enhance tolerance to various abiotic stresses by improving antioxidant capacity, maintaining photosynthetic performance, and regulating cellular redox homeostasis (Hayat et al., 2007; Sharma et al., 2020). Recent studies have demonstrated that exogenous application of SA can alleviate arsenic-induced toxicity and improve plant growth under contaminated conditions (Bano et al., 2022).

Although SA-mediated protection against metal/metalloid stress has been reported, integrated evidence linking growth, photosynthetic gas exchange, PSII photochemistry, ROS localization, SEM-EDX-based structural/nutrient changes, and root cellular damage in As-stressed *B. juncea* remains limited. Therefore, the present study was designed to elucidate the potential of salicylic acid in alleviating arsenic-induced toxicity in Indian mustard (*Brassica juncea*), with particular emphasis on growth, photosynthetic performance, oxidative stress, antioxidant defense mechanisms, and cellular and ultrastructural responses.

2. Materials and Methods

2.1 Plant Material and Growth Conditions

The experiment was conducted in the net house of the Department of Botany, Aligarh Muslim University, Aligarh, India. Seeds of *Brassica juncea* L. cv. Varuna was obtained from the Indian Agricultural Research Institute (IARI), New Delhi, India. Healthy, uniform seeds were surface-sterilized and sown in earthen pots.

2.2 Chemicals and Soil Analysis

Sodium arsenate ($Na_3AsO_4 \cdot 7H_2O$) was used as the source of arsenic (As). Salicylic acid (SA) was procured from Sigma-Aldrich (India) and prepared as a 10^{-5} M solution by dissolving the required amount in ethanol, then diluting with double-distilled water. Tween-20 (0.05%, v/v) was added as a surfactant. The SA concentration was selected based on previous studies (Yusuf et al., 2012). Before sowing, the soil was analyzed at the Soil Testing Laboratory, Government Agriculture Farm, Aligarh, India. The soil was sandy loam with a pH of 7.2 and 1.58% organic matter. Available N, P, and K contents were 48.51, 41.60, and 142 mg kg^{-1} soil, respectively. No detectable arsenic was present in the soil.

2.3 Experimental Design and Treatments

The experiment was conducted in 10-inch earthen pots containing sandy loam soil and farmyard manure at a 1:1 (w/w) ratio. Treatments were arranged in a completely randomized design (CRD) with eight treatments and four replicates per treatment. Ten seeds were sown per pot and later thinned to five uniform plants. The treatments were: water (T0), As25 (T1), As50 (T2), As75 (T3), 10^{-5} M SA (T4), As25 + 10^{-5} M SA (T5), As50 + 10^{-5} M SA (T6), and As75 + 10^{-5} M SA (T7). Arsenic was applied to the soil at 10 days after germination (DAG), whereas 50 ml of SA was applied as a foliar spray from 25 to 29 DAG. These plants were irrigated regularly to maintain adequate soil moisture.

2.4 Growth Measurements

At harvest, plants were carefully uprooted and washed thoroughly. Shoot length, root length, fresh mass, and dry mass were recorded. Dry mass was determined after oven-drying at 80°C until a constant weight was reached. Leaf area was measured using a portable leaf area meter (ADC Bioscientific, UK).

2.5 Physiological Measurements

Leaf chlorophyll content was estimated using a SPAD chlorophyll meter (SPAD-502, Konica Minolta, Japan). Gas-exchange parameters, including net photosynthetic rate (Pn), stomatal conductance (gs), transpiration rate (E), and intercellular CO_2 concentration (Ci), were measured using a portable infrared gas analyzer (LI-6400, LI-COR, USA).

Chlorophyll fluorescence parameters were determined using a PAM fluorometer (PAM-200, Walz, Germany) after 30 min of dark adaptation. Minimum fluorescence (Fo), maximum fluorescence (Fm), maximum quantum yield of PSII (Fv/Fm), effective quantum yield of PSII (ΦPSII), photochemical quenching (qP), non-photochemical quenching (NPQ), and electron transport rate (ETR) were recorded. ETR was calculated as:

$$ETR = \Phi PSII \times PAR \times 0.5 \times 0.84$$

2.6 Biochemical Analyses

Carbonic anhydrase (CA) and nitrate reductase (NR) activities were assayed following the methods of Dwivedi and Randhawa (1974) and Jaworski (1971), respectively. Superoxide radical ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) contents were determined according to Wu et al. (2010) and Patterson et al. (1984), respectively. For antioxidant enzyme analysis, fresh leaf tissue was homogenized in an extraction buffer containing phosphate buffer, EDTA, PMSF, Triton X-100, and polyvinylpyrrolidone. The homogenate was centrifuged, and the supernatant was used for enzyme assays. Activities of superoxide dismutase (SOD), catalase (CAT), and peroxidase (POX) were determined following the methods of Beauchamp and Fridovich (1971), Aebi (1984), and Chance and Maehly (1955), respectively.

2.7 Histochemical Detection of Reactive Oxygen Species

In situ localization of $O_2^{\bullet -}$ and H_2O_2 was performed using nitroblue tetrazolium (NBT) and 3,3′-diaminobenzidine (DAB) staining, respectively, following Kaur et al. (2016). Stained leaves were decolorized in ethanol and photographed to visualize ROS accumulation.

2.8 Microscopic Analyses

Stomatal morphology and elemental composition of leaves were examined using scanning electron microscopy coupled with energy dispersive X-ray spectroscopy (SEM–EDX). Root cellular damage was assessed using confocal laser scanning microscopy after staining with propidium iodide (PI). All microscopic analyses were performed at the University Sophisticated Instrumentation Facility (USIF), Aligarh Muslim University, India.

2.9 Statistical Analysis

Data were obtained from four biological replicates per treatment. Normality and homogeneity of variance were checked using Shapiro–Wilk and Levene's tests, respectively. One-way ANOVA was performed, followed by Tukey's HSD multiple comparison test at $p \leq 0.05$. Different lowercase letters above bars indicate statistically significant differences among treatments. Error bars represent ± SE

3. RESULTS

3.1 Growth Characteristics

The effect of As and SA on plant growth parameters is presented in Figs. 1- 4. As stress caused a dose-dependent decline in plant growth compared with the control. The greatest reductions in shoot and root length (33.5% and 37.2%, respectively) were recorded at T3 (75 mg kg^{-1}). Biomass production was also adversely affected under As stress. The largest decreases were observed at T3, with reductions of 40.4% and 44.3% in shoot and root fresh mass, and 37.5% and 47% in shoot and root dry mass, respectively. Foliar application of SA mitigated the inhibitory effects of As stress, with the most pronounced recovery observed at T5. Under this treatment, shoot and root length increased by 19.5% and 35%, respectively, and fresh mass by 21% and 28%, respectively, and dry mass by 36.8% and 34%, respectively, compared with the corresponding stressed plants.

3.2 Leaf Area and SPAD Value

Arsenic application significantly reduced leaf area (LA) and SPAD values compared with the control (Fig. 5). The greatest reductions were observed at T3, where LA and SPAD decreased by 37% and 36.1%, respectively. In contrast, foliar application of SA alone enhanced LA and SPAD by 22.1% and 12.8%, respectively. Under As stress, the SA application alleviated the reduction in these parameters, with the greatest improvements observed at T5, where LA and SPAD increased by 20.3% and 12%, respectively, relative to the corresponding stressed plants.

3.3 Photosynthetic Gas Exchange Parameters

As stress significantly reduced net photosynthetic rate (Pn) across all treatments compared with the control. The largest decline (37%) was recorded at T3. Foliar supplementation with SA improved Pn, with the greatest increase (38.2%) observed at T5 relative to the corresponding As-stressed treatment. Similarly, stomatal conductance (gs), transpiration rate (E), and intercellular CO_2 concentration (Ci) declined under As stress. The maximum reductions of 35.7%, 36.3%, and 33.1%, respectively, were observed at T3. Application of SA significantly restored gs, E, and Ci, with the greatest improvements observed at T5, increasing 16%, 18.2%, and 15%, respectively, compared with the corresponding stressed plants (Fig. 6).

3.4 Chlorophyll Fluorescence Parameters

As stress adversely affected chlorophyll fluorescence parameters, including Fv/Fm, ΦPSII, qP, and ETR (Fig. 7). The largest reductions (21.1%, 35.3%, 37.0%, and 35.4%, respectively) were observed at T3 compared with the control. In contrast, non-photochemical quenching (NPQ) increased significantly under As stress, with the highest increase (36.9%) observed at T3. Foliar application of SA partially alleviated As-induced photoinhibition, with the greatest improvements recorded at T5. Under this treatment, Fv/Fm, ΦPSII, qP, and ETR increased by 4.6%, 4.5%, 3.1%, and 4.6%, respectively, relative to the corresponding stressed treatments. Additionally, NPQ decreased by 14% at T5, indicating partial recovery of photosystem II efficiency.

3.5 Enzymatic Activities of CA and NR

Activities of carbonic anhydrase (CA) and nitrate reductase (NR) declined markedly under As stress compared with the control (Fig. 8). The greatest reductions, 44.5% and 25.8%, respectively, were observed at T3. Application of SA significantly improved these enzyme activities in As-stressed plants. The highest recovery was observed at T5, where CA and NR activities increased by 39.9% and 23.9%, respectively, compared with the corresponding stressed treatments.

3.6 Oxidative Stress and Histochemical Staining of $O_2^{\bullet-}$ and H_2O_2

Exposure to As caused a progressive increase in reactive oxygen species (ROS) accumulation. The highest increases in $O_2^{\bullet-}$ and H_2O_2 (82.5% and 68.4%, respectively) were observed at T3 compared with the control (Fig. 9). Foliar application of SA significantly reduced ROS accumulation, with the greatest reductions observed at T5, where $O_2^{\bullet-}$ and H_2O_2 decreased by 14.08% and 12.09%, respectively, relative to the corresponding stressed treatments. Histochemical staining further supported these biochemical observations. NBT staining

revealed intense blue formazan deposition, indicating higher $O_2^{\bullet-}$ accumulation in As-stressed leaves, particularly in T3 plants (Fig.11). Similarly, DAB staining showed strong brown precipitates, indicating elevated H_2O_2 accumulation in T3 leaves compared with the control. In contrast, leaves treated with SA (T5) showed markedly reduced blue and brown staining, indicating mitigation of As-induced oxidative stress (Fig.11)

3.7 Oxidative Stress and Histochemical Staining of $O_2^{\bullet-}$ and H_2O_2

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3.8 Antioxidant Enzyme Activities and Proline Accumulation

Activities of antioxidant enzymes increased markedly in response to As stress (Fig. 10). The highest increases in SOD, CAT, and POX activities (102.3%, 52.2%, and 111%, respectively) were recorded at T3 compared with the control. Application of SA further enhanced antioxidant enzyme activities in As-stressed plants. The greatest increases were observed at T5, where SOD, CAT, and POX activities increased by 33.5%, 16.1%, and 47.2%, respectively, relative to the corresponding stressed treatments. Similarly, proline accumulation increased significantly under As stress. This accumulation was further enhanced by SA application, resulting in increases of 35%, 29.9%, and 19.3% in T5, T6, and T7, respectively, compared with the corresponding stressed plants.

3.9 Stomatal Morphology and Elemental Analysis in Leaves

SEM revealed clear differences in stomatal characteristics among treatments (Fig. 12a). Observations at 500× magnification showed that the number of stomata under higher As stress (T2) were very less compared to the control (T0) and stomata appeared both closed and partially closed and deformed (Fig. 12a). Plants treated with SA alone (T4) showed an increased number of stomata with normal guard cell structure (Fig. 4). In contrast, plants treated with SA under As stress (T6) showed partial recovery, compared with the corresponding As-stressed plants.

Further observations at higher magnifications (3000×) revealed clear differences in stomatal morphology among treatments. In control plants (T0), stomata were well developed with normal guard cell structure (Fig. 12a). In contrast, leaves exposed to As stress (T2) exhibited pronounced stomatal closure and deformation compared with the control (Fig. 12a). Plants treated with SA alone (T4) showed normal and well-formed stomata. However, the SA

application under As stress (T6) improved stomatal structure, indicating partial recovery from As-induced damage relative to the corresponding As-stressed plants (Fig. 12a).

Elemental composition of leaf tissues was analyzed using EDX analysis (Fig. 12b). The EDX spectrum of control leaves indicated the presence of major elements such as C, O, Na, Mg, K, Ca, P, and S, with relative abundances of 35.09%, 56.03%, 0.76%, 0.57%, 3.62%, 3.12%, 0.36%, and 0.42%, respectively. In comparison, the SA alone treatment showed relative abundances of 30.42%, 60.46%, 0.81%, 0.97%, 3.90%, 2.53%, 0.43%, and 0.48%, respectively. In contrast, leaves exposed to As stress showed the presence of As (0.78%), along with noticeable alterations in elemental composition. It was characterized by a decline in essential nutrient elements, including Na, K, and P, which decreased to 0.06%, 1.98%, and 0.01%, respectively, while Mg and S increased to 1.48% and 0.55%, respectively. However, foliar application of SA under As stress partially restored the levels of these elements, indicating an improved nutrient status compared with the corresponding As-stressed plants.

3.10 Cellular Damage Detection in Roots

Roots exposed to As stress exhibited pronounced cellular damage, as indicated by strong PI-induced red fluorescence in root tissues compared with the control (Fig. 13). In contrast, roots from plants treated with SA under As stress showed markedly reduced fluorescence, indicating mitigation of As-induced cellular damage (Fig. 13).

4. Discussion

Heavy metal and metalloid toxicity is a major abiotic constraint to crop productivity, and its effects are generally concentration-dependent (Farooq et al., 2016; Wang et al., 2016). In the present study, increasing As concentrations markedly inhibited growth, photosynthetic performance, carbon and nitrogen metabolism, and membrane stability in *Brassica juncea*, while enhancing ROS accumulation, antioxidant defense, and proline content. Exogenous salicylic acid (SA) substantially alleviated these effects, indicating that SA enhances As tolerance through coordinated regulation of growth, photosynthesis, redox homeostasis, osmoprotection, and detoxification.

Arsenic stress significantly reduced shoot and root length and biomass, with the inhibitory effect being more pronounced in roots. As the primary site of contact with As in the growth medium, roots are particularly vulnerable to As-induced disruption of cellular metabolism, membrane integrity, cell division, and elongation (Farooq et al., 2015; Hasanuzzaman et al., 2024). Root impairment can subsequently restrict water and nutrient acquisition, thereby imposing secondary limitations on shoot growth and biomass accumulation. The diversion of metabolic resources toward stress defense and detoxification, including antioxidant and thiol-containing compounds, may further contribute to growth inhibition (Farooq et al., 2015). Similar dose-dependent reductions in growth have been reported in wheat, B. oleracea, and B. juncea under As stress (Liu et al., 2012; Karimi et al., 2019; Ahmad et al., 2021).

SA application substantially restored shoot and root growth and biomass under As stress. This protection may involve improved hormonal regulation, membrane stabilization, enhanced antioxidant capacity, and reduced As movement from roots to shoots (Bano et al., 2022). SA-

mediated modulation of auxin signaling may also contribute to the restoration of cell division and elongation (Singh et al., 2015). Thus, the growth-promoting effect of SA appears to result from improved cellular homeostasis rather than from a single physiological process.

Marked declines in leaf area, chlorophyll content, gas-exchange characteristics, and PSII photochemical efficiency accompanied the reduction in growth. As-induced impairment of root function can restrict water and nutrient uptake, while direct damage to leaf tissues and chloroplasts can further compromise photosynthesis. The decline in SPAD values observed here may reflect impaired chloroplast organization and inhibition of chlorophyll biosynthesis under As stress (Rahman et al., 2007; Nahar et al., 2022). Altered elemental composition observed by EDX further suggests that As stress disturbed nutrient homeostasis, including elements associated with photosynthetic metabolism.

The reductions in PN, gs, E, and Ci indicate that As stress impaired both CO_2 diffusion and carbon assimilation. Stomatal limitation was supported by SEM observations showing alterations in stomatal morphology. As-induced disruption of stomatal development and function may restrict CO_2 diffusion into the leaf, while impaired chloroplast activity limits its subsequent fixation (Gupta and Bhatnagar, 2015; Nahar et al., 2022). Similar reductions in photosynthetic traits have been reported in Pistia stratiotes, soybean, rice, mustard, and mung bean exposed to As stress (Gusman et al., 2013; Farnese et al., 2017; Vezza et al., 2022; Jalil et al., 2024; Bhat et al., 2023; Khan et al., 2025).

As stress also impaired PSII photochemistry, as reflected by reductions in ΦPSII and ETR and an increase in NPQ. Reduced CO_2 fixation limits $NADP^+$ regeneration, causing over-reduction of the photosynthetic electron transport chain and increasing the dissipation of excess excitation energy through photoprotective mechanisms such as thermal dissipation, photorespiration, and the water–water cycle (Weng et al., 2008). The increase in NPQ, therefore, represents an important protective response to excess excitation energy, but its occurrence, together with reduced ΦPSII and ETR, indicates substantial impairment of photosynthetic function.

SA markedly improved chlorophyll content, gas exchange, and PSII photochemical performance under As stress. This protection may result from preservation of chloroplast and membrane integrity, improved electron transport, maintenance of Rubisco activity, and better regulation of stomatal function (Sahu et al., 2002; Arfan et al., 2007; Krantev et al., 2008; Wang et al., 2010; Poor et al., 2011). SA may therefore interrupt the cascade linking As-induced root dysfunction, stomatal limitation, impaired CO_2 assimilation, and PSII photoinhibition.

The decline in photosynthetic performance under As stress was accompanied by reduced activities of carbonic anhydrase (CA) and nitrate reductase (NR), indicating disruption of both carbon and nitrogen metabolism. The reduced CA activity may be associated with reduced CO_2 availability and impaired photosynthesis, whereas inhibition of NR may reflect As-induced disruption of enzyme structure or catalytic activity (Hayat et al., 2008; Singh et al., 2009). Because CA facilitates CO_2/HCO_3^- interconversion and NR is central to nitrate assimilation, their inhibition may further restrict carbon fixation and nitrogen metabolism.

The SA application significantly restored CA and NR activities under As stress. Similar SA-mediated enhancement of CA and NR has been reported in B. juncea and other plant species (Yusuf et al., 2012; Fariduddin, 2003; Hayat et al., 2009; Amooaghaie and Nikram, 2025). The recovery of these enzymes is consistent with the improved photosynthetic performance and growth observed following SA treatment, suggesting that SA helps maintain coordinated C and N metabolism under As stress.

A major component of As toxicity was the accumulation of ROS, as indicated by increased $O_2^{\bullet -}$ and H_2O_2 levels and stronger NBT and DAB staining. Disruption of photosynthetic electron transport, reduced CO_2 assimilation, and impaired cellular redox balance can promote electron leakage to O_2 and enhance ROS production (Yadav et al., 2014; Mishra et al., 2017; Sachdev et al., 2021). Mitochondrial electron transport may also contribute to ROS formation under stress (Blokhina et al., 2003). Thus, the increased ROS observed in the present study likely reflects disturbances in both photosynthetic and respiratory electron transport.

The increased activities of SOD, CAT, and POX under As stress indicate activation of the enzymatic antioxidant system. SOD converts $O_2^{\bullet -}$ to H_2O_2, which is subsequently detoxified by CAT and POX, thereby limiting oxidative damage (Das and Roychoudhury, 2014; Zaidi et al., 2026). However, the simultaneous accumulation of ROS suggests that the induced antioxidant response was insufficient to completely prevent oxidative stress.

SA substantially reduced NBT and DAB staining and moderated ROS accumulation, indicating improved redox homeostasis. SA-mediated protection is likely associated with more effective antioxidant defense and enhanced detoxification capacity. SA may additionally promote sulfur metabolism and the production of non-protein thiols and phytochelatins, which can bind As and reduce its cytotoxicity and translocation (Bano et al., 2022). The increased S content observed in SA-treated plants supports a possible contribution of sulfur-dependent detoxification to As tolerance. Similar reductions in As-induced oxidative stress following SA application have been reported in Arabidopsis, rice, and B. napus (Odjegba, 2012; Singh et al., 2015; Bano et al., 2022).

The As stress caused substantial accumulation of proline, indicating activation of osmoprotective and antioxidant mechanisms. Proline contributes to osmotic adjustment, membrane and protein stabilization, ROS scavenging, and maintenance of cellular redox balance under stress (Khan et al., 2015). SA further increased proline accumulation under As stress, suggesting that SA strengthens osmotic and redox protection. Enhanced proline biosynthesis, together with improved regulation of its degradation and NADPH metabolism, may help maintain cellular energy and reduce oxidative injury during stress and recovery (Bano et al., 2022; Youssef et al., 2023). Similar SA-induced increases in proline under As stress have been reported previously (Kumar et al., 2014; Chandrakar et al., 2016).

Microscopic observations supported the physiological responses. As-induced alterations in stomatal morphology and cellular structure provide structural evidence for impaired gas exchange and increased cellular injury. Conversely, the comparatively improved structural characteristics following SA treatment are consistent with its ability to stabilize membranes, maintain cellular organization, and reduce oxidative damage. Together with EDX evidence of

altered elemental composition, these observations indicate that SA-mediated tolerance involves both structural preservation and improved physiological regulation.

Overall, the present findings indicate that As toxicity in B. juncea operates through a connected chain of effects: root dysfunction and impaired nutrient/water acquisition → disruption of stomatal and chloroplast function → reduced CO_2 assimilation and PSII activity → enhanced ROS production and oxidative injury → impairment of C/N metabolism and growth. SA interrupts this cascade by maintaining photosynthetic machinery and C/N metabolism, strengthening antioxidant and osmoprotective defenses, and potentially enhancing sulfur- and thiol-mediated As detoxification. The combined physiological, biochemical, elemental, and microscopic responses therefore demonstrate that SA provides an integrated mechanism of protection against As-induced toxicity in B. juncea.

Conclusion

Arsenic stress severely impaired growth, photosynthetic efficiency, nutrient balance, and cellular integrity in *Brassica juncea* by inducing excessive accumulation of reactive oxygen species. Histochemical, microscopic, and elemental analyses confirmed substantial oxidative and structural damage under increasing arsenic concentrations. Foliar application of salicylic acid effectively alleviated arsenic toxicity by enhancing antioxidant defense mechanisms, improving photosynthetic performance, maintaining nutrient homeostasis, and reducing ROS accumulation and cellular injury. The protective effects of salicylic acid were particularly evident under moderate arsenic stress levels. Overall, salicylic acid functions as an effective stress-mitigating phytohormone and may represent a promising strategy for improving arsenic tolerance and sustaining mustard productivity in contaminated soils.

Author contribution

U.N. performed the experimental work. S.Z. prepared the first draft of the manuscript. M.H.Y and S.A. contributed to the experimental procedures and assisted with data analysis. S.H., given the research idea, supervised the overall study and revised the manuscript for its final version. A.B. critically reviewed the manuscript and carried out language editing and proofreading. All authors read, reviewed, and approved the final manuscript before submission.

Funding: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Data availability: Data will be made available on request

Declarations

Conflict of interest: The authors declare no conflict of interest.

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Figures Legends

Fig. 1. Effect of arsenic stress and salicylic acid on root and shoot length of *Brassica juncea* at 30 days after germination (DAG). Plants were treated with water (T0), As25 (T1), As50 (T2), As75 (T3), 10^{-5} M SA (T4), As25 + 10^{-5} M SA (T5), As50 + 10^{-5} M SA (T6), and As75 + 10^{-5} M SA (T7). Bars represent mean ± SE, n = 4. Different lowercase letters indicate significant differences among treatments according to Tukey's HSD test at $p \leq 0.05$

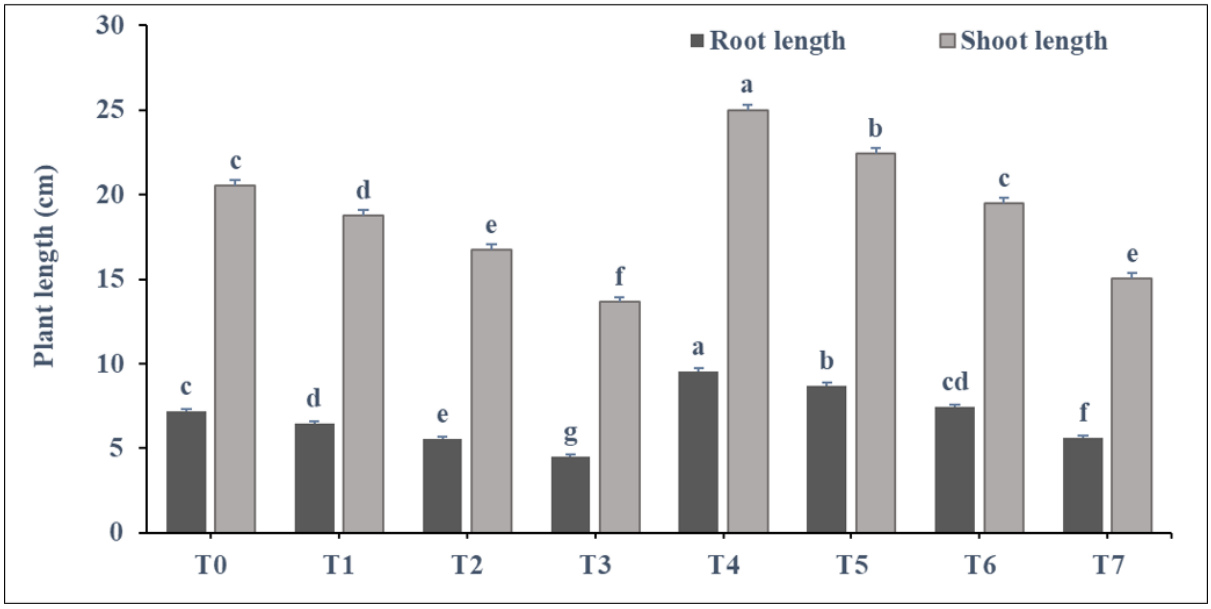

Fig 2: Effect of arsenic stress and salicylic acid on root fresh and dry mass of *Brassica juncea* at 30 DAG. Plants were treated with water (T0), As25 (T1), As50 (T2), As75 (T3), 10^{-5} M SA (T4), As25 + 10^{-5} M SA (T5), As50 + 10^{-5} M SA (T6), and As75 + 10^{-5} M SA (T7). Bars represent mean ± SE, n = 4. Different lowercase letters indicate significant differences among treatments according to Tukey's HSD test at $p \leq 0.05$.

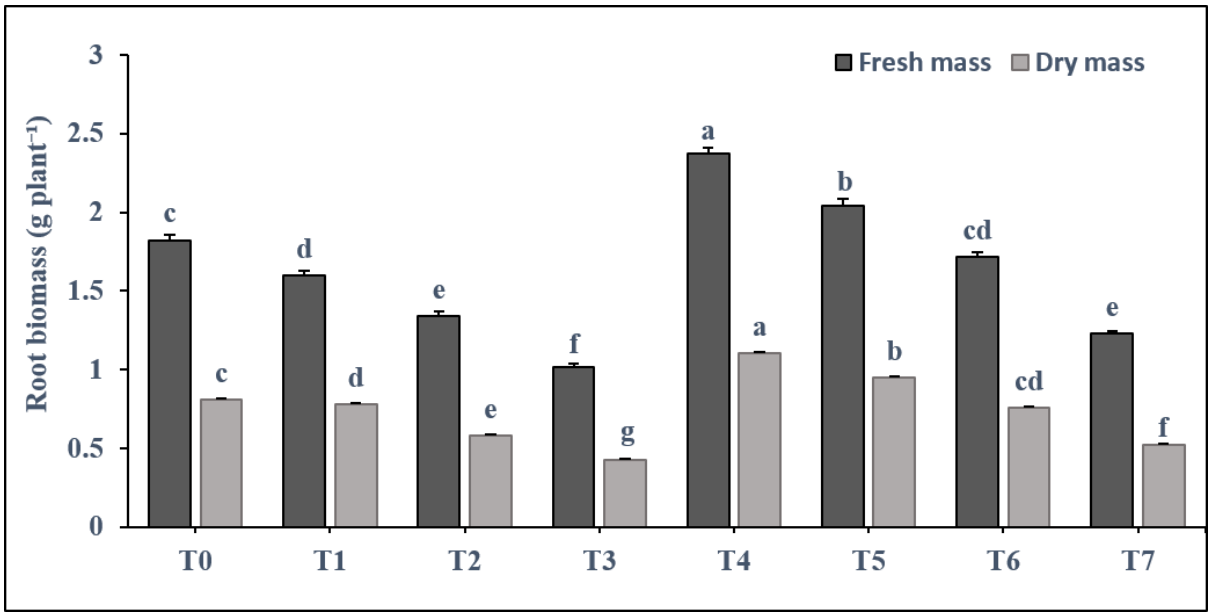

Fig 3: Effect of arsenic stress and salicylic acid on shoot fresh and dry mass of *Brassica juncea* at 30 DAG. Plants were treated with water (T0), As25 (T1), As50 (T2), As75 (T3), 10^{-5} M SA (T4), As25 + 10^{-5} M SA (T5), As50 + 10^{-5} M SA (T6), and As75 + 10^{-5} M SA (T7). Bars represent mean ± SE, n = 4. Different lowercase letters indicate significant differences among treatments according to Tukey's HSD test at $p \leq 0.05$.

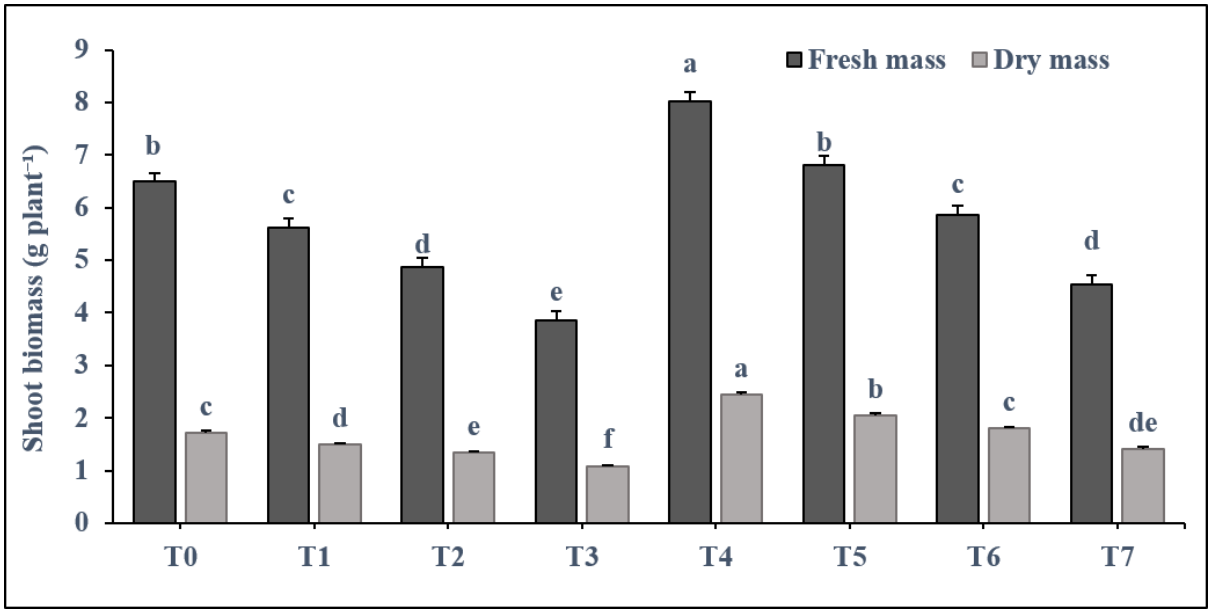

Fig 4: Uprooted Indian mustard (*Brassica juncea* L.) plants showing comparative shoot and root growth at 30 days after germination (DAG) with water (T0), As25 (T1), As50 (T2), As75 (T3), 10^{-5} M SA (T4), As25 + 10^{-5} M SA (T5), As50 + 10^{-5} M SA (T6), and As75 + 10^{-5} M SA (T7).

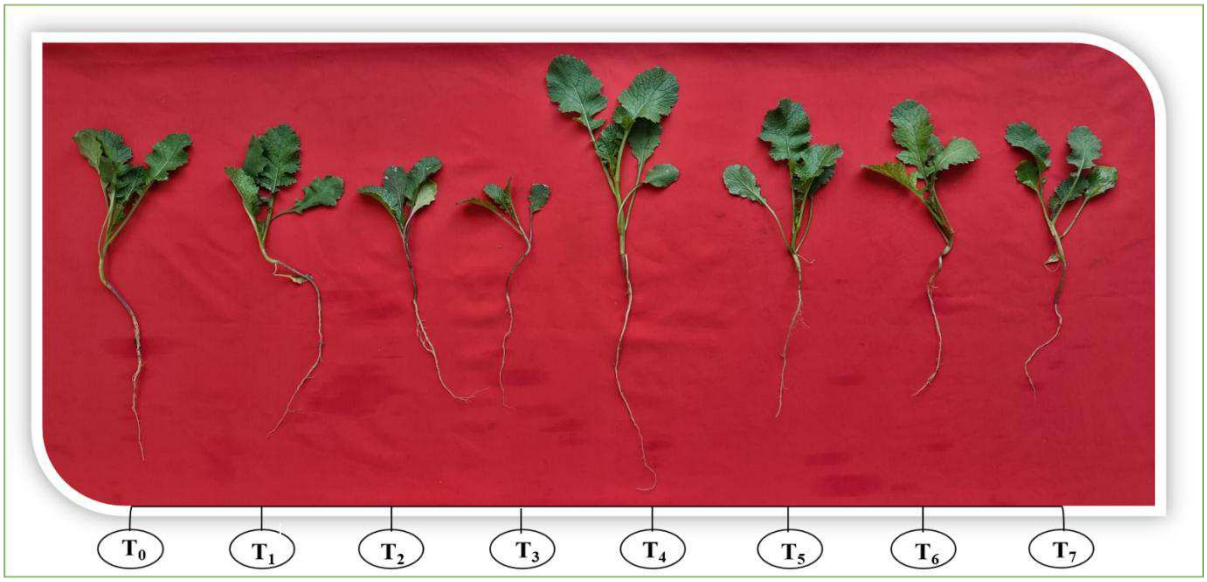

Fig 5: Effect of arsenic stress and salicylic acid on (a) leaf area (cm^2 $plant^{-1}$) and (b) SPAD chlorophyll index of *Brassica juncea* at 30 DAG. Plants were treated with water (T0), As25 (T1), As50 (T2), As75 (T3), 10^{-5} M SA (T4), As25 + 10^{-5} M SA (T5), As50 + 10^{-5} M SA (T6),

and As75 + 10^{-5} M SA (T7). Bars represent mean ± SE, n = 4. Different lowercase letters indicate significant differences among treatments according to Tukey's HSD test at p ≤ 0. 05.

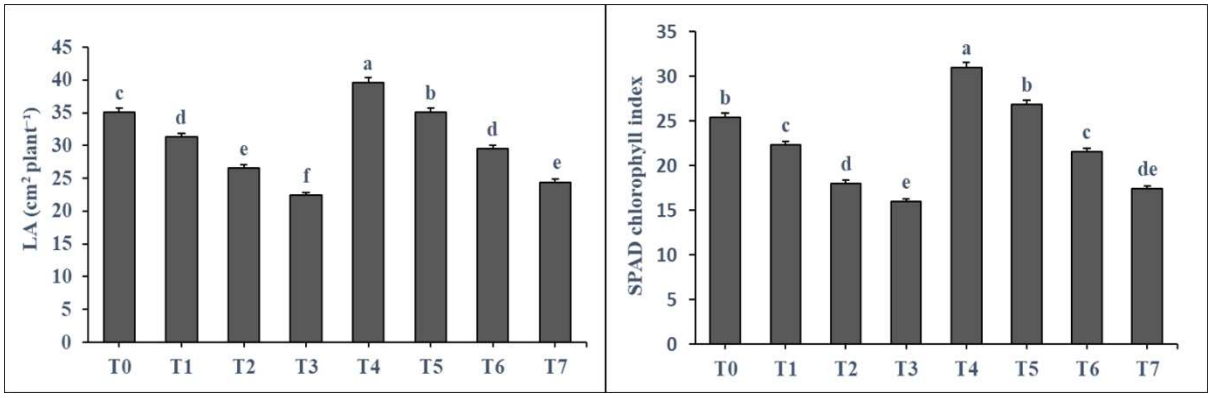

Fig 6: **Effect of arsenic stress and salicylic acid** on (a) Pn (µmol CO_2 m^{-2} s^{-1}), (b) gs (mol H_2O m^{-2} s^{-1}), (c) Ci (µmol CO_2 mol^{-1} air), and (d) E (mmol H_2O m^{-2} s^{-1}) of *Brassica juncea* at 30 DAG. Plants were treated with water (T0), As25 (T1), As50 (T2), As75 (T3), 10^{-5} M SA (T4), As25 + 10^{-5} M SA (T5), As50 + 10^{-5} M SA (T6), and As75 + 10^{-5} M SA (T7). Bars represent mean ± SE, n = 4. Different lowercase letters indicate significant differences among treatments according to Tukey's HSD test at p ≤ 0.05.

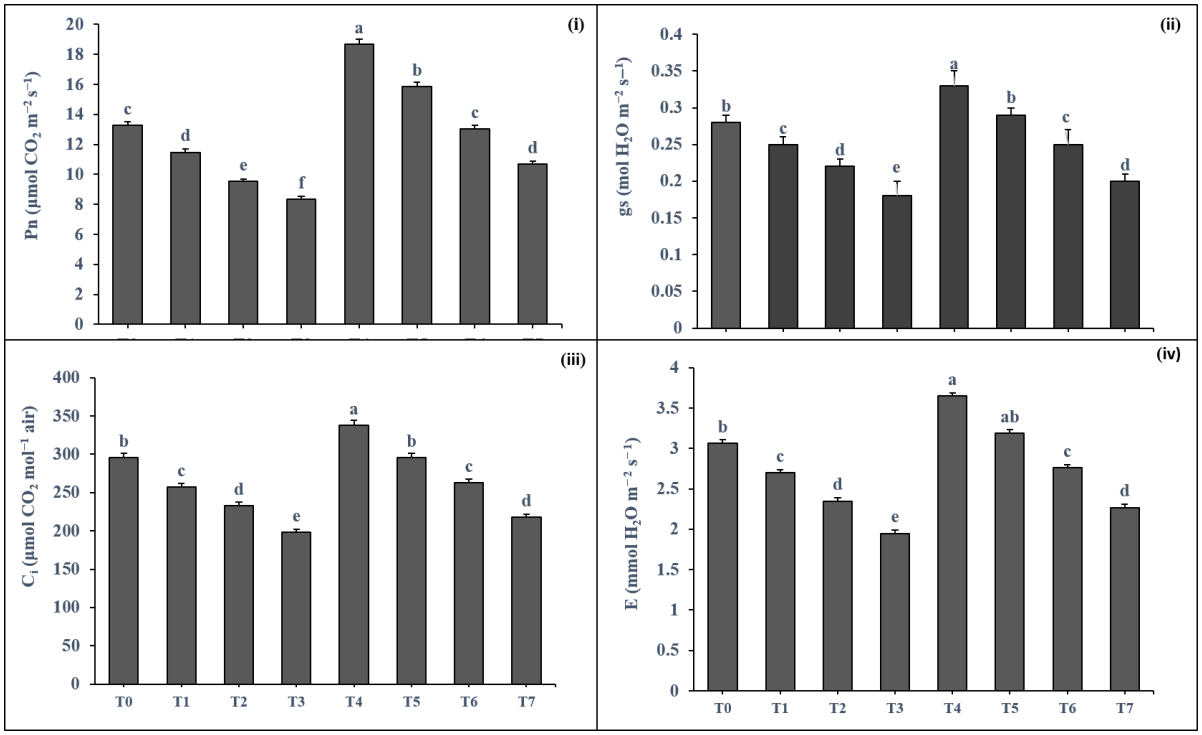

Fig 7: **Effect of arsenic stress and salicylic acid** on chlorophyll fluorescence parameters (Fv/Fm, PSII, qP, ETR, NPQ) of *Brassica juncea* at 30 DAG. Plants were treated with water (T0), As25 (T1), As50 (T2), As75 (T3), 10^{-5} M SA (T4), As25 + 10^{-5} M SA (T5), As50 + 10^{-5} M SA (T6), and As75 + 10^{-5} M SA (T7). Bars represent mean ± SE, n = 4. Different lowercase letters indicate significant differences among treatments according to Tukey's HSD test at p ≤ 0.05.

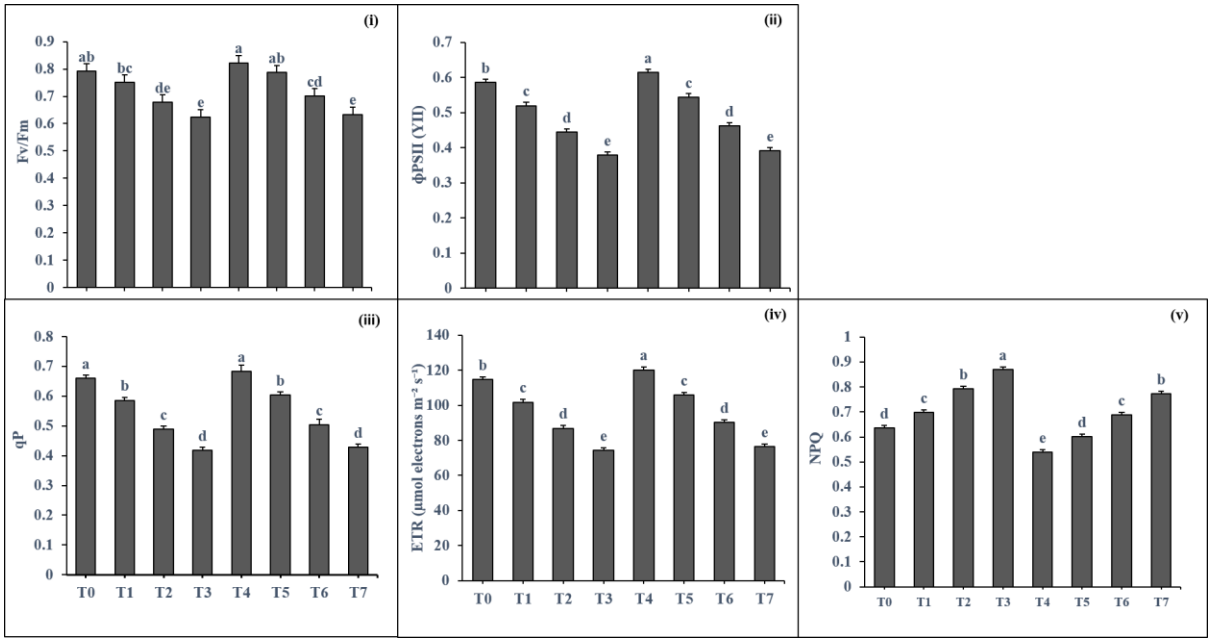

Fig 8: **Effect of arsenic stress and salicylic acid** on (a) CA activity (µmol CO_2 kg^{-1} FW s^{-1}) and (b) NR activity (µmol NO_2 g^{-1} FW h^{-1}) of *Brassica juncea* at 30 DAG. Plants were treated with water (T0), As25 (T1), As50 (T2), As75 (T3), 10^{-5} M SA (T4), As25 + 10^{-5} M SA (T5), As50 + 10^{-5} M SA (T6), and As75 + 10^{-5} M SA (T7). Bars represent mean ± SE, n = 4. Different lowercase letters indicate significant differences among treatments according to Tukey's HSD test at $p \leq 0.05$.

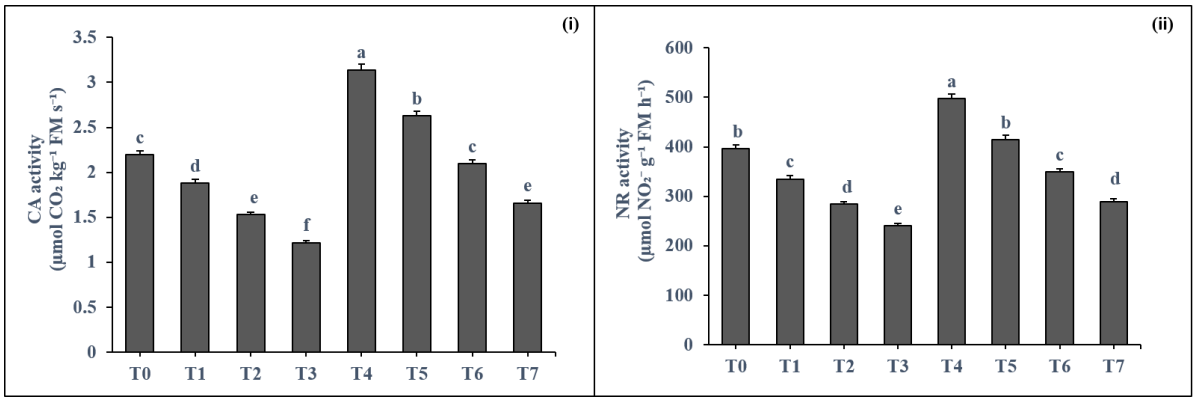

Fig 9: **Effect of arsenic stress and salicylic acid** on $O_2^{\cdot-}$ content (µmol g^{-1} FW) and H_2O_2 content (µmol g^{-1} FW) of *Brassica juncea* at 30 DAG. Plants were treated with water (T0), As25 (T1), As50 (T2), As75 (T3), 10^{-5} M SA (T4), As25 + 10^{-5} M SA (T5), As50 + 10^{-5} M SA (T6), and As75 + 10^{-5} M SA (T7). Bars represent mean ± SE, n = 4. Different lowercase letters indicate significant differences among treatments according to Tukey's HSD test at $p \leq 0.05$.

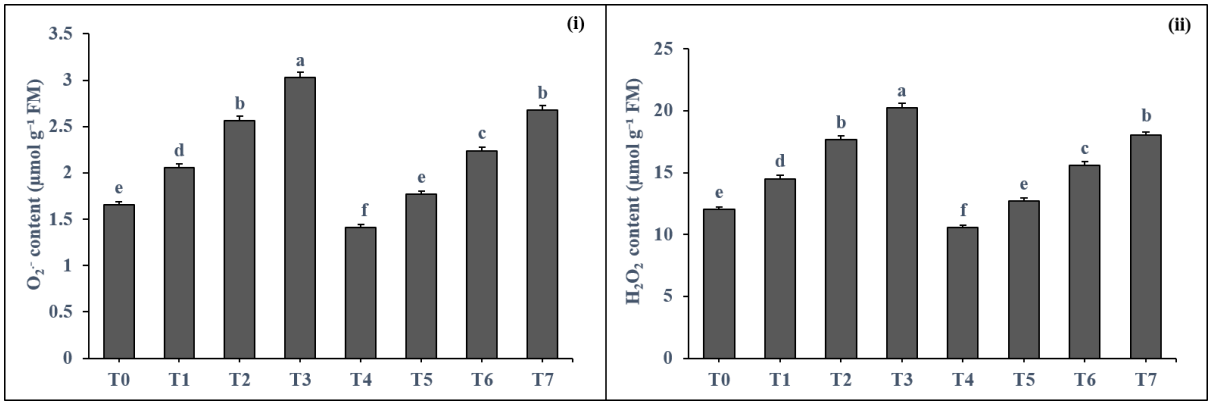

Fig 10: **Effect of arsenic stress and salicylic acid** on (a) SOD (U g^{-1} FW), (b) CAT (U g^{-1} FW), (c) POX (U g^{-1} FW), and (d) proline content (µmol g^{-1} FW) of *Brassica juncea* at 30 DAG. Plants were treated with water (T0), As25 (T1), As50 (T2), As75 (T3), 10^{-5} M SA (T4), As25 + 10^{-5} M SA (T5), As50 + 10^{-5} M SA (T6), and As75 + 10^{-5} M SA (T7). Bars represent mean ± SE, n = 4. Different lowercase letters indicate significant differences among treatments according to Tukey's HSD test at $p \leq 0.05$.

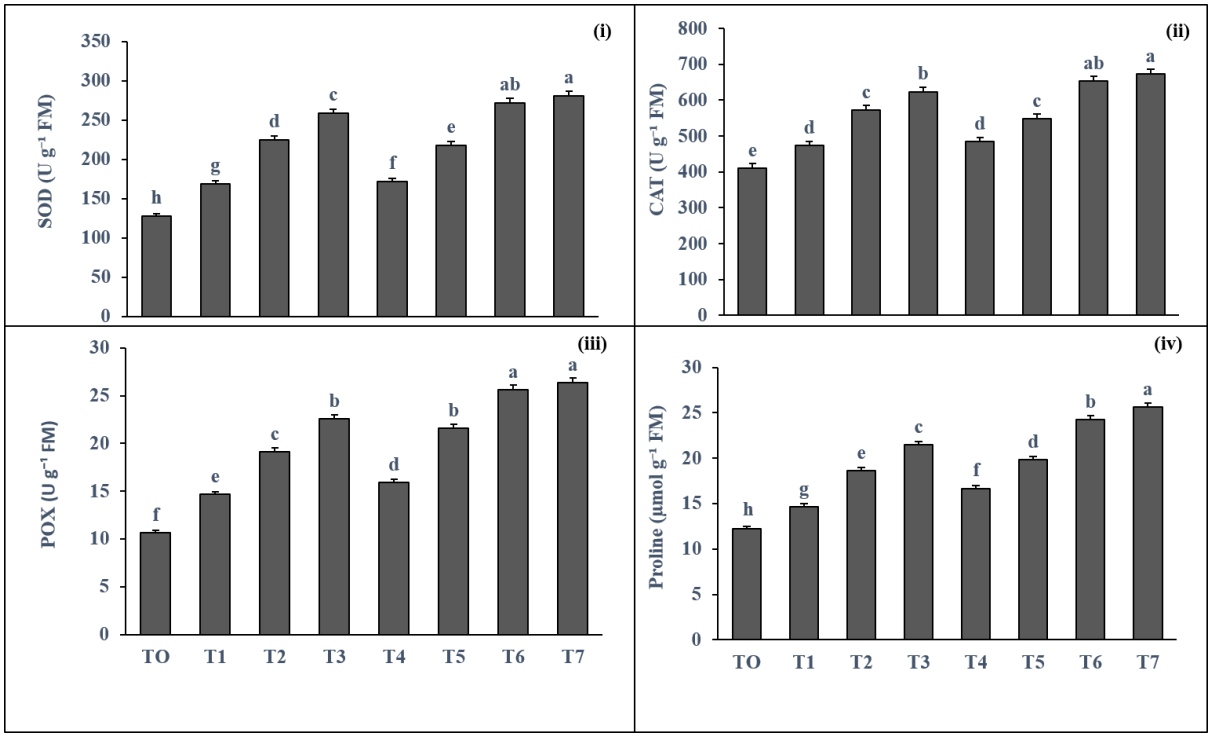

Fig 11: Histochemical detection of reactive oxygen species in *Brassica juncea* leaves at 30 DAG under different treatments. (i–iv) Detection of hydrogen peroxide (H_2O_2) as brown staining by DAB in leaves of water (T0), 50 mg As (T2), SA (T4), and 50 mg As + SA (T6), respectively. (v–viii) Detection of superoxide radicals ($O_2^{\cdot-}$) as blue by NBT in leaves of water (T0), 50 mg As (T2), SA (T4), and 50 mg As + SA (T6), respectively.

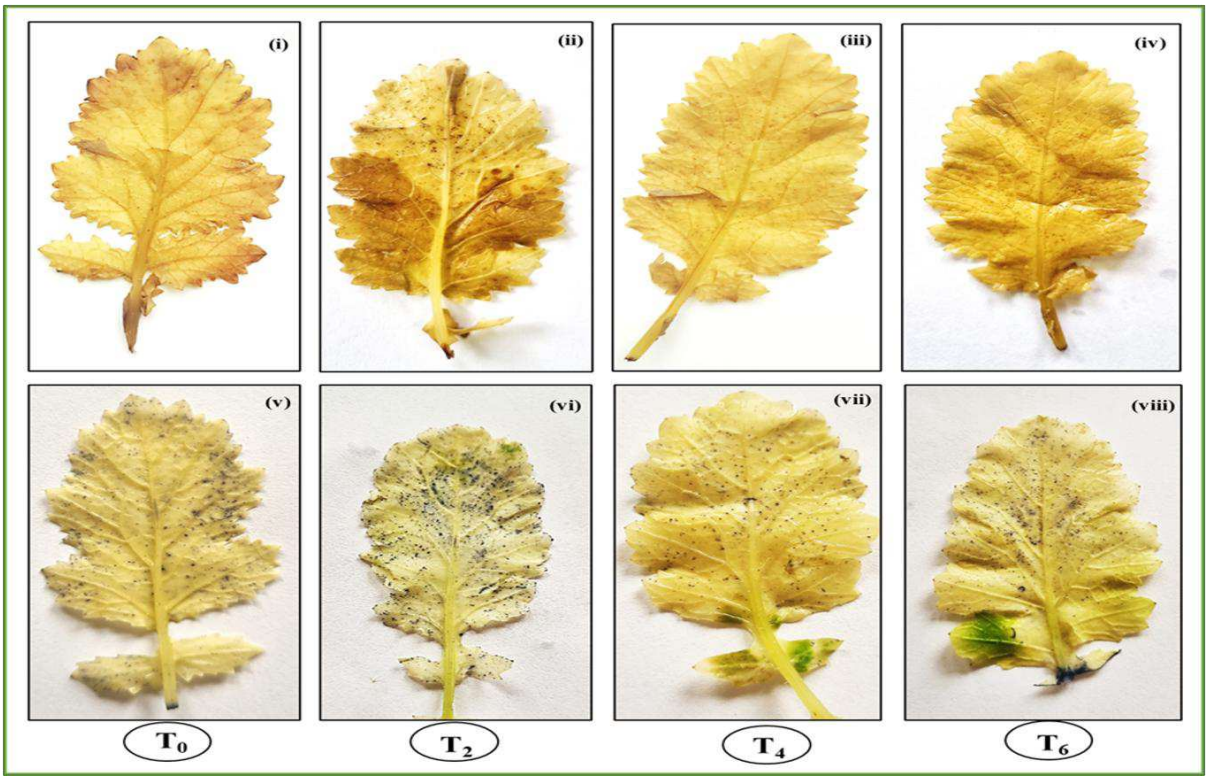

Figure 12a: Scanning electron microscopy (SEM) of *B. juncea* leaves at 30 DAG under different treatments. SEM micrographs showing stomatal characteristics at 500× magnification for water (I), 50 mg As (II), SA (III), and 50 mg As + SA (IV), respectively. SEM images of stomata at 3000× magnification for water (V), 50 mg As (VI), SA (VII), and 50 mg As + SA(VIII), respectively.

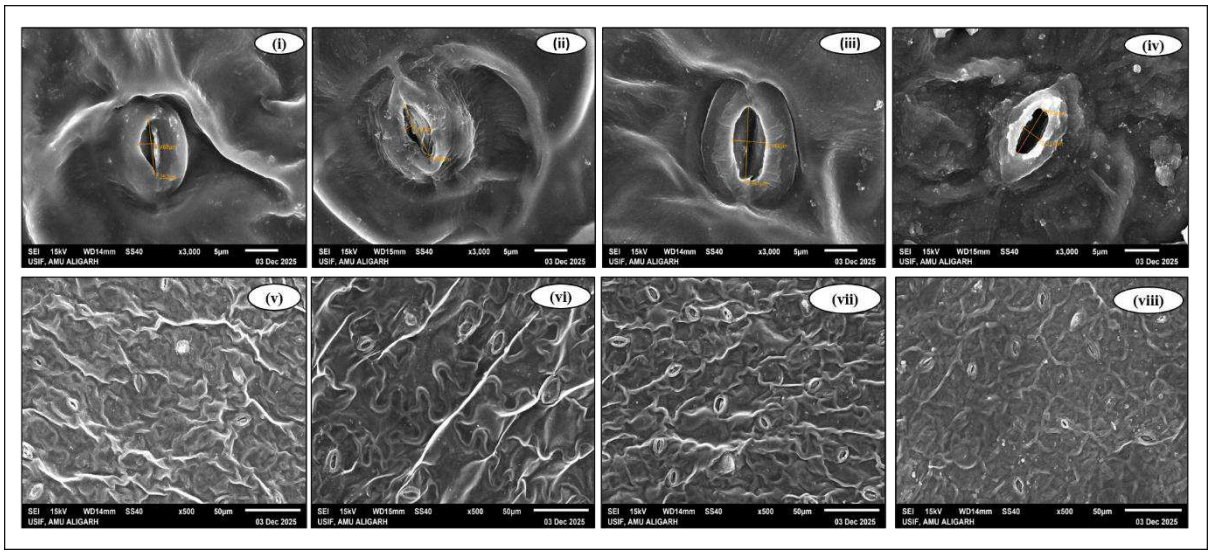

Figure 12b: Energy dispersive X-ray (EDX) analysis of *B. juncea* leaves at 30 DAG under different treatments.EDX spectra showing the elemental composition of leaf tissues for water (i), 50 mg As (ii), SA (iii), and 50 mg As + SA (iv), respectively

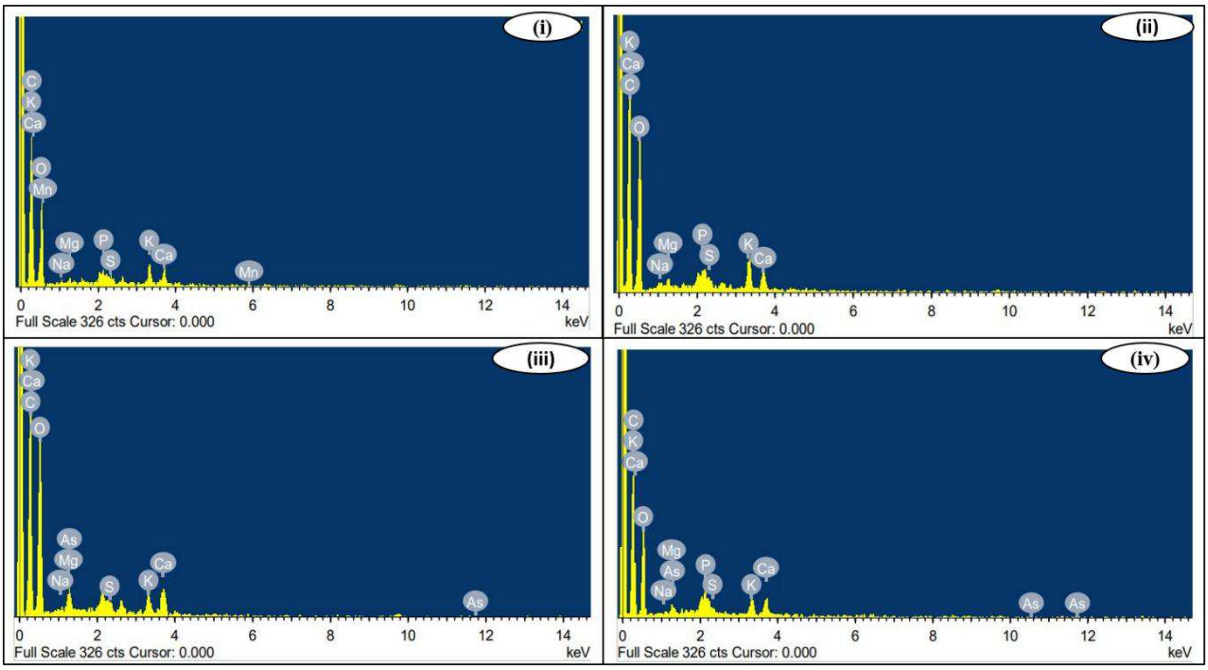

Figure 13: Propidium iodide (PI) staining with higher red fluorescence indicates greater cellular damage in root tissues of Brassica juncea at 30 DAG under different treatments. (I–IV) Confocal images of roots from the water (T0) 50 mg As (T2), SA (T4), and 50 mg As + SA (T6), respectively.

