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RESEARCH

Foliar Application of Ascorbic Acid and α-Tocopherol Enhances Salinity Tolerance in *Hordeum vulgare* L. by Regulating Antioxidant Enzymatic Activity and Improving Physiological and Biochemical Responses

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Abstract

Soil salinity severely impairs crop productivity by disrupting physiological and biochemical processes. This study investigated the efficacy of foliar-applied ascorbic acid (AsA) and α-tocopherol (α-toc) in enhancing salinity tolerance in barley (*Hordeum vulgare* L. cv. AJJ). A pot experiment using a randomized complete block design with three replicates was conducted under controlled conditions. Seedlings at the 4–5 leaf stage were exposed to 0, 40, or 80 mM NaCl salinity stress, followed by foliar treatments of AsA and α-toc (150 mg L^{-1}). Germination, agronomic, physiological, and biochemical parameters were analyzed. Salinity stress at 40 and 80 mM NaCl significantly reduced germination percentage (GP) by 18.6% and 18.4%, respectively, compared to the control (86.6%). Foliar application of AsA + α-toc under 40 and 80 mM stress restored GP to 82.3% and 81.7%, reflecting recoveries of 16.8% and 15.6% overstressed groups. Water content in leaves, shoots, and roots declined by 10–30% under salinity but improved by 15–25% with AsA + α-toc. Photosynthetic pigments were severely affected: chlorophyll a and total chlorophyll content (TCC) decreased by 45.7% (1.75 to 0.95 mg g^{-1} FW) and 10.9% (1.75 to 1.56 mg g^{-1} FW) at 40 and 80 mM NaCl, respectively. AsA + α-toc mitigated these losses, elevating TCC by 42.6% under 80 mM stress. Soluble protein content increased by 186% (0.50 to 1.43 mg g^{-1}) under 80 mM stress but was stabilized by antioxidant treatments. Antioxidant enzymes, including superoxide dismutase (SOD) and peroxidase (POD), surged by 40–60% under salinity, while AsA + α-toc reduced hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) levels by 30–50%. The combined application of AsA and α-toc proved most effective, enhancing stress tolerance through improved osmotic regulation, antioxidant defense, and chlorophyll preservation. These findings highlight the potential of AsA and α-toc as sustainable agronomic tools for cultivating barley in saline soils, with AsA showing marginally greater efficacy. Further field studies are warranted to validate these results under different climatic conditions.

Keywords Plant nutrition · Plant stress · Osmotic regulation · Plant defense mechanisms · Oxidative stress

Introduction

Ionic toxicity inhibits plant growth and development, negatively affecting agricultural production. Salinity induces both ionic toxicity and osmotic stress, reducing available water for plants and disrupting metabolic reactions, including enzyme inactivation (Safdar et al. 2019). Salinization is projected to cause a 50% loss of arable land worldwide by 2050, primarily impacting South Asia and the Mediterranean region. Currently, it has already affected 20% of the world's total irrigated land (Negrão et al. 2017). Major agricultural crops such as wheat, maize, rice and barley have shown a significant yield reduction of 70% due to salinity stress (Acquaah 2009). High salinity concentrations in growth media adversely affect plant biomass, physiology, mineral ion accumulation, and the disruption of PSII processes (Liu et al. 2020), leading to biochemical damage, all due to the production of reactive oxygen species (ROS) (Li

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et al. 2020). Ultimately, this leads to impaired metabolism and stunted plant growth. The imbalance between ROS production and antioxidant defense mechanisms is one of the prominent effects of salinity that causes oxidative stress and excessive ROS accumulation in plants (Saleem et al. 2020).

Especially under high salinity conditions, both enzymatic and non-enzymatic antioxidant defense systems maintain the balance between ROS generation and detoxification (Shafiq et al. 2020). Reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide anion ($O2^{\bullet -}$), hydroxyl radical ($HO^{\bullet}$), alkoxyl radical ($RO^{\bullet}$), peroxyl radical ($RO_2^{\bullet}$), organic hydroperoxide (ROOH), and singlet oxygen ($^{1/2}O_2$), can cause oxidative damage in plants under stress conditions. To mitigate this, plants activate a range of antioxidants, including enzymatic antioxidants like superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and ascorbate peroxidase (APX), which play key roles in scavenging and neutralizing these ROS (Javed et al. 2021). Recently, it was reported that numerous external non-enzymatic compounds are employed to mitigate the adverse impacts caused by salt stress and restore plant physiology (Shah et al. 2021). Ascorbic acid (AsA), α-tocopherol (α-toc), phenolics, and other compounds with the ability to neutralize singlet oxygen and hydroxyl radicals are among the enzymatic and non-enzymatic antioxidants regulated by the activities of numerous enzymes in plants to detoxify ROS (Mohamed et al. 2018).

Salinity affects over 6% of the world's land, causing crop losses. Barley, the fourth most important cereal crop, has outstanding salinity tolerance during germination. The genetic diversity of dehydration-responsive element-binding (DREB) protein, somatic embryogenesis receptor-like kinase, and aquaporin genes are discussed by (Mwando et al. 2020). Salinity tolerance in barley is influenced by ionic, osmotic, and oxidative stress components. Plant tissue tolerance, root K^+ retention, and reduced hydroxyl radical production are key. Transcriptional changes in genes play a small role in plant adaptive responses to salinity. Targeting these traits simultaneously is crucial for progress (Adem et al. 2014). The study reveals that barley genotypes Giza 129 and 135, which are sensitive to salt stress, experience decreased growth and yield, suggesting potential for genetic enhancement programs, and that grain yield is the only reliable indicator of salt tolerance (Abdelrady et al. 2024) therefore, sustainable solutions are required for salinity tolerance (Munir et al. 2022).

Ascorbic acid (AsA), commonly known as vitamin C, serves as a crucial non-enzymatic antioxidant present in plants, playing a vital role in regulating oxidative stress induced by both biotic and abiotic stresses (Sharma et al. 2019). Ascorbic acid (AsA) can enhance both plant growth and its resilience to stress, necessitating the synthesis of proteins containing hydroxyproline (Bilska et al. 2019). In addition, AsA protects plants against oxidative stress by effectively neutralizing various free radicals, such as H_2O_2. Its primary role is to act as a substrate for APX, a crucial enzyme within the ascorbate-glutathione pathway (Sharma et al. 2019). Similarly, the addition of AsA from exogenous sources inhibits lipid peroxidation, reducing malondialdehyde (MDA) levels in plants and boosting antioxidant capacity. Additionally, vital cellular enzymes like violaxanthin de-epoxidase, which is crucial for photoprotection through the xanthophyll cycle, rely on ascorbate as a cofactor (Zhu 2016). In addition to regenerating enzymatic antioxidants and regulating metabolism, AsA functions as a cofactor for enzymes involved in the production of plant hormones, such as gibberellic acid (GA) (Lalarukh and Shahbaz 2020).

In addition to being a non-enzymatic antioxidant, α-tocopherol has physiological effects on processes such as germination, photoassimilate translocation, and leaf senescence (Szarka et al. 2012). α-tocopherol supports plant development and yields under abiotic stress by reducing oxidative damage and increasing nutrient and water availability (Noreen et al. 2021). Studies conducted in the past suggest that foliar application of α-tocopherol plays an important role in reducing abiotic stress by quenching ROS and decreasing ion leakage and lipid peroxidation (Saeed et al. 2020).

Barley (*Hordeum vulgare* L.) is a nutrient-dense cereal with vigorous growth and strong abiotic stress tolerance, making it a prime model in agronomy and plant physiology (Noreen et al. 2021). It delivers health benefits like β–glucan–mediated cholesterol reduction and sustains productivity in saline drylands by balancing vacuolar NaCl osmotic potential with cytoplasmic solutes (Agami 2014; Behall et al. 2004; Ullah et al. 2016). These robust salt-tolerance traits position barley as an ideal metabolomic model for transferring resilience to crops such as rice (*Oryza sativa* L.) and winter wheat (*Triticum aestivum* L.).

Previous studies have been conducted using barley as a cereal crop under salt-stressed conditions and using various strategies to offset the negative effect (Sharma et al. 2021). However, to date, few studies have demonstrated the effects of exogenous applications of Ascorbic acid (AsA) and α-tocopherol (α-toc) on various morpho-physiological traits and nutrient uptake in plants under salinity. Understanding how AsA and α-toc influence nutrient acquisition opens new avenues for their use in agriculture and provides a strong foundation for increasing plant tolerance and resistance to salt stress. It was hypothesized that the exogenous application of AsA and α-tocopherol could protect barley from the detrimental effects of salt stress by modifying morpho-physio-biochemical characteristics and regulating the antioxidant defense system. The present study was undertaken with the following specific objectives: (i) To

Table 1 Experimental design for *Hordeum vulgare* under induced salinity stress

Treatments	Description
Control (T0)	Salinity (0 mM) + Ascorbic Acid (0 mM) + α-Tocopherol (0 mM)
S_{40} (T1)	Salinity (40 mM)
S_{40} + AsA (T2)	Salinity (40 mM) + Ascorbic Acid (40 mM)
S_{40} + α-toc (T3)	Salinity (40 mM) + α-Tocopherol (40 mM)
S_{40}(AsA + α-toc) (T4)	Salinity (40 mM) + Ascorbic Acid (40 mM) + α-Tocopherol (40 mM)
S_{80}(T5)	Salinity (80 mM)
S_{80} + AsA (T6)	Salinity(80 mM) + Ascorbic Acid (80 mM)
S_{80} + α-toc (T7)	Salinity(80 mM) + α-Tocopherol (80 mM)
S_{80}(AsA + α-toc) (T8)	Salinity (80 mM) + Ascorbic Acid (80 mM) + α-Tocopherol (80 mM)

evaluate the agro-physiological, biochemical and antioxidant responses of barley to varied concentrations of foliar applied AsA and α-toc grown under salt stress (ii) To understand the mechanisms by which AsA and α-toc confer tolerance to salt stress (iii), and to compare and recommend the most effective foliar treatment for economically important barley under salinity conditions. This study explores the synergistic effects of ascorbic acid and α-tocopherol in mitigating salinity stress in barley, providing insights for improving crop resilience and highlighting the superior efficacy of AsA over α-toc, thereby guiding future sustainable agricultural practices.

Materials and Methods

Plant Material

In this study, *Hordeum vulgare* cv. AJJ, a robust and mature barley variety, was employed as the test crop. Healthy seeds with 95% viability were obtained from the Nuclear Institute for Food and Agriculture (NIFA), Peshawar, Pakistan. Although previous studies have reported the salinity responses of various barley genotypes (Abdelrady et al. 2024; Hassan et al. 2021; Sharma et al. 2021; Noreen et al. 2021), specific information regarding stress tolerance or susceptibility of the AJJ variety under salinity conditions remains unavailable. Therefore, the current study not only explores the effects of foliar-applied ascorbic acid (AsA) and α-tocopherol (α-toc) on barley under salinity stress but also provides novel insights into the salinity tolerance potential of the AJJ variety.

Study Area

During the growing season of November 2021 to February 2022, this experiment was conducted at the net house of the Department of Botany, University of Peshawar, KP, Pakistan. The experimental site had a sub-humid climate with severe weather (intense summer; 40.8 °C and mild winter; 18.4 °C) and average humidity of 27 ± 5%, located 450 m above sea level (Rahman et al. 2021).

Treatments and Experimental Setup

In this study, a randomized complete block design (RCBD) pot experiment along with three replicates were utilized. Specifics of the treatments used in this study are shown in Table 1. The cumulative and daily germination data in each pot were recorded from the 3rd day of sowing while the agronomic data (shoot length, root length, fresh and dry weight of leaf, shoot and root) were recorded after 55 days at harvest. In addition, the plants were refrigerated at 4 °C for one week to analyze their physiological characteristics. After 5 days, three pots were kept under control and watered regularly. Each treatment had three replicates, with 15 plants initially planted in each pot. The plants were then thinned, leaving only five plants per pot. To prevent surface fungal/bacterial contamination, surface sterilization with 95% ethanol along with distilled water was performed on seeds which were then sown in pots containing 3 kg of soil. Standard laboratory techniques were used to characterize the soil physically and chemically before the experiment (Jamal et al. 2023). Aligned with the USDA-NRCS classification, the experimental soil texture is classified as clay loam (33% clay, 18% sand, and 49% silt). It is naturally calcareous (162.32 g kg^{-1} lime), has low organic matter content (8.7 g kg^{-1}), has an electrical conductivity of 0.26 dS m^{-1}, and is alkaline in reaction (pH 8.2). In addition, total nitrogen (0.05 g kg^{-1}), exchangeable potassium (AB-DTPA) (128 mg kg^{-1}) and low levels of extractable phosphorus (4.45 mg kg^{-1}) were observed in the soil. Using a pressure pump, exogenous growth mediators (AsA and α-toc) were applied to the plants after seedling emergence (4–5 leaf stage) at a dose of 150 mg L^{-1}. The other plants were treated with salinity stress after the emergence of seedlings at the 4–5 leaf stage (early growth stage), using 40 mM and 80 mM NaCl solutions. These solutions were prepared by dissolving 2.34 g and 4.68 g of NaCl, respectively, in distilled water and diluting each to a final volume of 1 liter, while control conditions were kept in three pots and watered regularly. The salinity stress and exogenous growth mediators were applied to the plants twice a week to ensure consistent exposure throughout the experiment. Germination and agronomic data of the vegetative growth stage were recorded.

Measurements

Germination and Agronomic Parameters

Using the procedure of Ullah et al. (2022), germination and agronomic indices were calculated by the following formulas:

Germination Percentage (GP)

$$GP = \frac{\text{Final number of seedlinsg emerged}}{\text{Total number of seeds sown}} \times 100 \quad (1)$$

Mean Germination Time (MGT)

$$MGT = \frac{\sum (\mathbf{n} \times \mathbf{d})}{\mathbf{N}} \quad (2)$$

where *n* is the number of seeds germinated each day, *d* is the number of days from the beginning of the test, and *N* is the total number of seeds germinated at the termination of the experiment.

Mean Germination Rate (MGR)

$$MGR = \frac{1}{\text{Mean Germination Time}} \quad (3)$$

Coefficient of the Velocity of Germination (CVG)

$$CVG = \frac{N1 + N2 + N3 \ldots Nx}{100} \times N1T1 \ldots .NxTx \quad (4)$$

where *N* is the percentage of seeds that sprout each day, and *T* is the period between planting and seed germination for N.

Germination Index (GI)

$$GI = (10 \times n1) + (9n \times n2) \ldots, (1n \times 10) \quad (5)$$

where *n1, n2...n10* represent the frequency of seeds that germinated on the first, second, and subsequent days until the final day.

Germination Rate Index (GRI)

$$GRI = \frac{G1}{1} + \frac{G2}{2} + \frac{G3}{3} \ldots . \frac{Gx}{x} \quad (6)$$

where *G1* and *G2* denote percent germinations on the first and second days after sowing and *Gx* is the final germination percentage on the last day.

Timson Germination Index (TGI)

$$TGI = \frac{\in G}{T} \quad (7)$$

where *G* represents the daily germination rate as a whole and *T* denotes the germination time.

Germination Energy (GE)

$$GE = \frac{X1}{Y1} + \left(\frac{X2 - X1}{Y2}\right) + \left(\frac{Xn - Xn - 1}{Yn}\right) \quad (8)$$

In the above equation, X_1, X_2 and X_n denote the frequency of seeds that emerged on the 1st day, 2nd day, and so on. The days from sowing to the 1st, 2nd, and final days are denoted by Y_1, Y_2, and Y_n, respectively.

Time to 50% Germination (T50%)

$$T50\% = \frac{ti + (N/2 - ni)(tj - ti)}{(nj - ni)} \quad (9)$$

where *N* is the total number of seeds that eventually emerged, while *nj* and *ni* are the total number of seeds that emerged following adjacent counts during *tj* and *ti*, respectively.

Root-shoot Ratio (RSR)

$$RSR = \frac{\text{root dry weight}}{\text{shoot dry weight}} \quad (10)$$

Seed Vigour Indices (SVI-I and II)

$$SVI - I = \text{Seedlings length} \times \%\ \text{Germination} \quad (11)$$

$$SVI - II\ \text{Seedling dry weight} \times \%\ \text{Germination} \quad (12)$$

Coefficient of Variation of Germination Time (CVt)

The method proposed by Ranal et al. (2009) calculates the coefficient of variation of the germination time.

$$CV_t = \frac{St}{t} \times 100 \quad (13)$$

where *St* represents the standard deviation of the germination time, and *t* denotes the mean germination time.

Percent Moisture Content (PMC)

The percent moisture content of each plant part (leaf, shoot and root) was calculated individually. The PMC index was calculated by knowing the corresponding fresh and dry weights using the method of Mubeen et al. (2021).

$$\text{Root Moisture Content (RMC)} = \frac{\text{Wet weight of root} - \text{Dry weight of root}}{\text{wet Weight of root}} \quad (14)$$

$$\text{Shoot Moisture Content (SMC)} = \frac{\text{Wet weight of shoot} - \text{Dry weight of shoot}}{\text{wet Weight of shoot}} \quad (15)$$

$$\text{Leaf Moisture Content (LMC)} = \frac{\text{Wet weight of leaf} - \text{Dry weight of leaf}}{\text{wet Weight of leaf}} \quad (16)$$

Physiological and Biochemical Parameters

Photosynthetic Pigment

Chlorophyll (Chl a and Chl b) and total chlorophyll were measured by immersing 200 mg w/w of leaf material in 8 mL of acetone 80% solution (v/v) for 48 h at –4 °C in the dark. UV-visible spectrophotometer (Ultraviolet-2600, Shimadzu, Japan) was employed to check the absorbance of carotenoid content and chlorophyll in the extract. For the calculation of Total photosynthetic pigments following formulas were utilized by Sumanta et al. (2014):

$$\text{Chlorophyll}\, a \left(\text{mg}g^{-1}\text{FW}\right) = (0.0127 \times A663) - (0.00269 \times A645) \quad (17)$$

$$\text{Chlorophyll}\, b \left(\text{mg}g^{-1}\text{FW}\right) = (0.0229 \times A645) - (0.00468 \times A663) \quad (18)$$

$$\text{Total Chlorophyll}\, a + b \left(\text{mg}g^{-1}\text{FW}\right) = (0.0202 \times \text{A645}) + (0.00802 \times \text{A663}) \quad (19)$$

The following equation was used for the calculation of the chlorophyll *a/b* ratio:

$$Chl\frac{a}{b}\, ratio = \text{chlorophyll, a/b ratio} \quad (20)$$

As suggested by Ahmad and Anis, (2019) Carotenoid content (CAR) was determined by the formula:

$$\text{Carotenoid} = \text{A480} + (0.114 \times \text{A663}) - (0.638 \times \text{A645}) \quad (21)$$

where A is the absorbance at the respective wavelength and *V* represents the volume of extract in mL.

Soluble Protein Content (SPC), Soluble Sugar Content (SSC), Total Proline Content (TPC) and Glycine Betaine Content (GB)

We used El-Beltagi et al. (2022a) procedure to determine soluble protein quantity. Young leaves were collected when the leaf is the youngest fully expanded leaf (YFEL) on the plant. Fresh leaves (0.2 g) were crushed in 1 mL of phosphate buffer (pH 7.5) with the help of mortar and pestle. 1 mL total volume was prepared by mixing distilled water and 0.1 mL of the above extract. 3.0 mL of the reagent containing 1.5 g Na-K tartrate and 0.125 g $CuSO_4.5H_2O$ dissolved in 25 mL of distilled water was added. The reagent also contained 3 g sodium carbonate (Na_2CO_3) and 0.6 g NaOH (0.1 N). 0.1 mL of Folin phenol reagent was added, after shaking for 10 min. After incubation for 30 min, the absorbance of each sample was measured at 650 nm. The contents were calculated with the formula:

$$\text{Protein\%}\left(\frac{\text{W}}{\text{W}}\right) = \text{Cp} \times \text{V} \times \frac{\text{DF}}{\text{wt}} \quad (22)$$

where *wt* is leaf weight (mg), *Cp* is the concentration of protein (mg L^{-1}), *DF* is the dilution factor, and *V* is buffer lysis volume.

Using the youngest completely developed leaf, the El-Beltagi et al. (2022b) methodology was used to determine the amount of soluble sugars. Using an Ultraviolet-2600, Shimadzu, Japan, spectrophotometer, the amount of soluble sugar was measured at 420 nm. Proline was quantified spectrophotometrically (Ultraviolet-2600, Shimadzu, Japan) at 520 nm and expressed on a fresh matter basis. For the standard, L-proline was used.

With slight modifications, glycine betaine (GB) content was quantified using the methods given by Zhang et al. (2013). After 7 days of treatment, samples were collected from each flask, cleaned with distilled water, and dried at 80 °C to a constant weight. Dry tissue material was used to extract GB. After storage at 4 °C for 24 h, 1 mL of extract was combined with 1 mL of H_2SO_4 (2 N) and 0.4 mL of potassium tri-iodide solution. It was then centrifuged at 15,000 g for 15 min at 0 °C. The supernatant was carefully aspirated using a fine-tipped glass tube and 9 mL of 1, 2-dichloroethane was used to dissolve the periodide crystals. After 2 h, the absorbance at 365 nm was determined spectrophotometrically (Ultraviolet-2600, Shimadzu, Japan). The GB content was calculated using the following equation:

$$\text{GB} = \frac{\text{A} \times \text{DF} \times \text{MW} \times 1000}{\varepsilon \times \text{L}} \quad (23)$$

where *A* represents the change in absorbance. The dilution factor is denoted by *DF*, the molecular weight is denoted by *MW*, and the extinction coefficient is denoted by ε.

Hydrogen Peroxide (H_2O_2) and Malondialdehyde (MDA)

According to Velikova et al. (2006), the amount of hydrogen peroxide was determined following the reaction with potassium iodide (KI). The reaction mixture was kept in the dark for 1 h. Using a standard curve generated with H_2O_2 solutions, the amount of H_2O_2 was determined spectrophotometrically (Ultraviolet-2600, Shimadzu, Japan) at 390 nm. To calculate the total protein content (TPC) of leaves, the technique described by Ouzounidou et al. (2016) was used, where 100 mg of leaves were used for TPC determination.

Malondialdehyde (MDA) content was determined using the technique of Zhang and Kirkham, (1996). Briefly, 1 mL of the extract was mixed with a tube containing 20% (v/v) trichloroacetic acid and 0.5% (v/v) thiobarbituric acid. After cooling to room temperature, the mixture was heated in a water bath for 30 min at 95 °C and centrifuged for 10 min at 10,000 g. At 530 and 600 nm wavelengths, the absorbance of the supernatant was measured. The absorbance at 600 nm for non-specific absorption was subtracted from the result at 530 nm. MDA content was determined as follows:

$$\text{MDA} = D(A532nm - A600nm/1.56 \times 105 \tag{24}$$

Antioxidant Enzyme Assays

Firstly, the powdered samples were centrifuged for 10 min at 14,000 g maintaining the temperature at 4 °C. after extracting 0.1 g of fresh leaves from each sample in 1 mL of 50 mM potassium phosphate buffer at pH 7.4. The resulting supernatant was isolated and used for various enzymatic and non-enzymatic analyses. All analyses were performed in duplicate.

The activity of Superoxide dismutase (SOD) was checked by the method suggested by Flohe, (1984), the mixture was exposed to white light for a time of 15 min while the temperature was maintained at room level so that the SOD reaction could be initiated. At 560 nm, the absorbance of the solution was measured after 15 min of incubation for the control and blank samples.

Peroxidase (POD) extraction was also performed as described by Piechowiak et al. (2020). The reaction mixture contained 0.05 mL of 20 mM guaiacol, 3 mL of 0.1 M phosphate buffer (pH 7.0), 0.5 mL enzyme and 0.03 mL of H_2O_2. An increase in absorbance at 420 nm indicated enzymatic activity (Ultraviolet-2600, Shimadzu, Japan).

Activity of Ascorbate peroxidase (APOX) was detected by decrease in absorbance at 240 nm in alignment with the method of Livingstone et al. (1992) with certain modifications. The 3 mL reaction medium contained 100 mm HOAc-NaOAc buffer (pH 5.8), 100 µL enzyme extract, 3 µM EDTA, 5 mm H_2O_2, and 10 mm ascorbic acid. The extracted leaves were also used to measure ascorbate peroxidase enzyme activity.

$$\text{Enzyme Activity} = \Delta\text{A} \times \text{Total assay}\frac{\text{volume}}{\Delta\text{t}} \times \in \times\text{i} \times \text{Enzyme sample volume} \tag{25}$$

where *A* is the change in absorbance, *t* is the incubation time, and *E* is the absorbance coefficient of the substrate.

The initial rate of quinone formation helped in detecting the activity of polyphenol oxidase (PPO) since it showed a rise in absorbance units at 420 nm; therefore, we adopted the procedure of Cho and Ahn, (1999) with some modifications.

The catalase activity (CAT) of the leaf extract was measured according to the method of Livingstone et al. (1992). The 3 mL of reaction mixture contains 15 mm, 50 mm phosphate buffer (pH 7.0) and $H_2O_2$100 µL enzyme extract. The reaction was started after the addition of enzyme extract. Changes in absorbance were measured every minute based on the linear decrease in absorbance at 240 nm during the first 3 min.

The technique used to extract glutathione reductase was modified from that described by Piechowiak et al. (2020). Briefly, 0.2 mL of 0.2 M potassium phosphate buffer (pH 7.5), 0.1 mL of 0.2 mM EDTA, 1.5 mL of $MgCl_2$, 0.2 mL of 0.5 mM NADPH, 0.2 mL of enzyme extract, and 0.2 mL of 2 mM glutathione were added to a quartz cuvette to initiate the reaction. Enzyme activity was calculated by reading the decrease in absorbance at 340 nm at intervals of 30 s to 3 min.

Data Analysis

Microsoft Excel 2010 (Redmond, DC, USA) was used to calculate and analyze the standard error and mean of data. Co-Stat Window version 6.3 was used to perform an analysis of variance (ANOVA) to determine significant differences between treatments. Standard procedures were used to calculate means and standard errors. The Least Significant Difference (LSD) test was also performed at (±0.001). The XLSTAT statistical package software (ver. 2014, Excel Add-ins software SARL, New York, NY, USA) was used for graphical presentation, Pearson correlation matrix, principal component analysis (PCA) and heat map between the measured variables of barley.

Table 2 Effect of ascorbic acid and α-tocopherol on germination percentage (GP), mean germination time (MGT), mean germination rate (MGR), coefficient of velocity of germination (CVG), germination index (GI), germination rate index (GRI), Timson germination index (TGI), germination energy (GE) of barley under induced salinity stress

Treatments	GP		MGT		MGR		CVG		GI		GRI		TGI		GE	
Control	86.6$^{\pm 9.4}$	a	5.3$^{\pm 0.1}$	abc	0.1$^{\pm 0.0}$	b	4.0$^{\pm 0.8}$	b	49.0$^{\pm 5.7}$	abc	85.9$^{\pm 17.0}$	bcd	49.0$^{\pm 5.7}$	abc	2.4$^{\pm 0.6}$	b
S_{40}	70.5$^{\pm 8.3}$	c	4.5$^{\pm 0.1}$	abcd	0.2$^{\pm 0.0}$	b	3.9$^{\pm 0.8}$	c	40.7$^{\pm 4.5}$	abc	91.3$^{\pm 12.4}$	abc	41.0$^{\pm 5.7}$	abc	2.7$^{\pm 0.4}$	b
S_{40} + AsA	80.5$^{\pm 9.5}$	b	3.3$^{\pm 0.2}$	bcd	0.2$^{\pm 0.0}$	b	3.2$^{\pm 1.2}$	c	50.3$^{\pm 8.1}$	abc	99.3$^{\pm 9.8}$	abcd	48.3$^{\pm 9.2}$	abc	2.9$^{\pm 0.3}$	b
S_{40} + α-toc	74.7$^{\pm 5.6}$	c	4.9$^{\pm 0.1}$	a	0.2$^{\pm 0.0}$	a	2.9$^{\pm 0.4}$	a	52.0$^{\pm 4.3}$	abc	97.7$^{\pm 12.0}$	ab	47.7$^{\pm 3.3}$	a	2.9$^{\pm 0.4}$	a
S_{40}(AsA + α-toc)	82.3$^{\pm 9.9}$	ab	5.0$^{\pm 0.3}$	ab	0.2$^{\pm 0.0}$	a	2.7$^{\pm 0.5}$	b	51.3$^{\pm 4.5}$	a	99.0$^{\pm 20.1}$	a	49.7$^{\pm 12.5}$	a	2.9$^{\pm 0.4}$	a
S_{80}	70.7$^{\pm 9.4}$	c	2.1$^{\pm 0.1}$	ab	0.1$^{\pm 0.0}$	a	3.0$^{\pm 0.9}$	b	41.0$^{\pm 5.7}$	ab	90.2$^{\pm 13.9}$	ab	40.0$^{\pm 5.6}$	ab	2.2$^{\pm 0.5}$	a
S_{80} + AsA	81.0$^{\pm 16.3}$	b	3.9$^{\pm 0.2}$	cd	0.1$^{\pm 0.0}$	c	3.2$^{\pm 0.3}$	c	43.3$^{\pm 11.0}$	c	97.8$^{\pm 2.2}$	d	42.3$^{\pm 8.8}$	bc	2.2$^{\pm 0.7}$	c
S_{80} + α-toc	73.7$^{\pm 4.7}$	c	3.7$^{\pm 0.2}$	cd	0.1$^{\pm 0.0}$	c	2.0$^{\pm 1.4}$	d	47.7$^{\pm 3.3}$	abc	91.0$^{\pm 8.8}$	cd	43.7$^{\pm 3.3}$	bc	2.7$^{\pm 0.2}$	c
S_{80}(AsA + α-toc)	81.7$^{\pm 12.4}$	b	3.6$^{\pm 0.6}$	d	0.2$^{\pm 0.0}$	c	2.0$^{\pm 1.5}$	d	49.7$^{\pm 12.4}$	bc	93.3$^{\pm 28.8}$	cd	46.7$^{\pm 12.5}$	c	2.8$^{\pm 0.9}$	c

Control (0 mM salinity +0 mM Ascorbic Acid +0 mM α-Tocopherol), S40 (Salinity 40 mM), S40+AsA (Salinity 40 mM+ Ascorbic Acid 40 mM), S40 + α-toc (Salinity 40 mM+ α-Tocopherol 40 mM), S40 (AsA + α-toc) (Salinity 40 mM+ Ascorbic Acid 40 mM+ α-Tocopherol 40 mM), S80 (Salinity 80 mM), S80 + AsA (Salinity 80 mM+ Ascorbic Acid 80 mM), S80 + α-toc (Salinity 80 mM)+ α-Tocopherol (80 mM), S80 (AsA + α-toc) (Salinity 80 mM+ Ascorbic Acid 80 mM+ α-Tocopherol 80 mM). Data represents means of 3 replications or $n=3$ and the lower-case letters a–d denoted significant and non-significant changes in the mean values

Table 3 Effect of ascorbic acid and α-tocopherol on time to 50% germination (T50%), root shoot ratio (RSR), seed vigor index-I (SVI-I), seed vigor index-II (SVI-II), coefficient of variation of germination time (CVt), leaf fresh weight (LFW), leaf dry weight (LDW) of barley under induced salinity stress

Treatments	T50%		RSR		SVI-I		SVI-II		CVt		LFW		LDW	
Control	3.9$^{\pm 0.3}$	a	0.001$^{\pm 0.010}$	a	4.0$^{\pm 0.2}$	bc	12.6$^{\pm 4.5}$	ab	46.9$^{\pm 7.5}$	ab	0.2$^{\pm 0.07}$	ab	0.005$^{\pm 0.006}$	a
S_{40}	2.4$^{\pm 0.4}$	c	0.002$^{\pm 0.000}$	a	3.1$^{\pm 0.1}$	cd	4.3$^{\pm 0.7}$	bc	51.6$^{\pm 3.3}$	ab	0.1$^{\pm 0.02}$	ab	0.002$^{\pm 0.002}$	a
S_{40} + AsA	3.2$^{\pm 0.5}$	b	0.003$^{\pm 0.001}$	a	3.6$^{\pm 1.3}$	c	8.7$^{\pm 1.7}$	bc	59.6$^{\pm 1.3}$	bc	0.2$^{\pm 0.01}$	ab	0.004$^{\pm 0.003}$	a
S_{40} + α-toc	3.6$^{\pm 0.2}$	ab	0.005$^{\pm 0.003}$	a	3.2$^{\pm 0.2}$	a	4.8$^{\pm 2.1}$	a	57.6$^{\pm 5.4}$	a	0.1$^{\pm 0.01}$	a	0.003$^{\pm 0.002}$	a
S_{40}(AsA + α-toc)	3.6$^{\pm 0.7}$	ab	0.006$^{\pm 0.002}$	a	3.4$^{\pm 0.9}$	ab	5.3$^{\pm 1.2}$	abc	53.3$^{\pm 1.6}$	a	0.2$^{\pm 0.02}$	ab	0.004$^{\pm 0.002}$	a
S_{80}	2.3$^{\pm 0.3}$	c	0.001$^{\pm 0.002}$	a	3.1$^{\pm 0.1}$	a	3.3$^{\pm 0.8}$	abc	41.6$^{\pm 2.3}$	ab	0.1$^{\pm 0.01}$	ab	0.001$^{\pm 0.002}$	a
S_{80} + AsA	3.1$^{\pm 0.4}$	b	0.002$^{\pm 0.001}$	a	3.3$^{\pm 1.4}$	de	6.8$^{\pm 1.6}$	abc	49.6$^{\pm 1.3}$	bc	0.1$^{\pm 0.02}$	ab	0.003$^{\pm 0.003}$	a
S_{80} + α-toc	3.4$^{\pm 0.1}$	ab	0.002$^{\pm 0.002}$	a	3.3$^{\pm 0.3}$	e	3.9$^{\pm 2.2}$	c	47.6$^{\pm 1.4}$	bc	0.12$^{\pm 0.01}$	b	0.002$^{\pm 0.002}$	a
S_{80}(AsA + α-toc)	3.5$^{\pm 0.6}$	ab	0.005$^{\pm 0.003}$	a	3.3$^{\pm 0.9}$	e	4.2$^{\pm 1.3}$	c	43.3$^{\pm 2.6}$	c	0.18$^{\pm 0.02}$	b	0.003$^{\pm 0.001}$	a

Control (0 mM salinity +0 mM Ascorbic Acid +0 mM α-Tocopherol), S40 (Salinity 40 mM), S40+AsA (Salinity 40 mM+ Ascorbic Acid 40 mM), S40 + α-toc (Salinity 40 mM+ α-Tocopherol 40 mM), S40 (AsA+ α-toc) (Salinity 40 mM+ Ascorbic Acid 40 mM + α-Tocopherol 40 mM), S80 (Salinity 80 mM), S80+AsA (Salinity 80 mM + Ascorbic Acid 80 mM), S80 + α-toc (Salinity 80 mM)+ α-Tocopherol 80 mM), S80 (AsA+ α-toc) (Salinity 80 mM + Ascorbic Acid 80 mM + α-Tocopherol 80 mM) and T1-T8 represents the treatments. Data represents means of 3 replications or $n=3$ and the lower-case letters a-e denoted significant and non-significant changes in the mean values

Table 4 Effect of ascorbic acid and α-tocopherol on barley Leaf moisture content (LMC), Shoot fresh weight (SFW), Shoot dry weight (SDW), Shoot moisture content (SMC), Root fresh weight (RFW), Root dry weight (RDW), Root moisture content (RMC) under induced salinity stress

Treatments	LMC		SFW		SDW		SMC		RFW		RDW		RMC	
Control	$0.09^{\pm 0.002}$	ab	$0.5^{\pm 0.1}$	a	$0.068^{\pm 0.012}$	a	$0.086^{\pm 0.008}$	ab	$0.16^{\pm 0.08}$	bc	$0.80^{\pm 0.05}$	ab	$0.06^{\pm 0.02}$	a
S_{40}	$0.09^{\pm 0.001}$	a	$0.2^{\pm 0.1}$	a	$0.027^{\pm 0.009}$	a	$0.074^{\pm 0.027}$	ab	$0.04^{\pm 0.01}$	ab	$0.25^{\pm 0.01}$	ab	$0.04^{\pm 0.02}$	ab
S_{40} + AsA	$0.09^{\pm 0.001}$	a	$0.9^{\pm 0.4}$	a	$0.078^{\pm 0.036}$	a	$0.092^{\pm 0.003}$	a	$0.06^{\pm 0.02}$	a	$0.32^{\pm 0.01}$	ab	$0.06^{\pm 0.02}$	a
S_{40} + α-toc	$0.09^{\pm 0.001}$	a	$0.3^{\pm 0.1}$	a	$0.032^{\pm 0.008}$	a	$0.091^{\pm 0.002}$	a	$0.06^{\pm 0.02}$	a	$0.30^{\pm 0.02}$	ab	$0.05^{\pm 0.03}$	a
S_{40}(AsA + α-toc)	$0.09^{\pm 0.002}$	ab	$0.5^{\pm 0.1}$	a	$0.044^{\pm 0.010}$	a	$0.091^{\pm 0.001}$	a	$0.08^{\pm 0.01}$	ab	$0.39^{\pm 0.00}$	ab	$0.07^{\pm 0.02}$	a
S_{80}	$0.08^{\pm 0.001}$	ab	$0.2^{\pm 0.1}$	a	$0.024^{\pm 0.009}$	a	$0.064^{\pm 0.021}$	a	$0.03^{\pm 0.01}$	abc	$0.23^{\pm 0.01}$	abc	$0.04^{\pm 0.01}$	a
S_{80} + AsA	$0.09^{\pm 0.002}$	a	$0.9^{\pm 0.4}$	a	$0.068^{\pm 0.036}$	a	$0.082^{\pm 0.002}$	ab	$0.05^{\pm 0.02}$	d	$0.29^{\pm 0.02}$	a	$0.05^{\pm 0.02}$	a
S_{80} + α-toc	$0.09^{\pm 0.001}$	a	$0.3^{\pm 0.1}$	a	$0.030^{\pm 0.008}$	a	$0.087^{\pm 0.001}$	a	$0.05^{\pm 0.01}$	d	$0.30^{\pm 0.02}$	bc	$0.04^{\pm 0.03}$	a
S_{80}(AsA + α-toc)	$0.09^{\pm 0.001}$	a	$0.5^{\pm 0.1}$	a	$0.039^{\pm 0.010}$	a	$0.089^{\pm 0.002}$	a	$0.06^{\pm 0.02}$	a	$0.37^{\pm 0.01}$	bc	$0.06^{\pm 0.02}$	a

Control (0 mM salinity +0 mM Ascorbic Acid +0 mM α-Tocopherol), S40 (Salinity 40 mM), S40+AsA (Salinity 40 mM+ Ascorbic Acid 40 mM), S40 + α-toc (Salinity 40 mM+ α-Tocopherol 40 mM), S40 (AsA+ α-toc) (Salinity 40 mM+ Ascorbic Acid 40 mM + α-Tocopherol 40 mM), S80 (Salinity 80 mM), S80+AsA (Salinity 80 mM + Ascorbic Acid 80 mM), S80 + α-toc (Salinity 80 mM)+ α-Tocopherol 80 mM), S80 (AsA+ α-toc) (Salinity 80 mM + Ascorbic Acid 80 mM + α-Tocopherol 80 mM) and T1-T8 represents the treatments. Data represents means of 3 replications or $n = 3$ and the lower-case letters a-d denoted significant and non-significant changes in the mean values.

Results

Agronomic Characteristics

The present experiment yielded significant results, elucidating that the growth parameters of barley exhibit significant enhancement when subjected to induced salinity stress and treated with α-toc and AsA. Conversely, a considerable decrease in the morphological functioning of barley was noticed under varying levels of salinity stress, particularly at 40 mM and 80 mM, as indicated by data pertaining to growth and germination parameters (Table 2, 3 and 4; Fig. 1). A marked decrease ($p \leq 0.001$) was noted in various parameters, including germination percentage (GP), root-shoot ratio (RSR), mean germination rate (MGR), germination index (GI), Timson germination index (TGI), germination rate index (GRI), germination energy (GE), time to 50% germination (T50%), seed vigor indices (SVI-I, SVI-II), coefficient of variation of germination time (CVt), leaf dry weight (LDW), leaf fresh weight (LFW), leaf moisture content (LMC), shoot dry weight (SDW), shoot fresh weight (SFW), shoot moisture content (SMC), root dry weight (RDW), root fresh weight (RFW) and root moisture content (RMC) under the influence of 40 mM and 80 mM salinity stress.

Specifically, the most favorable outcomes in terms of GP, MGT, MGR, GI, TGI, T50%, and RSR were observed at 40 mM and 80 mM salinity levels when treated with S_{40} (AsA + α-toc) (T4) and S_{80}(AsA + α-toc) (T8) (Tables 2 and 3). Fig. 1 visually represents a continuous decline in these characteristics with escalating salinity levels in the growth media. Notably, plants on which AsA and α-toc were applied exhibited higher values for these characteristics in comparison with both control and stressed plants. Furthermore, the interaction analysis of α-toc and AsA revealed that plant growth regulators (PGRs) exert a positive influence on the agronomic characteristics and germination of barley. Importantly, AsA demonstrated greater efficacy than α-toc in reducing the detrimental effects of salinity over barley.

Data indicated a significant reduction in leaf moisture content (LMC), shoot dry weight (SDW), shoot fresh weight (SFW), shoot moisture content (SMC), root dry weight (RDW), root fresh weight (RFW), and root moisture content (RMC) under control salinity treatments T1 and T5 (Table 4). Maximum values for SFW (0.9 ± 0.4) and SDW (0.078 ± 0.036) were recorded in salinity treatments with AsA (T2), while maximum SMC was observed in T2 (0.092 ± 0.003) and T8 (0.089 ± 0.002). Additionally, maximum values for RFW, RDW, and RMC were recorded in salinity treatments with the combined application of α-toc and AsA (T4 and T8).

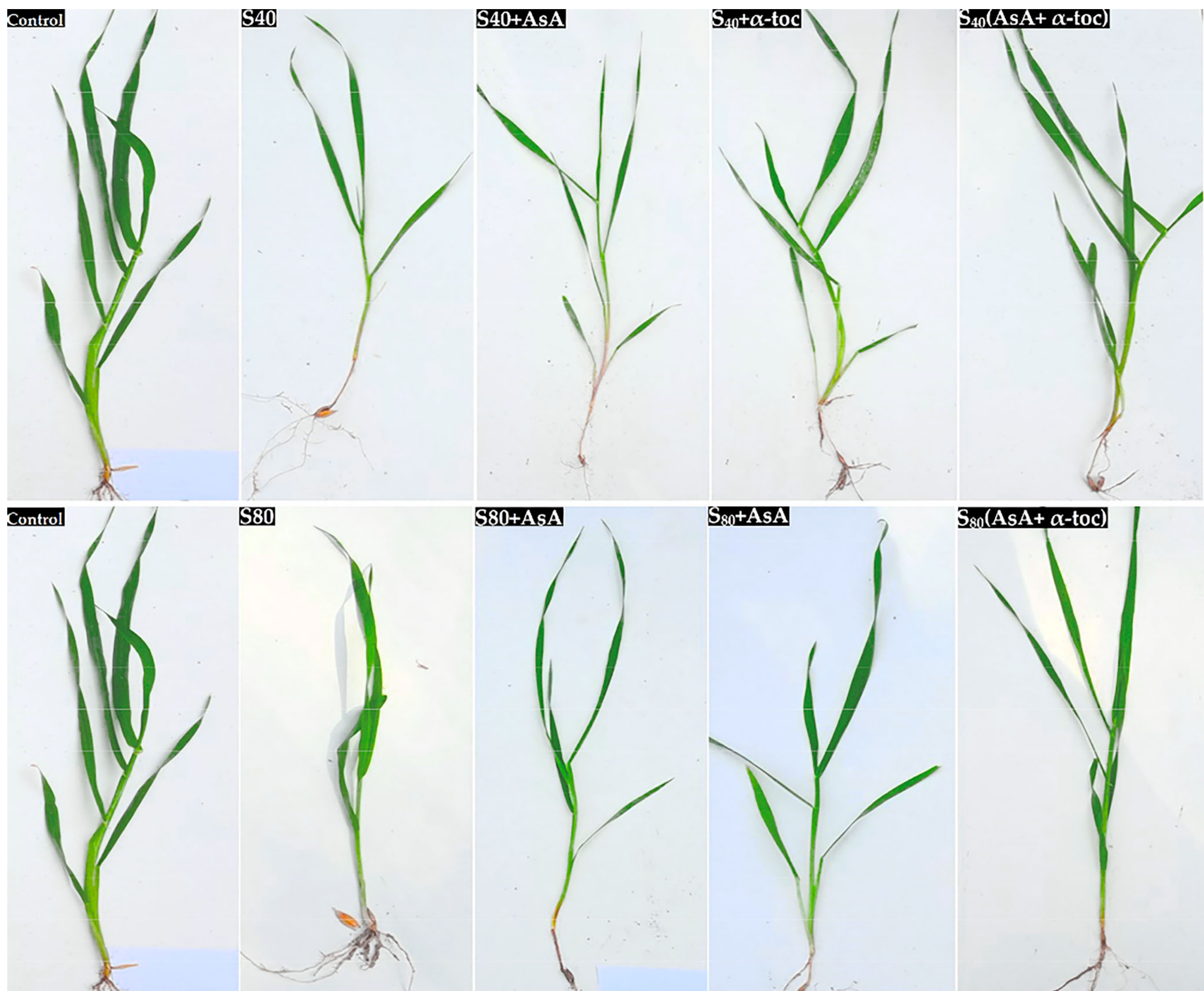

Fig. 1 Morphological response of barley to ascorbic acid and α-tocopherol under induced (40 and 80 mM) salinity stress. Control (0 mM salinity +0 mM Ascorbic Acid +0 mM α-Tocopherol), S_{40} (Salinity 40 mM), S_{40} + AsA (Salinity 40 mM+ Ascorbic Acid 40 mM), S_{40} + α-toc (Salinity 40 mM+ α-Tocopherol 40 mM), S_{40} (AsA + α-toc) (Salinity 40 mM+ Ascorbic Acid 40 mM+ α-Tocopherol 40 mM), S_{80} (Salinity 80 mM), S_{80} + AsA (Salinity 80 mM+ Ascorbic Acid 80 mM), S_{80} + α-toc (Salinity 80 mM)+ α-Tocopherol 80 mM), S_{80} (AsA + α-toc) (Salinity 80 mM+ Ascorbic Acid 80 mM+ α-Tocopherol 80 mM)

Physiological and Biochemical Properties

Salinity stress exerted a detrimental impact on various physiological attributes while concurrently enhancing antioxidant activities. Chlorophyll a and b (Chl. a & b) contents significantly decreased ($p \leq 0.001$; Table S1) to 0.35 and 0.31, respectively, under 80 mM salinity stress compared to the control treatment (Fig. 2a, b). Notably, the application of both ascorbic acid (AsA) and α-tocopherol (α-toc) demonstrated a positive effect on chlorophyll content under both 40 mM and 80 mM salinity stress. Salinity significantly ($p \leq 0.001$) reduced total chlorophyll content (TCC) in the control from 1.75 to 0.95 and 1.56 at 40 mM and 80 mM, respectively. However, AsA and α-toc ameliorated TCC in both treatments (Fig. 2c).

Barley treated with only ascorbic acid and/or combined with α-Tocopherol produced significant results, such as minimizing the damage to photosynthesis pigments induced by NaCl. For example, when barley was treated with AsA+α-toc and subjected to S40 and S80 salt stress, there was a decrease of 5.76% and 30.59% in the damage to photosynthesis pigments compared to just applying salt stress alone. When exposed to high salt levels, applying AsA+α-toc to the leaves reduced the negative effects and plant damage caused by NaCl, leading to a 9.61% and 42.62% rise in chlorophyll levels in plants subjected to S40 and S80, respectively. A significant ($p \leq 0.001$) reduction

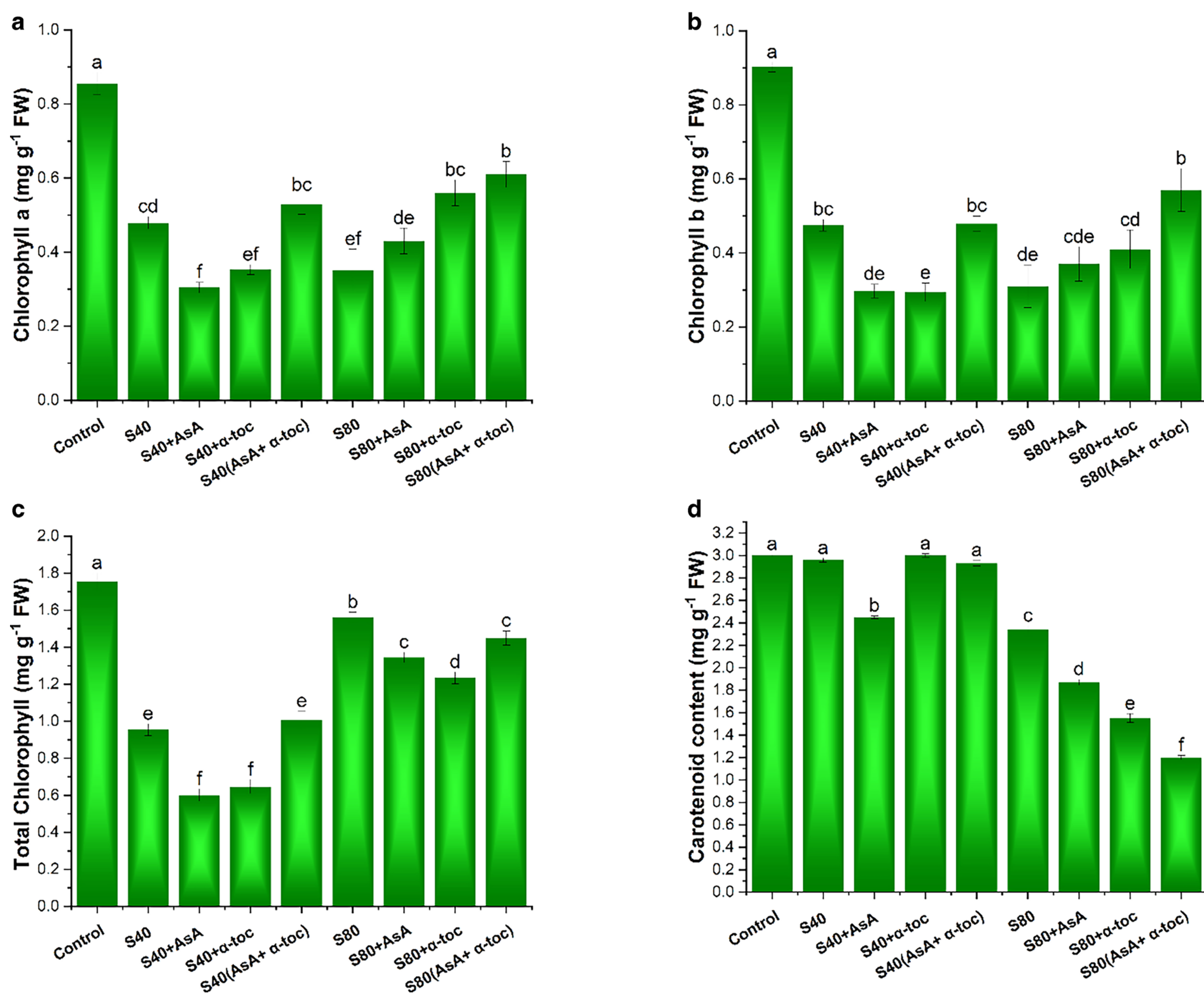

Fig. 2 Effect of ascorbic acid and α-tocopherol on barley **a** Chlorophyll *a* (Chl. *a)*, **b** Chlorophyll *b* (Chl. *b)*, **c** Total Chlorophyll (TCC) and **d** carotenoid content (CAR) under induced salinity stress. Control (0 mM salinity +0 mM Ascorbic Acid +0 mM α-Tocopherol), S40 (Salinity 40 mM), S40+AsA (Salinity 40 mM+ Ascorbic Acid 40 mM), S40 + α-toc (Salinity 40 mM+ α-Tocopherol 40 mM), S40 (AsA+ α-toc) (Salinity 40 mM+ Ascorbic Acid 40 mM + α-Tocopherol 40 mM), S80 (Salinity 80 mM), S80+AsA (Salinity 80 mM + Ascorbic Acid 80 mM), S80 + α-toc (Salinity 80 mM) + α-Tocopherol 80 mM), S80 (AsA+ α-toc) (Salinity 80 mM + Ascorbic Acid 80 mM + α-Tocopherol 80 mM). Vertical lines in bars denoted ± SE. Data represents means of 3 replications or $n = 3$ and the lower-case letters a–f denoted significant and non-significant changes in the mean values

in carotenoid content (CAR) was observed under salinity stress, with the control experiencing a decrease from 3.00 to 2.96 and 2.34 at 40 mM and 80 mM, respectively (T1 and T5). Application of AsA and α-toc exhibited a positive effect on CAR at salinity level S_{40} (Fig. 2d).

Furthermore, a significant increase in soluble protein content (SPC) and soluble sugar content (SSC) was observed, rising from 0.50 to 1.43 and from 0.82 to 0.89, respectively, under 80 mM salinity stress (T5) (Fig. 3a, b). This indicates an augmentation of SPC and SSC with both salinity and exogenous application of α-toc and AsA. Total proline content (TPC) decreased under salinity stress compared to the control; however, relatively high concentrations were observed in all treatments receiving foliar sprays of AsA, α-toc, or both (Fig. 3c). Additionally, glycine betaine (GB) content increased significantly under salt stress, reaching its highest concentration (1.67) under 80 mM salinity stress, and the lowest concentrations were recorded when AsA and α-toc were applied topically under 40 mM salinity stress (Fig. 3d).

The maximum hydrogen peroxide (H_2O_2) levels, significant at $p \leq 0.001$ (Table S1), were recorded in plants grown under 80 mM salinity stress (T5), while the minimum levels were observed in plants treated with ascorbic acid under 40 mM salinity stress (T3) (Fig. 4a). Moreover, malondialdehyde (MDA) levels were significantly elevated in bar-

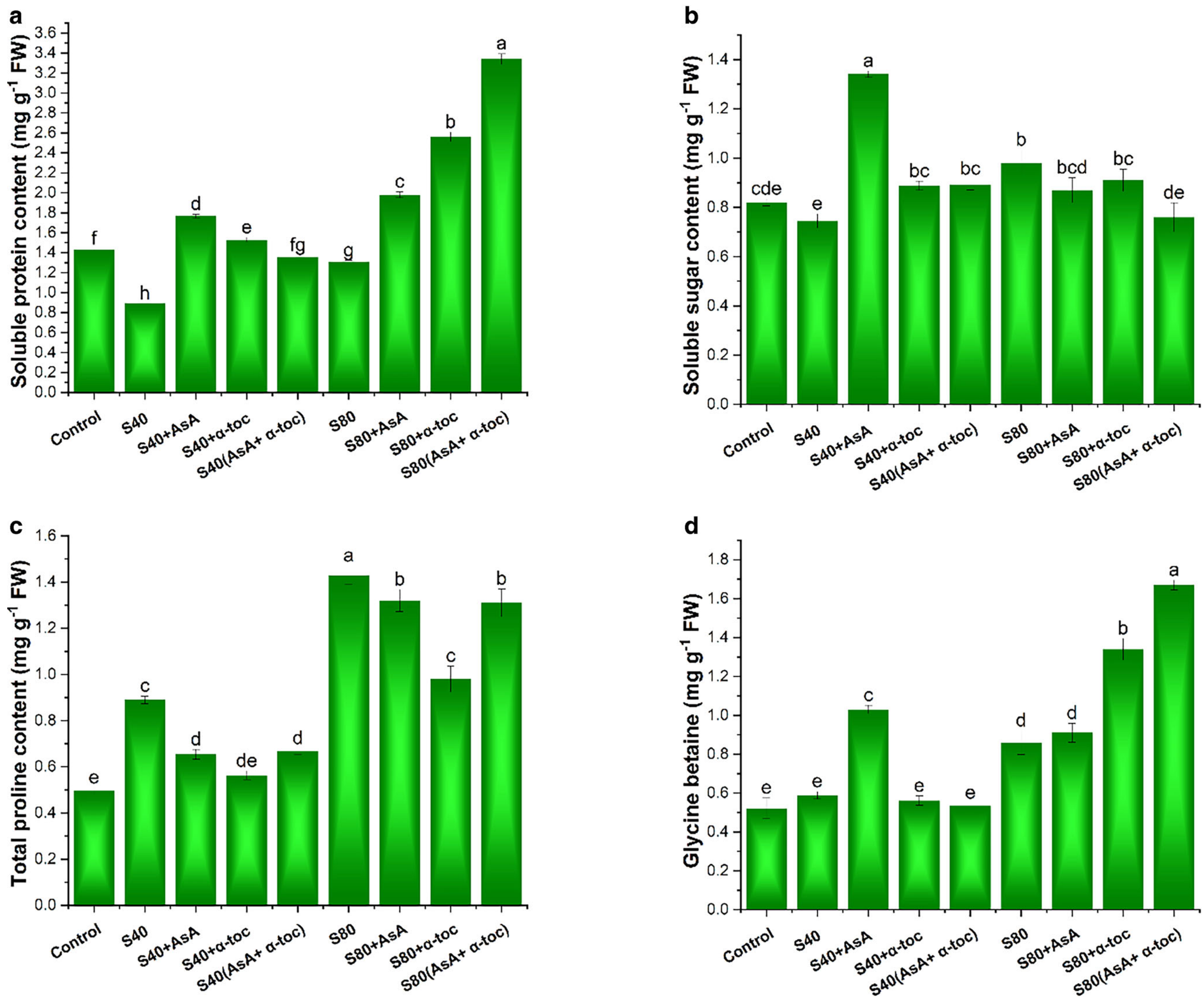

Fig. 3 Effect of ascorbic acid and α-tocopherol on **a** soluble protein content (SPC), **b** Soluble Sugar Content (SSC), **c** Total proline content (TPC) and **d** Glycine betaine (GB) under induced salinity stress. Control (0 mM salinity +0 mM Ascorbic Acid +0 mM α-Tocopherol), S40 (Salinity 40 mM), S40+AsA (Salinity 40 mM+ Ascorbic Acid 40 mM), S40 + α-toc (Salinity 40 mM+ α-Tocopherol 40 mM), S40 (AsA+ α-toc) (Salinity 40 mM+ Ascorbic Acid 40 mM + α-Tocopherol 40 mM), S80 (Salinity 80 mM), S80+AsA (Salinity 80 mM + Ascorbic Acid 80 mM), S80 + α-toc (Salinity 80 mM) + α-Tocopherol 80 mM), S80 (AsA+ α-toc) (Salinity 80 mM + Ascorbic Acid 80 mM + α-Tocopherol 80 mM). Vertical lines in bars denoted ± SE. Data represents means of 3 replications or $n = 3$ and the lower-case letters a–f denoted significant and non-significant changes in the mean values

ley grown under salinity conditions, with the highest MDA content recorded under 80 mM salinity stress (T5) and the lowest under 40 mM salinity stress (T4) (Fig. 4b).

At a significance level of $p \leq 0.001$, peroxidase (POD) and superoxide dismutase (SOD) levels were significantly elevated in barley plants subjected to 80 mM salinity stress (T5) (Fig. 5a, b). Conversely, plants with application of AsA and α-toc (T4) at 40 mM salinity showed the lowest levels of SOD, and the lowest POD content was recorded in 40 mM salinity plants treated with ascorbic acid (T2). Ascorbate peroxidase (APOX) levels increased significantly under salinity stress in all 30 mM and 40 mM stress treatments (Fig. 5c). The highest APOX concentration of 1.78 was observed under 80 mM salinity stress, while the lowest concentration of 0.40 was noticed under 40 mM salinity stress with the exogenous application of AsA and α-toc. Polyphenol oxidase (PPO) levels in barley grown under salinity conditions were significantly high, reaching 0.89 in 80 mM salinity stress (T5), while the lowest PPO was recorded in 40 mM salinity (T4) (Fig. 5d). Comparatively, catalase (CAT) activity was prominently enhanced in all stress treatments (Fig. 5e), with the treatments receiving foliar sprays of AsA, α-tocopherol, or both showing relatively high CAT concentrations (Table 4). Plants grown

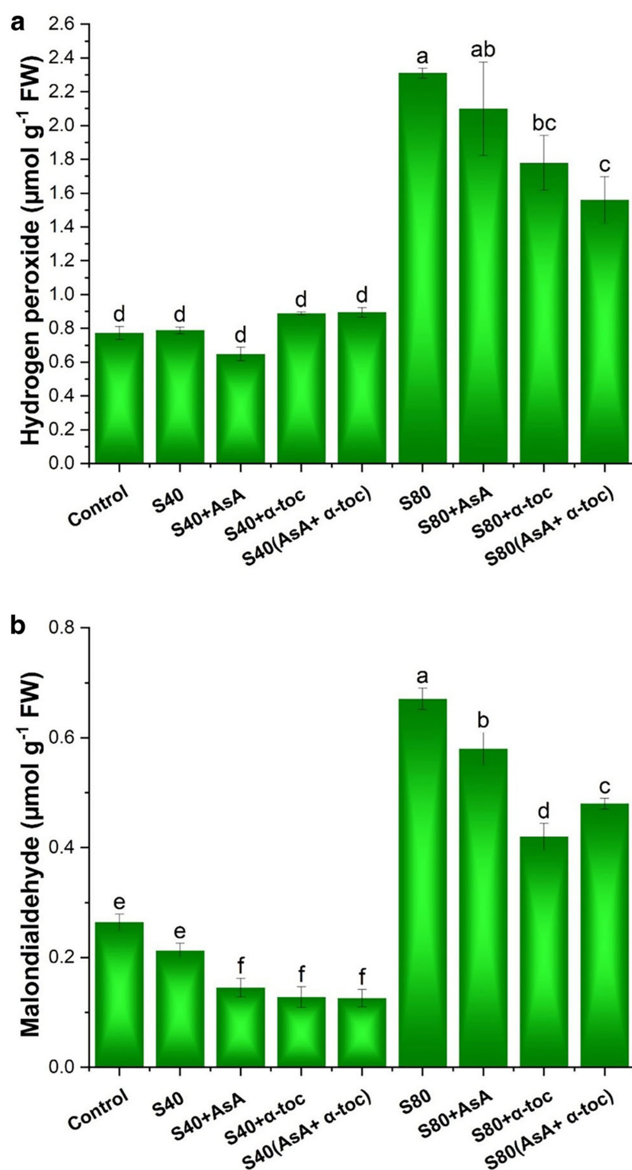

Fig. 4 Effect of ascorbic acid and α-tocopherol on **a** Hydrogen peroxide (H_2O_2) and **b** Malondialdehyde (MDA) under induced salinity stress. Control (0 mM salinity +0 mM Ascorbic Acid +0 mM α-Tocopherol), S40 (Salinity 40 mM), S40+AsA (Salinity 40 mM+ Ascorbic Acid 40 mM), S40+α-toc (Salinity 40 mM+ α-Tocopherol 40 mM), S40 (AsA+ α-toc) (Salinity 40 mM+ Ascorbic Acid 40 mM + α-Tocopherol 40 mM), S80 (Salinity 80 mM), S80+AsA (Salinity 80 mM + Ascorbic Acid 80 mM), S80+α-toc (Salinity 80 mM) + α-Tocopherol 80 mM), S80 (AsA+ α-toc) (Salinity 80 mM + Ascorbic Acid 80 mM + α-Tocopherol 80 mM). Vertical lines in bars denoted ± SE. Data represents means of 3 replications or $n=3$ and the lower-case letters a–f denoted significant and non-significant changes in the mean values

under 80 mM salinity stress (T5) exhibited significant CAT levels at $p \leq 0.001$ (Table S1), whereas plants grown under 40 mM salinity stress and treated with ascorbic acid (T3) showed the lowest levels. Glutathione reductase (GR) activity (Fig. 5f) showed significant variation across treatments. The highest GR activity (~1.5 EU mg^{-1}) was recorded in T5, whereas the lowest (~0.8 EU mg^{-1}) was found in T6 (Fig. 5f).

Relationships Between Different Morpho-Physio-Biochemical Attributes and Multivariate Analysis

Relationships Between Measured Traits

The correlation analysis in Fig. 6 quantifies the relationship among traits assessed in barley plants growing in saline soil, with or without treatment with α-Toc and AsA. The correlation matrix, encompassing 39 measured traits across nine treatments, reveals linear relationships sensitive to outliers. Notably, growth traits such as leaf dry weight (LDW), leaf fresh weight (LFW), leaf moisture content (LMC), shoot dry weight (SDW), shoot fresh weight (SFW), shoot moisture content (SMC), root dry weight (RDW), root fresh weight (RFW), and root moisture content (RMC) exhibit a positive correlation (green color) with germination measurements such as germination percentage (GP), mean germination time (MGT), coefficient of germination velocity (CVG), mean germination rate (MGR), germination index (GI), Timson germination index (TGI), germination rate index (GRI), germination energy (GE), and time to 50% germination (T50%) (Fig. 6). Conversely, growth traits are negatively correlated (red color) with biochemical attributes including hydrogen peroxide (H_2O_2), superoxide dismutase (SOD) activity, glutathione reductase (GR), peroxidase (POD), malondialdehyde (MDA), ascorbate peroxidase (APOX) and polyphenol oxidase (PPO). The correlation matrix also highlights relationships with chlorophyll a (Chl.a), chlorophyll b (Chl.b), total proline content (SPC), total protein content (TPC) and total chlorophyll content (TCC). The Chl a/b ratio decreases throughout the experiment, while other factors, specifically enzyme activity, increase in stress circumstances owing to the hunting nature in addition to the generation of reactive oxygen species.

The variables from different treatments are visualized using a heat map histogram in Fig. 7, reflecting results similar to correlation analysis. Two distinct clusters emerge, with the first cluster comprising S40+AsA, S40 + α-toc, and S40 (AsA+ α-toc), including the control, while the second cluster includes S80+AsA, S80 + α-toc, S80 (AsA+ α-toc), and S40 S80 (Fig. 5). At the highest salinity level, antioxidant enzymes, including POD, GR, SOD, PPO, APOX, MDA, H2O2, and SPC, exhibit a high association with S80 treatment, indicating their close relationship with salt stress. Positive associations between morphological characteristics and treatments S40+AsA, S40 + α-toc, and S40 (AsA+α-toc) are observed, demonstrating similar responses to AsA and α-toc application under stress conditions.

Principal Component Analysis (PCA) is employed to correlate morpho-physiological traits and antioxidant en-

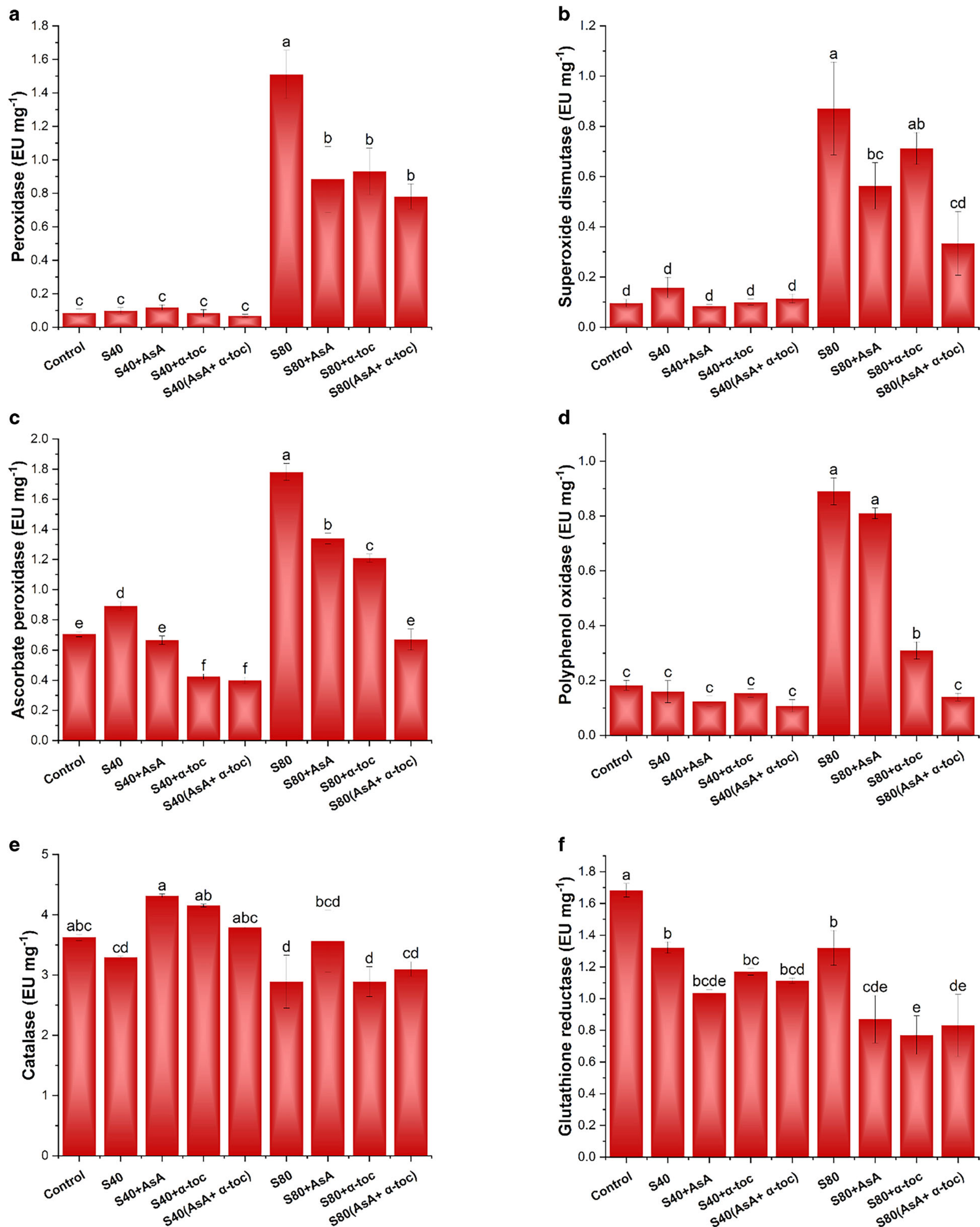

Fig. 5 Effect of ascorbic acid and α-tocopherol on enzyme activities (EU mg^{-1}). **a** Peroxidase (POD), **b** Superoxide dismutase (SOD), **c** Ascorbate peroxidase (APOX), **d** Polyphenol oxidase (PPO), **e** Catalase (CAT) and **f** glutathione reductase (GR) under induced salinity stress. Control (0 mM salinity +0 mM Ascorbic Acid +0 mM α-Tocopherol), S40 (Salinity 40 mM), S40+AsA (Salinity 40 mM+ Ascorbic Acid 40 mM), S40 + α-toc (Salinity 40 mM+ α-Tocopherol 40 mM), S40 (AsA+ α-toc) (Salinity 40 mM+ Ascorbic Acid 40 mM + α-Tocopherol 40 mM), S80 (Salinity 80 mM), S80+AsA (Salinity 80 mM + Ascorbic Acid 80 mM), S80 + α-toc (Salinity 80 mM) + α-Tocopherol 80 mM), S80 (AsA+ α-toc) (Salinity 80 mM + Ascorbic Acid 80 mM + α-Tocopherol 80 mM). Vertical lines in bars denoted ± SE. Data represents means of 3 replications or $n = 3$ and the lower-case letters a–f denoted significant and non-significant changes in the mean values

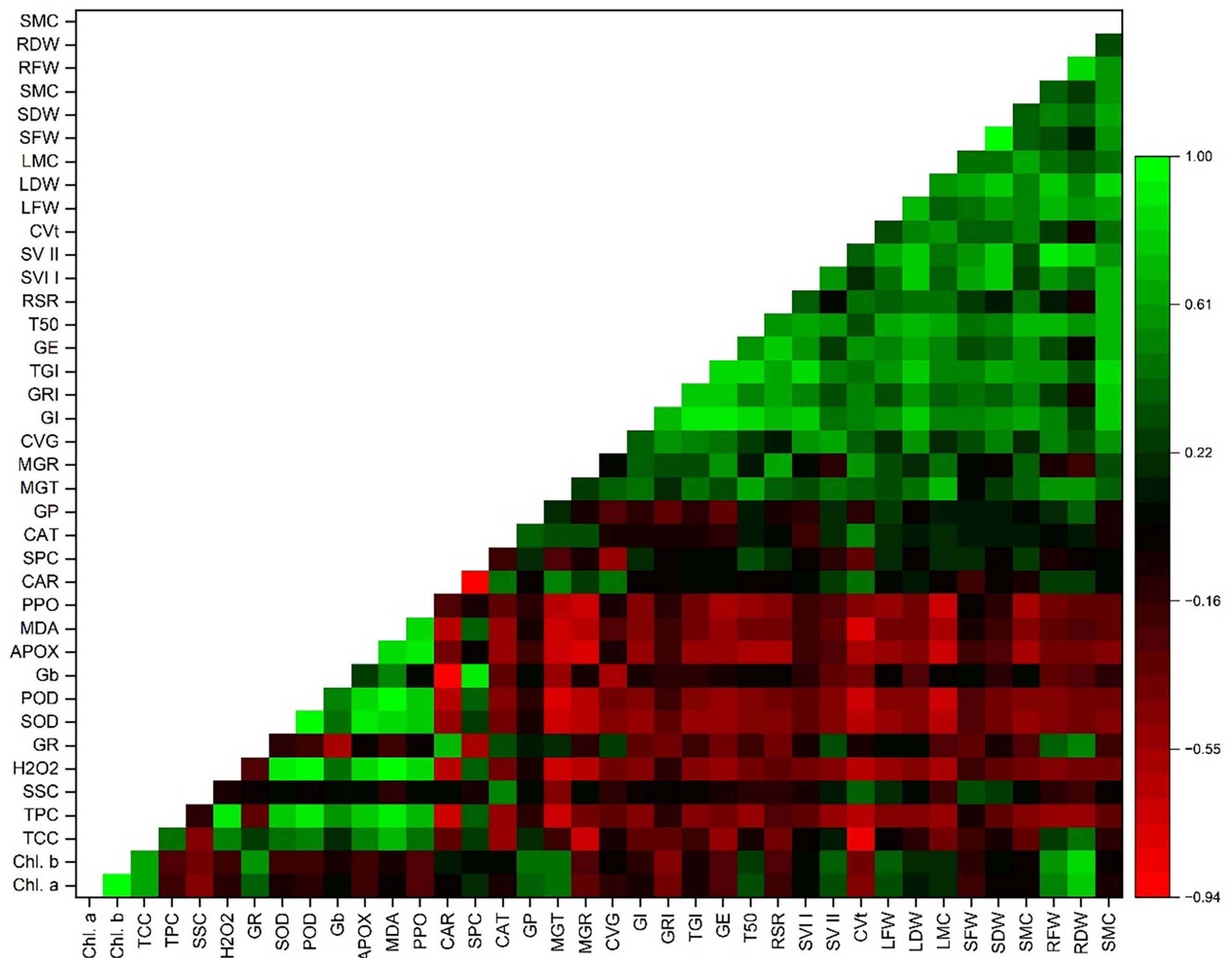

Fig. 6 Correlation between various morpho-physio-biochemical attributes of barley under induced salinity stress with the application of ascorbic acid and α-tocopherol. germination percentage (GP), mean germination rate (MGR), coefficient of the velocity of germination (CVG), germination index (GI), germination rate index (GRI), timson germination index (TGI), germination energy (GE), time to 50% germination (T50%), root shoot ratio (RSR), seed vigour indices (SVI-I and II), Coefficient of variation of germination time (CVt), leaf fresh weight (LFW), leaf dry weight (LDW), leaf moisture content (LMC), shoot fresh weight (SFW), shoot dry weight (SDW), shoot moisture content (SMC), root fresh weight (RFW),root dry weight (RDW), root moisture content (RMC), chlorophyll *a* (Chl.a), chlorophyll *b* (Chl.b), total chlorophyll content (TCC), ratio of chlorophyll *a* & *b* (Chl a/b), total proline content (SPC), soluble sugar content (SSC), hydrogen peroxide (H_2O_2), glutathione reductase (GR), superoxide dismutase activity (SOD), peroxidase (POD), glycine betaine content (GB), ascorbate peroxidase (APOX), malondialdehyde (MDA), polyphenol oxidase (PPO), carotenoid (CAR), total protein content (TPC) and catalase activity (CAT)

zymes under induced salinity stress. In Fig. 8a, b, PCA is applied to 22 morphological and 17 physio-biochemical traits, respectively, across nine treatments, including the control. The results reveal that salinity considerably affects multiple morpho-physio-biochemical traits in every treatment. The first two components (PCs) explain 70% of the total variation, and the PCA-based biplot using these components aligns with the heat map histogram, illustrating associations between morphological traits (SMC, SFW, RMC, GI, LMC, MGR, CVt, GE, RSR, and GRI) and treatments S40+AsA, S40 (AsA+α-toc), and S80 (AsA+α-toc) (Fig. 8a). This reflects the positive effects of AsA in conjunction with α-toc on barley growth and related parameters. Conversely, S40, S80, S80+AsA, and S80 + α-toc treatments show negative relationships with all measured traits. Photosynthetic pigments and protein contents decrease under salinity stress but show a positive relationship with S80 (AsA+α-toc) and S80 + α-toc. Additionally, PCA highlights that SOD, POD, APOX, MDA, PPO, and CAT are associated with the high salinity level of S80, indicating increased enzyme activities in response to stress owing to the elevated production of ROS (Fig. 8b).

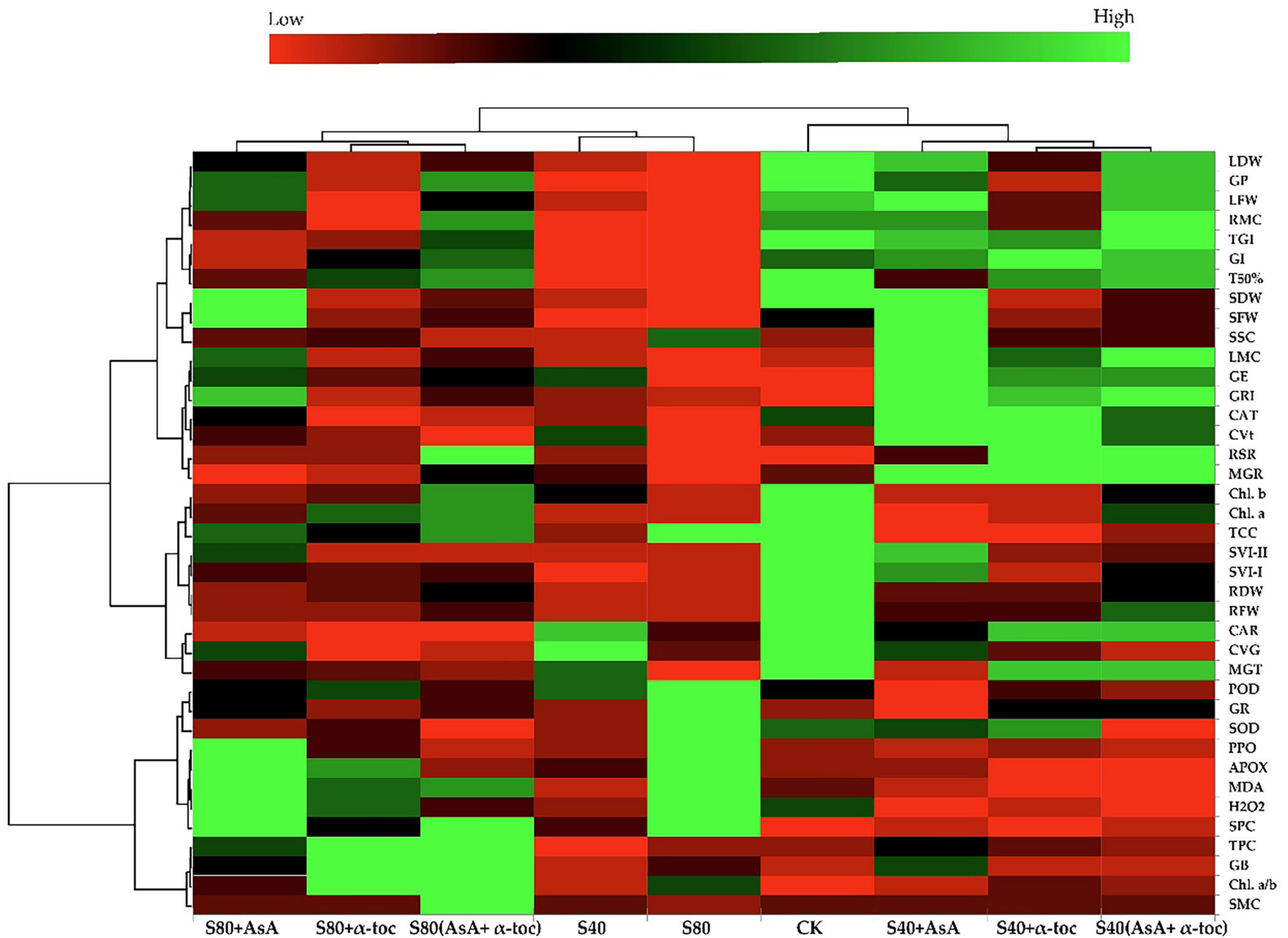

Fig. 7 Heatmap histogram correlation between various morphological and physio-biochemical attributes of *Hordeum vulgare* studied in this experiment. Control (0 mM salinity +0 mM Ascorbic Acid +0 mM α-Tocopherol), S40 (Salinity 40 mM), S40+AsA (Salinity 40 mM+ Ascorbic Acid 40 mM), S40+ α-toc (Salinity 40 mM+ α-Tocopherol 40 mM), S40 (AsA+ α-toc) (Salinity 40 mM+ Ascorbic Acid 40 mM + α-Tocopherol 40 mM), S80 (Salinity 80 mM), S80+AsA (Salinity 80 mM + Ascorbic Acid 80 mM), S80+ α-toc (Salinity 80 mM)+ α-Tocopherol 80 mM), S80 (AsA+ α-toc) (Salinity 80 mM + Ascorbic Acid 80 mM + α-Tocopherol 80 mM)

Discussion

Plants face various ecological challenges, including drought, heavy metal toxicity, temperature changes, floods, and salt stress. Salinity stress can lead to toxic effects and reduce crop productivity. Researchers are exploring nanoparticles as a solution to address ionic toxicity and improve plant growth. Salinity stands as a prominent abiotic stressor, adversely impacting plants through constraints on physiological, biochemical, and biomass (Zia et al. 2022). This stressor is pervasive, exerting a substantial negative influence on crop productivity (Ahmad et al. 2022). Furthermore, the anticipated effects of global climate change are projected to intensify salinity stress across various regions (Iqbal et al. 2022). Plant responses to salinity stress are contingent upon factors such as stress intensity, duration, plant species, and phenological phases. Barley plants subjected to treatments involving ascorbic acid (AsA) and/or α-tocopherol (α-Toc) exhibited capacity to maintain their physiological, biochemical, and agronomic characteristics despite challenging environmental conditions.

This study found a significant reduction in various germination and growth parameters, including germination percentage, rate index, and root-shoot ratio, under both 40 mM and 80 mM salinity stress conditions, as shown in Tables 2 and 3, and 4. These findings are in accordance with the results found by Jaleel et al. (2008), indicating that salinity adversely affects morphological parameters and diminishes overall growth performance. Similar outcomes have been reported in the existing literature, including studies by Abid et al. (2020), Zia et al. (2022) and Iqbal et al. (2022). Moreover, the inhibitory influence of salt stress on root development, including root length and weight, is evident in

Fig. 8 Score and loading plot of principal component analysis (PCA) on different morphological (**a**) and physio-biochemical attributes (**b**) of *Hordeum vulgare* under different levels of salinity with or without the application of AsA. Score plot represents separation of treatments as Control (0 mM salinity +0 mM Ascorbic Acid 0 mM α-Tocopherol), S40 (Salinity 40 mM), S40+AsA (Salinity 40 mM+ Ascorbic Acid 40 mM), S40 + α-toc (Salinity 40 mM+ α-Tocopherol 40 mM), S40 (AsA+ α-toc) (Salinity 40 mM+ Ascorbic Acid 40 mM + α-Tocopherol 40 mM), S80 (Salinity 80 mM), S80+AsA (Salinity 80 mM + Ascorbic Acid 80 mM), S80 + α-toc (Salinity 80 mM)+ α-Tocopherol 80 mM), S80 (AsA+ α-toc) (Salinity 80 mM + Ascorbic Acid 80 mM + α-Tocopherol 80 mM)

our findings, consistent with conclusions from Ahmad et al. (2022) and Zahra et al. (2020).

In our study, an increase in soil salinity adversely affected all germination parameters, and while the ameliorating effect of α-tocopherol (α-toc) and ascorbic acid (AsA) on these traits was significant, the highest values for GP, MGT, MGR, GI, TGI, T50%, and RSR were observed in treatments with AsA and α-toc under both 40 mM and 80 mM salinity (T4 and T8) as indicated in Tables 2 and 3. These findings suggest that the foliar supplementation of α-toc and AsA efficiently mitigated inhibitory influence of salinity on plant growth. Our results align with El Hariri et al. (2010), who noted an increase in plant growth following the foliar application of ascorbic acid and/or α-tocopherol. The observed decrease in germination rate with increasing salinity is in alignment with research held in past (Jacob et al. 2020), suggesting that the osmotic effect of high salinity hampers plant establishment by limiting the capacity of germinating seeds to engross water. High uptake of sodium and chloride ions during seed germination might contribute to cell toxicity, hindering seed establishment (Masuda et al. 2021). It is proposed that high osmotic stress and ion toxicity associated with salinity stress, as well as potential changes in cell wall structure, collectively contribute to the reduction in plant development (Hasanuzzaman et al. 2013).

In Fig. 1, a continuous decrease in the measured traits was observed with increasing salinity levels in the growth media. However, barley plants treated with AsA and α-Toc showed significantly higher values of these traits compared to both the salinity stressed and untreated control plants. The exogenous implication of α-Toc and AsA is associated with enhanced production of antioxidant enzymes and osmolytes, mitigating the limiting effects on the performance and development of barley. Notably, these parameters exhibited their peak values in plants which were treated with sprays of α-Toc and/or AsA at 150 mg L^{-1} concentration. The interactions observed between AsA and α-Toc indicate their positive influence as plant growth regulators (PGRs) on the germination and agronomic traits of barley. However, our study affirms that ascorbic acid is more effective while compared to α-Toc in mitigating adverse effects of salinity stress on barley, aligning with previous findings (Ishaq et al. 2021).

AsA and α-Toc play a crucial role in stabilizing biological membranes by scavenging reactive oxidative anions and their by-products, inhibiting lipid peroxidation (Noreen et al. 2021). For instance, a single α-Toc molecule can efficiently neutralize 120 singlet oxygen ($^1O_2$) molecules under stressful conditions, demonstrating their potent antioxidative capabilities (Srinivasan et al. 2019; Hasanuzzaman et al. 2023).

Salinity stress induced a decline in all physiological characteristics and heightened antioxidant activities. In Fig. 2a, a significant decrease ($p \leq 0.001$) in chlorophyll (Chl) content, both Chl a and Chl b, was noted under 80 mM salinity stress, possibly linked to stomatal closure (Mafakheri et al. 2010), generation of ROS (Lalarukh and Shahbaz 2020) and a reduction in intracellular water potential (Noreen et al. 2021). This decrease in chlorophyll content aligns with findings by Iqbal et al. (2022), they inquired about the impact

of NaCl on chlorophyll (Chl a, Chl b, and total carotenoids) in salt-stressed plants. Notably, barley treated with ascorbic acid (AsA) and α-tocopherol (vitamin E) has shown significant protective effects under both 40 mM and 80 mM salinity stress conditions, particularly in minimizing damage to photosynthetic pigments. Research indicates that the combination of AsA and α-tocopherol enhances the plant's ability to cope with abiotic stress, such as salinity, by improving various physiological and biochemical attributes. The co-application of AsA and α-tocopherol has been observed to mitigate the detrimental effects of NaCl on photosynthetic pigments in barley. This suggests a synergistic effect where both antioxidants work together to enhance the plant's resilience against oxidative stress caused by salinity (Iqbal et al. 2022). AsA acts as a major redox buffer and cofactor for enzymes involved in photosynthesis, while α-tocopherol is primarily known for its role in protecting cellular membranes from oxidative damage. Together, they bolster the plant's antioxidant defense system, reducing lipid peroxidation and improving overall plant health under stress conditions (Mesa and Munné-Bosch 2023). The application of these antioxidants not only protects photosynthetic pigments but also enhances ion uptake and the expression of stress response genes. This multifaceted approach allows barley to maintain better physiological function even in saline conditions (Hassan et al. 2021).

Salinity stress also significantly decreased carotenoid content, consistent with previous reports indicating a reduction in carotenoids compared to control conditions (Zia et al. 2022). Usage of sprays, particularly α-toc and AsA, has been known to enhance the photosynthetic pigment recovery under stress conditions, attributed to the control of hydrogen peroxide formation and an increase in phenolic levels (Masuda et al. 2021). The antioxidant properties of AsA, through direct interaction with various ROS, may contribute to the observed increase in photosynthetic pigments.

In Fig. 2c, concentrations of soluble sugar content (SSC) and soluble protein content (SPC) exhibited a significant increase under 80 mM salinity stress (T5). Furthermore, the elevation in proline content, consistent with findings from Zahra et al. (2020), suggests that high salinity induces an increase in proline levels. Comparable results for total proline content (TPC) were stated by (Dustgeer et al. 2021). The rise in soluble sugar and proline content in the shoot during salinity stress plays a role in counteracting osmotic pressure (Abid et al. 2020), and more tolerant plants tend to accumulate higher proline levels (Noreen et al. 2021).

Figure 2e illustrates that the maximum levels of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) were observed under salinity stress. These findings are in alignment with Dustgeer et al. (2021), who reported an increase in H_2O_2 with salinity stress. The loss of the plant's ability to manage reactive oxygen species (ROS) contributes to elevated malondialdehyde production, leading to membrane damage by lipid peroxidation or hydroxyl radicals' generation (El-Beltagi et al. 2022b).

In Fig. 3a, b, and 3c, the highest activities of peroxidase (POD) and superoxide dismutase (SOD), glycine betaine (GB), ascorbate peroxidase (APOX), malondialdehyde (MDA), and polyphenol oxidase (PPO) at a significance level of $p \leq 0.001$ were noted in barley plants subjected to 80 mM salinity stress (T5), while the lowest levels were observed in salinity-treated plants with ascorbic acid and α-tocopherol (T4), either individually or in combination. Comparable results were reported by Ali et al. (2011) for SOD, POD, and MDA contents and by Aly et al. (Aly et al. 2019), They found a notable rise in POD and PPO contents under salinity conditions. Elevated peroxidase content under salinity stress was also documented in previous studies (Akbari et al. 2020; Kibria et al. 2017). The literature indicates that salinity-induced stress leads to increased levels of antioxidant enzymes such as SOD, APOX, and MDA (Azeem et al. 2023), demonstrating augmentation of the antioxidant defense mechanism, which serves as a protective strategy in stressed plants (Kim et al. 2005). Khan et al. (2020) also reported an increase in glutathione reductase (GR) levels under salinity stress.

In the current study, the treatments receiving foliar sprays of AsA, α-tocopherol, or both showed relatively high antioxidant enzyme concentrations. Exogenous administration of AsA and α-tocopherol has been reported for the active regulation of antioxidant synthesis (proline, CAT, SOD, POD, APX,) to counteract the deleterious effects of salt stress by reducing ROS-driven lipid peroxidation (MDA) (Noreen et al. 2021). These alterations in plant physiology are pivotal strategies for enhancing salt tolerance in barley. The utilization of various osmoprotectants enhances enzymatic activity, bolstering the antioxidant response in conditions of stress, thereby enabling plants to maintain osmotic adjustment and integrity of membrane (Skłodowska et al. 2009). AsA functions not only as a direct antioxidant but also as a co-factor in various enzymatic reactions that enhance the plant's ability to cope with oxidative stress. Studies have indicated that exogenous application of AsA can increase the activities of antioxidant enzymes such as CAT, SOD, and APX, thereby improving the plant's tolerance to salinity and other abiotic stresses. For instance, AsA has been reported to induce higher transcript levels of these antioxidant enzymes in plants subjected to stress, leading to reduced levels of reactive oxygen species (ROS) and lipid peroxidation (Hasanuzzaman et al. 2019). Similarly, α-tocopherol (vitamin E) is recognized for its antioxidant properties, particularly in chloroplasts, where it protects against lipid peroxidation and scavenges singlet oxygen. The application of α-tocopherol has been shown to enhance the antioxidant capacity of plants, leading to improved growth

and physiological responses under salt stress. It increases the activities of various antioxidant enzymes and reduces oxidative stress markers such as malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) levels (Taie and Rady 2024).

Previous studies advocate for protective role of vitamins, antioxidants, and phytohormones in enhancing plant stress tolerance and, consequently, agricultural production (Hasanuzzaman et al. 2013). This study provides valuable insights into the potential of ascorbic acid and α-tocopherol to assist barley plants in coping with salinity stress. Furthermore, these results can inform practical strategies for the widespread application of α-tocopherol and AsA in the field, including assessments of financial viability, environmental impact, and benefits to growers. Given the absence of studies supporting the beneficial effects of exogenous tocopherols on crops grown in a natural field environment, there is a need for field trials to validate the observed findings under controlled conditions in real-world settings.

Conclusions

Results led to the conclusion that foliar application of AsA and α-toc could serve as a "shot-gun" approach for improving barley stress tolerance capacity through improving growth, physiological and biochemical characteristics under salinity stress. It was concluded during the comparison investigation that AsA is more relevant and efficient in barley for reducing the negative impacts of salinity stress. The results of this study showed that exogenous application of AsA and α-Tocopherol can effectively boost the antioxidant defense system and reduce ROS (H_2O_2) induced lipid peroxidation (MDA). Thus, enhanced physiological and biochemical responses to salinity stress result in increased barley seed production and are a step toward solving the malnutrition problem through the biofortification of vitamins C and E. Our best understanding of the literature leads us to believe that this is the first thorough and comparative study from Pakistan to choose one of the most efficient plant growth regulators for barley grown under salinity stress.

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Data availability The authors confirm that the data supporting the findings of this study are available within the article and/or its supplementary materials.

Conflict of interest Sadaf, S. Ullah, S. Shah, A. Jamal, A. Mihoub, M. Farhan Saeed, I. Ahmad, E. Radicetti, A. Zia, A. Mastinu and J. Černý declare that they have no competing interests.

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