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ORIGINAL ARTICLE

Pipecolic acid priming promotes salt stress tolerance via regulating antioxidant defense system and sugar metabolism in barley plants

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Abstract

Pipecolic acid (Pip) is the product of L-lysine metabolism and plays a role in the systemic acquired resistance (SAR) response under biotic stress factors in plants while there is limited research on abiotic stress related to its effect. To illuminate this, in the present study, barley (*Hordeum vulgare* L.) Bülbül89 seeds were primed with 2, 4 and 8 ppm pipecolic acid and distilled water as a control for 24 h. After germination, for salinity treatments, seedlings were treated with 300 mM NaCl for 48 h. The physiological (growth parameters, relative electrolyte leakage (REL), relative water content (RWC), chlorophyll content) along with stress markers (malondialdehyde (MDA), hydrogen peroxide (H_2O_2) and proline content) as well as antioxidant enzymes (SOD, CAT, POX, APX, GR) and sugar metabolism (glucose, fructose and maltose) and SEM analysis were used to determine the anatomical changes in the samples. In the results, all Pip doses maintained RWC and REL, and 8 ppm Pip was the most effective in reducing oxidative damage. Beside this, Pip priming treatment alleviated chlorophyll content under salinity, but only 8 ppm Pip reduced proline, glucose and fructose content. Only 2 and 4 ppm Pip induced SOD enzyme activity under salinity, whereas no change was observed in APX, POX and CAT enzyme activity. On the other hand, leaf area was increased by pipecolic acid with enlarged cells as supported by SEM observations. In summary, the present study firstly indicates that Pip (2, 4 and 8 ppm) could be used as an effective antioxidant molecule or ROS inhibitor to increase salt stress tolerance in barley plants.

Keywords Barley · Salinity · Sugar · N-hydroxyl pipecolic acid

Introduction

Salinity has become a global problem for agriculture with detrimental effects. By 2050, it is estimated that the world's population will increase to 9.7 billion and salt stress will affect soils worldwide by 30% (Otlewska et al. 2020). These two facts lead to together "food shortage" with decreasing crop production with the effect of salt stress (Hickey et al. 2019). Plant growth is affected by salinity through both an initial osmotic stress and a later ionic toxicity. Research has shown that salinity causes oxidative damage by causing overproduction of reactive oxygen species in plants. Osmotic stress causes water loss in cells, and ionic toxicity is caused on by excessive accumulation of sodium (Na^+) and chloride (Cl^-) ions in cells. Both of these situations disrupt water potential, ion homeostasis, photosynthesis activity and stomatal conductance.

Salt stress can impact plant carbohydrate metabolism contributing to their adaptation and tolerance to salt stress (Mansour and Hassan 2022). Under salt stress conditions, alterations in carbohydrate metabolism occur to maintain cellular homeostasis, regulate osmotic pressure, water uptake and alleviate the negative impact of salinity (Khan et al. 2021; Joshi et al. 2023). When stress begins, starch rapidly hydrolyzes and sucrose accumulation starts in plant cells. Otherwise, changed sugar level is related with signaling pathways in plant growth under salt stress.

Seed priming is one of the techniques which create more tolerant crops to stress conditions during the growth (Tanou et al. 2012) as well as improve seed germination under several abiotic stress factors like salinity, drought or heat. This technique is known as low cost, effective and environmentally friend (Johnson and Puthur 2021, Bojovic et al. 2022). In recent years, different kinds of seed priming techniques

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such as nano-, hydro-, hormonal, biostimulant, osmo-, halo-, mineral and chemical priming have been reported to enhance seed growth under stresses. Specifically, there are important reports that salt stress tolerance is increased with seed priming in crops through improved modifications in several physiological and molecular processes in plants (Farhoudi et al. 2011; Tabatabaei 2013, Karadag and Yucel 2017, Gonzalez Grande et al. 2020). These effects are related with improvement in reserve mobilization, starch metabolism, RNA and DNA synthesis, chlorophyll synthesis, enhancing carbon assimilation, membrane stability, antioxidant defense system and metabolites.

A systemic response to biotic stress is called systemic acquired resistance (SAR) and is typically initiated by interactions of particular cells with pathogens (bacteria, virus or fungi). Furthermore, during the first line of defense, there is an increase in the synthesis of plant hormones to activate two pathways: induced systemic resistance (ISR) and systemic acquired resistance (SAR). The SAR-induced plants are primed for induction of defense genes thereby activating plants to protect themselves more efficiently against the following pathogen attack (Klessig et al. 2018). SAR involves various effective small molecules [azelaic acid (AzA), dehydroabietinal (DA), salicylic acid (SA), methyl salicylate (MeSA), glycerol-3-phosphate (G3P) and pipecolic acid (Pip)] associated with long distance communication and signal amplification during SAR.

Pipecolic acid (Pip) plays main role in the SAR mechanism and is a product of the metabolism of L-lysine. Moulin et al. (2006) identified that Pip is an osmoregulatory amino acid in non-halophilic plants under various osmotic stress conditions. Also, it was determined that this molecule, an inherently occurring non-protein amino acid, accumulates locally and systemically during pathogen attack (Vogel-Adghough et al. 2013;). Results with pipecolic acid showed that it was originally determined in petiole exudates of SAR-induced plants (Wang et al. 2018). Pipecolic acid is synthesized in the chloroplast by two main enzymatic steps catalyzed by the aminotransferase ALD1 and the reducing factor SAR-DEFICIENT4 (SARD4) (Ding et al. 2016;). After this, N-hydroxylation by flavin-dependent monooxygenase1 (FMO1) in the cytosol results in the production of *N*-hydroxy-pipecolic (NHP) (Chen et al. 2018). Hartmann et al. (2021) reported that the NHP biosynthetic pathway is inducible by pathogens and leads to induce SAR mechanism. Pipecolic acid pretreated plants for optimal production of the defensive signal SA and mediates SAR and defense priming by both SA-dependent and SA-independent signal mechanisms.

In plants, there is limited work with the effects of pipecolic acid on abiotic stress although many studies were presented on biotic stress (Koc and Seckin Dinler 2022). When biotic stress studies were examined, it was determined that pipecolic and N-hydroxyl pipecolic acid was accumulated in infected and non-infected leaf tissues. Navarova et al. (2012) presented that exogenous pipecolic acid treatment induced resistance due to increased salicylic acid accumulation and signaling. Otherwise, in a study conducted on transgenic rice plants, overexpression of the *ALD1* gene, which is involved in pipecolic acid biosynthesis, was shown to increase the plant's protection mechanism against infection by the fungus *Magnaporthe* (Jung et al. 2016). Exogenous application with 10 µM D, L-Pip enhanced resistance against *Pseudomonas syringae* pv. tabaci in tobacco plants (Vogel-Adghough et al. 2013). In recent years, Xie et al (2020) reported that pipecolic acid accumulation was induced under salinity in rice plants. Additionally, Gao et al. (2021) showed that NHP plays important role in plant immunity. Lastly, it was shown that *ald1* mutant tomato plants were more tolerant to drought stress than *fmo1* mutant plants (Wang et al. 2021). Beside this, Zeng et al. (2023) found that treatment of *Pseudomonas fluorescens* improves biocontrol activity against various biotic stress by inducing pipecolic acid accumulation in wheat. Totally, several works indicate that NHP can be defined as a novel plant defense hormone with a induction of SAR.

Barley (*Hordeum vulgare* L.) is a cereal crop from the Poaceae family for animal feed, food production and fermentation industries (Sakellariou 2020). Beside this, Östman et al. (2006) reported that it is especially preferred by people due to its abundance in protein, β-glucan and dietary fiber, which are highly helpful to human health. It is ranking forth in production of after maize, wheat and rice. Compared these crops, it is more salt tolerant, but salinity causes alterations of development and quality of the barley crop as reported in recently work (Alsamadany et al. 2024). Therefore, it is important to find more eco-friendly and economic practices to reduce the negative impact of salt stress on barley.

In this study, the effects of pipecolic acid priming technique on barley seeds that have not been previously identified were analyzed. The physiological and biochemical parameters, regulation of reactive oxygen species, the antioxidant enzyme system, sugar metabolism, anatomical changes observed through SEM were determined in barley leaves. The results obtained through this work provide new insights into the mechanism of Pip-treated salt stress tolerance in plants.

Materials and methods

Experimental design

Barley (*Hordeum vulgare* L.) Bülbül89 seeds were taken from Field Crops Research Institute (Ankara). The seeds were dived in two groups: 1) primed with 2, 4 and 8 ppm

pipecolic acid and 2) primed with distill water for 24 h. Then the seeds were sown in plastic pots, each of which (width 20 cm, length 12 cm, height 18 cm) contained five barley seeds and the mixture of soil corrected) pH 6.5. Pots were kept in the dark for germination. After germination, seedlings were grown in a growth room at 25 °C (16 h day/8 h night photoperiod), light intensity of 350 µmol m^{-2} s^{-1}, and watered with half Hoagland solution, 100 ml per pot. (Hoagland and Arnon (added) 1950). After 24 h, for the stress application, the seedlings were in the Pip and control groups were exposed to 300 mM NaCl for 48 h. The design of the experiment contained eight groups: control (1), 2 ppm Pip (2), 4 ppm Pip (3), 8 ppm Pip (4), salt (300 mM NaCl) (5), 2 ppm + S (6), 4 ppm + S (7), 8 ppm + S (8). All solutions were treated within the Hoagland solution. Harvested plants were stored at -80 °C for future analysis.

For determining the concentrations, 2, 4, 8 and 12 ppm pipecolic acid solutions were used for barley seeds. For salt treatment, 250, 300 and 350 mM NaCl solutions were tested. The effective doses were determined according to stress analysis results. Lastly, 2, 4 and 8 ppm and 300 mM NaCl were selected. General view of barley leaves is shown in Fig. 1.

Physiological analysis

Relative water content (RWC)

RWC of barley leaves was measured by (Smart and Bingham 1974). Six leaf were collected from plants of eight group. Then the samples were floated on dI-H_2O for 5 h under low irradiance. The turgid tissues were then rapidly blotted dried prior to determining turgid weight. Then dry weight was assayed after incubator at 70 °C for 72 h, while constant weight was reached. The RWC was determined using the following formula as below.

$$RWC\ (\%) = [(FW - W)/(TW - W)]$$

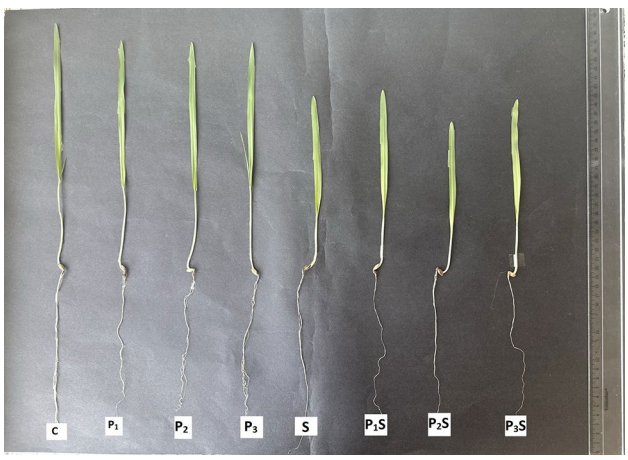

Fig. 1 *Hordeum vulgare* L. materials used in this study. Seedlings after 48 h of stress

Total chlorophyll content (CLH)

CLH content of barley leaves was estimated in accordance with Lichtenthaler and Buschmann (1987). The pigments of 1 g fresh leaves were extracted in 80% (v/v) acetone. The absorbance of chlorophyll content was measured at 645 and 663 nm using Genesys 10S UV VIS spectrophotometer (Thermo Fisher Scientific). The formulas used for the results are showed below:

Chlorophyll a (µg/ml) = 12.25 A663 – 2.798 A645.
Chlorophyll b (µg/ml) = 21.5 A645 – 5.1 A663.
Total chlorophyll (µg/ml) = chlorophyll a + chlorophyll b.

Leaf area measurement (LA)

The leaf area in barley leaves was measured at the 48 h after stress treatment using a CID Bio-Science CI-201 portable laser leaf area meter. The calculations were made by the program contained in the device, and the leaf area was calculated in cm^2.

Relative electrolyte leakage (REL)

REL of barley leaves was assayed in accordance with to Singh et al. (2008). The leaves were left to vibrate for 30 min in dI-H_2O. After that, the conductivity (C1) of the solution was measured. Then the tubes were boiled for 15 min, and again conductivity (C2) was measured. The REL was determined according to the following formula;

$$REL(\%) = (C1/C2) \times 100$$

Biochemical analysis

Malondialdehyde (MDA) content

The MDA value was checked in accordance with the procedure of Madhava Rao and Stresty (2000). 0.5 g barley leaves was homogenized by adding TCA (thiochloroacetic acid). Homogenates were centrifugated for 5 min at + 4 °C and 10.000 × g. The supernatants from samples were added to a reaction mix with TBA and TCA. The tubes were incubated to 30 min at 95 °C and centrifugated to 10.000 × g 15 min. Absorbance values of the supernatant at 600 and 532 nm

were checked. The concentration of (MDA) was calculated using the extinction coefficient (£ = 155 mM^{-1} cm^{-1}).

Superoxide ($O_2^{\bullet-}$) radical content

Superoxide radical content in barley leaves was established according to Ke and Sun (2004). 0.5 g barley leaf samples was homogenized in 2 ml 65 mM phosphate buffer (pH:7.8). The homogenate was then centrifugated by 5.000 × g. 0.9 ml phosphate buffer, and 0.1 ml hydroxylamine hydrochloride (10 mM) was added to the 1 ml supernatant. The solution was mixed and incubated at 25 °C for 20 min. Then, 1 ml from the solution and 1 ml of 17 mM aminobenzene sulfonic acid and 1 ml of 17 mM 1-naphthylamine were added and incubated for 20 min at 25 °C. Again, the mixture was incubated at 25 °C for 20 min. The absorbance at 530 nm was read after adding 3 ml N-butyl alcohol to the mixture. The standard was used absorbance of NaOH values at 530 nm at different concentrations.

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity was defined according to the modified method given by (Kim and Minamikawa, 1997)(1997). The method is formed on the ability of fusiformis extracts to scavenge this radical generated by Fenton reaction. The scavenging activity on hydroxyl radicals was calculated as below:

[(A0 – A1)/A0 × 100].

Hydrogen peroxide (H_2O_2) content

The H_2O_2 content was tested according to modified from Velikova et al. (2000). Fresh barley leaves (0.3 g) were homogenized in 5 ml of 0.1% TCA and centrifuged at 12.000 rpm for 15 min. The supernatant (0.5 ml) was then mixed with 0.5 ml of buffer (10 mM KH_2PO_4, pH 7) and 1 ml of 1 M potassium iodide (KI). The absorbance reading was checked at 390 nm.

Proline content

The content of free proline of barley leaves was analyzed according to Claussen (2005). Samples (0.5 g) were homogenized with 5 ml of 3% (w/v) sulfosalicylic acid. After the homogenization, 1 ml of ninhydrin and 1 ml of acetic acid were added. The mixture was incubated at 100 °C for 1 h. The post-incubation mixture was transferred to ice water and the reaction was stopped. The mixture was extruded with 4 ml of toluene and was shuffled in a tube mixer for 25–30 s. The colored liquid containing toluene was brought to room temperature and the absorbance value at 520 nm was taken. The amount of proline in plant tissues (fresh weight µmol/g) was calculated via a calibration curve.

Antioxidant enzymes activities

For protein and enzyme extractions, 0.5 g of fresh barley leaf samples was homogenized in 1.5 ml of 50 mM sodium phosphate buffer (pH 7.8) containing 1 mM Na_2-ethylenediaminetetraacetic acid (EDTA) and 2% (w/v) polyvinylpolypyrrolidone (PVPP). Samples were centrifuged at 14.000 g for 30 min, and supernatants were used for the determination of protein content and all enzyme activities. The total soluble protein contents of the enzyme extracts were analyzed according to Bradford (1976) using bovine serum albumin (BSA) as a standard.

Superoxide dismutase (SOD) activity

SOD enzyme (SOD; EC 1.15.1.1) activity was assayed by Beauchamp and Fridovich (1971) using nitro blue tetrazolium (NBT). 50 mM phosphate buffer (pH:7.8), 33 mM NBT, 10 mM L-methionine and 0.66 mM EDTA Na_2, 0.0033 mM riboflavin and 3 ml reaction mixture were added to supernatants. After the supernatants were diluted, the mixture was held under a light intensity of 300 mmol m^2s^1 for 10 min. The absorbance values at 560 nm were noted. One enzyme unit for SOD activity is defined as the amount of protein (mg) that causes 50% inhibition of light reduction.

Catalase (CAT) enzyme activity

CAT enzyme (CAT; EC 1.11.1.6) activity was measured according to Bergmeyer (1970)'s procedure. The reduction in the content of H_2O_2 was determined by the reduction in the maximum absorption at 240 nm. During the reaction, the drop in absorption was followed for 180 s. CAT activity was expressed as µmol H_2O_2 spent per minute.

Ascorbate peroxidase (APX) enzyme activity

APX enzyme (APX; EC 1.11.1.11) activity was determined according to Nakano and Asada (1981). The reaction mixture contained 50 mM Na-phosphate buffer (pH 7.0), 50 mM ascorbate, 0.1 mM EDTA Na_2, 1.2 mM H_2O_2 and 0.1 ml of enzyme extract in a final assay volume of 1 ml. The concentration of oxidized ascorbate was calculated by using an

extinction coefficient of 2.8 mM^{-1} cm^{-1}. One unit of APX was defined as 1 mmol ml^{-1} ascorbate oxidized min^{-1}.

Peroxidase (POX) enzyme activity

POX enzyme (POX; EC 1.11.1.7) activity was carried out according to the methods of Herzog and Fahimi (1973). The activity was calculated by measuring the increase in absorbance with the oxidation of tetrahydrochloride of 3, 3-diaminobenzidine at 465 nm. The reaction was initiated by adding H_2O_2 to the reaction mixture in the polystyrene cuvette (DAB solution, 0.6% H_2O_2, dl-H_2O and enzyme extrusion), and an increase in absorbance was measured for 3 min. A unit of POX activity was defined as 1 mmol ml^{-1} decomposed H_2O_2 min^{-1}.

Glutathione reductase (GR) enzyme activity

GR enzyme (GR; EC 1.6.4.2) activity was determined according to Foyer and Halliwell (1976). In NADHP, the reduction in the content of oxide glutathione was analyzed for 3 min at 340 nm. Calculations were done using the extinction coefficient of glutathione reduction enzymes (6.2 mm^{-1} cm^{-1}). One unit of GR was defined as 1 mmol ml^{-1} GSSG reduced min^{-1}.

Glucose, maltose and fructose content

For sugar analysis, 0.1 g of barley leaf was taken in a 15-ml volumetric flask and add 9 ml ultra-pure water and then sonicate for 5 min. A 0.5 ml of Carrez I reagent (0.25 M solution of potassium hexacyanoferrate (II) ($K_4Fe(CN)6.3H_2O$) and 0.5 ml of Carrez II reagent (1 M solution of zinc acetate ($Zn(CH_3COO)2.2H_2O$) were added; after making up to the mark the sample solution was centrifuged for 6 min at 9.000 rpm (modified from Eslamizad et al. 2020). After centrifugation, the liquid part of plant samples was placed in a 50-ml volumetric flask and add 10 ml ultra-pure water at 70 °C. The solutions were then filtered through a 0.45-µm polytetrafluoroethylene (PTFE) syringe filter and transferred to a 1.5 -ml HPLC vial for injection into an HPLC system.

HPLC

HPLC-RID analysis was performed on a Shimadzu LC-20AT Prominence Liquid Chromatograph, with a Shimadzu SIL-20AC HT Prominence Auto Sampler, a Shimadzu CTO-20A Column Oven, and a Shimadzu RID10A Refractive Index Detector. Sample preparation and chromatographic procedure were conducted as described in AOAC12. Samples were injected directly after filtration.

Chromatographic conditions: detector: RID, column: HPLC carbohydrate analysis column-NH_2 (5 mm, (250 mm × 4.6 mm) (mobile phase: ACN:ddH_2O (80:20), flow rate: 1.3 ml/min, analysis time: 35 min.

Scanning electron microscopy analysis

SEM analysis of this study was determined in accordance to Soumya et al. (2022). Barley tissue samples were harvested for 48 h and pre-fixed in formaldehyde-alcohol-acetic acid (FAA) for 4 h at room temperature. After fixation, the samples were washed in 0.1 M phosphate buffer, and then, the samples were postfixed in 70% methanol for 15 min at room temperature. Again, the samples were washed in 0.1 M phosphate buffer. After that, they were passed through a series of concentrations of ethanol (20%, 40%, 60%, 80% and 100%) for the dehydration process at room temperature for 10 min. The properties, shapes and particle sizes of barley leaves were observed using SEM (FEI, Quanta FEG 250, FEI, Quanta FEG 650) at Kastamonu University Central Research Laboratory. The samples were dried with the critical point drier (Quourm Technologies, E3100, Kastamonu University). After that, samples were coated with gold and platinum for 30 sn (30 milliampere) to ensure via sputter coating (Cressington, 108 Auto) to ensure their conductivity for SEM analysis. Pin stubs were then used to obtain cross sections of the barley leaves.

Statistical analysis

Descriptive statistics for continuous variables in the study is expressed as mean, standard deviation, median, minimum and maximum. "Mann–Whitney-U test" was used for pairwise comparisons, and "Kruskal–Wallis test" two-way ANOVA test was used for multiple comparisons. Following the Kruskal–Wallis test, the "Bonferroni corrected post hoc test" was used to compare different groups. The statistical significance level (a) was set at 5% in the calculations, and the SPSS (IBM SPSS for Windows, ver.26) statistical package program was used for the calculations.

Results

The results of pretreatment of pipecolic acid on physiological analysis

In the present study, no significant change was determined in root length under all treatments, but salinity reduced the

shoot length in barley plants by 25.86% according to control groups. Beside this, no significant effect was observed with Pip treatment on this parameter under salinity according to salt stress treated alone (Table 1).

When the root fresh weight measurements were analyzed, it was noticed that salinity leads to decrease by 37.7% according to control groups. Beside this, Pip treatment under salinity did not change this value according to salt stress alone. Moreover, salinity reduced the shoot fresh weight by 29.58% according to control groups, but 2 ppm Pip treatment increased this content by 16.27% as compared to salt stress alone, respectively, while there was no change with 4 and 8 ppm (Table 1).

In this work, salt stress caused a decrease in the root dry weight by 41.66% according to control groups. Beside this, 2 ppm Pip dose approached the content to the control levels significantly. However, salt stress reduced shoot dry weight by 25.84% according to control groups. Pip under salinity treatment did not change this content according to salt stress alone (Table 1).

Salinity reduced relative water content by 13.91% according to control groups, but Pip applied alone did not cause any alteration in this content. However, all Pip treatment under salinity increased RWC content, but this increase was not significantly according to statistical analysis (Table 1).

Relative electrolyte leakage was not affected with Pip treatment under nonstress conditions. But salinity induced this value by 2.8 fold according to salt stress alone. Beside this, Pip under salinity reduced REL values by 18.30%, 30.14% and 24.6% according to control groups, respectively (Table 2).

In the present study, CHL content was reduced by 59.01% according to control group while all Pip doses under salinity improved this content by 45.63%, 49.65% and 43.46% according to salt stress alone, respectively (Table 2).

When the leaf area measurements were checked, all pipecolic acid treatment did not change the leaf area under nonstress conditions as compared to control groups. Salt stress reduced this value by 34.91% according to control. Pip treatment under salinity increased this content by 32.39%, 12% and 43.89% according to salt stress alone (Table 2).

Table 2 Priming effects of Pip (2, 4 and 8 ppm) on leaf area

	Chlorophyll (mg/g(FW)	REL (%)	Leaf Area (cm^2)
C	53.08 ± 1.30^a	4.9 ± 0.04^a	14.60 ± 3.90^a
P_1	46.63 ± 3.18^a	3.85 ± 0.05^a	12.42 ± 1.40^a
P_2	41.35 ± 1.78^a	5.63 ± 0.03^a	13.84 ± 0.87^a
P_3	49.22 ± 0.41^a	4.43 ± 0.02^a	13.71 ± 1.55^a
Salt	21.75 ± 2.11^b	14.62 ± 0.03^b	9.60 ± 0.86^b
P_1S	40.01 ± 2.84^c	11.6 ± 0.04^c	14.18 ± 0.71^c
P_2S	43.21 ± 5.43^c	9.92 ± 0.02^c	10.91 ± 2.33^c
P_3S	38.48 ± 4.94^c	10.7 ± 0.04^c	17.14 ± 0.47^c

Chlorophyll content and relative electrolyte leakage under the salt stress in barley leaves. Control (1), Pip1; 2 ppm (2), Pip2; 4 ppm (3), Pip3 (8 ppm) (4) S, 300 mM NaCl (5), P1 + S (6), P2 + S (7), P3 + S (8)

The results of pretreatment of pipecolic acid on stress markers

Under nonstress conditions, superoxide radical content was induced by 4 and 8 ppm pipecolic acid according to control group. Beside this, salt stress induced this value by 2.6-fold, but combination treatment of salt and pipecolic acid increased by 6.71-, 4.99- and 9.73-fold according to control groups. From these results, it could be said that pipecolic acid could able to induce superoxide radical accumulation in barley leaves (Fig. 2).

When the hydrogen peroxide results were examined, salinity induced this content by 28.8% according to control group, while Pip treatment under salinity reduced by 44.32%, 34.04% and 33.53% according to salt stress alone (Fig. 2).

Table 1 Priming effects of Pip (2, 4 and 8 ppm) on lengths

	Root Length (cm)	Shoot Length (cm)	Root FW(gr)	Shoot FW(gr)	Root DW(gr)	Shoot DW (gr)	RWC (%)
C	21.00 ± 1.77^a	26.26 ± 0.89^a	0.196 ± 0.03^a	0.40 ± 0.02^a	0.012 ± 0.001^a	0.089 ± 0.008^a	92.86 ± 2.28^a
P_1	20.13 ± 1.75^a	25.20 ± 0.44^a	0.161 ± 0.03^a	0.36 ± 0.03^a	0.012 ± 0.002^a	0.065 ± 0.01^a	91.82 ± 4.36^a
P_2	20.50 ± 1.77^a	25.10 ± 0.94^a	0.172 ± 0.02^a	0.34 ± 0.04^a	0.01 ± 0.002^a	0.064 ± 0.008^a	88.23 ± 0.34^a
P_3	19.53 ± 1.61^a	25.50 ± 0.86^a	0.122 ± 0.03^a	0.30 ± 0.01^a	0.009 ± 0.001^a	0.054 ± 0.006^a	96.16 ± 0.05^a
Salt	20.66 ± 1.89^a	19.46 ± 1.06^b	0.122 ± 0.01^b	0.28 ± 0.03^b	0.007 ± 0.002^b	0.066 ± 0.009^b	79.94 ± 7.01^b
P_1S	21.43 ± 2.64^a	21.00 ± 1.95^b	0.158 ± 0.02^b	0.34 ± 0.05^a	0.013 ± 0.001^a	0.073 ± 0.01^b	81.31 ± 3.05^a
P_2S	19.23 ± 1.85^a	18.00 ± 1.24^b	0.114 ± 0.03^b	0.28 ± 0.02^b	0.007 ± 0.001^b	0.065 ± 0.009^b	84.34 ± 1.92^a
P_3S	19.93 ± 1.46^a	19.50 ± 1.46^b	0.117 ± 0.005^b	0.32 ± 0.03^b	0.011 ± 0.001^b	0.089 ± 0.01^b	83.64 ± 1.22^a

Fresh and dry weights, relative water content (RWC) under the salt stress in barley leaves. Control (1), Pip1; 2 ppm (2), Pip2; 4 ppm (3), Pip3 (8 ppm) (4) S, 300 mM NaCl (5), P1 + S (6), P2 + S (7), P3 + S (8)

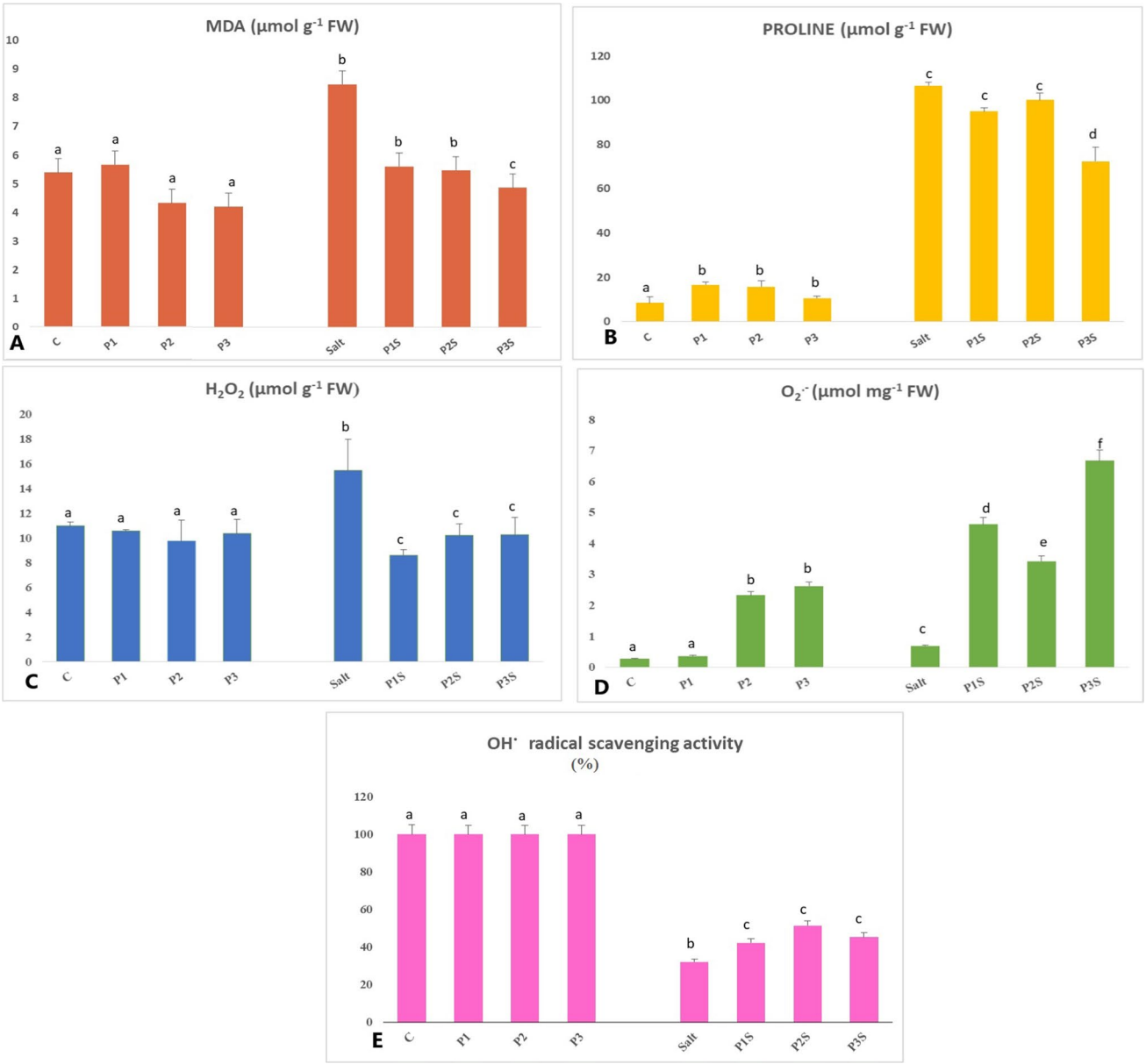

Fig. 2 Priming effects of Pip (2, 4 and 8 ppm) on stress markers under the salt stress in barley a) proline, b) MDA, c) H_2O_2, d) $O_2^{\cdot-}$, e) hydroxyl scavenging capacity

Proline content was induced significantly under Pip treatment alone (1.98-, 1.88-, 1.27-fold) while it was remarkable with salt treatment by 12.7-fold according to control group. Moreover, only 8 ppm Pip treatment reduced proline content significantly by 37.1% as compared salt application alone (Fig. 2).

In this work, salt stress induced malondialdehyde content by 36.22% according to control groups, but 8 ppm Pip treatment under stress conditions reduced this content by 42.51% according to salt stress alone, while the other inductions were not significant by 2 and 4 ppm. According to results, it could be said that the most effective dose was the 8 ppm pipecolic acid to decrease oxidative damage in barley leaves (Fig. 2).

In this study, hydroxyl radical scavenging capacity was reduced by 67.86% under salinity according to control groups while Pip treatment alone did not lead to change in this content. However, Pip treatment under salt stress induced this content 24.12%, 37.47%, 28.96%, respectively (Fig. 2).

The results of pipecolic acid on antioxidant enzymes

Totally, Pip application induced only SOD enzyme in this study, while no change was observed with other enzymes. Salinity reduced SOD enzyme activity according to control groups. This reduction was by 62.32%, while Pip treatment alone did not cause any change in this enzyme activity. 2 ppm and 4 ppm Pip treatment under salt stress induced this activity by 56.77% and 63.0%, while 8 ppm did not lead to change in this content according to salt stress alone. When the GR enzyme activity was examined, only 8 ppm under salt stress reduced this enzyme activity according to salt stress alone, while there was no change with other application. Beside this, APX, POX and CAT enzyme activities were not changed significantly by all treatment according to statistical analysis (Fig. 3).

The results of pipecolic acid on sugar metabolism

Glucose content was induced by pipecolic treatment (4.6-, 2.7- and 3.9-fold) under nonstress conditions, while the highest induction was at 2 ppm. 2 ppm and 4 ppm Pip treatment under salinity did not lead to change in this

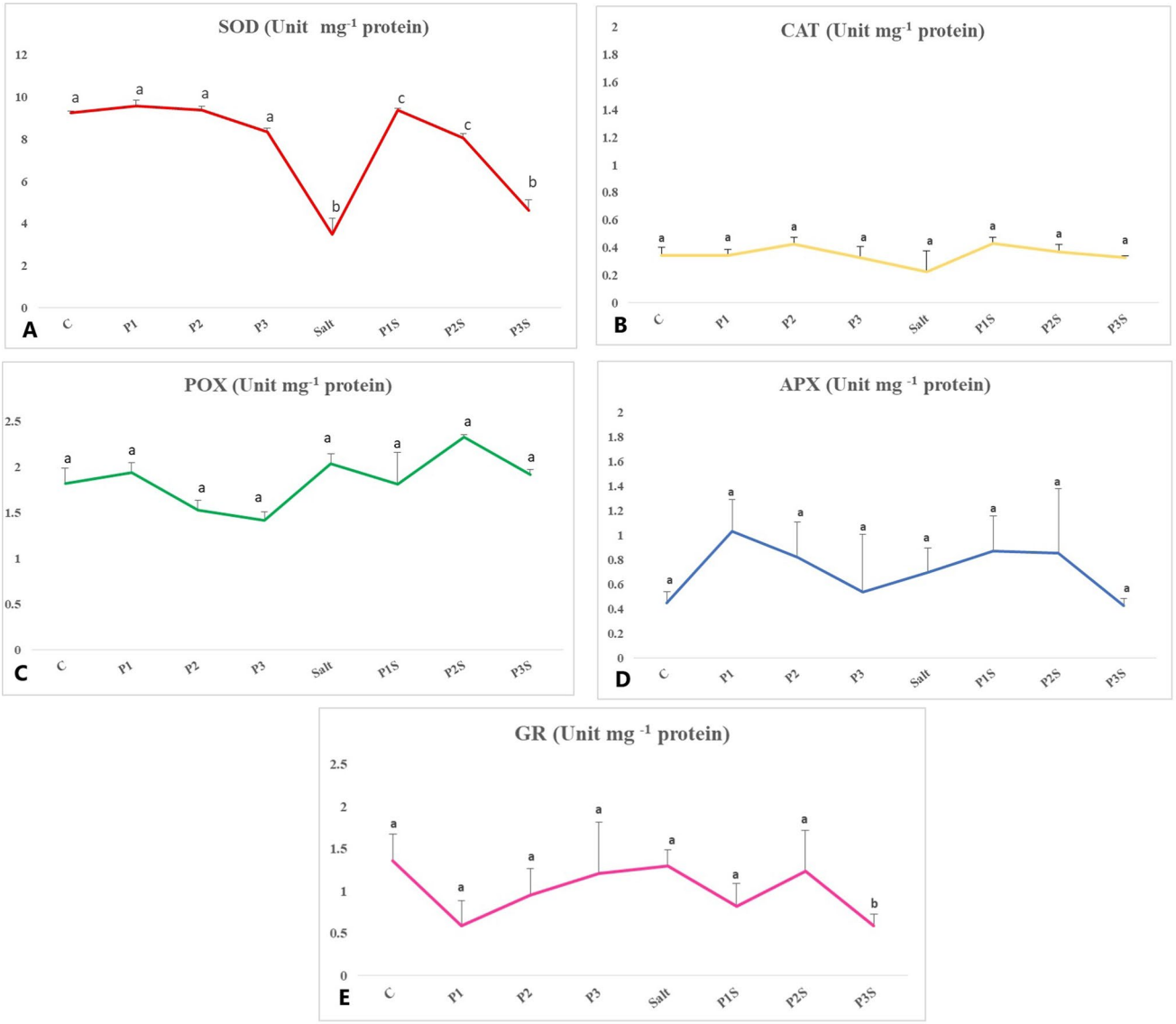

Fig. 3 Priming effects of Pip (2, 4 and 8 ppm) on antioxidant enzymes under the salt stress in barley leaves a) SOD, b) CAT, c) POX, d) APX, e) GR

content according to salt treatment alone, while there was a noticeably reduction (117.4 fold) with 8 ppm pipecolic acid (Fig. 4).

When the results of fructose were analyzed, only 2 ppm pipecolic acid increased this content by 2.3 according to control. Salt stress induced by 3.7-fold according to control group. Beside this, 2 ppm and 4 ppm Pip treatment under salinity did not effect this content, but 8 ppm reduced by threefold according to salt stress alone. 8 ppm Pip treatment induced maltose content by 2.1-fold, while salt stress increased this content by 5.1-fold according to control group. All Pip treatments under salinity lead to decrease by 4.53-, 4.17- and 4.17-fold significantly according to salt stress alone.

The results of pipecolic acid on structural change

As it is shown in Fig. 5, salinity leads to more leaf hair and increase stoma number in cells as compared to control groups. Beside this, Pip treatment under salinity showed a similar photograph to control groups. Otherwise, Pip application alone induced cell area and has wide spaces according to control groups.

Discussion

Regarding growth parameters, pipecolic acid (Pip) priming treatment alone did not produce changes in root or shoot lengths of barley seedlings, whereas only shoot length was inhibited under salinity (Table 1). These results are consistent with the reports by Abdelrady et al. (2024) and Sonia et al. (2024), who reported a reduction in the growth of barley plants under salt stress. In this work, salt stress may have reduced water uptake capacity and this suggestion is in agreement with the water content results (Table 1). The unchanged values in the lengths of root that were exposed to salinity may be related to the duration of stress (48 h). Our results reveal that Na^+ accumulation in leaves was greater than that in roots.

In addition, Pip treatment combined with salt stress did not lead to any changes in growth parameters compared with those under salt stress alone (Table 1). In the literature, Kim et al. (2020) reported that Pip biosynthesis retards plant growth in *Arabidopsis* mutants; this process is related to N-hydroxypipecolic acid (NHP) formation, which is catalyzed by flavin-dependent monooxygenase (FMO1) to convert Pip into NHP. Similarly, a high level of NHP leads to an enhanced systemic acquired response (SAR) as reported by Cai et al. (2023). In contrast, our results revealed that Pip concentrations (2, 4 and 8 ppm) applied to the seeds were not toxic to barley plants and this could be explained by converting Pip to NHP under high salinity. These results

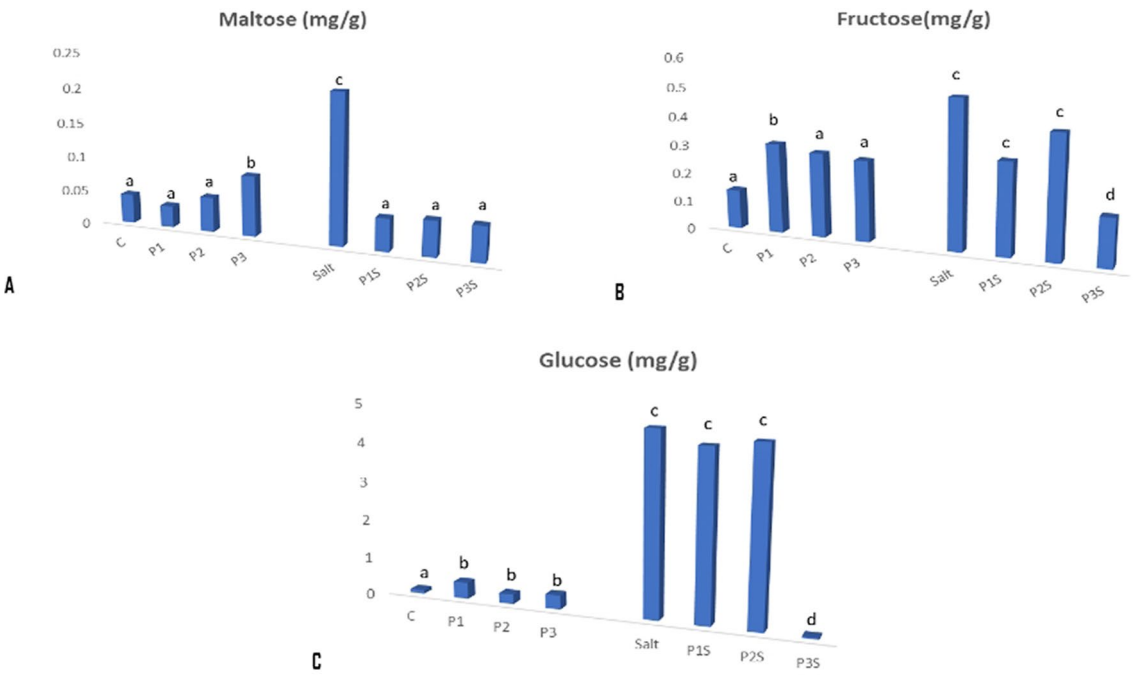

Fig. 4 Priming effects of Pip (2, 4 and 8 ppm) on sugar content under the salt stress in barley leaves a) glucose, b) fructose, c) maltose

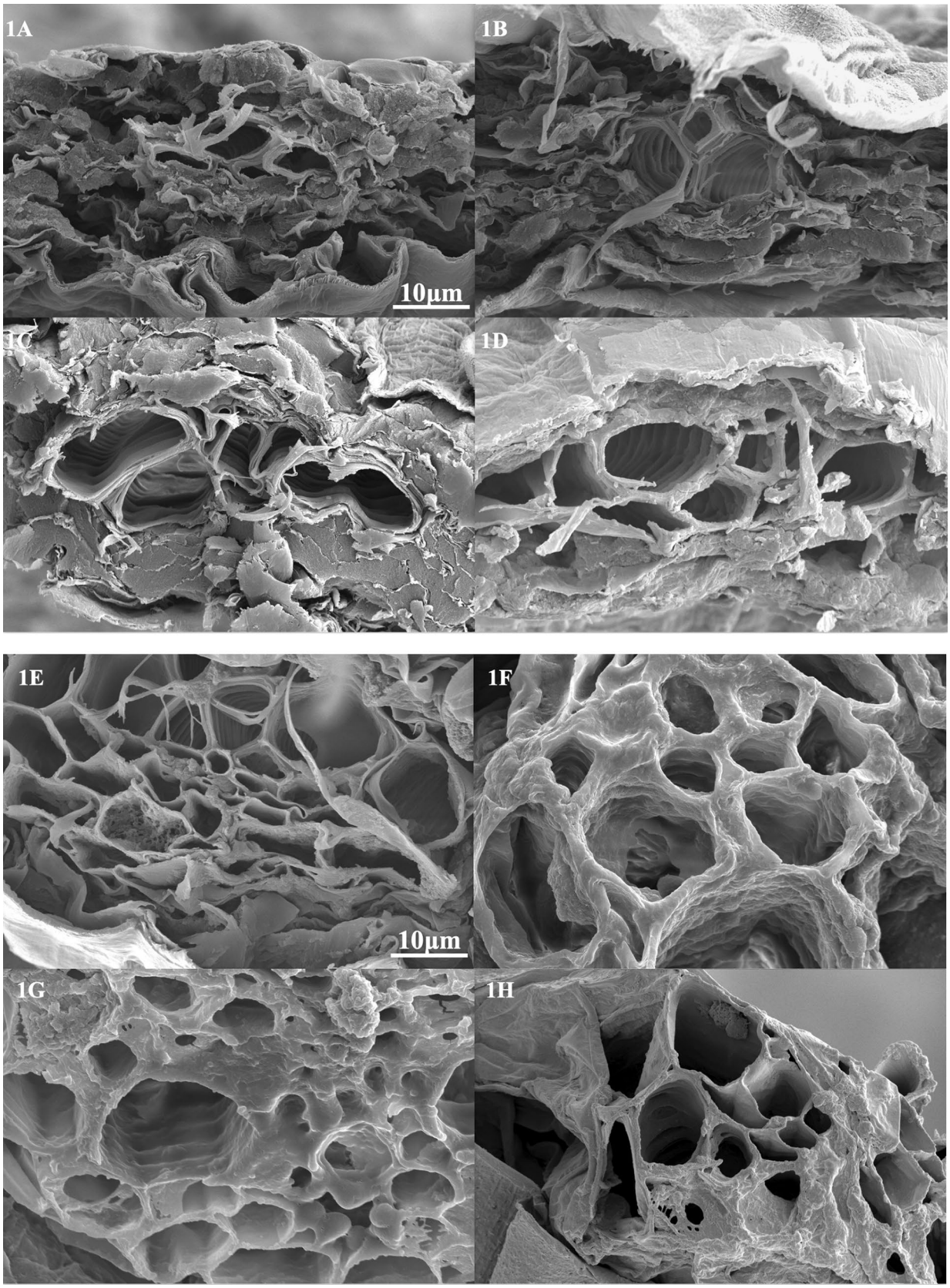

Fig. 5 SEM images of surface and cross section of barley leaves (1) epidermis cell, (2) adaxial surface. A) A, B, C, D columns 10 µm; B) E, F, G, H columns, 20 µm

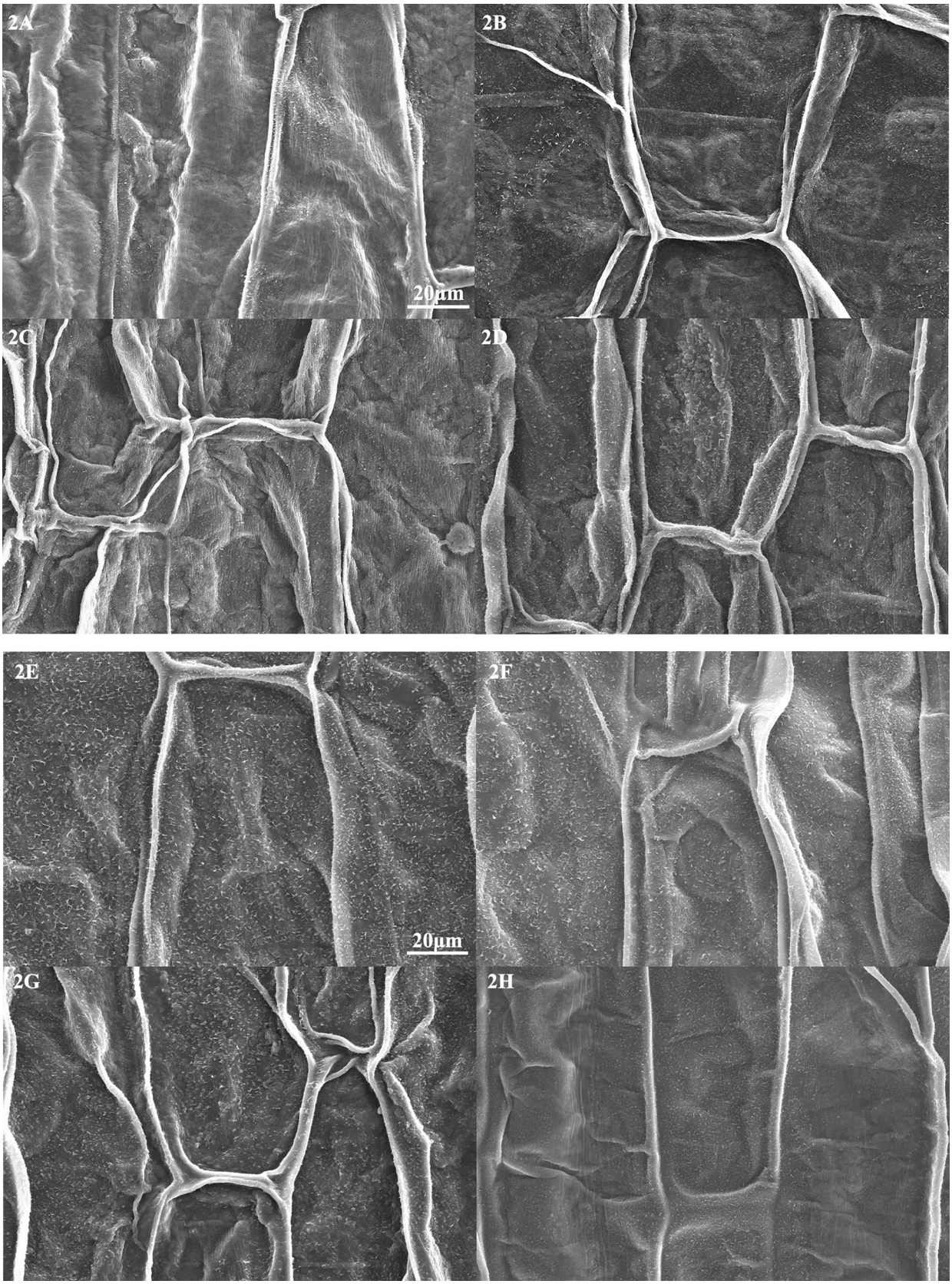

Fig. 5 (continued)

also suggest that priming or pretreatment with Pip may be more suitable than the use of concurrent treatments (Pip and stress) to cope with salinity. Nevertheless, Pazarlar (2024) presented that exogenous Pip induces stomatal closure in *Arabidopsis* leaves. In our work, exogenous Pip might have caused an increase in levels of the stress hormone, abscisic acid (ABA) and a reduction in the growth hormone, gibberellic acid (GA_3) levels in treated barley seeds. This study highlights the need to study the interplay between Pip and plant growth regulators in plants. This study highlights the need to study the interaction between Pip and plant growth regulators in plants.

When the fresh weights of the roots were measured, no changes in Pip or salinity were detected in the roots, whereas salt stress reduced these values (Table 1). Similarly, 100 mM NaCl strongly inhibited the fresh weight of roots during the hydroponic growth of barley (Qu et al. 2024). Considering the shoot values, only 2 ppm was effective, and this value significantly changed in response to salt stress alone. In parallel, Haghighi and Sheibanirad (2018) revealed that azelaic acid (AzA) treatment, which plays a role in the SAR mechanism such as Pip, leads to an improvement in fresh weights of shoots under increased salinity in tomato plants. In results, the dry weight of the roots increased with 2 ppm Pip, whereas 4 and 8 ppm produced a significant decrease in this parameter. These results suggest that Pip priming plays a role in adapting to increased salinity by facilitating maintenance of water transport, mineral uptake and membrane stability, but these effects differed with increasing Pip concentration. In parallel with our results, Pérez-García et al. (2019) reported that pipecolic acid could be described as an osmoprotectant in bacteria and plants.

The total chlorophyll content decreased after undergoing salt stress (Table 1), whereas Pip application leads to a significant increase in the total chlorophyll content under stress. This result was supported by photographs of barley, which showed more green leaves than salt-stressed barley did (Fig. 1). A decrease in chlorophyll content in barley is one of the most common types of damage caused by salinity (Sreesaeng et al. 2024; Bouhraoua et al. 2024). Considering the above results, it can be suggested that Pip could maintain the water and nutrient balance and lead to better photosynthetic capacity under salt stress. Similarly, Wang et al. (2021) noticed that the deletion of SlFMO1 in tomato plants that blocks flavin-dependent monooxygenase resulted in hydroxylated modifications of pipecolic acid to N-hydroxypecolic acid, which leads to produce sensitivity to drought stress and a more damaged photosynthetic system. In summary, the Pip cycle (Pip/NHP) is sensitive to drought stress in plants and can change drought tolerance. From this result, it could be suggested that when the conversion to NHP from Pip is triggered, salt tolerance might increase in barley leaves by increasing photosynthesis capacity.

With respect to the stress markers, the superoxide radical, hydrogen peroxide, proline and MDA contents were determined in barley leaves. The superoxide radical content was induced by high levels of salinity, but the accumulation of this content triggered by pipecolic acid was marked under nonstress (Fig. 2A). This result could be explained by the opening of pipecolic acid signal transduction, which enhances the defense system to cope with stress. Similarly, Pazarlar et al. (2021) reported that Pip treatment caused an increase in the reactive oxygen species content in cucumber (*Cucumber sativus* L.) plants in response to *P. xanthii* and *Pseudomonas syringae pv. lachrymans*. This idea could be supported by the previous report of Lenk et al. (2019) who determined that Pip stimulates NO production and primes the accumulation of reactive oxygen species to cause innate immune responses in barley. In relation to this finding, under nonstress conditions, no change in the activity of the SOD enzyme which catalyzes superoxide to less reactive ROS (hydrogen peroxide) was observed (Karpinska and Foyer 2024). In this work, the results showed that superoxide radical accumulation under nonstress conditions may be related to NADP oxidase which produces superoxide radical in the membrane system. In support of this second idea, Li et al. (2023) showed that exogenous Pip mediates eNADP (extracellular NADP) accumulation and induces the SAR system.

In this work, 300 mM NaCl leads to a decrease in SOD enzyme activity (Fig. 3A). In contrast, some studies have shown that this enzyme activity tends to increase in barley under salinity (Noren et al. 2021, Talaat et al. 2023; Rasouli et al. 2024), but this result could be explained by differences in the growth medium of barley, which was highlighted in previous studies (Maksimovic et al. 2013). In addition, decreased SOD enzyme activity was observed in rice (Dionisio and Tobita et al. 1998), wheat (Raza et al. 2007), barley (Seckin et al. 2010), soybean (Amirjani et al. 2010) and turnip (Noreen et al. 2010).

Under saline conditions, 2 and 4 ppm Pip leads to an increase in SOD enzyme activity, whereas 8 ppm Pip did not. These results reveal that a lower dose stimulated the antioxidant defense system. In support of this idea, NHP pretreatment was found to change the expression of genes related to cellular redox homeostasis in wheat seedlings (Zhang et al. 2021). However, when the MDA results were examined, the 8 ppm Pip concentration had the lowest MDA content under salinity. This finding revealed that this concentration was the most efficient dose for coping with the oxidative damage triggered by salt stress. In this way, pipecolic acid mimics an antioxidant or osmoprotectant to modulate redox homeostasis in barley leaves. This finding is also in line with the findings for chlorophyll and the relative water content (Tables 1 and 2).

In the present study, salinity increased the hydrogen peroxide content (Fig. 2C), while there was no significant

change in the APX, POX or CAT enzyme activities of barley leaves. This result is consistent with findings of salt stress-induced oxidative damage, with unchanged or decreased (SOD) enzyme activity with increased MDA content. However, the hydrogen peroxide content was reduced after Pip treatment under saline conditions (Fig. 2C). Interestingly, no changes in enzymes related to catalyzing the conversion of hydrogen peroxide to water and oxygen (APX, POX or CAT) in barley leaves that are involved in ROS detoxification were found. This result may be related to the antioxidant role itself or the induction of other antioxidants (anthocyanin, flavonoid, or polyphenol, terpenes) without the need to enhance the enzymatic defense system. In accordance with this suggestion, Rahimova et al. (2024) reported that treatment with pipecolic acid leads to an increase in the concentrations of terpenoids in all tissues. Similarly, Pip treatment induced resistance against PstDC3000 *B. cinerea* in tomatoes by regulating ROS accumulation without causing a change in CAT activity and causing a decrease in SOD enzyme activity (Zhang et al. 2020). In contrast with this finding, Sabharwal et al. (2024) reported that 1 mM pipecolic acid induced SOD and CAT enzyme activities in tomato plants with *R. solanacerum* inoculation. Similarly, Gula et al. (2024) showed that Pip supplementation induced SOD enzyme activity under bacteria treatment in *M. crystallinum*.

Proline is induced in various plants to maintain water balance, modulate cell redox homeostasis and scavenge ROS under stress conditions (Gharsallah et al. 2016). In this situation, the proline content increased as the salinity increased in barley leaves, but Pip application leads to a decrease in proline content (Fig. 2B). Pip priming may act as an osmoprotectant and produce an increase in the defense system in barley as reported by Moulin et al. (2006). This finding is also supported by the hydroxyl radical scavenging capacity and relative electrolyte leakage, both of which were reduced under combined Pip treatment and salinity compared to salt stress alone (Fig. 2E). These findings were also consistent with those of dehydroabietic acid (DA, a SAR signaling molecule, such as Pip) treatment of soybean leaves under salinity (Tascı and Seckin Dinler 2020). In total, pipecolic acid could maintain membrane stability by ensuring the retention of Na^+ and Cl^- ions and facilitating a reduction in salt stress damage.

Sugars are soluble and different types, including fructose, glucose and sucrose, are found. Soluble sugars play crucial roles in sugar signaling, a process that regulates plant development and stress responses in plants (Shah et al. 2024). In this work, after carbohydrate metabolism was analyzed, it can be concluded that salt stress leads to an increase in the glucose, maltose and fructose contents under salinity with the greatest increase observed in the glucose content (Fig. 4C). Under salinity, photosynthesis is limited, and starch can be converted to soluble sugars for the purpose of producing an increase in the root carbon supply (Marron et al. 2002). Similarly, many reports have shown that carbohydrate metabolism is impaired and that starch generally decomposes to glucose in plants under saline conditions (Meng et al. 2023). On the other hand, these soluble sugars could act as osmoprotectants that are involved in the maintaining the water balance. In this study, 8 ppm pipecolic acid combined with salinity clearly leads to a reduction in the glucose and fructose contents, whereas 2 ppm leads to a reduction in the maltose content (Fig. 4). These results reveal that pipecolic acid treatment maintained sugar metabolism and alleviated photosynthetic activity under salinity to reduce stress damage. However, to the best of our knowledge, no reports of interactions between pipecolic acid and sugar metabolism under stress are available in the literature. This subject warrants advanced biochemical and molecular analysis in the future.

SEM analysis revealed that salt stress impaired tissue formation and caused shrinkage in cell formation. Additionally, pipecolic acid treatment caused an increase the thickness of vascular bundles and provoked the integrity of barley tissue under saline conditions (Fig. 5). These findings suggest that pipecolic acid could alleviate stress-induced damage as supported by SEM findings. This result is also consistent with the REL values and leaf area (Table 2). In parallel with this result, Eastmond (2004) reported that G3P (glycerol-3-P), which is involved in the effects of SAR-inducing chemicals, improved the leaf area under stress conditions.

Conclusion

Pipecolic acid is known as an important signal metabolite that induces the SAR mechanism upon pathogen and bacterial attack in plants. Recently, abiotic stress factors (salinity, drought) have been mentioned. In the present study, the priming effects of pipecolic acid in barley leaves under salinity were investigated. Pip-treated seeds were able to cope with salt stress to the same degree as the control seeds. This molecule increases leaf area and protects photosynthetic activity, maintains membrane stability and water content and scavenges ROS-induced oxidative damage. These findings suggest that pipecolic acid acts as an antioxidant in stress-treated barley leaves. In summary, this study revealed that pipecolic acid can also use signaling key molecule against abiotic stress in plants. Nevertheless, there is still a knowledge gap concerning the interactions among hormones, secondary metabolites, mineral nutrients and pipecolic acid.

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Author Contribution Seckin Dinler B involved in writing original draft, investigation, formal analysis, conceptualization, performed the experiments. Kücükkalyon SM performed the experiments.

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Declarations

Conflict of interest The authors declare that they have no conflicts of interest.

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