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Original Research

The Mitigation Effects of Exogenous Hydrogen Peroxide when Alleviating Seed Germination and Seedling Growth Inhibition on Salinity-Induced Stress in Barley

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Abstract

Our study focused on the mitigation role of exogenously applied hydrogen peroxide (H_2O_2) in eliminating toxicty caused by salt (NaCl). Barley seeds were pretreated with 30 µM (micromolal) H_2O_2 for 24 hours and then exposed to increasing salt concentrations (0.0, 0.25, 0.275, 0.30 M). Morphogical and physiological changes in seed germination and seedling growth stages were compared between different treatments of salt in laboratory conditions. Adverse effects of salt during both germination and seedling growth stages were dependent on the concentration of the salt treatment. We found that the application of H_2O_2 effectively alleviated the salt-induced inhibition, and reduced the negative effects of salt on germination (germination index and vigor index), seedling growth stages (radicle and coleoptile lengths, fresh weight), and leaf parameters (stomata and epidermis counts, stomatal index, stomata sizes of adaxial and abaxial surfaces). The differences were statistically significant. Alleviating the effects of H_2O_2 increased in parallel with salt concentration ($p \leq 0.05$). However, under non-stress conditions (control), H_2O_2 didn't have any effect on the investigated parameters ($p \geq 0.05$). Our results suggest that exogenous H_2O_2 application is involved in the resistance of barley to salt stress.

Keywords: hydrogen peroxide, salinity, stomata, germination, barley

Introduction

Plants have evolved to live in environments where they are exposed to a wide range of abiotic stresses such as drought, salinity, high temperature, and UV. Among these stresses, salinity is considered one of the major abiotic stresses that has a negative impact on plant growth [1], especially reduction in photosynthesis, respiration, and protein synthesis [2]. At the present time, approximately 20% of agricultural areas are salty [3], and it is estimated that by 2050 this ratio will reach 50% [4]. Ever increasing salinity negatively affects processes such as seed germination, seedling growth and vigour, vegetative growth,

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flowering and fruit set, ultimately causing diminished economic yield and production quality [5], and these circumstances could cause a worldwide famine in the future. Thus helping plants adapt to saline soils and increasing their yield and quality is a must. Increased salt stress causes water stress (physiological drought) resulting from the increase of osmotic potential by raised ion levels in the soil. Especially during water stress, which is triggered by salt stress, excess accumulation of leaf Na^+ and Cl^- may negatively affect photosynthesis – the driving force of plant growth and development [6]. Moreover, salt stress inhibits CO_2 assimilation by inducing stomatal responses [7]. Although the inhibiting effects of salt stress on the growth and development of plants have been known for a long time, the mechanisms of whole plant responses to salt stress have not yet been completely explained.

Hydrogen peroxide (H_2O_2) is an important, relatively stable non-radical reactive oxygen species (ROS) [8]. ROS, including H_2O_2, is known to increase under stress factors such as salinity [9] and high temperature [10] in plant tissues [11]. In other words, environmental stresses result in oxidative stress in plants [12], and they are generally considered harmful to plant cells due to the fact that they can cause extreme oxidative damage to biological molecules such as DNA, proteins, and lipids [13], whereas it has been recently discovered that subtoxic concentrations of H_2O_2 act as the main regulator of plants' responses to environmental stress [14]. Moreover, it functions as a signal molecule [15] in the regulation of plant growth and differentiation [16]. On the other hand, an intracellular increase of ROS under biotic and abiotic stress conditions activates antioxidant enzymes (AOE) such as catalases and peroxidases in the cell [17]. Thanks to this antioxidant defence mechanism [18], adaptation by plants to stressful environments becomes easier, and they can survive under negative environmental conditions.

H_2O_2, a second messenger in plant cells, plays a critical role in seed germination and seedling growth as well as the acquisition of stress tolerance [13]. However, there are few studies about the effects of H_2O_2 on seed germination and seedling growth under normal and saline conditions. Some experimental studies have shown that the exogenous application of H_2O_2 stimulates the germination of *Zinnia elegans* [19], rice [20], sunflower [21], wheat [22], and maize [23] seeds in both distilled water and saline medium. Moreover, foliar spray of H_2O_2 significantly reduced salinity-induced effects by increasing the activites of catalase in maize [24].

The investigation of modifications in germination and growth parameters of plants under various stress conditions (which causes changes in morphological, anatomical, physiological processes, and their complex interactions) [25], can be determined by anatomical and morphological anomalies that may be visible signs of these changes [26]. Therefore, in this study, direct and indirect effects of salt on the growth and development of barley were evaluated via some growth parameters. And, to measure the potential mitigation effects of the exogenous application of H_2O_2 on seed germination, seedling growth and stomatal responses of barley plants exposed to increasing salt concentrations were investigated.

Materials and Methods

Plant Material and Plantation

In this study, barley (*Hordeum vulgare* cv. Bülbül 89) seeds were used as the material. The seeds were surface sterilized with 1% sodium hypochloride. Salt (NaCl) concentrations applied in the study were 0.0, 0.25, 0.275, and 0.30 M. Hydrogen peroxide (H_2O_2) concentration was 30 µM. H_2O_2 and NaCl concentrations used in the study were determined in a preliminary investigation. Barley seeds were pretreated in beakers that contained a sufficient volume of distilled water (control) or aqueous solution of H_2O_2 for 24 h at room temperature. Subsequently, for each experiment 25 seeds with the same size were carefully selected and placed on a whatman paper soaked with 20 ml distilled water and then incubated at 20ºC for seven days. Each treatment was replicated three times. The ledge of radicle through the seed coat was taken as the criteria of seed germination. For analysis of growth parameters (stomatal index, stomata length, and width), seedlings were transplanted into pots with perlite, and were cultivated in a growth chamber. Hoagland's nutrient solution was regularly added to each pot for 45 days.

Measurements

On the seventh day after sowing, relevant parameters (root length, stem height, number of germinated seeds) were calculated to determine germination and vigor indexes. Germination indices (GI) [27] and vigor indices (VI) [28] of each treatment were calculated using the following equations:

GI = (% Relative seed germination
X % Relative root growth) / 100

...where:

– *% Relative seed germination*: (Number of seeds germinated in Salt concentration / number of seeds germinated in control) X 100
– *% Relative root growth*: Mean root length in Salt concentration / Mean root length in control X 100
– VI = (Seedlings length (cm) X Germination percentage (%)) / 100

Stomatal index was calculated according to Rengifo et al. [29]. Stomata sizes (length and width) were measured using an ocular micrometer under a light microscope (40x object and 10x ocular).

Statistical Analysis

Statistical analysis of variance (ANOVA) was performed on all experimental data reported in the present paper and statistical significance was taken as $P \leq 0.05$.

Duncan's multiple range test was applied on means of at least two independent assays with three replicates using SPSS Software 13.0. All experimental data are expressed as means ± standard deviation (SD).

Results

Germination Index, Vigor Index, and Other Growth Parameters

The effects of H_2O_2, salt, and their interactions during seed germination and seedling growth stages of barley were examined in terms of germination index (GI) and vigor index (VI) (Figs 1a, b), and other growth parameters (radicle and coleoptile length, plant fresh weight) (Figs 2a, b, c). Under normal conditions (control), the GI rate of barley plants that were exposed to salt stress decreased as the salt concentration increased, to as much as 51% (the most devastating effect) in a 0.30 M salt concentration. VI was also reduced by 88% in the highest applied salt concentration. This suggests that VI was affected more than GI. Furthermore, the observed decreases in GI and VI in parallel with increasing salt concentrations were found to be statistically significant ($p \leq 0.05$). Negative effects of salt stress were greatly reduced in barley plants pretreated with H_2O_2 ($p \leq 0.05$). For example, the pretreatment of H_2O_2 increased the GI from 16.71 to 25.07 in 0.25 M salt concentration, from 12.25 to 21.23 in 0.275 M, and from 8.19 to 14.65 in 0.30 M compared to control. Also, H_2O_2 increased VI by 56%, 33%, and 16% in increasing salt concentrations compared to control. On the other hand, H_2O_2 had positive effects on GI and VI not only under stress conditions, but also when applied alone.

Growth of the barley plants was severely impaired by salt stress in terms of all examined seedling growth parameters (radicle and coleoptile length, plant fresh weight). Salt stress decreased radicle length by 82%, 84%, and 85% (Fig. 2a), coleoptile length by 39%, 62%, and 76% (Fig. 2b), and fresh weight by 27%, 47%, and 66% (Fig. 2c), in 0.25, 0.275, and 0.30 M salt concentrations, respectively. Radicle length reflected the most suppressing effect of salt stress ($p \leq 0.05$). However, pretreatment

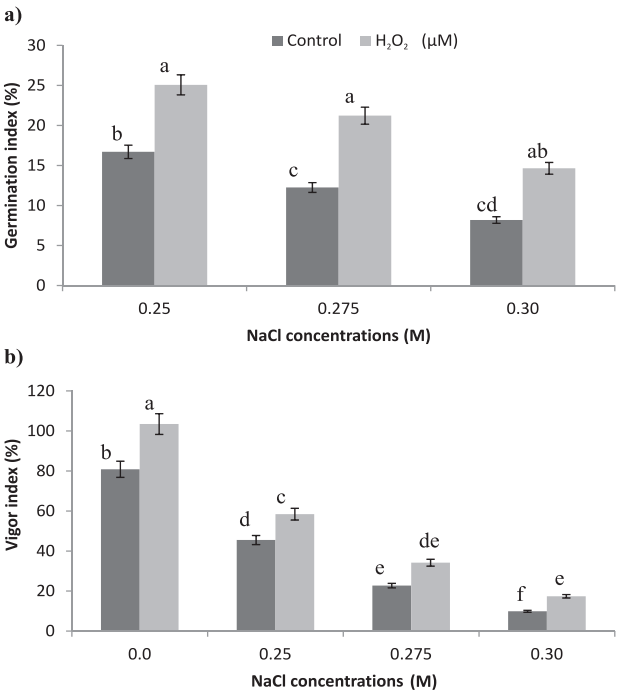

Fig. 1. Germination index a) and Vigor index b) values of barley seeds exposed to various concentrations of salt after H_2O_2 pretreatment. Data represent the means and vertical bars indicate the standard error. The values that are followed by the same letter do not differ statistically at a significance level of $P \leq 0.05$.

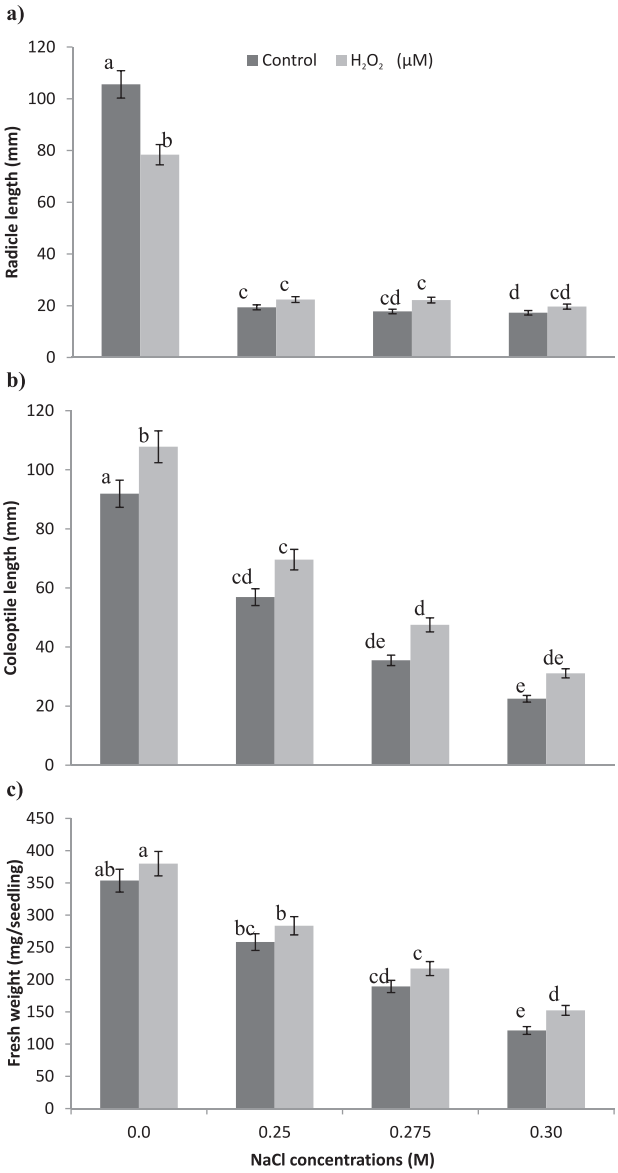

Fig. 2. Effects of H_2O_2 on various characters of early seedling growth in salt stress. Radicle length a), coleoptile length b), and fresh weight c). Data represent the means and vertical bars indicate the standard error. The values that are followed by the same letter do not differ statistically at a significance level $P \leq 0.05$.

Table 1. The structure of adaxial and abaxial epidermis in leaves of barley plants treated with different concentrations of salt after H_2O_2 (μM) pretreatment.

	NaCl (M)	Stomata number (mm^2)		Epidermal cells number (mm^2)		Stomatal index		Stomata width (μm)		Stomata length (μm)	
		C	H_2O_2 (μM)	C	H_2O_2 (μM)	C	H_2O_2 (μM)	C	H_2O_2 (μM)	C	H_2O_2 (μM)
Adaxial surfaces	0.0	*5.6±0.5^{a}	5.8±0.1^{a}	13.1±0.1^{a}	13.5±0.2^{a}	29.9±0.1^{a}	30.2±0.1^{a}	9.0±0.5^{a}	9.8±0.3^{a}	21.1±0.5^{a}	21.6±0.2^{a}
	0.25	4.8±0.1^{b}	5.4±0.4^{ab}	12.4±0.3^{ab}	13.1±0.6^{a}	27.9±0.2^{b}	29.1±0.1^{ab}	8.4±1.0^{ab}	9.4±1.2^{a}	19.3±0.3^{b}	20.6±1.0^{b}
	0.275	4.0±1.1^{c}	5.1±0.3^{b}	11.9±0.2^{b}	12.8±0.3^{ab}	25.1±0.1^{c}	28.4±0.1^{b}	7.5±0.2^{b}	8.1±0.4^{ab}	18.1±1.2^{c}	19.3±1.1^{bc}
	0.30	3.1±0.1^{d}	4.5±1.1^{c}	11.1±1.1^{bc}	12.1±0.2^{b}	21.4±0.1^{d}	27.1±0.2^{bc}	6.7±0.3^{c}	7.6±0.2^{b}	16.2±0.3^{d}	17.9±1.3^{c}
Abaxial surfaces	0.0	5.1±0.2^{a}	5.3±0.5^{a}	12.2±1.2^{a}	12.8±0.3^{a}	29.4±0.1^{a}	29.8±0.1^{a}	7.9±0.1^{a}	8.6±1.1^{a}	20.6±1.2^{a}	20.8±0.5^{a}
	0.25	4.0±1.1^{b}	4.8±0.6^{ab}	11.2±0.3^{ab}	12.6±0.5^{a}	26.3±0.1^{b}	27.5±0.1^{b}	7.4±0.6^{ab}	8.1±0.2^{ab}	20.1±0.5^{ab}	20.4±0.5^{a}
	0.275	3.1±0.7^{c}	4.5±0.2^{bc}	10.4±0.7^{b}	11.9±0.2^{ab}	22.9±0.2^{c}	27.4±0.1^{b}	6.7±1.1^{b}	7.6±1.2^{b}	18.0±1.1^{b}	19.4±0.6^{ab}
	0.30	2.2±0.4^{d}	4.1±1.1^{c}	9.5±0.8^{c}	11.3±1.3^{b}	18.8±0.1^{d}	26.6±0.1^{c}	6.1±0.2^{bc}	7.2±1.1^{bc}	17.2±1.1^{c}	18.3±1.3^{b}

*Values followed by the same letter do not differ statistically at significance level P≤0.05.

of H_2O_2 alleviated the inhibitory effect of salt stress on the parameters in a dose-dependent manner. Coleoptile length increased by 15%, 19%, 26%, and 21%, and fresh weight by 7%, 9%, 13%, and 10% in 0.0, 0.25, 0.275, 0.30 M salt concentrations, respectively. However, even though H_2O_2 significantly improved radicle length under salt stress ($p\leq0.05$), it didn't have much effect under control conditions ($p\geq0.05$). Moreover, H_2O_2 increased radicle length by 14%, 20%, and 13% in 0.25, 0.275, and 0.30 M salt concentrations, but it didn't have any effect in the control medium (0.0 M).

Stomatal Responses

Barley plants exposed to salt stress showed reduced growth in terms of photosynthetic apertures (stomata and epidermis number, and stomata sizes) on both adaxial and abaxial surfaces of the leaves (Table 1). Salt stress decreased the stomata count by 15%, 29%, and 45%, and epidermis count by 6%, 10%, and 16% on adaixal surfaces under 0.25, 0.275, and 0.30 M salt stress, compared to control, respectively. The decrease in stomata and epidermis counts of barley seeds exposed to increasing salt stress also decreased stomatal index on adaxial surfaces ($p\leq0.05$). Negative effects of salt stress on abaxial surfaces were nearly identical to those observed on adaxial surfaces. For example, stomata and epidermis numbers on abaxial surfaces decreased by 22%, 40%, and 57%, and 9%, 15%, and 23% at 0.25, 0.275, and 0.30M salt concentrations, respectively, compared to control. On the other hand, the stomatal index of abaxial surfaces decreased by 11%, 23%, and 37%, respectively. The negative impact of increasing salt concentrations manifested as a decrease in stoma sizes in both surfaces of leaves ($p\leq0.05$). This was most dramatically observed in the adaxial surface of the leaves treated with 30M salt concentration as a 26% decrease in stomata widths. Effects of increasing salt concentrations on stomata lengths on adaxial surfaces were determined as a 24% decrease at the highest applied salt concentration compared with control. However, pretreatment of H_2O_2 alleviated the adverse effect of salt stress on phosynthetic apertures on both surfaces of leaves. H_2O_2 pretreatment increased stomata and epidermis counts on adaxial surfaces by 4% and 3% in control conditions, 12% and 6% in 0.25 M salt, 22% and 8% in 0.275 M, and 32% and 9% in 0.30 M, respectively ($p\leq0.05$). The success of H_2O_2 pretreatment that increased stomata and epidermis counts in increasing salt concentrations were also reflected by stomatal index (1%, 5%, 12%, and 22% increase on adaxial surfaces, 1%, 2%, 8%, and 7% on abaxial surfaces). Similar results were observed for stomata sizes – especially stomata width, which significantly increased with H_2O_2 pretreatment on both surfaces of leaves ($p\leq0.05$). This positive effect was determined as 12% on adaxial surfaces and 16% on abaxial surfaces at the highest applied salt concentation. In simillar concentrations, stomata length was determined to be increased by 10% on adaxial and 7% on abaxial surfaces.

Discussion of Results

Although the germination process is governed by cellular differantiation, the process can negatively or positively [30] alter due to various environmental conditions (water, nutrition, soil characteristics, etc.). Soil salinity [31] particularly affects the germination process [32] – one of the most important periods of plant growth – by delaying germination and decreasing the germination rate [33]. Since germination is the most sensitive growth stage of plants to salinity [34], it is only natural to think that being exposed to stress conditions during this stage will negatively impact plant development and differentiation in later stages. In our study, GI and VI decreased with increasing salt concentrations. This negative impact was especially destructive at the highest applied salt concentration (0.30 M), reducing the parameters by 51% and 88%, respectively. Therefore, the delay in growth of barley can be explained by the suppression of germination under salt stress. Various plant growth regulators and osmoprotectans are used to alleviate the detrimental effects of salt stress on plants [35-37]. They act by either increasing nutrient intake and transfer, or protecting osmotic balance [36]. The exogenous application of H_2O_2 significantly alleviated salt stress-induced growth inhibition. For example, the pretreatment of H_2O_2 increased GI by 66%, 57%, and 55% at 0.25, 0.275, and 0.30 M salt concentrations, respectively. While simillar effects were observed for VI ($p \leq 0.05$), H_2O_2 was more effecive on GI. This also points to the fact that VI is more sensitive to salt stress than GI. In other words, it is evidence of the alleviating effect of H_2O. At the same time, the observation that H_2O_2 has very little effect on radicle length of the plants grown in control medium indicated that it is more active under stress conditons. But H_2O_2 wasn't really effective at the highest applied salt concentration (30 M). The negative effects of salt stress on the various parameters of plant development [38] were observed in our study with the application of increasing salt concentrations (radicle and coleoptile length, fresh weight). At seed germination stage, the redistribution of toxic Na and Cl ions affects the embryo and impairs germination, and creates an osmotic imbalance affecting root growth [39]. H_2O_2 was most effective on radicle length at 0.25 M salt concentration (20%), on coleoptile length at 0.275 M (26%), and on fresh weight at 0.275 M (13%). The negative impact of salt on seed germination and seedling growth stages is caused by the decreased activity of antioxidant enzymes such as catalase (CAT) and peroxidase (POX) when NaCl reaches toxic levels in the environment and triggers reactive oxygen species (ROS) [40].

Stomata are epidermal structures that are very important to a plant's vital functions and productivity. Many environmental factors such as biotic and abiotic stresses can modulate stomatal reactions [41]. The variation of stomatal responses under abiotic stress [42] made it possible to use these structures in determining stress tolerance [43]. We determined that in parallel with increasing salt concentrations the amount of photosynthetic apertures on both surfaces of barley leaves decreased. The highest applied salt concentration (30 M) significantly affected photosynthetic apertures on both sides of the leaves. At this concentration, reductions by 45% and 57% were observed in stomata count, 16% and 23% in epidermis count, and 29% and 37% in stomatal index on adaxial and abaxial surfaces, respectively, compared to control ($p \leq 0.05$). Various environmental factors such as salt stress [44] and drought [45] cause changes in the development of stomatal apertures (e.g., stomata density and size). Salt stress affected the abaxial surfaces of the leaves significantly more than the adaxial surfaces. Especially, a reduction in stomata count on abaxial surfaces of leaves by 57% was observed at the highest applied salt concentration, which means that stomata count has decreased by more than half. The decrease in stomata and epidermis counts on both surfaces of the leaves also negatively affects the stomata index and leads to a shrinking of leaf surface area [46]. However, although the highest applied salt concentration greatly reduced stomata counts (45% on adaxial, 57% on abaxial surfaces) ($p \leq 0.05$), there weren't significant reductions in epidermis counts on both surfaces ($p \geq 0.05$). On the other hand, the negative impact of increasing salt concentrations manifested as a decrease in stomata sizes on both surfaces of leaves ($p \leq 0.05$). This was most dramatically observed on the adaxial surface of the leaf treated with 30 M salt concentration as a 26% decrease in stomata widths. The effects of salt stress on stomata lengths on adaxial surfaces were determined as a reduction by 24% at the highest salt concentration compared with control. Reductions in counts and sizes of photosynthetic apertures are important indicators that the photosynthesis mechanism is also adversely affected [42, 47]. At the same time, it was determined that drought stress reduces the stomata sizes of maize plants, and gas exchange rates decreased in parallel with increasing drought stress [48]. A reduction of stomata sizes under salt stress protects the balance between photosynthesis and carbon intake, and prevents excessive water loss with transpiration [49]. These processes give the plant a xerophytic character [50] and help it to adapt to drought conditions caused by salt stress [51]. Besides, reductions in stomata counts and sizes of plants exposed to various stress conditions negatively affect photosynthetic activity, causing regressed growth and development [52]. Furthermore, abnormalities in the shapes and numbers of these structures in plants exposed to salt stress can reduce carbon fixation capacity [53], and ultimately disturb the photosynthetic process [12]. It is thought that reductions in sizes of stomatal structures (width/length) on both surfaces of leaves can have simillar outcomes. It is also thought that these reductions can be caused by impaired cell elongation or a decrease in elongated cells [54].

H_2O_2 pretreatment significantly improved the sizes and counts of photosynthetic apertures on both surfaces of leaves and had a positive effect on plant growth. The observation that this positive effect increased in parallel with increasing salt concentrations is remarkable. Stomata

indices, which reflect the stomata and epidermis counts per unit area of leaf surfaces, significantly increased with H_2O_2 pretreatment under salt stress ($p \leq 0.05$). This positive effect was more evident on adaxial surfaces (22%) than abaxial surfaces at the highest applied salt concentration. The most devastating effect of salt stress was observed on adaxial surfaces of leaves. H_2O_2 was also the most succesful in reducing salt stress on the same surface (adaxial), indicating that its effect increases with increasing stress. A similar situation was observed for the sizes of stomatal apertures. Increasing salt stress affected stomata width the most on both surfaces of leaves, especially on adaxial surfaces (26% decrease at 30M). H_2O_2 also had positive effects on stomata widths on both surfaces of leaves, especially abaxial surfaces (16% increase at 30M). Our results show that H_2O_2 activates antioxidant defence mechanism under stress conditions and triggers certain adaptive mechanisms of plants. This stimulating effect of H_2O_2 demonstrates that it can prevent or reduce the negative impact of stress conditions on the growth of plants. However, even though barley seeds pretreated with H_2O_2 sustained development in increasing salt concentrations, concentrations above 0.30M were out of H_2O_2's effective range.

Conclusions

These results show that H_2O_2 can alleviate the negative effects of salt stress and positively affect plant growth and development. GI, VI, radicle and coleoptile lengths, fresh weight, and stomatal responses on both surfaces of leaves were decreased with increasing salt concentrations compared to control. The adverse effects of salt stress on plant growth were alleviated with H_2O_2 application. H_2O_2 application significantly decreased the inhibitory effect on plant growth, and thus the application of H_2O_2 stimulated the growth of plants. Moreover, H_2O_2 activates antioxidant enzymes in the cell such as catalases and peroxidases at subtoxic concentrations, and triggers plants' antioxidant defence mechanisms, and in this way it is thought that it provides significant adaptation to salinity. The present study showed that the growth parameters of barley plants were enhanced by adding H_2O_2 under salt stress conditions, proving the positive and beneficial effects of H_2O_2 on barley growth.

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