



Salt stress alleviation by seed priming with silicon in lettuce seedlings: an approach based on enhancing antioxidant responses

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ABSTRACT: Seed germination and seedling growth are the stages most sensitive to salt stress, which can induce ion-specific effects and oxidative stress. Although silicon (Si) has been known to improve plant defense systems against stressful-conditions, little is known about seed priming with Si and mechanisms of Si-mediated alleviation of salt stress in seedlings. To further the modulation of salt-stress responses and their relation with Si, seed priming with calcium silicate (0, 0.05 and 0.1 mM Ca_2SiO_4) was used in lettuce seeds to improve seed germination and tolerance on subsequent salt-stress exposure (0 and 50 mM NaCl). The effect of Si-priming was assessed on germination, germination rate index and mean germination time and survival of seedlings in salt-stressful conditions supported by biochemical approach involving antioxidant responses. Overall results indicated that Si plays a role in alleviating the negative effects of salt stress by improving germination performance and displayed increased SOD, CAT and GR activities and reduced malondialdehyde and H_2O_2 contents. Thus, our findings demonstrate that seed-priming with Si is an efficient management technique that can be used to alleviate deleterious effects of salt-stressful on germination of lettuce seeds and enhance salt tolerance of seedlings due the increased activity of ROS-scavenging enzymes.

Key words: antioxidant enzymes, *Lactuca sativa*, oxidative damages, salinity.

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INTRODUCTION

Seed germination is one of the most important phases in the life cycle of higher plants. It is the sum of all physiologic processes inside the seed, when water is taken up for respiration, protein synthesis and other metabolic activities (Ouji et al. 2015). However, under unfavorable growth conditions, such as salinity, the seed viability will be affected by osmotic potential outside and obstructing of water absorption, or Na^+ and Cl^- toxicity effects (Yacoubi et al. 2013), which results in osmotic stress and generation of reactive oxygen species (ROS) (Gratão et al. 2015). However, the effects of salt-stress on plants depend on the concentration, time of exposure to salt, plant genotype and environmental factors (Alves et al. 2018; Astaneh et al. 2018).

Plants are able to detoxify excessive oxidation caused by salt-stress conditions by complex enzymatic and nonenzymatic mechanisms that protect plant cells against oxidative damage (Alves et al. 2018): Superoxide dismutase (SOD, EC 1.15.1.1) dismutates O_2^- to H_2O_2 and subsequently H_2O_2 may be detoxified to H_2O by the action of ascorbate peroxidase (APX, EC 1.11.1.11), catalase (CAT, 1.11.1.6) (Gill and Tuteja 2010; Shekari et al. 2017; Alves et al. 2018), glutathione peroxidase (EC 1.11.1.9), guaiacol-type peroxidase (GPOX, EC 1.11.1.7), and thioredoxin (TRX, EC 1.8.1.9), which can act directly on plant signaling processes and define the redox state of transcription factors linked to the promoter region of DNA; whereas

peroxiredoxins (PRX, EC 1.11.1.15) are responsible for detecting stress and redox status of the cell, acting on plant signaling (Liu et al. 2013; Liebthal et al. 2018; Laxa et al. 2019).

The reduction of ROS may occur through the action of the antioxidant defense system, which is also composed of nonenzymatic compounds that maintain the cellular redox status and involves the regeneration of reduced glutathione (GSH) through the enzyme glutathione reductase (GR, EC 1.6.4.2), and the ascorbate (AsA) that can regulate biochemical reactions and improve the synthesis of stress-responsive proteins (Ahmed et al. 2016; Alves et al. 2018).

In addition to the antioxidant defense system, H_2O_2 acts as a signaling molecule by directly affecting stomatal closure, the activity of a target molecule, cell signaling transduction pathways and gene expression in plants (Suhita et al. 2004; Niu and Liao 2016).

Plants grow in dynamic environments where they can be exposed to stressful conditions and are able to further fortify their defense systems. Therefore, some management strategies have been developed to improve plant performance under unfavorable conditions to guarantee better rate of germination and plant development (Shekari et al. 2017; Singh et al. 2018).

In fact, seed priming is a potentially able method to improve the seed performance and overcome the negative effects associated with stressful conditions. During seed-priming process, physicochemical changes that modify the protoplasmic characters occur, increasing the embryo physiological activity and associated structure, leading to higher water absorption and cell elasticity (Singh et al. 2018).

An alternative recently studied to decrease the oxidative damage on plants is to use silicon (Si) fertilization in plants subjected to environmental stresses (Khan et al. 2016). Therefore, Si has been shown to be able to improve early growth and establishment of plants under stressful conditions through increasing antioxidant enzymes, photosynthetic capacity and low transpiration coefficient (Sivanesan et al. 2011; Imtiaz et al. 2016). However, few studies have investigated the use of Si with emphasis on ROS generation and activation of antioxidant defense system in plants under stressful-salt conditions (Coskun et al. 2016; Silva et al. 2018).

In view of the promising application of seed priming method, this work aimed to further address the modulation of salt-stress responses and their relation with Si in lettuce seeds, subsidized by the antioxidant systems responses of the seedlings to stressful-salt conditions.

MATERIAL AND METHODS

Plant material

Lettuce (*Lactuca sativa* L.) cultivar Vera was used in the present experiment. The choice of this cultivar was due to its characteristics: it can be cultivated throughout the year and it can be cultivated in various regions of the country.

Priming treatment and growth conditions

The seed-priming treatments were performed by the combination of two calcium silicate concentrations (0, 0.05 and 0.1 mmol·L⁻¹ Ca₂SiO₄) and salt-stress exposure (0 and 50 mmol·L⁻¹ NaCl). NaCl concentrations were established through the literature review, and studies have demonstrated that lettuce plants exhibit growth reduction to the exposure of 50 mmol·L⁻¹ NaCl (Al-Maskri et al. 2010; Bartha et al. 2015; Hniličková et al. 2019). Si concentrations were established according to laboratory pre-tests, which showed responses to avoid oxidative damages (data not shown). Four replicates of 50 seeds were placed on a layer of 10.5 cm diameter germination paper moistened in plastic boxes by direct immersion in distilled water (control) or 15 mL of the respective 0.05 and 0.1 mmol·L⁻¹ Ca₂SiO₄ solution for 1 h. After priming period, seeds were submitted to salinity (0 and 50 mmol·L⁻¹ NaCl) and germination was carried out in a germination chamber in gerbox with germitest paper under 12/12 h photoperiod (light/darkness), light fluence of 51.2 μmol·m⁻²·s⁻¹ photosynthetic photon flux density (PPFD), 60% relative humidity and temperature of 25 °C. The saline solution along with germitest sheet changing were performed weekly. Treatments were arranged in a completely randomized design in a 2 × 3 factorial, with four replications. An unprimed control treatment was also maintained for comparison.

Germination parameters determination

Seed germination was recorded every 12 h for a week. The germination (G), germination rate index (GRI) and mean germination time (MGT) were measured according to Maguire (1962) and Laboriau (1983). Eqs. 1-3 and the parameters there in were employed to express the process of seed germination.

$$Ge = (N/A) \times 100 \quad (1)$$

where G = germination; N = number of seeds germinated and A = number of seeds placed per box.

$$GRI = (E1/N1) + (E2/N2) + (En/Nn) \quad (2)$$

where GRI = germination rate index; E = number of germinated seeds and N = days of counting (or hours)

$$MGT = \sum ni \times ti / \sum ni \quad (3)$$

where MGT = mean germination time; ni = number of germinated seeds per day and ti = incubation time.

Seedlings growth analyses

Following 15 days after germination, totaling 22 days of NaCl exposure and of the duration, the seedlings were harvested, washed in distilled water, immersed in liquid N₂ and stored at -80 °C, for further biochemical analyses. Samples of seedlings were placed in an oven for 72 h at 70 °C and dry mass measurements were carried out using an analytical balance accurate to 0.01 g and the results expressed in g.plant⁻¹.

Biochemical analysis

Lipid peroxidation

Lipid peroxidation was estimated by the content of thiobarbituric acid reactive substances (TBARS). The concentration of malondialdehyde (MDA) equivalents was calculated using an extinction coefficient of $1.55 \times 10^{-5} \text{ mol}^{-1} \cdot \text{cm}^{-1}$, with readings between 535 and 600 nm (Gratão et al. 2012). The results are expressed in $\mu\text{mol} \cdot \text{mg}^{-1}$ fresh weight.

Hydrogen peroxide content

The content of hydrogen peroxide (H₂O₂) was determined by reaction with 100 mmol·L⁻¹ potassium phosphate buffer (pH 7.5) and 1 mol·L⁻¹ potassium iodide. This solution was incubated on ice for 1 h and the absorbance was measured at 390 nm (Alexieva et al. 2001). The results were expressed in $\mu\text{mol} \cdot \text{mg}^{-1}$ fresh weight.

Enzyme extraction and protein determination

To perform the quantification of total proteins and specific activities of the SOD, CAT and GR enzymes in the seedlings, the enzymatic extraction was performed using a methodology described by Boaretto et al. (2014): The seedlings were homogenized in a extraction buffer containing 100 mmol·L⁻¹ potassium phosphate buffer (pH 7.5), 1 mmol·L⁻¹ ethylene diaminetetraacetic acid (EDTA), 3 mmol·L⁻¹ DL-dithiothreitol and 5% (w/v) insoluble polyvinylpyrrolidone (Boaretto et al. 2014). The homogenate was centrifuged at 10,000 xg for 30 min, and the supernatant was stored at -80 °C for further analyses. The protein concentration was assayed following the method of Bradford (1976).

Superoxide dismutase (SOD, EC 1.15.1.1) assay

Superoxide dismutase (SOD) activity was carried out in a chamber under illumination of 15 W fluorescent lamps at a temperature of 25 °C for 5 min, and the reaction medium consisting of: Plant extract, 50 mM sodium PBS buffer (pH 7.8), 50 mmol·L⁻¹ methionine, 10 mmol·L⁻¹ ethylene diaminetetraacetic acid (EDTA), 10 mmol·L⁻¹ nitrotriazolium blue chloride and 0.1 mmol·L⁻¹ riboflavin (Giannopolitis and Ries 1977). Superoxide dismutase activity was determined in spectrophotometer at 560 nm, through the formation of blue formazan compound due to photoreaction of NBT present in the reaction medium and values were expressed as U SOD mg⁻¹ protein.

Catalase (CAT, EC 1.11.1.6) assay

Catalase (CAT) activity was determined by H₂O₂ decomposition monitored in spectrophotometer over 1 min at 240 nm. The reaction medium was composed by 100 mmol·L⁻¹ potassium phosphate buffer (pH 7.5) and H₂O₂ (30% solution) (Cia et al. 2012). Values were expressed as μmol·min⁻¹·mg⁻¹ protein.

Glutathione reductase (GR, EC 1.6.4.2) assay

Glutathione reductase (GR) activity was determined spectrophotometrically at 30 °C in a mixture consisting of 100 mmol·L⁻¹ potassium phosphate buffer (pH 7.5), containing 1 mmol·L⁻¹ 5,5'-dithiobis (2-nitrobenzoic acid), 1 mmol·L⁻¹ GSSG and 0.1 mmol·L⁻¹ NADPH. The rate of reduction of GSSG was followed by monitoring the increase in absorbance at 412 nm over 1 min (Carvalho et al. 2013). GR activity was expressed as μmol·min⁻¹·mg⁻¹ protein.

Statistical analyses

The parameters evaluated were submitted to normality test using the Kolmogorov-Smirnov and Shapiro-Wilk tests to verify absence of normality. A multiple comparison between Tukey's test means followed by an individual ANOVA for each character at 0.05 level of significance was applied. The statistical analysis was performed using the Sisvar 5.3 software (Ferreira 2011).

RESULTS AND DISCUSSION

No changes were observed in seed germination under 0 mmol·L⁻¹ NaCl, irrespective of Si application. However, germination was affected by Si concentrations following NaCl application (50 mmol·L⁻¹ NaCl), whereas seeds with priming of 0.1 mmol·L⁻¹ Si exhibited the highest germination (Fig. 1a). With respect to GRI analysis, primed seeds (0.05 and 0.1 mmol·L⁻¹) exhibited an increase in GRI values following 50 mmol·L⁻¹ NaCl exposure, in contrast to observed to 0 mmol·L⁻¹ NaCl (Fig. 1b).

Following MGT analyses, nonprimed seeds exhibited an increase in MGT without NaCl exposure. On the other hand, primed seeds with Si showed similar germination performance to the non-primed ones (Fig. 1c).

The salinity can prevent or delay seed germination through various factors, such as the reduction of water availability, changes in the mobilization of stored reserves and affecting the structural organization of proteins (Keshavarzi 2012; Sharma et al. 2014; Ibrarim 2016). According to the results, unprimed seeds submitted to salt stress exhibited increased GRI and MGT (Fig. 1b and c). Although, when seed priming management was adopted, the seeds did not show reduction in GRI and MGT parameters, being similar to non-salt stress condition.

Although Si is not considered an essential nutrient for most plants, a lot of works have demonstrated the beneficial effects of Si in reduction of damages caused by stress-inducing agents (Imtiaz et al. 2016; Alsaeedi et al. 2017). Silicon plays a role

in alleviating the negative effects of salt stress by improving germination performance and its ability to withstand adverse conditions, since that Si may be involved in silicon-mediated plant tolerance (Zang et al. 2015; Maghsoudi and Emam 2016). Silicon is a known precursor for lignin synthesis, and may play therefore an essential role in seed coat resistance (Toledo et al. 2011). It can also deposit on the walls of epidermis and vascular tissues of the stem, leaf sheath and hull, regulating physiological and biochemical functions of plants (Parven and Ashraf 2010; Siddiqui and Al-Whaib 2014).

Over 15 days postgermination, seedlings did not exhibit differences in dry mass, not only for Si concentrations but also NaCl application (Fig. 2a). When plants are subjected to environmental stresses, their development can be affected, and their growth and yield may be reduced due to changes in metabolism.

When plants are subjected to environmental stresses, their development can be affected, and their growth and yield may be reduced due to changes in metabolism. Over 15 days postgermination, seedlings did not exhibit growth differences, irrespective of Si and NaCl concentrations (Fig. 2). Salinity effects on plant growth may be associated with low osmotic potential of solution, which reduces both the ability of plants to take up water and the processes of cell division (Yacoubi et al. 2013; Ozhan and Hajibabaei 2014). No changes observed in seedlings growth could be related to short time of exposure to salinity for toxic ions accumulation, since that Na^+ and Cl^- can affect plant metabolism (Morais et al. 2012; Panuccio et al. 2014).

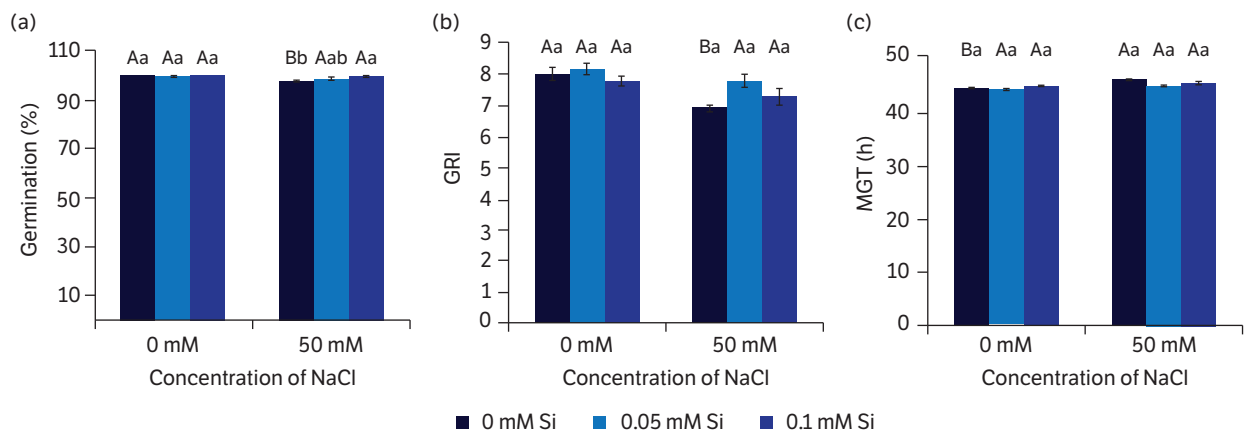


Figure 1. (a) Germination, (b) germination rate index (GRI) and (c) mean germination time (MGT) in lettuce seeds. Different lowercase letters above the bars indicate significant differences among Si concentrations within each NaCl concentrations and different capital letters indicate significant differences of Si concentrations among NaCl concentrations, according to the Tukey's test ($\alpha = 0.05$).

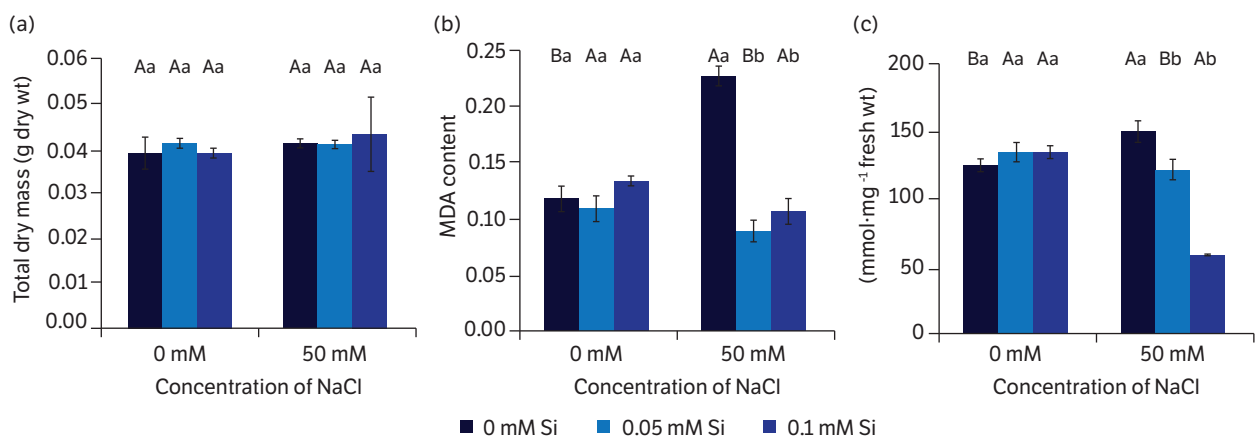


Figure 2. (a) Total dry mass, (b) MDA and (c) H_2O_2 contents in lettuce seedlings. Different lowercase letters above the bars indicate significant differences among Si concentrations within each NaCl concentrations and different capital letters indicate significant differences of Si concentrations among NaCl concentrations, according to the Tukey's test ($\alpha = 0.05$).

Although Si can improve water status and water-use efficiency by reducing leaf transpiration being able to maintain normal plant growth (Sivanesan et al. 2011; Wang et al. 2015; Coskun et al. 2016), no changes were observed by our results with Si application. Regardless of the mechanism involved, Si can play a role in reducing the effects of abiotic stresses through induction of defense antioxidant system, coprecipitation of toxic metal ions, immobilization of toxic metal ions in growth media and uptake processes (Liang et al. 2007; Alseedi et al. 2017).

Lipid peroxidation, expressed as MDA content, was pronounced in seedlings submitted to salt stress. In the presence of NaCl, unprimed treatment exhibited pronounced increases in the MDA content compared with priming treatments (Fig. 2b). Among NaCl treatments, unprimed treatment increased lipid peroxidation rates, whereas seedlings with seed priming treatment of 0.05 mmol·L⁻¹ Si appeared to be less affected by salt exposure (Fig. 2b).

When H₂O₂ content is concerned, no significant differences were observed for priming treatments to 0 mmol·L⁻¹ NaCl, irrespective of Si concentration (Fig. 2c). However, H₂O₂ content was higher in seedlings exposed to NaCl application, whereas seedlings with seed priming of 0.1 mmol·L⁻¹ Si appeared to be less affected by NaCl exposure (Fig. 2c).

Despite the deleterious effect of salinity to seedlings growth was not observed, salt stress caused induction of oxidative stress. Salt stress can induce ionic stress and osmotic stress in plant cells. A direct result of these primary effects is the overproduction of reactive oxygen species (ROS) leading to oxidative effects on key and sensitive biomolecules, including proteins, nucleic acids and membrane lipids (Nasibi et al. 2014; Gratão et al. 2015; Alves et al. 2018). One of the consequences of uncontrolled oxidative stress is direct damage to lipids and malondialdehyde (MDA) appears to be the most mutagenic product of lipid peroxidation (Nasibi et al. 2014). According to the results, lipid peroxidation was pronounced in seedlings submitted to salt stress and priming treatments exhibited decreases in the MDA content compared with unprimed treatments (Fig. 2b).

Furthermore, the decreased MDA (Fig. 3a) and H₂O₂ (Fig. 2c) contents on tissues submitted to salinity stress can be dependent on the regulation of the antioxidant defense system (Kibria et al. 2017). Plants counteract the stress-induced changes by regulating their defense systems such as antioxidants (Alves et al. 2018).

CAT (Fig. 3b), and GR (Fig. 3c) activities are crucial for the detoxification of any excess H₂O₂ produced by SOD (Fig. 3a) and/or by other metabolic processes. The overall results showed significant effects of SOD activity in seedlings with priming treatments following 0 and 50 mmol·L⁻¹ NaCl applications. When Si treatments were analyzed, seedlings with 0.1 mmol·L⁻¹ Si exhibited the highest SOD activity when compared to 0.05 mmol·L⁻¹ Si treatment, irrespective to NaCl concentrations (Fig. 3a).

Concerning CAT and GR enzymes, seedlings with seed priming of 0.1 mmol·L⁻¹ Si exhibited an increase in CAT activity, following 0 and 50 mmol·L⁻¹ NaCl applications, whereas seedlings with seed priming treatment of 0.05 mmol·L⁻¹ Si appeared to be less affected by salt exposure (Fig. 3b). On the other hand, seedlings with seed priming

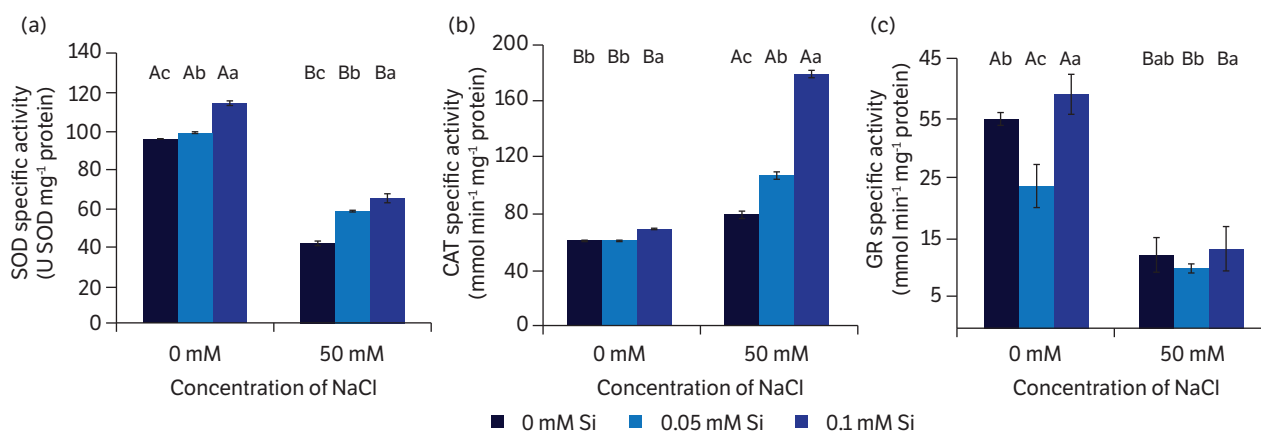


Figure 3. Antioxidant total enzyme activity. (a) SOD specific activity, (b) CAT specific activity and (c) GR specific activity in lettuce seedlings. Different lowercase letters above the bars indicate significant differences among Si concentrations within each NaCl concentrations and different capital letters indicate significant differences of Si concentrations among NaCl concentrations, according to the Tukey's test ($\alpha = 0.05$).

exhibited pronounced CAT activity when submitted to salt exposure, compared with seedlings following 0 mmol·L⁻¹ NaCl. In contrast, seedlings submitted to salt exposure maintained low GR activity when compared to control seedlings (0 mmol·L⁻¹ NaCl) (Fig. 3c).

Seedlings with seed priming of 0.1 mmol·L⁻¹ Si exhibited an increase in GR activity following 0 and 50 mmol·L⁻¹ NaCl applications, whereas seedlings with seed priming of 0.05 mmol·L⁻¹ Si exhibited the lowest GR activity (Fig. 3c).

Overall results indicated that Si (0.05 and 0.1 mmol·L⁻¹) plays a role in alleviating the negative effects of salt stress by improving SOD (Fig. 3a), CAT (Fig. 3b) and GR (Fig. 3c) activities and reduced MDA (Fig. 2a) and H₂O₂ (Fig. 2b) contents. Silicon can reestablish the activity of the photosynthetic apparatus by activation of ROS-metabolizing enzymes (Sivanesan et al. 2011), indicating the protective role of Si against membrane damage and improved the chloroplast structure (Abdalla 2011; Silva et al. 2018).

The increased activity of antioxidant enzymes (Fig. 3a-c) by seed priming with Si may be considered as one of the primary mechanisms against salt stress (Shi et al. 2013; Soundararajan et al. 2014; Manivannan et al. 2016), since that unprimed seedlings to salt exposure exhibited decreased enzyme activities and high MDA content (Fig. 2a and b, Fig. 3a-c).

Superoxide dismutase plays an important role as the first line of defense mechanism and is responsible for catalyzing the dismutation of the superoxide radical in hydrogen peroxide (Manivannan et al. 2016). H₂O₂ produced in response to SOD activity or other metabolic activities can subsequently be reduced to H₂O by the action of CAT, GR and other peroxidases. Superoxide dismutase, which converts O₂⁻ into H₂O₂, has been shown to be induced in a number of plant species exposed to salt (Alves et al. 2018). Catalase is an oxidoreductase enzyme that regulates H₂O₂ to the signaling process (Mittler 2002). Catalase activity was increased by seed priming with Si (Fig. 3b).

A pronounced increase in SOD, CAT and GR activities of salt-stressed plants by Si suggests that Si may be involved in the metabolic or physiological activities, thus helping to maintain membrane permeability (Soylemezoglu et al. 2009; Liang et al. 2015; Shi et al. 2016; Coskun et al. 2016). Furthermore, the activity of GR was not affected by salt stress in priming seedlings with 0.05 mmol·L⁻¹ Si, while CAT activity was increased for both priming seedlings (0.05 and 0.1 mmol·L⁻¹ Si) to salt exposure (Fig. 4b and c).

Glutathione reductase plays an important role in maintaining the metabolic balance between GSH, ascorbate (AsA) contents and H₂O₂ degradation (Alves et al. 2017). Overall results indicated that GR enzyme can be damaged by salt stress, where these responses may be related to other mechanisms acting directly on H₂O₂ degradation.

According to the results, seed germination seems to be a major factor limiting the establishment of plants under salt-stress conditions, which can affect germination rate and seedling growth in different ways depending on plant species. Nevertheless, seed priming with Si was effective in improving salt stress tolerance by activation of enzymatic antioxidant defense. Seed priming with Si can be a good management strategy to reduce the deleterious effects caused by salt stress, since that Si can be deposited or polymerized in the endodermal and exothermic layers of the apoplastic by inhibiting Na uptake by the roots (Azeem et al. 2015). Little is known about the mechanisms triggered by the use of seed priming, although a huge number of works have been carried out to improved tolerance of plants against salt stress (Azeem et al. 2015; Coskun et al. 2016; Sing et al. 2018). Further analysis is needed for a better understanding about the mechanisms connecting antioxidant responses and stressful conditions, since Si is directly involved in such a mechanism (Coskun et al. 2016; Rios et al. 2017; Liu et al. 2018). Such studies may contribute to explore various ways to improve crop productivity through the alleviation of salt stress.

CONCLUSION

Seed priming with Si is an efficient management technique that can be used to alleviate deleterious effects of salt-stressful conditions on germination of lettuce seeds and enhance salt tolerance of seedlings due to the increased activity of ROS scavenging enzymes.

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