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ORIGINAL ARTICLE

Osmopriming-induced salt tolerance during seed germination of alfalfa most likely mediates through H_2O_2 signaling and upregulation of heme oxygenase

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Abstract The present study showed that osmopriming or pretreatment with low H_2O_2 doses (2 mM) for 6 h alleviated salt-reduced seed germination. The NADPH oxidase activity was the main source, and superoxide dismutase (SOD) activity might be a secondary source of H_2O_2 generation during osmopriming or H_2O_2 pretreatment. Hematin pretreatment similar to osmopriming improved salt-reduced seed germination that was coincident with the enhancement of heme oxygenase (HO) activity. The semi-quantitative RT-PCR confirmed that osmopriming or H_2O_2 pretreatment was able to upregulate heme oxygenase *HO-1* transcription, while the application of *N*,*N*-dimethyl thiourea (DMTU as trap of endogenous H_2O_2) and diphenyleneiodonium (DPI as inhibitor of NADPHox) not only blocked the upregulation of HO but also reversed the osmopriming-induced salt attenuation. The addition of CO-saturated aqueous rescued the inhibitory effect of DMTU and DPI on seed germination and α-amylase activity during osmopriming or H_2O_2 pretreatment, but H_2O_2 could not reverse the inhibitory effect of ZnPPIX (as HO inhibitor) or Hb (as CO scavenger) that indicates that the CO acts downstream of H_2O_2 in priming-driven salt acclimation. The antioxidant enzymes and proline synthesis were upregulated in roots of seedlings grown from primed seeds, and these responses were reversed by adding DMTU, ZnPPIX, and Hb during osmopriming. These findings for the first time suggest that H_2O_2 signaling and upregulation of heme oxygenase play a crucial role in priming-driven salt tolerance.

Keywords CO · Salt acclimation · Hydrogen peroxide · Heme oxygenase · Signal transduction · Osmopriming

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Introduction

Salinity is a devastating environmental stress for seed germination and plant growth. It can affect seed germination and stand establishment through osmotic stress, ion toxicity, and oxidative stress. The salinity delays or prevents seed germination through reducing water availability, changing the mobilization of stored reserves, and affecting the structural organization of proteins. Thus, various techniques are applied to improve seed germination and stand establishment under salt conditions. Among these, seed priming is considered as a simple procedure that not only enhances seed germination but also mitigates the adverse effects of environmental stresses (Ibrahim 2015; Jisha et al. 2013).

Seed priming is a presowing technique that exposes seeds to a certain solution for a certain period and promotes partial hydration and physiological process during early phase of germination called "pregerminative metabolism," but prevents from radicle emergence and complement of seed germination (Jisha et al. 2013; Amooaghaie et al. 2010). Seed priming enhances germination rate and seedling establishment by weakening endosperm and modulating the activity of many enzymes involved in the stored reserves mobilization (such as α and β amylases) prior to the emergence of the radicle (Anese et al. 2011; Amooaghaie et al. 2010; Varier et al. 2010). These enzymes are involved in seed storage degradation that is essential process for the development and growth of the embryo, and thus they contribute to early and higher seedling emergence

(Farooq et al. 2006; Varier et al. 2010). The priming is also triggering DNA repair and epigenetic changes as well as activating transcription factors that led to better seed germination (Paparella et al. 2015; Tanou et al. 2012).

Seed priming also enhances defense responses including the activities of antioxidant enzymes and proline accumulation in seedlings grown from primed seeds under stress condition (Amooaghaie 2011; Khodabakhsh et al. 2014). It has been suggested that seed priming increases stress tolerance by inducing "stress imprint" (Bruce et al. 2007). The primary source of stress could be the priming strategy itself. For example, hydropriming that is shortening the hydration duration and osmopriming, matric priming, or drum priming that are exposing seeds to relatively low external water potential; subject seeds to partial drought or osmotic stress. Polyethylene glycol (PEG), as a usual osmopriming agent, not only triggers osmotic stress but can also impose anoxia (Hardegree and Emmerich 1994) and the oxidative stress on primed seeds (Balestrazzi et al. 2011). H_2O_2 priming directly induces oxidative injuries in cells. Halopriming causes both osmotic stress and ionic toxicity. Thus, the priming itself can cause moderate abiotic stresses (such as osmotic, drought, and oxidative). Furthermore, seeds were exposed to stress during the post priming drying. Drying of primed seeds that have partially lost desiccation tolerance constitutes a stress (Chen and Arora 2012). These experiences activate an array of defense processes in primed seeds that establishes resistance to various environmental stresses in subsequent exposures (Amooaghaie 2011; Chen and Arora 2011; Khodabakhsh et al. 2014; Ibrahim 2015). In other words, seed priming acts as a pregermination stress exposure and creates a "stress memory" in cells that facilitates the expression of stress responses in subsequent exposures (Bruce et al. 2007; Chen and Arora 2011; Paparella et al. 2015; Pastor et al. 2013). From this point of view, priming is similar to acclimation phenomena during early seed imbibition.

Recently, much evidence has documented that in many plant species, H_2O_2, NO, and CO play role of important signal molecules in the perception of environmental factors in normal or stress conditions (Amooaghaie et al. 2015; Amooaghaie and Nikzad 2013; Amooaghaie and Roohollahi 2016; Bailly et al. 2008). Moreover, several lines of evidence exhibited that these signal molecules mediate the phenomena of acclimation and cross-tolerance, in which previous exposure to low level of one stress can lead to tolerance in subsequent exposure to higher level of the same or different stresses (Neill et al. 2002). Therefore, it is possible that the first step in activating molecular and cellular responses by priming can be stress experience and relaying stress-related information through signal transduction pathways.

It has been shown that reactive oxygen species (ROS) accumulate during seed germination, and the exogenous application of H_2O_2 can improve seed germination in many plant species. These results emphasize the requirement for the crucial levels of ROS for seed germination proposed by the model of the "oxidative window" (Bailly et al. 2008). The interplay between ROS and hormone signaling pathways that induces changes in gene expression or in cellular redox status has an important role in the perception of environmental factors by seeds during their germination (Ishibashi et al. 2012; Sarath et al. 2007). Several lines of evidence suggest that H_2O_2 pretreatment of seeds increases the tolerance of plants to abiotic stresses and H_2O_2 plays role of a signal molecule in triggering plant responses to stresses (Gondim et al. 2010; Wahid et al. 2007). On the other hand, the high dose of H_2O_2 which is generated in adverse environmental conditions in seeds is deleterious and ROS-induced oxidative damage inhibits or delays seed germination (Bailly et al. 2008). However, there is very little knowledge concerning on how priming affects H_2O_2 levels in seeds and ultimately leads to the change of the developmental and physiological programs of plants in response to stresses.

Recently, a number of studies on plants have also demonstrated that endogenous H_2O_2 production can enhance abiotic stress tolerance via the upregulation of heme oxygenase (Chen et al. 2009; Wei et al. 2012). Heme oxygenase (HO) is one of enzymes involved in phytochrome chromophore biosynthesis (Muramoto et al. 2002) that catalyzes the cleavage of heme to biliverdin-IXα, iron (Fe^{2+}) and carbon monoxide (CO). In addition, HO-1, as an inducible form of heme oxygenase and the potential source of CO production in plants, has a strong antioxidant enzyme against different abiotic stresses (Xu et al. 2006; Liu et al. 2007). For example, the application of the exogenous hematin as HO-1 inducer and a CO aqueous solution dose dependently alleviated the inhibition of wheat seed germination and seedling growth under osmotic stress (Liu et al. 2010). Several authors confirmed the role of HO in acclimation to stresses (Chen et al. 2009; Wei et al. 2012; Xie et al. 2011). According to our literature review, no information exists on the changes of HO gene expression or HO activity in seeds after priming.

Proteomic analyses of primed seeds of *Medicago sativa* L. has emphasized that metabolic and biochemical processes are involved in the increment of seed vigor of primed seeds (Yacoubi et al. 2013). The global proteome and transcriptome expression profiling of PEG-primed seeds of *Brassica napus* L. also demonstrated abundant genes and numerous proteins were differentially expressed during the main phases of priming technique and final germination of primed seeds (Kubala et al. 2015). However, which signal transduction pathways are involved in activating pregerminative metabolism and gene expression, by priming, is still unknown. Therefore, we investigated assumption that priming similar to acclimation process may act to improve seed germination and salt tolerance of alfalfa, through ROS signaling and HO/CO upregulation.

Materials and methods

Pretreatment with various concentrations of H_2O_2

Seeds of alfalfa (*M. sativa*, Hamedani cv.) were sterilized in 2% NaClO for 5 min and washed in distilled water. In the first experiment, seeds were treated with different H_2O_2 concentrations (0, 1, 2, 4, 8 mM) for 6 h. Then, laboratory seed germination tests were conducted in 9-cm Petri dishes (50 seeds per each plate and five replicates for each treatment) on a layer of filter paper moistened with distilled water at 25 °C with a continuous light intensity of 300 μmol m^{-2} s^{-1}. The seeds treated with distilled water under the same conditions were considered as controls. Seed germination was counted daily for 5 days. Seeds were considered germinated when radicles were protruded for more than 2 mm.

Osmopriming and other treatments

For osmopriming, seeds were primed in polyethylene glycol solution (PEG 6000; Shanghai Chemical Reagent Co. Ltd., Shanghai, China) at 300 g L^{-1} with continuous aeration. After 6 h at 25 °C, the seeds were removed and rinsed in distilled water and wiped free of water and air dried at 25 °C for 24 h. It is worth to note that in the preliminary study, 150 mM NaCl was chosen as optimal stress conditions and measurements with concentration gradients of PEG revealed this PEG concentration was the best treatment for improving seed germination under 150 mM salt stress (data not shown).

Carbon monoxide aqueous solution was prepared according to the method described by Han et al. (2008). For the preparation of CO-saturated aqueous solution, CO gas was bubbled into 500 mL of distilled water by a glass tube for 50 min. Then, the required concentration of CO aqueous solution was prepared by the dilution of this saturated stock solution (100% of saturation) with distilled water.

Preliminary studies using various concentrations (1, 10, 20, 40, and 80% of saturation) of CO aqueous solution showed that 10% CO-saturated aqueous solution was the suitable treatment due to its better effect on improving seed germination under 150 mM salt stress (data not shown).

Hematin (H: C34H33N4O5Fe) and zinc protoporphyrin IX (ZnPPIX), were purchased from Sigma and applied as a CO donor and inhibitor of HO-1, respectively (Liu et al. 2010). The seeds were treated with various concentrations (0, 2.5, 5, 10, and 20.0 μM) of hematin, and the data showed that 2.5 μM hematin was the best treatment. ZnPPIX was used at 100 μM concentration. Furthermore, hemoglobin (Hb) as the scavenger of CO and *N*,*N*-dimethylthiourea (DMTU) as the scavenger of H_2O_2 (Chen et al. 2009) were purchased from Fluka. Additionally, the remaining chemicals were of analytical grade from Chinese companies.

In various experiments, osmoprimed seeds or seeds pretreated with 100 μM H_2O_2, as well as control seeds, directly were exposed with 1 mM DMTU, 1 mM DPI, AT, 5 μM hematin, 100 μM ZnPPIX, 10% CO-saturated aqueous solution, and 0.1 g L^{-1} Hb singly or combination of two or three of the previous chemicals for 6 h then transferred to Petri dishes containing of distilled water or 150 mM NaCl and seed germination was evaluated as mentioned previously. The seeds were treated with distilled water under the same conditions considered as controls.

After various treatments, biochemical parameters (NADPHox, SOD, HO activity, H_2O_2 content) were measured 6 h after salt exposure in germinating seeds before any radicle protrusion occurred or 24 h after salt exposure as described in the "Results" section. Also, H_2O_2 content and the activities of SOD, CAT, APX, proline content, and *SOD, CAT, APX,* and *P5CS* (D-1-pyrroline-5-carboxylate synthetase enzyme) transcripts were assessed in 5-day-old seedlings. The biochemical parameters and gene expressions were evaluated as described in following sections.

H_2O_2 content determination

The H_2O_2 content of seeds was determined according to method described by Oracz et al. (2007). The extracts of seed obtained from the homogenization of seed with perchloric acid were centrifuged at 10,000×*g* at 4 °C for 15 min, and supernatant was used for H_2O_2 determination. The assay mixture (1.5 ml final volume) contained 12 mM 3-dimethylaminobenzoic acid in 0.375 M phosphate buffer (pH 6.5), 1.3 mM 3- methyl-2-benzothiazolidone hydrazone, 20 μl (0.25 U) horseradish peroxidase (Sigma), and 70 μl of the collected supernatant. The spectrophotometric absorbance at 590 nm was read after 5 min at 25 °C, and results are reported as nmol H_2O_2 g^{-1}DW.

Assays of enzyme activities

Heme oxygenase activity was measured as described by Muramoto et al. (2002), with minor modifications. The concentration of biliverdin IX (BV) was determined using a molar absorption coefficient at 650 nm of 6.25 mM^{-1} cm^{-1} in 0.1 M HEPES–NaOH buffer (pH 7.2). One unit of activity (U) was defined by calculating the quantity of the enzyme to produce 1 nmol BV per 30 min.

Total SOD activity was assayed on the basis of its ability to reduce nitroblue tetrazolium (NBT) by the superoxide anion generated by the riboflavin system under illumination. One unit of SOD (U) was expressed as the amount of crude enzyme extract required to inhibit the reduction rate of NBT by 50%.

The activities of CAT, SOD, and APX in roots of seedlings were determined as described by Amooaghaie and Roohollahi (2016).

NADPH oxidase were analyzed using the method described by Van Gestelen et al. (1997) using nitroblue tetrazolium (NBT) and NADPH. NADPH-ox activity was calculated from the difference in NBT reduction using an extinction coefficient of 12.8 mM^{-1} cm^{-1} in the absence or presence of 50 U mL^{-1} superoxide dismutase (bovine erythrocytes, Sigma-Aldrich, St. Louis, MO, USA).

Protein was evaluated by the method of Bradford (1976), using bovine serum albumin (BSA) as a standard.

Semi-quantitative RT-PCR analysis

Samples were homogenized with mortar and pestle in liquid nitrogen and after total RNA isolation; cDNA was amplified by PCR using the following primers: amplifying a 476-bp fragment for HO-1 (accession number HM212768), forward (5′-TACATACAAAGGACCAGGCTAAAG-3′) and reverse (5′-GTCCCTCACATTCTGCAACAACTG-3′), for *P5CS* gene (accession number: CAA67069.1) forward (5′-CATC CCTGTTTCTCTCCACC-3′) and reverse (5′- CCATCTCG CGTACATCAACC-3′), for *Cu-Zn SOD* gene (accession numbers: XM003626314.2, XM013565057.1) forward (AATGTCACCGTCGGTGATGATG) and reverse (GTTCATCCTTGCAAACCAATAATACC), for *CAT* gene (accession numbers: XM013606824.1, XM013606823.1) forward (CCTATTTGATGATGTGGGTGTCC) and reverse (GTCTTGAGTAGCATGGCTGTGGT) and for *APX* gene (accession numbers: XM003615115.2, CU928858.2) forward (ACCAACCTCGTTCAGTGTCCAT) and reverse (AGAGCGCTGTCTGCGTTCTATT) and finally amplifying a 505-bp fragment; for actin, forward 5′- GTGACAAT GGAACTGGAATGG-3′ and reverse 5′-AGAC GGAGGATAGCGTGAGG-3′ designed based on primary sequence with accession number NM_001316010.1 in the NCBI genomic data bank.

To standardize the results, the relative intensity of electrophoretic bands belong to genes PCR products against ones of actin gene as internal control were calculated in exponential phase of PCR reactions (22–28 cycles). The aliquots of the PCR reactions were loaded on 1.5% agarose gels with the use of ethidium bromide. Ethidium bromide-stained gels were scanned and analyzed using the Total Lab v1.10 software (Nonlinear Dynamics, Newcastle-upon-Tyne, UK).

Proline measurement

The proline content of roots was determined as described by Bates et al. (1973). Briefly, root samples were homogenized in 1 mL of 3% sulphosalicylic acid solution on an ice bath and were filtrated. The test tubes including the filtrate, 2.5% ninhidrine solution, and glacial acetic acid were laid in a water bath at 100 °C for 50 min and then were cooled in ice bath and toluene was added. The absorbance of the pink-red upper phase was recorded at 520 nm, against toluene blank. The proline concentration was calculated using a standard curve and expressed as micromole proline per gram fresh weight.

Statistical analysis

Some experiments were carried out as factorial experiment with a completely randomized design, and other trials were conducted as completely randomized design. For all tests, five replications were utilized. The differences among treatments were analyzed by one-way ANOVA using SPSS version 13, and Duncan's multiple range tests at $P < 0.05$ were used to compare the treatment means.

Results

Effects of pretreatment at various concentrations of H_2O_2 on seed germination

As shown in Fig. 1, pretreatment with lower H_2O_2 doses (1, 2 mM) mitigated the salt inhibition of seed germination and 2 mM H_2O_2 was the most effective concentrations, which was then used to investigate the role of H_2O_2 in acclimation to salt stress in subsequent experiments. However, higher concentrations (4 and 8 mM) reduced seed germination not only under salt stress but also in normal condition. For example, under salinity stress, seed germination in seeds pretreated with 2 mM H_2O_2 increased approximately 30%, but the pretreatment with 8 mM H_2O_2 not only did not show any positive effect but also damaged seeds.

Effects of osmopriming and exogenous H_2O_2 pretreatment on seed germination

In the second experiment, seeds were pretreated with 2 mM H_2O_2 or osmoprimed with PEG and then exposed to 150 mM NaCl solution.

Figure 2 shows that 150 mM NaCl alone (without osmopriming or H_2O_2 pretreatment) significantly decreased seed germination in comparison with salt-free control (C). Both osmopriming and H_2O_2 pretreatment were able to rescue the salt-induced inhibition of seed germination partly (Fig. 2). The stimulatory effect of conferred by osmopriming or H_2O_2 pretreatment on seed germination could be fully repressed by *N*,*N*′-dimethylthiourea (DMTU), as scavenger of H_2O_2 or with DPI as an inhibitors of NADPHox, while the responses induced by osmopriming or H_2O_2 pretreatment only briefly were reversed by the addition of AT as the inhibitor of SOD (Fig. 2).

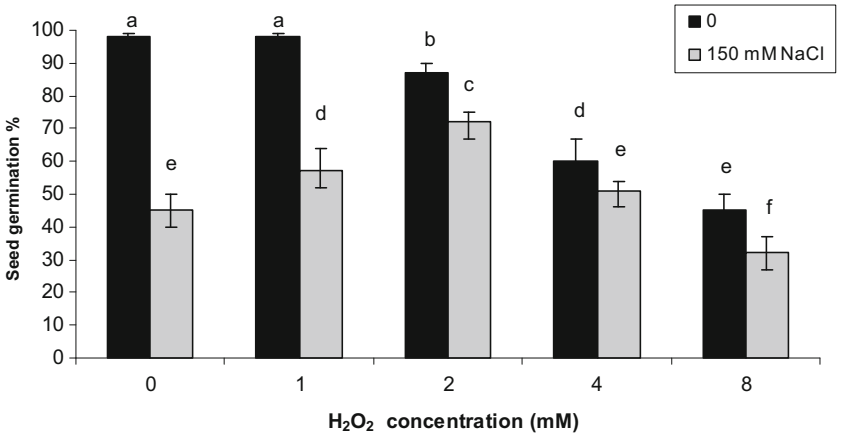

Fig. 1 Effects of pretreatment at various concentrations of H_2O_2 on alfalfa seed germination under salinity stress. Seeds were pretreated with various H_2O_2 concentrations (0, 1, 2, 4, 8 mM) for 6 h and then exposed to 150 mM salt solution or distilled water for 5 days. Values are means ± *SE* of five replications. *Different letters above the bars* indicate significant differences ($P < 0.05$) according to Duncan's multiple range test

To examine whether the acclimation to salinity caused by H_2O_2 pretreatment or osmopriming is due to the increase of internal H_2O_2 in the plants, endogenous H_2O_2 levels were measured in the seeds of each treatment after 6 and 24 h of imbibition in the presence of water or a 150 mM NaCl solution.

Data showed that H_2O_2 contents significantly increased in germinating alfalfa seeds under salt stress as compared to the control (Fig. 3). Both osmopriming and H_2O_2 pretreatment significantly increased endogenous H_2O_2 levels after 6 h of imbibition. When DMTU, DPI, and AT were combined with the osmopriming or H_2O_2 pretreatment, endogenous H_2O_2 levels were dramatically declined, reaching to a level lesser than the treatment of salt stress alone. These results indicate that an increase in endogenous H_2O_2 could be part of the mechanism triggered by H_2O_2 pretreatment or osmopriming for salt acclimation.

Interestingly, both osmopriming and H_2O_2 pretreatment significantly reduced endogenous levels of H_2O_2 after 24 h of imbibition. In other words, these treatments induced a fast burst of endogenous H_2O_2 production at 6 h followed by a sharp decrease at 24 h of imbibition. Conversely, in salinity treatment alone, endogenous H_2O_2 levels increased after 24 h when they were compared to it at 6-h imbibition (Fig. 3).

The assessment of two H_2O_2 producing enzymes revealed that salinity stress after 6-h imbibition had no significant effect on SOD activity but increased NADPH oxidase activity significantly. Conversely, after 24-h imbibition, SOD activity increased and NADPH oxidase activity decreased sharply (Fig. 4). H_2O_2 pretreatment or osmopriming after 6-h

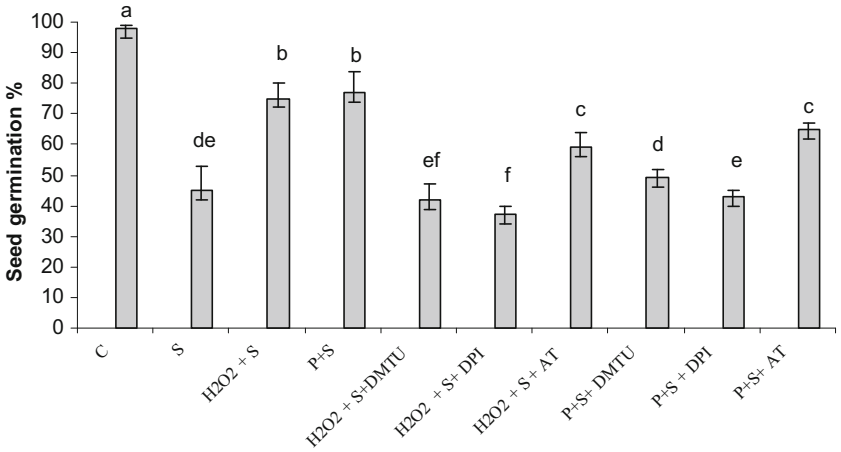

Fig. 2 Effect of osmopriming and exogenous H_2O_2 pretreatment singly or in combination with DMTU, DPI, and AT on alfalfa seed germination under salinity stress. Seeds pretreated with 2 mM H_2O_2 or primed in polyethylene glycol solution (PEG 6000) after preincubation with 1 mM DMTU, DPI, and AT for 6 h were exposed to water (C) or 150 mM salinity for 5 days. Values are means ± *SE* of five replications. *Different letters above the bars* indicate significant differences ($P < 0.05$) according to Duncan's multiple range test. *P* osmopriming, *S* salinity, *C* control

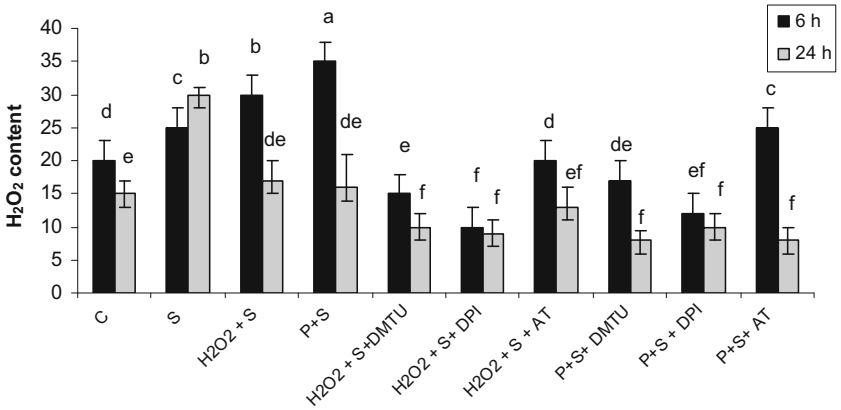

Fig. 3 Effect of osmopriming and H_2O_2 pretreatment singly or in combination with DMTU, DPI, and AT on endogenous levels of H_2O_2 in germinating seeds of alfalfa under salinity stress. Seeds pretreated with 2 mM H_2O_2 or primed in polyethylene glycol solution (PEG 6000) after preincubation with 1 mM. DMTU, DPI, and AT for 6 h were exposed to water (C) or 150 mM salinity, The endogenous H_2O_2 levels were measured in germinating seeds of alfalfa after 6- and 24-h exposure to 150 mM salinity. Values are means ± *SE* of five replications. *Different letters above the bars* indicate significant differences ($P < 0.05$) according to Duncan's multiple range test. *P* osmopriming, *S* salinity, *C* control

imbibition markedly increased NADPH oxidase activity in germinating seeds under salt stress. The combination of DPI with the osmopriming and H_2O_2 pretreatment repressed this increment. In contrast, SOD activity after 6-h imbibition was increased only by H_2O_2 pretreatment significantly (Fig. 1) and osmopriming had no significant effect on SOD activity.

After 6-h imbibition, AT partly suppressed the effect of H_2O_2 pretreatment and also reduced SOD activity and

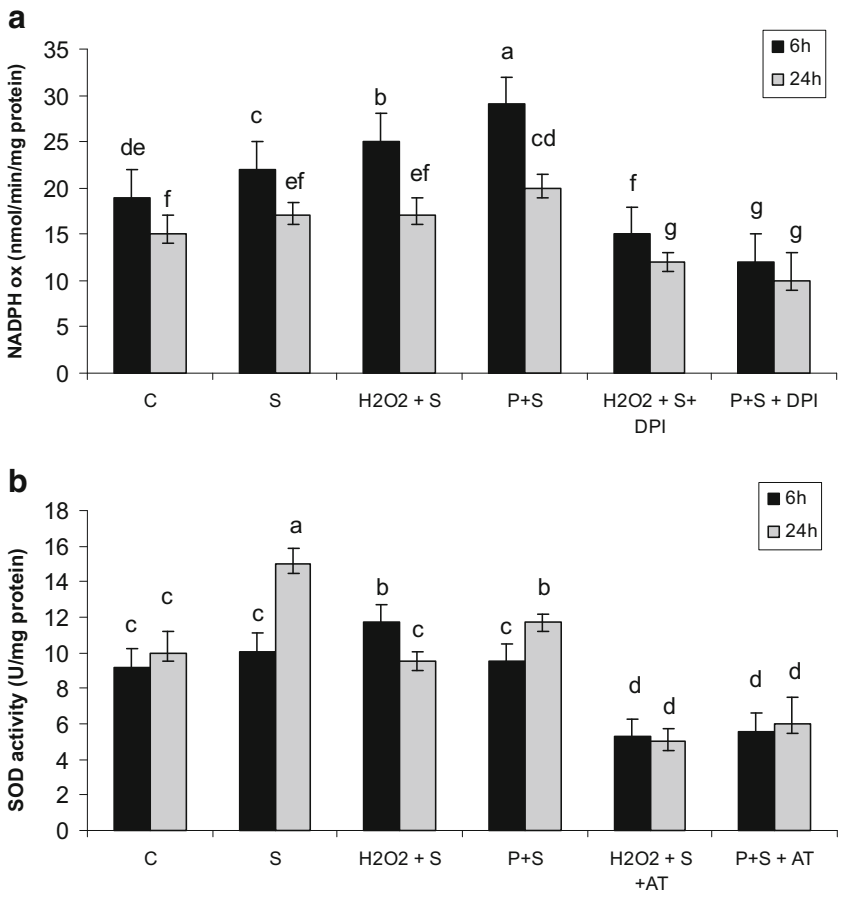

Fig. 4 Effect of osmopriming and H_2O_2 pretreatment singly or in combination with DPI and AT on NADPH oxidase (NADPHox) and superoxide dismutase (SOD) activities in germinating seeds of alfalfa under salinity stress. Seeds pretreated with 2 mM H_2O_2 or primed in polyethylene glycol solution (PEG 6000) after preincubation with 1 mM. DPI and AT for 6 h were exposed to water (C) or 150 mM salinity and then enzyme activity levels were measured in germinating seeds of alfalfa after 6- and 24-h exposure to 150 mM salinity. Values are means ± *SE* of five replications. *Different letters* indicate significant differences ($P < 0.05$) according to Duncan's multiple range test. *P* osmopriming, *S* salinity, *C* control

production of endogenous H_2O_2 in germinating seeds of alfalfa subjected to osmopriming or H_2O_2 pretreatment. Thus, SOD activity might be a secondary potential source of H_2O_2 production during osmopriming.

Interestingly, both osmopriming and H_2O_2 pretreatment reduced activities of NADPH oxidase and SOD after 24 h of imbibition. In other words, these treatments induced a fast burst of NADPH oxidase activity at 6 h of imbibition followed by a sharp decrease after 24 h of imbibition.

Role of HO/CO system in responses conferred by osmopriming on seed germination

Figure 5 shows that the pretreatment of hematin improved seed germination under salt stress in a dose-dependent manner, and 2.5 μM hematin was the best concentrations that could rescue the inhibition of seed germination under salt stress, which was then used in subsequent tests. However, high concentration (≥5 μM) reduced seed germination under both normal and salt stress condition. For example, under salinity stress, seeds pretreated in 2.5 μM hematin germinated 25% further than non-pretreated seeds, but the pretreatment with 20 μM hematin not only did not show any protective effect but also damaged the seeds.

To evaluate whether *HO-1* is involved in the salt acclimation induced by osmopriming, the effects of hematin pretreatment and osmopriming on HO activity and seed germination under salt stress were compared. The results showed that both osmopriming and hematin pretreatment significantly increased HO activity and seed germination. Interestingly, the elevation of HO activity and seed germination, conferred by osmopriming or hematin pretreatment, could be completely repressed by ZnPPIX, as an inhibitor of heme oxygenase (Fig. 6).

Interplay HO-1 and H_2O_2 in responses conferred by osmopriming on seed germination

To assess whether interplay HO-1 activity and H_2O_2 is involved in the acclimation to salt stress induced by osmopriming, the effects of H_2O_2 pretreatment and osmopriming on HO activity and *HO-1* transcripts were investigated. As expected, both osmopriming and H_2O_2 pretreatments significantly increased HO activity and *HO-1* transcripts in germinating alfalfa seeds under salt stress as compared to the control (Fig. 7). Application of DMTU and DPI significantly reversed effects of H_2O_2 pretreatment and osmopriming on HO activity and *HO-1* transcripts. The results also demonstrated a positive correlation between HO enzyme activity and *HO-1* transcript level (Fig. 7).

The results shown in Fig. 8 revealed that the alleviation of the salt-induced inhibition of seed germination, conferred by osmopriming, could be reversed by DMTU, DPI, ZnPPIX, and Hb. Interestingly, the addition of CO aqueous solution mitigated the negative effects conferred by DMTU and DPI during osmopriming (Fig. 8). In contrast, the addition of H_2O_2 could not alleviate the inhibitory effects of ZnPPIX or Hb on osmopriming-induced seed germination completely.

Role of H_2O_2 and HO/CO system in salt tolerance of seedlings grown from the primed seeds

An increase of H_2O_2 content following salinity stress was observed in the control plants. The H_2O_2 content was lower in primed seedlings when compared to controls (Table 1).

Data also showed that salinity upregulated antioxidant enzymes and proline biosynthesis in seedlings slightly. In addition, osmopriming, as well as pretreatment with hematin or

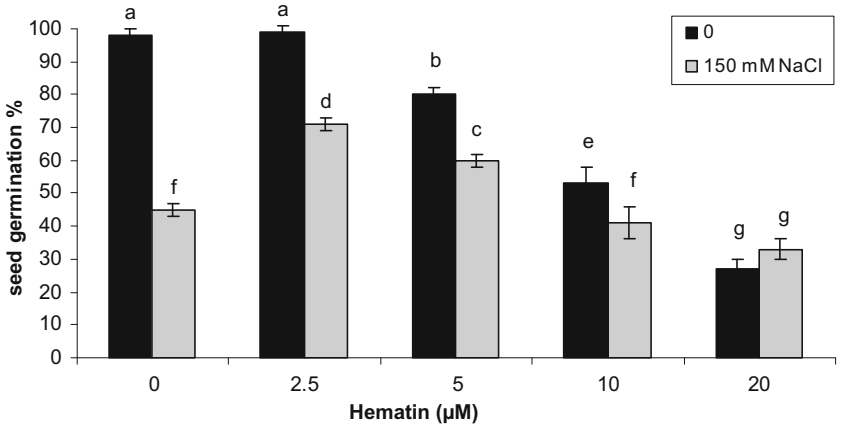

Fig. 5 Effects of pretreatment at various concentrations of hematin on alfalfa seed germination under salinity stress. Seeds were pretreated at various hematin concentrations (0, 2.5, 5, 10, 20 μM) for 6 h and then exposed to water or 150 mM salinity for 5 days. Values are means ± *SE* of five replications. *Different letters above the bars* indicate significant differences ($P < 0.05$) according to Duncan's multiple range test. *P* osmopriming, *S* salinity, *C* control

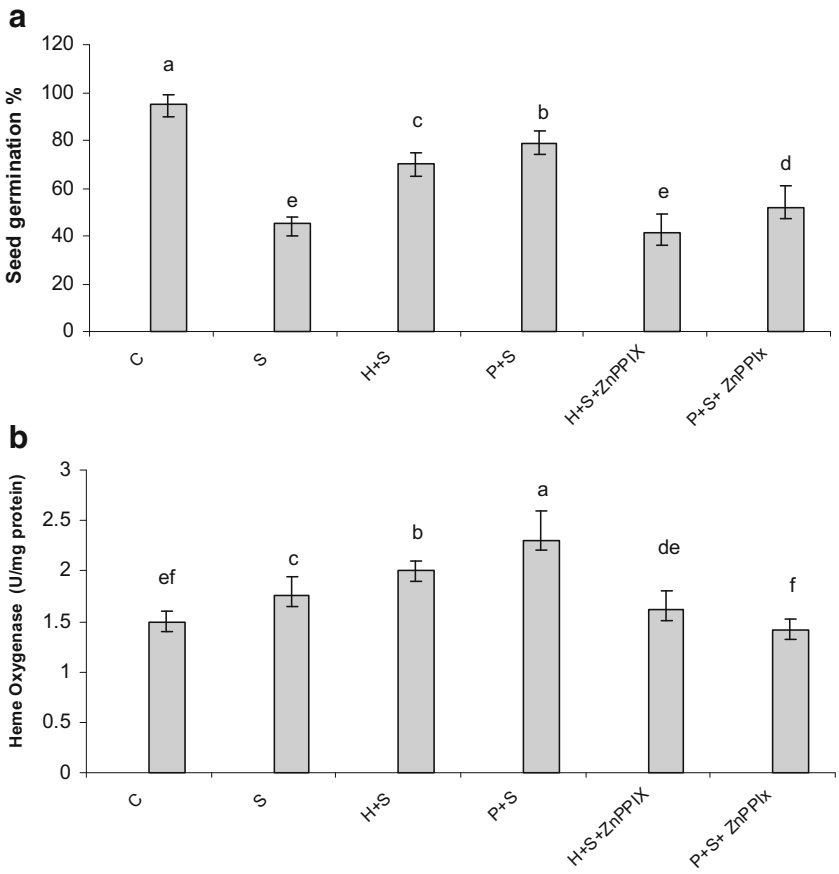

Fig. 6 Effect of osmopriming and hematin pretreatment singly or in combination with ZnPPIX on seed germination (**a**) and heme oxygenase activity (**b**) in germinating seeds of alfalfa under salinity stress. Seeds pretreated with 2.5 mM hematin or primed in polyethylene glycol solution (PEG 6000) after preincubation with 100 μM ZnPPIX for 6 h were exposed to water or 150 mM salinity for 5 days. Enzyme assays were performed at germinating seeds of alfalfa after 6-h imbibition. Values are means ± *SE* of five replications. Different letters above the bars indicate significant differences ($P < 0.05$) according to Duncan's multiple range test. *P* osmopriming, *S* salinity, *C* control

H_2O_2, considerably enhanced antioxidant enzymes at both levels of activity and transcription and also increased *P5CS* (D-1-pyrroline-5-carboxylate synthetase) transcripts and proline accumulation in the roots of seedlings grown from the primed seeds. Adding DMTU, ZnPPIX, and Hb during osmopriming, reversed the previous responses in seedlings grown from the primed seeds (Table 1 and Fig. 9).

Discussion

Results clearly confirmed that H_2O_2 pretreatment caused oxidative stress at high concentrations, while low levels of H_2O_2 conferred a protective effect (Fig. 1). These results support a dual role of H_2O_2 in seed physiology, as signal messenger at lower doses and as toxic and detrimental molecule at high doses that accumulates under biotic and abiotic stress conditions (Bailly et al. 2008).

The present study confirmed that osmopriming as well as lower doses of H_2O_2 increased seed germination percentage under salt stress (Fig. 2) and led to the acclimation to salt stress. The improvement of seed germination and salt acclimation by pretreatment with lower doses of H_2O_2 (Wahid et al. 2007; Gondim et al. 2010) and osmopriming of seeds (Farooq et al. 2006; Pradhan et al. 2014; Sivritepe et al. 2008) has been reported in many plants. Pandolfi et al. (2012) described that plant exposure to low-level stress activates multiple defense responses leading to an improvement of plant stress tolerance. Chen et al. (2009) also demonstrated that H_2O_2 is involved in the acclimation tolerance against oxidative stress in wheat seedling leaves.

As expected, salt stress reduced seed germination and increased H_2O_2 accumulation. Salt stress is deleterious for plant cells through inducing oxidative stress (Sivritepe et al. 2008). Interestingly, a burst of endogenous H_2O_2 in seeds subjected to both osmopriming and H_2O_2 pretreatment occurred after 6 h of imbibition (Fig. 3). In consistent with these results, Chen et al. (2009) illustrated that 0.5 mM H_2O_2 pretreatment triggered the biphasic production of H_2O_2 during a 24-h period. Naglreiter et al. (2005) also reported that the combined priming of PEG and GA induced the accumulation of free radicals in the seeds of Scotch pine (*Pinus sylvestris*) and larch (*Larix deciduas*). It seems that the pretreatment with lower doses of H_2O_2 elevated endogenous ROS levels and induced a moderate oxidative stress by the disruption of cellular ROS homeostasis. Similarity, seed priming can be considered as a brief stress since water is not provided in a sufficient amount to allow radicle protrusion through the seed coat (Chen and Arora 2012) and reduction of water availability can lead to

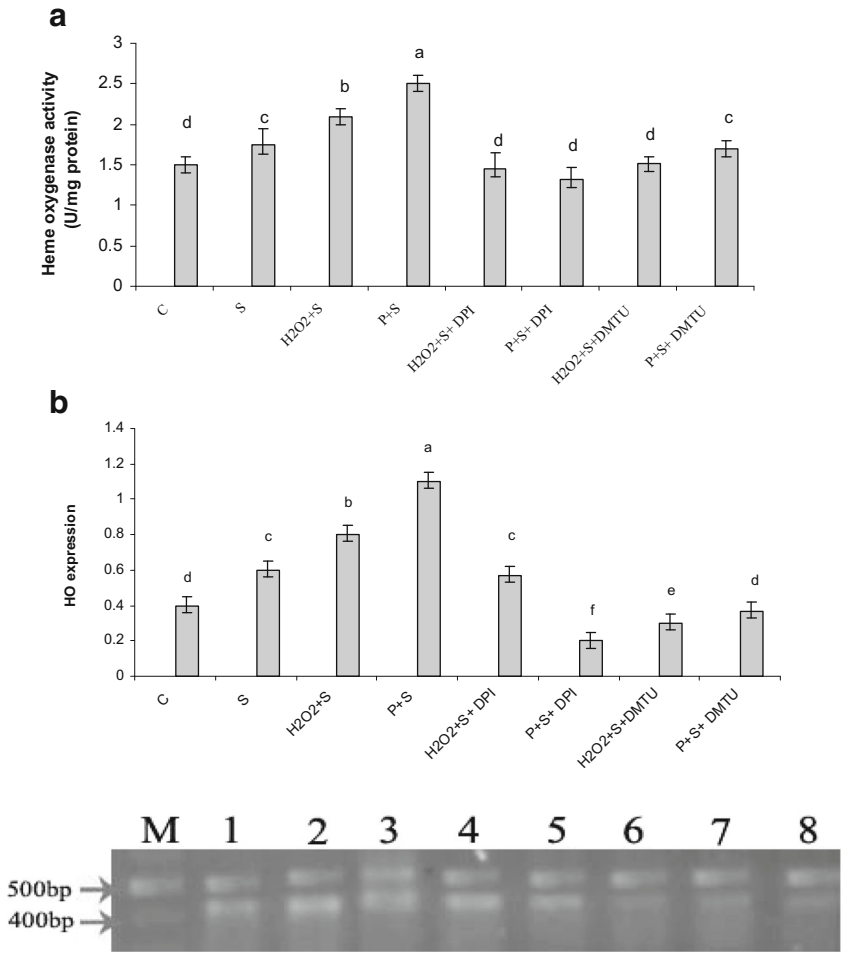

Fig. 7 Effects of osmopriming and H_2O_2 pretreatment singly or in combination with DMTU and DPI on HO activity (**a**) and *HO-1* transcripts (**b**, **c**) in seeds under salt treatment. Seeds were pretreated with 2 mM H_2O_2 or primed in polyethylene glycol solution (PEG 6000) after preincubation with 1 mM DMTU and DPI for 6 h were exposed to water or 150 mM salinity. HO activity and *HO-1* transcripts were measured after 6-h imbibition. *HO-1* transcripts were analyzed by semi-quantitative reverse transcription-polymerase chain reaction. The relative intensity of electrophoretic bands belong to *HO-1* transcripts against ones of actin gene (as internal control) was calculated as relative abundance of transcripts. Values are means ± *SE* of five replications. *Different letters above the bars* indicate significant differences ($P < 0.05$) according to Duncan's multiple range test. *P* osmopriming, *S* salinity, *C* control

producing excessive ROS. It is known that plants mediate their developmental patterns to reprogram their gene expression in response to ROS level in cells for acclimation to their environment. Moreover, data of this survey illustrated the application of DMTU as a scavenger of H_2O_2 and DPI as a NADPH oxidase inhibitor could repress the positive effects of osmopriming and H_2O_2 pretreatment on seed germination under salt stress (Fig. 2) that coincided with less content of H_2O_2 in these treatments (Fig. 3). These findings suggest that the success of osmopriming as well as H_2O_2 pretreatment largely depends on H_2O_2 signaling.

Interestingly, the induced burst of endogenous H_2O_2 production after 6 h of imbibition by both osmopriming and H_2O_2 pretreatment was followed by a sharp decrease after 24 h of imbibition (Fig. 3). Although ROS burst during priming known to be a certainty, only Naglreiter et al. (2005) have reported such increase, while the decrease of ROS content during post priming in mature plants has been reported more frequently (Khodabakhsh et al. 2014; Chiu et al. 2006; Sivritepe et al. 2008). The decrease of H_2O_2 content after 24 h of imbibition may be the result of rapid response of antioxidant system that scavenges ROS. This assumption further supported by these findings that the reduction of H_2O_2 content in seedlings grown from the primed seeds was coincident to more upregulation of antioxidant enzymes and proline synthesis in them (Table 1 and Fig. 9). The similar results have been reported about effect of priming on the elevation of antioxidant system and declining ROS content in many plants (Chiu et al. 2006; Chen and Arora 2011; Khodabakhsh et al. 2014; Li and Zhang 2012). Wahid et al. (2007) also reported that the pretreatment of wheat seed with H_2O_2 reduced H_2O_2 content in the seedlings under saline conditions in comparison with H_2O_2 content in seedlings grown

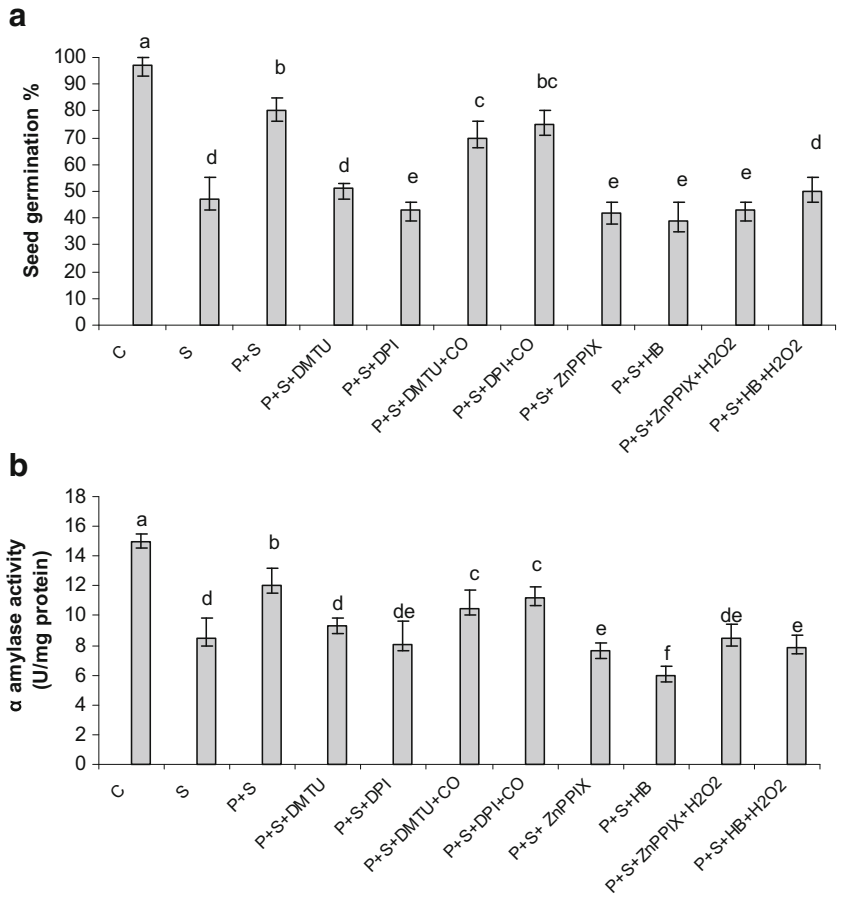

Fig. 8 Effects of osmopriming, *N*,*N*′-dimethylthiourea (DMTU), DPI and ZnPPIX, Hb and CO saturated aqueous solution, and H_2O_2 singly or in combinations on germination of alfalfa seeds under salinity stress. Seeds primed in polyethylene glycol solution (PEG 6000) after preincubation with 1 mM DCMU and DPI, 100 μM ZnppIX and 0.1 g L^{-1} Hb, 10% CO-saturated aqueous solution, H_2O_2 or a combination of treatments for 6 h were exposed 150 mM salinity for 5 days. The α-amylase activity was measured after 6-h imbibition. Values are means ± *SE* of five replications. The *different letters above the bars* indicate statistically significant differences ($P < 0.05$) according to Duncan's multiple range test. *P* osmopriming, S salinity, *C* control

from control seeds. Overall, these findings suggest that early ROS burst during osmopriming can be considered as mild stress exposure at the pregermination stage, which may activate cellular antioxidant system and enhance the detoxification of ROS in subsequent developmental stages and induce cross-tolerance during early seedling establishment.

Table 1 Effect of Osmopriming, H_2O_2 or hematin or osmopriming singly or in combination with DMTU, ZnPPIX, and Hb on the activities SOD, APX, CAT, and proline content in roots of seedlings grown from seeds

	H_2O_2 content nmol g^{-1} F.W	Enzyme activity (U g^{-1} F.W)			Proline content μmol g^{-1} F.W
		SOD	APX	CAT	
Control	20.13f	13.05e	5.61e	28.24f	16.26f
Saliniy	65.92a	22.42d	8.15d	35.36e	22.43e
P + S	32.80e	49.31a	15.69a	67.82a	46.17a
H_2O_2 + S	36.51e	36.67b	12.43b	60.95ab	38.75b
H + S	43.06d	44.58a	10.29c	56.38b	42.62ab
P + DCMU + S	54.23c	30.96c	3.53f	42.66cd	27.91cd
P+ ZnPPIX+ S	51.65c	26.55cd	3.28f	46.45c	30.44c
P+ Hb + S	60.09b	23.47d	4.21ef	39.74de	24.85de

Values are means ± *SE* of five replications. Different letters per each column indicate significant differences ($P < 0.05$) according to Duncan's multiple range test

P osmopriming, S salinity, *H* hematin, *Hb* hemoglobin

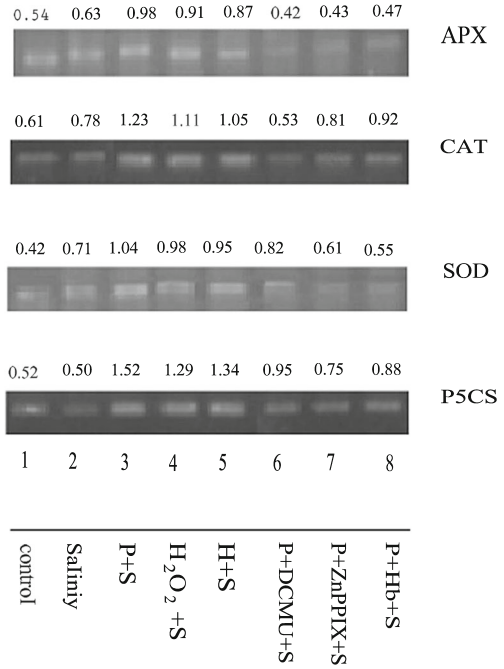

Fig. 9 Effects of osmopriming, H_2O_2 or hematin or osmopriming singly or in combination with DMTU, ZnPPIX, and Hb on relative abundance of *SOD, APX, CAT,* and *P5CS* transcripts in roots of 5-day-old seedlings under salinity stress. Seeds primed in polyethylene glycol solution (PEG 6000) or pretreated with hematin or 2 mM H_2O_2 after preincubation with 1 mM DMTU and ZnPPIX or Hb for 6 h were exposed to water (control) or 150 mM salinity. The transcripts were analyzed by semi-quantitative reverse transcription-polymerase chain reaction in roots of 5-day-old seedlings grown from seeds. The relative intensity of electrophoretic bands belong to gene PCR products against ones of actin gene (as internal control) was calculated as relative abundance of transcripts were indicated above each band. Values are means of five replications. *P* osmopriming, *S* salinity, *H* hematin, *Hb* hemoglobin

Data showed that the alterations of H_2O_2 content in germinating seeds were coincident with the effect of osmopriming and H_2O_2 pretreatment on the activity of NADPHox and partly SOD. The activities of these enzymes increased at early 6 h of imbibition and reduced after 24 h of imbibition (Fig. 4). NADPH oxidase (NADPHox), a plasma membrane-bound enzyme, catalyzes the production of superoxide that is quickly converted to H_2O_2 (Sarath et al. 2007), and SOD is one other of enzymes responsible for the generation of H_2O_2 (Hossain et al. 2015). Results exhibited the osmopriming-induced accumulation of H_2O_2 and is further mediated through the activation of NADPH oxidase in alfalfa germinating seeds. Xie et al. (2011) also observed the exposure with mild salt stress caused biphasic increases in NADPHox-dependent ROS production that led to the improvement of tolerance to higher salt concentration in seedlings. Despite a briefly and non-significant enhancement of SOD activity in following osmopriming, the significant effect of AT as an inhibitor of SOD on the germination of primed seeds (Fig. 4) suggests that it might be the secondary source of H_2O_2 production in beside of NADPHox enzyme. The elevation of SOD activity, endogenous H_2O_2 content and the improvement of growth by H_2O_2 pretreatment and alleviation these acclamatory responses by DMTU, has been also reported for wheat seedlings grown under oxidative stress (Chen et al. 2009). According to the literature review, this is *the first report* in regard to role of the NADPHox-dependent production of H_2O_2 in salt acclamatory responses induced by osmopriming.

Data showed that lower doses of hematin enhanced seed germination under salt stress, while high doses of hematin had deleterious effects (Fig. 5). Although several lines of evidence confirmed the antioxidative effects of heme compounds against various stresses (Amooaghaie et al. 2015; Cui et al. 2011; Liu et al. 2010; Xu et al. 2011), it has been also reported that heme compounds act primarily as a prooxidant in vivo, which can promote the production of oxygen radicals and thus cause an oxidative damage of biologically active molecules (Schmitt et al. 1993).

Hematin pretreatment similar to osmopriming was able to alleviate the inhibition of salt-driven seed germination (Fig. 6a). The similar role of hematin in salt acclimation has been reported in wheat (Xu et al. 2006) and rice (Liu et al. 2007) seeds. As expected, results showed that both osmopriming and hematin pretreatment enhanced HO activity, and ZnPPIX, as an inhibitor of HO, could repress their stimulatory effects on seed germination (Fig. 6a) and HO activity under salt stress (Fig. 6b). These findings suggest that HO could act as an antioxidative barrier against salt induced oxidative damage in seeds, and endogenous HO activity might play a pivotal role in salt-protective effects induced by osmopriming or hematin pretreatment.

The salinity increased H_2O_2 accumulation (Fig. 3) and enhanced HO activity and *HO-1* messenger RNA (mRNA) levels moderately (Fig. 7). Both osmopriming and H_2O_2 pretreatment induced the considerable elevation of HO activity and *HO-1* mRNA levels (Fig. 7). Importantly, these changes were correlated with seed germination responses (Fig. 2). It has been also reported that H_2O_2, as a downstream regulator of HO, participated in regulating rice seed germination under salinity stress (Wei et al. 2012). These findings suggest that osmopriming and lower dose of H_2O_2 act as slight stressors that induce H_2O_2 generation, and H_2O_2 signaling triggers the elevation of HO activity and shortens the time to achieve the required HO activity levels for subsequent seed germination and seedling growth under severe salinity stress. This conclusion was further supported by observations that the increments of HO activity and *HO-1* mRNA levels as well as seed germination induced by osmopriming and H_2O_2 pretreatment could be blocked by DPI and DMTU (Fig. 7).

These findings are consistent with the report of Xie et al. (2011) who suggested the participation of NADPHox-driven ROS is necessary for HO upregulation and HO plays an important role in salt acclimation. Chen et al. (2009) also reported that SOD-driven H_2O_2 induced the upregulation of

HO activity and *HO-1* expression in wheat leaves. However, to the best of our knowledge, the present paper is the first report that is suggesting NADPHox-driven H_2O_2 might be involved in the upregulation of HO by osmopriming. Certainly, the application of mutants and other molecular techniques underlying their gene expression must provide more evidence to confirm this hypothesis.

On the other hand, the application of 10% CO-saturated aqueous solution was able to mimic the effect of osmopriming on the alleviation of the negative effect of salt on seed germination and α-amylase activity (Fig. 8). The priming-mediated response on improving seed germination and α-amylase activity appeared to be CO dependent, as it was completely blocked by treatment of the scavenger of CO. In other words, hemoglobin (Hb as scavenger of CO) was able to mimic the effects of DMTU on the reversal of priming effects (Fig. 8). These results again suggested that HO and its product, i.e., CO were involved in cytoprotective effects induced by osmopriming. Wu et al. (2012) also found that CO mediates *Amyc2* expression and activates amylase activity in seeds. Similar results about effect of HO and CO on stress tolerance were also reported previously (Amooaghaie et al. 2015; Liu et al. 2007; Han et al. 2008; Xie et al. 2008). The enhancement of amylase activity by H_2O_2 (Ishibashi et al. 2012), hematin (Amooaghaie et al. 2015), hemin (Wu et al. 2012), CO (Liu et al. 2010), and priming (Amooaghaie and Nikzad 2013; Farooq et al. 2006) has been reported by other authors. However, our paper is the first report on the role of heme oxygenase activity and CO signaling in responses induced by osmopriming.

Interestingly, the addition of CO-saturated aqueous solution after osmopriming alleviated the inhibitory effect of DMTU or DPI on salt acclimatory responses. Conversely, H_2O_2 could not reverse the inhibitory effect of ZnPPIX and Hb on seed germination and α-amylase activity. This pharmacological evidence again proposed that the maintenance of homeostatic H_2O_2 and CO is necessary for the cytoprotective effects of osmopriming and probably CO acts in the downstream of H_2O_2 in the signal transduction network promoted by osmopriming. These results suggest that osmopriming induced H_2O_2 production and H_2O_2 signaling triggered HO upregulation. CO, as the product of HO activity, promoted the enhancement of amylase activity and consequently resulted in a higher rate of seed germination under salt stress.

It is known that enhancing the activity of D-1-pyrroline-5-carboxylate synthetase enzyme, (P5CS catalyze the rate-limiting step of proline biosynthesis) and also antioxidant enzymes contribute to stress tolerance in plants. Therefore, to explore the role of signal molecules (H_2O_2 and CO) in priming-induced salt tolerance, we examined the changes of antioxidant enzymes and proline content. Our results revealed that osmopriming as well as pretreatment with hematin or H_2O_2 upregulated genes of *SOD, APX, CAT,* and *P5CS* (Fig. 9) that led to the increase of proline content and elevating the activities of antioxidant enzymes (Table 1) in seedlings grown from the primed and hematin- or H_2O_2- pretreated seeds. Importantly, these effects were reversed when DMTU, ZnPPIX, and Hb were added after osmopriming (Table 1 and Fig. 9). These results are indicating that H_2O_2 and HO/CO burst during osmopriming are essential for establishing subsequent stress tolerance in seedlings grown from the primed seeds.

In conclusion, our data suggest that osmopriming as a mild stress induced H_2O_2 production and HO activity; signal molecules (H_2O_2 and CO) might shared a pathway that led to creating stress memory in cells and primed cells using this stress imprint could upregulate defense responses more effectively than unprimed cells under subsequent exposure with salt stress.

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