



# Hydrogen peroxide-induced salt tolerance in relation to antioxidant systems in pistachio seedlings

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## ABSTRACT

In this study, the effects of H<sub>2</sub>O<sub>2</sub> pretreatment on growth and antioxidant system were investigated in pistachio rootstock Badami Rize Zarand during short term salt stress. The plants were subjected to NaCl stress (120 or 240 mM) for 7 days after pretreatment with 0, 1, 5 or 10 mM H<sub>2</sub>O<sub>2</sub>. Salinity stress significantly decreased seedling growth and ascorbic acid (ASA) and carotenoid (CAR) contents, whilst exogenous H<sub>2</sub>O<sub>2</sub> application improved the growth and helped to reduce the percent reduction of total ASA and CAR contents under salt stress conditions. Ascorbate peroxidase (APX) and catalase (CAT) activities were strongly induced by salt stress after 48 h and reached the highest level at 168 h after spraying. H<sub>2</sub>O<sub>2</sub>-treated plants showed enzymatic activity higher than untreated plants, during the entire experimental period. Salinity treatments caused increase in plants GSH content with or without H<sub>2</sub>O<sub>2</sub> pretreatment. GSH levels in the plants were previously treated with H<sub>2</sub>O<sub>2</sub> were elevated even higher. Overall, better performance of pistachio seedlings under salt stress due to H<sub>2</sub>O<sub>2</sub> pretreatment might be due to improved better osmotic adjustment through coordination with CAT, and APX and enhanced non-enzymatic antioxidant accumulation under salt stress leading to a higher ability to withstand salt-induced effects.

## 1. Introduction

Iran is one of the two major centers of *Pistacia* diversity and the main producer of pistachios in the world. Salinity has so far been a limiting factor in plant productivity in most Iranian ecosystems. The main reason for higher soil salinity in the pistachio nut production regions is low rainfall and use of poor quality irrigation waters. Evidence suggests these areas have suffered enormously as a result of salinity problems in recent years. Poor quality of irrigation water in association with salt build-up soils has reduced the yields of pistachio over recent years, especially in Kerman, and in central Iran (Tavallali et al., 2009). On the other hand some important local genetic resources of pistachio cultivars and rootstocks have been lost (Esmailpour et al., 2015).

Along with the importance of potentially negative impacts of salinity on plant productivity in marginal lands (Karimi and Tavallali, 2017), in recent years, some methods have been progressed for improving the salinity tolerance instead of traditional inbreeding programs (Kamali et al., 2014). For example, Fekri et al. (2016) suggest that application of pistachio waste, at low concentration, can reduce some adverse effects of salinity on pistachio seedlings growth.

Researchers showed that zinc (Tavallali et al., 2009), boron (Karimi and Tavallali, 2017), phosphorus (Fekri et al., 2015), or nitrogen (Razavi Nasab et al., 2014) application can strongly improve pistachio performance under salinity conditions. The results of Kamali et al. (2014) showed the promising potential use of polyamines for improving the growth of pistachio seedlings and alleviating the negative effects of salinity stress. Azarmi et al. (2016) used plant growth promoting rhizobacteria as a new strategy to reduce the destructive effects of salinity and reported that inoculation pistachio plants by rhizobacteria significantly increased the protein concentration, proline and the soluble sugars, whereas, reduced the hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) levels at all NaCl levels.

Mild oxidative stress induced by either chemical or physical treatments appears to be beneficial to subsequently stressed seedlings (Hu et al., 2009). Exogenous use of various chemicals to alleviate the toxic effects of saline stress may have implications both from theoretical and practical perspectives (Manaa et al., 2014). Various stressors lead to H<sub>2</sub>O<sub>2</sub> formation in plant cells. Some authors suggested that H<sub>2</sub>O<sub>2</sub> plays a dual role in plants: acts as a messenger that involved in signaling and in triggering tolerance against various stresses at low concentration,

**Abbreviations:** APX, ascorbate peroxidase; ASA, ascorbic acid; CAR, carotenoids; CAT, catalase; GSH, reduced glutathione; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; MDA, malondialdehyde; ROS, reactive oxygen species

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and as an oxidative stress inducer at high concentrations (Fedina et al., 2009). Recent studies have indicated that pretreatment of plants with exogenous  $H_2O_2$  consults abiotic stress tolerance. Pretreatment with  $H_2O_2$  improves plants tolerance against oxidative damage caused by stress conditions including waterlogging (Andrade et al., 2018), heat (Sarwar et al., 2017), Salt (Kong et al., 2017), pathogenic micro-organisms (Vargas-Hernández et al., 2017), drought (Sun et al., 2016), heavy metal (Wen et al., 2013), low light (Zhang et al., 2011) and chilling (Yu et al., 2003). Using pretreatment conditions in which a moderate stress condition is applied, results in dormant signals accumulation in different plant parts. Then, when a stress condition occurs, the stored signals will drive molecular adjustments resulting in progression of harder and more efficient mechanisms of tolerance (Savvides et al., 2016). According to Liu et al. (2010) pretreatment with low concentrations of  $H_2O_2$  increases the enzymatic antioxidant activity and accumulation of ascorbate and reduced glutathione in plants. The effect of salinity-induced oxidative stress on some agricultural crops is well documented, however, such type of information is lacking with respect to some fruit rootstocks. We hypothesized that  $H_2O_2$  pretreatment would improve the tolerance of pistachio rootstock Badami Rize Zarand to salt stress. We investigated the possible role of the antioxidant defense system in this process leading to protect pistachio plant from salt-induced injury. It has not been previously reported that  $H_2O_2$  pretreatment alleviates the effects of salt stress on this rootstock.

## 2. Materials and methods

### 2.1. Plant materials and treatments

Two replicate experiments with potted seedlings were conducted in two consecutive years. Certified fresh seeds of pistachio cultivar, Badami Rize Zarand, was obtained from the Pistachio Research Institute, Kerman, Iran in September 2016. For cold stratification, surface sterilized seeds were kept on moist peat moss at 4 °C refrigerator for 20 days and then sown in 7-L plastic pots, which were filled with sterilized sand. Five seeds were sown in each pot and two pots were kept as one replication. After germination the seedlings were thinned to two uniform plants per pot. The plants were grown for 40 days in a greenhouse and watered once each day with distilled water before they were sprayed with  $H_2O_2$ . Mean average day- and night-time temperature and photosynthetic photon flux density in the greenhouse were 25–29 °C, 11–13 °C and about 1200  $\mu\text{mol m}^{-2} \text{s}^{-1}$  at midday, respectively. The aboveground organs of plants were then sprayed with either freshly prepared 1, 5, 10 mM  $H_2O_2$  or distilled water. At 24 h after the  $H_2O_2$  spray, the seedlings were treated with NaCl solutions. Three salinity levels were applied: 0, 120, and 240 mM NaCl. Treatments were completely randomized and replicated four times. Four harvests were performed to measure plant antioxidant defense responses. The first harvest was forty days after sowing and before the spray; the second harvest was at 24 h after spraying and before salt addition; the third harvest was at 48 h after the spraying, when the plants were subjected to saline conditions; and the fourth harvest was at 168 h (7 days) after the spraying (144 h after NaCl application).

### 2.2. Measurements of growth parameters

Plant growth parameters (i.e., plant relative height, plant relative diameter and dry plant and root weights), from all treatments, were evaluated at the end of experiment. Plant were harvested at the end of experiment and their shoots and roots were separated. Relative plant height and diameter were calculated from plant length and diameter data obtained from 40 and 48 days after sowing. Shoot diameter was measured by digital caliper. Dry weight of shoot and roots were determined by oven-drying at 70 °C to constant weight.

### 2.3. Determination of hydrogen peroxide ( $H_2O_2$ ) and lipid peroxidation

Hydrogen peroxide ( $H_2O_2$ ) was spectrophotometrically measured with KI following the protocol of Sergiev et al. (1997). Leaf tissues (500 mg) were homogenized in the ice bath with 5 mL 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 12,000 g for 15 min; 0.5 mL of the supernatant was added to 0.5 mL 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1 M KI. The absorbance of the supernatant was read at 390 nm. The amount of  $H_2O_2$  was calculated using an external calibration curve prepared with known concentrations of  $H_2O_2$ .

As the indicator of leaf lipid peroxidation, malondialdehyde (MDA) content was determined according to Soliman et al. (2012). Frozen leaf samples (500 mg) were homogenized with a prechilled mortar and pestle with 5 mL of 0.1% TCA and centrifuged for 20 min at 15,000 g. One mL aliquot of the supernatant was mixed with 3 mL of 0.5% thiobarbituric acid solution containing 20% (w/v) trichloroacetic acid. The mixture was heated at 95 °C for 30 min. Then, the reaction was interrupted and the mixture was immediately placed in an ice-bath. After centrifugation (10,000 g for 5 min at 4 °C), the supernatant absorbance was read at 532 nm using a Shimadzu UV 160 A spectrophotometer (Shimadzu Corp., Kyoto, Japan). Subsequent to subtracting the non-specific absorbance at 600 nm, MDA concentration was determined by the following formula using the extinction coefficient of  $155 \text{ mM}^{-1} \text{ cm}^{-1}$ .

$$\text{MDA } (\mu\text{mol g FW}^{-1}) = [(A_{532} - A_{600}) / 155] \times 10^3$$

### 2.4. Antioxidant enzyme activity assays

The methods for the determination of the activities of catalase (CAT) and ascorbate peroxidase (APX) were those reported by Jiang and Zhang (2002). Frozen leaf segments (0.5 g) were homogenized in 10 mL of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1% polyvinylpyrrolidone. However, for APX, 1 mM ascorbate was included while EDTA was excluded from the extraction buffer. The homogenate was centrifuged at 15,000 g for 20 min at 4 °C and the supernatant was immediately used for the following enzyme assays. APX activity was measured by monitoring the decrease in the absorbance at 290 nm while ascorbate was oxidized as described by Nakano and Asada (1981). The 3 mL-reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.1 mM EDTA, 0.1 mM  $H_2O_2$  and 0.1 mL enzyme. The reaction was started by the addition of 0.1 mM  $H_2O_2$ . CAT activity was assayed by measuring the rate of the decomposition of  $H_2O_2$  at 240 nm as described by Aebi (1984). The 3.0 mL-reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 12.5 mM  $H_2O_2$ , 0.05 mL enzyme and water to make up the volume of 3.0 mL. Adding  $H_2O_2$ , the researchers started the reaction and traced a decrease in the absorbance for 1 min.

### 2.5. Estimation of non-enzymatic antioxidants

Reduced glutathione (GSH) content was determined following the method proposed by Anderson (1985). Leaf tissue (0.5 g) was homogenized in 2 mL of 5% (w/v) sulphosalicylic acid under cold conditions. The homogenate was centrifuged at  $10,000 \times g$  for 10 min and the supernatant was collected. 0.5 mL of supernatant, 0.5 mL of phosphate buffer (100 mM, pH 7.0), 0.5 mM EDTA and 50  $\mu\text{L}$  of 3 mM 5,5-dithiobis (2-nitrobenzoic acid) (DTNB) were added. After 2 min, the absorbance was read at 412 nm. A standard curve was prepared from varying concentrations of reduced GSH.

Ascorbic acid (ASA) levels were determined as described by Liu et al. (2015). Frozen leaf powder (0.1 g) was homogenized in 1 mL of 5% trichloroacetic acid (TCA). The extracts were centrifuged at  $15,000 \times g$  for 10 min at 4 °C, and then the supernatant was used to

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