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Physiological and growth responses of *Calendula officinalis* L. plants to the interaction effects of polyamines and salt stress

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ABSTRACT

Salt and water stresses are the most important factors limiting plant growth and productivity in the world. Polyamines are shown to have a role in adaptation of plants to salt stress condition. Effects of exogenous spermine (Spm) and spermidine (Spd) on growth and physiological traits were investigated in *Calendula officinalis* L. plants under salt stress condition. At all levels of salt stress application of Spd at 1 mmol concentration affected significantly shoot dry matter, peroxidase activity (POD) and proline, protein and total chlorophyll contents compared to the other levels of polyamines. Oxidative activities of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) were decreased when plants were treated by 1mM Spm concentration under salt stress conditions. Whatever was the salt stress level, application of Spm at 1mmol increased catalase activity (CAT) and maximum photochemical quantum yield (F_V/F_M) of calendula plants. Results of this study suggest that polyamines may increase plant tolerance to salt stress by decreasing oxidative damages and increasing enzyme activities.

1. Introduction

Soil salinity is one of the factors, particularly in the arid and semi-arid regions, which limits crop plants growth and productivity. It is the result of in part incorrect irrigation practices without drainage systems and concentration of salt in top soil layers. Soils with electrical conductivity (EC) of higher than 4 dS m^{-1} are considered as saline soils and more than 6% of total arable lands around the world are classified as saline (FAO, 2008). To cope with the problem of salinity, crop plants usually implement different approaches. Osmotic effect of salts in the soil solution makes it difficult for plants to absorb water. Shoot parts are usually more affected by salt compared to the roots (Fariduddin et al., 2013). Absorbed ions in saline soils is usually high enough to be toxic for plants and in some cases they prevent the absorption of nutrient element as in the case of Na^+ instead of K^+ (Zhu, 2002). When plants are exposed to salt stress condition, reactive species of oxygen (ROS) such as superoxide (O^{2-}), hydrogen peroxide (H_2O_2), singlet oxygen (O_2) and hydroxyl radical (OH^-) concentration increase inside plant tissues (Diao et al., 2015). Increasing levels of ROS damage the lipids, carbohydrates and nucleic acid molecules and accelerates plant senescence (Velikova et al., 2000). Damages to the lipids in the membrane structures increase ion leakage from the cells (Ahmad Jallel et al., 2009). Resistant plants usually use antioxidants to reduce the detrimental effects on plant cells. In addition enzymes such as catalase (CAT), peroxidase (POD), superoxide dismutase (SOD), ascorbate peroxidase (APX) and glutathione reductase (GR) which are existing in the cells particularly in chloroplast and mitochondria are used to scavenge ROS (Rasool et al., 2013).

Polyamines (PAs), including putrescine (Put), spermidine (Spd) and spermine (Spm) are aliphatic nitrogenous groups with low molecular mass. They contribute to different physiological and biochemical processes involved in plant growth (Roychoudhury et al., 2011). Effect of polyamines on different plant species under abiotic stresses have been reported (Unal et al., 2008; Ahmad Jallel et al., 2009; Farooq et al., 2009; Radhakrishnan and Lee, 2013; Diao et al., 2015). Application of exogenous polyamines is considered as an effective method in not only the determination of their roles in response to salinity but also as a way to increase plant resistance to salinity (Chattopadhayay et al., 2002). PAs are able to interact with proteins, nucleic acids, membrane phospholipids and cell wall constituents, thereby stabilizing these molecules (Roychoudhury et al., 2011). Exogenous application of polyamines has been shown to improve the reduction of photosynthetic efficiency due

Abbreviations: Ec, electrical conductivity; ROS, reactive oxygen species; H_2O_2, hydrogen peroxide; O_2-, superoxide radical; CAT, catalase; POD, peroxidase; SOD, superoxide dismutase; APX, ascorbate peroxidase; GR, glutathione reductase; PAs, polyamines; Spd, spermidine; Spm, spermine; Put, putrescine; F_V/F_M, maximum photochemical quantum yield; MDA, malondialdehyde; TCA, trichloroacetic acid; TBA, thiobarbituric acid

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to salt stress in most plant species. However the effect is highly dependent on the level of stress and concentration and the polyamine type (Duan et al., 2008). It has been reported that Photosystem II (PSII) photosynthetic efficiency and maximum photochemical quantum yield (F_V/F_M) increase in cucumber plants under salt stress condition (Shu et al., 2013).

Pot marigold or calendula (*Calendula officinalis* L.) belongs to Asteraceae family and native to southern Europe. It grows as bedding plant, cut flower, or potted plant. The petals color is different from yellow to orang and aromatic scent. Calendula is a sun plant which and grow also under half-shade condition (Dole and Wilkins, 2005).

There is little information regarding the effects of polyamines on salt stress tolerance of calendula plants. More detailed information is necessary regarding the responses of this plant to salt stress condition. In this study we examined the effects of salt stress and antioxidant enzymes which are responsible for degeneration of ROS and also the levels of lipid peroxidation, proline content and F_V/F_M on responses of calendula plants under salt stress condition.

2. Materials and methods

2.1. Plant material and growth conditions

Seeds of calendula plant were prepared from Pakanbazr seed production company (Isfahan-Iran). Seeds sown in pots containing a mixture of cocopeat and perlite (1:1) (Azarrahab Co., Karadj, Iran), perlite particle 0.5–1 mm were used. Soil salinity was measured as electrical conductivity (EC- $dS.m^{-1}$) at water saturated soil extract (Winduas-Germany). At four leaf stage seedlings were transplanted into another growing media containing sandy-loam soil with EC at 1 $dS\,m^{-1}$. Plants were irrigated by half-strength Hoagland solution every 10 days (Hoagland and Arnold, 1938). Temperature ranged between 20–30 °C, and the relative humidity fluctuated between 50% and 65% under fluorescent light with intensity of 400 $\mu mol\,m^{-2}s^{-1}$ in 14 h photoperiod. The experiment was a factorial based on CRD with 4 replications conducted at the glass greenhouse of SB university of Kerman. Two weeks after transplanting plants were divided into 3 groups with 5 treatments per group. These groups included three levels of saline solutions (NaCl) including 1, 5 and 9 $dS\,m^{-1}$. Saline solutions were applied at several steps in order to prevent salt stress shock on plants. Plants were then sprayed with Spm and Spd at 3 levels (0, 0.5 and 1mmol). Polyamines treatments were repeated 10 days later. Pots were weighted regularly every days and the amount of water lost was added. Sixty days after transplanting, at flowering stage, leaf samples were taken and immediately transferred into liquid nitrogen for later biochemical measurement.

2.2. Determination of shoot and dry weight

At the end experiment, root and shoot were sampled and dried at 65 °C for 48 h and weighted.

2.3. Determination of total chlorophyll

The concentration of total chlorophyll was determined following the method of Lichtenthaler (1987). Fresh samples (0.1 g) were homogenized with 10 mL of 80% (w/v) acetone. The homogenate was centrifuged at 2700*g* for 10 min. Total chlorophyll concentration were measured using a UV–vis spectrophotometer (Cary50 device-Germany) at 663 and 645 nm. Spectrophotometric readings were converted into total chlorophyll concentrations using the following equations.

$$Chla = (12.25 \times D663 - 2.79 \times D645) \times \frac{V}{1000 \times W}$$

$$Chla = (21.5 \times D645 - 5.10 \times D663) \times \frac{V}{1000 \times W}$$

Total chlorophyll (Chl$a$ + b) = Chl$a$ + Chl$b$

In which W is weight and V is volume.

2.4. Determination of F_V/F_M

Fluorescence parameters including maximum and minimum fluorescence (Fm, F0) were determined on pre-dark adapted leaves by exposing it to a light at 400 µmol photon $m^2\,s^{-1}$ for 5 s that was measured by fluorometer (Junior PAM-Waltz-Germany). Measurements were replicated 5 times on fully expanded leaves. F_V/F_M of PSII was determined using the following equation according to Genty et al (1987).

$$Fv/Fm = (Fm - F0)/Fm$$

2.5. Determination of malondialdehyde content (MDA)

MDA content was measured by method Heath and Packer (1969). Frozen plant tissues (0.2 g leaf and root) were pulverized in 5 ml of 1% trichloroacetic acid (TCA). The solution was centrifuged for 5 min at 10,000*g*. 4ml of 20% TCA containing thiobarbituric acid (TBA) was added to the supernatant. The solution was incubated for 30 min in water bath 95 °C and immediately cooled in ice. The mixture was then centrifuged in 10,000*g* for 10 min. absorbance of solution was measured in 532 nm using spectrophotometer. Absorbance of other non-specific pigments was determined in 600 nm and deducted from this value. Extinction coefficient equal to 155 $mM^{-1}\,cm^{-1}$ was used to determine MDA concentration, and the results were calculated as nanomole per gram of fresh weight.

2.6. Determination of H_2O_2 content

H_2O_2 was also determined according to Velikova et al (2000). Fresh samples (0.5 g) were homogenized with 5 mL of 0.1% (w/v) trichloacetic acid. The homogenate was centrifuged at 12,000*g* for 15 min, and 0.5 mL of the supernatant was added to 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1 M potassium iodide. The absorbance of supernatant was recorded at 390 nm. The content of H_2O_2 was calculated by comparison with a standard calibration curve, plotted using different concentrations of H_2O_2.

2.7. Protein and antioxidants assays

Bradford method (1976) was used in order to determine leaf protein content. Buffer pH 7.0 (0.01 M) was used then centrifuged at 4 °C for 10 min at 12,000*g*. POD and CAT activity were expressed as activity per mg protein. In order to determine the POD activity, Kar and Mishra (1976) method was used. Here, the degree of inhibition of pyrogallol auto-oxidation by supernatant was measured. To perform the test, 0.1 ml enzyme extract was also prepared and added to 50 µM solution of H_2O_2 and 50 µM pyrogallol. POD activity was determined by the amount of purpurogallin formed in 420 nm. CAT activity was also measured by the method of Aebi (1984). 0.5 ml of 0.2 M H_2O_2 in 10-mM phosphate buffer adds to the enzyme extract. CAT activity was measured by the reduction in absorbance of H_2O_2 at 240 nm and 1 unit of CAT was determined as l g of H_2O_2 released per mg $protein^{-1}\,min^{-1}$.

2.8. Determination of proline content

The proline content was estimated using the acid ninhydrin method (Bates et al., 1973). The leaf tissue was homogenized with 6 ml of 3% (w/v) sulfosalicylic acid than the extract was centrifuged for 5 min at 10,000*g*. Two ml of the centrifuged extract was taken for the analysis to which 2 ml acid ninhydrin and 2 ml of glacial acetic acid were added. The reaction mixture was incubated in a boiling water bath for 1 h and the reaction was finished in an ice bath. Four ml of toluene was added