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Salicylic acid improves salinityalkalinity tolerance in pepper (*Capsicum annuum* L.)

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Abstract: Salinization and alkalization of soils are agricultural problems in arid and semiarid regions of the world such as Iran. In this experiment the effects of salicylic acid (SA) on resistance of pepper plants under salt stress (SS) and alkali stress (AS) were evaluated. Treatments include 0 and 150 mM of SS, 0, 50 and 100 mM of AS and 0, 0.75 and 1.5 mM SA. Results showed that SS and AS imposed negative effects on pepper plant growth and productivity. Reduction in growth and yield in SS was higher than AS and maximum reduction occurred in high mixed stresses. SA application improved growth parameters and increased yield, relative water content (RWC) and chlorophyll of plants subjected to SS and AS and provided significant protection against stress compared to non-SA-treated plants. For most traits, 0.75 mM of SA was more effective than 1.5 mM concentration. SA ameliorated the injury caused by SS and AS by increasing chlorophyll and RWC and inhibiting proline accumulation and leaf electrolyte leakage (EL). In general, results indicate that salinity and alkalinity have negative effects on growth and yield of pepper plants and these negative effects can be ameliorated by application of SA.

1. Introduction

Salinity and alkalinity of soil seriously affect about 932 million hectares of land globally, reducing productivity in about 100 million hectares in Asia (Rao *et al.*, 2008). In Iran 12.5% of the agricultural lands in arid and semi-arid areas are alkaline. While salt stress (SS) in a soil generally involves drought stress and ion-induced injury (Munns, 2002), alkali stress (AS) exerts the same salt stress influences with the added effects of high-pH stress (Shi and Yin, 1993). The high-pH caused by AS directly affects the mineral absorption and interferes with the re-establishment of ionic balance. It can strongly affect the absorption of inorganic anions such as

 Cl^- , NO_3^- and $H_2PO_4^-$, and can disrupt the selective absorption of K⁺-Na⁺, and pH homeostasis in the plant tissues (Yang et al., 2007). To resist AS, plant not only have to regulate the intercellular pH to maintain ionic balance, but also have to spend material and energy to regulate pH in their root environment. Thus osmo-regulation and ion balance play key influence in plants SS and AS resistance (Yang et al., 2007). There has been considerable study of SS on plants growth and development, however, relatively little attention has been given to AS despite its importance. Like to other environmental adverse conditions, AS can lead to emerge reactive oxygen species (ROS), as a result of closing stomata and reducing CO₂ into cells and consequently blocking photosynthesis activities (Yan et al., 2011). AS caused metabolic disturbance, lipid peroxidation and chlorophyll breakdown and proline accumulation in plant tissue (Gao et al., 2012).

Various techniques have been tested to improve saline-alkali soils, including chemistry, physics, biology and engineering improvements to increase soil fertility and crop yield (Li-Ping et al., 2015). In recent studies a number of plant growth regulators (PGRs) have been under trial to alleviate the environmental stresses in plants. Salicylic acid (SA) is a phenolic compound which is considered as a PGR and plays an important role in defensive mechanisms against biotic and abiotic stresses in plants. Flowering induction, plant growth and development, synthesis of ethylene, opening and closure of stomata and respiration are some of the important roles of SA in plants (Raskin, 1992). SA protects plants from damages caused by oxidative stresses through increasing antioxidants enzymes activities (El-Tayeb, 2005; Idrees et al., 2011). SA has received much attention due to its function in plants' responses to environmental stresses. Literature exists about some beneficial effects of SA on plants under drought (Jafari et al., 2015), low temperature, high temperature (Wang and Li, 2006; Sayyari, 2012), salinity (Shakirova et al., 2003; El-Tayeb, 2005; Stevens et al., 2006; Idrees et al., 2011), heavy metal (Metwally et al., 2003) and biotic stresses (Makandar et al., 2012). However, no information exists on the effects of the mentioned compounds on salt-alkali stress defense mechanisms, until now. Thus the purpose of this experiment was to examine the possibility that application of SA would protect pepper plants from damaging effects of salt-alkali stresses. Specific objectives of this research were: (1) to compare SS and AS on pepper

plant growth and development (2), to determine some physiological responses of pepper plant to SS and AS and (3) evaluate the protective effect of a pre-treatment with SA.

2. Materials and Methods

Plant material and growing conditions

Seeds of *Capsicum annuum* L. cv. Plenty were obtained from Pakan Bazr Co. (Isfahan, Iran) and cultured in bed to obtain seedlings for experiment. When the seedlings had 2-4 true leaves, the seedling with similar size were selected and transferred into plastic pots (20 cm height, and 23 cm diameter) which were filled with about 8 kg of 1:1:2 mixture of fine sand, leaf mound and garden soil. The pots were then transferred to the greenhouse with average temperature of 25.5/19.5°C (day/night) and natural light.

Salt stress, alkali stress and salicylic acid treatments

When the seedlings have been established in pots (4-6 true leaves), they were sprayed with 0 (as control), 0.75 and 1.5 mM SA until both sides of the leaves were completely wet. Three days later, plants were subjected to SS and AS treatments until end of each experiment. Two neutral salts (NaCl and Na_2SO_4) and two alkaline salts (NaHCO₃ and Na_2CO_3) were selected for salinity and alkalinity stresses imposition. The two neutral salts were mixed in a 9:1 molar ratio (NaCl:Na₂SO₄), and applied in 0 mM and 150 mM to the SS group, and two alkaline salts were also mixed in a 9:1 molar ratio (NaHCO₃:Na₂CO₃) in 0 mM, 50 mM and 100 mM, and applied to the AS group. SS and AS treatment were conducted with daily watering of plants by mentioned treatment. Control plants (0 mM SS and 0 mM AS) were watered with distilled water (Yang et al., 2007; Rao et al., 2008).

Chlorophyll determination

Chlorophyll was assessed by taking fresh leaf samples (0.1 g) of plants in each replicate from young and fully-developed leaves. The samples were homogenized with 5 ml of acetone (80% v/v) using pestle and mortar and centrifuged at 3000 rpm. The absorbance was measured with a UV/visible spectrophotometer at 663 and 645 nm and chlorophyll were calculated (mg/g FW) using the equations proposed by Strain and Svec (1966) given below:

Total chlorophyll = $20.2 \times (A645) + 8.02(A663)$.

Electrolyte leakage

Electrolyte leakage was determined according to Lutts *et al.* (1995) method. Ten leaf discs of randomly chosen plant were taken from the youngest fullyexpanded leaf. The leaf discs were then placed in test tubes containing 10 ml of distilled water. These samples were incubated at 25°C on a shaker for 24 h. Electrical conductivity (EC) of bathing solution (EC1) was read after incubation. The same samples were then placed into a water bath (100°C) for 20 min and the second reading (EC2) was determined after cooling of the solution to room temperature. The EL was calculated as EC1/EC2 and expressed as a percentage.

Proline content determination

The proline was determined according to the method described by Bates *et al.* (1973). The amount of 0.5 g of samples were homogenized in 10 ml of 3% (w/v) sulfosalicylic acid. After centrifuging at 10000 rpm, 2 ml of the supernatant was mixed with 2 ml of acid ninhydrin and 2 ml of glacial acetic acid in a test tube. The mixture was placed in a water bath (100°C) for 1 h. The reaction mixture was extracted with toluene and the chromophore-containing toluene was aspirated and cooled to 25°C. The absorbance was measured at 520 nm with a UV/visible spectrophotometer. Proline concentration was expressed as $\mu g/g$ FW.

Measurement of relative water content

Relative water content was measured according to Costes *et al.* (2006). Leaves sample in each treatment was weighed (FW) and then immediately floated on distilled water for 5 h in the dark. Turgid weight (TW) of leaf disks was obtained after drying excess surface water with paper towels. Dry weight (DW) of disks was measured after drying at 75°C for 48 h. The RWC was calculated using the following formula:

Relative water content = (FW-DW)/(TW-DW)×100

Statistical analysis

Data were analyzed for significant differences using a factorial analysis of variance with SS and AS levels and SA concentrations as main factors with three replications and 5 seedlings per each. Statistical analysis was performed using SAS program and the means compared using the Duncan's Multiple Range Test at p=0.05.

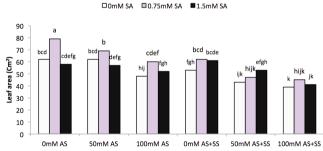
3. Results and Discussion

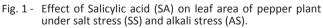
Leaf area and total yield

The leaf area and pepper yield displayed a signifi-

cant reduction in response to the increasing levels of SS and AS treatments. Among all stress treatments tested, 100 mM AS+SS exhibited the strongest reduction of leaf area and yield followed by 50 mM AS+SS. Foliar sprayed SA plants exhibited significant response to improve yield as compared to unsprayed ones in all stress levels (Fig. 1 and 2). The general effect of soil salinity in plants is to decrease the growth resulting in smaller leaves, shorter stature, and sometimes fewer leaves. The primary effect of salinity, especially at low to moderate levels, is due to its osmotic stress (Munns, 2002). The AS exerts the same stress factors as SS but with the added effect of high-pH stress. The effect of SS and AS in reducing the growth and yield of various plants was reported in (Yang et al., 2007; Chen et al., 2011) studies that are in agreement with the findings of this study. However, in the present study, adverse effects of AS on yield and leaf area were lower than that of SS. This results implies not only that SS and AS are different stresses, but also that resistance of pepper plant to AS is stronger that to SS.

The treatment of SA caused a significant increasing in yield and leaf area in comparison to control plants at all stresses levels (Fig. 1 and 2). The positive effect of SA on plants under salinity stress have been reported (Shakirova *et al.*, 2003; Stevens *et al.*, 2006; Idrees *et al.*, 2011) which their upshots are in agree-







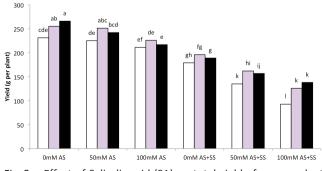


Fig. 2 - Effect of Salicylic acid (SA) on total yield of pepper plant under salt stress (SS) and alkali stress (AS).

ment with our findings. Application of SA improved wheat plants under water stress by activities of cell division in apical meristem (Shakirova *et al.*, 2003). Exogenous SA treatment also improved chlorophyll and photosynthesis rate and created stability of plant cell membrane, reduction of EL in barely plants and finally tolerance plants to water stress (El-Tayeb, 2005). The ability of SA to increase yield and growth parameters, ameliorating the adverse effects of stress, may have important implications in improving the plant growth and overcoming the growth barrier arising from SS and AS conditions.

Chlorophyll content

Some of the environmental stresses symptoms in the plants are reduction of chlorophyll content and this reduction depends upon the plant species (Colom and Vazzana, 2001). In this experiment, the reduction of chlorophyll was occurred due to SS and AS and 100 mM SS+AS treatment resulted in having the lowest rate of total chlorophyll (Fig. 3). In this respect, there are same reports over reducing chlorophyll due to exposing plants to SS and AS (Chen *et al.*, 2011; Gao *et al.*, 2012). Environmental stresses leads to increase the ROS production in cells. These free radicals cause peroxidation and consequently destructing the photosynthesis pigments (Schütz and Fangmeier, 2001). Because of these events, the growth of plant will be affected and declined.

Treated plants with SA as foliar spray increased chlorophyll content in all concentrations, but 0.75 mM SA treated plants showed significant increases in chlorophyll compared with control and 1.5 mM SA (Fig. 3). These results are in agreement with those of Sayyari (2012) who found that SA foliar and soil applications increased chlorophyll content in cucumber plants following chilling stress.

Salicylic acid by eliminating of ROS may improve chlorophyll content in pepper plants under stressful conditions. Chen *et al.* (1993) showed that in response to environmental stresses SA accumulates

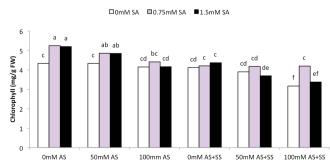


Fig. 3 - Effect of Salicylic acid (SA) on chlorophyll of pepper plant under salt stress (SS) and alkali stress (AS).

to high concentrations, and prevent antioxidant enzyme such as CAT activity, thereby leading to an enhancement in Hydrogen peroxide (H_2O_2) content, which could then induct the development of systemic acquired resistance (SAR), induce activity of ROSdetoxifying enzymes and antioxidant metabolites. Also, Idrees *et al.* (2011) reported that SA-induced salinity tolerance in periwinkle plants might be associated with an increase in the antioxidant activity. Therefore, impact of SA on plants chlorophyll under stresses condition may be related to its effect on the antioxidative enzyme activities and H_2O_2 metabolism (Idrees *et al.*, 2011).

Proline content

Proline is sensitive physiological index of plants responding to some stresses. Salinity and alkalinity treatments had significant effects on proline contents in pepper plant leaves. Proline accumulation in leaves was markedly increased in salt and alkali stressed plants in comparison with that of the control plants. The results showed that mixed SS-AS can cause heavy accumulation of proline content compared with SS and AS single stresses (Fig. 4). In general, the accumulation of proline, relates closely with osmotic stress intensity. Osmo-regulation is a physiological phenomenon during which osmosis potential of stressed tissues are reduced due to the accumulation of some material such as elements, sugar, amino acids (proline) and organic acids. Thus, turgor pressure of the cells is kept well (Irigoyen et al., 1992). Proline by osmosis control, maintaining enzymes

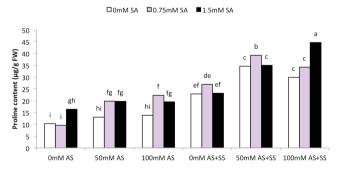


Fig. 4 - Effect of Salicylic acid (SA) on proline content of pepper plant under salt stress (SS) and alkali stress (AS).

activity and removal of hydroxyl radicals, increases the tolerance of the plants against stresses (Kuznetsov and Shevyakova 1999). The results showed that proline accumulation increased not only with increasing SS, but also when AS imposed (Fig. 4). This suggested that the proline accumulation, as the major osmolyte, correlates closely with the intensity of the osmotic stress induced by SS and AS.

Results showed that SA application increased proline content in pepper plants under SS and AS. A highest amount of proline (44.67 μ M/gFW) was achieved in 1.5 mM SA treatment and 100 mM AS+SS, and lowest amounts (9.54 µM/gFW) were observed in 0.75 mM SA and 0 mM AS (Fig. 4). These results are in agreement with those of El-Tayeb (2005) who showed that SA treatment increases the proline content in the leaves of barely plant subjected to salinity stress. In the present study, SA induced an accumulation of proline in the leaves under SS and AS, and when SA was applied, a stress tolerance occurred in the pepper plant. Thus, osmo-regulation can be considered to be one of the important phenomenon involved in SA induced protective mechanism in pepper leaves in response to salt and alkali stresses.

Relative water content

One of the indices showed the water status of plants is RWC. Measuring of relative water content is an important physiological parameter in evaluation of plant response to environmental stresses (Nautiyal et al., 2002). Results indicated that by increasing SS and AS, RWC in pepper leaves decreased slightly. Reduction under SS was greater than those under AS and greatest RWC reduction was achieved in highest mixed stress level (100 mM AS+SS) (Fig. 5). These results are in agreement with Yang et al. (2007) who found that salt-alkali stresses decreases the RWC in the leaves of Kochia sieversiana. Plants can reduce RWC as a quick and economical approach to osmotic adjustment in response to osmotic stress (Lissner et al., 1999). Reduction of RWC of the plants due to stress is related to the reduction of soil humidity; in these conditions, plants close the stomata to avoid more water waste. The reason of stomata closure is ABA that is made in the root in stress conditions and is accumulated in stomata cells (Chaves et al., 2002). Therefore, maintaining a high RWC might be a key

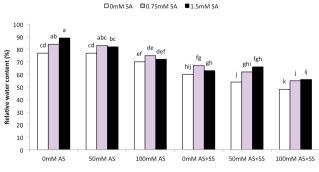


Fig. 5 - Effect of Salicylic acid (SA) on relative water content of pepper plant under salt stress (SS) and alkali stress (AS).

characteristic of plants that allow it to absorption osmolytes with minimum energy consumption.

Results showed that SA treatment increased leaf RWC in pepper plants under SS and AS. A highest amount of RWC (89%) was achieved in 1.5 mM SA treatment and 0 mM AS, and lowest (54%) were observed in 0 mM SA and 100 mM AS+SS (Fig. 5). Parida and Das (2005) reported that the RWC, water potential and osmotic potential of plants under stress become more negative with an increase in salinity. This study showed that SA treatments induced an increase in RWC of plants as compared to the un-treated SA plant. Increases in RWC of plants treated with SA were also reported for other crops grown under stress including tomato (Stevens et al., 2006) and barely (El-Tayeb, 2005). Increasing of RWC may be related to the role of SA in accumulation of compatible osmolytes in plants subjected to stress, as, this effect was observed in the results of proline.

Electrolyte leakage

In order to assess membrane permeability, electrolyte leakage (EL) was determined. Its relative conductivity can be used to evaluate the damage on structure and function of cell membranes under stresses. Results showed that EL significantly higher under SS than AS. On the other hand, both stresses increased the EL but the extent of the increment under SS was much greater than under AS and maximum of EL was achieved in highest mixed stress level (100 mM AS+SS) (Fig. 6). The results of the present study are in agreement with Gao et al. (2012) who determined that EL of oat (Avena sativa L.) was intensively increased by AS. Gao et al. (2012) showed that the EL of alfalfa seeds gradually increased with increasing salinity and alkalinity, this was attributed to the damage seeds cell membranes resulting from mixed salt-alkali stress. These results suggested cell membrane structure of pepper leaves under SS and AS received damage after treatment with salts.

Application of SA significantly decreased leaf EL in salt and salt × alkali stressed plants. Plants treated by SA foliar spray at 0.75 and 1.5 mM had shown significantly less EL than control plants (Fig. 6). These results indicated that, SA reversed the adverse effects of stress and caused a significant decrease in EL. The results of the present study are in agreement with Stevens *et al.* (2006) who showed that SA facilitated the maintenance of membrane functions in tomato under salinity stress. This effect could be attributed to the stimulation of antioxidant responses and elevated calcium absorption that protects the plant from the oxidative damage (El-Tayeb, 2005). Also, Jafari *et al.* (2015) showed that exogenous application of SA in cucumber (*Cucumis sativus* L.) subjected to osmotic stress lead to a decrease in EL and induced drought tolerance. These results suggested that pepper leaves cell membrane structure in during salt and alkali stresses received less damage after application of SA.

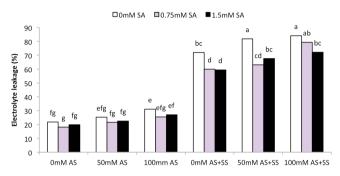


Fig. 6 - Effect of Salicylic acid (SA) on leaves electrolyte leakage of pepper plant under salt stress (SS) and alkali stress (AS).

4. Conclusions

In summary, our study clearly showed that saline and alkaline stresses as two types of abiotic stresses, have negative effects on pepper plant growth and productivity. On the other hand, the negative effect of mixed salt-alkali stress is more severe than that of only salt or alkali. The results of this research demonstrated that treating plants with 0 mM AS (control) is resulted in increasing plant growth parameters and RWC in comparison to other treatments. The harmful effect of saline stress on the pepper plants was significantly greater than that of alkaline; this harmful effect might have resulted from the higher concentration of salinity or higher sensitivity of pepper to SS compared to AS. SA pre-treatment of pepper seedlings via foliar spray was effective in saline and alkaline resistance. In most of evaluated treatments employed in this research, they did not show significant differences on SA concentrations. The best protection was obtained in plants treated with 0.75 mM SA, however 1.5 mM SA is more effective in 100 mM AS+SS in some trait for example proline content. This SA effect was associated with alter of physiological parameters such as increase of photosynthetic pigments and proline accumulation and decrease EL of plants by addition of SA. Further, the results indicate that SA can be considered as a potential growth regulator for improving plant growth and yield under SS

and AS, and it may be recommended in arid and semiarid regions.

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