

## Chilling Tolerance Improving of Watermelon Seedling by Salicylic Acid Seed and Foliar Application

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### Abstract

Chilling temperatures lead to numerous physiological disturbances in the cells of chilling-sensitive plants and result in chilling injury and death of tropical and subtropical plants such as watermelon. In this study, the possibility of cold stress tolerance enhancing of watermelon seedling (*Citrullus lanatus*) by exogenous application of Salicylic acid (SA) was investigated. SA was applied through seed soaking or foliar spray at 0, 0.5, 1 and 1.5 mM concentration. After SA treatment, the seedlings were subjected to chilling 5 h/day at 4°C for 5 days. Statistical analysis showed significant effects of the application methods and SA concentrations on plant growth parameters, photosynthetic pigments, electrolyte leakage, proline and chilling injury index. SA application improved growth parameters and increased chlorophyll content of watermelon seedling subjected to chilling stress and provided significant protection against chilling stress compared to non-SA-treated seedlings. Although two SA application methods improved chilling stress tolerance, seed soaking method provided better protection compared to foliar spray method. SA ameliorated the injury caused by chilling stress via inhibiting proline accumulation and leaf electrolyte leakage. The highest cold tolerance was obtained with 0.5 mM SA application. Results indicate that SA could be used effectively to protect watermelon seedling from damaging effects of chilling stress at the early stages of growth.

**Keyword:** chilling index, cold stress, electrolyte leakage, growth parameters, proline content

### Introduction

Plants are subjected to various abiotic stresses including water, high and low temperatures, salinity, and light stresses. Cold stress, defined as the temperature in a range low enough to suppress growth without ceasing cellular functions, is known to induce several abnormalities at various levels of cell organization (Balestrasse *et al.*, 2010). The optimum temperature for watermelon (*Citrullus lanatus*) growth ranges from 20 to 32°C (Bates and Robinson, 1995). Exposure of tropical and subtropical plants, such as cucurbits, to chilling temperatures may stunt the plant's growth, induce wilting and necrotic lesions on leaves, and increase susceptibility to diseases and pathogens (Hällgreen and Öquist, 1990). The symptoms of stress-induced injuries in these plants appear from 48 to 72 h, however, this duration varies from plant to plant and also depends upon the plant sensitivity to cold stress. Various phenotypic symptoms in response to chilling stress include reduced leaf expansion, wilting as well as chlorosis (yellowing of leaves) and may lead to necrosis (death of tissue). Chilling also severely hampers the reproductive development of some plants (Mahajan and Tuteja, 2005).

Although many parts of Iran are ideal for watermelons growing, but targeting early harvests requires field planting in early spring before temperatures reach optimum ranges (20-32°C). Once the seedlings have been planted

in the field, due to temperature fluctuations in some years, they may be exposed to temperatures cycling between chilling and optimal for some days before the temperature begins to stabilize. This condition may retard growth, delay flowering, reduce total yields and quality, and even kill the plants (Baninasab, 2009).

Breeding, genetic engineering and use of plant growth regulators (PGRs) are some approaches to increase plants tolerance to chilling stress. In recent studies a number of PGRs have been under trial to alleviate the cold stress in plants (Balestrasse *et al.*, 2010; Baninasab, 2009; Korkmaz *et al.*, 2010). Salicylic acid (SA) is a common plant-produced phenolic compound that can function as PGR. Various physiological and biochemical functions of SA in plants have been reported (Raskin, 1992). SA has received much attention due to its role in plants' responses to biotic and abiotic stresses. Literature exists about some beneficial effects of SA on plants under environmental stresses. Janda *et al.* (1999) found that the addition of 0.5 mM SA to the hydroponic growth solution of young maize plants under normal growth conditions provided protection against subsequent low-temperature stress. Previous study (Sayyari, 2012) showed that SA application enhanced chilling tolerance of cucumber seedling during low temperature stress. Pre-treatment of chilling-sensitive banana seedlings with 0.5 mM SA solution by spraying the foliage or irri-

gating the roots for 1 day, induced an increase in chilling tolerance during subsequent 5°C chilling stress (Kang *et al.*, 2003). In tomato and bean plants, 0.1 mM and 0.5 mM concentrations of both SA and acetyl salicylic acid (ASA) proved effective not only against heat and drought stress, but also against low temperature stress (Senaratna *et al.*, 2000). Thus the purpose of this experiment was to test the possibility that application of SA would protect watermelon plants at the early stages of growth from damaging effects of chilling stress. Specific objectives of this research were: (1) to compare seed and foliar application methods and (2) to determine the optimum SA concentration that would provide the best protection against chilling stress.

## Materials and methods

### *Plant material and growing conditions*

Seeds of watermelon (*Citrullus lanatus*) cultivar Charleston Grey, which is one of the most important cultivars grown in Iran, were disinfected in 1% (active ingredient) sodium hypochlorite solution for 10 min to eliminate possible seed-borne microorganisms, then they were rinsed for 1 min under running water prior to drying for 30 min at room temperature. After that, the seeds were soaked with SA at 0 (as control), 0.5, 1 and 1.5 mM for 24 h (Still and Pill, 2004) at room temperature (23±2°C). Seeds were washed and planted immediately and then were planted into 1.5 L plastic pots filled with a 1:1:1 mixture of fine sand, leaf mould 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. A second batch of seeds was also soaked in distilled water under the same conditions prior to sowing to obtain seedlings for foliar application of SA, and these seedlings were also raised in greenhouse under the same conditions. When the seedlings developed two true leaves (25 days after sowing), they were sprayed with 0 (as control), 0.5, 1 and 1.5 mM SA solution until both sides of the leaves were completely wet. Irrigation was done twice a week to keep the optimum moisture level in the growth medium. The layout was a 2 × 4 factorial experiment with SA application method and SA concentration as the main factors in a complete randomized block design (CRBD) with three replications and four plants per replication.

### *Chilling stress imposition*

One week after the foliar SA application or 5 weeks after the seed treatment, all seedlings (seed soaked + foliar spray) were exposed to chilling in a growth chamber at 4±0.5°C for 5 h and then returned to the greenhouse. Chilling period was repeated for 5 days. All plants were assessed 72 h after the end of chilling stress to determine the extent of chilling injury (Banasab, 2009), and data were collected.

### *Chilling injury index*

The degree of Chilling injury (CI) was visually assessed on the wilting, dehydration and necrosis of the leaves and shoots and classified by using the following scale: normal, no visible symptoms; trace, small necrotic areas on shoots but without growth restrictions (less than 5% of leaf area necrotic); slight, small necrotic areas on shoots (less than 15% of leaf area necrotic); moderate, well-defined necrotic areas on shoots (less than 30% of leaf area necrotic); and severe, extensive necrotic areas and severe growth restrictions (more than 50% of leaf area necrotic but plant still alive). By assigning values of 1, 2, 3, 4 and 5 respectively to each group, the average injury for each treatment was calculated (Wang, 1985).

### *Chlorophyll content determination*

Chlorophyll (Chl) content was determined by taking fresh leaf samples (0.1 g) 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 contents were calculated using the equations proposed by Strain and Svec (1966) given below:

$$\text{Ch.a mg/g F.W} = \{12.7(\text{A663}) - 2.69(\text{A645})\}$$

$$\text{Ch.b mg/g F.W} = \{22.9(\text{A645}) - 4.68(\text{A663})\}$$

$$\text{Ch.a+b mg/g F.W} = \{20.2(\text{A645}) + 8.02(\text{A663})\}$$

### *Electrolyte leakage*

Electrolyte leakage (EL) was used to assess membrane permeability. This procedure was based on Lutts *et al.* (1995) method. EL was measured using an electrical conductivity meter (Met Rohm, 664). Six leaf discs of randomly chosen plant per replicate were taken from the youngest fully-expanded leaf. The leaf samples were then placed in test tubes containing 10 mL of distilled water. After three washes with distilled water to remove surface contaminations, these samples were incubated at room temperature 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 boiling water bath 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*

Proline content was determined according to the method described by Bates *et al.* (1973). Fresh leaf material (0.5 g) was homogenized in 10 ml of 3% aqueous sulfo salicylic acid and the homogenate was centrifuged at 10,000 rpm. Two milliliter 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 for

1 h at 100°C. The reaction mixture was extracted with 4 ml toluene and the chromophore containing toluene was aspirated and cooled to room temperature, then the absorbance was measured at 520 nm with a UV/visible spectrophotometer. Appropriate proline standards were included for the calculation of proline in the samples.

#### Shoot and root characters

After the chilling injury determination, shoots of seedlings were cut at the ground surface and their fresh weights were recorded. The roots of the seedling were carefully washed under running tap water to remove the growth medium and dried with paper towels to remove the surface water and their fresh weights were recorded. The shoots and roots were dried at 80°C for 72 h and their dry weights were determined.

#### Statistical analysis

Data from the analytical determinations were subjected to analysis of variance (ANOVA). Sources of variation were SA application methods and SA concentrations. Mean comparisons were performed using Duncan's test ( $p < 0.05$ ). All analyses were performed with SAS and MC-TATC software programs.

### Results and discussion

The data statistical analysis showed that application methods had significant effects on the growth parameters including shoot fresh weight (SFW), shoot dry weight (SDW), root fresh weight (RFW) and root dry weight (RDW) of watermelon seedling subjected to chilling stress (Tab. 1). Seedlings soaked by SA showed significantly more SFW, SDW, RFW and RDW in comparison

Tab. 1. Effect of SA and its application method on shoot fresh weight (SFW), shoot dry weight (SDW), root fresh weight (RFW) and root dry weight (RDW) of watermelon seedling subjected to chilling stress

Treatments	SFW (g)	SDW (g)	RFW (g)	RDW (g)
Application method				
Seed soak	3.23 <sup>a</sup>	0.559 <sup>a</sup>	0.568 <sup>a</sup>	0.086 <sup>a</sup>
Foliar spray	3.00 <sup>b</sup>	0.530 <sup>b</sup>	0.465 <sup>b</sup>	0.074 <sup>b</sup>
SA Concentration (mM)				
0	2.99 <sup>b</sup>	0.527 <sup>bc</sup>	0.505 <sup>ab</sup>	0.078 <sup>ab</sup>
0.5	3.32 <sup>a</sup>	0.580 <sup>a</sup>	0.478 <sup>b</sup>	0.084 <sup>a</sup>
1	3.24 <sup>a</sup>	0.559 <sup>ab</sup>	0.545 <sup>a</sup>	0.085 <sup>a</sup>
1.5	2.92 <sup>b</sup>	0.511 <sup>c</sup>	0.538 <sup>ab</sup>	0.073 <sup>b</sup>
ANOVA				
App. method (M)	**	*	**	**
SA Con. (C)	**	**	Ns	*
M×C	**	*	**	**

Ns, \*\* and \*, not significant, significant at  $p \leq 0.01$  and  $0.05$ , respectively. Values followed by the same letter within the column are not significantly different at  $p < 0.05$

with foliar-sprayed seedling. SA concentration affected shoot and root characters except RFW and SA treatment significantly increased all growth parameters when compared with untreated seedlings. All concentrations of SA (Except 1.5 mM) significantly increased shoot and root characters. This data showed that SA application in low concentration (especially in 0.5 mM) could effectively improve the growth of watermelon seedling under chilling stress condition. There is a significant interaction between application method and SA concentration in SFW, SDW, RFW and RDW (Tab. 2). Application of 0.5 mM SA in seed-soaked seedling increased about 10% and 13% SFW and SDW respectively compared with untreated seedling. Besides, all treatments with foliar spray application method increased SFW about 12% compared with controls (Fig. 1). Seedling treatment with 1 mM SA as seed soaking increased RFW and RDW about 20% and 28% when compared with controls, respectively. On the other hand, foliar spray of SA in all concentration had no significant effect on these characters (Fig. 2). These results are in agreement with previous study (Sayyari, 2012). In cucumber seedling, SA application as seed soaking improved the growth of cucumber seedling subjected to chilling stress. In SA treated plants cold stress tolerance increased. Imami *et al.* (2011) also reported similar results in growth of chickpea plants in response to salicylic acid treatment. The ability of SA to increase plant fresh and dry mass, ameliorating the adverse effects of chilling stress, may have significant implications in improving the plant growth and overcoming the growth barrier arising from chilling conditions.

Analysis of variance and mean compositions results of photosynthetic pigments including chlorophyll a (Chl a), Chlorophyll b (Chl b) and Chlorophyll a+b (Chl a+b) are shown in Tab. 2. Application method affected Chl a, Chl b and Chl a+b and SA concentration affected Chl

Tab. 2. Effect of SA and its application method on Chlorophyll a (Chl a), Chlorophyll b (Chl b) and Chlorophyll a + b (Chl a+b) of watermelon seedling subjected to chilling stress

Treatments	Chl a (mgg <sup>-1</sup> F.W)	Chl b (mgg <sup>-1</sup> F.W)	Chl a+b (mgg <sup>-1</sup> F.W)
Application method			
Seed soak	4.52 <sup>b</sup>	1.98 <sup>b</sup>	4.70 <sup>b</sup>
Foliar spray	5.43 <sup>a</sup>	2.35 <sup>a</sup>	5.61 <sup>a</sup>
SA Concentration (mM)			
0	4.71 <sup>b</sup>	1.98 <sup>b</sup>	4.80 <sup>b</sup>
0.5	5.10 <sup>a</sup>	2.22 <sup>a</sup>	5.28 <sup>a</sup>
1	5.03 <sup>ab</sup>	2.30 <sup>a</sup>	5.36 <sup>a</sup>
1.5	5.07 <sup>a</sup>	2.15 <sup>ab</sup>	5.18 <sup>a</sup>
ANOVA			
App. method (M)	**	**	**
SA Con. (C)	Ns	*	**
M×C	*	*	**

Ns, \*\* and \*, not significant, significant at  $p \leq 0.01$  and  $0.05$ , respectively. Values followed by the same letter within the column are not significantly different at  $p < 0.05$

b and Chl a+b, significantly (Tab. 2). Treated seedlings with foliar spray method had shown higher Chl a, Chl b and Chl a+b than seed soaked seedlings. All SA concentrations significantly increased photosynthetic pigments in comparison with controls, however 0.5 and 1 mM SA were more effective. There was a significant interaction between application method and SA concentration for Chl a, Chl b and Chl a+b (Tab. 2). Treated plants with SA as foliar spray increased photosynthetic pigments in all concentrations, but seed soaked plants showed significant increases in Chl b and Chl a+b compared with control (Fig. 3). These results are in agreement with those of Imami *et al.* (2011) who found that SA foliar and soil applications increased chlorophyll content in chickpea plants following chilling stress. Environmental stresses mainly reduces chlorophyll content and this reduction depend upon the plant's genotype (Colom and Vazzana, 2001). Based on the theory of Schutz and Fangmir (2001), the reduction of chlorophyll due to stress is related to high production of Reactive oxygen species (ROS) in the cell. These free radicals cause peroxidation, disintegration and reduction of chlorophyll content in plant under stressful conditions. SA with scavenging of ROS may increase Chl content in watermelon seedling in this experiment. As reported by Chen *et al.* (1993), in response to biotic stress SA accumulates to high level, binds and inhibits catalase (CAT) activity, thereby leading to an increase in H<sub>2</sub>O<sub>2</sub> content, which could then initiate the development of systemic acquired resistance, induce activity of ROS-detoxifying enzymes, and synthesis of antioxidant metabolites. Kang and Saltveit (2002) reported that SA-induced chilling tolerance in maize and cucumber plants might be associated with an increase in the activity of glutathione reductase and peroxidase. Therefore, impact of salicylic acid on chlorophyll may be related to its influence on the antioxidative enzyme activities and hydrogen peroxide metabolism.

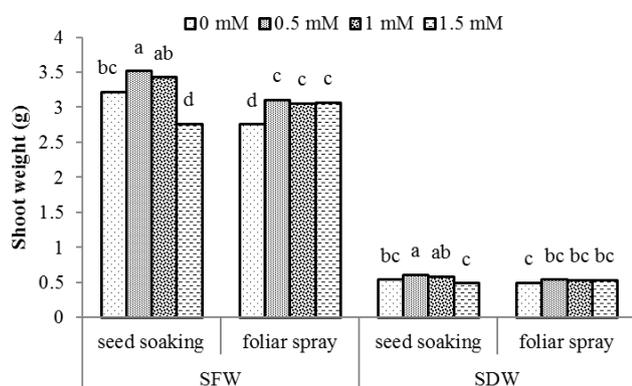


Fig. 1. Effect of SA and its application method on shoot fresh weight (SFW) and shoot dry weight (SDW) of watermelon seedling subjected to chilling stress. Values followed by different letters were significant difference according to Duncan's Multiple Range Test at  $p < 0.05$

Tab. 3. Effect of SA and its application method on chilling index (CI), electrolyte leakage (EL) and proline content of watermelon seedling subjected to chilling stress

Treatments	CI (Scores)	Electrolyte leakage (%)	Proline ( $\mu\text{mg}^{-1}\text{FW}$ )
Application method			
Seed soak	2.58 <sup>a</sup>	57.64 <sup>a</sup>	3.60 <sup>b</sup>
Foliar spray	2.79 <sup>a</sup>	54.47 <sup>a</sup>	2.13 <sup>a</sup>
SA Concentration. (mM)			
0	3.54 <sup>a</sup>	61.75 <sup>a</sup>	4.24 <sup>a</sup>
0.5	2.25 <sup>b</sup>	47.40 <sup>c</sup>	3.97 <sup>a</sup>
1	2.33 <sup>b</sup>	60.49 <sup>ab</sup>	3.24 <sup>b</sup>
1.5	2.62 <sup>b</sup>	54.58 <sup>b</sup>	3.57 <sup>b</sup>
ANOVA			
App. method (M)	Ns	Ns	*
SA Con. (C)	**	**	**
M×C	Ns	*	*

Ns, \*\* and \*, not significant, significant at  $p \leq 0.01$  and  $0.05$ , respectively. Values followed by the same letter within the column are not significantly different at  $p < 0.05$

The results showed that chilling injury (CI) was not affected by application method, but SA concentration significantly affected CI in 0.01 probability level (Tab. 3). After exposing watermelon seedlings to chilling, control plants exhibited typical chilling injury symptoms, while SA-treated seedlings were slightly damaged. All SA concentrations significantly decreased CI, the most effective concentration was 0.5 mM and seedlings treated with 0.5 mM SA exhibited the least CI symptoms. There was no interaction between application method and SA concentration. In previous study (Sayyari, 2012), application of 0.5 mM SA as foliar spray and 1 mM and 1.5 mM as seed soaking method decreased CI index in chilled cucumber seed-

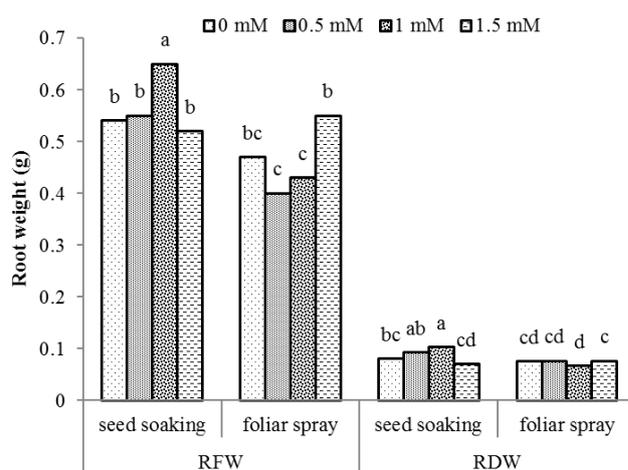


Fig. 2. Effect of SA and its application method on root fresh weight (RFW) and root dry weight (RDW) of watermelon seedling subjected to chilling stress. Values followed by different letters were significant difference according to Duncan's Multiple Range Test at  $p < 0.05$

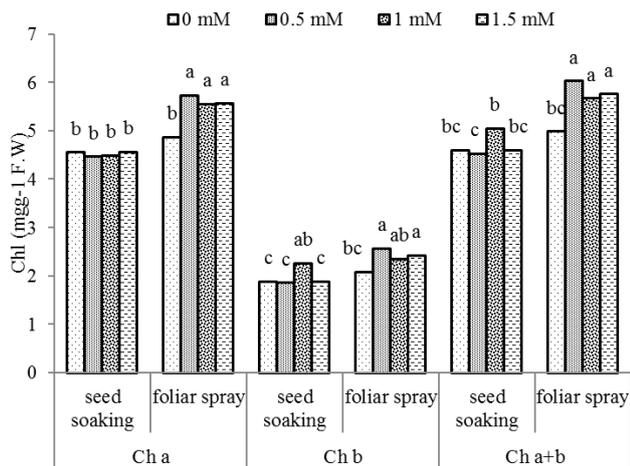


Fig. 3. Effect of SA and its application method on Chlorophyll a (Chl a), Chlorophyll b (Chl b) and Chlorophyll a + b (Chl a+b) of watermelon seedling subjected to chilling stress. Values followed by different letters were significant difference according to Duncan's Multiple Range Test at  $p < 0.05$

ling. These results correlate well with the findings of Lee and Hong (2002) that showed cucumber seedling treatment with SA in 0.5 mM concentration improved growth and survival of the non-acclimated chilled seedlings. Their results indicated that SA induced chilling tolerance and had common effects with acclimation. Similarly, Kang *et al.* (2003) reported that pre-treatment of chilling-sensitive banana seedlings with 0.5 mM SA solution by foliar spray or irrigating the roots for 1 day, induced an increase in chilling tolerance during subsequent 5°C chilling stress. In order to assess membrane permeability, electrolyte leakage (EL) was determined. Application method did not influence leaf EL, whereas SA concentration affected significantly this character (Tab. 3). Application of SA significantly decreased leaf EL; the least EL was obtained

from application of SA at 0.5 mM. There was interaction between application method and SA concentration in this trait. Seedling treated by seed soak or foliar spray methods at 0.5 mM SA had shown significantly less leaf EL than other treatment (Fig. 4). EL reflects the changes of cell membrane structure of plants under stress. Its relative conductivity can be used to evaluate the damage on structure and function of cell membranes under stresses. SA reversed the adverse effects of stress and caused a significant decrease in electrolyte leakage. The results of the present study are in agreement with Stevens *et al.* (2006) who determined that SA facilitated the maintenance of membrane functions in tomato. This facilitation could be attributed to the induction of antioxidant responses and elevated calcium uptake that protects the plant from the oxidative damage by SA (El-Tayeb, 2005). Also, Wang and Li (2006) showed that exogenous application of SA in grape plant exposed to chilling stress lead to a decrease in EL and induced cold tolerance. These results suggested that cell membrane structure of watermelon leaves during chilling stress received less damage after SA pre-treatment.

The proline content was significantly affected by application method, SA concentration and their interaction (Tab. 3). Plants treated by seed soak method showed higher proline accumulation (3.60  $\mu\text{mg}^{-1}$  FW) compared with plant treated by foliar spray method (2.13  $\mu\text{mg}^{-1}$  FW). The SA treatment reduced proline content in watermelon leaves, the least proline accumulation was observed in seedlings treated with 1 mM SA (3.24  $\mu\text{mg}^{-1}$  FW), which was 23.5% less than the control seedlings. SA application with two methods significantly decreased proline content of watermelon seedlings subjected to chilling stress; the least amount of proline obtained in SA-treated seedling with 0.5 mM by seed soak method (Fig. 5). Likewise, Shahba *et al.* (2010) showed that SA application in high levels of salinity (75 and 100 mM NaCl) decreased proline accu-

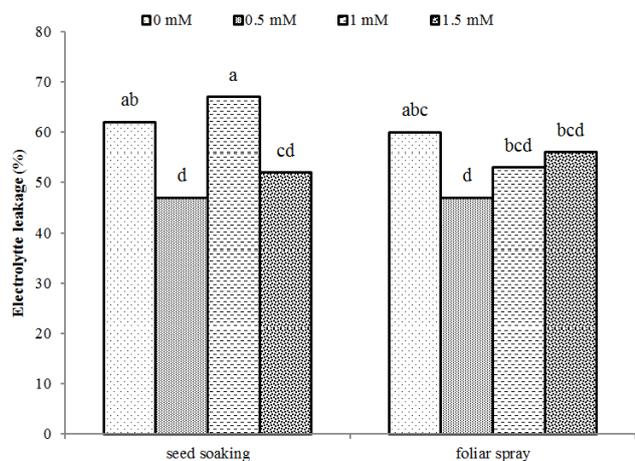


Fig. 4. Effect of SA and its application method on electrolyte leakage of watermelon seedling subjected to chilling stress. Values followed by different letters were significant difference according to Duncan's Multiple Range Test at  $p < 0.05$

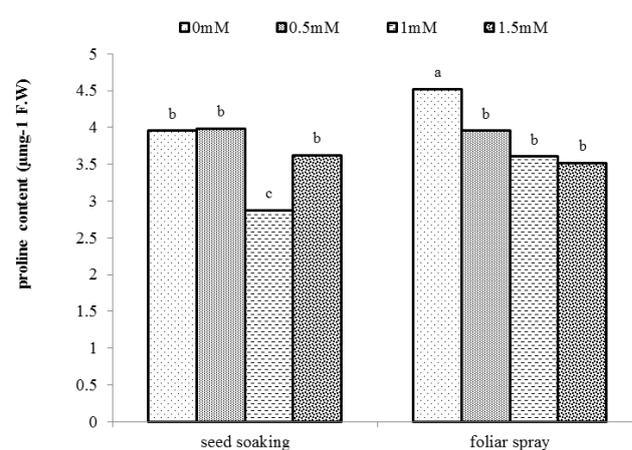


Fig. 5. Effect of SA and its application method on proline content of watermelon seedling subjected to chilling stress. Values followed by different letters were significant difference according to Duncan's Multiple Range Test at  $p < 0.05$

mulation in shoot and root of tomato plants. Also, Idrees *et al.* (2010) showed that SA treatment decreased proline content of lemongrass varieties under drought stress condition. The plants during the encounter with stressful conditions have various physiological mechanisms. As one of the most efficient mechanisms of the plant is osmotic adjustment. Osmotic adjustment is a physiological phenomenon during which osmotic potential of stressed tissues are reduced due to the accumulation of osmosis substances including mineral elements (e.g. potassium, sodium and calcium) and some of the metabolites as sugar, amino acids (proline) and organic acids. Thus, turgor pressure of the cells is kept well (Irigoyen *et al.*, 1992). These metabolites are not in contradiction to normal biochemical reactions of the cells and are called compatible solutes (Bohnert *et al.*, 1995). Increase of proline content helps osmotic control and is reported due to some factors including the prevention of proline disintegration, avoiding proline participate in protein structure or increase of protein disintegration (Kao, 2005). Proline via osmotic control, avoiding enzymes destruction and removal of hydroxyl radicals, increased the tolerance of the plants against stresses (Kuznetsov and Shevykova, 1999). According to these results, it can be inferred that reduction in proline content could be important for stress alleviation in watermelon seedlings and the variation in seedlings response to chilling stress may partly be dependent on the plant species and cultivars.

### Conclusions

The result of the present study revealed that SA pretreatment of watermelon seedlings via seed soaking and foliar spray was effective in chilling resistance at early stage of growth; the best protection was obtained in plants pretreated with 0.5 mM SA. SA application by seed soak method was more effective than foliar spray in providing chilling tolerance. However, since seed soak method is simpler and more convenient, it would be a more desirable method for SA application. The finding of this study showed that SA could be used as a seed treatment to prevent chilling damages in watermelon seedling may have significant practical applications.

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