

Improving Chilling Resistance of Cucumber Seedlings by Salicylic Acid

Mohammad Sayyari

Department of Horticulture, College of Agriculture, University of Ilam, Ilam 69315-516, Iran

Abstract: Salicylic acid (SA) has been reported to induce a number of defense responses and increase chilling tolerance in plants. Therefore, an experiment was conducted to test whether SA application at various concentrations (0, 0.5, 1 and 1.5 mM) through seed soaking or foliar spray would protect cucumber (*Cucumis sativus*) seedlings subjected to chilling stress. Thirty five-day old plants were exposed to chilling 5 h/day at 4°C for 5 days. SA improved growth rate of cucumber seedling subjected to chilling stress and increased proline content compared with the control at the end of chilling stress. SA ameliorated the injury caused by chilling stress via inhibiting increases in lipid peroxidation and leaf electrolyte leakage, which suggested that SA ameliorated the negative effect of chilling stress. SA was most effective in increased chilling tolerance of cucumber seedling when applied using the seed soak method than as a foliar spray. The best protection appeared to be obtained from seedlings that seed soaked with SA at 0.5 and 1mM.

Key words: Chilling stress • Lipid peroxidation • Electrolyte leakage • *Cucumis sativus*

INTRODUCTION

Temperature is one of the major determinants of the occurrence and spread of natural plant associations. Tropical and sub-tropical species show characteristic damage symptoms when subjected to low, above zero temperatures [1]. Plants native to warm regions such as maize, tomato, cucumber and watermelon are generally injured at temperatures below 10°C [2]. Apart from genetic factors, cold sensitivity depends on the stage of development and the level of metabolic activity and they are more sensitive to cold in the early phases of development. The symptoms of chilling injury include stunted growth, reduced photosynthetic capacity, necrosis and discoloration, wilting, acceleration of senescence and death. In addition, the effects of chilling on plant metabolism are numerous and have been reported in relation to respiration, photosynthesis, phenolic and sugar metabolism and redox regulation [3].

Many parts in Iran are ideal for growing of cucumber, but targeting early harvests required field planting in early spring before temperatures stabilization. Due to temperature fluctuations the seedlings may be exposed to temperatures cycling between chilling and optimal for some days that may retard growth, delays flowering, reduce total yields and quality, and even kills the plants [4].

Many approaches have been developed to reduce chilling injury in growing plants such as genetic engineering, modifying crop management practices and application of chemicals [5-7]. Salicylic acid (SA), an ubiquitous plant phenolic, has been reported to induce a number of defense responses and increase chilling tolerance in plants. It was shown 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 [8, 9]. Treating mustard seedlings with exogenous SA was found to improve their thermotolerance and heat acclimation [5]. Pre-treatment of the leaves of chilling-sensitive banana seedlings with 0.5 mM SA solution by spraying the foliage or irrigating the roots for 1 day, induced an increase in chilling tolerance during subsequent 5°C chilling stress [10]. Pre-soaking seeds before sowing could also be an effective way of improving cold tolerance. In tomato and bean plants, 0.1 mM and 0.5 mM concentrations of both SA and acetyl SA proved effective not only against heat and drought stress, but also against low temperature stress [11]. Thus the purpose of this experiment was to test the possibility that application of SA would protect cucumber plants at early stage of growth from damaging effects of chilling stress.

MATERIALS AND METHODS

Plant Material and Cultural Practice: Cucumber seeds (*Cucumis sativus*) cultivar Super Dominus, were used in this research. Seeds were soaked with SA at 0 (control), 0.5, 1 and 1.5mM for 24 h [7, 12] at room temperature (23-28°C) after which the seeds were washed and planted immediately. Seeds were planted into 1.5 L plastic pots filled with a 1:1:1 mixture of fine sand, leaf mould and soil. The pots were then transferred to the greenhouse with average temperature of 25/20°C (day/night) and natural light (April and May, 2010). 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 had two true leaves (25 days after sowing), the seedlings were sprayed with 0 (control), 0.5, 1 and 1.5mM SA solution until both sides of the leaves were completely wet. Irrigation was done two times in a week to keep the optimum moisture level in the growth medium.

The layout was a factorial experiment (2×4) with SA application methods and SA concentrations as main factors in a complete randomized design with four replications and three plants per replication.

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. Stress imposition was repeated for 5 days. All plants were assessed 72 h after the end of chilling stress to determine the extent of chilling injury [4] and data were collected.

Chilling Injury Rating Values: 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 [13].

Electrolyte Leakage: Electrolyte leakage was used to assess membrane permeability. This procedure was based on Lutts *et al.* [14]. Electrolyte leakage 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. Leaf samples were then placed in test tubes containing 10 mL of distilled water after three washes with distilled water to remove surface contamination. 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 in a boiling water bath for 20 min and the second reading (EC2) was determined after cooling of the solution to room temperature. The electrolyte leakage was calculated as EC1/EC2 and expressed as percent.

Proline Determination: Proline content in leaves was measured by rapid colorimetric method as suggested by Bates *et al.* [15]. Proline was extracted from 0.5 g of leaf samples by grinding in 10 ml of 3% sulphosalicylic acid and the mixture was then centrifuged at 10,000×g for 10 min. 2 ml of the supernatant was added into test tubes to which 2 ml of freshly prepared acid-ninhydrin solution and 2 ml of glacial acetic acid was added. Tubes were incubated in a water bath at 90°C for 1h and then the reaction was terminated in ice-bath. The reaction mixture was extracted with 4 ml of toluene and vortexed for 15 s. The tubes were allowed to stand at least for 20 min in darkness at room temperature for separation of toluene and aqueous phase. The absorbance of chromophore containing toluene was measured at 520 nm with a Shimadzu UV 160A spectrometer (Shimadzu Corporation, Kyoto, Japan). Proline concentration in the sample was determined from a standard curve using analytical grade proline and calculated (on fresh weight basis) as follows:

$$\frac{((\mu\text{g proline/ml} \times \text{ml Toluene}) / (115.5_{\mu\text{g}/\mu\text{mole}}) / ((\text{g sample}) / 5))}{\text{g}} = \mu\text{mole proline} \square \text{resh weight}$$

Malondialdehyde (MDA) Content: The lipid peroxidation was measured in terms of malondialdehyde content, the product of lipid peroxidation [16]. The leaf samples (1 g) were homogenized in 10 ml of 0.1% trichloroacetic acid and homogenate was centrifuged at 15,000×g for 5 min. Four milliliters of 0.5% thiobarbituric acid in 20% trichloroacetic acid was added to a 1ml aliquot of the supernatant. Thereafter, the mixture was heated at 95°C

for 30 min and then quickly cooled in an ice bath. After centrifugation at 10,000×g for 10 min, the absorbance was recorded at 532 nm. The value for nonspecific absorption at 600 nm was subtracted. The content was calculated using its absorption coefficient of 155 mmol⁻¹ cm⁻¹ and expressed as micromole per gram fresh weight.

Shoot and Root Characters: After the determination of chilling injury, shoots of the 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 software package.

RESULTS AND DISCUSSION

The results showed that all measured parameters were affected by SA concentration, but among them, shoot fresh weight and chilling index were not affected by application method. There was interaction between application method and SA concentration in stem diameter, shoot fresh weight, root fresh and dry weight and electrolyte leakage. A summary of the statistical results is shown in Table 1.

Table 1: ANOVA for dependent variables for application methods (A), SA concentrations (C) and their interactions (A×C) in cucumber seedling ^a.

	A	C	A×C
Electrolyte leakage	*	**	**
Chilling index	ns	*	ns
Proline content	*	**	NS
Malondealdehyde content	**	**	*
Stem diameter	**	*	*
Shoot fresh weight	**	ns	*
Root fresh weight	**	**	*
Root dry weight	**	**	**

^a ** and * represent significance at the 0.01 and 0.05 levels, respectively and NS represents non-significance at *P* < 0.05.

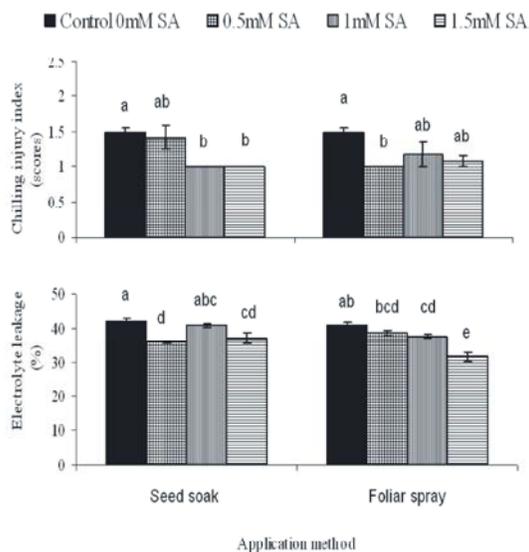


Fig. 1: Effect of SA treatment and application method on the Electrolyte leakage (EL) and chilling index (CI) in cucumber seedlings subjected to chilling stress. The data are displayed with mean ± standard error (n=4). Values with same letters are not significantly different at *P* < 0.05

SA application decreased chilling index and electrolyte leakage of cucumber seedlings. After exposure to chilling, the seedlings not applied with SA (0 mM) exhibited typical chilling injury symptoms in moderate to severe level, while SA treated seedlings were slightly damaged. 1 and 1.5 mM of SA with seed soaking method and 0.5 mM with foliar spray method caused significant reduction of seedling chilling index (Fig. 1). However, the least electrolyte leakage was obtained from the application of SA at 1.5 mM in foliar application method. Lee and Hong [5] showed that exogenous treatment of cucumber with SA (0.5 mM) resulted in improved growth and survival of the non-acclimated chilled seedlings, indicating that SA induced chilling tolerance and also, SA and acclimation had common effects. These results correlate well with the findings of Kang *et al.* [10] who reported that pre-treatment of the leaves of chilling-sensitive banana seedlings with 0.5 mM SA solution by spraying the foliage or irrigating the roots for 1 day, induced an increase in chilling tolerance during subsequent 5°C chilling stress.

Application of SA significantly decreased leaf electrolyte leakage, with the largest decrease in leaf electrolyte leakage by using 1.5 mM SA (Fig. 1). Seedlings treated by the seed soak method had shown significantly less leaf electrolyte leakage than with foliar

spray method. This result is in agreement with those reported by Kang and Saltveit [7] who found that the chilling tolerance of leaves or hypocotyls significantly increased by the application of 0.5 mM SA not only in maize, but also in cucumber and rice. Similar to our results Wang and Li [17] showed that SA treatment of grape plants exposed to low temperature stress led to a decrease in the rates of lipid peroxidation and electrolyte leakage and induced cold tolerance. Chilling tolerance was manifested as a reduction in the chilling-induced electrolyte leakage from excised maize and rice leaf discs and from excised cucumber hypocotyls segments in plants pre-treated with SA. An increase in ion leakage indicates leakiness of ions due to a loss of membrane integrity. This is an inherent feature of plants which are exposed to stresses such as low temperature [18].

Proline content was affected by increasing SA concentration in two application methods.

Plants treated by the foliar spray method had shown greater proline compared with seed soak method. However, 1.5 mM SA was more effective (Figure 2). Similarly, Lee and Hong [5] showed that exogenous treatment of cucumber seedling with 0.5 mM SA resulted in improved growth and survival of the non-acclimated chilled seedling. Their results indicated that SA induced chilling tolerance and had common effects with acclimation. In addition, elevated proline level observed in cold-treated and SA-treated seedlings in the mentioned experiment was more pronounced, indicating that endogenous proline may play a role in chilling tolerance by stabilizing the water status in response to chilling.

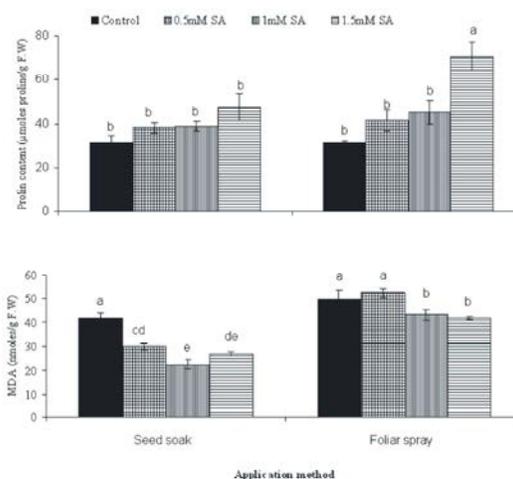


Fig. 2: Effect of SA treatment and application method on the malonaldehyde (MDA) and Proline content in cucumber seedlings subjected to chilling stress. The data are displayed with mean \pm standard error (n=4). Values with same letters are not significantly different at $P < 0.05$.

From our results and other reports it is suggested that SA can protect plants against low temperature stress by increasing the proline accumulation. Pre-treatment with SA may induce antioxidant enzymes and detoxify ROS in stress condition leading to increased chilling tolerance. Hence more studies suggest proline role in turgor generation and in stabilisation of membranes and proteins as antioxidant and buffering cellular redox potential during stress [19].

Table 2: Effect of SA and its application method on stem diameter (SD), shoot fresh weight (SFW), root fresh weight (RFW) and root dry weight (RDW) of cucumber seedlings subjected to chilling stress

Application method	Seed soak	SA (mM)	SD (mm)	SFW (g)	RFW (g)	RDW (g)
Seed soak		0	5.11c	9.49bc	5.49d	0.25c
		0.5	5.22bc	8.94c	8.22bc	0.76b
		1	5.75b	10.47bc	9.05b	0.78b
		1.5	6.33a	14.50a	11.61a	1.23a
Foliar spray		0	4.94c	10.52bc	6.17cd	0.48bc
		0.5	5.20bc	11.14b	6.77bcd	0.52bc
		1	5.04c	10.44bc	7.55bcd	0.34c
		1.5	5.10c	11.37b	7.157bcd	0.32c
Means for application method Seed soak			5.6a	10.84a	8.59a	0.76a
Foliar spray			5.06b	10.86a	6.91b	0.41b
Means for SA concentration		0	5.02b	10b	5.83c	0.37c
		0.5	5.20b	10.03b	7.49b	0.64ab
		1	5.39ab	10.45b	8.30ab	0.56bc
		1.5	5.71a	12.93a	9.38a	0.78a

For each parameter, values with same letters in the same column are not significantly different at $P < 0.05$.

MDA was affected by both application method and SA concentration (Table 1 and Fig. 2). Plants treated by the seed soak method had shown lower MDA compared with foliar spray method (Fig. 2). All concentrations of SA significantly decreased MDA compared with the control. However, 1mM SA was more effective. There was a significant interaction between the application method and SA concentration; there was a decrease in the MDA when using 1mM SA by the seed soaks method (Fig. 2). Chilling imposes severe oxidative stress and reactive oxygen species (ROS) formation which may be responsible for chilling-associated damages. Under chilling condition over-production of ROS, including H_2O_2 , induces and their accumulation causes oxidative damage in plants because they oxidase organic compound and induce membrane lipid peroxidation in the cellular environment. Polyunsaturated fatty acids within the lipids are a preferred target of ROS attack. The toxicity of ROS has often been monitored by measuring lipid peroxidation. MDA is one of the final products of stress-induced lipid peroxidation of polyunsaturated fatty acids and has been considered as a marker for cold sensitivity [20]. SA application with scavenging of ROS may decrease MDA content and chilling injury in cucumber seedling in this experiment. As reported by Chen *et al.* [21], in response to biotic stress SA accumulates to high level, binds and inhibits catalase (CAT) activity, thereby leading to an increase in H_2O_2 content, which could then initiate the development of systemic acquired resistance, induce activity of ROS-detoxifying enzymes and synthesis of antioxidant metabolites. Treatment of bean and tomato plants with SA or aspirin increased their tolerance against heat, chilling and drought stress [11]. Kang and Saltveit [7] 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. Results of these works suggest that the role of SA in chilling tolerance is related with its influence on the antioxidative enzyme activities and hydrogen peroxide metabolism.

Application method affected shoot and root characters except SFW, with increasing SA concentration all these parameters increased. Plants treated by the seed soak method had shown significantly greater SD, RFW and RDW compared with foliar spray method. All concentrations of SA significantly increased shoot and root characters compared with the control (Table 2). There was a significant interaction between the application method and SA concentration; there was an

increase in the shoot and root characters when increasing SA concentration by the seed soak method. Seed soaked plants with high concentration of SA (1.5 mM) had greater growth rate (SD = 6.33mm, SFW = 14.5g, RFW = 11.61g and RDW = 1.13g) (Tables 2).

CONCLUSION

From the results of this investigation, it can be concluded that the application of SA would protect cucumber seedlings partially against chilling stress. SA applied by seed soaking was most 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 fact that SA, readily available, could be used to prevent crop losses due to chilling stress may have a significant practical application.

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