

Induction of Drought Tolerance in Sweet Basil (*Ocimum basilicum* L) by Salicylic Acid

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Abstract

Drought stress is a major constraint for crop production in arid and semiarid regions, such as Iran. In this study, the possibility of enhancing drought stress tolerance of sweet basil plant (*Ocimum basilicum* L.) by exogenous application of Salicylic acid (SA) was investigated. The layout was factorial experiment in randomized complete block design (RCBD) with 3 levels of drought stress including stress-free conditions, mild stress and severe stress and 3 concentrations of SA (including 0, 0.75 and 1.5 mM) as main factors and 3 replicate. The statistical analysis showed that drought stress and application of SA had significant effects on the morphological, physiological and biochemical parameters of the plant such as plant height, fresh and dry weight, relative water content (RWC), chlorophyll (Chl), proline and electrolyte leakage (EL). Result showed that drought stress imposed negative effects on plant growth and productivity. In drought conditions, growth parameters, Chl content, RWC reduced, but proline content and EL increased. SA application significantly caused increasing of plant growth characters, photosynthetic pigments, RWC and proline and decreased EL. In this experiment, SA application with increasing growth rate and changing plant physiological process decreased adverse effects of drought stress on sweet basil plants.

Keywords: Chlorophyll, Electrolyte leakage, Growth, Proline, Relative water content

1. Introduction

Abiotic stresses, such as drought, salinity, extreme temperatures, chemical toxicity and oxidative stress are serious threats to agriculture and result in the deterioration of the environment. Abiotic stress is the primary cause of crop loss worldwide, reducing average yields for most major crop plants by more than 50% (Boyer, 1982; Bray *et al.*, 2000). In many regions of the world, including Iran, drought stress is one of the most important factors that decrease agricultural crop production (Zahedi *et al.*, 2009). Drought stress causes an increase in solute concentration in environment, leading to an osmotic flow of water out of plant cells. This in turn causes the solute concentration

inside plant cells to increase, thus lowering water potential and disrupting membranes along with essential processes like photosynthesis. These drought-stressed plants consequently exhibit poor growth and yield (Taheri-Asghari *et al.*, 2009). Breeding, genetic engineering and use of plant growth regulators (PGRs) are some approaches to increase plants tolerance to stresses. In recent studies a number of plant growth regulators have been under trial to alleviate the drought stress in plants, such as Mepiquat Chloride (Ahmed *et al.*, 2009), Brassinosteroids (Bajguz and Hayat, 2009), Caronatine (Ai *et al.*, 2008), Jasmonate (Wang,

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1999), 5-aminolevulinic acid (Liu *et al.*, 2011) and Salicylic acid (Senaratna *et al.*, 2000).

Salicylic acid (SA) is a common plant-produced phenolic compound that can function as a plant growth regulator. Various physiological and biochemical functions of SA in plants have been reported (Raskin, 1992), and SA has received much attention due to its role in plant responses to biotic and abiotic stresses. Literature exists about some beneficial effects of SA in plant 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. 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 (Senaratna *et al.*, 2000). Several methods of SA application (soaking the seeds prior to sowing, adding to the hydroponic solution, irrigating or spraying with SA solution) have been shown to protect various plant species against abiotic stress factors by inducing a wide range of processes involved in stress tolerance mechanisms (Horvath *et al.*, 2007). Several studies also supported a major role of SA in modulating the plant response to several abiotic stresses including drought (Senaratna *et al.*, 2000; Yazdanpanah *et al.*, 2011). Thus, the purpose of this experiment was to test the possibility that application of SA would protect sweet basil plants from damaging effects of drought stress.

2. Materials and Methods

2.1. Plant Material and Treatments

This study was carried out in factorial experiment layout in randomized complete block design (RCBD) with drought stress levels and SA concentrations as main factors in the greenhouse of agricultural faculty of Ilam University during 2010-2011. Seeds of sweet basil (*Ocimum basilicum* L.) were obtained from Agriculture university of Ilam and cultured in plastic pots with 20 cm height, and 23 cm diameter which were filled with 1:1:1: mixture of fine sand, leaf mould and garden soil. When the 4-6 true leaves of seedling were expanded SA was sprayed in ratio 0, 0.75 and 1.5 mM until both sides of the leaves

were completely wet. 72 h after foliar spray, all plants were subjected to three levels of drought stress including stress-free conditions (irrigation within the field capacity), mild stress (humidity of soil about 60% of field capacity) and severe stress (humidity of soil about 30% of field capacity) until the end of experiment.

2.2. Properties Measurement

2.2.1. Plant Mass Determination

The measurement of dry and fresh weight of shoots and roots was done at the end of experiment. The fresh weight of shoot was recorded after the complete cut of plants from the soil surface by digital scale. To measure exact fresh weight of the root, they were weighted after washing. The shoots and roots were put in the oven at 70 °C for 48 h and after weighting, their dry weight was recorded.

2.2.2. Relative Water Content

Discs (1cm in diameter) from middle portion of fully developed leaf were randomly taken from chosen plants of each replicate. Discs were weighed (FW) and then immediately floated on distilled water for 5 h in the dark. Turgid weights (TW) of leaf discs were obtained after drying excess surface water with paper towels. Dry weights (DW) of discs were measured after drying at 75 °C for 48 h. Relative water content (RWC) was calculated using the following formula (Korkmaz *et al.*, 2010):

$$RWC = \left(\frac{FW - DW}{TW - DW} \right) \times 100$$

2.2.3. Photosynthetic Pigments 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 5ml of acetone (80% v/v) using pestle and mortar and centrifuged at 3,000 rpm. The absorbance was measured with a UV/visible spectrophotometer at 663 and 645nm and chlorophyll contents were calculated using the equations proposed by Strain and Svec (1966) given below:

$$Ch.a = mg/g F.W = \{12.7(A_{663}) - 2.69(A_{645})\}$$

$$Ch.b = \{22.9(A_{645}) - 4.68(A_{663})\}$$

$$Ch a+b = \{20.2(A_{645}) + 8.02(A_{663})\}$$

Table 1

ANOVA for dependent variable for treatment applied, drought, salicylic acid and their interactions for sweet basil plant.

Sources of Variation	Shoot fresh weight	Shoot dry weight	Root fresh weight	Root dry weight	Means of Squares						
					Plant height	Chl a	Chl b	Chl a+b	RWC	Electrolyte leakage	Proline
Drought (D)	77.98**	2.49**	2.57**	0.073**	1345**	0.592**	0.079*	1.05**	1289**	49.75**	340**
Salicylic(SA)	56.82**	3.10**	1.86**	0.051**	183**	0.635**	0.294**	1.76**	147**	42.82**	102**
D*SA	1.55 ^{ns}	0.10 ^{ns}	0.17**	0.001 ^{ns}	5 ^{ns}	0.042 ^{ns}	0.015 ^{ns}	0.053 ^{ns}	16 ^{ns}	1.35 ^{ns}	9*
C.V	9.46	13.25	14.51	13.06	14.18	11.36	12.73	9.79	5.36	6.18	8.29

** and * represent significant at the 0.01 and 0.05 levels, respectively, and ns represent non- significant.

2.2.4. Electrolyte Leakage

In order to assess membrane permeability, electrolyte leakage was determined according to the method described by Korkmaz *et al.* (2010). Leaf discs (1cm in diameter) from randomly chosen plants per replicate were taken from the middle portion of fully developed leaf and washed with distilled water to remove surface contamination. The discs were placed in individual vials containing 10 ml of distilled water. After incubating the samples at room temperature on a shaker (150 rpm) for 24 h, the electrical conductivity (EC) of the bathing solution (EC1) was determined. The same samples were then placed in an autoclave at 121 °C for 20 min and a second reading (EC2) was determined after cooling the solution to room temperature. The electrolyte leakage was calculated as EC1/EC2 and expressed as percent.

2.2.5. Proline Content

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 sulfosalicylic acid and the homogenate was centrifuged at 10,000 rpm. 2 milliliter of the supernatant was mixed with 2ml of acid ninhydrin and 2ml 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 4ml toluene and the chromophore containing toluene was aspirated, cooled to room temperature, and 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.

2.3. Statistical Analysis

Data were analyzed for significant differences using a factorial analysis of variance with drought stress levels and SA concentrations as main factors. Statistical analysis was performed using SAS and MSTATC software programs and the means compared using the Duncan's Multiple Range Test at $p=0.05$. Figures were drawn using Microsoft Excel 2003 and bars in the figures show standard errors (SE) of the means with 3 independent assays.

3. Results and Discussion

3.1. Fresh and Dry Weight and Plant Height

The statistical analysis showed that drought stress and SA application had significant ($p<0.01$) effects on the growth parameters including shoot fresh weight (SFW), shoot dry weight (SDW), root fresh weight (RFW), root dry weight (RDW) and plant height of sweet basil plants (Table. 1). Simple effects of drought (Table 2) showed that drought stress significantly decreases fresh and dry weight and plant height. Highest SFW (11.79g), SDW (2.37g), RFW (1.74g), RDW (0.321g) and plant height (55.93 Cm) were appeared under non stress condition (100% FC) and lowest SFW (6.09g), SDW (1.37g), RFW (0.68g), RDW (0.14g) and plant height (31.48Cm) achieved in severe stress condition. The effect of water stress in reducing the growth of various parts of plant and yield was reported in Yazdanpanah *et al.* (2011) and Liu *et al.* (2011) studies that are in agreement with the findings of this study.

Table 2

Effect of drought stress levels on some measured parameters of sweet basil plant.

Drought treatment	Shoot fresh weight (g)	Shoot dry weight (g)	Root fresh weight (g)	Root dry weight (g)	Plant height (Cm)	Traits			RWC (%)	Electrolyte leakage (%)	Proline (µm/g FW)
						Chl a (mg/g F.W)	Chl b (mg/g F.W)	Chl a+b (mg/g F.W)			
100% FC	11.79 ^a	2.37 ^a	1.74 ^a	0.321 ^a	55.93 ^a	2.13 ^a	1.14 ^a	3.28 ^a	81.29 ^a	16.75 ^c	15.40 ^c
60% FC	10.90 ^b	2.15 ^a	1.12 ^b	0.238 ^b	43.95 ^b	1.79 ^b	1.10 ^a	2.89 ^b	66.66 ^b	19.73 ^b	21.51 ^b
30% FC	6.09 ^c	1.37 ^b	0.68 ^c	0.140 ^c	31.48 ^c	1.62 ^b	0.96 ^b	2.59 ^c	57.58 ^c	21.40 ^a	27.70 ^a

Means with the same letters within rows are not significantly different at $p < 0.05$ using Duncan's Multiple Range Test.**Table 3**

Effect of salicylic acid (SA) treatments on some measured parameters of sweet basil plant.

SA treatment	Shoot fresh weight (g)	Shoot dry weight (g)	Root fresh weight (g)	Root dry weight (g)	Plant height (Cm)	Traits			RWC (%)	Electrolyte leakage (%)	Proline (µm/g FW)
						Chl a (mg/g F.W)	Chl b (mg/g F.W)	Chl a+b (mg/g F.W)			
0 mM	6.78 ^c	1.38 ^c	0.70 ^c	0.156 ^c	39.93 ^b	1.54 ^b	0.86 ^b	2.41 ^b	64.05 ^b	21.27 ^a	18.14 ^c
0.75 mM	9.48 ^b	1.95 ^b	1.24 ^b	0.235 ^b	42.68 ^{ab}	1.98 ^a	1.20 ^a	3.18 ^a	69.52 ^a	19.65 ^b	21.59 ^b
1.5 mM	11.80 ^a	2.56 ^a	1.61 ^a	0.307 ^a	48.76 ^a	2.02 ^a	1.14 ^a	3.17 ^a	71.95 ^a	16.96 ^c	24.89 ^a

Means with the same letters within rows are not significantly different at $p < 0.05$ using Duncan's Multiple Range Test.

Water stress is characterized by wilting, closure of stomata and decrease in cell enlargement and growth due to reduction of water content, turgor and total water potential. Cell division, enlargement and differentiation, are the main processes that determine the quality and quantity of plant growth, affected by various internal and external factors, such as water stress (Patel and Golakia, 1988). The observed reduction in growth parameters in drought stress condition in this study is due to the disturbance in metabolic process of the plant including photosynthesis and transpiration, chlorophyll destruction and the cell division.

Plants treated by SA showed significantly more SFW, SDW, RFW, RDW and plant height than with control plants (Table 3). This data showed that SA application in low concentration could effectively improve the growth of sweet basil plants under water stress. There is a significant ($p < 0.01$) interaction between the drought stress and SA in RFW (Table 1). The highest RFW (2.44g) in treatment composition of 100% FC and 1.5mM SA application and the lowest value (0.42g) in treat-

ment composition of 30% FC and applying 0mM SA were achieved (Fig. 1). SA-treated plants exhibited an increase in tolerance to water stress. This increase in water stress tolerance was reflected in the measured growth criteria. Gutierrez-Coronado *et al.* (1998) also reported a similar increase in the growth of shoots and roots of soybean plants in response to salicylic acid treatment. Imami *et al.* (2011) also reported a similar increase in the growth of chickpea plants in response to salicylic acid treatment. Senaratna *et al.* (2000) have suggested a similar mechanism to be responsible for SA induced multiple stress tolerance in bean and tomato plants. The ability of SA to increase plant dry mass, ameliorating the adverse effect of water stress, may have significant implications in improving the plant growth and overcoming the yield barrier arising from conditions of limited water availability.

3.2. Photosynthetic Pigments

Analysis of variance results of photosynthetic pigments chlorophyll a (Chl a), chlorophyll b (Chl b) and chlorophyll a+b (Chl a+b) are shown in Table 1. Drought stress and SA application

affected photosynthetic pigments significantly. The interaction effect of drought and SA on Chl a, Chl b and Chl a+b was not significant. Simple effects of drought (Table 2) showed that drought stress significantly decreases all photosynthetic pigments. Some signs of environmental stresses in the plants are reduction of chlorophyll and this reduction depends upon the plant genotype (Colom and Vazzana, 2001). Chlorophyll content reduction was reported in drought stressed cotton (Massacci *et al.*, 2008) and *Catharanthus roseus* (Jaleel *et al.*, 2008). Decreases in photosynthetic pigments were due to instability of protein complexes and destruction of chlorophyll by increased activity of chlorophyll degrading enzymes and chlorophyllase under stress condition. Results showed that SA treatment significantly increased photosynthetic pigments compared with control; however 1.5mM concentration were more effective. This result is in agreement with those reported by Imami *et al.* (2011), who found that SA foliar and soil applications increased chlorophyll content in chickpea plants following chilling stress. Moreover, Khan *et al.* (2003) showed that SA increased photosynthetic rate in corn and soybean. Based on the theory of Schutz and Fangmir (2001), the reduction of chlorophyll due to stress is related to the increase of production of Reactive oxygen species (ROS) in the cell. These free radicals cause peroxidation, disintegration and reduction of chlorophyll content in plants under stressful conditions. SA application with scavenging of ROS may increase Chl content in sweet basil 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_2O_2 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 with its influence on the antioxidative enzyme activities and hydrogen peroxide metabolism.

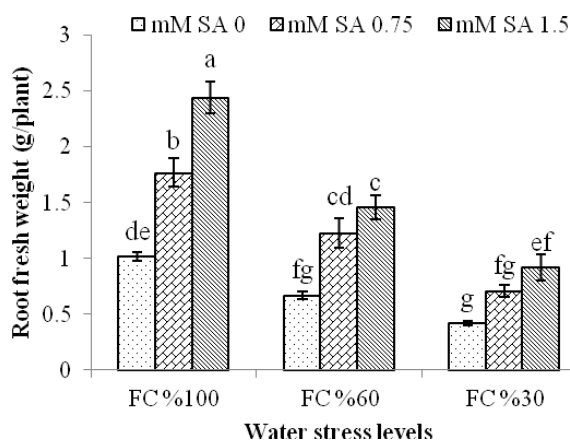


Figure 1: Effect of Salicylic acid on root fresh weight of sweet basil plants under drought stress.

3.3. Relative Water Content (RWC)

Measuring plant water status is an important physiological index in identification of plant response to drought stress. One of the indices showed that the status of water of plant is Relative water content (RWC). Result showed that drought stress and SA treatment significantly affected leaf RWC in $p < 0.01$, but, interaction effect was not significant. RWC was decreased by increasing drought intensity (Table 2), it is in line with the results of Khadem *et al.* (2010). Also, Saneoka *et al.* (2004) reported that RWC in lenti decreased by drought stress as compared with non-stress conditions. Reduction of RWC of the leaf due to drought stress is related to the reduction of soil humidity, in these conditions the stomata should be closed to avoid more water waste. The reason of stomata closure is Absciscic acid that is made in the root in drought stress conditions and is accumulated in stomata cells (Chaves *et al.*, 2002). In this experiment when plants were sprayed with 0.75 and 1.5mM, SA led to a significant increase in leaf RWC as compared to unsprayed SA plants. Similarly, Agarwal *et al.* (2005) reported that RWC increased in wheat with SA treatment.

3.4. Electrolyte Leakage

In order to assess membrane permeability, electrolyte leakage (EL) was determined. Drought stress and SA treatment affected EL, but their interaction effect was not significant (Table 1). Decrease of soil humidity from 100% to 30% FC significantly increased 22% EL. The results showed that the

highest (21.40%) and lowest (16.75%) amounts of EL were achieved in severe and non- stress conditions, respectively (Table 2). EL is an index, which can quantify the damage conceived by plant cell membrane. Its relative conductivity can be used to evaluate the damage on structure and function of cell membranes under stresses. Application of SA significantly decreased EL, the largest decrease in EL obtained by using 1.5 mM SA. SA reversed the adverse effect of stress and caused a significant decrease in EL. Wang and Li (2006) showed that exogenous application of SA in grape plant exposed to cold stress lead to a decrease in EL and induced chilling tolerance. The results of the present study are in agreement with Stevens *et al.* (2006) who determined that SA facilitated the maintenance membrane functions in tomato plants. This facilitation could be attributed to the induction of antioxidant responses and elevated Ca uptake that protects the plant from the oxidative damage by SA (El-Tayeb, 2005). These results suggested cell membrane structure of sweet basil leaves under drought stress received less damage after pretreatment with SA.

3.5. Proline Accumulation

The proline content was significantly affected by drought stress, SA treatment and their interaction (Table 1). Results showed that the highest proline content ($27.70\mu\text{m/g F.W}$) was achieved in severe stress condition and lowest amount ($15.40\mu\text{m/g F.W}$) achieved in non-stress condition. Proline as an amino acid is known to occur widely in higher plants and normally accumulates in large quantities in response to environmental stresses. There are many reports about increasing of proline under environmental stress conditions in plants. For example, in basil plants subjected to salt stress, the concentration of proline was increased in the leaves (Delavari *et al.*, 2010). The increase of the proline content helps osmosis control and is reported due to some factors including the prevention of proline disintegration, avoiding proline participate in protein structure and 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). SA treatment increased proline content in sweet basil leaves, the maximum proline accumulation was observed from plants

treated with 1.5mM SA, which was 27% more than the control plants (Table. 3). These results are in agreement with those of Yazdanpanah *et al.* (2011) who found that SA treatment increases the proline content in the leaves of *Satureja hortensis*. Similarly, SA treatment increases proline content of basil plant under salinity stress (Delavari *et al.*, 2010). In the current experiment SA induced an accumulation of proline in the plants under drought stress (Fig 2). Thus, proline can be considered to be one of the important factors involved in SA induced protective mechanism in sweet basil leaves in response to drought stress.

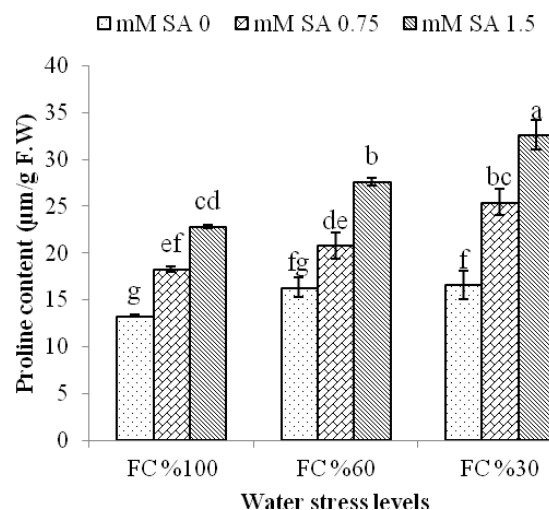


Figure 2: Effect of Salicylic acid on proline content of sweet basil plants under drought stress.

4. Conclusion

In general, results showed that drought stress imposed negative effects on plant growth and productivity of sweet basil plant which were ameliorated by SA application. This was associated with the changing of physiological processes such as increase of photosynthetic pigments and proline content and decrease in electrolyte leakage of plants by addition of SA. Further, results indicate that SA can be considered as a potential growth regulator for improving plant growth and yield under limited soil water availability, and it may be recommended in arid and semiarid regions.

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