



## Effects of salicylic acid on the induction of physiological and biochemical changes in *Brassica napus* L. under water stress

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### Abstract

Drought stress is considered as a restricting factor in plant production and salicylic acid (SA) has been reported to minimize the harmful effects of many stresses including drought. In this study, salicylic acid was sprayed on the leaves of *Brassica napus* at the 4-leaf stage at concentrations of 0.5, 1 and 1.5 mM for two days. Plants were subjected to drought stress (withholding water for five days). Drought stress significantly reduced photosynthetic pigments (Chlorophyll and carotenoids) and anthocyanins content but increased lipid peroxidation, proline and ethylene. SA at 1 mM alleviated damage caused by drought stress on all parameters measured. It is concluded that salicylic acid can considerably alleviate damages caused by drought.

**Keywords:** chlorophyll; drought stress; ethylene; lipid peroxidation

### Abbreviations:

ACC: 1-aminocyclopropane-1-carboxylic acid; FW: fresh weight; GC: gas chromatography; LSD: least significant difference; MDA: Malondialdehyde; ROS: reactive oxygen species; SA: Salicylic acid; TBA: Thiobarbituric acid; TCA: Trichloroacetic acid

**Mazaheri Tirani, M., F. Nasibi and Kh. M. Kalantari.** 2012. 'Effects of salicylic acid on the induction of physiological and biochemical changes in *Brassica napus* L. under water stress'. *Iranian Journal of Plant Physiology* 2 (2), 371-379.

### Introduction

Drought stress is defined as a condition where water availability to the plants is so low that it is unfavorable for the growth of a given plant species (Egert and Tevini, 2002; Zhu, 2001). Plants respond to drought stress through a number of physiological and developmental changes (Inze and Montagu, 2000). Under

stressful conditions, the stress factor or toxic molecules derived from stress, attack the most sensitive molecules (primary targets) in cells to impair their function (Ingram and Bartels, 1996; Inze and Montagu, 2000). The damaged targets are recovered by either repair or replacement via *de novo* biosynthesis. When the stress is too intense and severely damaging target molecules, catastrophic cascades of events set in, leading to cell death (Inze and Montagu, 2000). Cells are protected by the endogenous molecular systems that mitigate the stress (Ingram and Bartels, 1996; Inze and Montagu, 2000). Agricultural productivity worldwide has been greatly

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Received: November, 2011

Accepted: January, 2012

restricted by environmental stresses and a high capacity to tolerate environmental stresses is the prerequisite to the immobile life of plants (Dat et al., 1998; Sakabudinova et al., 2003; Senaranta et al., 2002). Drought stress is considered as a restricting factor for plant product synthesis and many compounds have been applied to minimize the harmful effects of stress. One of these compounds, which has antioxidative characteristic is salicylic acid. Salicylic acid (SA) is a common plant-produced phenolic compound that can function as a growth regulator (Popova et al., 1997). In addition, it has been suggested that SA could be included in the category of phytohormones (Raskin, 1992). Exogenous application of SA may influence a range of diverse processes in plants, including seed germination (Popova et al., 1997), stomata closure (Raskin, 1992), ion uptake and transport (Harper and Balke, 1981), membrane permeability (Popova et al., 1997), photosynthetic and growth rate (Davis, 2005; Popova et al., 1997). El-Tayeb (2005) found that SA treatment increased the chlorophyll a, b and carotenoid content in barley under salinity stress. SA is also known as an important signal molecule for modulating plant responses to environmental stress (Senaranta et al., 2002). It is now clear that SA provides protection against a number of abiotic stress, e.g., heat stress in mustard seedlings (Dat et al., 1998), heavy metal stress in barley seedlings (Metwally et al., 2003), and salt stress in barley plants (El-Tayeb, 2005). In addition, it is becoming clear that SA interacts both negatively and positively with other major signaling pathways including those regulated by jasmonic acid and ethylene (Raskin, 1992). Aspirin, a trade name for acetylsalicylic acid, undergoes spontaneous hydrolysis in plants to form SA (Popova et al., 1997) when exogenously applied to plants; it is rapidly converted to SA. Despite the fact that aspirin is not considered as a natural product, it is widely used by many plant scientists in their experiments. It has been reported that salicylic acid may act as a component of the signal transduction system, and be important in defense mechanisms against pathogen attack (Dat et al., 1998; Raskin, 1992). SA is also known as an important signal molecule for modulating plant responses to the environmental stresses. It is now clear that SA

provides protection against a number of biotic and abiotic stresses (Horvath et al. 2007; Gue et al. 2007; Zhou et al. 2009). The alleviation of oxidative damage and increase resistance to the environmental stresses are often correlated with an efficient anti oxidative system.

Although many compounds have been used to help plants to overcome disturbances caused by stresses in the laboratory, little is known about their effectiveness in the field. This investigation was also an attempt to understand the effect of salicylic acid on the water stress responses of canola plants in controlled chamber.

### Materials and Methods

Canola (*Brassica napus* L. cv. option) was used in this study. Seeds were sown in plastic pots containing sand, loam and peat (2:1:1) and the seedlings were transferred to green house with day/night temperature of 22° C / 18° C and a 16 h photoperiod with a relative humidity of 50%. Preliminary screening identified that optimum responses occurred between the concentrations of 0.5 to 1.5 mM salicylic acid. The seedlings were irrigated with water once a day and half-strength Long Ashton solution once a week (pH of nutrient solution was adjusted about 6.7).

After one month (at 4-leaf stage) salicylic acid was sprayed to the leaves at 0 (as control for SA), 0.5, 1 and 1.5 mM concentration for 2 days. For this purpose, salicylic acid was dissolved in distilled water and pH was adjusted about 5.5 by KOH. Triton X-100 (0.01%) was used as a surfactant. After 2 days of SA treatment, two levels of water regime (control and 5 days withholding water) were applied. Light intensity was 11000 Lux at the plant level. Three replications were assigned for each treatment. After 5 days of drought treatment, the plants were harvested, divided into shoot and root and samples were either rapidly dried in an oven at 80° C to constant weight, which was used for determination of dry weight and further analyses, or were frozen in liquid nitrogen and stored at -20° C for subsequent biochemical analysis.

The amount of photosynthetic pigment, (chlorophyll a, b, total and carotenoids), were determined according to the method of Lichtenthaler et al., (1987). The pigment extract

was measured against a blank of 80% (V/V) acetone at wavelengths of 646.8 and 663.2 nm for chlorophyll assays and 470 nm for carotenoid assays.

Chl. a, Chl. b, total Chl. and carotenoids contents were calculated using the following formulas:

$$\text{Chl. a} = (12.25A_{663.2} - 2.79A_{646.8}) \times \text{volume of supernatant (ml)} \times \text{dilution factor/sample weight (g)}$$

$$\text{Chl. b} = (21.21A_{646.8} - 5.1 A_{663.2}) \times \text{volume of supernatant (ml)} \times \text{dilution factor/sample weight (g)}$$

$$\text{Car} = [(1000A_{470} - 1.8 \text{ Chl. A} - 85.02 \text{ Chl. b}) / 198] \times \text{volume of supernatant (ml)} \times \text{dilution factor/sample weight (g)}$$

For determination of anthocyanins content, frozen tissue samples (100 mg) were soaked immediately in 10 ml of acidified methanol (methanol: HCl 99:1 (v/v)). Tissues were crushed using a glass pestle and kept at 25 °C for 24 hours in the dark. The extract was then centrifuged at 4000 × g for 5 min at room temperature (22 °C) and absorption at 550 nm of the supernatant was read by a UV-VIS spectrophotometer (Cary 50). For the calculation of the amount of anthocyanins, the extinction coefficient of 33000 mM<sup>-1</sup>cm<sup>-1</sup> was used (Wagner, 1979).

To determine the content of flavonoids, 0.1 g of leaf tissue was extracted in 15 ml glass centrifuge tubes containing 10 ml of acidified ethanol (ethanol: acetic acid, 99:1 (v/v)). The samples were gently boiled for 10 min in a water bath at 80° C and brought up to volume. Absorbance was measured at three wavelengths: 270, 300 and 330 nm with UV-VIS spectrophotometer (Krizek et al., 1998).

The level of lipid peroxidation in plant tissues was measured by determination of Malondialdehyde (MDA) (Heath and Packer, 1969) which is known to be breakdown product of lipid peroxidation. The MDA content was determined with the thiobarbituric acid (TBA) reaction. Briefly, a 0.2 g tissue sample was homogenized in 5 ml 0.1% TCA. The homogenate was centrifuged at 10000 × g for 5 min. To 1 ml

aliquot of the supernatant, 4 ml of 20% TCA containing 0.5% TBA was added. The mixture was heated at 95° C for 15 min and cooled immediately in ice. The absorbance was measured at 532 nm. The value for the non-specific absorption at 600 nm was subtracted. The level of lipid peroxidation was expressed as μmol of MDA formed using an extinction coefficient of 155 mM<sup>-1</sup>cm<sup>-1</sup> (Heath and Packer, 1969).

Ethylene released from detached leaves was determined on three replicates, each in a 120 ml flask, capped with a rubber stopper for 2h. One ml of the gas phase samples was removed from the headspace using an airtight syringe. The 1 ml gas samples were injected into a gas chromatograph (GC) (Agilent USA) fitted with a flame ionization detector and a glass column packed with (30-100 mesh) activated alumina (180 cm × 0.34 cm OD). The GC was operated at injector, detector and oven temperatures of 90, 200 and 250° C respectively. Nitrogen was used as the mobile phase. Pure ethylene (99.9%) was used as a standard (Kalantari et al., 2000).

The free proline was determined according to Bates et al. (1973). The data were statistically analyzed by one-way analysis of variance using both SPSS and MSTATC software and the least significant difference (LSD) was used to test the significant differences between treatments.

## Results

### Contents of photosynthetic pigments

The photosynthetic pigments (chlorophyll a, b, total and carotenoids) were measured in this research. Chlorophyll a content decreased in drought stressed plants as compared with the control plants (Figs. I a, b, c & d). Pretreatment of plants with 1 mM salicylic acid following exposure to 5 days drought stress caused significant increases in chlorophyll a (Fig. I a). The total chlorophyll content showed the same pattern as chlorophyll a ( $\alpha \leq 0.05$ ) (Fig. I c). Carotenoids increased significantly in 1 mM salicylic acid treated plants in comparison to the drought stressed plants (Fig. I d).

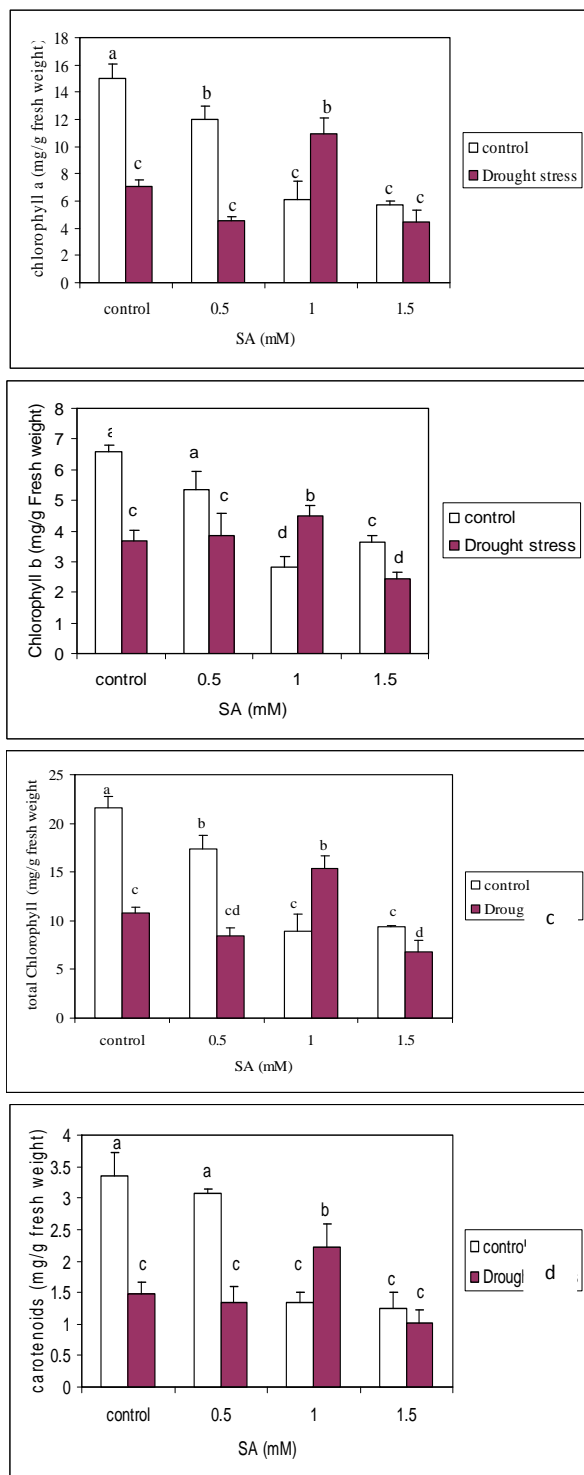


Fig. I. Effects of SA and drought stress on photosynthetic pigment content of *B. napus*. Values are the means of three replicates, and bars indicate SEM significant difference at  $P > 0.05$  according to LSD test.

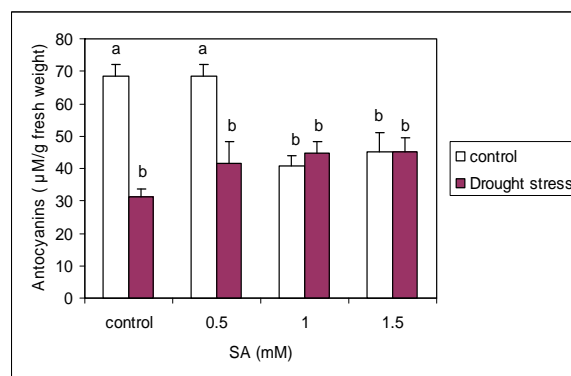


Fig. II. Effects of SA and drought stress on anthocyanin content of *B. napus*. Values are the means of three replicates, and bars indicate SEM significant difference at  $P > 0.05$  according to LSD test.

### Anthocyanin content

Figure (II) shows anthocyanin content during drought stress and SA treatment. During drought stress there was a significant decrease in anthocyanin content, but salicylic acid treatment significantly increased anthocyanin content in plants under drought stress compared with plants grown under drought stress ( $\alpha \leq 0.05$ ).

### Flavonoids

In this study, methanolic extracts of control plants and plants just under salicylic acid treatment showed a significantly lower absorbance of extracts (presumably flavonoids) at 270, 300 and 330 nm than those of plants grown in the presence of both water stress and 1 mM salicylic acid ( $\alpha \leq 0.05$ ) (Fig. III).

### Lipid peroxidation

Figure (IV) shows the changes in MDA content in leaves of canola. Cytotoxic end-product of lipid peroxidation (MDA content) was significantly increased in drought stressed plants. Plants pretreated with SA had much lower MDA content than drought stress treated plants ( $\alpha \leq 0.05$ ) (Fig. IV).

### Proline content

Our studies showed that the content of proline progressively increased in plants (Fig. V). SA treatments in drought stress treated plants

induced an increase in the content of this compound comparison to the control plants. proline accumulated in 1mM SA-treated plants which were under stress conditions( $\alpha \leq 0.05$ ).

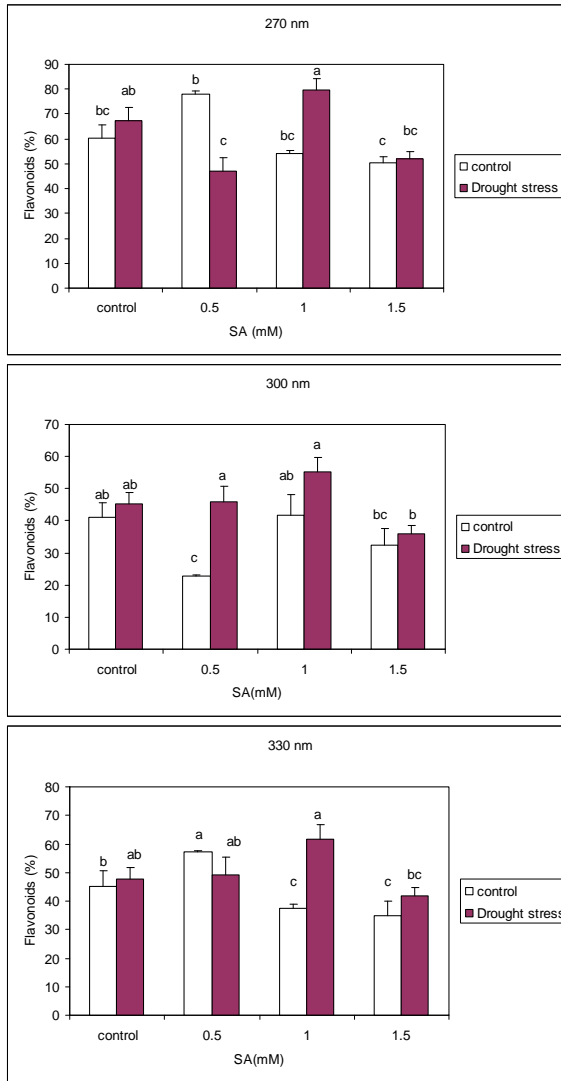


Fig. III. Effects of SA and drought stress on flavonoids content of *B. napus*. Values are the means of three replicates, and bars indicate SEM significant difference at P>0.05 according to LSD test.

### Ethylene production

Our study showed that drought stress caused an increase in ethylene release significantly after 5 days water holding (Fig. VI). During drought stress there was a significant increase in ethylene production compared to control plants, but 1 mM salicylic acid treatment

significantly decreased ethylene production in plants under drought stress in comparison with the control plants.

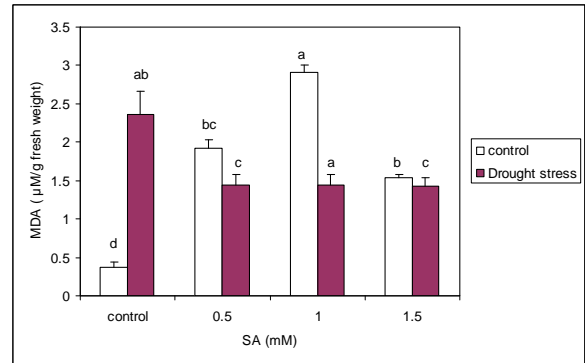


Fig. IV. Effects of SA and drought stress on MDA content of *B. napus*. Values are the means of three replicates, and bars indicate SEM significant difference at P>0.05 according to LSD test.

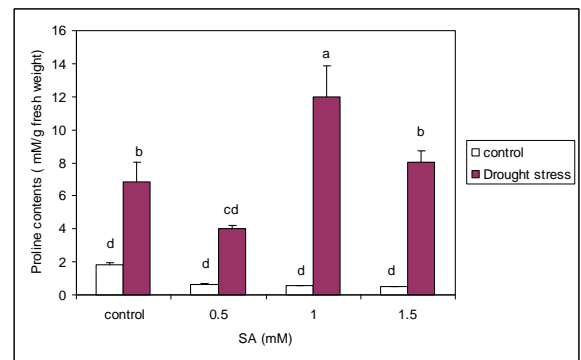


Fig. V. Effects of SA and drought stress on proline content of *B. napus*. Values are the means of three replicates, and bars indicate SEM significant difference at P>0.05 according to LSD test.

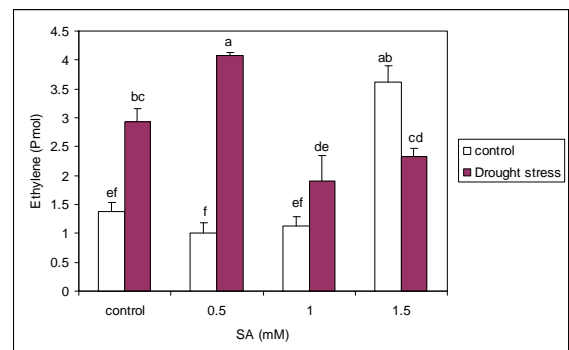


Fig. VI. Effects of SA and drought stress on ethylene production content of *B. napus*. Values are the means of three replicates, and bars indicate SEM significant difference at P<0.05 according to LSD test.

## Discussion

The photosynthetic pigments (chlorophyll a, b, total, and carotenoids) significantly decreased in drought stressed plants, compared to the controls ( $p \leq 0.05$ ) (Fig. I). Costa et al. (2005) reported similar results in water stressed broccoli. It has been reported that the photosynthetic pigments reduction under drought and salinity could be due to the destruction of chloroplast and photosynthetic apparatus, photo oxidation of chlorophyll, interaction of chlorophyll with singlet oxygen, degradation of chlorophyll substrates, biosynthesis inhibition of new chlorophyll, and the increase of chlorophyllase enzyme (Sultana et al., 2005; El-Tayeb, 2005; Neocleus and Nasilakakis, 2007). However, 1.0 mM SA significantly increased the pigment contents in plants under drought stress. These results are also in agreement with those of Singh and Usha (2003) and Horveth et al. (2007) in wheat under water stress. In addition, it has been reported that, SA treatment reduced the stress-induced loss in chlorophylls contents and photosynthetic rate in maize under water stress (Zafar et al., 2010) and barley under salinity stress (El-Tayeb 2005). Previous research showed that SA could increment the antioxidant ability of cell and had protective role in photosynthetic apparatus by induction of new protein synthesis in this machinery (Popova et al., 2003). Result of our experiment showed that 1 mM salicylic acid increased the carotenoids content under drought stress. Induction of carotenoids synthesis by SA could be related to the protecting role of these compounds in photosynthetic machinery (Koyro, 2006). Carotenoids could scavenge or quench the singlet oxygen and protect the chloroplast from lipid peroxidation and oxidative damages (Loggini, 1999).

Results of this study showed that drought stress decreased anthocyanin content, while SA alleviated the damaging effect of drought on anthocyanins losses (Fig II). It has been reported that phenolic compounds protect the cell through scavenging the ROS, breakdown of peroxidation chain, hydrogen donation, quenching of singlet oxygen and peroxidase enzyme (Chu et al., 2000). Results of the present study showed that drought

stress and SA pre-treatment had no effects on flavonoids content (Fig III). Several reports have shown that phenolic compounds accumulate in cells of plants subjected to cadmium or other environmental stresses, and are thought to have an anti oxidative role in plant cells (Ganesan and Thomas, 2001; Kang et al., 1973; Metwally et al., 2003; Smallwood et al., 1999). However it seems that in our study, flavonoids had no effect on canola plant to cope with water stress.

MDA and other aldehyde formations in plants exposed to water stress are reliable indicators of free radical formation in the tissue, and are currently used as indicators of lipid peroxidation (Borsanio et al., 2001; Nasibi et al., 2009; Yazdanpanah et al., 2010; Daneshmand et al., 2010). In this study, the content of MDA increased significantly in drought stressed plants. However, SA pretreated plants under drought stress had much lower MDA content when compared with those plants which were not treated with SA but still were under drought stress (Fig IV). Similarly, Metwally et al. (2003) found treatment with salicylic acid significantly decreased the MDA content in cadmium-treated plants. In barley seedlings, foliar spray with SA decreased MDA content as well as electrolyte leakage under salt stress (El-Tayeb, 2005), which well accords with our findings. It has been reported that salicylic acid could decline lipid peroxidation through the inhibition of lipoxygenase activity and decline of  $H_2O_2$  content and so maintaining the integrity of cellular membranes under stress conditions (Hayat and Ahmad, 2007). These results suggest that under water stress, SA can induce antioxidant defense activity in plants to remove the possible toxic effects of free radicals, making the plants more resistant to water stress.

The content of proline progressively increased in plants under drought stress (Fig V). Only 1 mM SA increased the content of this compound compared with those plants not treated with SA but under stress. Salicylic acid had no effect on proline content in control plants. El-Tayeb (2005) and Yazanpanah et al. (2010) reported that SA treatments induced a higher accumulation of the free amino acids, including proline, in the stressed plants. Shakirova and Sahabutdinova et al. (2003) also found that SA

increased proline accumulation in normal or stressed wheat seedlings. In previous investigations increase of proline content under different environmental stress such as cold (Wanner and Junttila, 1999), salinity (Ali et al., 1999), drought (Nayyar, 2003) and heavy metal (Ali and Saradhi., 1991) was reported. Proline has been reported to play a different role in cell protection. In addition to osmolyte characteristic, and protection of the equilibrium of the cell water, proline could maintain protein and enzymes stability stabilizes the cell membrane, scavenge the ROS and quench the singlet oxygen (Matysik et al., 2002; Rai, 2002; Verbuggen and Hermans, 2008). Shakirova et al. (2005) reported that SA treatment induced change in phytohormones and increased the ABA level, which is responsible for the induction of proline biosynthesis in the stress condition. It is assumed that SA treatment may also stimulate hydrolysis of proteins, providing a pool of compatible osmolyte, which is important in osmotic adjustment in drought stress (El-Tayeb, 2005; Sakihama et al., 2002; Shakirova and Sahabudinova, 2003). Our data also showed that proline accumulated in 1 mM SA-treated plants, which were under stress conditions. The role of proline as an important component of protection against drought is well known and therefore treated plants with SA can better cope with water stress.

During drought stress there was significant increase in ethylene production, but salicylic acid pretreatment significantly decreased ethylene production in plants under drought stress (Fig VI). The mechanism of SA action in ethylene emanation of plants has not been clarified, although published data and our results suggest that SA has some association with ethylene production (Popova et al., 1997; Raskin, 1992). Zhang et al. (2003) showed that SA inhibits ethylene biosynthesis at low concentrations. This suppression was believed to be due to its inhibitory effect on the conversion of ACC into ethylene (Beltrano et al., 1997; Popova et al., 1997). The degree of inhibition may both be concentration and pH dependent. With decreasing pH, the inhibitory effect of SA increased, suggesting that the protonated form of SA was more active than its charged form

which is partly due to the absorption of SA into cells which is pH dependent (Popova et al., 1997).

## Conclusion

Our results show that SA treatments ameliorate the adverse effects of drought stress via decreasing the oxidative damage of plant membranes, possibly by the induction of a compatible solute for osmotic adjustment in canola plants. Finally, the results hint that SA may find application for improving plant growth and yield in dry areas in future.

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