Changes in growth and antioxidant capacity of canola by salinity and salicylic acid under in vitro

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ABSTRACT: The study was conducted to evaluate alterations in growth and antioxidant capacity of salt stressed canola plants in present of salicylic acid (SA). Seeds of Brassica napus cultivar Ocapy were cultured in MS (Murashig and Skoog) medium containing 0, 50 and 100 mM NaCl and different levels of SA (0, 2 and 5 µM) under in vitro condition. After 4 weeks, the effect of salinity and SA treatments were studied on growth and the antioxidant enzymes activity, ascorbate peroxidase (APX) and catalase (CAT), in roots and shoots. The percentage of seed germination was decreased with increase in salt levels. SA significantly increased it under salinity. Shoot length and fresh weight decreased not only under salt and SA treatments but also in response to NaCl and SA interactions, whereas enhancement of root length and fresh weight was presented under salt stress in the absence of SA. Treatment with SA and interaction of NaCl and SA reduced root length. Total anthocyanin content was significantly reduced under salt concentrations and SA in comparison to the control. Salt stress increased significantly reducing sugars in roots, while SA-treated plants exhibited high accumulation of reducing sugars in shoots under salinity. Individual NaCl treatment did not show a significant effect on antioxidant activity (CAT and APX) while the presence of SA decreased CAT activity in root and APX activity in shoot under salt stress.

Keywords: antioxidant enzymes; Brassica napus L.; canola; salicylic acid; salinity.

Abbreviations: APX-ascorbate peroxidase; CAT-catalase; DHAR-dehydroascorbatereductase; EDTA-ethylenediaminetetraacetate; FW-fresh weight; GR-glutathione reductase; MS-Murashig and Skoog; SA-salicylic acid; PVP-polyvinylpyrrolidone; ROSs-reactive oxygen species; SOD-superoxide dismutase

INTRODUCTION

Stresses, biotic and abiotic, have always threatened different life forms. Environmental conditions such as drought, salinity and temperature changes can induce water-deficit stress, limit plant growth and development like leaf area, root and shoot length and root and shoot fresh and dry weight. Also agricultural productivity significantly reduces by salinity (Khodary, 2004; Ashrafuzzaman et al., 2002). Salinity, as a major abiotic stress conditions in crop species, disrupts homeostasis in water potential, ion distribution and induces inhibition of growth and oxidative changes as a secondary stress. Formation and accumulation of reactive oxygen species (ROSs) can be induced by salt stress (Erdal et al., 2011). Higher plant cells have evolved enzymatic and non-enzymatic antioxidant defense systems in order to reduce ROSs accumulation and oxidative damages by detoxifying free radicals (Borsani et al., 2001). Superoxide dismutase (SOD), Ascorbateproxidases (APX) and catalase (CAT) are some of the antioxidant enzymes which can participate in elimination of ROSs. CAT and non-specific peroxidases destroy the generated H2O2 in different cell compartments (Moran et al., 1994; Anderson et al., 1995). APX, dehydroascorbatereductase (DHAR) and glutathione reductase (GR) can participate in Halliwell-Asada pathway (Ascorbate-glutathion cycle) which removes H2O2 in cyanobacteria and plant chloroplasts (Dalton et al., 1986; May et al., 1998).

Increase in salinity tolerance under salicylic acid (SA) was reported in wheat (Shakirova and Bezrukova, 1997), Maize (Khodary, 2004; Hussein et al., 2007), tomato (Stevens et al., 2006; Shahba et al., 2010) and mungbean cultivars (Nazar et al., 2011). SA, as an antioxidant compound and plant growth regulator, is known to relate on different physiological processes in plants including growth (Gutierrez–Coronado et al., 1998; Khodary, 2004; Hussein et al., 2007), defense responses (Delaney et al, 1994; Silverman et al, 1995; Choudhury and Panda, 2004) and
regulating the activities of antioxidant enzymes and increase plant tolerance to abiotic stress (Li et al., 1998; He et al., 2002; Noreen et al., 2009; Erdal et al., 2011).

Because of food and economic importance of canola (Brassica napus L.) and its enhanced cultivation even on salt-affected soils arid and semi-arid regions, there is an emergency need for further improvement in the salt tolerance of canola. The main objective of this study was to investigate whether SA can influence on salt stress’s effects on growth and antioxidant defense in B. napus, and thereby increasing its salt tolerance.

**MATERIAL AND METHODS**

**Plant material and culture conditions**

The dry mature seeds of B. napus cultivar Ocapy were studied for percentage of germination, growth parameters, total anthocyanin, reducing sugar and the antioxidant enzymes activities, APX and CAT, in shoots and roots. The seeds were provided by the Oil Seed Cultivation Company, Isfahan-Iran. The mature and sterilized seeds were grown on MS (Murashig and Skoog, 1962) medium containing concentrations of 0 (control), 50 and 100 mMNaCl and 0, 2 and 5 μM SA. All cultures then were kept in the culture room with a 16/8-h light/dark photoperiod at 25 ± 2 °C for 4 weeks.

**Measurement of Percentage of germination and growth parameters**

Germination percentage of seeds was determined after 5 and 10 days after culture. Shoot and root lengths, shoot and root fresh and dry weights were measured as the growth parameters of canola plants in response to salt and SA treatments. Shoot and root lengths were measured using a centimeter scale. For dry biomass measurement, shoot and root after weighting were dried at 70 °C for 3 days and weighed.

**Measurement of anthocyanin**

Measurement of total anthocyanin was determined according to modified Wagner (1979) method using acidified ethanol (ethanol: HCl 99: 1 v/v). 0.05 g of frozen leaf was homogenized in 2.5 ml acidified ethanol and then kept at 25°C for 24 h in the dark. The extract was centrifuged at 4000 g for 10 min at room temperature. The absorbance of each supernatant was read at 550 nm. The extinction coefficient 33,000 (mol⁻¹ cm⁻¹) was used to calculate the amount of total anthocyanin and it was expressed as μ mol g⁻¹ FW.

**Measurement of reducing sugars**

Reducing sugar content was measured by adapting Somogyi-Nelson’s method (Nelson, 1944; Somogyi, 1952). Approximately 0.1 g of fresh stem-leaf and 0.03 g of root from 4 weeks old plants were extracted with 10 ml distilled water. The mixture was boiled in a boiling water bath, cooled and filtered. Then 2 ml of the extract was mixed with 2 ml of alkaline copper tartarate and the reaction mixture was heated for 20 min (Alkaline copper tartarate was prepared by dissolving 4 g anhydrous sodium carbonate, 0.75 g tartaric acid and 0.45 g hydrated cupric sulphate in 80 ml of distilled water and finally made up to 100 ml). 2 ml of phosphomolibdate solution was added and the intensity of blue color was measured at 600 nm using spectrophotometer. D-glucose was used as standard. The reducing sugar content was expressed in terms of percentage on fresh weight basis.

**Enzyme extraction and assay**

For enzyme extraction 0.1 g of shoot and 0.03 g of root from 4 weeks old plants were homogenized using a mortar and pestle with 1 ml of 100 mM sodium phosphate buffer (pH 7.8) containing 0.1 mM EDTA and 1% polyvinylpyrrolidone (PVP). The whole extraction procedure was carried out on ice. The homogenates were then centrifuged for 30 min at 14000 rpm at 4°C and supernatants were used for protein and enzyme activity measurement.

Ascorbate peroxidase (APX, EC 1.11.1.11) activity was determined according to the method of Nakano and Asada (1981). The reaction buffer for APX activity contained 50 mM sodium phosphate buffer (pH 7), 0.5 mM ascorbic acid, 0.1 mM EDTA, 1.25 mM H₂O₂ and 0.05 ml enzyme extract in a final volume of 1 ml. Ascorbate oxidation was measured at 290 nm for 1 min with extinction coefficient of 2.8 mM⁻¹ cm⁻¹.

Catalase (CAT, EC 1.11.1.6) activity assay was also carried out according to the method of Aebi (1984). The decrease in H₂O₂ was measured at 240 nm and activity was calculated as μM H₂O₂ consumed per minute (extinction coefficient 39.4 mM⁻¹ cm⁻¹).

**Statistical analysis**

All experiments were carried out in three replications and mean values ± standard deviation were presented. Data were subjected to ANOVA using the statistical package Sigmastat 2.01 and the mean differences were compared by Dunkan test at p < 0.05.
RESULTS AND DISCUSSION

SA, as a plant phenolic compound, is now considered as a hormone-like endogenous regulator, and there is a great interest to clarify its role in the defense mechanisms against biotic and abiotic stress (Edral et al., 2011). Therefore, in the present study, the effect of exogenously treated SA on canola seedlings growing upon medium containing NaCl was investigated under in vitro conditions.

Salinity, SA and their interactions significantly affected the germination of B. napus seeds. As figure 1 shows germination percentage of canola seed cultivar Ocapy decreased significantly due to the addition of salt to medium, while exogenous application of SA improved significantly salt stress effect on seed germination. The maximum seed germination was observed under individual SA treatments as compared to control plants.

The results demonstrated that SA alleviated salt stress effect on seed germination of canola. Similar to our result has been reported in Arabidopsis (Rajjou et al., 2006) grown under salinity stress. Plants are continually exposed to biotic and abiotic stresses. Salt stress, as one of the most severe abiotic stresses, is limiting plant productivity (Edral et al., 2011). Salt stress can reduce seed germination and early seedling growth due to low availability of water, and change the activities of certain enzymes because of intake of Cl⁻ and Na⁺ as toxic ions (Filho and Sodek, 1988; Guerrier, 1988) and reduction in hydrolysis and utilization of food reserves (Ahmad and Bano, 1992; Mondal et al., 1988). The exogenous application of SA improved the seed germination with increase in salt concentrations. It is likely that SA might have protected the cellular membranes against ions toxicity and salt-induced oxidative damages and/or have maintained a better water status under salt-induced water limited conditions in germinating seeds of canola plants. Sakhabutdinova et al., (2003) concluded that the SA treatment reduced the damaging action of salinity and water deficit on wheat seedling growth and accelerated a restoration of growth processes.

Application of the concentrations of NaCl and SA to canola plants variously influenced their growth parameters (shoot and root length, fresh weight of shoots and roots) as compared to control plants (dry weight data are not shown). Salinity, SA treatments and combination of them caused significant reduction in growth parameters like shoot length, fresh weight of shoot (Figure 2 A and B) while enhancement of root length, fresh weight of root were presented under salt stress in the absence of SA treatment (Figure 3 A and B). Although individual treatment of SA and the interaction of NaCl and SA reduced root length, they did not change root fresh weight in comparison to control. However, despite high level of salinity, inclusion of SA in the media did not improve fresh weight of root, compared to non SA-containing treatments.

Figure 1. Germination percentage of canola seed under salt and SA treatment after 5 days (A) and 10 days (B). Values are means of three replications ± Std.

Figure 2. Growth characteristics of canola seedlings under salt and SA treatment. Shoot length (A) and shoot fresh weight (B). Values are means of three replications ± Std.
In the present study, all growth attributes such as length and fresh weight of shoot decreased with increase in salinity levels in contrast, root length increased with increase in the salinity levels. This result indicated that higher salinity increased the level of cell division within the apical meristem of seedling roots causing an increase in root growth. Some of these results are in agreement with the earlier findings by Ghoulam et al. (2001) and Noreen et al. (2009), who showed that salinity caused a marked reduction in growth parameters (fresh and dry weight of shoots) of sugar beet and sunflower plants, respectively. Furthermore, significant reduction in growth parameters like shoot dry weight of maize was reported by Ashrafuzzaman et al. (2002). Previous studies have proposed that SA acts as endogenous signal molecule responsible for inducing abiotic stress tolerance in plants (Tari et al., 2002; Gunes et al., 2006; Sakhanokho and Rowena, 2009). They emphasized that exogenous application of SA increased plant growth significantly both in saline and non saline conditions. In contrast, Barba-Espín et al. (2011) have reported that NaCl-induced damage to leaves was increased by SA, which was correlated with a reduction in pea plant growth. The exogenous application of SA did not increase growth patterns of shoot in canola plants in comparison to control plants. The slightly lower values for both shoot length and fresh weight of shoots might be due to the toxic effect of SA which has been reported in some plant species (Roustan et al., 1989; Sakhanokho and Rowena, 2009). The enhancement of root length was lower when plants were treated with both SA and salt concentrations. Overall, it was found that SA treatment could increase significantly seed germination of canola cultivar Ocapy under salinity, but could not improve plant growth. It has been indicated that salt stress adversely influenced growth parameters of root as compared to control while the present study showed that shoots of canola seedlings were more sensitive to salinity and SA than roots which could be related to the variation in salt adaptation and tolerance of different plant species. These results are similar with the earlier findings in maize (Izzo et al., 1996), black seeds (Hussain et al., 2009). Also the increased root length under salt stress was reported by Munns and Termaat (1986) and Al-Neimi et al. (1992). The reason for growth reduction under salt stress can be due to water shortage and ionic toxicity. The increase in plant growth may be due to turgor potential which is decreased by water deficit produced by higher salinity (Ashraf and Naqvi, 1996). The variation in salt adaptation and growth during early seedling growth among different plants can be mainly attributable to differences in: 1) Na\(^+\) and Cl\(^-\) uptake by root, translocation to shoot and accumulation; and 2) osmoregulation (Reggiani1 et al., 1995).

Higher salinity and SA induced a significant decrease in total anthocyanin content separately in comparison to the control. Similar results were obtained when plants were treated by NaCl in the present of SA (Figure 4).
In the present study, negative correlations were observed between salinity, SA and combined effect of them with anthocyanin in leaves. Although some of the previous researches have demonstrated that SA enhanced anthocyanin content in Capsicum annuum (Mahdavian et al., 2008) and Zingiber officinale (Ghasemzadeh et al., 2012), the result of present study has indicated that SA was not effective in increasing of total anthocyanin content in canola leaves and significantly decreased it than control. On the other hand, AhmadianChashmi et al. (2010) determined that total anthocyanin was not affected by SA treatment in Atropa belladonna (article in Persian with an abstract in English). Moreover, an insignificantly increased amount of anthocyanin than control was reported by Rahimi et al. (2013) in Coriandrum sativum. Similarly, decreased total anthocyanin content was reported by Hashemi et al. (2010) in Lepidium sativum under SA treatment (article in Persian with an abstract in English). Saw et al. (2010) also reported that the anthocyanin concentration in SA treated sample was not different from the control one during the first 7 days of cell growth in grape (Vitis vinifera) cell cultures while it increased again at the end of cell suspension culture. They suggested that the anthocyanin synthesis increased when there was no more cell growth. Furthermore, it seems the presence of growth regulators in medium might interfere with SA effect on induction or accumulation of anthocyanin under SA treatment in this research (Šesták and Ullmann, 1960; Sunderland, 1966). In addition, decrease in total anthocyanin might due to inhibition of ethylene biosynthesis (Qinghua and Zhujun, 2008). It is also indicated that the contents of anthocyanin is dependent on the plant genotype (Hamouz et al., 2007; Nadernejad et al., 2012).

Salinity induced a significant decrease in reducing sugar in shoots, whereas it was increased in roots under salt stress. Moreover, in the presence of SA without NaCl, the reducing sugars content was considerably reduced both in shoots and roots. However, SA-treated plants exhibited high accumulation of reducing sugars in shoots especially at 5 µM SA and 100 mM NaCl. Reducing sugars content decreased markedly not only under SA treatments but also in response to SA and salt interactions (Figure 5 A and B).

The present results demonstrated that interactive effect of SA with NaCl was significantly improved the reducing sugars production or/and accumulation in shoot of canola only. However, we found a positive correlation between reducing sugars content and growth parameters of canola cultivar Ocapy in response to salt stress both in shoots and roots. Some of these findings are similar to those of Barakat (2011) who showed a decrease in reducing sugars as salinity increased in only NaCl stressed wheat and a considerable improvement in reducing sugars due to foliar applied SA in shoot than root of salinity-stressed wheat. In this regard, reducing sugar content was also increased in wheat plants in relation to salt stress (Hamid et al., 2010). Sugars play a key role in the acclimatization of plant roots by production the precursors of most chemical synthases, production of metabolic energy and consequently maintained osmoregulation in roots (Barakat, 2011). Accumulation of reducing sugar could be acting as activators of carbohydrates synthesis (Kodandaramaiah, 1983). In addition, accumulation of sugars play a key role in alleviating the salinity stress, either via osmotic adjustment, as Ackerson, (1985), or by conferring some desiccation resistance to plant cells according to Srivastava et al. (1995). It is suggested that exogenous application of SA might activate the metabolic production or concentration of reducing sugars in shoot cells than root cells as plant protective mechanisms under salt stress in this study. The interactive effect of SA and NaCl treatments might also be assumed to active enzymatic systems of reducing sugars biosynthesis in shoot. However, the accumulation of reducing sugars in root might reflect a salt protective mechanism in response to salinity to form new cell constituents as a mechanism to stimulate the growth of plants (Khodary, 2004).
The CAT activity of salinized canola plants was not different significantly as compared to untreated plants in shoots although it decreased slightly in roots. In contrast, SA treatment resulted in an insignificant stimulation of CAT activity in shoot and a marked decrease in root as compared to control plants. The interactive effect of SA and salinity induced a meaningful increase in CAT activity in shoot especially at 50 mM NaCl and 2 µM SA. On the other hand, treatments with SA in most salinization levels resulted in a pronounced reduction of CAT activity as compared with only NaCl and control plants (Figure 6 A and B).

APX activity did not show any significant difference in shoots and roots under salt stress without SA. By increasing the SA concentrations in the absence NaCl in the culture media, the activity of the APX enzyme increased in shoot only while remained without significant changes in roots in comparison to the control plants. The highest value of APX activity was recorded at the higher salinity level with 2 µM SA in shoots. Overall, the interactive SA and salinity resulted in a considerable reduction in APX activity in shoot, but did not change significantly it in root (Figure 7 A and B).

There are many evidences for the involvement of oxidative stress during salt stress. The generation of ROSs during salt stress is mainly attributed to increased leakage of electrons to O$_2$, ensuing from a decline in CO$_2$ fixation (Dat et al., 2000). The results indicated that the activity levels of antioxidant enzymes as CAT and APX were affected variously under salinity, SA levels and interaction of them. Our experiments showed that salt stress independently had no significant effects on the enzymes activity in shoot and root. NaCl in combination with SA led to a significant decrease in the activity of CAT in root and APX in shoot. Previously role of SA was indicated in the induction of antioxidant defenses, maintaining the redox state of the gluthatione pool and plant protection against oxidative stress (Borsani et al., 2001). Therefore the effect of exogenously treated SA on canola seedlings growing under salt stress was investigated in the present study. Different abiotic stresses may provoke oxidative stress, which lead to cellular adaptive responses such as acceleration of ROSs scavenging systems and alteration in antioxidant enzyme activities in plants (Hamdia and Shaddad, 2010). Modulation of antioxidant systems and the levels of substrates can correlate to tolerance to salinity stress in higher plants (Jahnke and White, 2003). Changes in the levels of antioxidant molecules and the activity of antioxidant enzymes, which are signals of plant tolerance/adaptation to stress conditions, are correlated into oxidative stress tolerance of plants (Lee et al., 2001; Sudhakar et al., 2001). Variations in the antioxidant levels can serve as a signal for the modulation of ROSs scavenging mechanisms and ROSs signal transdution (Mittler, 2002).
Regarding to CAT activity, there are some investigations describing the decrease of CAT activity by exogenous SA treatment (Shim et al., 2003; Shi et al., 2006) while some other studies also showed that SA treatment did not inhibit CAT activity (Tenhaken and Rubel, 1997; Ding et al., 2007), and also induced it (He et al., 2005; Agarwal et al., 2005; Mutlu et al., 2009). It seems that the plants response to SA may be different according to the intensity of the stress, plant parts and species, time assayed after stress treatment, and induction of new isozyme(s) (Shim et al., 2003). The CAT activity remained constant in shoot while reduced slightly in root under salt stress and this indicated that CAT activity was not affected by oxidative stress as well as salt stress. Similar our resultsNoreen et al. (2009) reported that CAT activity slightly increased under salt stress in sunflower (Helianthus annuus). Choudhury and Panda (2004), Hashemi et al. (2010) and Mutlu et al. (2013) reported that treatment with SA decreased the activity of CAT in wheat (Triticum aestivum) cultivar Bezostaya, Lepidium sativum “(article in Persian with an abstract in English)” and Oryza sativa, respectively. Moreover, Mutlu et al. (2009) demonstrated the SA treatments significantly inhibited apoplastic CAT activity under salinity. The non-significant change in CAT activity in the present SA might have been due to increase in endogenous level of SA, which might have inhibited the CAT activity. Previous studies were indicated that it is a phenomenon that occurs in many plant species exposed to oxidative (Shim et al., 2003). In addition, the decrease in CAT activity by salt stress is a phenomenon that occurs in many kinds of plant species, which has been reported not only in the gramineous species like wheat (Edral et al., 2011) but also in pea and rye treated with NaCl (Shim et al., 2003). Furthermore, the reduction of CAT activity was also due to CAT protein degradation by salt-induced endogenous proteases (Hertwig et al., 1992), increase in degradation rate of CAT protein rather than biosynthesis (Feierabend and Engel, 1986), increase in H$_2$O$_2$ content (Hashemi et al., 2010) and induced-toxicity by ROSs. On the other hand, CAT activation by salt stress and SA treated plants might be due to synthesis of new enzymes, (Feierabend and Dehne, 1996). The present results implied that SA could induce a concentration-dependent temporary increase and decrease in the CAT activity.

A significant increase was observed in APX by SA treatment in shoot while levels of SA treatment had no significant effects on the enzyme activity in root tissue. Similarly, Popova et al. (2003) reported that APX activity remained no significant changes in barely (Hordeum vulgare. L., cv. Alfa) leaves treated with 500 μM SA for 24 h in the dark. However, increased APX activity caused by SA application is well established (Agarwal et al., 2005; Yordanova and Popova, 2007; Kabiri et al., 2012), which is might be related to the key role of the enzyme in ROSs detoxification under these conditions (Kabiri et al., 2012) and could appear to be caused by over-expression of genes coding for peroxidases (Mittal and Dubey, 1991). On the other hand, it has been demonstrated that SA also can inhibit APX results in an increased level of endogenous H$_2$O$_2$ (Durner and Klessig, 1995).

In conclusion, the results revealed that SA was involved variously in growth and defense reactions of canola plants to salt stress under in vitro culture. Most probably its beneficial effect was exerted on seed germination and antioxidative defense system. The concentration-dependent changes in antioxidant enzymes activities were induced by SA treatment in salt-stressed canola plants. On the other hand, it is still difficult to separate any presumable beneficial effect of SA from the adaptive reactions to salt in plant species like canola.

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REFERENCES


