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Impact of Foliar Application with Salicylic Acid on Biochemical Characters of Canola Plants under Cold Stress Condition

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Abstract

In this study the effect of foliar application of salicylic acid on the chlorophyll content, antioxidant enzymes activity, and the content of solute protein and proline were investigated in two canola varieties (*Brassica napus* L., cv 'RGS' and 'Licord') leaves during 0, 24, and 48 hours after salicylic acid treatment. The results showed that the content of total chlorophyll was decreased in 'RGS' cultivar during the experiment and this process was related with increasing of salicylic acid concentration. The activity of superoxide dismutase, peroxidase, and also lipid peroxidation were increased significantly after 48 hours compared with the first day. The results of catalase activity showed that, this trait was decreased 24 hours after salicylic acid treatment and this decrease was related with salicylic concentration. The content of protein in both cultivars slightly changed and plants treated with salicylic acid had more protein content, by contrast proline was greatly affected by salicylic acid treatment and its content was the highest 24 hours after treatment. According to the present findings the application of salicylic acid has useful effects on the biochemical traits of *Brassica napus* cultivars. Therefore it may be effective for the improvement of plant growth in cold regions.

***Abbreviation*:** CAT-Catalase; Chl-Chlorophyll; SA-Salicylic acid; SOD-Superoxide dismutase; POX-Peroxidase; ROS-Reactive Oxygen Species.

Keywords: antioxidant enzymes, cold stress, oxidative damage, photosynthetic pigments, salicylic

Introduction

It is a well know that, low concentration of phytohormones could control the wide range of growth and development process (Yamamuro *et al*., 2016). However, the metabolic aspects of plants grown under applied phytohormones changed to varied degrees depend on the plant type and action mode (Yamamuro *et al*., 2016). Salicylic acid belongs to a group of plant phenolics which has an aromatic ring and natural product of phenylpropanoid metabolism. SA is involved in plant growth, flower induction, termogenesis and affects ions uptake (Raskin, 1992). Enhancement the pigments content of photosynthetic rate (chlorophyll and carotenoids) and modifying the activity of some important enzymes are other roles of SA (Ruelland *et al*., 2016). In terms of stress physiology, SA was first demonstrated to play a role in responses to biotic stress and is involved in signal transduction process of biotic stress tolerance (Ruelland *et al*., 2016). In addition, acts as a signal for development of hypersensitive reaction (Horváth *et al.,* 2007). However, it was gradually found to have more effects that could be of importance for other stress. Several studies show the ability of SA to produce protective effects in plant response to abiotic stress factors (Arivalagan and Somasundaram, 2015). The results of several studies shown that exogenous application of SA can aid plant tolerance with many abiotic stresses, such as: induced the increase in resistance to salinity (Szepesi *et al.,* 2005), low temperature (Janda *et al.,* 1997), water deficit (Singh and Usha, 2003), freezing (Tasgin *et al.,* 2003) and heavy metal (Mishra and Choudhuri, 1999). Exogenous application reduced the inhibitory effect of heavy metal in rice (*Oryza sativa* L.) (Mishra and Choudhuri, 1999). In wheat, seeds were soaked in acetyl salicylic acid and the plants had better resistance to drought stress (Singh and Usha, 2003). In tobacco growth in medium containing SA was observed an increase in heat tolerance (Dat *et al.,* 2000). It was shown that SA treatment applied to hydroponics growth solution of maize plants provide protection against low temperature stress (Mishra and Choudhuri, 1999). In addition SA is involved in resistance to salinity stress in tomato plants (Szepesi *et al.,* 2005).

The genus *Brassica* is an important agriculture crop grown primarily for edible oil and used for various purposes, as vegetable, fodder, and condiments. Furthermore, it is known that *Brassica* species are significant plant for investigation in resistance of abiotic stress, especially cold resistance, and great advances have been made in term of cold induced genes and antioxidant mechanisms (Wang *et al.,* 2007).

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In stress condition (low temperature especially), the primary target of damages are related to the photosynthesis and photoinhibition of photosynthesis causes the generation of ROS in the thylakoid membranes (Arivalagan and Somasundaram, 2015). These free radicals are able to damage the photosynthetic protein and pigments. In order to alleviated or prevent damage of free radicals, plants have evolved mechanisms by accumulation of low molecular weight solutes (Horváth *et al.,* 2007) and antioxidant compounds (Tasgin *et al.,* 2003), such as: SOD, CAT, and POX that scavenge the ROS. SOD is metallo-enzymes that scavenge the toxic superoxide radicals and catalyze the conversion of two superoxide anions into oxygen and H_2O_2 (Qaiser *et al.,* 2010). Then, CAT converts the H_2O_2 into water and oxygen. POD decomposes H_2O_2 by oxidation of cosubstrates, such as: phenolic compounds and antioxidants (Ebrahimia *et al*., 2014).

The current research was conducted in order to determine the effect of salicylic acid treatment in two canola varieties and to add more information on the physiological traits under cold stress. For this goal, it has been investigated the changes in enzymes activity status, proline accumulation, protein solution, lipid peroxidation and chlorophyll pigments, related to defence strategies in each variety.

Materials and Methods

Biological material and treatments

Seeds of canola (*Brassica napus* L.) used in this experiment were surface sterilized, then washed several times with distilled water. Ten sterilized canola seeds were sown in 20 cm diameter of plastic pots filled with clay/organic/perlit (3/1/1, v/v/v) mixture and placed under controlled environment conditions with a day/night temperature of 22/20 °C for one month with a 16 h daily light period (supplemented, if necessary, by 400 W sodium lamps). For SA treatment, thirty day old plants were spraying by salicylic acid. For cold stress treatment the plots were performed in a freezing chamber at -2 °C for 3 days (after 24 h of salicylic treatment). The cold treatment was carried out in a Conviron PGV-36 chamber (Controlled Environments Ltd) at -2 °C with a photosynthetic photon flux density of 250 µmol $m^{-2}s^{-1}$ provided by metal halide lamps and a 12 h photoperiod. Based on a preliminary experiment, the optimum concentrations for pre-treatments were: 100, 200 and 400 µM salicylic acid. Plants treated with similar volume of distilled water were used as controls. Some of the plants were pre-treated with 50, 100, 500 and 5000 µM SA by spraying for 1 d at 22/20 °C. Preliminary experiments showed that treatment with 50 and 500 µM SA was not reproducibly effective, while higher concentrations (5000 µM) often caused visible damage to the plants after 5 d of treatment under normal growth conditions. The plants were firstly sprayed by 100 cm^3 of the appropriate solution or water and then were transferred to the chamber. To determine the biochemical changes, the leaves were sampled after 0, 24 and 48 h of chilling.

Chlorophyll assay

According to the method of Arnon (1949), chlorophyll was extracted in 80% acetone from leaves. Extracts were filtrated and content of total Chl was determined by spectrophotometry at 645 and 663 nm. The content of Chl was expressed as $mg.g^{-1}$ FW.

Total chlorophyll=(0.0202)×(A.645)+(0.00802)×(A.663)

Malondialdehyde assay

The level of lipid peroxidation was analysed in terms of malondialdehyde (MDA) contents reacting to thiobarbituric acid (TBA) reactive substance using the method of De Vos *et al.* (1991). Samples were homogenized in an aqueous solution of TBA (10% w/v) and 1 ml aliquot of appropriately diluted sample was added to a test tube with an equal volume of either thiobarbituric acid (TCA) solution containing 25% (w/v) TCA, then mixtures were heated at boiling water (95 °C) for 25 min. The amount of MDA was determined from the absorbance of the supernatant at 532 and 600 nm. The content of MDA was determined using the extinction coefficient of MDA (ε=155 µ$M^{-1}cm^{-1}$).

Preparation enzyme of extracts

Leaf samples (0.2 g) were homogenized in a mortar and pestle with 3 ml ice cold extraction buffer (50 mM potassium phosphate, pH 7). The homogenate was centrifuged at 18000 g for 30 min at 4 °C then the supernatant was filtered through filter paper. The supernatant fraction was used as a crude extract for the assay of enzyme activity and PROT content. All operations were carried out at 4 °C.

Assay of antioxidant enzymes activity

Enzyme activities were measured at 25 °C using a spectrophotometer model Variam Cary Win UV 6000i, Australia.

Superoxide dismutase activity was determined according to the method of Giannopolitis and Ries (1977). The reaction mixture contained 100 µl 1 µM riboflavin, 100 µl 12 mM L-methionine, 100 µl 50 mM Na_2CO_3 (pH 10.2), and 100 µl 75 µM nitro blue tetrazolium (NBT) in 2.3 ml 50 mM potassium phosphate buffer (pH 7), with 200 µl crude enzyme extract in a final volume of 3 ml. SOD activity was assayed by measuring the ability of the enzyme extract to inhibit the photochemical reduction of NBT (with some modification). Glass test tubes containing the mixture were illuminated with a fluorescent lamp (120 W); identical tubes that were not illuminated served as blanks. After illumination for 15 min, the absorbance was measured at 560 nm. Blank were run in the same way but without illumination. One unit of SOD was defined as the amount of enzyme activity that was able to inhibit by 50% the photoreduction of NBT to purple formazan. The SOD activity of the extract was expressed as SOD units per milligram of protein.

Peroxidase activity was determined by the oxidation of guaiacol in the presence of H_2O_2. The increase in absorbance at 470 nm was recorded for 1 min (Ghanati *et al*., 2002). The reaction mixture contained 100 µl crude enzyme, 500 µl 5 mM H_2O_2, 500 µl guaiacol 28 mM, and 1.9 ml 50 mM potassium phosphate buffer (pH 7). POX activity of the extract was expressed as POX units per milligram protein.

Catalase activity was estimated by the method of Cakmak and Horst (1991). The reaction mixture contained 100 µl crude enzyme extract, 500 µl 10 mM H_2O_2, and 1.9 ml 50 mM potassium phosphate buffer. The decrease in the absorbance at 240 nm was recorded for 1 min. CAT activity of the extract was expressed as CAT units per milligram of protein.

Protein assay

Total protein content was determined using bovine serum albumin (BSA) as a standard, as described in Bradford (1976),

using 1 ml Bradford solution and 100 µl crude extract. The protein concentration was calculated from a BSA standard curve.

Proline assay

Proline content was determined according to the method of Bates *et al.* (1973) which was modified as follows. Samples of leaves (0.2 g) were homogenized in a mortar and pestle with 3 ml sulphosalicylic acid (3 % w/v), and then the homogenate was centrifuged at 18000 g for 15 min. 2 ml of the supernatant were then put into a test tube which 2 ml contained glacial acetic acid and 2 ml freshly prepared acid ninhydrin solution (1.25 g ninhydrin dissolved in 30 ml glacial acetic acid and 20 ml 6 M orthophosphoric acid) were added. Tubes were incubated in a water bath for 1 h at 100 °C, and then allowed to cool at room temperature. After that 4 ml of toluene were added and mixed on a vortex mixer for 20 s. The test tubes were allowed to stand for at least 10 min to allow the separation of the toluene and aqueous phases. The toluene phase was carefully pipetted out into a glass test tube, and its absorbance was measured at 520 nm in a spectrophotometer. The content of proline was calculated from a PROL standard curve and was expressed as mg g^{-1} per fresh weight.

Statistical analysis

All data were analysed using SAS software (SAS Institute Inc. 2002). Each treatment was analysed in three replications and the analyses of the variance (ANOVA) were perform to test the difference between cultivar, salicylic concentration, cold stress and their interaction. When ANOVA showed significant treatment effects, Duncan's multiple range test was applied to compared the means at ($P< 0.05$) (Steel and Torrie, 1980).

Results

The cold stress and SA treatments, as well as the interaction of them, showed that there was a significant effect on canola physiological traits (Tables 1, 2 and 3). These traits significantly decreased by cold stress treatment and increased by SA application.

As shown in Fig. 1 total Chl content declined during the experiment and the maximum content was observed in first day in both cultivars but cold stress dramatically reduced this parameter in plants. Plants treated with SA showed less decline than non-treated, and concentration of SA was directly related to Chl concentration. The Chl level was greater in 200 µM than water spraying; conversely 400 µM SA treatment resulted in decreasing this trait.

The content of MDA (as a key for evaluating the membrane oxidation) was upward during the experiment and 'RGS' cultivar in the third day in non-SA treatment had the highest content of MDA. The lowest content of MDA was observed in 'Licord' cultivar, with 200 µM SA in first day (Fig. 2).

Antioxidant enzyme activity of SOD was significantly increased in stress condition also the enzyme activity increased with increasing concentration of SA and resistant cultivar in 200 µM SA treatment had the highest activity of this enzyme. In third sampling, activity of SOD had slightly increased or even decreased (Fig. 3).

Peroxidase enzyme activities as well as SOD increased in stress condition and during the experiment were an upward. In addition, the plants treated with SA increased the activity of this enzyme compared with no treatment. The highest activity of this enzyme was observed in the third day with 200 µM SA treatment in resistant cultivars ('Licord'), but 400 µM SA reduced the activity of this enzyme in this cultivar. By contrast, further increase in SA concentration (400 µM) in resistance cultivar ('Licord') showed no increase in POX activity at third day (Fig. 4).

The level CAT activity during the experiment was in a downward trend whereas the lowest activity was observed in the 'RGS' cultivars with 200 µM SA application. It is notable that in this treatment, the enzyme activity was increased in third day (Fig. 5).

Protein concentration also decreased affected cold stress and SA spraying was effective in protein content, too. The lowest content was in the 'RGS' cultivar and in non-SA treatment. The content of protein was little

Table 1. Analysis of variance on total Chl, MDA, SOD, CAT,POX, protein and proline of canola genotypes, treated by SA in first day

SOV	df	Total Chl	MDA	SOD	CAT	POX	PROT	PROL
Rep	2	0.0038 ^{ns}	0.0008 ^{ns}	0.0257 **	0.000066 ^{ns}	0.000087 ^{ns}	0.415 ^{ns}	0.018 ^{ns}
Variety	1	0.0620 **	0.0040 ^{ns}	0.000016 ^{ns}	0.00041 ^{ns}	0.00010 ^{ns}	3.62 *	0.0057 ^{ns}
Concentration	3	0.0199 ^{ns}	0.00091 ^{ns}	0.0079 ^{ns}	0.00016 ^{ns}	0.000048 ^{ns}	0.74 ^{ns}	0.0316 ^{ns}
Interaction	3	0.1445 **	0.00108 ^{ns}	0.0044 ^{ns}	0.00002 ^{ns}	0.0015 **	3.28 **	0.0033 ^{ns}
Error	14	0.0068	0.0016	0.0026	0.000104	0.00013	0.57	0.028
CV %		5.68	8.54	11.25	7.26	9.36	3.86	11.70

*, ** and ns significant at 0.05, 0.001 probability level and no significant, respectively. *CAT-Catalase; Chl-Chlorophyll; SA-Salicylic acid; SOD-Superoxide dismutase; POX-Peroxidase*

Table 2. Analysis of variance on total Chl, MDA, SOD, CAT,POX, protein and proline of canola genotypes treated by SA in second day

SOV	df	Total Chl	MDA	SOD	CAT	POX	PROT	PROL
Rep	2	0.0105 ^{ns}	0.00030 ^{ns}	0.0021 *	0.00000038 **	0.000028 ^{ns}	0.42 ^{ns}	0.0062 ^{ns}
Variety	1	0.0133 ^{ns}	0.00018 ^{ns}	0.891 **	0.00010 **	0.000497 **	8.56 **	20.97 **
Concentration	3	0.024 **	0.00046 ^{ns}	0.043 **	0.0012 **	0.00174 **	3.586 **	0.74 **
Interaction	3	0.026 **	0.0035 *	0.053 **	0.00037 **	0.0019 **	1.08 ^{ns}	0.147 ^{ns}
Error	14	0.0039	0.00066	0.00052	0.000010	0.000024	0.44	0.0632
CV %		6.40	7.90	1.59	6.33	3.23	3.71	9.74

*, ** and ns significant at 0.05, 0.001 probability level and no significant, respectively. *CAT-Catalase; Chl-Chlorophyll; SA-Salicylic acid; SOD-Superoxide dismutase; POX-Peroxidase*

Table 3. Analysis of variance on total Chl, MDA, SOD, CAT, POX, protein and proline of canola genotypes treated by SA in third day

SOV	df	Chl total	MDA	SOD	CAT	POX	PROT	PROL
Rep	2	0.0048 ^{ns}	0.00089 ^{ns}	0.0068	0.000040 *	0.0000018 ^{ns}	1.28 ^{ns}	0.35 ^{ns}
Variety	1	0.0025 ^{ns}	0.034 **	1.147 **	0.0001 **	0.0015 **	0.58	11.10 **
Concentration	3	0.027 *	0.025 **	0.099 **	0.00087 **	0.0012 **	9.668 **	0.96 **
Interaction	3	0.039 **	0.0047 **	0.0025 ^{ns}	0.00002 ^{ns}	0.00071 **	11.35 **	0.273 ^{ns}
Error	14	0.0049	0.00033	0.0033	0.0000089	0.000013	0.46	0.13
CV %		7.02	6.83	4.13	6.98	2.47	3.99	11.86

*, ** and ns significant at 0.05, 0.001 probability level and no significant, respectively. *CAT-Catalase; Chl-Chlorophyll; SA-Salicylic acid; SOD-Superoxide dismutase; POX-Peroxidase*

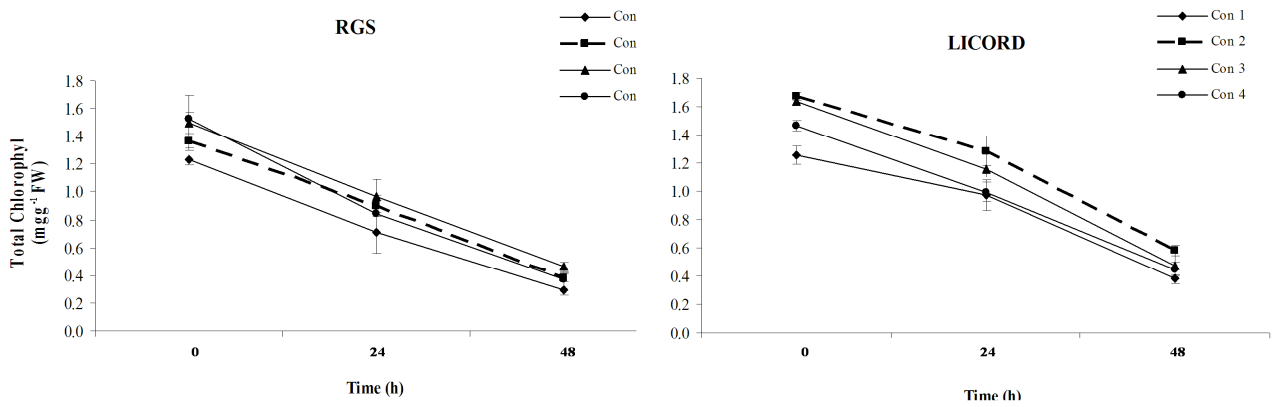

Fig. 1. Effect of different concentration of SA on total Chl content in 'RGS' and 'Licord' cultivars (Con 1-0 µM/L SA; Con 2-100 µM/L SA; Con 3-200 µM/L SA; Con 4-400 µM/L SA)

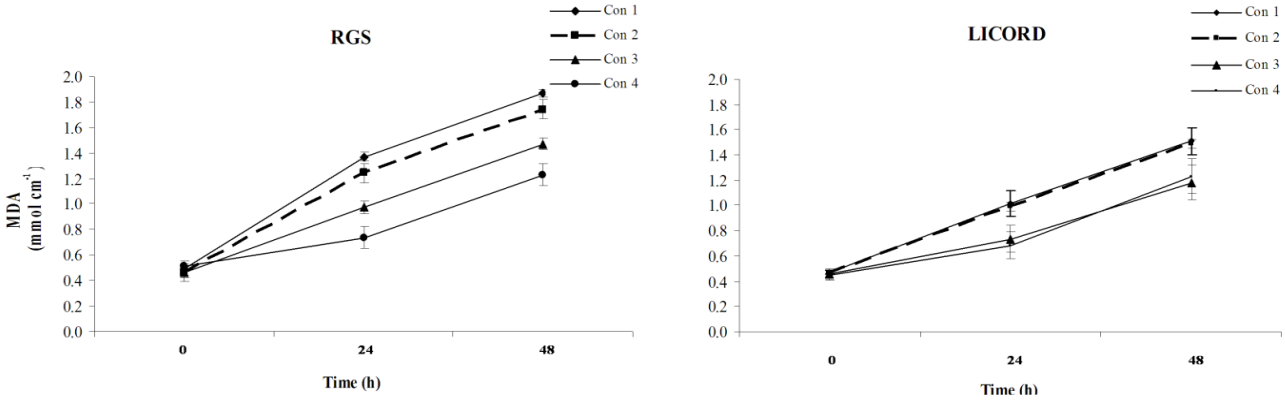

Fig. 2. Effect of different concentration of SA on MDA content in 'RGS' and 'Licord' cultivars (Con 1-0 µM/L SA; Con 2-100 µM/L SA; Con 3-200 µM/L SA; Con 4-400 µM/L SA)

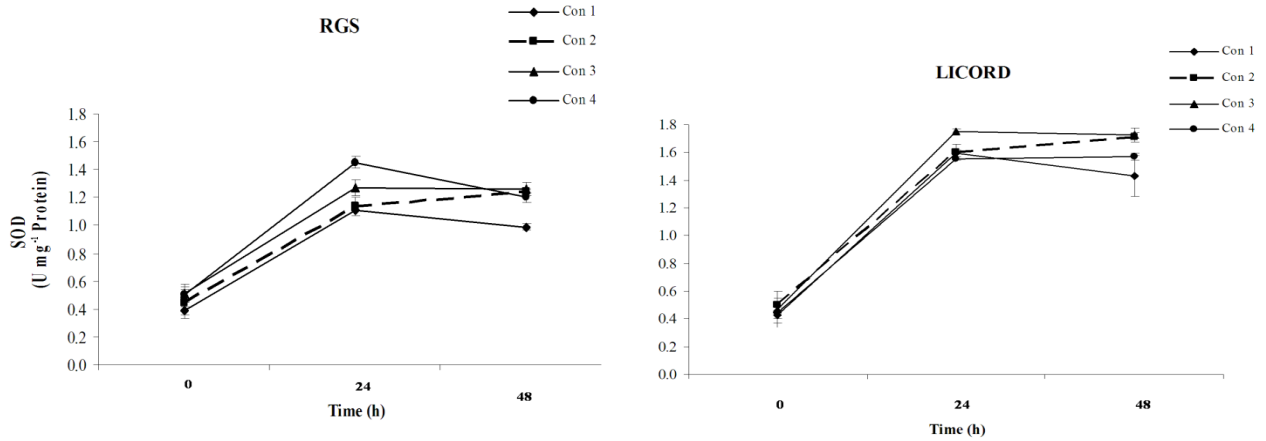

Fig. 3. Effect of different concentration of SA on SOD activity in 'RGS' and 'Licord' cultivars (Con 1-0 µM/L SA; Con 2-100 µM/L SA; Con 3-200 µM/L SA; Con 4-400 µM/L SA)

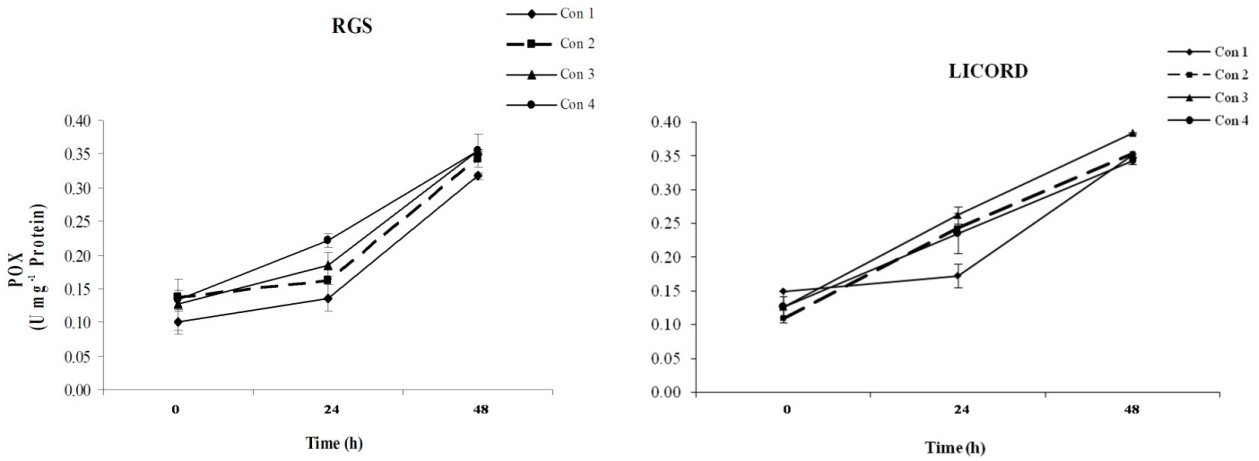

Fig. 4. Effect of different concentration of SA on POX activity in canola
(Con 1-0 µM/L SA; Con 2-100 µM/L SA; Con 3-200 µM/L SA; Con 4-400 µM/L SA)

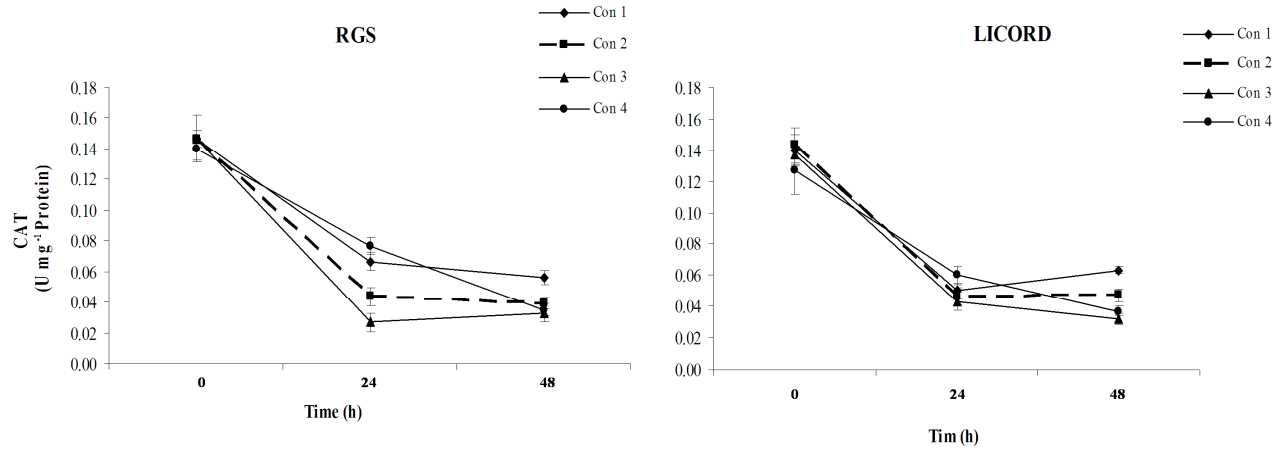

Fig. 5. Effect of different concentration of SA on CAT activity in 'RGS' and 'Licord' cultivars
(Con 1-0 µM/L SA; Con 2-100 µM/L SA; Con 3-200 µM/L SA; Con 4-400 µM/L SA)

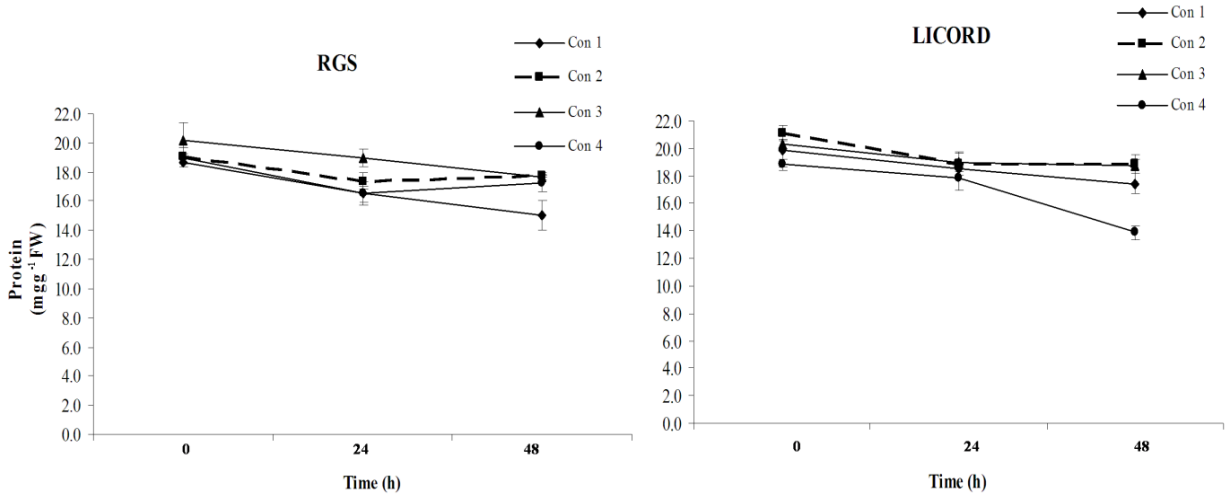

Fig. 6. Effect of different concentration of SA on protein content in 'RGS' and 'Licord' cultivars
(Con 1-0 µM/L SA; Con 2-100 µM/L SA; Con 3-200 µM/L SA; Con 4-400 µM/L SA)

changed in the second day and third day. Although, protein levels were reduced by 400 µM SA in 'Licord' cultivar (Fig. 6).

Proline was greatly affected by SA treatment and its content was the highest in third day after treatment with 200 µM SA in 'Licord'.

Other concentrations, similar 400 µM SA led to increasing in proline content in second day after treatment then decreased.

In 'RGS' genotype, proline content was higher in 400 µM SA than other concentrations and it was the highest in end of the experiment (Fig. 7).

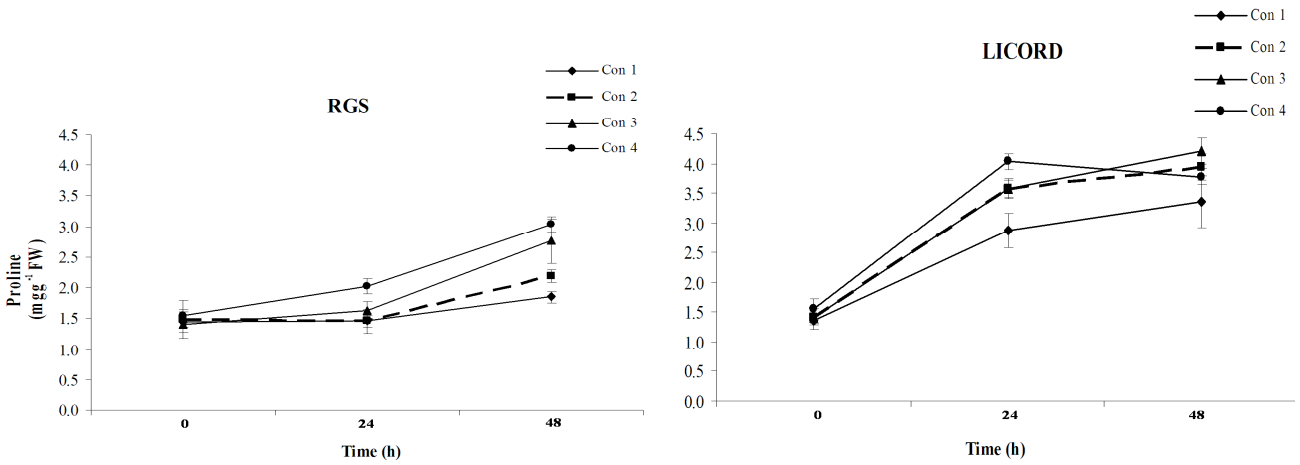

Fig. 7. Effect of different concentration of SA on proline content in 'RGS' and 'Licord' cultivars (Con 1-0 µM/L SA; Con 2-100 µM/L SA; Con 3-200 µM/L SA; Con 4-400 µM/L SA)

Discussion

The results showed that SA application increased the content of total Chl in both cultivars. The loss of Chl is one of the symptoms of oxidative stress (Manafi *et al*., 2015). Cold stress caused to an increase in free radicals in chloroplast which results in cellular damaging and decline in membrane permeability. Therefore certain metabolic such as carbon fixation might have been affected. Because the reaction of photosynthesis is hindered by stress condition and the excessive light energy cannot be used for the reduction of $NADP^+$, superoxide anion of the ROS is generated (Manafi *et al*., 2015). To avoid cellular damage, the Chl needs to be degraded quickly. Maybe low temperature leads to increase ROS in chloroplast and caused the Chl molecules destruction and damaged the chloroplast membrane system and photosynthetic reaction centre. Probably, SA as a detoxifier of ROS might prevent the activity of free radicals alleviated the superoxide radicals and can enhance leaf Chl content. Similar results were reported by Manafi *et al.* (2015) in soybean and Kang and Saltveit (2002) in maize. Chilling stress causes not only a substantial damage to photosynthetic pigments, but also leads to deterioration to thylakoid membrane. In severe chilling stress condition chlorophyllase increased as a result the chlorophyll content are decreased. The exogenous application of SA, significantly increased the activity of antioxidant system and it's forced a direct impact on the intensity of photosysthesis.

Stress conditions and unfavourable environmental factors induced oxidative stress in plants tissues (Manafi *et al*., 2015). When chloroplasts are expose to excessive excitation energy, it caused generation of ROS and induce oxidative stress (Tasgin *et al.,* 2003). To prevent damage of free radicals, plants have developed mechanisms by accumulation antioxidant systems (Tasgin *et al.,* 2003). Relatively higher activities of ROS scavenger enzymes have been reported in many plants which suggested that the antioxidant system play an important role in plants against environmental stress (Qaiser *et al.,* 2010).

Recent studies have demonstrated that SOD may function as a ROS scavenger by converting $O^{\cdot}$ to H_2O_2, and can lead to enhanced stress tolerance (Manafi *et al*., 2015). Similar findings were presented under stress condition in higher plants (Horváth *et al.,* 2007). The present results showed that SA induced SOD activation that consistent with results obtained with other various plants species (Kang and Saltveit, 2002). The activity of SOD increased in both cultivars during the whole experiment period that maybe caused of low temperature. Probably, cold condition directly or indirectly lead to production of ROS, which result in increased oxidative stress and SOD, which is one of the key enzymes antioxidant system that scavenger of free radical, converts one form of ROS to H_2O_2 (Manafi *et al*., 2015).

H_2O_2 is converted to oxygen and water by CAT and POX which use ascorbate as the hydrogen donor. It is considered that one of the functions of SA is the inhibition of CAT, resulting in H_2O_2 accumulation (Dat *et al.,* 1998; Horváth *et al.,* 2002). The current results showed that CAT activity decreased when plants were treated with SA (Fig. 5). Similar results shown that CAT was found to be inhibited in some plant (Dat *et al.,* 1998; Janda *et al.,* 1999). However other reports showed an increases in CAT activity after SA treatment (Horváth *et al.,* 2007). Activities of POX enzyme was also affected by SA treatment (Fig. 4). It has been found that SA has a protective role against ROS and acts as an activated antioxidant system and scavenging free radicals with activated the antioxidant system. These free radicals can damage the protein and nucleic acids (Manafi *et al*., 2015). It has been observed that leaf activity of SOD and POX increased with increasing in SA concentration that probably low temperature induced oxidative damage. Thus, induction of antioxidative defence mechanisms may reflect the plant response requirement to overcome oxidative injury induced by environmental stress. These findings suggest that an accumulation of ROS may be occurred in response to low temperature and SA by increasing the antioxidant enzymes activity and reduced oxidative injury. Thus, the obtained data demonstrated that activation of antioxidant enzymes induced by the treatment with SA and this contribute to its anti-stress effects in plants. Similarly increase in the activities of SOD (Horváth *et al.,* 2007) and POX (Janda *et al.,* 1999; Kang and Saltveit, 2002) and decrease in CAT activities (Senaranta *et al.,* 2000) has been reported.

Under cold stress conditions, oxidative damage may occur, due to overproduction of ROS (Dat *et al.,*, 2000) and rate of neutralizing of ROS by enzymatic antioxidants are essential to maintain the concentration of ROS at relatively low level.

Many reports have confirmed that plants could employ their antioxidative system to alleviate chilling stress induced oxidative injury (Horváth *et al.,* 2007). This observation was supported by the increase of MDA or lipid peroxidation in cold condition. The role of SA has been studied in many physiological processes and reported that exogenous SA could increase environmental tolerance in some plants (Horváth *et al.*, 2007). The current results suggest that SA application may control the cold induced oxidative stress in canola plants and it is also associated with the raising of total SOD and POX activity and decreased in accumulation of MDA.

Low level of protein in treated plants with distillation maybe related to oxidative damage which mediated the degradation of proteins (Noctor and Foyer, 1998). Probably SA by neutralization of free radicals prevented the destruction of protein and leads to increasing in leaves protein solution.

In a wide range of environmental stress, free proline expands and it is established that proline is a highly water soluble amino acid (Manafi *et al*., 2015) and are involved in the succession resistant capability of plants (Parvanova *et al.,* 2004). Recently it has been reported that increasing the level of proline in transgenic tobacco lead to increased tolerance in high level of cold stress (Parvanova *et al.,* 2004). Winter wheat with high level of proline has greater tolerance in chilling stress (Doerffling *et al.,* 1993). In addition Galiba (1994) reported direct correlation between proline level and chilling tolerance in various species. The present results showed that the greatest level of obtained proline was in third and second day after treatment in 'Licord' and 'RGS', respectively.

The present findings showed that suitable concentration of SA activated antioxidant activities; however, higher concentration of SA, inversely depressed activity of antioxidative enzymes. The chilling stress affected the biochemical process and reduced photosynthetic pigments. Application of SA can increased the antioxidant activity in canola than compared to untreated plants. Use of SA can reduces the destructive effect of chilling stress and it was shown that 200 μM had better result on physiological process.

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