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Effects of foliar application with salicylic acid on the biochemical parameters and redox status in two Canola plant varieties exposed to cold stress

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Abstract

Low temperature is an important environmental factor that limits the survival, productivity and geographical distribution of plants. Oil seeds are the second global food resources among which *Brassica napus* L. is the third annual oil seed in the world. In cold stress, some biochemical and physiological reactions occur in response to reactive oxygen species (ROS). Hence, the effect of foliar application of salicylic acid (SA) on total chlorophyll content, malondialdehyde, and antioxidant enzymes activity and solute protein and proline contents were assessed in two canola varieties (*Brassica napus* L., cv RGS and LICORD) leaves exposed to cold stress during 0, 24, and 48 hours after salicylic acid treatment. They were first grown in a controlled growth room at 22/20 °C (day/night) for one month followed by SA spraying application (100, 200 and 400µM) and then plots were transferred to a cold environment (-2 °C) for 3 days. The results showed that the total chlorophyll content was decreased in RGS cultivars related to high salicylic acid concentration during the experiment. The results of antioxidant status showed that superoxide dismutase (SOD), peroxidase (POX), and also lipid peroxidation were increased significantly after 48 hours compared first day. Catalase (CAT) activity was decreased 24 hours after salicylic acid treatment. Results showed an increase in protein content in both cultivars treated with SA, by contrast proline was greatly affected by salicylic acid treatment and its content was the highest 24 hours after treatment. According to the results of the present study indicated that application of salicylic acid has useful effects on the biochemical traits. Thereupon it may be effective for the improvement of plant growth in cold regions.

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Introduction

It is a well know that, low concentration of phytohormones could regulation the wide rang of growth and development process. However, the metabolic aspects of plants applied with phytohormones shifted to varied degrees depend on the plant type and action mode. Salicylic acid belongs to a group of plant phenolics which has an aromatic ring and natural product of phenylpropanoid metabolism. SA involved in plant growth, flower induction, and Termogenesis (Raskin, 1992). It also has lots of affects on ions uptake (Raskin, 1992).

Enhancement of the content pigments photosynthetic rate (chlorophyll and carotenoids), and modifying the activity of some of the important enzymes are other roles of SA. In terms of stress physiology, SA was first demonstrated to play a role in responses to biotic stress and involved in signal transduction process of biotic stress tolerance. 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. The results of several studies shown that exogenous application of SA can aid plant tolerance with many abiotic stresses, such as; induced increase in the 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; the plants had better resistance to drought stress (Singh and Usha 2003). In tobacco that growth in medium containing, SA caused to increased 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). SA involved in resistance to stress form salinity for example in tomato plants (Szepesi *et al.,* 2005).

The genus *Brassics* is an important agriculture crop grown primarily for edible oil and used for various purposes, e.g., 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).

In stress condition (low temperature especially), the primary target of damages related to the photosynthesis. In this situation, photoinhibition of photosynthesis is causes of the generation of Reactive Oxygen Species (ROS) in the thylakoid membrance. These free radicals are able to damage to protein and pigments photosynthetic. 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.

The recent work was conducted in order to determined salicylic acid treatment and its effect in two canola varieties to give more information on the physiological traits under chilling stress. For this purpose, we investigated the change in enzymes activity status, proline accumulation, protein solution, lipid peroxidation and chlorophyll pigments to related them to defense strategies in each variety.

Materials and methods

Plants and treatments

Seeds of canola (*Brassica napus* L.) used in this experiment were surface sterilized with sodium hypochlorite solution for 5 min and ethanol 96 % for 30 s, 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 after growing plants were thinned to five plants per pot. All pots were placed into greenhouse located in Faculty of natural and life sciences, University of Oran 1 Ahmed BenBella, Oran, Algeria under environment controlled conditions with at 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. The pots were watered to field capacity every other day. For SA treatment, thirty day old plants were spraying by salicylic acid and for cold stress treatment, twenty four hours after salicylic treatment the plots were performed in a freezing chamber at -2°C for 3 days. 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 400µM 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 activities of antioxidant activity (SOD, POX and CAT), content of protein solution, total chlorophyll, proline accumulation and malondialdehyde (MDA) leaves were sampled after 0, 24 and 48 h of chilling. Plant material for biochemical analysis was fresh frozen in liquid N_2 and, stored at -80 °C. Each measurement was done independently.

Chlorophyll assay

According to the method of Arnon (1949), Chlorophyll was extracted in 80% acetone from the leaf samples. 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 ($mg.g^{-1}$) = (0.0202) × (A.645) + (0.00802) × (A.663)

Malondialdehyde assay

The level of lipid peroxidation was analyzed 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 (TBA) solution containing 25% (w/v) of trichloro-acetic acid (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 µM $^{-1}cm^{-1}$).

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 (EC 1. 15. 1. 1) 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 (EC 1. 11. 1. 7) 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 (EC 1. 11. 1. 6) 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 to room temperature then 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 PROL was calculated from a PROL standard curve and was expressed as mg g^{-1} per fresh weight.

Statistical analysis

All data were analyzed using SAS software (SAS Institute Inc. 2002). Each treatment was analyzed in three replications and when analyses of the variance (ANOVA) were performance to test for 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

Analysis of variance showed significant interaction between SA treatment and cold stress on Chl and POX activity ($p= 0.001$) and total protein ($p=0.05$) in cultivars, in first day of experiment (table 1). Furthermore, the Chl and total protein content show a different variety to SA treatment. These parameters appear to be actively involved in response to SA application in plant exposed to cold stress. In second day, significant interaction effects on antioxidant enzymes (SOD, CAT, POX) activity, protein and proline content were shown in response to SA in cultivars (Table 2). Whereas, both physiological traits and antioxidant enzymes activity underwent changes in term of concentration with the exception of MDA content which does not seem to be affected with SA treatment.

Table 1. Analysis of variance on Chl total, MDA, SOD, CAT,POX, protein and proline of canola 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

Table 2. Analysis of variance on Chl total, MDA, SOD, CAT,POX, protein and proline of canola 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 *	0.0000038 **	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.

In the third day of SA application, the variance analysis (table 3) demonstrated a high interaction effect (P=0.001) in all traits with the exception of SOD, CAT and proline. In addition, Chl, protein, proline and MDA content and also antioxidant enzymes activity (P=0.001) were significantly affected by SA treatment in cultivars. Taken together, these results suggest that there was a significant effect of the cold stress and SA treatments as well as the interaction of them on physiological traits. These traits significantly decreased by cold stress treatment and increased by SA application.

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

SOV	df	Total Chl	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.

As shown in Fig.1 total chl content declined during the experiment and the maximum content was observed at first day in both cultivars but cold stress dramatically reduced this parameter in plants. The content of chl was lowest on the third day in sensitive cultivar in non- SA treatment. SA treatment was effective in chl reducing and plants treated with SA showed less decline than non-treated, also

concentration of SA was directly related to chl concentration and the chl level was greater in 200 µM than water spraying, conversely 400 µM SA treatment resulting in decreased this trait again. The content of MDA (as an indicator for evaluating of the oxidation of membrane) was upward during the experiment and RGS cultivar at third day in non-SA treatment had the highest of MDA and the lowest content of MDA was observed in Licord cultivar, with 200 µM SA at 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 but in 400 µM SA treatment activity was decreased. 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; also, 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. 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 showed no increase in POX activity at third day (Fig. 4). The level activity of CAT during the experiment was a downward trend whereas the lowest activity was observed in the RGS cultivars with 200mm SA application. It is notable that in this treatment, the enzyme activity was increased in third day (Fig. 5).

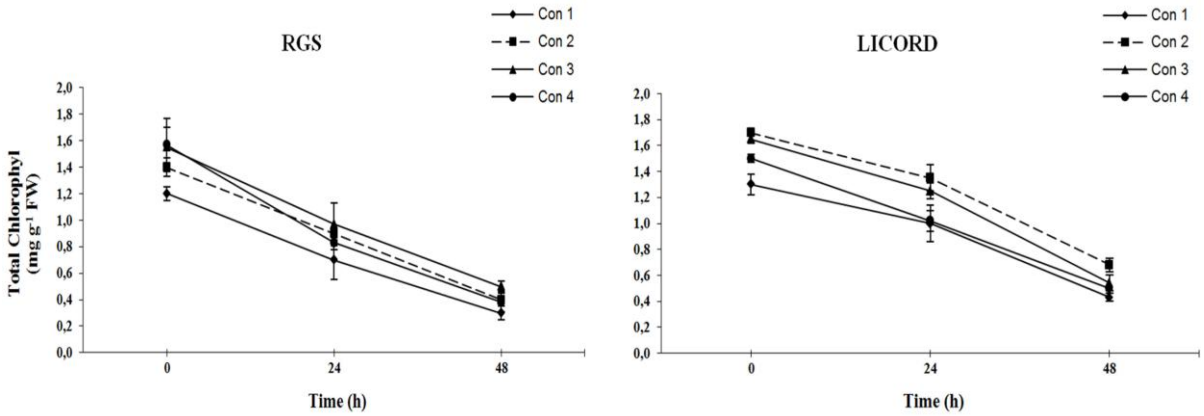

Fig. 1. Effect of different concentration of SA on total chl content in RGS and Licord cultivar. (Con 1) 0 µM/L SA; (Con 2) 100 µM/L SA; (Con 3) 200 µM/L SA; (Con 4) 400 µM/L SA.

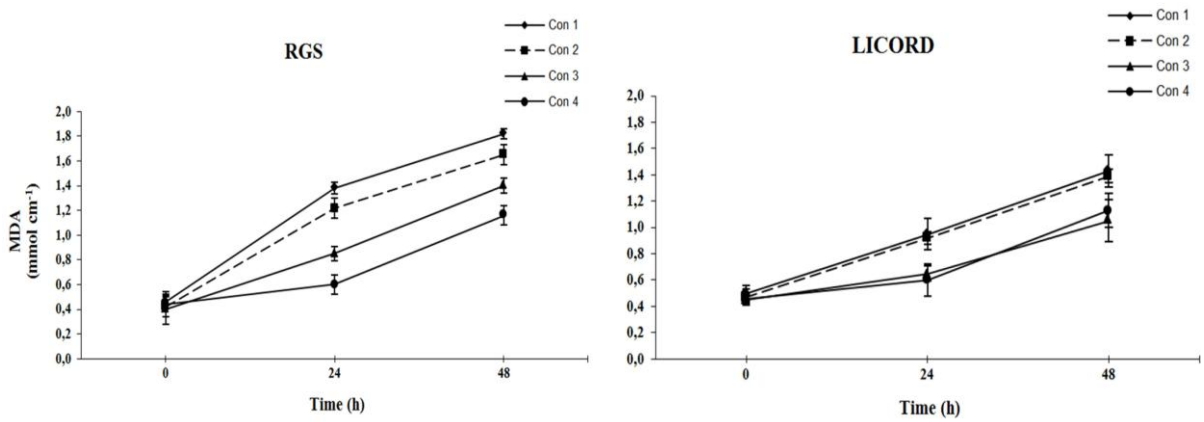

Fig. 2. Effect of different concentration of SA on MDA content in RGS and Licord cultivars. (Con 1) 0 µM/L SA; (Con 2) 100 µM/L SA; (Con 3) 200 µM/L SA; (Con 4) 400 µM/L SA.

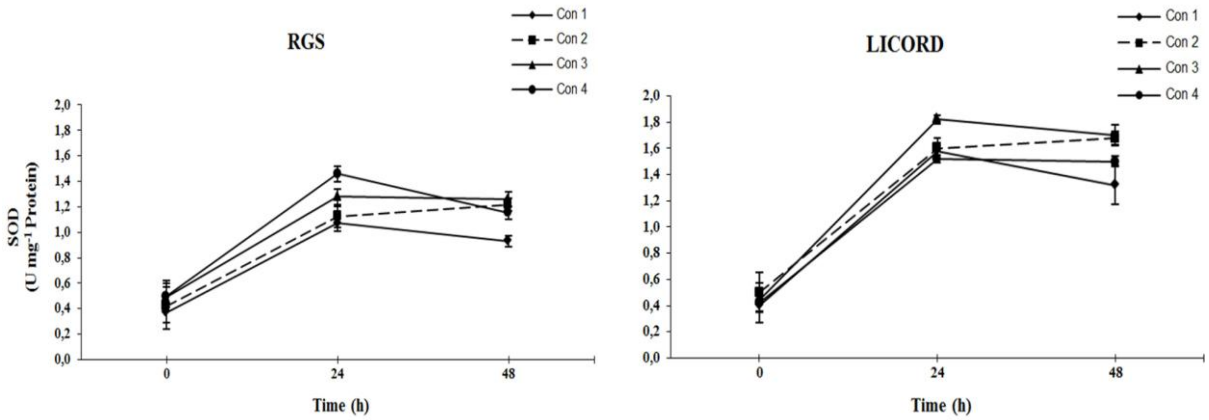

Fig. 3. Effect of different concentration of SA on SOD activity in RGS and Licord cultivars. (Con 1) 0 µM/L SA; (Con 2) 100 µM/L SA; (Con 3) 200 µM/L SA; (Con 4) 400 µM/L SA.

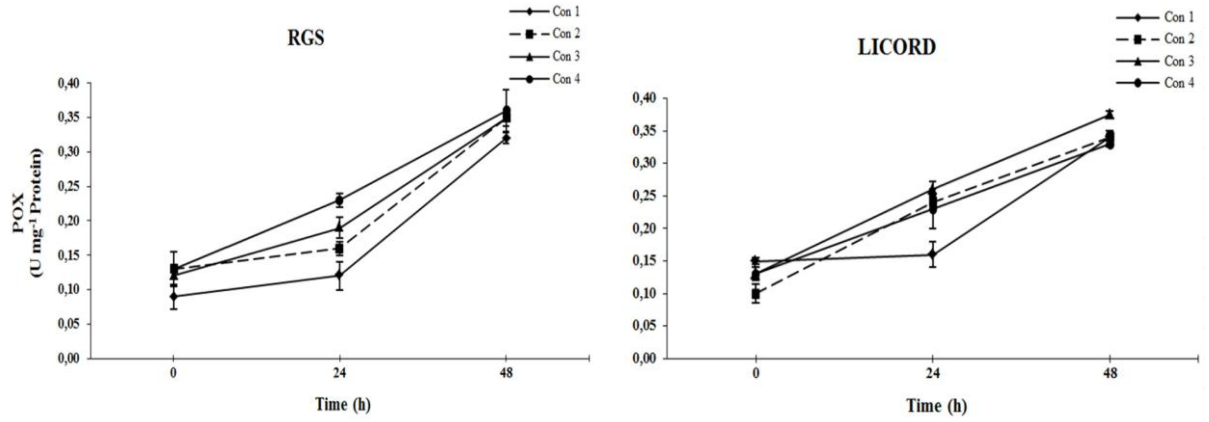

Fig. 4. Effect of different concentration of SA on POX activity in canola. (Con 1) 0 µM/L SA; (Con 2) 100 µM/L SA; (Con 3) 200 µM/L SA; (Con 4) 400 µM/L SA.

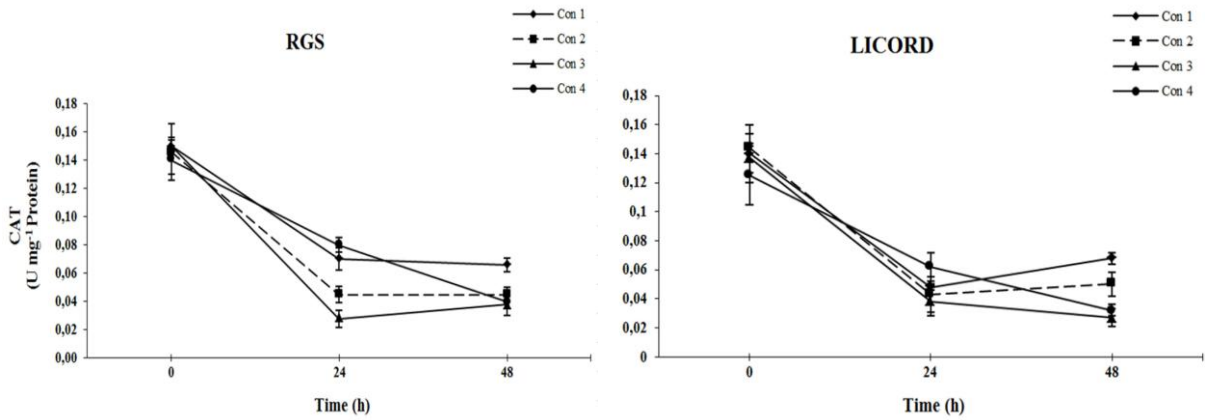

Fig. 5. Effect of different concentration of SA on CAT activity in RGS and Licord cultivars. (Con 1) 0 µM/L SA; (Con 2) 100 µM/L SA; (Con 3) 200 µM/L SA; (Con 4) 400 µM/L SA.

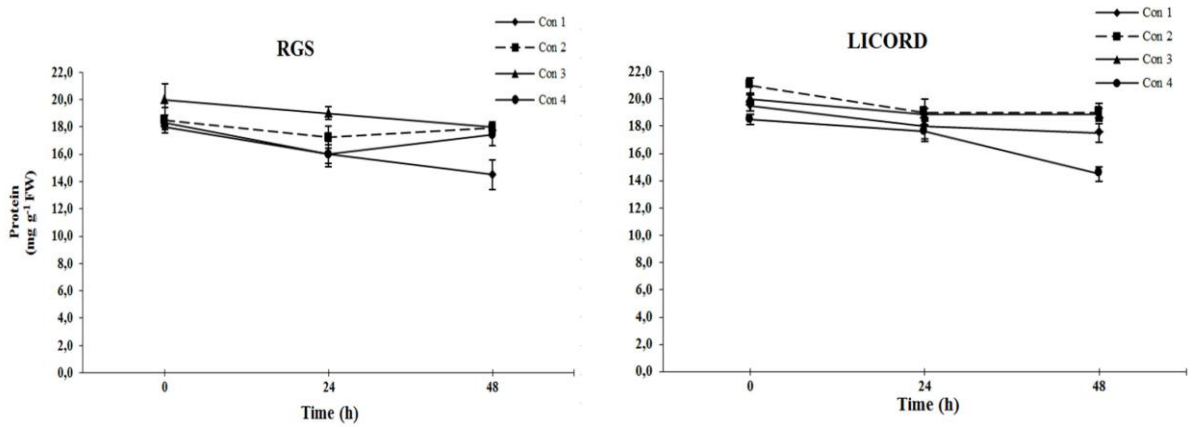

Fig. 6. Effect of different concentration of SA on protein content in RGS and Licord cultivars. (Con 1) 0 µM/L SA; (Con 2) 100 µM/L SA; (Con 3) 200 µM/L SA; (Con 4) 400 µM/L SA.

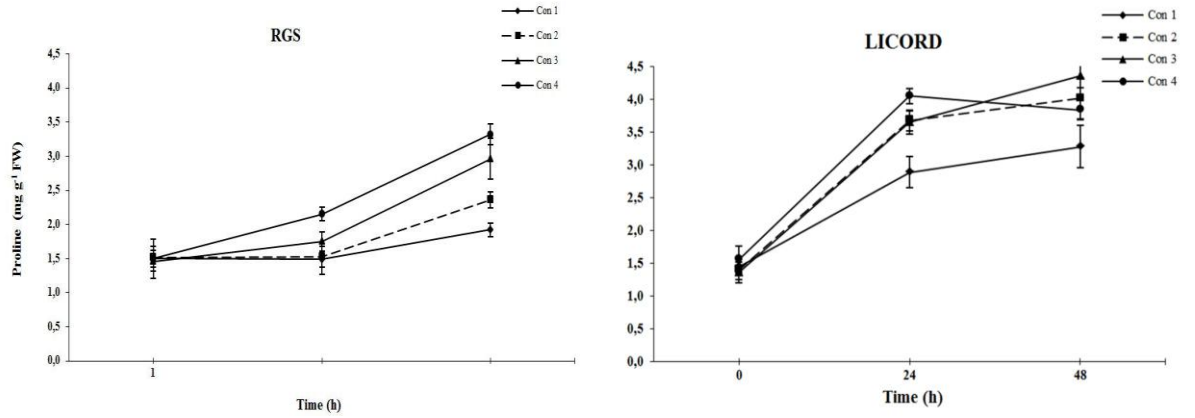

Fig. 7. Effect of different concentration of SA on proline content in RGS and Licord cultivars. (Con 1) 0 µM/L SA; (Con 2) 100 µM/L SA; (Con 3) 200 µM/L SA; (Con 4) 400 µM/L SA.

Protein concentration also decreased affected cold stress and SA spraying was effective in protein content, too. The lowest was in the RGS cultivar and non-SA treatment. The content of protein was little changed on the second day to 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 3 days after treatment with 200 µM SA in LICORD. Other Concentrations, similar 400 µM SA led to increasing in proline content in 2 days after treatment then decreased. As to RGS, proline content was higher in 400 µM SA than other concentrations and it was the highest in end of the experiment (Fig. 7).

Discussion

The results showed that SA increased the content of total Chl in both cultivars. The loss of chl is one of the symptoms of oxidative stress. Cold stress caused to an increase in free radicals in chloroplast which results in cellular damaging and decline in membrance permeability. So 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 $NADP^+$ so, superoxide anion of the ROS is generated. To avoid cellular damage, the chl needs to be degraded quickly. Maybe low temperature leads to increase ROS in chloroplast and caused to destruction Chl molecules and damaging the chloroplast membrance system and photosynthetic reaction center. Probably, SA as a detoxifier of ROS by prevented of the activity of free radicals alleviated the superoxide radicals and can enhance leaf chl content.

It has been demonstrated that stress conditions and unfavorites environmental factors induced oxidative stress in plants tissues. When chloroplasts exposes to excessive excitation energy, it caused to generation of ROS and induce oxidative stress (Tasgin *et al.,* 2003). To prevent damage of free radicals, plants have evolved 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 faction as a ROS scavenger by converting $O^{\cdot}$ to H_2O_2, and can lead to enhanced stress tolerance. Similar findings were presented under stress condition in higher plants (Horváth *et al.,* 2007). Our results showing that SA induced SOD activation that consistent with the 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.

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). Our results showed that CAT activity decreased when plants 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 to protein and nucleic acids. We 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 defense mechanisms may be to reflect the plant response required 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 in antioxidant enzymes activity, reduced oxidative injury. Thus, the data obtained by us demonstrate that activation antioxidant enzymes induced by the treatment with SA probably 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 decreased of CAT activities (Senaranta *et al.,* 2000) have been reported.

Under stress conditions caused by chilling stress, oxidative damage can happening, that 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 had 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 data of the increased 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). These results suggest that SA may regulate the cold induced oxidative stress in canola plants and SA spraying reduction of cold induced toxicity to the canola plants 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 may be related to oxidative damage which mediated by degradation of proteins (Noctor and Foyer, 1998). Probably SA by neutralization of free radicals,

prevented by destructive of protein and leads to increasing in leaves protein solution.

Free proline accumulated in the wide range of environmental stress in many plants and it's established that proline is a highly water soluble amino acid. When plants are stressed the accumulation of proline is a common metabolism response to adversity and is involved in the succession resistant capability of plants (Parvanova *et al.,* 2004). Recently it has been reported that increasing accumulation high level of proline in transgenic tabacco caused to increased tolerance in high level of cold stress (Parvanova *et al.,* 2004). Winter wheat with high level of proline have greater tolerance in chilling stress (Doerffling *et al.,* 1993) and observed direct correlation between proline level and chilling tolerance in various species (Galiba, 1994). Our results showed that the greatest level of obtained proline in 3 and 2 days after treatment in LICORD and RGS, respectively. Maybe usage of SA led to activation of biosynthesis of proline and preservation of enzyme structure and activity and protection of membrance from damage by ROS.

Conclusion

The present study showed that suitable concentration of SA activated antioxidant activities; however, higher concentration of SA, inversely depressed activity of antioxidative enzymes, consequently, caused to weaken the capacity of cold tolerance that this activity is depended on plant species, cultivar, age and also action mode of SA. Also, it has been recognized that the level of plant response has a close relationship with resistance of plant species.

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