SALT-INDUCED VARIATION OF INORGANIC NUTRIENTS, ANTIOXIDANT ENZYMES, LEAF PROLINE AND MALON-DIALDEHYDE (MDA) CONTENT IN CANOLA (*BRASSICA NAPUS* L.)

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Abstract. Present experiment was performed to determine the effect of salinity stress on canola (*Brassica napus* L.) using the parameters of inorganic nutrients, malondialdehyde (MDA) and leaf proline content and the activity of antioxidant enzymes. Four canola cultivars viz., Oscar, Ac Excel, Cyclone and Dunkled and two levels of salt (0 mmol/L NaCl, 120 mmol/L NaCl) were used. Root fresh and dry weight of four canola cultivars markedly declined due to salinity stress. Salt stress significantly increased leaf proline content in four canola cultivars and higher values were recorded in cultivar Dunkled and Ac Excel than those in Cyclone and Oscar. Salt stress significantly enhanced antioxidant enzyme activities including SOD, POD and CAT in all canola cultivars. SOD showed highest value in Cyclone and Dunkled, POD in Ac Excel, Cyclone and Dunkled while CAT in Ac Excel and Dunkled. Malondialdehyde (MDA) showed variable response in all canola cultivars under saline conditions. Shoot and root (Na^+, Cl^-, K^+, and Ca^{2+}) were significantly affected under salt regimes. Salt stress increased Na^+ content in root and shoot in canola cultivars. High shoot Na^+ was found in Oscar and Cyclone, root Na^+ in Oscar, Ac Excel and Cyclone. Value of K^+ and Ca^{2+} in shoot and root was markedly reduced under saline conditions. It is concluded that high salt tolerance of canola cultivar "Dunkled" could be accredited due to exclusion of Na^+ and accumulation of K^+ ions, enhanced leaf free proline content and also antioxidant enzyme CAT activity.
Keywords: *oilseed crop, salt stress, enzyme assay, biomass production, Na^+ and K^+*

Introduction

Stresses including biotic and abiotic limits life forms all over the world. Abiotic stresses such as drought, salinity, waterlogging, low light intensity, and extreme in temperature inhibit plant growth and development like biomass production. Overall, agriculture productivity markedly reduces by salinity stress (Khodary, 2004; Ashrafuzzaman et al., 2002). Salinity, as a major abiotic stress in crop species, disrupts homeostasis, water potential, ion distribution and induces inhibition of growth and oxidative changes as a secondary stress. Formation and accumulation of reactive oxygen species (ROS) can be induced by salinity stress (Erdal et al., 2011). Reactive oxygen species include singlet oxygen, hydroxyl radicals and hydrogen peroxide which destroy mitochondria and DNA. Enzymatic as well as non-enzymatic antioxidant defence mechanisms reduce the accumulation of reactive oxygen species by detoxifying free radicals (Siddiqi et al., 2011). The activities of antioxidant enzymes, such as catalase, peroxidase, superoxide dismutase and ascorbate have a key role in the removal of reactive oxygen species (Anderson et al., 1995).

Canola (*Brassica napus* L.) is a moderately salt tolerant oilseed crop which is mostly cultivated for edible oil purpose (Francois, 1994). In literature, it is widely reported that requirements of canola oil as oilseed crop has increased since the last four decades due to

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increased awareness about health benefits of canola. Attention increased to cultivate canola in soils where salinity issues already exist (Ashraf and McNeilly, 2004). To improve salt tolerant crop, it is necessary to increase the productivity of saline soils, different approaches have been proposed by various plant scientists (Ashraf et al., 2008).

Major objectives of the present research work were to explore the response of canola cultivars to salt stress through their antioxidant enzymes, leaf proline and nutrients content at vegetative growth stage.

Materials and methods

Objective of the present investigation were to determine the effect of salt stress on canola (*Brassica napus* L.) cultivars. Pot experiment was performed in the Botanical Garden, Department of Botany, University of Gujrat, Pakistan. Seeds of four canola cultivars were acquired from Ayub Agriculture Research Institute (AARI), Faisalabad, Pakistan. Seeds were treated using sodium hypochlorite (5% NaClO) solution for surface sterilization for 5 min. Seeds were rinsed with double distilled water after the process of sterilization. Plastic pots (24 cm diameter) were filled with 10 kg fresh river washed sand. Eight (8) healthy seeds were sown in each plastic pot. Each pot was irrigated with Hoagland nutrition (2 L) solution. Thinning was done to maintain five plants in each pot after ten days. Treatment of the experiment was control (0 mmol/L NaCl) and salt stress (120 mmol/L NaCl). After twenty days of initiation seed germination, salt treatment was started with full strength Hoaglands nutrient solution. Objective of the Hoagland nutrient solution application to plants is to maintain mineral nutrients requirements. Step wise salt treatment was given by adding 40 mmol/L NaCl on a daily basis to maintain final salinity level. Also, distilled water (250 ml) was applied on a daily basis to each pot to avoid evaporation loss. Data of the followings attributes were noted after the start of seven weeks of salt treatment.

Biomass production

One plant from each replicate was removed and separated into their respective shoot and root. Samples of shoot and root were oven dried at 65 °C for five (05) days and their dry weight was recorded by using an analytical balance.

Malondialdehyde (MDA)

Method of Carmak and Horst (1991) was followed for the determination of leaf malondialdehyde (MDA) contents. Fresh leaf sample (0.5 g) was grinded, trichloroacetic acid (10 ml) was added and then that mixture was centrifuged for 10 min at 12,000 × g. Four ml of 0.5% thiobarbituric acid prepared in 20% trichloroacetic acid was added in 1 ml of the supernatant. After that reaction mixture was placed in water bath at 90 °C for three min. These samples were again centrifuged for 10 min at 12,000 × g. Absorbance of the samples was noted at two wavelengths 532 and 600 nm with a spectrophotometer (U2020 IRMECO).

Antioxidant enzyme activities

Fresh leaf sample (0.5 g) was grinded in 0.5 ml phosphate buffer for the measurement of antioxidant enzyme activities. Homogenate was centrifuged at 15,000 × g for 15 min at 4 °C. Values of superoxide dismutase, peroxidase and catalase was determined using the supernatant. Superoxide dismutase activity was determined by enzymatic photoreduction of

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nitroblue tetrazolium (NBT) following the method of Giannopolitis and Ries (1977). Reaction mixture of 3 ml contained methionine (13 mmol/L), riboflavin (1.3 μmol L^{-1}), phosphate buffer and NBT (50 μmol L^{-1}). In test tube 20–50 μL enzyme extract was homogenized. Under white fluorescent light, solution mixture was illuminated. Catalase and peroxidase activities were determined using the method of Chance and Maehly, 1995. Solution mixture was prepared containing guaiacol (20 mmol/L), hydrogen peroxide (40 mmol/L), phosphate buffer (50 mmol/L phosphate) and enzyme extract (0.1 ml). Catalase activity was determined using hydrogen peroxide (5.9 mmol/L), phosphate buffer (50 mmol/L) and 0.1 ml enzyme extract. To determine catalase activity, enzyme extract was mixed with reaction mixture and absorbance was recorded at 240 nm after every 20 sec. Concentration of protein was determined using the method of Bradford (1976).

Determination of free proline

Method of Bates et al. (1973) with some amendments was used for the measurement of leaf proline content. 0.5 g of fresh leaf sample was grinded and extracted using sulfosalicylic acid (3%). Filtrate of the reaction mixture was obtained using filter paper (Whatman No. 2). Two ml of filtrate was added in the test tube with 2 ml of acidic ninhydrin solution and 2 ml of glacial acetic acid and further homogenized. Then it was heated in water bath at 75 °C for 60 min. After that mixture was placed in ice water bath for the termination of reaction. Toluene (4 ml) was added to the reaction mixture to isolate proline as supernatant. Absorbance of the proline supernatant was recorded at 520 nm using a spectrophotometer. Only toluene was used as a blank.

Nutrient analysis

Method of Wolf (1982) was followed for the determination of different nutrient contents. Dried sample (0.1 g) was grinded and digested in H_2SO_4 and H_2O_2 for the determination of nutrients (Na^+, K^+ and Ca^{2+}). Sulphuric acid (2.5 ml) was added in the digestion tube and further tubes were incubated overnight at room temperature. Then hydrogen peroxide (1 ml) was added to each digestion tube. Tubes was set in digestion block and heated to 350 °C until fumes were produced. Heating continued for a further 30 min and after that digestion tubes were removed from the digestion block. Appearance of colorless material in the digestion tube is the indication of completed digestion. Using deionized water, volume of the digestion material was made to 50 ml. Values of Na^+, K^+ and Ca^{2+} were determined with a flame photometer (PFP-7 ELE, Jenway Instrument Co. Ltd, Stone, UK).

Experimental design and statistical analysis

Experiment was set as a completely randomized design (CRD) with four replicates. Two factorial experiments consist of two levels of salt treatment and four canola cultivar. The data obtained were analyzed statistically by using Analysis of Variance (ANOVA) technique using the MSTAT- C computer package (MSTAT Development Team, 1989).

Results

Growth parameter

Salinity in the rooting growth medium markedly reduced root fresh and dry weight in four canola cultivars. A notable variation was recorded in this set. Maximum value of

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root fresh weight was found in cultivar "Oscar and Dunkled" and minimum in "Ac excel and Cyclone" while root dry weight showed highest value in "Dunkled" as compared to all other canola cultivar under saline regimes (*Fig. 1*). Analysis of variance of the data showed that salt stress significantly reduced the root fresh and dry weight of four canola cultivars and a clear variable response was recorded (*Table 1*).

Leaf proline and MDA content

Addition of salinity in the root zone significantly increased leaf proline content in all canola cultivar. A significant variation was found in this regard. Highest value of leaf proline was found in "Ac excel and Dunkled" as compared to other cultivar under salt regimes (*Fig. 1*).

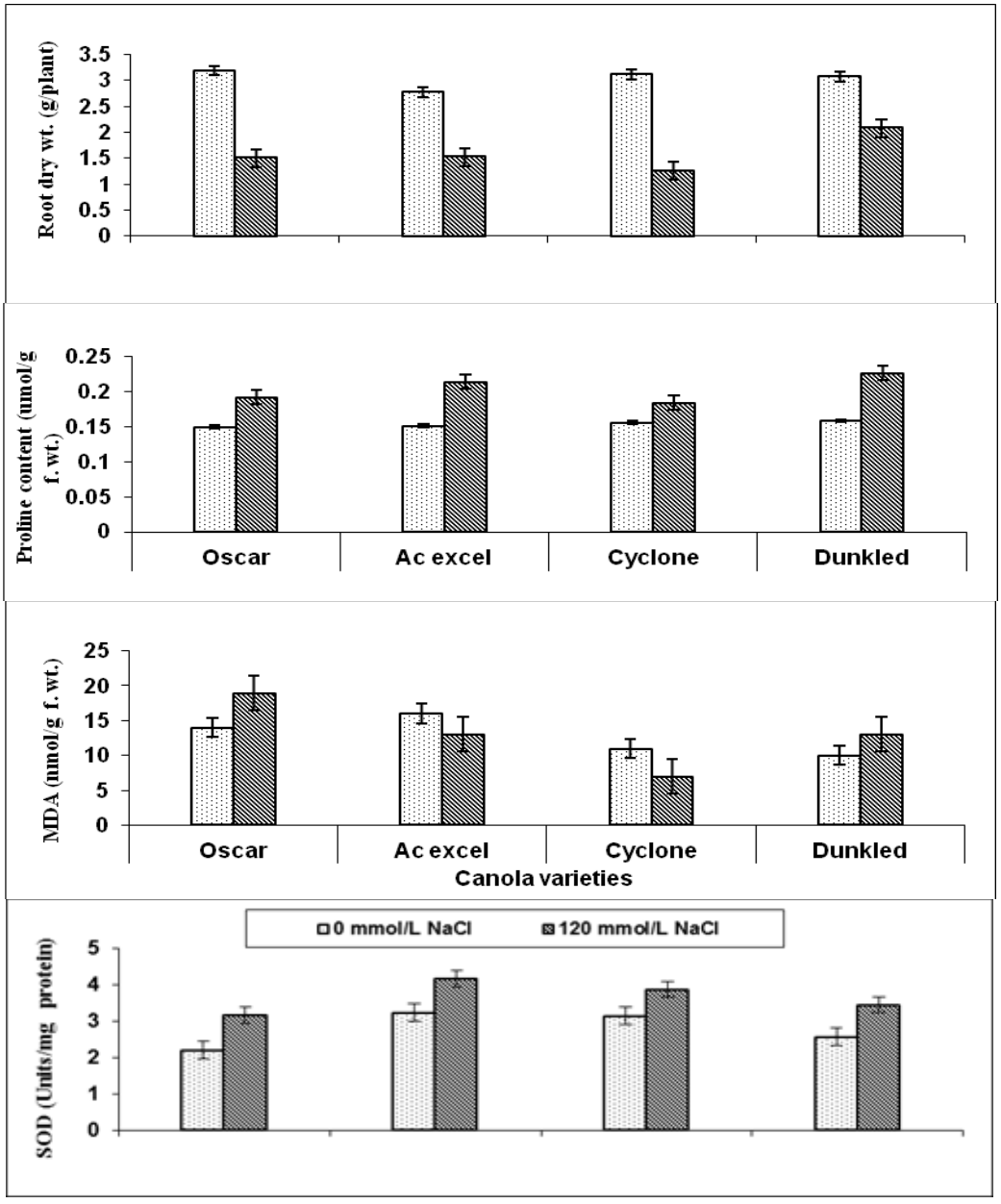

Figure 1. *Fresh and dry weight of root, leaf proline and malondialdehyde (MDA) content of four canola cultivars when treated to salinity stress for 48 days*

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Addition of salt to rooting growth medium showed variable response in malondialdehyde (MDA) values in four canola cultivars. Cultivar "Oscar and Dunkled" showed increased while Ac excel and Cyclone showed decreased values of MDA under saline conditions (*Fig. 1*). Analysis of variance of the data showed that salt level and cultivars showed significant result in the set of leaf proline and MDA content (*Table 1*).

Nutrient analysis

Salinity in the growth medium increased Na^+ content in shoot and root of four canola cultivars. A variable response was noted in this attribute. Cultivar "Oscar and Cyclone" showed higher values in shoot Na^+ content while Ac Excel, Oscar and Cyclone in root Na^+ under saline conditions (*Fig. 2*).

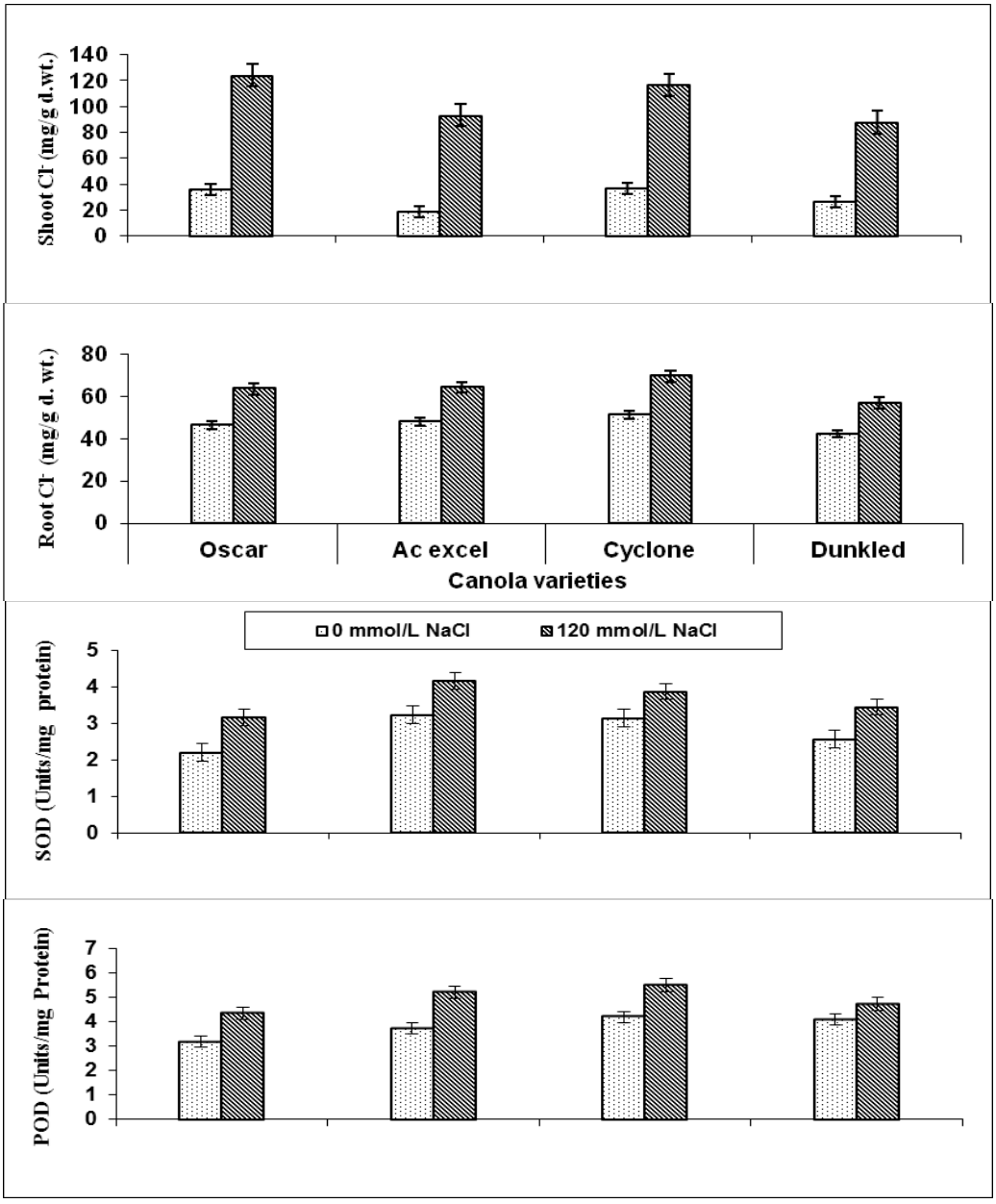

Figure 2. *Shoot and root Na^+ and Cl^- of four canola cultivars when treated to salt stress for 48 days.*

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Addition of salinity in the rooting medium highly increased the values of root and shoot chloride (Cl^-) in four canola cultivars. A clear variation was found in this attribute. Higher conc. of chloride was found in the shoot of Oscar and Cyclone and root of Oscar, Ac Excel and Cyclone than those of other cultivars as compared to control (*Fig. 2*).

Increasing salinity in the rooting medium significantly decreased values of K^+ in four canola cultivars. A variable response was found in this set of parameter. Less reduction of shoot K^+ was found in Ac excel and Cyclone while root K^+ in Oscar, Ac excel and Cylone under saline conditions (*Fig. 3*). Salt stress significantly decreased Ca^{2+} in shoot and root in all canola cultivars. Less variation was observed in shoot Ca^{2+}. Less reduction in shoot Ca^{2+} was noted in Cyclone while in root Ca^{2+} in Oscar and Cyclone under salt regimes (*Fig. 3*). Analysis of variance of the data showed that salt level and cultivars showed significant result in the parameters of shoot and root Na+, Cl^-, K^+ and Ca^{2+} (*Tables 2* and *3*).

Table 1. *Mean squares from ANOVA of the data for root fresh and dry weight, leaf proline and MDA content of four canola (Brassica napus L.) cultivars when subjected to salt-stress and non-stress conditions*

SOV	d.f.	Root fresh weight		Root dry weight		Leaf proline		MDA	
		F	P	F	P	F	P	F	P
Salt level	1	643.793	.000***	235.715	.0000***	95.713	.0000***	29.779	.0000***
Cultivar	3	12.194	.000***	4.355	.0111*	4.266	.012*	.003**	.003**
Salt level × cultivar	3	12.113	.000***	4.363	.0110*	3.248	.0345*	.786 ns	.786 ns
Error	32								

Table 2. *Mean squares from ANOVA of the data for shoot and root Na^+ and Cl^- content of four canola (Brassica napus L.) cultivars when subjected to salt-stress and non-stress conditions*

SOV	d.f.	Shoot Na^+		Root Na^+		Shoot Cl^-		Root Cl^-	
		F	P	F	P	F	P	F	P
Salt level	1	116974.540	.0000***	2961.794	.0000***	116974.540	.0000***	691.538	.0000***
Cultivar	3	4918.852	.0000***	213.738	.0000***	4918.852	.0000***	43.601	.0000***
Salt level × cultivar	3	3992.863	.0000***	7.363	.0007***	3992.863	.0000***	4.373	.0109*
Error	32								

Table 3. *Mean squares from ANOVA of the data for shoot and root K^+ and Ca^{2+} content of four canola (Brassica napus L.) cultivars when subjected to salt-stress and non-stress conditions*

SOV	d.f.	Shoot K^+		Root K^+		Shoot Ca^{2+}		Root Ca^{2+}	
		F	P	F	P	F	P	F	P
Salt level	1	472.804	.0000***	593.249	.0000***	4921.891	.0000***	365.492	.0000***
Cultivar	3	49.435	.0000***	166.376	.0000***	58.012	.0000***	22.676	.0000***
Salt level × cultivar	3	26.375	.0000***	21.658	.0007***	99.384	.0000***	5.406	.0040**
Error	32								

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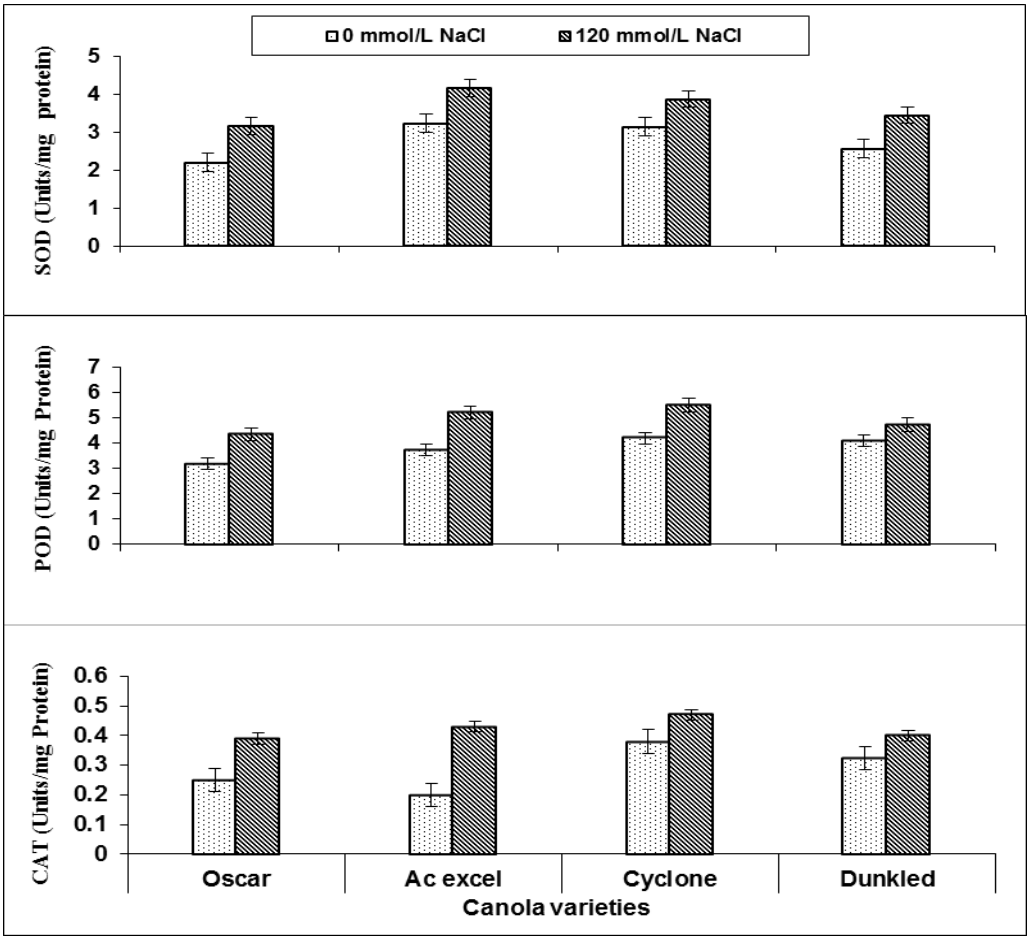

Figure 3. *Shoot and root K^+ and Ca^{2+} of four canola cultivars when treated to salt stress for 48 days*

Antioxidant enzyme activities

Values of antioxidant enzyme activity (SOD, POD. CAT) were increased in four canola cultivars under saline conditions. A notable variation was found in these parameters. Higher value of SOD was found in cultivar "Cyclone and Ac excel" than in "Oscar and Ac excel" under salt stress. POD showed higher value in Ac excels and Cyclone while lower in Ac excel and Oscar under salinity stress. Less reduction in catalase activity was found in Oscar as compared to Ac excel, Cyclone and Dunkled under salt regimes (*Fig. 4*). ANOVA of data showed that salt level, cultivar and salt level × cultivar interaction showed significant results in the value of antioxidant enzyme activities (SOD, POD. CAT) (*Table 4*).

Table 4. *Mean squares from ANOVA of the data for SOD, POD and CAT content of four canola (Brassica napus L.) cultivars when subjected to salt-stress and non-stress conditions*

SOV	d.f.	SOD		POD		CAT	
		F	P	F	P	F	P
Salt level	1	5017.421	.0000***	5017.421	.0000***	5017.421	.0000***
Cultivar	3	379.241	.0000***	379.241	.0000***	379.241	.0000***
Salt level × cultivar	3	71.190	.0000***	71.190	.0007***	71.190	.0000***
Error	32						

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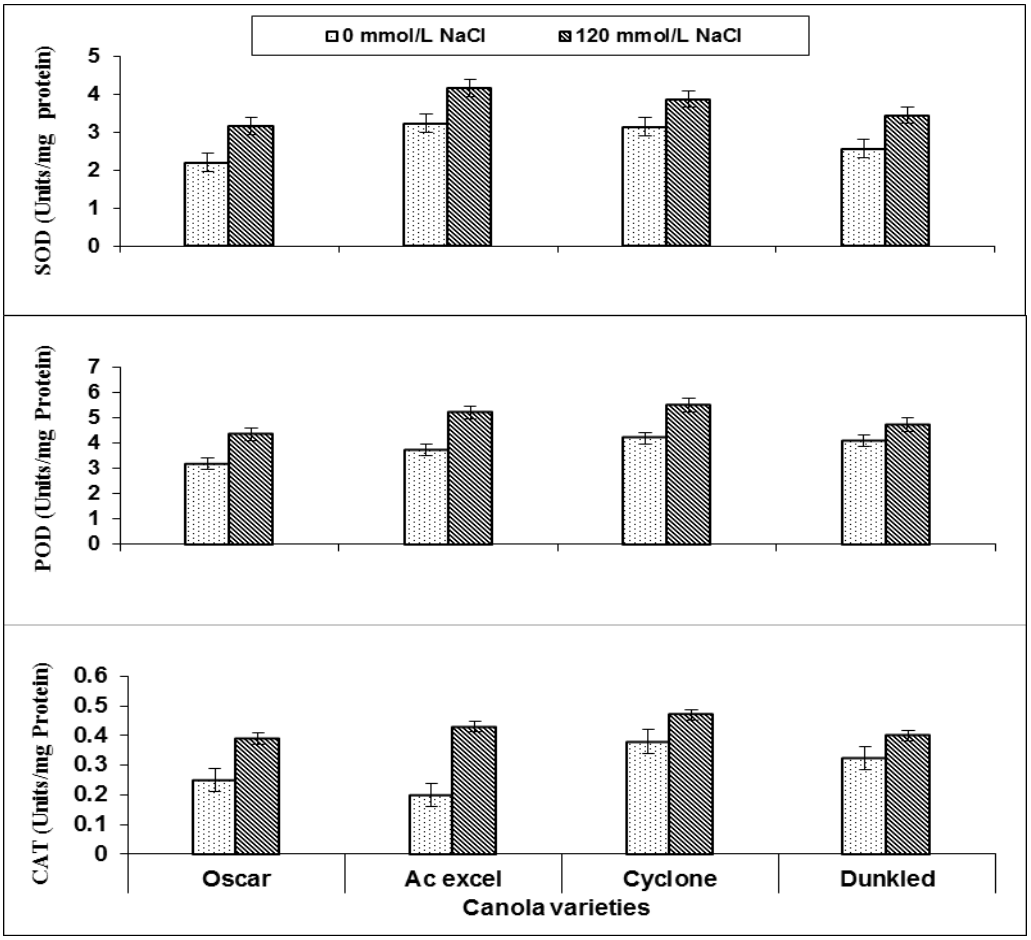

Figure 4. *Antioxidant enzyme (SOD, POD and Catalase) of four canola cultivars when treated to salt stress for 48 days*

Discussion

To explore variation at inter and intra-cultivar level is a prime importance for a breeding program needing crop improvement for different abiotic stresses including salinity (Munns, 2007). Results of the present research work are in accordance with the finding of different researchers where biomass production (root fresh and dry weight) were significantly reduced in different crops e.g., mangrove plant (Parida et al., 2004), tomato (Foolad and Lin, 1997) and safflower (Siddiqi et al., 2011).

Salinity stress has severe damaging effect on plants through ion toxicity or nutrient imbalance which occur due to higher accumulation of sodium and chloride in differentparts of plant in different plants (Munns et al., 2002; Ashraf, 2004). Higher quantity of salinity (NaCl) in soil or water hinders the uptake of different essential nutrients, such as potassium and calcium due to which plants face the problem of nutrient deficiency (Parida et al., 2004; Siddiqi et al., 2011). In the present investigation, accumulation of salt in the rooting zone medium markedly increased the concentration of Na^+ and Cl^- while decreased that of K^+ and Ca^{2+} in four canola cultivars. In literature, it is reported that low accumulation of Na^+ and high K^+ showed salt tolerant cultivars of the different crop species such as barley (Wei et al., 2003) and safflower (Siddiqi et al., 2011). Plant vacuoles maintained the balance of various nutrients due to accumulation

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of active osmolyte through osmotic adjustment. Of different osmolytes, proline is the essential one which amasses in various plants under saline conditions (Ashraf, 1994; Abbas et al., 2010). Present investigation showed that salt-stress increased the concentration of proline in the leaf of all canola cultivars. These results are in accordance with earlier findings where accumulation of proline under salt stress conditions has a key contribution to salt tolerance of various plant species (Maggio et al., 2002; Abbas et al., 2010). Furthermore, it is clearly reported in literature that proline accumulates more in salt-sensitive than in salt-tolerant cultivars (Ozturket al., 2012; Abbas et al., 2010).

Abiotic stresses including salt stress generate reactive oxygen species in plants (Turkyilmaz et al., 2014; Flowers et al., 2010). Plants have ability to counterpoise salinity-induced reactive oxygen species with the help of various antioxidant enzyme activities. In literature, it is reported that activities of antioxidant enzymes have a key role in salt tolerance of plant in different plant species. Findings of the present research work showed that addition of salinity in the growth medium increased the values of superoxide dismutase, peroxidase and catalase in all canola cultivars. Our results are in agreement with the findings on different crops such as wheat, safflower, finger millet and tomato where antioxidant enzymes produced under salinity stress (Raza et al., 2007; Ediga et al., 2013; Shalata et al., 2001).

In the present research work, values of MDA showed variable response in all canola cultivars under saline conditions, which are in accordance with the findings in the case of e.g. wheat (Ashraf et al., 2010), and finger millet (Ediga et al., 2013).

Conclusion

It is concluded that salt stress significantly increased sodium (Na^+), chloride (Cl^-) and leaf proline content of root and shoot, and enhanced the activities of SOD, POD and CAT, while it decreased the values of potassium (K^+) and calcium (Ca^{2+}). Overall, tolerance of canola to salt stress could be accredited to exclusion of Na^+ and Cl^-, higher accretion of K^+ and free leaf proline boosting antioxidant enzyme activities in canola cultivars.

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