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LEAF PROTEOME ANALYSIS SIGNIFIED THAT PHOTOSYNTHESIS AND ANTIOXIDANTS ARE KEY INDICATORS OF SALINITY TOLERANCE IN CANOLA (*BRASSICA NAPUS* L.)

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Abstract

Growth and yield reduction in different crops including canola is predicted to rise due to salinity stress in coming years. Understanding responses to salt stress will help in selecting and breeding salt tolerant canola cultivars. Physiological and leaf proteomic responses of 13 cultivars of canola were investigated under salt stress. In a pot experiment, three-week-old plants were grown under normal or salt stress (150 mM NaCl) for further two weeks. Out of 13 canola cultivars, cvs DGL, Dunkled, Faisal Canola and Punjab Canola were categorized as salinity stress tolerant cultivars, while cvs Bulbul-98, Oscar, Legend and Cyclone were considered as salt sensitive. Wide genotypic variations in canola cultivars have been observed in accumulation of potassium and sodium ions in the leaves and roots. Salt tolerant cultivars accumulated low Na^+ in their leaves than in in roots indicating limited uptake of Na^+ at root level with subsequent its transport to shoot. Moreover, salt tolerant cultivars had greater Na^+ discriminating capacity against K^+. Salt tolerant cultivars were higher in leaf relative water content. Although Fv/Fm did not change in canola cultivars due to salt stress, Fv/Fo and PI_{ABS} decreased considerably in all cultivars indicating important indicators of salt stress. Salt stress increased V_J, V_I, ABS/RC, TRo/RC and DIo/RC but it decreased ETo/RC which indicated that salt stress damaged the donor end of PSII (oxygen evolving complex) and reaction centers. Such adverse effects were maximal in salt sensitive cultivar Legend while minimal effects were observed in salt tolerant canola cultivars Faisal Canola, DGL and Dunkled. From comparative proteome analysis, it is obvious that 18 differentially expressed proteins in canola cultivars are mainly related with antioxidative defense system, photosynthesis and gene regulation. In addition, expression of these proteins was greater in salt tolerant cultivars. Cellular enegetics related proteins were downregulated particularly in salt sensitive cultivars due to salt stress. It is concluded that antioxidative defence system and photosynthesis are major componens of salt tolerance in canola in addition to salt exclusion. In addition, physiological studies complemented with proteomics will help in understanding detailed mechanism of salt tolerance.

Key words: Ion-exclusion, K^+/Na^+ ratio, JIP-test, Performance index, Proteomics, Photosynthesis, Antioxidants.

Introduction

Salinity stress adversely affects crop productivity worldwide (Athar & Ashraf, 2009; Munns & Gilliham, 2015). The problem of salinity is consistently becoming worst as the extent of salinized land is increasing all over the world (Munns & Gilliham, 2015). However, to judiciously utilize salt affected lands, development of salt tolerant cereals and oil-yielding crops seems to be very plausible approach (Kanwal *et al*., 2019). Developing salt tolerant lines will help in meeting global food demand which will increase 70% by 2050 (Ray *et al.*, 2013; Long *et al.*, 2015). Under saline conditions, plants adapt themselves by osmotic adjustment, accumulation of osmoprotectants, modifications in ion transport to avoid ion toxicities, and activation of enzymes by changing gene expression (Akram *et al.*, 2006; Athar *et al.*, 2015). Various genes or proteins form a regulatory network to modulate such physiological or biochemical processes under abiotic stress conditions (Ibrahim *et al.*, 2016; Li *et al.*, 2017; Farooq *et al.*, 2018). Under saline conditions, plants may synthesize and accumulate certain stress specific proteins which directly reveal alteration or switching on/off the possible number of salt stress related genes (Zhang *et al.*, 2012; Li *et al.*, 2017). Identification of new proteins responsible for salinity stress tolerance may help in understanding detailed mechanism of salt tolerance in plants (Li *et al.*, 2017).

Direct proteome analysis for identification of components of salt tolerance pathways is advantageous over transcriptome analysis because mRNA levels do not always correlate with protein expression (Gygi *et al.*, 1999). Over the past few years, analysis of proteins from a whole organism, tissue or cell has been used to study salt stress responsive proteins expression in different crops including rice (Liu *et al.*, 2012), wheat (Huo *et al.*, 2004), barley (Witzel *et al.*, 2014), tomato (Manaa *et al.*, 2011), pea (Kav *et al.*, 2004), and cucumber (Du *et al.*, 2010). Several salt responsive proteins, particularly those played role in Na^+ transport and distribution have been extensively studied such as high affinity plasma membrane K^+ transporter (HKT) that regulates Na^+ and K^+ uptake in roots (Rus *et al.*, 2004). It has been reported that a variety of calcium binding proteins (CaBs) such as calmodulin (CaM) and calcinurin could participate in Ca^{2+} homeostasis under saline conditions (Zhao *et al.*, 2013). Similarly, 367 photosynthesis related proteins have been reported under saline conditions. Among them, 12 light reaction related and 14 Calvin cycle related UPs are affected by salinity (Zhang *et al.*, 2012). Furthermore, under saline conditions multiple isoforms of chloroplast ATP synthases and ferridoxin NADP(H) oxidoreductases (FNR) have been reported to be regulated by salinity (Zörb *et al.*, 2009). Plant salinity tolerance is positively or negatively correlated with the change in number and amount of proteins

which are part of complex regulatory network. For example, expression of proteins related with photosynthetic process (Rubisco activase, larger sub unit of Rubisco etc.) increased in salt sensitive mutants of rice plants as compared to salt tolerant mutants or salt tolerant wild type rice plants. In addition, expression of proteins related with ROS scavenging is enhanced in salt tolerant mutant or wild plants than in salt sensitive mutants (Ghaffari *et al.,* 2014). Similarly down-regulation of photosynthetic proteins in canola are associated with growth reduction (Jia *et al.,* 2015). These studies indicated that salt tolerant species had greater levels of proteins related with reactive oxygen species (ROS) scavenging, ion transport, stress signaling and photosynthesis than those in salt sensitive species. Moreover, salt sensitive species had more catabolism related proteins such as glycolytic and respiratory enzymes. Thus, these and other proteomics studies have provided direct understanding of salt adaptive mechanisms in crop plants. However, all components of these biochemical and physiological processes are not well identified yet and thus need to be further explored. Furthermore, identification of such proteins could be a potential tool in producing salt resistant genotypes (Kumar *et al.*, 2009).

Various proteome approaches used to assess mechanism of salinity tolerance in different plant species. Advanced proteomic approaches such as SDS-PAGE complemented with LC-MS techniques may help in identification of new proteins which are responsible for salinity tolerance and/or identification of proteins which are components of various pathways that lead to salinity tolerance (Li *et al.,* 2017). Thus, physiological responses of 13 canola cultivars to salt stress was examined. In addition, the relationships will be drawn between physiological responses and salt responsive protein expressions using SDS-PAGE and LC-MS techniques with subsequent bioinformatic analysis. In addition, ion exclusion along with other physiological processes do not always a key to salt tolerance mechanism (Alagoz & Toorchi, 2018). Bioinformatics analysis is believed to comprehensively reveal the linkage between changes in protein expression and various metabolic pathways under salt stress.

Materials and Methods

To assess genetic variability of salt tolerance in local and exotic canola cultivars at different phenological stages, seeds of 13 canola cultivars (Cyclone, Faisal Canola, Bulbul-98, Dunkeld, Shiralee, Rainbow, Ac-Excel, DGL, Punjab canola, Legend, Oscar, CON-II and CON-III) was collected from Ayyub Agriculture Research Institute, Faisalabad Pakistan. Plastic pots having 28 cm diameter were filled with 9 kg river washed sand. Seeds of each canola cultivar were disinfected by incubating seeds in 1% sodium hypochloride solution for 10 minutes. Seeds were incubated in distilled water for 5 minutes and rinsed with water. Healthy seeds (20) of each canola cultivar were sown in sand. Thinning was done after one week of planting to maintain four plants in each pot. Three-week-old plants were subjected to salt stress by applying 0 and 150 mM NaCl salinity. Salinity level was gradually increased in aliquots of 50 mol m^{-3} on alternate day till desired salinity stress level attained. Two liters of Hoagland's nutrient solution without 150 mM NaCl salinity was added to each pot on weekly basis to avoid any nutrient deficiency. Plants were grown further for two weeks under control and saline conditions and then data for the following attributes was obtained.

After two weeks of salt stress, plants were harvested and plant biomass (Fresh and dry weights of shoots and roots) was measured. After measuring fresh weights, shoots and roots were oven-dried 75°C for three days and their dry weights were measured.

Ion analysis: For Na^+ and K^+ analysis, a young fully developed leaf was sampled from each plant and oven dried at 70°C for 72 h. Oven dried leaves and roots samples were grounded and 0.1gram oven-dried and grounded leaf powder was taken in 25 ml conical Pyrex flask. Digestion mixture amounting 1 ml was added and placed overnight. Next day, flasks were placed on hot plate. Temperature of the hot plate was gradually increased upto 100°C for 1 hour and then temperature was raised to 250°C. After one hour, 0.5 ml of perchloric acid was added and continued heating the samples until material became colorless. Samples were diluted with distilled water and their volumes were made up to 50 ml and filtered. Sodium and potassium concentrations were measured using flame photometer (Jenway, PFP-7).

Relative water contents (RWC): To measure RWC of the canola plants, 3rd leaf from top was taken from control and salt stressed plants of each canola cultivar. After recording its fresh weight, the leavs were immersed in distilled water for about 10 hours, and their weight was recorded as turgid weight. The leaves were oven-dried at 75°C and their dry weights were measured. Relative water contents were calculated using following formula:

$$\text{Relative water contents (RWC)} = \frac{\text{Leaf fresh weight} - \text{leaf dry weight}}{\text{leaf turgid weight} - \text{leaf dry weight}} \text{ x } 100$$

Total soluble proteins: Fresh green fully developed but the youngest leaf were selected and 200 mg leaf was grounded in liquid nitrogen and homogenized in 4 ml of potassium phosphate buffer (pH 7.8). Homogenized material was spun at 6000 x *g* for 12 minutes. A clear supernatant was taken in eppendorf tubes and stored at 4°C. Total soluble proteins from the leaf extract was measured following Bradford method (Bradford, 1976). Hundred microliters of leaf extract was added in 5 ml of Bradford reagent in the test tubes and allowed to develop a bluish colour in the dark for few minutes. Absorbance was noted at wavelength of 595 nm using singlebeam spectrophotometer (UV-1900 BMS). Amount of total soluble proteins in each sample were calculated using standard curve.

Proteome analysis of canola cultivars

One-dimensional SDS-PAGE: Total soluble proteins the leaves of each canola cultivars were extracted sing phosphate buffer. Proteins extracted so far were resolved on one dimensional 10% SDS-PAGE following Laemmli (1970). In stacking gel, 5% acryl amide was used while in resolving gel 12% acrylamide was used. From each sample, 20 uL containing 20 ug proteins were loaded on gel (VE-180, Tanon, China). Initially, SDS-PAGE was run at low voltage i.e., 70 V till protein tracking dye reached at the bottom of stacking gel. After this, SDS-PAGE was continuously run at 100 V till the protein tracking dye reached at the bottom. Separated protein bands in the gel were stained with Coomassie brilliant blue R-250.

LC-MS/MS of trypsin-digested proteins: Protein and peptide samples were analyzed using bottom-up mass spectrometry (MS) approach. In this approach protein or peptide samples were digested with enzymes, usually an endo-protease trypsin, to produce much smaller protein fragments with subsequent analysis through low resolution mass spectrometer (MS) such as peptide mass finger printing (PMF). Trypsin cleaves the amino acids at carboxyl or C-terminal end of lysine and arginine amino acids which must be linked at C-terminal end with proline residue. Due to this characteristic predictable nature of digestion, peptide products with unique masses used to identify the protein. However, it requires protein isolation and separation using SDS-PAGE or 2-D electrophoresis, otherwise in simply extracted protein samples it leads to miscalculation of expected peptide masses. Thus, in the present study, differentially expressed proteins as bands in SDS-PAGE gel were cut from the gel and solublized by InGel digestion method for protein identification. following Ibrahim *et al*., (2016). Protein bands were de-stained with 25 mM NH_4HCO_3 (in 50% CH_3OH, methanol) and were then dehydrated with acetonitrile for 2-10 minutes. Protein bands extracts were air dried for ten minutes and then were reduced with 10 mM DTT (25 mM NH_4HCO_3) at 60°C for 1 hour to break the disulfide bonds. Protein bands samples were alkylated by addition of 5.5 uL of 200 mM iodoacetamide (55 mM iodoacetamide in reaction mixture) for 45 minutes. Protein bands samples were digested with trypsin (2.5 ug trypsin/sample) by incubating at 37°C overnight. Thus, tryptic peptides were obtained from each band of SDS-PAGE.

Trypsin-digested peptides amounting 10 uL were first desalted on reverse phase trap column (PepMap C18, Dionex, UK) using isocratic solvent A (2% acetonitrile with 0.025% TFA trifluoroacetic acid in water). This work was carried out on nano-liquid chromatography coupled with MS (Ultimate 3000 Dionex nano-LC system, Dionex, UK). Trypsin-digested peptides were then separated on nano-analytical column C18 (PepMap C18, Dionex UK) by 45-minute gradient elution. The flow rate of mobile phase was 250 nL/minute while the temperature of the column was kept at 25°C. The 45-minute gradient was of 0-90% with solvent B (0.1% formic acid in 40% acetonitrile) versus solvent A (Solvent mobile phase A, 0.1% formic acid in 5% acetonitrile). The full scan for mass spectrometry MS (300-1400 m/z) and MS/MS (50-2200 m/z) were acquired at amaZon ETD ion trap MS.

Bioinformatics analysis: Based on this information and genomic information from different databases such as Swiss-Prot, PDB, ExPASy etc. proteome profile can be developed. Now a day, databases of peptide spectra generated from LC-MS analysis with search engines are available such as SEQUEST and MASCOT to match experimental peptide fragment spectra.

The obtained raw data files for LC/MS spectra of tryptic peptides from *Brassica napus* cultivars under normal or saline conditions were matched with theoretical trypsin-digested peptides of *Arabidopsis thaliana* databases containing genomic data available at NCBI using the MASCOT search software (Matrix Sciences, UK). Following search parameters were applied during evaluation/matching spectra: peptide mass tolerance, 10 ppm; fragment tolerance, 0.35 Da; precursor tolerance, 0.35 Da; variable modification, oxidation of methionine; fixed modification, carboxyamidomethylation of cysteine; missed cleavage per peptide, 2.

For identification of proteins, data for tryptic peptides were filtered as follows: cross-correlation scores (Xcorr) greater than 1.8 (for +1 charged peptides), 2.5 (for +2 charged peptides), 3.5 (for +3 charged peptides) were fixed for protein identification. Several peptide sequences having the highest Xcorr values were identified. Moreover, some other parameters were also chosen to filter anticipated peptides such as $\Delta CN > 0.1$; $Rsp < 4$; peptide probability < 0.0005. Moreover, proteins were positively identified when two or more than peptides were assigned to the same protein. Proteins or peptides below the set threshold or non-annotated hypothetical proteins were also considered to enhance sequence coverage and assigned putative functions after BLAST with NCBI database. After verifying mass to charge ratio value and corresponding protein sequence, redundant peptides and proteins were removed from the final list of all proteins. List of proteins expressed in different canola cultivars under normal or saline conditions was prepared. Each protein was assigned a major biochemical or physiological function and grouped as regulatory proteins, photosynthesis related, oxidative stress related, ion transport related, signaling process related.

Statistical analysis

Data collected from above all experiments was statistically analyzed by using software COSTAT version 6.4 (Cohort Software, Berkeley, California, USA). Means of physiological and biochemical attributes of each canola cultivar were compared using Least Significant Difference (LSD) as a yard stick when interaction term salt x cultivars was significant. Values of LSD were calculated following Snedecor & Cochran (1980). Means and percent of control bar graphs were drawn as two-axis charts using MS Excel-2010. Percent of control values were drawn on secondary y-axis as a line chart.

Results

Imposition of salinity stress caused significant reduction in shoot fresh and dry weights of 13 canola cultivars (Table 1). Canola cultivars also differed significantly in their shoot fresh and dry weight under both saline and non-saline conditions. Canola cultivar DGL followed by cvs. Dunkeld and Faisal Canola had higher fresh and dry weights of shoots under saline conditions (Fig. 1) whereas cultivar Oscar was the lowest in these growth variables (Fig. 1). A significant reduction in fresh and dry weights of roots was found in salt stressed plants of canola (Table 1). Results showed that cvs AC-Excel, Punjab Canola and DGL accumulated higher roots dry biomass while minimum root fresh weight and dry weight was found in cv. Oscar followed by cvs. Bulbul-98, CON-II and CON-III (Table 1; Fig 1).

Data for leaf ion analysis showed that values of leaf K^+ decreased significantly due to salinity stress and leaf Na^+ increased significantly in leaves of all 13 canola cultivars. Although cultivars did not differ significantly in leaf K^+, cultivars significantly differed in accumulation of K^+ in root, leaf and root sodium accumulation, and K^+/Na^+ ratio in leaves (Table 1; Fig. 2). Salt tolerant cultivar Faisal Canola followed by Dunkled were higher in leaf K^+/Na^+ ratio under saline conditions, while cultivar Oscar, Rainbow and Cyclone were the lowest in leaf K^+/Na^+ ratio under salt stress conditions.

Addition of salinity stress in the growth medium caused significant reduction in accumulation of total soluble proteins and total free amino acids in all canola cultivars (Table 1). Cultivar Legend had more total soluble proteins under saline conditions while it was lower in cv Ac-Excel. All the cultivars had a similar response to control and saline conditions in terms of accumulation of total free amino acids (Fig. 3). Leaf relative water content (RWC) was decreased due to NaCl salinity stress and all 13 canola cultivars differed significantly in their RWC (Fig. 3). Salt tolerant cultivars Faisal Canola, DGL and Dunkled were higher in RWC.

From OJIP curves, JIP-test of the 13 canola cultivars was assessed to draw the relationship between PSII structural integrity and salt tolerance in canola cultivars. Percent increase or decrease in various JIP-test parameters show that imposition of salt stress decreased the quantum efficiency of PSII (Fv/Fo) and performance index (PI_{ABS}) in canola cultivars (Fig. 4), whereas relative variable chlorophyll fluorescence at J and I steps (V_J and V_I) significantly increased in salt stressed plants of all canola cultivars. In addition, maximal decrease in quantum efficiency of PSII and performance index was found in Salt sensitive cultivar Legend followed by cvs Cyclone, Shiralee and Oscar. Smaller dcrease in quantum efficiency and performance index has been observed in cvs Punjab Sarson and DGL due to salt stress. Similarly, maximum percent increase in relative variable fluorescence at J step (V_J) due to salinity stress was found in cvs Legend, Cyclone and AC-Excel (Fig. 4). However, maximum percent increase in V_I was found in salt stressed plants of cv CON-II. Salt stress caused an increase in energy fluxes (more than 5%) for absorption (ABS/RC) in cvs Legends, Cyclone and Rainbow. There was no significant increase or decrease in energy fluxes for trapping (ETo/RC). Energy fluxes for electron transport decreased due to salt stress only in cvs AC-Excel, Cyclone, Rainbow, CON-II, Bulbul-98 and Dunkled. Maximum energy fluxes for heat dissipation (DIo/RC) increased in Salt sensitive cvs Legend, Cyclone, AC-Excel whereas there was a least increase in this attribute in Salt tolerant cultivars DGL, Dunkled, and Faisal Canola.

Results revealed the differential expression of proteins under saline and control conditions in 13 canola cultivars (Cyclone, Faisal Canola, Bulbul-98, Dunkeld, Shiralee, Rainbow, Ac-excel, DGL, Punjab Canola, Legend, Oscar, CON-II, CON-III). Proteins expressed in all 13 canola cultivars under control or saline conditions were presented in the Table (Table 2). Among the list of these proteins, various differentially expressed proteins were identified under various conditions. The distributions of these proteins were further characterized using various computational analysis. These results indicated that the differntial expression of these peptides or proteins might have played role in salt tolerance in plants.

Table 1. Mean squares from analysis of variance (ANOVA) of data for fresh and dry biomasses, soluble proteins, free amino acids, leaf and root Na^+, K^+ and relative water contents (RWC) of 13 canola cultivars grown under non-saline or saline conditions.

SOV	df	Shoot Fwt	Shoot dwt	Root fwt	Root dwt	Leaf K^+	Root K^+
Salt	1	725.00***	8.90***	105.35***	17.53***	6558.06***	3057.5***
Cultivars	12	11.30***	0.10***	1.127***	0.195**	24.44ns	11.62**
Salt x Cvs	12	1.93ns	0.02ns	0.593*	0.103ns	21.58ns	5.83ns
Error	52	3.13	0.02	0.303	.057	13.19	3.76
SOV	**df**	**Leaf Na^+**	**Root Na^+**	**Leaf K^+/Na^+ ratio**	**RWC**	**Free amino acids**	**Soluble proteins**
Salt	1	2810.6***	8.15**	2455.9***	1947.6***	233.93***	411.74***
Cultivars	12	4.10*	1.70*	7.0739***	54.09**	5.50***	5.75**
Salt x Cvs	12	3.54ns	0.97ns	7.0963***	34.35ns	2.077ns	3.61*
Error	52	2.10	0.77	0.7063	22.86	1.18	1.83

**, ***Significance at 0.01 and 0.001 level respectively ns=Non-significant

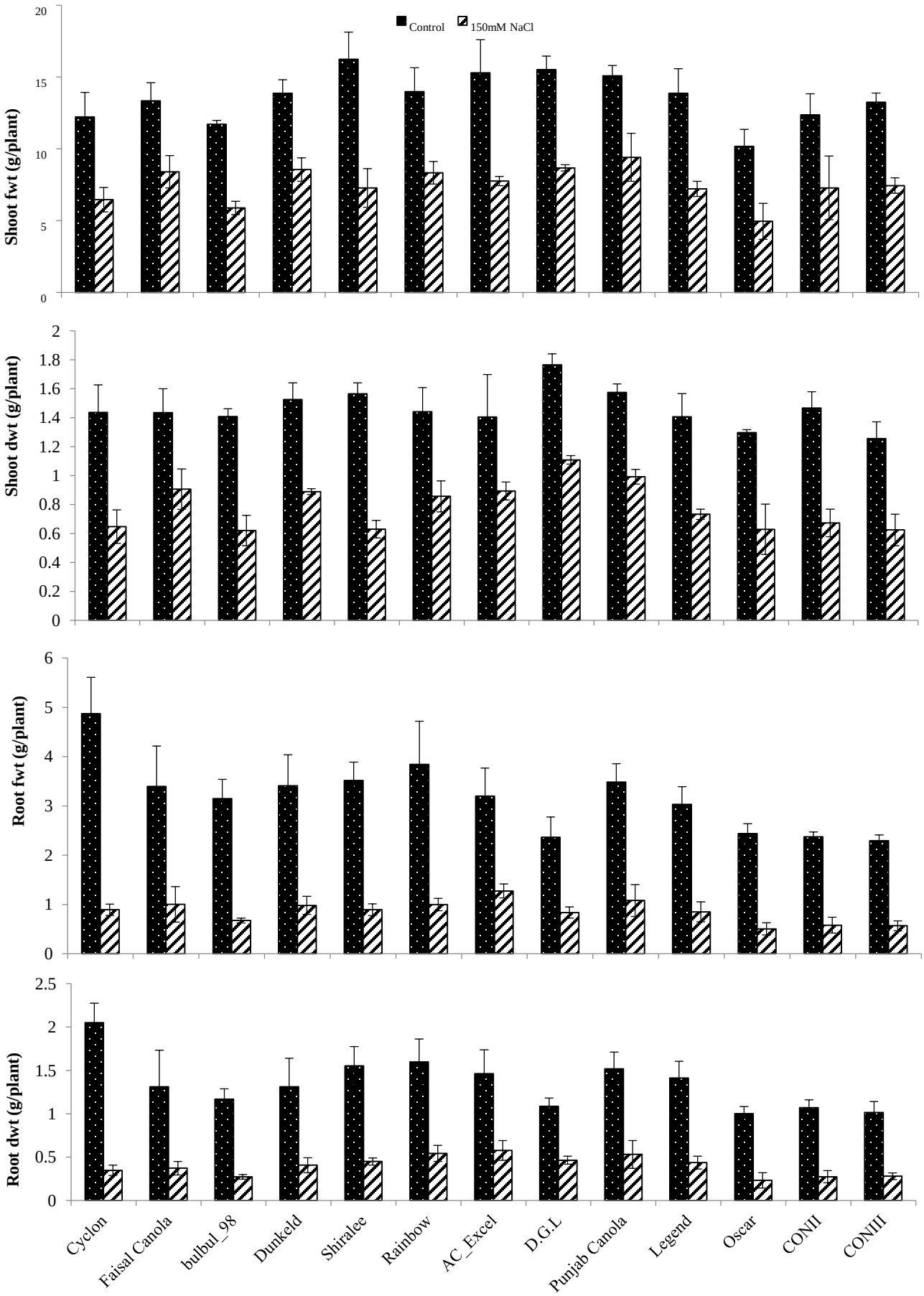

Fig. 1. Fresh and dry weights of shoots and roots of thirteen canola (*Brassica napus* L.) cultivars differing in salinity tolerance when three weeks old plants were subjected to 0 and 150 mM NaCl salinity.

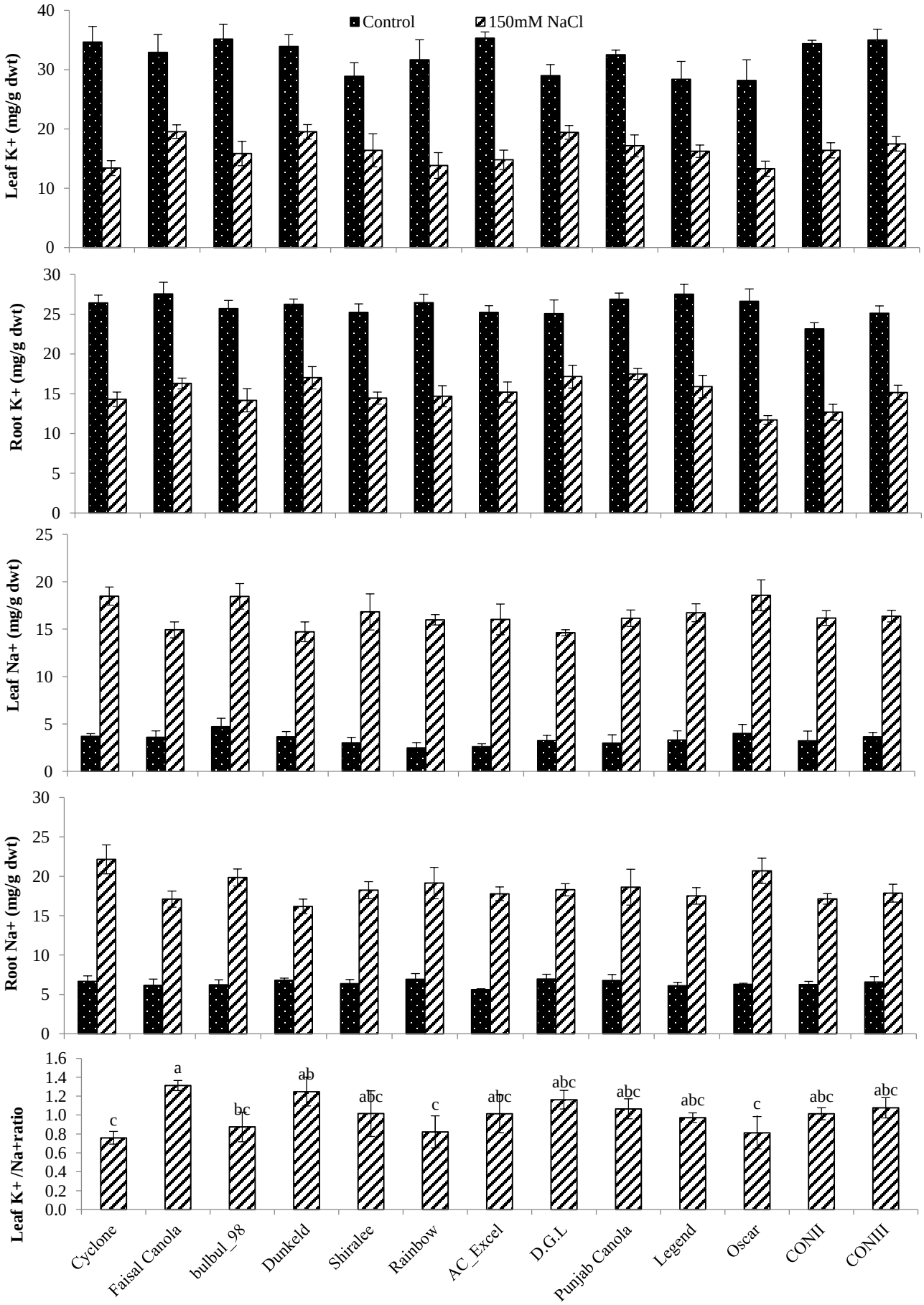

Fig. 2. Na+ and K+ of shoots and roots of thirteen canola (*Brassica napus* L.) cultivars differing in salinity tolerance when three weeks old plants were subjected to 0 and 150 mM NaCl salinity.

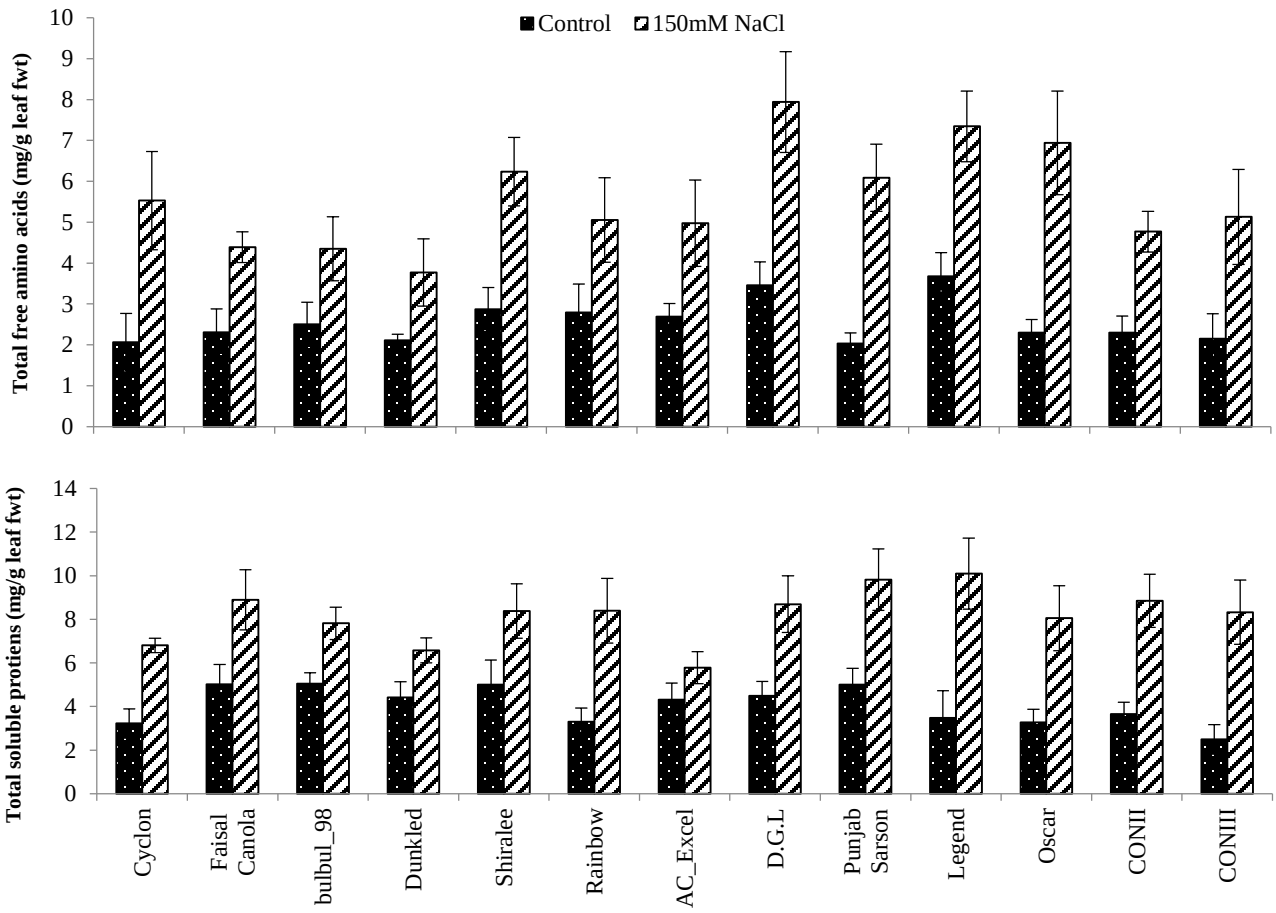

Fig. 3. Total soluble proteins, total free amino acids of thirteen canola (*Brassica napus* L.) cultivars differing in salinity tolerance when three weeks old plants were subjected to 0 and150 mM NaCl salinity.

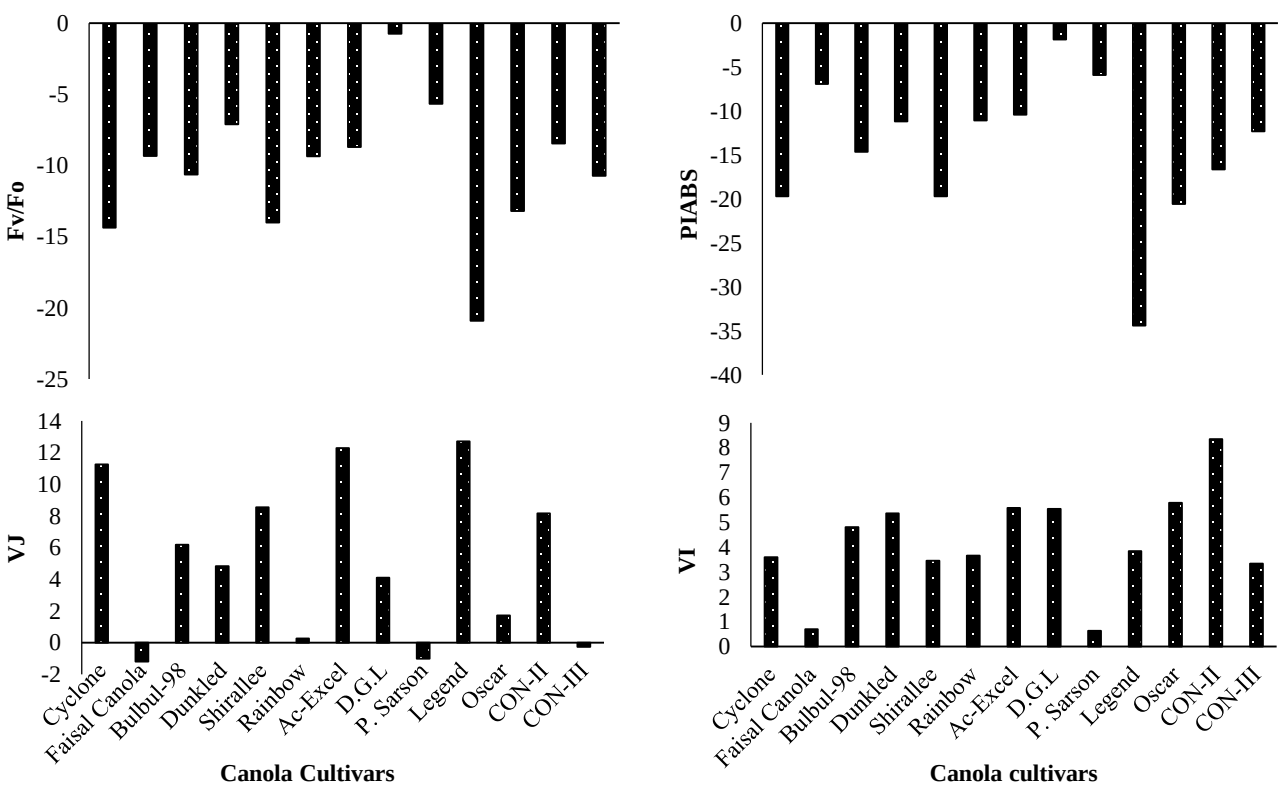

Fig. 4. Percent increase or decrease in quantum efficiency of PSII (Fv/Fo), performance index (PIABS), relative variable fluorescence at J and I step (VJ, VI), various JIP-test parameters of 13 canola (*Brassica napus* L.) cultivars when subjected to 0 and 150 mM NaCl salinity.

Table 2. Mean values for root fresh and dry weights of thirteen (13) canola (*Brassica napus* L.) cultivars differing in salinity tolerance when subjected to 0 and 150 mM NaCl salinity.

	Root fwt		Total soluble proteins	
	Control (0 mM NaCl)	Saline (150 mM NaCl)	Control (0 mM NaCl)	Saline (150 mM NaCl)
Cyclone	4.87 ± 0.736a y	0.894 ± 0.115a x	3.223 ± 0.668ab y	6.801 ± 0.327def x
Faisal canola	3.395 ± 0.820bc y	1.003 ± 0.360a x	5.017 ± 0.905a y	8.887 ± 1.379abc x
Bulbul-98	3.147 ± 0.393bc y	0.675 ± 0.049a x	5.045 ± 0.496a y	7.814 ± 0.736cde x
Dunkeld	3.41 ± 0.626bc y	0.981 ± 0.185a x	4.417 ± 0.719a y	6.573 ± 0.570ef x
Shiralee	3.513 ± 0.376bc y	0.893 ± 0.117a x	5.006 ± 1.126a y	8.376 ± 1.246abcde x
Rainbow	3.841 ± 0.878b y	0.998 ± 0.124a x	3.306 ± 0.630ab y	8.396 ± 1.481abcde x
Ac-Excel	3.198 ± 0.567bc y	1.275 ± 0.143a x	4.318 ± 0.764ab x	5.781 ± 0.734f x
D.G.L	2.364 ± 0.411bc y	0.837 ± 0.113a x	4.484 ± 0.664a y	8.693 ± 1.298abcd x
Punjab canola	3.481 ± 0.374bc y	1.081 ± 0.324a x	5.009 ± 0.744a y	9.820 ± 1.410ab x
Legend	3.028 ± 0.360bc y	0.851 ± 0.204a x	3.473 ± 1.246ab y	10.093 ± 1.631a x
Oscar	2.438 ± 0.202bc y	0.505 ± 0.122a x	3.274 ± 0.599ab y	8.048 ± 1.492bcde x
CON-II	2.375 ± 0.097bc y	0.58 ± 0.161a x	3.647 ± 0.545ab y	8.846 ± 1.214abc x
CON-III	2.293 ± 0.118c y	0.568 ± 0.100a x	2.496 ± 0.677b y	8.327 ± 1.474abcde x

LSD = 0.774; LSD = 1.90

Means with the same letters in each row (x-y) and in each column (a-b) do not differ significantly at the 5% level LSD 5% (salinity × cultivars); Means ± S.E

SDS-PAGE and 1-D protein profiling: Expression pattern of peptides or proteins in salt stressed plants of canola cultivars was significantly different from those of non-stressed plants of canola. In addition, all common major protein bands having same size such at about 14 kD and 20 kD were not selected for LCMS/MS. Differentially expressed protein bands were cut from the gel and digested with tryptic digestion method before further LC-MS/MS analysis (Table 3).

Identifications and *in silico* characterizations of shared proteins under control conditions: It has been noted that differentially expressed proteins under control conditions in all 13 canola cultivars were mainly associated with defense system, photosynthesis, ion transport and regulation of cellular metabolism. For example, ATP synthase subunit beta-1 COG COG0055 and Unknown protein having Pectinesterase inhibitor domain proteins were identified under control conditions in Faisal Canola cultivar. E3 ubiquitin-protein ligase COP1 that acts as an integrator of photoperiod and ambient temperature signaling expressed in the leaves of cvs. Cyclone, Dunkeld and Legend. Further computational analysis revealed that both proteins belong to unknown COG with Zinc Finger and START like domain proteins. Histone-lysine N-methyltransferase having Zinc finger family expressed only in cv Dunkled under non-saline conditions, whereas, DNA replication licensing factor MCM5 having protein domain MCM N-terminal domain were expressed in cvs Shiralee and DGL. In addition, proteins associated with defense and antioxidative potential were also difrentially expressed in some canola cultivars. For example, polyamine oxidase 1 with unknown COG expressed in cv. Legend, while Ubiquinol oxidase 1c with unknown COG was identified in cvs. Bulbul-98 and CON-III. Similarly, other proteins identified under control conditions were Histone-lysine N-methyltransferase only in Dunkeld having Zinc Finger family as in E3 ubiquitin-protein ligase COP1, and DNA replication licensing factor MCM5 having protein domain MCM N-terminal domain in cvs. Shiralee and DGL. Plant metabolism and cellular signaling related differentially expressed proteins are fructokinase-like 2 COG0524, wall-associated receptor kinase 1 COG0515 and NAC domain-containing protein 8 in cvs. Rainbow and Punjab. Mitochondrial uncoupling protein 1 that create proton leaks across the inner mitochondrial membrane and ubiquinol oxidase 1a were expressed in cv. AC-Excel under non-saline conditions. Glucan endo-1,3-beta-glucosidase 13 and mitochondrial outer membrane protein were identified in cv. CON-III.

Table 3. List of differentially expressed proteins identified using LC-MS/MS in 13 canola (*Brassica napus* L.) cultivars when grown for three weeks under non-saline or saline conditions.

Cultivars	Accession No.	Protein name	Differential proteins		Molecular mass	Pi
			Control	Saline		
Cyclone	P43254	E3 ubiquitin-protein ligase COP1	✓	×	76187	6.38
	Q7X9V7	PR10 protein (Pinus monticola)	✓	×	17791	5.3
	P83483	ATP synthase subunit beta-1	×	✓	59670	6.18
	Q9SR19	Rubisco accumulation factor 2	×	✓	50199	5.77
	Q43873	Peroxidase 73	×	✓	35927	9.44
Faisal canola	P83483	ATP synthase subunit beta-1	✓	×	59670	6.18
	Q9FNA2	Polyamine oxidase 1	✓	×	35927	5.3
	Q43873	Peroxidase 73	×	✓	35927	9.4
	Q9SRZ4	Peroxiredoxin-2C	×	✓	17414	5.33
	Q45FP5	Chloroplast CuZn-SOD	×	✓	834	5.28
Bulbul-98	P83483	ATP synthase subunit beta-1	×	✓	59670	6.18
	O22048	Ubiquinol oxidase 1c	✓	×	37816	6.91
	F4IY62	UDP-glucose pyrophosphorylase 3	×	✓	99042	5.97
	Q39241	Thioredoxin H5	×	✓	13122	5.19
Dunkeld	P43254	E3 ubiquitin-protein ligase COP1	✓	×	76187	6.38
	Q7X9V7	PR10 protein (Pinus monticola)	✓	×	17791	5.3
	Q2LAE1	Histone-lysine N-methyltransferase	✓	×	193228	5.34
	Q43873	Peroxidase 73	×	✓	35927	9.44
	F4IY62	UDP-glucose pyrophosphorylase 3	×	✓	17414	5.97
	Q39241	Thioredoxin H5	×	✓	13122	5.19
Shirallee	O80786	DNA replication licensing factor MCM5	✓	×	81014	7.17
	Q680P8	40S ribosomal protein S29	✓	×	50199	10.07
	Q9FN47	Thymidine kinase	×	✓	30703	8.43
	Q9FWW6	Flavin-containing monooxygenase	×	✓	23416	6.23
	Q9FRL8	ATP synthase subunit alpha	×	✓	27406	5.79
Rainbow	F4I0K2	Fructokinase-like 2	✓	×	68980	5.14
	Q39191	Wall-associated receptor kinase 1	✓	×	81211	5.46
	Q6NQK2	NAC domain-containing protein 8	✓	×	50288	4.91
	Q9FRL8	ATP synthase subunit alpha	×	✓	23406	5.79
Ac-Excel	Q39191	Wall-associated receptor kinase 1	×	✓	81211	5.46
	O81845	Mitochondrial uncoupling protein 1	✓	×	32662	9.62
	Q39219	Ubiquinol oxidase 1a	✓	×	39979	8.56
	O04331	Peroxidase 73	✓	×	30399	6.99
	P22197	Fructose-bisphosphate aldolase	×	✓	38810	6.85
DGL	O80786	DNA replication licensing factor MCM5	✓	×	81014	7.17
	Q680P8	40S ribosomal protein S29	✓	×	6429	10.07
	O65719	Heat shock 70 kDa protein 3	✓	✓	71147	4.96
	P0DH99	Elongation factor 11A	×	✓	49502	9.19
	Q42449	ATP synthase subunit alpha	×	✓	35927	4.7
Punjab canola	F4I0K2	Fructokinase-like 2	✓	×	68980	5.14
	Q6NQK2	NAC domain-containing protein 8	✓	×	50288	4.91
Legend	P43254	E3 ubiquitin-protein ligase COP1	✓	×	50199	4.38
	Q9FNA2	Polyamine oxidase 1	✓	×	52866	5.3
	Q9LZP9	Calvin cycle protein CP12-2	✓	×	14166	4.82
	Q43873	Peroxidase 73	×	✓	35927	9.44
	Q9S7C9	ATP – Hook motif nuclear-localized protein 27	×	✓	31842	6.7
	Q9LHG9	Nascent polypeptide-associated complex subunit alpha like protein	×	✓	219882	4.3
Oscar	O81845	Mitochondrial uncoupling protein 1	✓	×	32662	9.62
	O65719	Heat shock 70 kDa protein 3	×	✓	71147	4.96
	Q42449	ATP synthase subunit alpha	×	✓	35927	4.7
CON-II	P83483	ATP synthase subunit beta-1	✓	×	59670	6.18
	Q9M2M3	Beta-1, 3-glucanase	✓	×	30700	
	Q9FJU9	Glucan endo-1,3-beta-glucosidase 13	✓	×	55603	7.91
	P14713	Phytochrome B	×	✓	129331	5.62
	Q9C5R8	2-Cys peroxiredoxin BAS1-like	×	✓	29779	5.55
	P56757	ATP synthase subunit alpha	×	✓	55927	5.19
CON-III	O22048	Ubiquinol oxidase 1c	✓	×	37816	6.91
	Q9LZP9	Calvin cycle protein CP12-2	✓	×	14166	4.82
	Q9SRH5	Mitochondrial outer membrane protein porin 1	✓	×	29425	8.77
	P83483	ATP synthase subunit beta-1	×	✓	35927	6.18
	F4IY62	UDP-glucose pyrophosphorylase 3	×	✓	99042	5.97
	Q39241	Thioredoxin H5	×	✓	13122	5.99

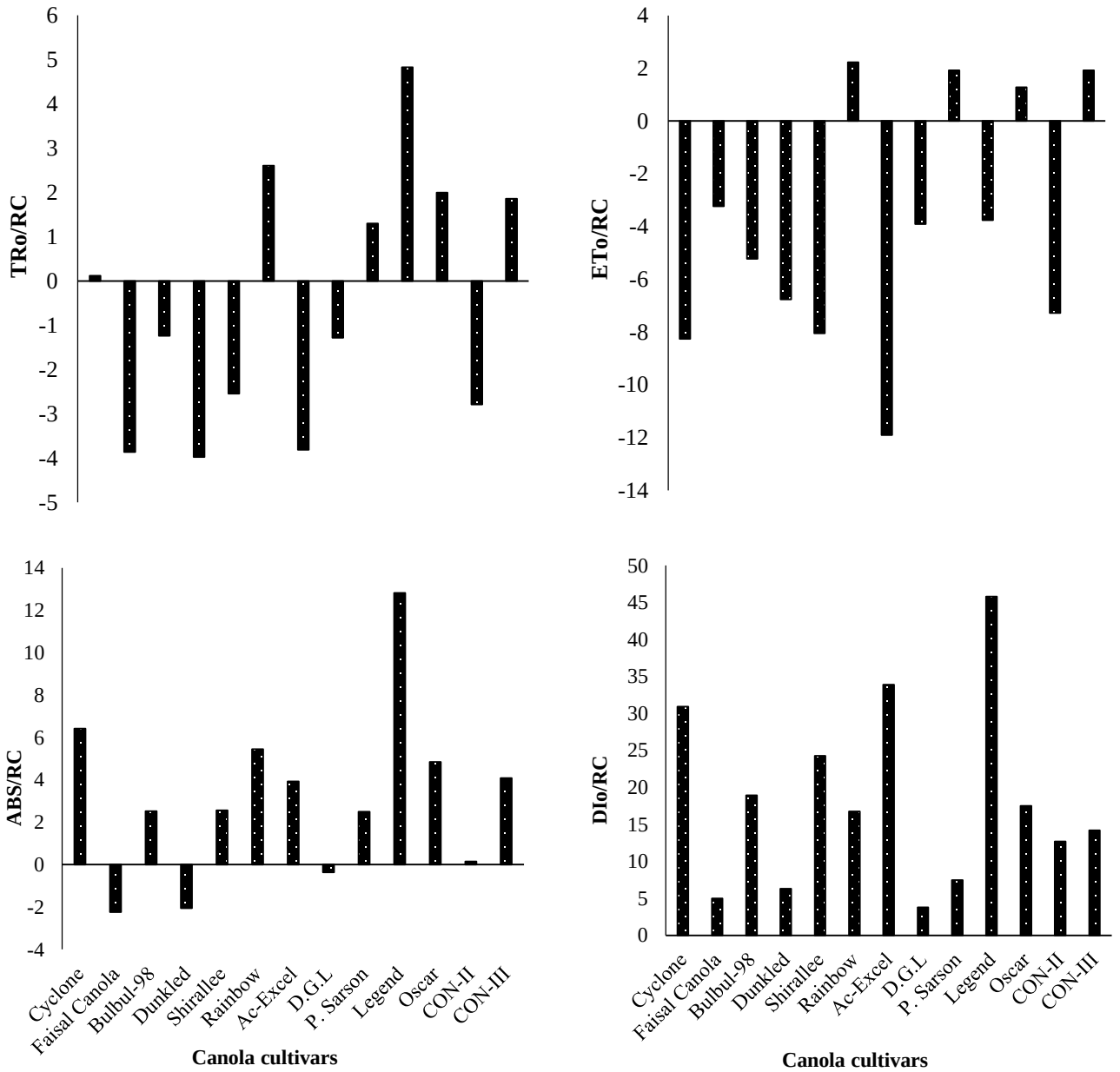

Fig. 5. Percent increase or decrease in various JIP-test parameters of 13 canola (*Brassica napus* L.) cultivars when subjected to 0 and 150 mM NaCl salinity.

Identifications and *in silico* characterizations of proteins identified under salt stress: Under salt stress conditions, differentially expressed proteins are mainly related with antioxidative potential and photosynthetic capacity such as Peroxidase 73, Peroxiredoxin-2C, Chloroplast CuZn-SOD, Thioredoxin H5, Polyamine oxidase 1, Ubiquinol oxidase 1c, rubisco accumulation factor, 2-Cys peroxiredoxin BAS1-like. In addition, salt stress caused the over-expression of proteins related with energy metabolism, and cell signaling. For example, wall associated receptor like kinase, UDP-glucose phosphorylase 3, mitochondrial outer membrane porin3, ATP synthase subunit beta-1 and ATP-synthase subunit alpha were identified in salt stressed plants of cvs. Cyclon, Bulbul-98 and CON-III. It seemed that these proteins were indispensible for the growth of plants under salt stress conditions. However, degree of salt tlerance in canola cultivars could be related with differentiall expression of proteins as salt tolerant canola cultivars had greater number of differentially expressed proteins related with antioxidant and photosynthetic capacity as compared to those of salt sensitive cultivars. For example, salt tolerant cultivar Faisal Canola had three differentially expressed proteins related with antioxidants such as Peroxidase 73, Peroxiredoxin-2C, Chloroplast CuZn-SOD. Similarly, salt tolerant canola cultivar Dunkled had two differentially expressed proteins Peroxidase 73, Thioredoxin H5. Moreover, abiotic stress tolerance conferring protein heat shock 70-kDa protein 3 COG0443 and rubisco activase (activation of Calvin Cycle) were only identified in salt tolerant cultivar DGL. In contrast, salt sensitive cultivars Cyclone and Legend had only one protein. Moreover, salt sensitive cultivar Oscar had no differentially expressed protein related with antioxidants.

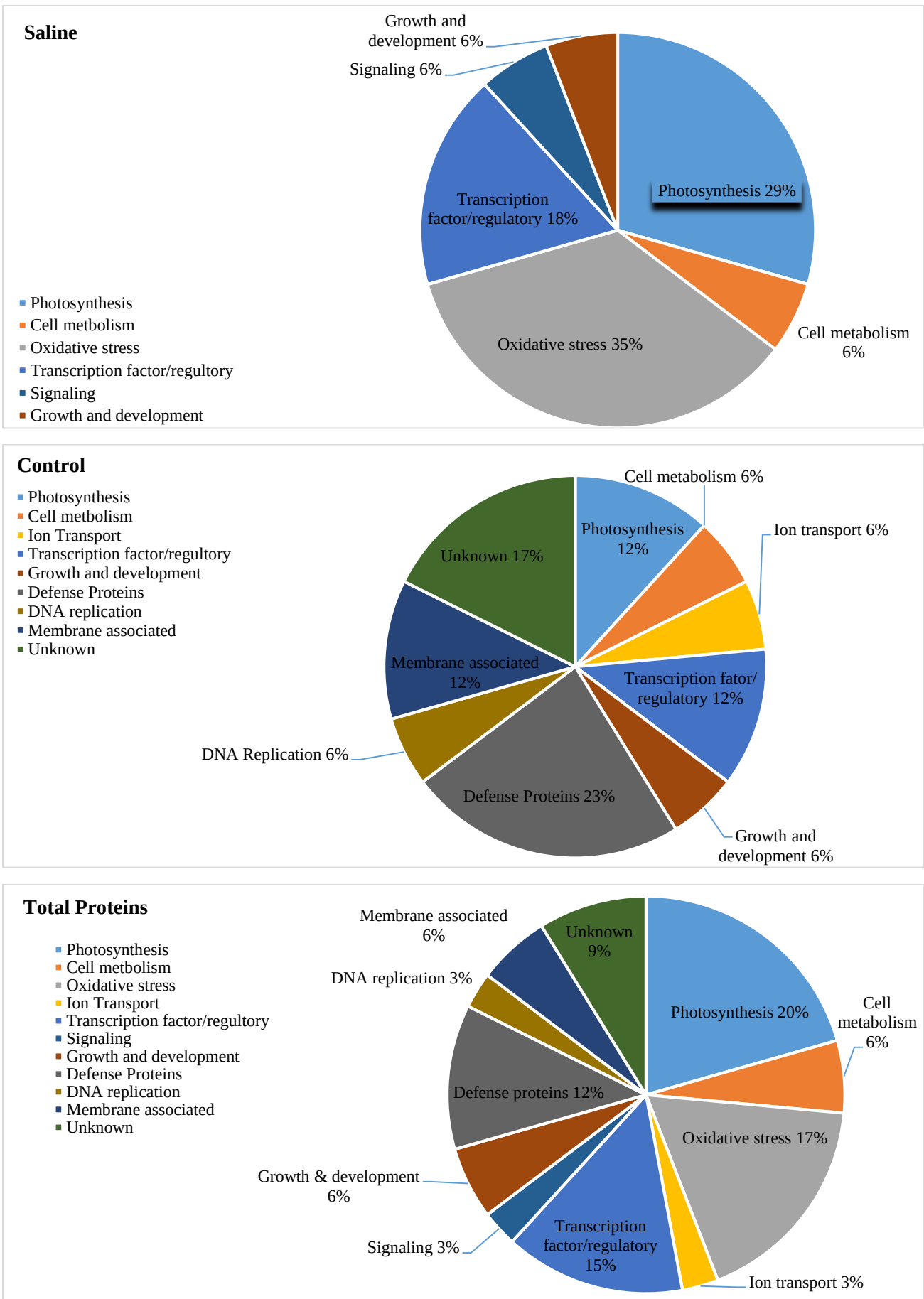

Fig. 6. Percentage of LC-MS/MS commonly identified proteins in 13 canola (*Brassica napus* L.) under control and saline conditions.

Discussion

Cultivars differing in salinity tolerance may have been due to difference in one or combination of various physiological or biochemical processes. Selecting cultivars using physiological selection criteria is one of the important strategies to develop salt tolerant cultivars. Poor understanding about detailed mechanism of salt tolerance is one of the major obstacles to use physiological indicators in breeding for stress tolerance programs (Ashraf *et al.*, 2008; Bose *et al.*, 2017; Zafar *et al.*, 2017; Ogbaga *et al.*, 2018). Physiological studies complemented with proteomic studies can help us in understanding mechanism of salt tolerance (Khalid *et al.*, 2015; Messedi *et al.*, 2016). It is proposed that greater salt tolerance in cultivars DGL, Dunkled, Faisal Canola and Punjab Canola out of 13 canola cultivars might have been due to greater photosynthetic capacity, ion exclusion, and antioxidant enzymes activities.

Maintaining water and ion homeostasis is challenging for plants in general and particularly in crops growing under saline conditions. In the present study, RWC was decreased in all canola cultivars under saline conditions. To maintain leaf turgor, plants accumulate inorganic solutes as a cheap source such as K^+ and Na^+. However, preferential accumulation of K^+ over Na^+ become inhibited under salt stress conditions because of sodium toxicity. Generally, all plants limit uptake of Na^+ in roots and leaves (Munns & Gilliham, 2015; Bose *et al.*, 2017). However, extent to which these salts are accumulated in a specific plant parts depend upon the plant capacity to limit uptake of these elements (Ali *et al.*, 2006). Results from current study demonstrate that cultivars Cyclone, Bulbul-98 and Oscar accumulated higher Na^+ concentration as compared to cvs. Dunkeld, DGL, Faisal Canola and Punjab canola. In addition, cultivar DGL accumulated higher K^+ concentration whereas reverse was true for cv. Cyclone. Furthermore, higher K^+/Na^+ ratio was obtained in cvs. Dunkeld and DGL and lowest in cv. Cyclone and Bulbul-98. These results indicated that salt tolerant canola cultivars particularly DGL and Dunkled had salt exclusion mechanism as discussed elsewhere that reduction in cytosolic Na^+ concentration favored salt exclusion and salt tolerance (Munns & Tester, 2008; Munns & Gilliham, 2015; Ismail & Horie, 2017).

Fast chlorophyll a kinetic analysis OJIP test was used to assess the structural and functional ability of photosystem-II (PSII) in plants under normal or stress conditions (Kalaji *et al.*, 2018). In the present study, quantum yield of PSII measured as Fv/Fm remained same due to salt stress in all canola cultivars (Data not shown) but it decreased Fv/Fo (quantum efficiency of PSII). It is suggested that quantum efficiency of PSII measured as Fv/Fo is more sensitive to salt stress than Fv/Fm and more reliable (Baker, 2008; Oukarroum *et al.*, 2015; Kalaji *et al.*, 2018). Further PSII activity can be analyzed by accessing relative variable fluorescence at different steps. Relative variable chlorophyll fluorescence at J step denoted as V_J represents number of closed reaction centers. Higher values of V_J was observed in cvs. Cyclon, Ac-Excel and Legend indicated increase in closed reaction centers in PSII. V_I is for normalized variable fluorescence at point of OJIP transient and reflects the ability of PSI and its acceptors to reoxidize the reduced plastoquinone. We observed minimum ability of PSI to oxidizing the PQ in salt sensitive canola cultivars Legend, Cyclone, AC-Excel. To assess the extent of adverse effects of salt stress on primary photochemistry in canola cultivars, energy fluxes (*ABS/RC*, *TRo/RC*, *ETo/RC* and *DIo/RC*) were calculated following JIP-test. Salinity stress did not change the *ABS/RC* and *TRo/RC in all canola cultivars except in Legend where it increased ABS/RC*. Moreover, *ETo/RC* energy flux for electron transport decreased in moderately salt sensitive or salt sensitive canola cultivars viz. AC-Excel, Shiralee, CON-II and Cyclone. However, *DIo/RC* was maximal in Legend, AC-Excel and Cyclone. From these results, it is suggested that salt tolerant canola cultivars-maintain energy conversion efficiency by down-regulating PSII activity with increase in *DIo/RC.* However, moderately salt sensitive and salt sensitive cultivars Legend and Cyclone were unable to sufficiently down-regulate PSII activities and thus excess excitation damaged PSII (Greater decrease in PI_{ABS}). However, cultivars AC-Excel down-regulate PSII activity with transfer of excitation pressure to PSI, thus PSII protected by dissipating heat as reflect greater PI_{ABS} values. From the results of the present study, it can be suggested that salt stress reduced energy fluxes for absorption, trapping and energy conversion efficiency resulting in photoinhibition in cultivar dependent manner.

Among plant metabolites, proteins play a vital role in initiating, regulating and inducing stress tolerance responses plants ranging from accumulation stress related proteins such as dehydrins, heat shock proteins to antioxidant enzymes, ion transporters and transcription factors. Over the past decade, a number of studies have been published in assessing crop stress tolerance in model species using proteomic approaches (Sugimoto & Takeda, 2009; Zhang *et al.*, 2012; Kosová *et al.*, 2014; Xu *et al.*, 2016; Li *et al.*, 2017). However, a very few attempts in assessing salt tolerance have been made so far in crop species like canola. Comparison of contrasting canola cultivars differing in salinity tolerance will provide insight information for salt tolerance. These results suggested that 18 proteins differentially expressed under saline conditions in a set of 13 canola cultivars. Of 18 differentially expressed proteins, six proteins were related with antioxidant activities such as peroxidase 73, chloroplastic Cu-Zn SOD, thioredoxin H5, Glutathione S-transferase DHAR2. Several studies signified the role of fine tuning of ROS level and antioxidants in salt tolerance in plants (Pang *et al.*, 2010; Manaa *et al.*, 2011; Wang *et al.*, 2015; Maršálová *et al.*, 2016). In addition, five identified proteins were responsible for light reaction and CO_2 fixation (chlorophyll biosynthesis and CO_2 fixation in Calvin cycle). These results are parallel to the findings of Pang *et al.*, (2010) who reported that a number of stress related proteins differentially over-expressed in salt tolerant *Thelungiella halophilla* and *Arabidopsis thaliana*. In some of the earlier studies, salt stress caused profound alterations in protein with photosynthetic metabolism including oxygen evolving complex, PSII subunits, rubisco and rubisco activase (Caruso *et al.*, 2008; Sobhanian *et al.*, 2010). In the present study, two proteins related with ATP metabolism were identified in salt

stressed plants of canola such as mitochondrial ATP synthase. From these results, it validates the arguments that ATP metabolism has a principal contribution in inducing salt tolerance because salt tolerance is an energetic process. For example, salt exclusion in durum and hexaploid wheat depends on H-ATPase activity (Cuin *et al*., 2011). Similarly, Ayala *et al*., (1997) found that salt tolerant wheat cultivars had greater H-ATPase activity than in salt sensitive one. Regarding chlorophyll biosynthesis, magnesium chelatase subunit ChlH differentially expressed in salt stressed plants of canola. During chlorophyll biosynthesis, this enzyme catalyzes the incorporation of Mg^{2+} in protoporphyrin IX. Moreover, this process is also ATP-dependent. These results were similar to the findings of Maršálová *et al*., (2016) in which they identified in salt tolerant species of *Hordeum vulagre*. In contrast, Cheng *et al*., (2015) found that magnesium chelatase decreased in salt stressed plants of *Tangut nitraria*. Only one protein UDP-glucose for carbohydrate anabolism was differentially expressed in salt stressed canola cultivars. From the results of the present study, it was also observed that salt tolerant cultivars Faisal Canola, Dunkled and DGL had greater number of differentially expressed proteins related with antioxidants and photosynthesis compared with those of salt sensitive cultivars Oscar, Legend, and Cyclone. However, variations in different canola cultivars with respect to these proteins relative abundance can be explained as poor detection in some samples or sequencing smaller number of peptides do not warrant good identification under strict criteria for identification.

In conclusion, salt tolerance in 13 canola cultivars were associated with multiple sub-components of salt tolerance such as salt exclusion, antioxidant capacity and photosynthesis. Although, salt exclusion is major component of salinity tolerance in number of crops and being used as selection criteria, antioxdants and photosynthetic capacity have major role in determining degree of salinity tolerance in canola cultivars.

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