

AN INVESTIGATION OF SOME KEY MORPHO-PHYSIOLOGICAL ATTRIBUTES AND LEAF PROTEOME PROFILE IN CANOLA (*BRASSICA NAPUS* L.) UNDER SALINITY STRESS

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Abstract

A serious abiotic stress in the world is salinity, which leads to great losses in the quality and quantity of products in agriculture. To investigate the tolerance mechanisms to salinity, a two-dimensional electrophoresis approach was adopted to visualize proteins achieved from the second-freshly-developed leaf of canola cv. Safi-7 seedlings, after plants had been treated with 0 and 300 mmol/L NaCl. Treatment with NaCl (300 mmol/L) significantly decreased the fresh and dry weights as well as the plant height, and promoted the proline content compared with the control group. Any changes were not significant neither quality nor activities of catalase and peroxidase. Out of 110 repeatable protein spots, 37 spots were differentially changed according to induction factor index. Among differentially changed proteins, five were significantly showed the changes. These proteins have roles, to produce the energy, and scavenge and suppress ROS, and in photosynthesis.

Key words: Down-regulation, Proteome, Salt stress, Two-dimensional electrophoresis (2DE), Up-regulation.

Introduction

Salinity is an abiotic stress, which imposes undesirable effects on the quality and yield of products in agriculture, so much that 20% of cultivation in the world is affected by salinity (Zhao *et al.*, 2007). Besides negative effects, salinity causes to over produce oxygen reactive species, commonly known as ROS, in the plant cells (Khan & Panda, 2008). These are very active and toxic molecules which lead the death of cells, through damaging proteins, lipids in the plasma membrane and nucleic acids (Morant-Manceau *et al.*, 2004). In early stages of growth, salinity reduces attributes such as the length of rootlet and young stem, dry and fresh weight of rootlet and young stem as well as the weight and leaf area index (Sexcion *et al.*, 2009; Jianjie *et al.*, 2013).

Correlation between anti-oxidant activity of enzymes and salt tolerance, through comparisons in many sensitive and tolerant plant cultivars disclosed the fact that the anti-oxidant defense system includes both enzymatic (including superoxide dismutase (SOD), catalase (CAT), peroxidase (POX or POD, and Ascorbate peroxidases (APX) and non-enzymatic anti-oxidants (such as proline, betaine, ascorbate) (Silva *et al.*, 2008). Mitigating oxidative damage and increasing the tolerance against environmental stresses are often associated with oxidative system (Raskin, 1992). Besides enzymes scavenging Reactive oxygen species (ROS), another approach, namely synthesizing organic compounds, including proline and betaine is utilized by plant defense system in response to a myriad stresses (Serraj & Sinclair, 2002). It is well established by now that these compounds contribute to balance the osmotic pressure (Rhodes & Hanson 1993), enzyme activity (Mansour, 2000), ROS detoxification (Ashraf & O'Leary 1994), and plasma integrity protection (Bohnert & Jensen, 1996).

Notwithstanding, identifying proteins involved in salt tolerance and their functions can help to set up an efficient breeding program (Shavrukov *et al.*, 2010). In the meantime, proteomics which was developed

sequential to develop other techniques, such as extraction, separation, and identification of proteins, played an undeniable role in the collection of proteins with biological functions (Wang *et al.*, 2011). This is an approach, based on two-dimensional electrophoresis (2DE) method, which provides reliable tools for identifying the candidate genes coding for tolerance (Chen & Harmon 2006). However, it needs to investigate the dynamic expression of proteins under salinity condition (Guo *et al.*, 2012). Comparative proteomics provides useful information concerning defense mechanisms against salinity before and after stress imposition (Mustafa Kamal *et al.*, 2010). A lot of researches, so far, have used this approach to tackle the issue, and many proteins have been identified their expressions in sync with protein concentration (Jiang *et al.*, 2007). Bandehagh *et al.*, (2011), for example, studied salt tolerance in canola by extracting proteins from seedlings. The results indicated that Na tends to accumulate in sensitive cultivars more than tolerant ones. Also, when detected proteins were analyzed, researchers found that out of 900 spots, 44 and 31 repeatable spots in tolerant and sensitive cultivars bore significant changes in expression, respectively. They concluded that photosynthetic proteins play important biochemical roles to tolerate salt stress (Bandehagh *et al.*, 2011).

Oilseeds, in general, are placed third, after cereals and pulses, in terms of food. Brassicaceae family, which canola belongs to, holds the third rank in all category of oil seeds category (Shirazi *et al.*, 2011). Among different species, canola is the first in terms of importance, because its oil has less erucic acid, hence giving good quality to cooking oil. Yet, its production is the minimum due to different stresses including salinity (Francois *et al.*, 1994). Therefore, in the current study, the aim was to tackle some of the issues related to the effects of salinity stress on the morphological, physiological and protein profile as well as identifying proteins and other compounds involved in the defense mechanisms, using 2DE and other laboratory techniques.

Plant materials and trait measurements: This study was implemented as a completely randomized design and seven replications under greenhouse condition in 2014. Treatments were included salinity in two levels (control or 0 and 300 mmol NaCl) and canola cultivar. The canola cultivar "Safi 7" was used in this experiment. Plants were grown in hydroponic culture system as explained by Bandehagh *et al.*, (2008). 34 day-old seedlings were treated with 300 mmol of NaCl. Ten days after imposing salt stress treatment, leaves were collected and immersed into liquid N₂ and stored at -80°C for protein extraction. Fresh weight and plant height were measured at the sampling moment. An oven was used to dry plants at 70°C for 48h and dry weight was determined.

Measurement of free proline: Proline content was measured by using ninhydrin reaction method (Bates *et al.*, 1973). Homogenization of 0.2 g leaves was conducted with 5 ml of 3% sulphosalicylic and centrifuged at 25°C for 7 min. Then, the filtered extract (1 mL) was taken to analyze with 1 ml ninhydrin reagent and 1 ml glacial acetic acid. The mixture was kept in 100°C water bath. The extraction of reaction mixture was conducted with 2 ml toluene, and the measurement of chromophore absorption was conducted at 250 nm against toluene as blank with spectrophotometer (Beckman, Fullerton, CA, USA). Proline content was calculated from an L-proline standard curve.

Enzyme extraction: 0.15 g fresh leaf was ground up by the liquid N₂ to a fine powder, then, 1.5 ml buffer phosphate 50 mmol (PH=7.5) containing PVP 1% and 2 mmol EDTA were added, and ground some more. The obtained extract was centrifuged at 20000 g at 4°C for 20 min. The clear and floating supernatant was used to assay catalase (CAT) and peroxidase (POX).

Peroxidase assay: POX activity was evaluated according to (MacAdam *et al.*, 1992). 0.5 g of the root specimen was homogenized in cold potassium phosphate buffer 0.1 M (PH=7.5) containing 0.5 mmol EDTA. Homogenized samples were centrifuged at 15000 g for 15 min. 20 µl of the homogenized supernatant was added to 0.81 ml potassium phosphate buffer 0.1 M (PH=6.6). 90 µl of 1% guaiacol was added to the product. The solution was poured in a cuvet. and, 90 µl of 3% H₂O₂, as the electron receptor, was added a seconds before measuring the reaction speed. Absorbance measurement was conducted at 470 nm at 25°C for 60 seconds in a spectrophotometer. Enzymatic changes recorded as the changes of absorption in minute mg⁻¹ protein.

CAT assay: For CAT, the assessment was carried out according to Pereira *et al.*, (2002). First, 3.5 ml of peroxide hydrogen 3% was taken and mixed with 50 ml double distilled water. From the solution, then, 17.5 µl was taken and mixed with 707.5 µl PVP- and EDTA-free potassium phosphate buffer. Afterward, 20 µl of the sample was added and, finally, absorption was recorded at 240 nm at 25°C for 60 seconds with spectrophotometer.

Leaf proteins extraction and quantitation: Total protein was extracted from approximately 0.5 g of frozen leaf per each biological replicate and ground to a fine powder in cold acetone containing 10% TCA and 0.07% 2-Mercaptoetanol. The resultant powder was dissolved in Lysis buffer, which contained 7 M urea, 2 M thiourea, 2% chaps, 60 mmol DDT and 1% ampholyte (pH: 3-10). The amount of protein was determined by Bradford assay (Bradford, 1976).

Two-dimensional electrophoresis and image analysis: The crude protein (400 mg, 100 µL) was separated by 2-DE (O'Farrell 1975) in the first dimensional isoelectric focusing (IEF) in tube gels and, in the second stage, by SAS-PAGE. An IEF tube gel of 11 cm length and 3 mm diameter consisted of 8 M urea, 3.5% polyacrylamide, 2% NP-40, 2% ampholyte (pH: 3-10 and 5-8), ammonium persulfate and TEMED was prepared. The voltage set for the IEF was 200 v for 30 min, 400 v for 16 h and 600 v for 1 h. Then, tube gels were retrieved from the glass tubes and subjected to the second dimensional (SDS-PAGE) by transferring onto a 15% acrylamide separating gel and 5% acrylamide stacking gel. The staining of gels was conducted with coomassie brilliant blue (G-250).

The scanning of analytical gels was implemented using GS-800 calibrated densitometer (Bio-Rad) at 600 dpi resolution. The scanned gels were saved as TIFF images for subsequent analysis using PDQuest software (version 8.0, Bio-Rad). Spot detection, spot measurement, background subtraction and spot matching were performed. After the automated spot detection, images of gel were correctly edited. Three well-separated gels of each treatment were applied to make replicate groups. Statistic, quantitative and qualitative "analysis sets" were made between each of control group and corresponded treated group. The quantity of the protein spot was explained as the intensities of all the proteins on gels determined by the migration of protein spots along the 11 cm IEF (5-8 linear). Protein identification was performed using searching program against the NCBI canola protein database, and matching the Isoelectric Point (pI) and Molecular Weight (MW) values of the changed spots with those from the protein databank.

Statistical analysis: Statistical analysis of traits were performed using IBM.SPSS Statistics v21 computer software package. Means comparison were tested using the t-test at the 1% probability level.

Results

Summary of mean comparisons is provided in Table 1. Reduction in aerial fresh weight (g), dry weight (g), plant height (cm) and proline content (µg/g fresh weight) are significant at 1% probability, according to the results.

It turned out that CAT and POX didn't undergo significant changes under stress conditions. Applying 300 mmol NaCl changed the average for above-mentioned traits, according to the Fig. 1. Out of 110 repeatable protein spots, which were detected in cultivar Safi 7, 37 spots showed significant changes in expression, based on Induction Factor (IF) index. After performing the t-test, a

total of 5 protein spots (assigned as A, B, C, D and E on the Fig. 2) were recognized significant at 5%. Spot A was overregulated, while the others showed down-regulation. Table 2 shows the features of protein spots illustrated in Fig. 3. The changes of expression for proteins in the treatments and control.

Table 1. Mean Comparisons of NaCl influence on the measured parameters of the cultivar of Safi 7.

	Fresh weight	Dry weight	Plant height	Proline	CAT	POX
Normal	17.33 ^a	1.30 ^a	34.26 ^a	33.34 ^b	1.99	0.30
Stress	5.90 ^b	0.74 ^b	21.69 ^b	305.85 ^a	1.31	0.40
t statistic	8.248**	4.514**	8.699**	-10.966**	0.824 ^{ns}	0.430 ^{ns}

** and ns represent significant at 1% and non-significant, respectively (a: maximum mean and b: minimum mean)

Salinity stress causes the reduction in the quantity of fresh and dry weights of plants, which is due to the presence of toxic ions, osmotic effects and imbalanced hormone secretion and nutrition distribution (Ashraf, 2009). Farhoudi (2011) reported that the fresh and dry weights in canola were decreased under salinity condition. Other researchers indicated that toxic ion accumulation in the rhizosphere imposed osmotic stress, dehydration, and reduction in water uptake in plants. Resistance to water uptake, retarded cell growth and development are primary causes, following salt stress, to restrict root growth (Cavalcanti *et al.*, 2007).

Salinity influences aerial parts of canola as well as the roots, as it causes the reduction in fresh and dry weights (Jamil *et al.*, 2014). In that condition, root and shoot growth are impeded as turgor shrinks as a consequence of the reduction in water potential in rhizosphere (Alam *et al.*, 2004). The same was reported for plant height (Ghuge *et al.*, 2011).

Ashrafijou *et al.*, (2010) reported that proline accumulation in canola was increased with NaCl concentration. Maximum and minimum amount of proline were produced with 300 mmol treatment and the control, respectively. Proline is an important osmolyte, which is common in microorganisms and plants under stress conditions, and play a role as a protectant. Some believe that its accumulation in plants is due to low anti-oxidative activity of antioxidant enzymes (Sudhakar, 2001).

Salinity causes oxidative stress through over producing ROS, including H₂O₂, OH⁻ and O₂ (Gomez *et al.*, 2004). To eliminate ROS, plants produce, through evolution, antioxidant defense mechanism which includes the enzymatic and non-enzymatic processes (Apel & Hirt, 2004). H₂O₂ production is dangerous, because it damages lipids in cell plasma membrane as well as other biomolecules (Mittler, 2002). Therefore, it has to be eliminated immediately after synthesis, an action performed mostly by CAT in glyoxysomes and peroxisomes, and by POX in chloroplasts, mitochondria, and apoplast (Shigeoka *et al.*, 2002). SOD, another important antioxidant enzyme, eliminates O₂ molecules, producing H₂O₂ and O₂ (Tuna *et al.*, 2008).

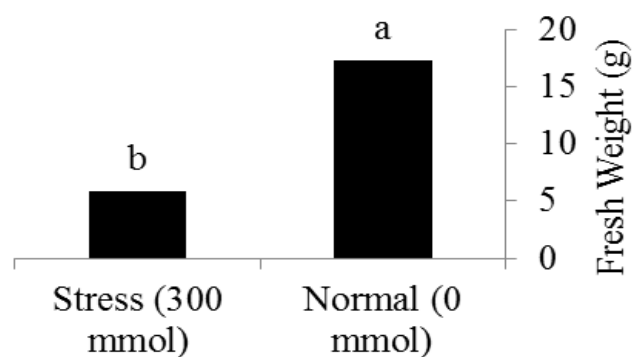


Fig. 1a. Average values of fresh weight measured in the leaves of canola grown under two treatments, 0 and 300 mmol NaCl. (a: maximum mean and b: minimum mean).

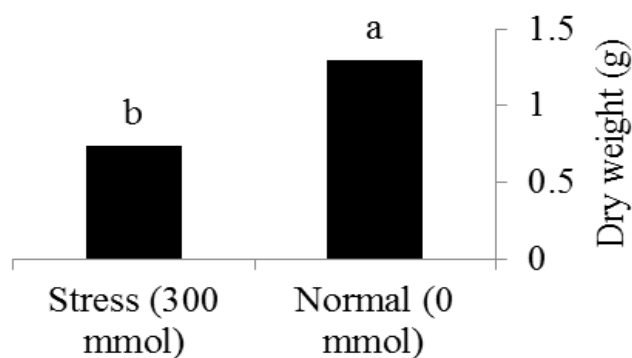


Fig. 1b. Average values of dry weight measured in the leaves of canola grown under two treatments, 0 and 300 mmol NaCl. (a: maximum mean and b: minimum mean).

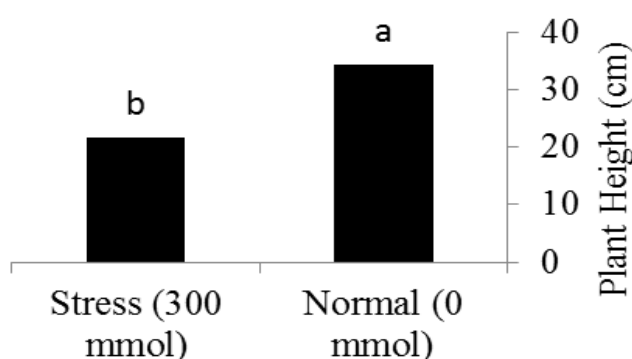


Fig. 1c. Average values of plant height measured in the leaves of canola grown under two treatments, 0 and 300 mmol NaCl. (a: maximum mean and b: minimum mean).

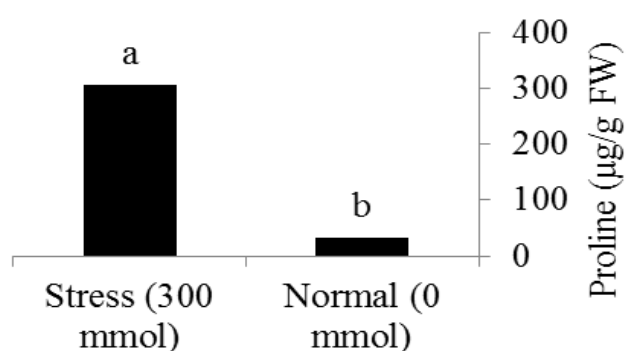


Fig. 1a. Average values of proline measured in the leaves of canola grown under two treatments, 0 and 300 mmol NaCl. (a: maximum mean and b: minimum mean).

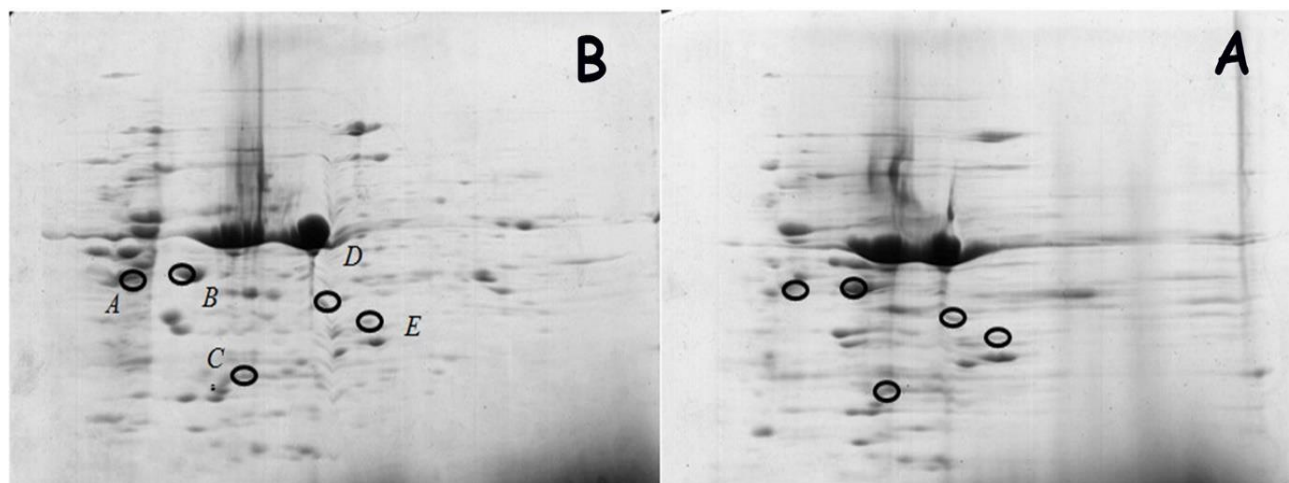


Fig. 2. An illustration of proteins detected with 2DE under normal (no salt, A) and stress (300 mmol salt, B) conditions.

Table 2. Detailed characteristics for the protein spots with changes in expression detected under 300 mmol influence of NaCl.

Change in expression type	Protein name	pI/MW Theo.	pI/MW Exp.	Accession number	Spot designated
Increase	Cytosolic Malate dehydrogenase	5.7/35.80	5.72/33	gi/49343245	(A)
Decrease	Chloroplastic fructose-bisphosphate aldolase	5.95/42.21	5.85/42.31	gi/223018643	(B)
Decrease	Copper/Zinc SOD	6.28/22	6.03/24/96	3273753	(C)
Decrease	PSBO-2/PSBO ₂ (PhotosystemII subunit O-2) oxygen evolving	5.92/35	6.43/36.03	15230324	(D)
Decrease	Triosephosphate isomerase	6/31.95	6.6/31.47	gi/1174745	(E)

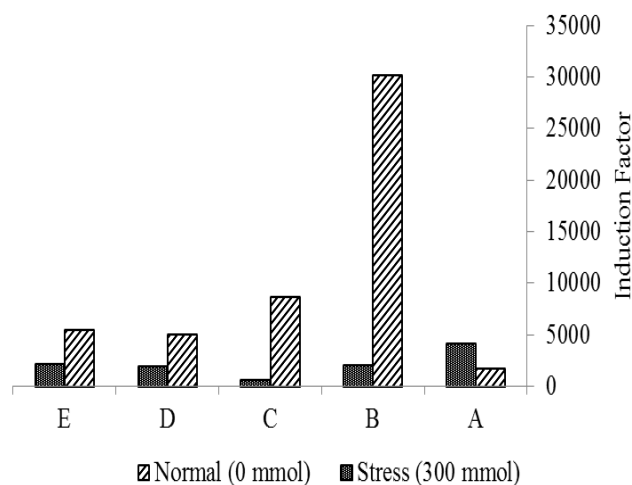


Fig. 3. The changes in expression of proteins detected in canola leaves under the Control and 300 Mmol NaCl stress conditions.

Increased anti-oxidative activity of enzymes upon salt stress, gives the idea that these are involved in salt tolerance in plants (Gao *et al.*, 2008). However, influenced by some mechanisms, which are unknown, amount and activity of these enzymes remained unchanged. In experiments on potatoes (Benavides *et al.*, 2000), and rice (Lin & Kao, 2000), for example, researchers found no change in CAT activity. Cultivars of SIm and Opera in rapeseed showed no change in the activity of POX under salinity stress (Farhoudi *et al.*, 2015), which was consistent with our results herein.

Protein spot A, which is likely cytosolic malate dehydrogenase, was over-regulated (Fig. 2). This is the enzyme which catalyzes malate to oxaloacetate by transformation and reverse reactions. In cytosol,

mitochondria and peroxisome, the enzyme is dependent on NAD⁺, while in chloroplast, it is dependent on NADP⁺ for its functions. Cytosolic NAD⁺-dependent malate dehydrogenase catalyzes oxaloacetate to malate by transformation, then, it is transported into mitochondria through dicarboxylate carriers, therein, consumed in the tricarboxylic acid cycle (TCA). The process enhances the cycle. Besides, oxaloacetate is used as a precursor for a few amino acids. It is said that enhancement to malate dehydrogenase activity is meant to keep the TCA and to synthesize amino acids as well (Kumar *et al.*, 2000). Protein spot B, probably chloroplastic bisphosphate aldolase, is down-regulated in the cultivar studied. There are two isoforms of this enzyme in plants, namely, cytosolic and chloroplastic. The chloroplastic isoform is involved in the starch production, in the process of producing fructose1, 6 bisphosphates from D-gliseradehid-3-phosphate and dihydroxy acetone. The reduction in production may lead to ATP and NADPH accumulation in chloroplast, hence protect plants from photo-oxidative damage (Michaelis & Gepstein, 2000). Chloroplastic bisphosphate aldolase showed down-expression in wheat under salinity stress, likewise (Caruso *et al.*, 2008).

Protein spot C, a Cu/Zn SOD showed down-expression (Fig. 2). It is related to a group of antioxidant enzymes, under the name of SOD involved in transforming superoxide radicals into oxygen molecules, which account for the main process in plants for scavenging ROS. It is also said that they constitute the first mechanism against ROS, which changes superoxide to H₂O₂, a less toxic molecule. In the lack of enough CO₂ as an electron receptor, electrons flow across photosynthetic membranes to join oxygen molecules to form superoxide ions, in the Mehler process (Cakmak, 2005).

There are three identified distinct groups of SODs. One SOD is dependent on Cu and Zn and unavailability of Zn, for whatever reason, may put a dent in enzyme's activity (Sun *et al.*, 2006). In response to salinity stress, the availability of Cu/Zn SOD is reduced (Komatsu & Tanaka, 2004). Congruent to our findings, the activity of Cu/Zn-SOD was also reduced in a sensitive cultivar under salinity stress (Ashraf & Harris, 2004). The plunge, however, is thought associating with a sudden peak in the production of OH⁻ radicals in the chloroplast of the leaves stressed by salinity. In other words, over- production of ROS, following salinity stress, interrupts normally-established balance between ROS production and scavenging under oxidative stress (Sun *et al.*, 2006).

Spot D, likely a Photosystem II subunit O-2 oxygen evolving (PSBO-2/PSBO2), also showed down-expression (Fig. 2). Based on oxygen-evolving family, this enzyme stabilizes manganese group as the main center of water hydrolysis (Kumar *et al.*, 2003). Downsized activity of this enzyme showed that salinity stress affect the photosynthesis processes, namely, oxygen release and photosystem complex II. Therefore, it plays a critical role in leaves senescence and gradual die (Komatsu & Tanaka, 2004).

Finally, the E spot, probably a Triose-phosphate isomerase, likewise, showed down-regulation in our study. It catalyzes the two-way transformation of dihydroxy acetone phosphate and D-Glyceraldehyde tri-phosphate. Two isomers of this enzyme were identified; one cytosolic and one chloroplastic. The last is involved in kelvin cycle (Gao *et al.*, 2011). It seems that over-production of chloroplastic Triose-phosphate isomerase is to maintain energy force required for detoxification of ROS and recovering from the damages caused by thereof (Wang *et al.*, 2008; Gao *et al.*, 2011). It also seems as though, for cytosolic isoform, the synthesis increases in response to methylglyoxal, according to Sharma *et al.*, (2012), on transgenic rice. In contrary, its activity reduces in occasions when stress is followed by an unexpected increase in the production of ROS, hence the expression is based on how to regulate its transcription. Our findings are consistent with the stated researches.

Conclusion

In this research, application of salinity on a Canola cultivar created significant changes in dry and fresh weights, plant height as well as proline content at 1% probability according to the results. Results showed that salinity stress (300 mmol) decreased fresh and dry weights and plant height, whereas proline content was increased. Any significant changes were not found in CAT and POX. Among differentially changed proteins, five spots were statistically recognized significant at 5% probability level. It seems that the activities of SOD and triose phosphate isomerase (OscTPI) are down-regulated under the influence of ROS compared to the control, according to 2DE results. Increased activity of Malate dehydrogenase helped to maintain TCA cycle and amino acid synthesis. Considering the destroying effects of salinity on the productivity of crop plants, many other investigations seem to be necessary to thoroughly comprehend the mechanisms attendant to mitigate stress influence, understand plants' short and long-term response to salinity as well as identifying proteins.

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