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RESEARCH ARTICLE

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Mitigation of salinity stress in canola plants by sodium nitroprusside application

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Abstract

Salinity is a global issue threatening land productivity and food production. The present study aimed to examine the role of sodium nitroprusside (SNP) on the alleviation of NaCl stress on different parameters of canola (*Brassica napus* L.) plant growth, yield as well as its physiological and anatomical characteristics. Canola plants were grown under greenhouse conditions in plastic pots and were exposed to 100 mM NaCl. At 50 and 70 days from sown, plants were sprayed with SNP (50 and 100 µM) solutions under normal or salinity condition. Growth and yield characters as well as some biochemical and anatomical changes were investigated under the experimental conditions. Salinity stress caused an extremely vital decline in plant growth and yield components. A significant increase was found in membrane permeability, lipid peroxidation, hydrogen peroxide, sodium, chloride, proline, soluble sugars, ascorbic and phenol in canola plants under salinity stress. Under normal conditions, SNP application significantly increased all studies characters, except sodium, chloride, hydrogen peroxide, lipid peroxidation, membrane permeability that markedly reduced. Application of SNP to salt-affected plants mitigated the injuries of salinity on plant growth, yield, and improved anatomical changes. The present investigation demonstrated that SNP has the potential to alleviate the salinity injurious on canola plants.

Additional keywords: antioxidants; *Brassica napus* L.; osmoprotectants; ultrastructure; yield.

Abbreviations used: ARC (Agricultural Research Centre); AsA (ascorbic acid); DFS (days from sowing); DW (dry weight); EC (electrical conductivity); FBN (flowering branches number/plant); ELP (electrolyte leakage percentage); FN (fruit number/plant); FW (fresh weight); LA (leaf area); LBT (leaf blade thickness); LN (leaf number); LP (lipid peroxidation); LRWC (leaf relative water content); MDA (malondialdehyde); MP (membrane permeability); PPT (palisade parenchyma thickness); ROS (reactive oxygen species); SDW (shoot dry weight); SFM (seedling fresh weight); SI (seed index '100 seed weight'); SL (seedling length); SN (seed number/pod); SNP (sodium nitroprusside); SPT (spongy parenchyma thickness); SY (seed yield/plant); TMR (thickness of midrib region); TP (total soluble phenol); TSC (total soluble carbohydrates); TW (turgid weight); VBT (vascular bundle thickness); VBW (vascular bundle width); WC (water content).

Authors' contributions: Both authors contributed equally in the design and performance of the experiments, statistical analysis of the experimental data, drafting and critical revision of the manuscript for important intellectual content.

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Introduction

Vegetable oils are an important part of the human diet. Nowadays, cultivation of vegetable-oil producing crops covers about 270 million hectares and produces 170 million tons, which accounts for approximately 75% of world production (FAO, 2014). Egypt's self-sufficiency of vegetable oil declined from 95 % in early 1960's to less than 30 % in 2004, resulting in an increase in the importation of processed vegetable oil (Hassan & Shafique, 2011). In 2011, Egypt spent about US$ 1.875 billion on the importation of vegetable oils (FAOSTAT, 2011). The United States Department of Agriculture, Division of Foreign Agriculture Service, reported in 2013-2014 that Egypt's total oilseeds planted area only covers 3-5% of Egypt's total edible oil consumption. Expanding into edible-vegetable oil production in Egypt is limited due to the limitation of agricultural land and water supplies. Therefore, expanding cultivation into newly reclaimed areas, while developing new high-yielding varieties, as well as minimizing agricultural inputs, might be the only formula for Egypt to fulfill its vegetable oil need. Canola (*Brassica napus* L.) is considered a specialty crop in Canada and USA. In Egypt, canola can contribute to reducing oil deficiency gaps. Additionally, it grows

vigorously within the northern part of Egypt, newly reclaimed soil.

Salinity is a global issue threatening land productivity and food production. Globally, about 900 million ha are affected by salinity and salinization is predicted to impact 50% of all arable lands by 2050 decreasing the major crop yield by over 50% (FAO, 2014). Under salinity stress, reduction in crop productivity, as well as changes in anatomy and ultra-structure, are sometimes interlinked with concomitant changes in a variety of biochemical, physiological, and molecular responses (Ahmad *et al.*, 2018). Salinity also acts as pro-oxidant and induces oxidative burst through hyper-accumulation of reactive oxygen species (ROS) that cause injuries to cellular ultra-structure, organic compounds and impaired a variety of metabolic reactions (Gupta *et al.*, 2017; Ahmad *et al.*, 2018; Kaya *et al.*, 2018; Kholghi *et al.*, 2018; Rahmani *et al.*, 2018).

Currently, there are many strategies for improving plant productivity under salinity, like genetic modifications, and/or using some inducers (Gupta *et al.*, 2017; Akram *et al.*, 2018). Sodium nitroprusside (SNP, nitric oxide donors) is a small diffusible bioactive signaling molecule, plays multifunctional roles in plant growth, productivity under normal or stressed conditions (Akladious & Mohamed, 2017; Ahmad *et al.*, 2018; Hanafy *et al.*, 2018). SNP can alleviate salt stress by directly scavenging ROS throughout the formation of peroxynitrite (ONOO-) (Beligni & Lamattina, 2002), that is less toxic than peroxides and conjointly by enhancing antioxidant enzyme activities and metabolites (Sheokand *et al.*, 2010). Moreover, SNP application stimulates activities of proton-pump within the tonoplast and increasing the K^+/Na^+ ratio (Beligni & Lamattina, 2002), additionally regulates proteins at the post-translational level needed for cellular division (Sheokand *et al.*, 2010).

Due to very few reports on inducing salt tolerance of canola plants by SNP, the present study is of special interest for improving canola production. The hypothesis of work is that SNP would improve canola salt tolerance by activating the processes would lead to attenuation of the cellular damage caused by salinity.

The aim of the current investigation was to assess the protective role of SNP for the alleviation of NaCl stress on different parameters of canola plant growth, yield as well as its physiological and anatomical characteristics.

Material and methods

Experimental layout and treatments

Two pot experiments were carried using a clay loam soil throughout 2014/2015 and 2015/2016 winter seasons at the Agricultural Botany Department Experimental Farm, Mansoura University. Water content (WC) at field capacity (30.8% and 31.0%), WC at the permanent wilting point (24.4 and 24.6%), dry bulk density (1.38 and 1.37 g/cm^3), pH (7.1 and 7.2), organic matter content (1.2% and 1.1%), and electrical conductivity (EC) (1.10 and 1.11 dS/m) of the experimental soil in both years respectively were determined. The experiments were performed in a completely randomized design.

Plastic pots (40 cm in diameter) containing homogeneous air-dried soils were divided into six groups consisting of 10 pots for each. Phosphorous as calcium super-phosphate at the rate of 2 g/pot and 1 g/pot potassium fertilizers as K^+ sulfate was added and mixed into the upper 3 cm of the soil of all pots before planting. We used canola cv Pactol seeds certified by the ARC, Ministry of Agriculture, Egypt. Ten sterilized (soaking in 0.001 $HgCl_2$ for 1 min, then washed several times with distilled water) air-dried seeds were planted in each pot on 10th November in both seasons. After full emergence (20 days from sowing), the seedlings were thinned to leave 4 healthy and homogenous plants per pot. The nitrogen fertilizer was added at three intervals after 20, 30 and 60 days from sowing as ammonium nitrate at the rate 0.5, 2 and 0.5 g/pot, respectively.

The pots were divided into six groups. The first group, irrigated with water alone, served as control. The second and third groups were foliar sprayed separately with 50 and 100 µM SNP respectively. The fourth group was salinized with 100 mM NaCl through the irrigation water. The fifth and sixth groups were salinized with 100 mM NaCl and sprayed with 50 and 100 µM SNP respectively. Tween 20 at 0.05% as a surfactant was added to the SNP solution before application, the spraying was done twice at 50 and 70 days from sowing (DFS) until runoff throughout late afternoon hours using the hand-held sprayer. Plant samples were randomly harvested at 90 DFS to assess morphological, physiological and anatomical trials and at 150 DFS for yield and its components.

Morphological characteristics

Ten randomly canola plants were harvested for determination of shoot length, number of leaves/plant, leaf area, shoots fresh and dry weights. Leaf area (cm^2) was measured using Leaf Area Meter, AM 300 (ADC Bioscientific Ltd).

Physiological and biochemical characteristics

— Photosynthetic pigments were extracted from the 3rd terminal upper leaf for 24 h at the lab. temperature with

methanol, then the optical density of the extracts was recorded at 470, 653, 666 nm spectrophotometrically (Spekol 11, UK); photosynthetic pigment concentration was calculated by the equation introduced by Lichtenthaler & Wellburn (1985) and expressed as mg/g fresh weight (FW).

— Water status and osmoprotectants: The WC% was assessed according to Fernandez-Ballester *et al.* (1998). Meanwhile, leaf relative water content percentage (LRWC%) was assayed using the Kaya *et al.* (2013) methodology through the equation LRWC (%) = [(FW-DW/(TW- DW)]×100, where DW is dry weight and TW turgid weight. In brief, leaf discs were immediately weighted to record FW, then the discs were submerged in distilled water for 6 h, and then weighted again to record TW, and finally, the leaf discs were oven-dried at 60ºC for 24 h to record DW. Proline concentration (mg/g FW) was assessed using the procedure described in Arbona *et al.* (2003) using acid ninhydrin reagent. Total soluble carbohydrate concentration (mg/g FW) was estimated using the anthrone reagent (Watanabe *et al.*, 2000).

— Leaf potassium, sodium and chloride concentration: K^+, Na^+ and Cl^- ions were extracted with boiling water for 2 h followed by the methods of Chaudhary *et al.* (1996). Na^+ and K^+ quantities were estimated by a Flame Photometer. Na^+ and K^+ concentrations were calculated based on a standard calibration curve and expressed as a percentage. On the other hand, chloride concentration in the water extract was calculated by titration against 0.01 N silver nitrate solution using 5% K^+ dichromate (K_2CrO_4) as an indicator as described by Fatma *et al.* (2014) and expressed as mg/g DW.

— Oxidative impairment and stress injury: Lipid peroxidation was quantified by assessing malondialdehyde (MDA) concentration, a breakdown product of lipid peroxidation (Shao *et al.*, 2005), using thiobarbituric acid reaction. The concentration of MDA in µmol/g of FW was estimated using the extinction coefficient of 155 mM^{-1} cm^{-1}. Hydrogen peroxide (mg/g FW) was assessed by the formation of a titanium-hydro peroxide complex (Rao *et al.*, 1997).

Electrolyte leakage percentage (ELP) assessment was employed to examine membrane permeability following the protocol of Goncalves *et al.* (2007). Leaf disks were placed in a test tube containing 25 mL of distilled water then placed at room temperature for 4 h, and the electrical conductivity (EC_1) of the solution was determined; after that the samples were subjected to 100ºC for 1 h, then the sample was brought down to laboratory temperature and the EC_2 measured. The ELP was calculated using the equation: $FLP=EC_1/EC_2\times 100$.

— Assay for ROS scavenging (non-enzymatic): Ascorbic acid (mg/g FW) was extracted and determined following Sadasivam & Manickam (1996) by titration against 2,6-dichlorophenol indophenol. Total phenolic compounds were quantified by the protocol of Julkenen-Titto (1985), using Folin-Ciocalteau reagent, with concomitant formation of a blue complex; the values were expressed as mg gallic acid/g FW.

Leaflet anatomy and transmission electron microscopy

Leaf segments (1-2 mm^2) were fixed overnight in cold 5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2), washed twice within the same buffer for 30 min and postfixed with 1% osmium tetroxide (OsO_4) for 4 h at 4ºC. Further samples were dehydrated in an ethanol series, then in propylene oxide, then infiltrated in a mixture of Durcupan resin andpropylene oxide (1/1, v/v), finally embedded in Durcupan resin. The semi-thin and ultrathin sections were cut with a glass knife, mounted on copper grids (400 meshes) and stained for 10 min with toluidine blue for semi-thin sections and uranyl acetate and lead citrate for ultrathin sections, and observed by light microscope and transmission electron microscope (Jeol JEM 1010) operating at 100 kV. At least 10 sections of each of the prepared samples were examined.

Yield and its components

At 150 DFS, the total yield per plant and yield components represented as flowering branch number/plant, silique number/plant, seed number/silique, seed index "100 seed weight", and seed yield/plant were determined. Seed oil percentage was calculated after extraction with Soxhelt's apparatus using hexane as an organic solvent according to AOAC (1990).

Statistical analysis

Data were statistically analyzed using the one-way analysis of variance (ANOVA) (CoHortSoftware, 2008). The standard error was calculated and an analysis of variance was performed to determine the least significant difference between treatment means with the level of significance of $p\leq 0.05$. Means marked with different letters are significantly different.

Results

Morphological characteristics

The impact of salinity and SNP, as well as their interactions on plant growth, was recorded (Table 1). We determined that salinity stress significantly (p<0.05) decreased canola plant growth characteristics, as compared to control treatment in each season. Salinity decreased the shoot length by 35% and 39%, leaf number per plant by 27% and 27% and leaf area per plant by 70% and 43%, shoot FW by 57% and 52% and shoot DW by 58% and 53% in the first and second season, respectively, as compared to control (Table 1). However, foliar application of SNP significantly (p<0.05) increased all tested growth traits compared with control plant. In saline conditions, with the lowest level of SNP (50 µM), plant growth increased almost up with the growth obtained from the non-saline control treatment or gave values of shoot FW and leaf area higher than control plants.

Photosynthetic pigments

The effect of salinity and SNP alone or in combination was assessed on the photosynthetic pigment concentration in canola 3rd upper fully expanded leaves (Table 1). Canola plants treated with salinity showed a significant reduction in chlorophyll a (by 28% and 19%), total chlorophyll (by 18% and 15%), total carotenoids (by 55% and 35%), and a non-significant decrease in chlorophyll b and the ratio of chlorophyll a:b as compared with control plants in either the first or second seasons, respectively.

The exogenous application of various concentrations of SNP (50, 100 µM) markedly increased the photosynthetic pigment concentration under normal conditions. Compared with salt treatment, exogenous application of SNP increased the photosynthetic pigment concentration. Moreover, plants treated with SNP alone additionally increased photosynthetic pigment concentration compared to the control.

Table 1. Effect of salinity and sodium nitroprusside (SNP) and their interactions on certain growth characters, photosynthetic pigment concentration (mg/g FW) and the ratio between chlorophyll a and chlorophyll b in the 3rd upper leaf of 90-day old canola plants on the first (1st) and second (2nd) season.

Treatments		Control	SNP		100 mM NaCl	100 mM NaCl+50 µM SNP	100 mM NaCl+100 µM SNP
			50 µM	100 µM			
SL (cm)	1st	42.3±1.20 b	56.6±1.85 a	52.6±1.45a	27.3±0.88 d	3.3±0.33 bc	34.3±0.96 c
	2nd	43.6±1.66 b	52.6±2.40 a	50.3±0.66 a	26.6±1.45 d	38.6±2.40 bc	35.6±1.45 c
LN./plant	1st	11.0±0.577b	14.3±0.333a	13.3±0.333a	8.0±0.577c	11.6±0.333b	10.3±0.333b
	2nd	10.6±0.333c	14.6±0.333a	13.3±0.333b	7.6±0.333d	11.0±0.577c	10.0±0.577c
LA/plant (cm^2)	1st	135.4±18.5b	247.4±6.51a	225.2±32.09a	65.8±3.20c	164.2±6.89b	156.5±14.65b
	2nd	124.2±5.10c	231.6±2.32a	222.7±9.50a	70.7±3.11d	157.5±2.03b	154.4±8.17b
SFW (g)	1st	80.1±2.57b	133.9±1.08 a	130.7±4.37a	34.2±1.41c	82.8±1.99b	77.8±2.72b
	2nd	77.5±0.75b	121.6±3.33a	120.6±4.46a	35.8±1.32c	82.5±2.02b	78.0±1.84b
SDW (g)	1st	23.3±0.43c	33.9±0.65a	31.8±0.56b	9.7±0.34f	20.9±0.51d	16.7±1.08e
	2nd	22.2±0.51c	35.8±1.22a	32.0±0.96b	10.3±0.50e	19.5±0.85 cd	17.5± 1.35d
Chlorophyll a	1st	1.703±0.131b	1.930±0.069ab	1.964±0.213ab	1.216±0.090c	2.141±0.042a	2.128±0.050a
	2nd	1.788±0.021b	1.82±0.029ab	1.896±0.101ab	1.448±0.072c	1.907±0.073ab	2.082±0.021a
Chlorophyll b	1st	1.021±0.027a	1.125±0.052a	0.912±0.083ab	0.995±0.003a	0.904±0.116ab	0.719±0.085b
	2nd	1.024±0.009ab	1.067±0.025a	0.965±0.055ab	0.940±0.013ab	0.917±0.058b	0.764±0.054c
Total chlorophyll	1st	2.725±0.159a	3.056±0.034a	2.876±0.198a	2.211±0.093b	3.045±0.151a	2.848±0.131a
	2nd	2.812±0.030a	2.960±0.034a	2.862± 0.064a	2.389±0.059 b	2.824±0.060a	2.847±0.063a
Total carotenoids	1st	0.445±0.016b	0.659±0.038a	0.508±0.022b	0.199±0.002c	0.530±0.024b	0.482±0.036b
	2nd	0.467±0.012c	0.620±0.20a	0.556±0.015ab	0.301±0.034d	0.503±0.027bc	0.498±0.011bc
Chl a:b ratio	1st	1.662±0.086bc	1.727±0.142bc	2.211±0.285ab	1.220±0.087c	2.451±0.320ab	3.033±0.320a
	2nd	1.746±0.008bc	1.774±0.055bc	1.987±0.216bc	1.541±0.098c	2.103±0.206b	2.751±0.197a

Means within columns followed by different letters are significantly different (p< 0.05); df for each analysis was 11, 35. Means (±SE) were calculated from five replicates for each treatment. SL: seedling length. LN: leaf number. LA: leaf area. SFW: shoot fresh weight. SDW: shoot dry weight.

Water status and osmoprotectants

Data presented in Table 2 show that WC and LRWC percentages significantly decreased with salinity stress, meanwhile increased with SNP application as compared with control. On the other hand, application of SNP under salinity stress counteracted the deleterious effect of salinity on WC% and LRWC% that increased it as compared with untreated salinized plants.

Proline and soluble carbohydrate concentration exhibited a rise in response to salinity, compared to the control (Table 2). The spray of SNP on unstressed plant significantly increased its concentration; the foremost effective in this concern was 50 µM. However, in association with NaCl, it elevated the quantity of proline and total soluble carbohydrates above the control or stressed conditions. The maximum concentration of each was found within the plants which were subjected to NaCl stress and subsequently sprayed with 50 µM SNP (Table 2).

Leaf K^+, Na^+ percentageand Cl^- concentration

The results represented in Table 2 discovered a substantial decrease in K^+% and K^+/Na^+ ratio concomitant to the accumulation of Na^+% and Cl^- concentration in shoot under the salinity stress. Conversely, the SNP application in both concentrations significantly (p<0.05) alleviated the impacts of salinity. SNP at 50 µM was the treatment most effective in lowering the Na^+ (56% and 46%) and Cl^- accumulation (44% and 36%) whereas increasing the K^+ (by 39% and 40%) and a K^+/Na^+ ratio (173% and 173%) over the plants exposed to salinity alone respectively in each year of experiments. Moreover, the data in Table 2 show that application of SNP, in special 50 µM under normal conditions, significantly (p<0.05) increased K^+ concentration and K^+/Na^+ ratio, but markedly decreased either Na^+ or chloride concentration in canola shoot.

Oxidative impairment and stress injury as well as some antioxidant metabolites

The exposure of canola plants to salinity significantly (p<0.05) increased the membrane permeability (80.494 and 84.800%); lipid peroxidation (22.478 and 17.799 µmol/g FW) and hydrogen peroxide concentration (61.25 and 63.36 mg/g FW) than control plant (45.98 and 50.28%; 10.021 and 8.653 µmol/g FW; 29.40 and 27.51 mg/g FW respectively in each growing season). A different pattern of response was observed once electrolyte leakage, lipid peroxidation,

Table 2. Effect of salinity and sodium nitroprusside (SNP) and their interactions on water status percentage, osmoprotectants concentration (mg/g FW), sodium and potassium percentage, potassium/sodium ratio and chloride concentration (mg/g DW) in the shoot of 90-day old canola plants on the first (1st) and second (2nd) season.

Treatments		Control	SNP		100 mM NaCl	100 mM NaCl+50 µM SNP	100 mM NaCl+100 µM SNP
			50 µM	100 µM			
WC %	1st	87.9±0.003 c	92.9±0.003a	93.2±0.003a	65.4±0.001d	89.8±0.016bc	91.8±0.090ab
	2nd	88.6±0.002ab	92.2±0.002a	93.1±0.001a	74.7±0.031c	86.3±0.006b	90.2±0.006ab
LRWC%	1st	81.09±2.66ab	87.02±2.34a	84.79±3.19ab	42.03±0.74c	83.83±3.53ab	76.12±3.30b
	2nd	80.23±1.08bc	88.78±1.30a	85.27±1.66ab	51.52±3.23d	75.03±2.67c	73.34±3.85c
Pro	1st	1.90±0.236d	4.02±0.256bc	3.08±0.771c	4.36±0.312b	6.60±0.049a	6.18±0.344a
	2nd	1.78±0.058e	3.61±0.136c	2.85±0.320d	4.15±0.138c	6.31±0.080a	5.55±0.302b
TSC	1st	24.73±2.358d	45.54±0.536c	39.25±0.700c	40.15±2.218c	78.08±1.574a	56.94±3.985b
	2nd	22.86±0.773d	40.34±0.328c	37.86±0.784c	41.77±0.582c	75.46±0.704a	64.24±0.049b
Cl^-	1st	32.66±1.63d	25.56±4.56d	28.87±1.70d	132.5±6.82a	73.84±7.37c	89.46±1.42b
	2nd	29.34±0.94d	25.56±0.81d	27.45±0.94d	123.0±4.20a	78.6±2.16c	86.14±1.25b
Na^+	1st	0.81±0.038c	0.72±0.022c	0.79±0.022c	2.80±0.251a	1.21±0.066b	1.55±0.176b
	2nd	0.92±0.044d	0.75±0.00e	0.77±0.022e	2.62±0.102a	1.39±0.022c	1.57±0.022b
K^+	1st	1.97±0.077ab	2.42±0.080a	2.26±0.146ab	1.41±0.089c	2.35±0.286ab	1.91±0.067b
	2nd	1.82±0.022c	2.29±0.022a	2.22±0.059a	1.26±0.022d	2.13±0.156ab	1.95±0.022bc
K^+/Na^+ ratio	1st	2.41±0.058c	3.33±0.051a	2.85±0.101b	0.50±0.170f	1.93±0.102cd	1.22±0.106e
	2nd	1.95±0.037c	3.05±0.010a	2.87±0.070b	0.48±0.063f	1.52±0.080cd	1.24±0.022e

Means within columns followed by different letters are significantly different (p< 0.05); df for each analysis was 11, 35. Means (±SE) were calculated from five replicates for each treatment. WC: water content. LRWC: leaf relative water content. Pro: proline. TSC: total soluble carbohydrates. Cl^-: chloride. Na^+: sodium. K^+: potassium.

and hydrogen peroxide accumulation were studied in SNP treated plants, in the presence and absence of NaCl (Table 3). Under control conditions, SNP, mainly in the concentration 50 µM, significantly decreased membrane permeability, hydrogen peroxide, and MDA concentration as compared to control treatment. However, the follow-up treatment of the stressed plants with SNP reduced the ionic leakage and lipid peroxidation and leveled the values with those of the control.

Data in Table 3 indicate that salinity caused significant increment ($p \leq 0.05$) within the total phenols and ascorbic acid in the canola shoot as compared with control plants. Application of SNP significantly rised ($p \leq 0.05$) total phenols and ascorbic acid concentrations. Generally, SNP at 50 µM treatment appears to be the foremost effective treatment in increasing total phenols and ascorbic concentrations in stressed canola plants.

Leaf anatomy

Canola plants sprayed with 50 and 100 µM SNP under non-salinized conditions had improved leaf anatomy compared to control plants (Table 3 and Fig. 1). In general, the most effective SNP level was 50µM, that increased thickness of midrib region, length and width of the main vascular bundle by 47.5, 60.7 and 160.7%, respectively, meanwhile application of 100 µM SNP increased leaf blade thickness, palisade parenchyma thickness, spongy parenchyma thickness by 47.6, 101.2 and 14.4% compared to the controls.

Salinity stress decreased all anatomical characteristics, additionally; the application of SNP under salinity stress helped in counteracting the deleterious effect of salinity on leaf blade and spongy parenchyma thickness as compared with salt-affected plants.

Leaf ultrastructure

Ultrastructural micrograph observation showed canola leaf mesophyll cells with a delimited cell wall, and continuous cell membranes, having a granular cytoplasm with numerous organelles (Fig. 2). Chloroplasts exhibited a typical structure, an ellipsoidal shape with well-arranged granum, well-arranged thylakoid membranes, together with many starch grains (1-2 per chloroplast) and an absence of plastoglobules (Fig. 2B). The mitochondrion structure observed was typical, with good membranes

Table 3. Effect of salinity and sodium nitroprusside (SNP) and their interactions on oxidative impairment and antioxidants metabolites concentration on the first (1st) and second (2nd) season as well as leaf anatomical characteristics (µm) in the 3rd upper leaf on the second (2nd) season of 90-day old canola plants.

Treatments		Control	SNP		100 mM NaCl	100 mM NaCl+50 µM SNP	100 mM NaCl+100 µM SNP
			50 µM	100 µM			
H2O2 (mg/gFW)	1st	29.40±1.112b	23.58±0.275c	23.68±0.125c	61.25±0.688a	26.51±1.298bc	28.86±1.276b
	2nd	27.51±0.802cd	23.38±0.057d	24.66±0.617cd	63.36±2.109a	29.25±0.391c	34.75±3.191b
MP %	1st	45.98±3.972cd	39.55±1.937d	43.98±1.354cd	80.494±1.787a	49.82±4.314bc	55.26±2.058b
	2nd	50.28±1.939c	37.78±0.341e	40.302±1.417de	84.80±2.257a	47.16±3.684cd	57.43±2.484b
LP (µmol MDA/g FW)	1st	10.02±1.282b	2.58±0.310d	6.60±0.279c	22.47±0.35a	8.26±0.222bc	10.70±1.838b
	2nd	8.65±0.064bc	4.50±0.734d	5.72±0.296cd	17.79±1.86a	11.88±1.340b	10.12±0.911b
AsA (mg/g FW)	1st	0.315±0.004c	0.390±0.024bc	0.369±0.062bc	0.420±0.013b	0.547±0.020a	0.524±0.019a
	2nd	0.328±0.004e	0.363±0.00d	0.352±0.022de	0.409±0.008c	0.554±0.004a	0.515±0.005b
TP (mg gallic acid/g FW)	1st	9.564±2.077d	15.486±0.662bcd	10.619±0.933cd	17.528±4.284bc	28.362±1.301a	19.703±1.615b
	2nd	8.936±0.522e	12.615±0.161d	11.740±0.530d	15.217±0.696c	25.670±0.741a	21.924±1.336b
LBT	2nd	164±2.60b	165±14.52b	254±23.09a	123±3.84c	165±5.77b	145±4.61bc
PPT	2nd	78±0.57bc	93±8.81b	157±11.54a	67±1.73c	90±2.88b	90±2.30b
SPT	2nd	76±2.02ab	62±5.77bc	87±11.54a	45±1.73c	65±2.88b	45±2.30c
TMR	2nd	427±5.77e	640±5.77a	540±5.77c	337±4.04f	562±1.15b	495±2.88d
VBT	2nd	112±1.15d	180±5.77a	157±4.04b	90±5.77e	135±2.88c	137±4.04c
VBW	2nd	112±3.48d	291±6.35a	202±1.15c	67±4.04e	247±4.04b	292±1.15a

Means within columns followed by different letters are significantly different ($p < 0.05$); df for each analysis was 11, 35. Means (±SE) were calculated from five replicates for each treatment. H_2O_2: hydrogen peroxide. MP: membrane permeability. LP: lipid peroxidation. AsA: ascorbic acid. TP: total soluble phenol. LBT: leaf blade thickness. PPT: palisade parenchyma thickness. SPT: spongy parenchyma thickness. TMR: thickness of midrib region. VBT: vascular bundle thickness. VBW: vascular bundle width.

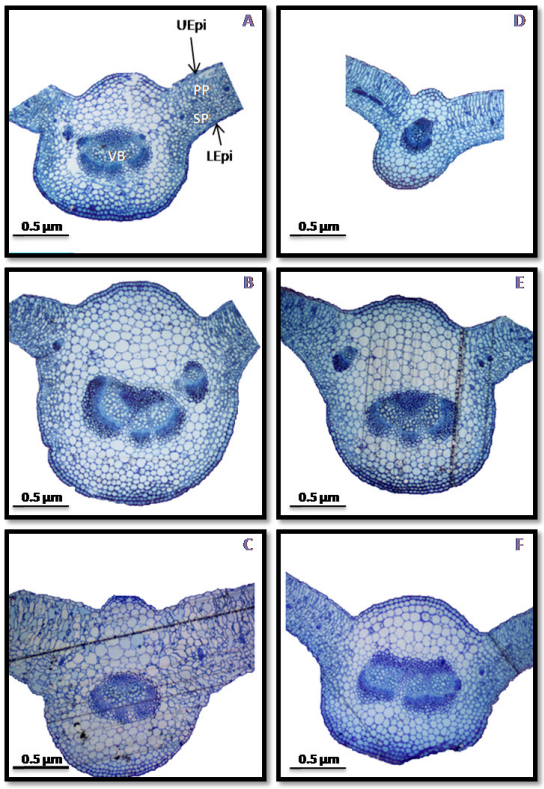

Figure 1. Cross section through the 3rd upper canola leaf (stained with toluidine blue O) as affected by salinity or sodium nitroprusside (SNP) concentration and their combinations, at 90 days from sowing in the second season (LEpi, lower epidermis; PP, palaside parenchyma; SP, spongy parenchyma; UEpi, upper epidermis; VB, vascular bundle. A, control; B, 50 µM SNP; C, 100 µM SNP; D, 100 mM NaCl; E, 100 mM NaCl plus 50 µM SNP; F, 100 mM NaCl plus 100 µM SNP).

(Fig. 2C). A clear nucleolus and well-developed nuclear envelope were noticed in the nucleus (Fig. 2C).

Cells from salt-affected plants showed some noticeable ultrastructural alteration of the organelles and cellular injuries (Fig. L-P), like nucleus condensation, protoplasm degeneration, and smaller organelles. The chloroplasts were nearly elongated and swelling, with an increased plastoglobules number and a very small sized starch grain (Fig. 2M). The chloroplasts became deformed, grana stacking were less frequent, and therefore the thylakoids were loosened and distorted (Fig. 2M). The mitochondria were very small and its number similar to control. The nucleus was a triangle in shape with a distorted nuclear (Fig. 2N).

Plants treated with SNP alone, showed a typical chloroplast ultrastructure with no significant changes, having large number (1-3) of big size starch grains, with some plastoglobules (Fig. 2F). The highest number of chloroplasts per cell was observed under low concentration of the SNP (Fig. 2E). Also, ultrastructure changes in mitochondria were inconspicuous. The number of mitochondria per cell was increased and elongated in the high SNP concentration (Fig. 2H). The nucleus was elongated, with the nucleolus and smooth and continuous envelope (Fig. 2G).

Foliar application of SNP to NaCl stressed plants reversed the structural changes of micro-organelles. Observation assessed that the form of chloroplast changed slightly from the elongated ellipse to almost oval, well aligned internal lamellar system and fewer plastoglobules (Fig. 2T-U). Ultrastructural changes in mitochondria were inconspicuous. The cells had a large number of mitochondria in special with 50 µM SNP (Fig. 2T). The mitochondria elongated under 50 µM SNP, meanwhile at 100 µM SNP appeared with oval shape and internal structures (Fig. 2T-W). Additionally, cell nucleolus was clear and apparent along with intact nuclear membranes (Fig. 2S-V).

Yield and its components

Exogenous application of SNP significantly increased the yield and its components represented as flowering branch number/plant, fruit number per plant, seed number per fruit, seed index, seed yield per plant and oil percentage compared to the controls (Table 4). The SNP level of 50 µM was most effective, exceeding the control values. Salinity stress significantly decreased all yield and its components as compared with untreated control plants. Meanwhile, under salinity stress application of SNP, especially at 50 µM alleviated the bad effect of salinity on yield and its components.

Discussion

Salinity considerably hampered crop growth that is in harmony with earlier reports (Gupta *et al.*, 2017; Ahmad *et al.*, 2018; Hanafy *et al.*, 2018; Kaya *et al.*, 2018). These could be attributed to a multifaceted action of salinity on vital processes like water absorption, ion uptake, photosynthesis, etc.

This reduction may be due to: (1) accumulation of salts within the leaves that causes an irreparable damage to the chloroplasts as observed in the current investigation, resulting in metabolic limitations of photosynthesis (Gong *et al.*, 2018); (2) the osmotic effects resulting from salt stress, that causes increases in abscisic acid and decline in indole acetic acid and gibberellic acid, and consequently inhibition of growth (Gupta *et al.*, 2017; Hanafy *et al.*, 2018); (3) decreasing in cell elongation ensuing from the repressing impact of water shortage on growth promoting hormones (Banon *et al.*, 2006). SNP supplementation counteracts the

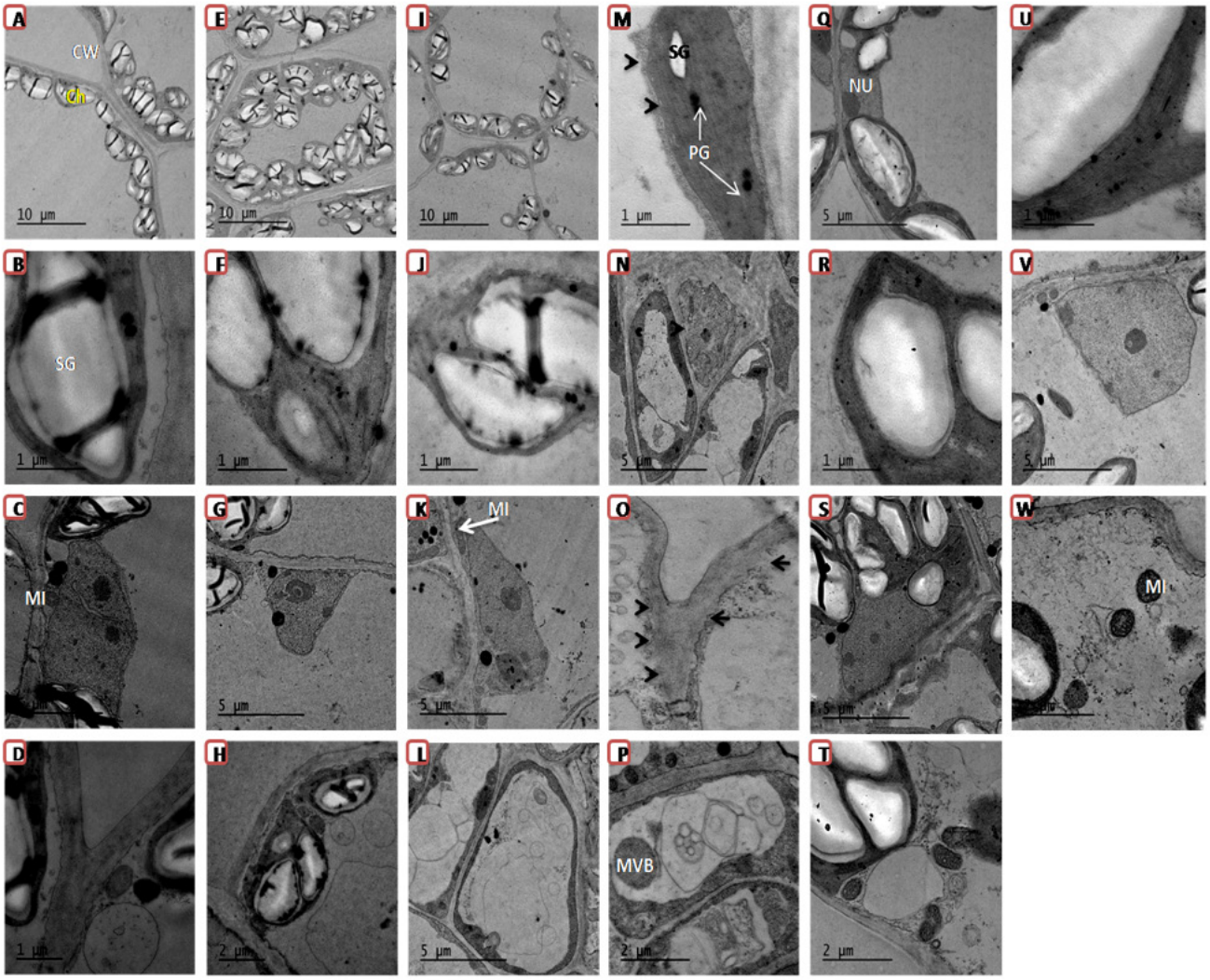

Figure 2. Ultrastructure of leaf mesophyll cells from the canola grown under control treatment (A-D), 50 µM sodium nitroprusside (SNP) (E-H); 100 µM SNP (I-K); 100 mM NaCl (L-P); 50 µM SNP and 100 mM NaCl (Q-T); and finally 100 µM SNP and 100 mM NaCl (U-W). A: General view and detail of control cells, showing many chloroplasts and smooth cell walls. B: Well organized chloroplasts with larger starch grains. C: Dispersion of nucleolus and chromatin in nucleus matrix of control with smooth and continuous membrane. D: Smooth and continuous cell wall as well as mitochondria. E: A general view of mesophyll cells having a huge number of chloroplasts with numerous starch grains. F: Chloroplasts with numerous starch grains. G: A nucleus with nucleolus and good nuclear envelop. H: Numerous mitochondria within the cell. I: General view of mesophyll cell having a less number of chloroplasts. J: Chloroplasts with starch grains. K: Elongated ellipsoidal nucleus with continuous nuclear envelope: L: overview of salt affected cells showing a destruction of cell organelles. M: Vacuolated and swelling chloroplasts with dilations of the thylakoid membranes, reduction in starch granules, relatively more plastoglobules. N: Mesophyll cell having a destroyed nucleus. O: Destruction of cell wall. P: multivesicular bodies in the vacuoles. Q: Mesophyll having a well-organized nucleus. R & U: Well-structured chloroplasts. S & V: Well organized nucleus with smooth nuclear envelop. T & W: Well organized mitochondria.

injurious impact of salinity on canola plant development that is in agreement with previous findings in different crops (Ghadakchiasl *et al.*, 2017; Gupta *et al.*, 2017; Ahmad *et al.*, 2018; Akram *et al.*, 2018; Hanafy *et al.*, 2018; Rai *et al.*, 2018). SNP plays a very important role in inducing plant growth of many plants exposed to environmental stresses by modulating a variety of metabolic alterations affecting plant's tolerance to salt stress, including: (1) scavenging ROS through induction of antioxidant system (Ahmad *et al.*, 2018; Akram *et al.*, 2018; Rai *et al.*, 2018); (2) SNP as signal molecule induces changes in the expression of stress-responsive genes, involved in enhancing crop growth (Gill *et al.*, 2013); (3) inhibition of ethylene biosynthesis (Zhu *et al.*,2008).

The reduction in chlorophyll concentration under salinity stress may be due to one or more of the following processes: (1) disruption chloroplasts ultrastructure and

Table 4. Effect of salinity and sodium nitroprusside (SNP) and their interactions on yield and its components of 150 day old canola plants on the first (1st) and second (2nd) season.

Treatments		Control	50 µM SNP	100 µM SNP	100 mM NaCl	100 mM NaCl+50 µM SNP	100 mM NaCl+100 µM SNP
FBN	1st	10.33±0.333bc	12.66±0.881a	11.33±0.333ab	4.66±0.666d	9.00±0.577c	8.66±0.333c
	2nd	8.00±0.577b	12.00±0.000a	11.66±0.333a	5.33±0.333c	8.00±0.577b	8.66±0.333b
FN	1st	182±3.055c	241±12.19a	205±7.76b	99.6±4.66e	171±4.509cd	158±5.925d
	2nd	187±3.480c	227±7.549a	208±2.403b	109±2.185e	161±1.660d	153±5.033d
SN	1st	23.66±0.881b	27.00±0.577a	25.66±0.881ab	11.66±1.201d	18.00±1.154c	17.33±0.333c
	2nd	21.00±0.577b	26.00±0.577a	25.00±0.577a	11.66±0.881d	17.66±0.333c	16.33±0.333c
SI (g)	1st	0.393±0.019c	0.553±0.017a	0.458±0.018b	0.151±0.022e	0.347±0.011cd	0.291±0.230d
	2nd	0.405±0.009c	0.515±0.014a	0.471±0.013b	0.158±0.015e	0.310±0.000d	0.285±0.007d
SY (g)	1st	6.80±0.057c	8.70±0.305a	7.30±0.173b	4.26±0.120e	6.26±0.066d	5.96±0.033d
	2nd	6.80±0.173b	8.16±0.145a	7.60±0.173a	4.73±0.185d	6.33±0.333bc	5.76±0.033c
Oil %	1st	30.56±0.470b	37.10±1.497a	34.86±0.133a	21.86±1.225c	30.46±0.829b	28.16±0.578b
	2nd	31.73±0.674b	36.06±0.622a	34.86±0.185a	21.60±0.461e	29.86±0.545c	27.76±0.348d

Means within columns followed by different letters are significantly different ($p< 0.05$); df for each analysis was 11, 35. Means (±SE) were calculated from five replicates for each treatment. FBN: flowering branches number/plant. FN: fruit number/plant. SN: seed number/pod. SI: seed index (100 seed weight). SY: seed yield/plant.

activated chlorophyllase enzyme (Nazar *et al.*, 2014); (2) reduced uptake of some micronutrients like Fe that plays a vital role in chlorophyll biosynthesis (Santos, 2004); (3) oxidation of chlorophylls coupled with the instability of the pigment-protein complex (Tariq *et al.*, 2011). Increased photosynthetic pigments due to SNP treatment has already been observed in salt-affected plants (Ghadakchiasl *et al.*, 2017; Gupta *et al.*, 2017; Ahmad *et al.*, 2018; Hanafy *et al.*, 2018). It was noted that SNP impairs the activity of the chlorophyllase and Mg-dechelatase that might have decreased the chlorophyll degradation (Shi *et al.*, 2016). Moreover, SNP might control Fe uptake in root and increased Fe availability within the cell organelles, by modulating the chlorophyll synthesis gene expression (Simontacchi *et al.*, 2012).

It is well documented that salinity stress perturbs water status and reduces either WC or LRWC in many plants (Ahmad *et al.*, 2018; Kholghi *et al.*, 2018), that we also observed in this experiment. This decrease can be attributed to lower water availability; or to lose the plant roots their ability to catch up on the water through a reduction of the absorbing surface (Yildirim *et al.*, 2008). However, supplementation of SNP has a positive impact on WC and LRWC of canola plants under salt stress, fact supported with previous reports (Ghadakchiasl *et al.*, 2017; Ahmad *et al.*, 2018). That is still unclear, however SNP is able to maintain LRWC in stressed plants and the rise in water retention induced by exogenous applied SNP in stress-treated plants could also be connected to stomatal closure, a reduced transpiration rate, and/or promoted adventitious root development.

Crop plants accumulate compatible osmolytes that facilitate to regulate turgor and water acquisition under stress conditions (Ahmad *et al.*, 2018; Kaya *et al.*, 2018; Kholghi *et al.*, 2018). Proline has been related to the relief of cellular osmotic stress, stabilization of proteins and/or membranes and improving the enzyme stability (Reddy *et al.*, 2015). It is additionally served as a safe guard chloroplast and acts as energy storage under salinity (Reddy *et al.*, 2015). The regulatory function of SNP in proline biosynthesis has conjointly been verified by inducing of 1-pyrroline -5- carboxylate synthetase activity, the key enzyme of proline biosynthesis; additionally by proline dehydrogenase activity impairment against salinity stress (Ahmad *et al.*, 2018). Accumulation of total soluble carbohydrates has been widely reported in plants as a response to salt stress (Rahmani *et al.*, 2018) or SNP application (Hanafy *et al.*, 2018). Soluble carbohydrates have a vital role in plant metabolism not only by acting as osmoprotection, stabilizing cellular membrane, but also as signal molecules, involved in ROS balance and responses to oxidative stress in plants (Nasrin *et al.*, 2010).

Salinity causes the impairment of ion equilibrium mechanism by hyper-accumulating toxic ions (Na^+ and Cl^-) and remarked reduction in useful ions (K^+) leading to decreasing K^+/Na^+ ratio, in numerous plant species (Gupta *et al.*, 2017; Kaya *et al.*, 2018; Kholghi *et al.*, 2018). Sodium toxicity is related to its competitive nature with K^+ for binding essential sites that carry normal cellular function. Additionally, Na^+ accumulation contributes to depolarization of the plasma membrane

and subsequent opening of K^+ outward rectifier channels that triggers an excessive loss of K^+ from the interior of the cell (Bose *et al.*, 2015). Exogenously, applied SNP lowered toxic ions (Na^+ and Cl^-), yet enhanced K^+ in canola plants under saline conditions that are in keeping with many earlier reports (Gupta *et al.*, 2017; Hanafy *et al.*, 2018). SNP, additionally maintains a high K^+/Na^+ ratio within the cytosol (Table 2) by inducing expression of H^+-ATPase, and Na^+/H^+ antiporter which facilitate Na^+ compartmentation under salinity (Shi *et al.*, 2016).

Our investigation assessed that salt-stress provokes an over-accumulation of H_2O_2, which clearly explains salt-mediated oxidative burst. However, the application of SNP effectively eliminated the high levels of H_2O_2, and thus played an important role in mitigating oxidative stress. These findings also demonstrate the direct ROS quenching role of SNP under salt stress, reported in several plants (Ghadakchiasl *et al.*, 2017; Gupta *et al.*, 2017; Ahmad *et al.*, 2018). The protective role of SNP may be explained due to (1) reaction with lipid radicals, that stops the generation of lipid peroxidation; (2) SNP might operate as a signal molecule, that activates the cellular antioxidant enzyme-encoding genes leading to higher enzyme activities possible by post-translational modification that renders the plants stress tolerant (Graß *et al.*, 2013); (3) SNP acts as an antioxidant that reacts with ROS resulting in generation of short lived peroxynitrite ($ONOO^-$), which is less toxic in the cellular environment and thus limits cellular damage.

The hyper-accumulation of ROS in plants is among the most important injuries induced by environmental stresses. The cellular components most degraded by ROS are the membrane lipids. As a consequence, MDA concentrations can increase, which is a symptom of membrane injury in a plant cell (Kaya *et al.*, 2018; Rahmani *et al.*, 2018). This increment in MDA could also result in the short coming of antioxidants to neutralize ROS ensuing from salt stress. In contrast, exogenous SNP reduced MDA and H_2O_2 concentrations within the plant exposed to salinity. This finding was in accordance with those of other studies that indicated that MDA and H_2O_2 were reduced by SNP treatments throughout abiotic stress as salt (Gupta *et al.*, 2017; Akram *et al.*, 2018; Hasanuzzaman *et al.*, 2018; Rai *et al.*, 2018).

The cell membrane is one of the most important cellular targets common to different stress conditions. Salt stress induces lipid peroxidation, thus making the membranes leaky as evidenced by increase electrolyte leakage, while SNP alleviates the injuries of NaCl on MDA concentration and membrane permeability percentage. These results are in accordance with those obtained by Ahmad *et al.* (2018), Kaya *et al.* (2018) and Kholghi *et al.* (2018). These studies suggest a reduction in electrolyte leakage that may be explained on the basis of the role of SNP facilitated maintenance of membrane functions through induction of antioxidant metabolites, thereby protecting the plants against oxidation damage on membrane permeability (Akram *et al.*, 2018; Rai *et al.*, 2018).

Under salt stress plant posses a range of non-enzymatic antioxidant molecules (Shafiq *et al.*, 2014; Ghadakchiasl *et al.*, 2017) to scavenge ROS. Phenolic substances will serve as molecular antioxidants through their ability to destroy free radicals as well as substrates of many antioxidant enzymes (Blokhina *et al.*, 2003). Accordingly, Franco *et al.* (2008) stressed that the existence of many hydroxyl groups bonded to associate an aromatic ring provides the molecule with the ability to donate a proton to a radical, thus acting as potential chain-breaking molecules or antioxidants upon secondary oxidation.

Salinity excited the build-up of ascorbic acid (AsA) as compared with that of the non-salinized control. These results harmonize with those reported by Shafiq *et al.* (2014). Moreover, the application of SNP markedly induced the accumulation of AsA in canola plants under normal or salinized conditions. Hasanuzzaman *et al.* (2018) and Rai *et al.* (2018) assessed that SNP raises the amount of AsA as the results of an increase within the capability of SNP to scavenge ROS and should account partially for the lower concentration of H_2O_2.

Investigating the plant cell ultra-structural changes under salinity may be a useful tool for unveiling the fundamental strategies involved in conferring salt tolerance (Atkin & Macherel, 2009). Salinity caused the major changes in chloroplasts including swollen of thylakoids, loose shapes of the part of internal lamellae thylakoids, although most of granal thylakoids were destroyed. These results were confirmed by Naeem *et al.* (2012). The distortions of grana stacking and swelling of thylakoids caused by salinity within this investigation were probably due to a change within the ionic composition of the stroma.

The increment in the plastoglobule number assessed in the present study may be a formal indicator of environmental stress effects (Austin *et al.*, 2006). The physical coupling between the plastoglobules and thylakoid membranes permits the free exchange of lipid molecules among the plastoglobules and thylakoids (Austin *et al.*, 2006). The increased plastoglobule size and number observed within the salt-treated plants may be one of the adaptive mechanisms which prevent the oxidative damages caused by severe salinity. However, reduction in starch grains will even be attributed to increased conversion of starch into soluble sugars, which are considered as compatible solutes and play a

vital role in osmotic adjustment. On the other hand, SNP relieved this structural damage by protecting the photosynthetic membrane system from oxidative stress. Large chloroplasts with no swelling and only minor dilations of the thylakoids in SNP and NaCl treated plants are the concrete indication of less oxidative stress. Another distinction within the chloroplast of the plants grown under salinity and SNP application along was that the dimension of starch grains increased. This means that the plants accumulate starch under favorable growth conditions which they metabolize a large amount of starch under saline to possess an appropriate pool of osmolytes (Ahmad *et al.*, 2018). Comparatively less number of plastoglobuli in chloroplasts of plants treated with NaCl and SNP along is another indication of lesser oxidative stress (Zhang *et al.*, 2010).

Under control conditions, the mitochondria had well-organized cristae and an intact structure and were of comparable size and appearance. On the other hand, cells from salt-affected plants had a very small and the lowest number of mitochondria as well as a loss of the integrity of the outer mitochondrial membranes. Similar results have been reported by Zhang *et al.* (2010). However, SNP application increased the dimensions and number of mitochondria under control or stressed conditions. The higher mitochondrial number and size meet the increased needs of ATP under unfavorable conditions, when photosynthesis is generally suppressed (Silva *et al.*, 2010), and additionally these organelles responses to stress by synthesis of various specific mitochondrial stress proteins (Rizhsky *et al.*, 2004).

Salinization severely affects the agricultural productivity of crop plants. The annual global income losses due to salinization of agricultural land were estimated in US$ 11.4 billion in irrigated land and US$ 1.2 billion in non-irrigated areas (Zhu *et al.*, 2008). The reduction in seed yield is basically due to a reduction in seed set within the fruit which can be attributed to a decrease in the pollen viability or in the stigmatic surface receptivity, or both (Khatun & Flowers, 1995). A decreasing in the supply of carbon assimilate produced by decreased leaf area per plant (Table 1) is additionally responsible for the reduction of the yield components. Abscission of flowers or young fruit due to ethylene induction by salinity could explain the reduced pod set observed (Chrominski *et al.*, 1989). SNP-improving stress tolerance and rise yield have been observed in numerous crops (Kausar & Shahbaz, 2013). Improvement salinity tolerance in numerous plants/crops has been shown to be connected with an increased growth and yield as well as multiple metabolic adaptations, including low accumulation of ROS (Fayez & Bazaid, 2014), low uptake of toxic ions and high accumulation of vital osmoprotectants (Kaya *et al.*, 2013). The present study asserted that exogenous application of SNP significantly increased yield and its components of the canola plant under normal and saline conditions. This increase could also be due to reduced accumulation of Na^+ and increased that of K^+ enhancing photosynthetic pigments, resulting in increased dry matter accumulation. It has been found that exogenous application of SNP causes stronger stem and roots, improved branching, earlier flowering and a greater number of silique due to increasing water use efficiency. Moreover, SNP-induced high biomass production could also be ascribed to low uptake of Na^+ as well as low accumulation of ROS (Ahmad *et al.*, 2018; Hasanuzzaman *et al.*, 2018; Rai *et al.*, 2018). Finally, application of SNP induced some structural protecting changes within the conductory tissues through increasing the phloem and xylem area, particularly with 50 µM resulting in a fast translocation of photoassimilates from the leaves towards the developing seeds.

In conclusion, exogenous application of SNP proved to be useful in enhancing plant growth and yield characters under normal or salinity conditions. The growth increment under SNP application was found to be associated with increasing photosynthetic pigment concentration, reduced lipid peroxidation-linked membrane deterioration, reduced the accumulation of Na^+ and Cl^- and prevented salt-induced K^+ leakage thereby maintaining a higher K^+/ Na^+ ratio. Based on the results of the current work, it can be concluded that SNP sprayed plants can postpone the salinity injuries by peroxide/phenolic/ascorbate system which is involved in scavenging the ROS produced during salt stress and improved biochemical processes and anatomical traits that increased plant growth and yield.

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