Contractile roots are the most sensitive organ in *Crocus sativus* to salt stress

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Abstract

Crocus sativus corms were grown in *Perlite* and watered by half-strength modified Hoagland nutrient solution containing 0, 50, 100, 150, 200 mM NaCl. Growth parameters and contents of proteins, proline, polyphenols, minerals and saccharides were studied in fibrous roots, contractile roots, corms and leaves. All plants remained alive and did not display any sign of foliar damage even at 200 mM NaCl. However, the salinity decreased growth, relative water content and increased contents of proline and Na^+ in all organs. Total protein content was increased in corms and contractile roots but decreased in fibrous roots. Changes in protein pattern were also observed. Polyphenol content was increased by salinity in all organs except the leaves. As salinity increased, content of soluble saccharides decreased except in the contractile roots.

Additional key words: hydroponic culture, NaCl, polyphenols, proline, protein, saffron crocus, sugars.

Introduction

Salinity affects almost all plant functions (Greenway and Munns 1980) and results in a wide variety of physiological and biochemical changes in plants (Yancy *et al.* 1982). Because of severe damages of plant growth and development, considerable attempts have been made in discovering physiological and biochemical processes contributing to adaptation to salinity in plants (Ashraf and Harris 2004). Some of these processes include osmotic adjustment by accumulation of compatible solutes (*e.g.* Tripathi *et al.* 2007) or soluble saccharides, and changes in regulatory mechanism for ion transport (*e.g.* Agarwal and Pandey 2004). The contents of proline, saccharides and proteins will provide potential biological markers useful in the identification and genetic manipulation of salt resistant plants (Shonjani 2002). Moreover, there is accumulating evidence that production of reactive oxygen species (ROS) is a major damaging factor in plants exposed to different environmental stresses, including salinity (Hernandez *et al.* 1995).

The aim of this study was to investigate the changes in growth parameters, relative water content, contents of proteins, proline, polyphenols, K^+, Na^+ and saccharides in different organs (fibrous roots, contractile roots, corms and leaves) of *C. sativus*, when subjected to NaCl stress. According to our knowledge, this is the first study on salinity induced physiological and biochemical changes in *C. sativus*.

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Abbreviations: MS - Murashige and Skoog; RWC - relative water content; ROS - reactive oxygen species; SDS - sodium dodecyl sulfate.
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Materials and methods

Crocus sativus L. corms were collected from Kashan in the central region of Iran. Corms (0.5 - 1.0 g each) were transferred into pots (3 dm^3) containing fine *Perlite* saturated with half-strength Hoagland nutrient solution (pH 5.8) for two weeks in order to leaf initiation and root emergence. For salt stress treatments, plants were irrigated with the same nutrient solution supplemented with 0, 50, 100, 150, 200 mM NaCl for 4 weeks. To avoid osmotic shock, NaCl concentrations were increased gradually by 25 mM every week until the desired concentration was reached. Plants were grown during summer time in a greenhouse under 14-h photoperiod (maximum irradiance about 2000 μmol m^{-2} s^{-1}), temperature of 27 ± 3 °C, air humidity of 75 % and irrigation every day for 4 weeks.

Relative water content (RWC) was estimated according to Wheatherley (1950) and calculated as follows: RWC = [(fresh mass - dry mass)/(saturated mass - dry mass)] × 100. Saturated mass of the plant was determined by keeping them in water at 4 °C in dark for 24 h, followed by their drying in hot air oven (60 °C for 48 h) till constant mass achieved.

The K^+ and Na^+ contents were determined by flame photometer (*Model Jenway PFP7*, Essex, UK). Before measurement, 0.5 g dry sample was digested by *Digesdahl Digestion Apparatus* (*Hach Company,* Ames, USA) using 4 cm^3 of concentrated H_2SO_4 and 10 cm^3 of 50 % (v/v) H_2O_2.

Free proline content was determined according to Bates *et al*. (1973) using L-proline as a standard as described elsewhere (Niknam *et al.* 2006).

For determination of saccharides content, 0.5 g of dry powder was extracted using 10 cm^3 of 80 % (v/v) ethanol, and supernatant was collected after twice centrifugation at 1 480 *g* for 15 min. The residue from ethanol extraction was subsequently used for polysaccharide extraction by boiling water (Seyyednejad *et al.* 2001). Total saccharides content was estimated by the method of Dubois *et al*. (1956). Reducing saccharides were quantified according to Nelson (1944). Oligosaccharide content was obtained from difference between soluble and reducing saccharides contents.

Polyphenols were extracted with 80 % (v/v) methanol at 70 °C water bath for 3 h (Niknam and Ebrahimzadeh 2002). The suspensions of methanolic extraction were filtered, the methanol was removed by vacuum distillation and then the aqueous solutions were used for quantitative determination. Polyphenol content was determined by the Folin-Denis method (Waterman and Mole 1994). In this procedure appropriate volumes of aqueous solutions were diluted to final volume of 17 cm^3 by distilled water then l cm^3 of Folin reagent and 2 cm^3 of saturated solution of sodium carbonate were added. After 30 min the absorbance was measured at 760 nm. Aqueous solutions of tannic acid (0 - 6.25 μg cm^3) were used as standards for plotting working curve (Ranganna 1986).

For determination of protein content, 0.1 g fresh sample was homogenized in a chilled (4 °C) mortar using a 50 mM Tris-HCl buffer (pH 7.0) containing 10 mM EDTA, 2 mM Mg SO_4, 20 mM cysteine, 10 % (v/v) glycerol and 2 % (m/v) PVPP (Jaaska 1996). After centrifugation at 13 000 *g* for 45 min at 4 °C, the supernatant was filtered and then transferred to Eppendorf tubes and the sample kept on ice at 4 °C. A portion of eluent was stored at -70 °C. Total protein content was measured by the spectrophotometric method of Bradford (1976) using bovine serum albumin (BSA) as the standard. High-speed centrifuge (*Beckman J2-21M*, Palo Alto, USA) and UV-visible spectrophotometer (*Shimadzu UV- 160*, Tokyo, Japan) with 10 mm matched quartz cells were used for centrifugation of the extracts and determination of the absorbance, respectively.

Discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970) with 12 % acrylamide gels. For detection of proteins, gels were stained with 0.03 % Coomassie Briliant Blue G250. A vertical electrophoresis apparatus (*model LKB,* Bromma, Sweden) was used. The electrophoretic run was carried out with 140 mV per plate towards the cathode.

The data determined in triplicate were analyzed by analysis of variance (*ANOVA*) using *SPSS* (version *11.0.0*). The significance of differences was determined according to Duncan's multiple range test (DMRT). P values < 0.05 are considered to be significant.

Results and discussion

Corms, that normally are used for *C. sativus* propagation, produce two structurally and functionally different roots; thin fibrous roots that play a central role for plant nutrition, and thick contractile roots that pull the corm down to its desired depth in the soil (Choi *et al.* 1996). Plant growth was clearly affected by the NaCl treatments. The hydroponic culture of *C. sativus* plants under salt stress did not induce a decrease in survival in comparison with control plants and also did not lead to appearance of necrosis on the young and old leaves, even at 200 mM NaCl. In contrast, contractile roots fresh mass significantly reduced at 50 mM NaCl and higher concentrations. The fresh mass of fibrous roots, contractile roots, corms and leaves at 200 mM NaCl were 24.9, 82.2, 5.4 and

43.3 % lower than the control values, respectively (Fig. 1*A*). Moreover, relative water content (RWC) of leaves declined to 88.0, 85.0, 80.2 and 79.0 % at 50, 100, 150 and 200 mM NaCl, respectively.

Protein content in contractile roots and corms increased significantly under NaCl stress (Fig. 1*B*). Moreover, protein content in leaves increased slightly when NaCl increased from 50 to 100 mM. The increase in protein content can be the result of the enhanced *de novo* synthesis of proteins for cell protection against the stress as suggested Chen and Plant (1999) and Chandrashekar and Sandhyarani (1995). In contrast, by increasing the NaCl concentrations, protein content in the fibrous roots decreased significantly. The decrease detected in the stressed fibrous roots has already been described in similar stress situations (Soussi *et al.* 1998, Chen and Plant 1999).

The highest and lowest proline content almost at all concentrations of NaCl was obtained in fibrous roots and leaves, respectively (Fig. 1*C*). This could be explained by the fact that root is the first organ to deal with this stress and is considered to be the main organ responsible for plant tolerance to salt stress by taking an active part in excluding ions in excess. All the organs respond to salt stress by increasing the proline content and this increment in contractile roots (*ca.* 127.7 % of the control value) was even higher than in the fibrous roots (*ca.* 27.2 % of the control value). It is well known that proline content in many plants gets enhanced by several stresses including salt stress (*e.g.* Tripathi *et al.* 2007). In plant tissues, proline accumulation has been suggested to result from *1*) a decrease in proline degredation, *2*) an increase in proline biosynthesis, *3*) a decrease in protein synthesis, and *4*) hydrolysis of proteins (Lin *et al*. 2002).

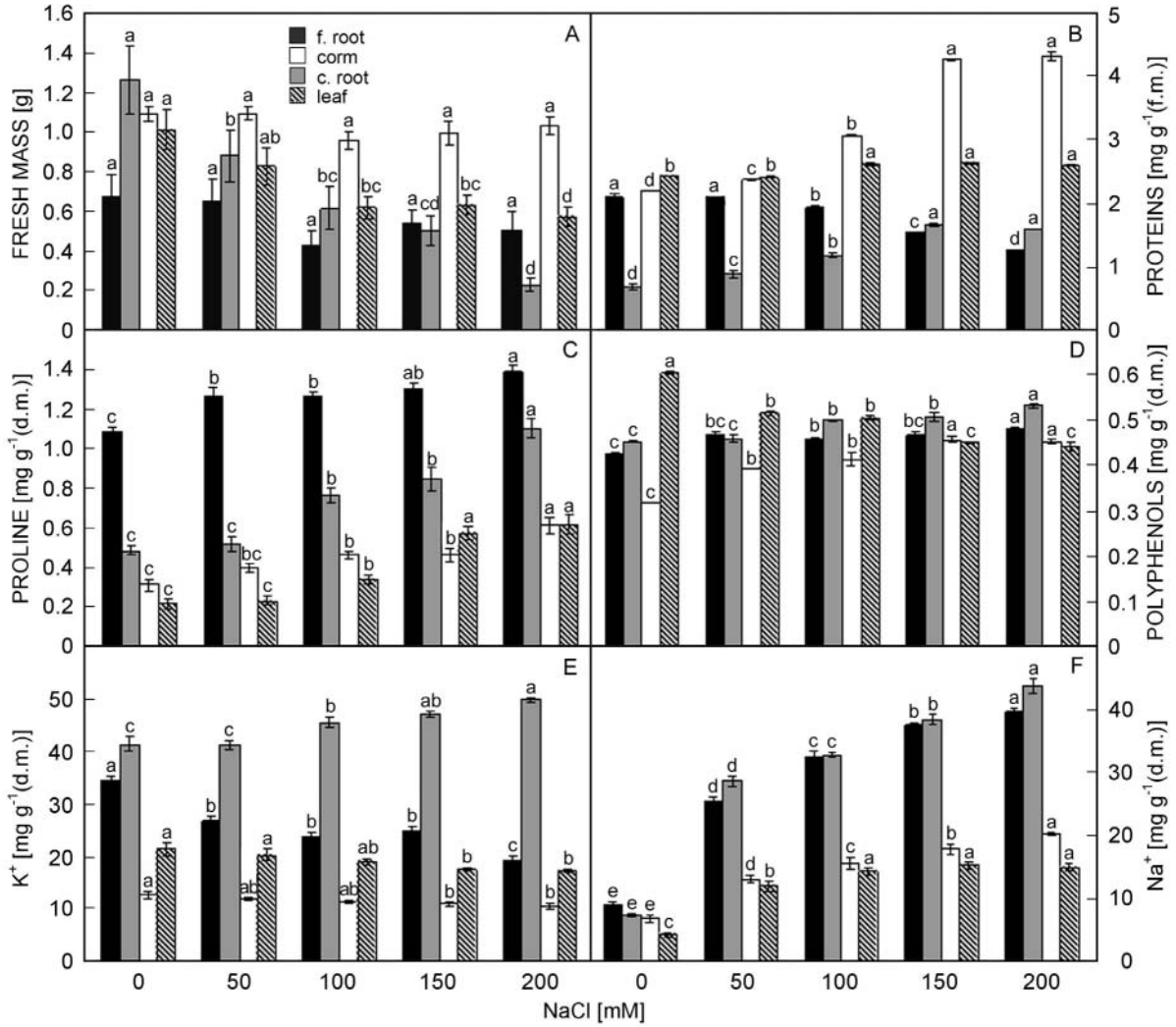

Fig. 1. Effect of different NaCl concentrations on fresh mass (*A*) and contents of protein (*B*), proline (*C*), polyphenols (*D*), K^+ (*E*) and Na^+ (*F*) in fibrous root, contractile root, corm and leaf of *Crocus sativus*. The *vertical bars* represent standard errors. The mean values marked by the same letter are not significantly different at $P < 0.05$ as determined by Duncan's multiple range test.

Polyphenol content was increased by salinity in all the organs except the leaves (Fig. 1*D*). At the control, the highest and lowest polyphenol content was determined in leaves and corms, respectively. Increase in polyphenol content in different organs under increasing salinity has also been reported in a number of plants (*e.g.* Agastian *et al.* 2000). Muthukumarasamy *et al.* (2000) reported that increase in polyphenol in the tissue ameliorates the ionic effect of NaCl. This report could explain why the polyphenol content was enhanced specially in underground organs of saffron (fibrous roots, contractile roots and corms) under salt stress.

The K^+ contents in different organs of control plants, were much higher than that of Na^+ as expected, and reduced significantly in fibrous roots after NaCl treatment (Fig. 1*E*,*F*). This may subsequently lead to a reduction of metabolically important ions such as K^+ (Kwon *et al.* 1995). In contrast, K^+ content in contractile roots increased under salinity. A high concentration of K^+ in the presence of NaCl in this plant organ is regarded as a positive index. Fibrous roots and contractile roots showed higher K^+ contents than that of corms and leaves.

Na^+ content increased in all organs under NaCl stress (Fig. 1*F*). These increments in contractile roots and fibrous roots were higher than those of other organs. The results of this study indicated that under salt stress, Na^+ is preferentially accumulated in roots. This is probably due to the control of Na^+ transport. According to Marschner (1986) resorption of Na^+ from xylem sap is an effective mechanism of restricting translocation to the leaf. Na^+competes with K^+ for intracellular influx because these cations are transported by common proteins (Hasegawa *et al*. 2000). Unlike Na^+, K^+ play a key role in a vast array of physiological process vital to plant growth *e.g.* protein and starch synthesis, enzyme activation, ATP synthesis, osmotic adjustment and transport of sugars.

Accumulation of proline in different organs under NaCl treatments was accompanied by high uptake of Na^+ (Fig. 1*C,F*). This result is in consistence with the finding of Tripathi *et al.* (2007). It appears that in all the organs, Na^+ is taken-up and then sequestered inside the vacuole and proline is synthesized and accumulated in the cytoplasm to balance the osmotic potential of the ion accumulated in the vacuole.

By increasing NaCl concentrations, reducing saccharides content decreased in contractile roots and leaves and remained unchanged in corms (Fig. 2*A*). The decrease of reducing saccharides content in contractile roots might be attributable to their conversion to other forms of saccharides (Fig. 2*B,C*). The decrease in saccharides content under salt stress has already been reported (Gadallah 1999, Niknam *et al.* 2004). Salinity increased oligosaccharide and soluble saccharides contents proportionally in contractile roots. Polysaccharide content was promoted significantly in corms under salinity (Fig. 2*D*). The increase in soluble saccharides, oligosaccharides and

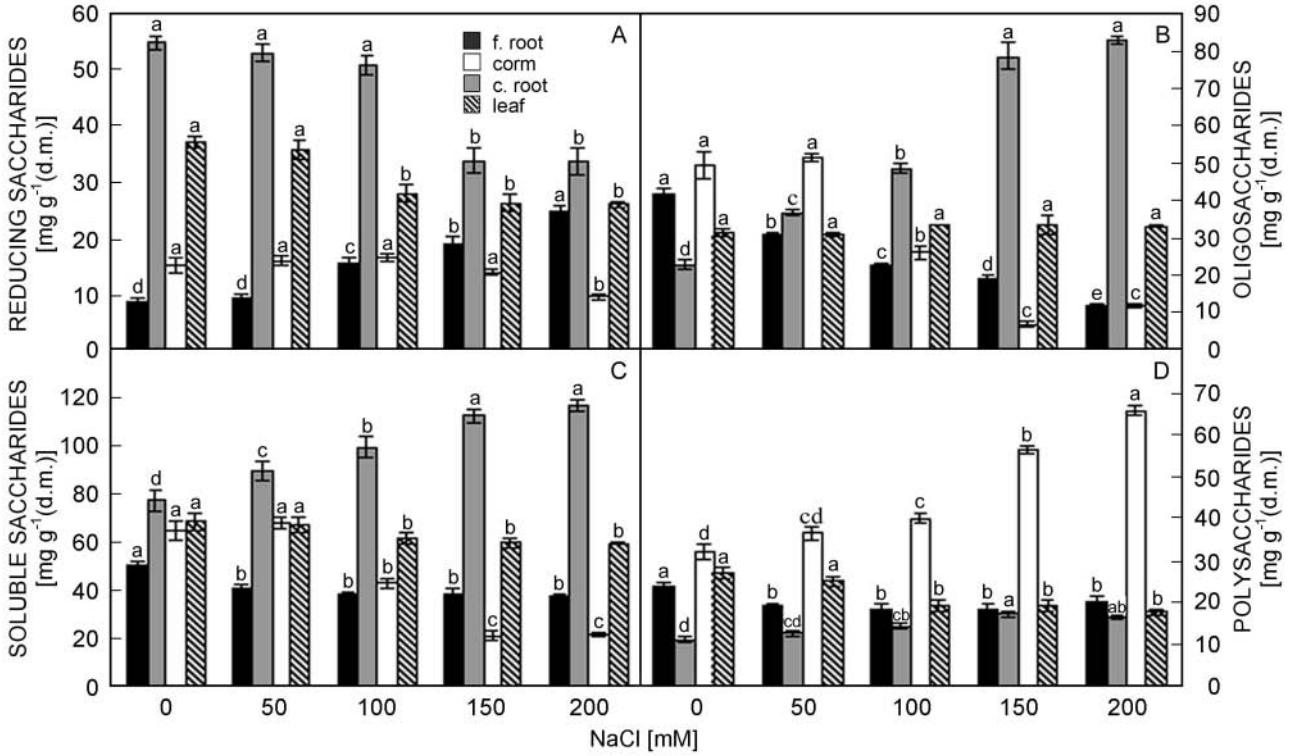

Fig. 2. Content of reducing saccharides (RS; *A*), oligosaccharides (OS; *B*) and soluble saccharides (SS = RS + OS; *C*) and polysaccharides (*D*), in different organs of *Crocus sativus* under NaCl stress. The *vertical bars* represent standard errors. The mean values marked by the same letter are not significantly different at $P < 0.05$ as determined by Duncan's multiple range test.

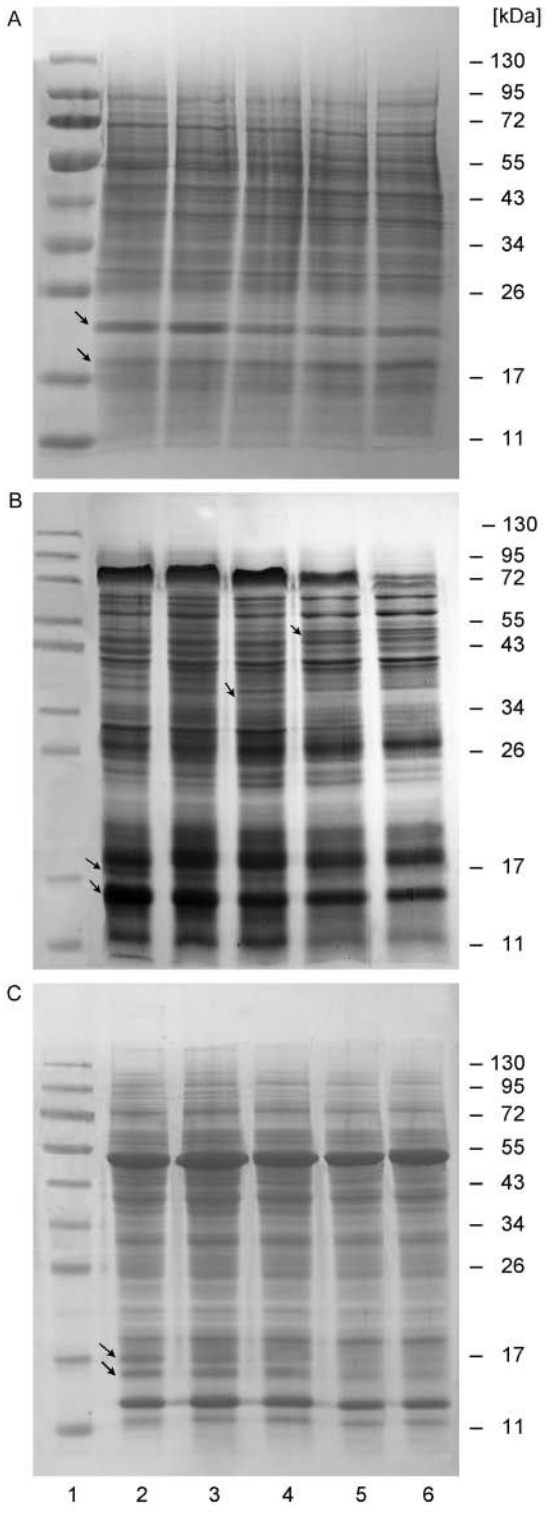

Fig. 3. SDS-PAGE pattern of proteins in contractile root (*A*), corm (*B*) and leaf (*C*) of *C. sativus* under NaCl concentrations of 0, 50, 100, 150 and 200 mM NaCl (lines 2 to 6); molecular mass markers (line 1). *Arrows* indicate some of the affected bands.

reducing saccharides has also already been reported in similar stress situations (Dubey and Singh 1999, Sotiropoulos 2007). During the course of salt stress, active accumulation of sugars is claimed to be an effective stress tolerance mechanism (*e.g.* Binzel *et al.* 1987, Fedina *et al.* 2002).

Patterns of polypeptides in contractile roots, corms and leaves under different concentrations of NaCl were not identical and the differences were both quantitative and qualitative (Fig. 3*A-C*). According to analysis of protein patterns in contractile roots, content of two protein bands with the molecular masses of about 19 and 21 kDa increased and decreased under increasing salinity, respectively. Proteins that accumulate in plants grown under saline conditions may provide a storage form of nitrogen that is re-utilized when stress is over and may play a role in osmotic adjustment. Content of some proteins with the molecular masses of about 12, 14, 15, 16, 17, 28, 59 and 72 kDa significantly decreased in corms of salt-stressed plant. Similarly, contents of 16 and 17 kDa proteins in leaves also decreased at 100 mM NaCl and higher. The decrease in proteins of different organs at higher concentrations of NaCl may be due to the release of them to the media due to osmotic shock or a decrease in the synthesis. In agreement to our result, Parida *et al.* (2004) reported the reduction in contents of several proteins in *Bruguiera parviflora*. Two new protein bands with the molecular masses of 36 and 50 kDa appeared in corm at 100 and 150 mM NaCl treatments, respectively. Moreover, intensity of a 23 kDa protein band increased up to 100 mM NaCl then decreased at higher salinities. To identify these polypeptides would have contributed to understanding the intracellular mechanisms by which saffron responses to salinity. Sousa *et al.* (2004) reported that cowpea seedlings subjected to NaCl stress showed increased concentration of 9 proteins, decreased concentration of one and *de novo* synthesis of one 21.2 kDa protein. The obtained results are also in agreement with the findings of Munoz *et al.* (1997), Hassanein (1999), Elshintinawy and Elshourbagy (2001) and El-Baky *et al.* (2003).

In conclusion, the present study demonstrated that the best salinity stress marker in *C. sativus* was growth of contractile roots. Our data indicated that there are significant differences between different organs in their responses to salinity. Corms and fibrous roots did not show significant negative growth for all the salinity levels and the resistance of these organs to salinity could be attributed to the increase in the content of metabolites such as proteins, proline and polyphenols. However, future studies on antioxidative enzymes, other antioxidants and lipid peroxidation are thus required in order to gain complementary information on these properties in *C. sativus*.

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