World Academy of Science, Engineering and Technology
International Journal of Agricultural and Biosystems Engineering
Vol:11, No:1, 2017

Adaptive Responses of *Carum copticum* to *in vitro* Salt Stress

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***Abstract*—**Salinity is one of the most widespread agricultural problems in arid and semi-arid areas that limits the plant growth and crop productivity. In this study, the salt stress effects on protein, reducing sugar, proline contents and antioxidant enzymes activities of *Carum copticum* L. under *in vitro* conditions were studied. Seeds of *C. copticum* were cultured in Murashige and Skoog (MS) medium containing 0, 25, 50, 100 and 150 mM NaCl and calli were cultured in MS medium containing 1 µM 2, 4-dichlorophenoxyacetic acid, 4 µM benzyl amino purine and different levels of NaCl (0, 25, 50, 100 and 150 mM). After NaCl treatment for 28 days, the proline and reducing sugar contents of shoots, roots and calli increased significantly in relation to the severity of the salt stress. The highest amount of proline and carbohydrate were observed at 150 and 100 mM NaCl, respectively. The reducing sugar accumulation in shoots was the highest as compared to roots, whereas, proline contents did not show any significant difference in roots and shoots under salt stress. The results showed significant reduction of protein contents in seedlings and calli. Based on these results, proteins extracted from the shoots, roots and calli of *C. copticum* treated with 150 mM NaCl showed the lowest contents. The positive relationships were observed between activity of antioxidant enzymes and the increase in stress levels. Catalase, ascorbate peroxidase and superoxide dismutase activity increased significantly under salt concentrations in comparison to the control. These results suggest that the accumulation of proline and sugars, and activation of antioxidant enzymes play adaptive roles in the adaptation of seedlings and callus of *C. copticum* to saline conditions.

***Keywords*—**Antioxidant enzymes, *Carum copticum*, organic solutes, salt stress.

I. Introduction

CROP production in the modern world is facing increasing challenges as it is strongly affected by both biotic and abiotic stresses. Salinity is one of the most widespread abiotic stress conditions that inhibit plant growth by disrupting homeostasis in water potential and disrupting ion balance. Salinity leads to the accumulation of reactive oxygen species (ROS) which seriously impair the normal function of cells and damage lipids and proteins, terpenoids, carbohydrates and nucleic acids [1]. Plants resort to both non-enzymatic and enzymatic defense systems for adaptation to salt stress [2]. Generally, acclimation of plant to drought stress is the result of many different physiological and biochemical mechanisms including a series of integrated events from stress signal perception and transduction to regulation of gene expression, leading to the adaptive changes in plant growth and physiological and biochemical processes [3]. The enzymatic systems that detoxify free radicals are divided into two categories: One that reacts with ROS and keeps them at low levels (including peroxidase, superoxide dismutase, and catalase), and one that regenerates the oxidized antioxidants (including ascorbate peroxidase and glutathione reductase) [4]. Moreover, in response to osmotic stress, plants accumulate large quantities of compatible solutes such as soluble sugars and proline. These solutes protect cells against damage from osmotic stress by contributing to cellular osmotic adjustment, ROS detoxification and protecting sub-cellular structures [5]. Therefore, the elucidation of physiological and biochemical mechanisms related to salt tolerance in crops is essential to reduce the adverse effects of salt stress and improve salinity tolerance in plants. Plant *in vitro* culture is one of the efficient methods for studying the effects of salt stress on plant responses under controlled conditions [6]. Stress imposition on production of medicinal plants during *in vitro* culturing is different from true stress conditions on the plants naturally exposed to these conditions. *In vitro* study holds a potential for the production of high quality plant based medicines.

With regards to side-effects of chemical drugs and increases in antibiotic resistant strains to conventional medicines, medicinal herbs such as *Carum capticum* with pharmacological and antibacterial properties can be served as an alternative for infection treatment. *C. copticum* L., commonly known as Ajwain, is one of the most important aromatic plants of Apiaceae family, with a variety of bioactive compounds. These compounds are commonly used for their carminative, antiseptic, amoebiasis expectorant, antimicrobial, antiparasitic and antilithiasis effects [7]. These compounds contain phenolics (carvacrol), flavonoids, saponins, glycosides, essential oils such as thymol, terpiene, paracymene, and beta-pinene [8]. Ajwain is grown in various regions such as Iran, Egypt, Pakistan, India, and Europe [9].

The present study aims to determine salt stress effects on physiological parameters and antioxidant activities in callus and seedlings of *C. copticum* by establishing conditions of salt stress using NaCl as an osmotic material under *in vitro* conditions.

II. Materials and Methods

A. Plant Materials

The mature and sterilized seeds of *C. c*opticum were cultured on MS medium [10] containing 0, 25, 50, 100 and 150 mM NaCl, and kept in the growth chamber (16 h light/8 h

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dark) at 25 °C and 95% relative humidity. 28 days after treatment, effects of salt stress were studied on proline accumulation, reducing sugars, protein contents and enzymatic mechanisms of roots and shoots of seedlings of *C. copticum.*

B. Callus Production and Salt Treatment

28 days old growing seedlings were used as source of explants. Stem explants about 0.5 cm were cultured on MS media containing of 1 µM 2, 4-dichlorophenoxyacetic acid (2, 4-D), and 4 µM of benzyl amino purine (BAP). After about 14 days, callus induction was observed. After 28 days, callus explants were subcultured on the same medium and kept at darkness and 25 ± 2 ºC for 28 days. Callus subculture was repeated monthly. After 3 subcultures, calli transferred into the medium supplemented with 4 µM BAP, 1 µM 2-4-D and 0, 25, 50, 100 and 150 mM NaCl, respectively. After 28 days, physiological parameters were analyzed in calli of *C. copticum* similar to seedlings.

C. Measuring Amount of Reducing Carbohydrates

Reducing sugar contents were determined according to the adapting Somogyi-Nelson's method [11]. 0.05 g fresh tissue (shoots, roots and calli) was homogenized with 10 mL distilled water. After boiling in a boiling water bath and passing through filter paper, 2 mL of the extract was mixed with 2 mL alkaline copper tartarate which was prepared by dissolving 4 g anhydrous sodium carbonate, 0.75 g tartaric acid and 0.45 g hydrated cupric sulphate in 80 mL distilled water and finally made up to 100 mL. Then, the reaction mixture was heated for 20 minutes. 2 mL of phosphomolibdate solution was added and the intensity of blue color was measured at 600 nm using spectrophotometer (U-6305 model; Jenway, Staffordshire, UK). The reducing sugar content was expressed as mg/g FW.

D. Measuring Amount of Proline

Proline content was measured using ninhydrin reaction according to Bates et al. [12]. Samples each containing 0.05 g of shoots, roots and calli were homogenized with 10 mL of 3% (w/v) sulphosalicylic acid and filtered by Whatman filter paper (no. 2; Whatman, Maidstone, UK). Afterwards, 2 mL ninhydrin reagent (Sigma, St. Louis, Missouri, USA) and 2 mL glacial acetic acid to 2 mL of the filtered extract, the mixture was incubated at 100 °C for 1 h. The reaction was terminated by placing it on ice. Then, 4 mL toluene was added to the reaction mixture and absorption intensity was measured at 520 nm using spectrophotometer. Proline content was calculated using L-proline (Sigma) as a standard curve.

E. Preparation of Protein and Enzyme Extracts

Samples containing 0.1 g of frozen shoots, roots and callus were homogenized in 2 mL 25 mM sodium phosphate buffer (pH 7). The homogenate was centrifuged at 15,000 × g for 20 min at 4 °C and supernatants were used for protein and enzyme activity measurement.

Measuring Protein Concentration

The amount of total protein of shoots, roots and callus was measured according to Bradford [13]. The absorbance of each extraction was measured at 595 nm and the measured amounts of proteins were expressed as mg/g FW.

Superoxide Dismutase Activity

Superoxide dismutase (SOD) activity was determined by adding 300 µL of the extracts to a mixture containing 50 mM sodium phosphate buffer (pH 7.8), 0.1 mM EDTA, 50 mM Na_2CO_3, 12 mM L-methionine, 1 µM riboflavin and 75 µM p-nitro blue tetrazolium chloride in dark conditions, following the procedure of Giannotolitis and Ries [14]. The reaction was carried out under illumination (30 W fluorescent lamp) at 25 °C for 10 min. The absorbance was measured at 560 nm and the activity was expressed as unit/g FW min.

Catalase Activity

Catalase (CAT) activity was assayed based on the rate of H_2O_2 decomposition (extinction coefficient 36 mM^{-1} cm^{-1}) as measured by the decrease of absorbance at 240 nm and expressed as unit/g FW min, following the procedure of Aebi [15]. The reaction mixture contained 2 mL of 25 mM sodium phosphate buffer (pH7), 100 µL of 37% H_2O_2 and 50 µL of extraction enzyme.

Ascorbate Peroxidase Activity

Ascorbate peroxidase (APX) activity was determined in a 1-mL reaction mixture containing 50 mM sodium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.2 mM EDTA, 0.1 mM H_2O_2 and 100 µL enzyme extract. The subsequent decrease in ascorbic acid was determined at 290 nm (E = 2.8 mM^{-1} cm^{-1}) and the activity was expressed as unit/g FW min [16].

F. Statistical Analysis

All experiments were performed in a completely random design with three replicates. One way ANOVA was used for treatment assay and Duncan tests were used to compare the mean values.

III. Results

A. Effects of NaCl on Accumulation of Compatible Osmolytes

To determine accumulation of compatible osmolytes under salt conditions, proline and reducing sugars contents were analyzed. Reducing sugar and proline contents as compatible osmolytes were measured in shoots, roots and calli after 28 days of NaCl treatment. These compatible osmolytes increased significantly in relation to the severity of the salt stress. As shown in Figs. 1 (a) and (b), maximum proline contents in the NaCl-treated seedlings and callus were observed at 150 mM NaCl. No statistical differences were observed in the proline contents of roots and shoots (Fig. 1 (a)). The highest accumulation of reducing sugars in seedlings was observed at 100 mM NaCl. However, the results of means comparisons revealed that sugar accumulation in shoots was the highest as compared to roots (Fig. 1 (c)). A similar trend in carbohydrate content was observed in the callus samples (Fig. 1 (d)).

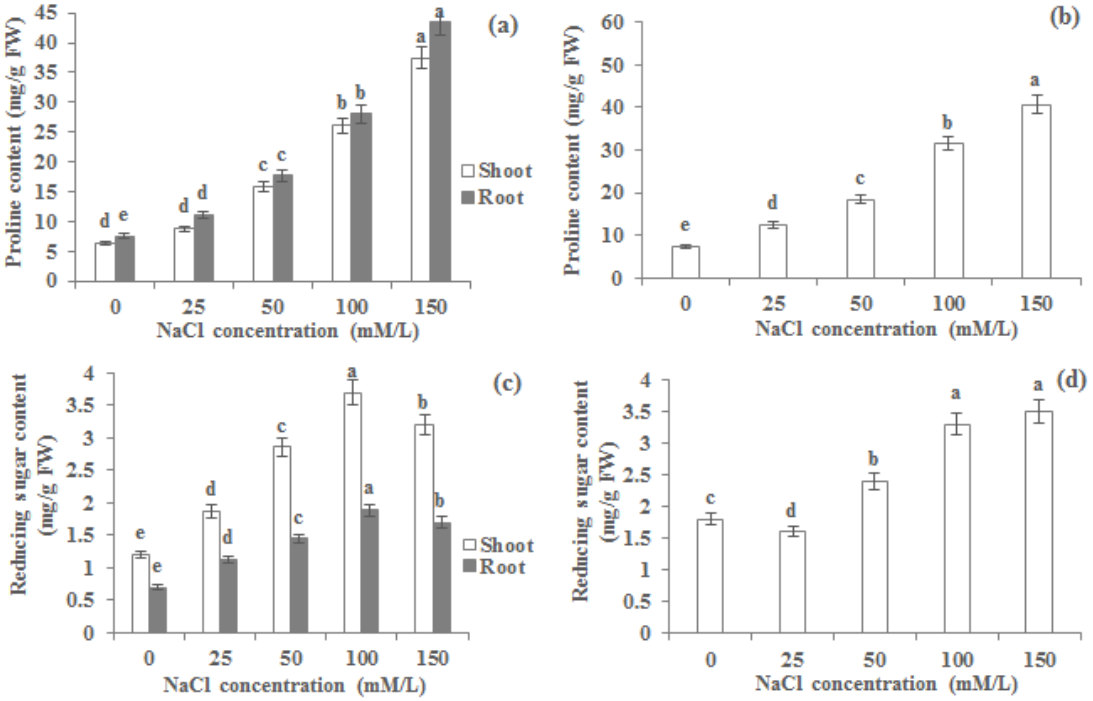

Fig. 1 The effect of NaCl on the proline content of seedlings (a), callus (b), reducing sugar content in seedlings (c) and callus (d) of *C. copticum:* Values are means of three replicates and dissimilar letters represent significantly differences according to Duncan's test ($P \leq 0.05$)

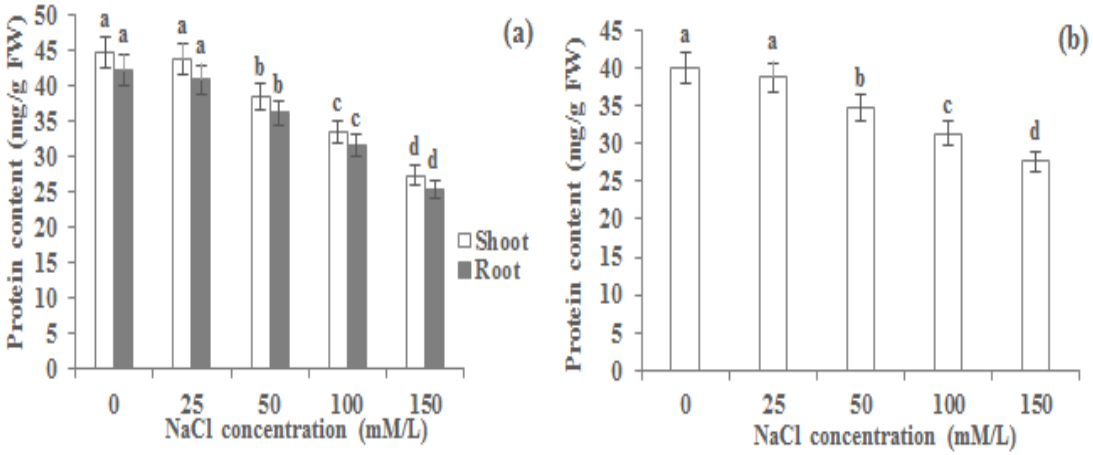

Fig. 2 The effect of NaCl on the protein content of seedlings (a) and callus (b) of *C. copticum*: Values are means of three replicates and dissimilar letters represent significantly differences according to Duncan's test ($P \leq 0.05$)

B. Effects of NaCl on Accumulation of Protein Contents

Fig. 2 shows a significant reduction in protein contents of calli and seedlings exposed to different concentrations of NaCl. Compared to the control, the protein contents decreased with increasing salt concentrations in the culture media. The lowest proteins contents were observed in seedlings and calli of *C. copticum* treated with 150 mM NaCl (Figs. 2 (a), (b)).

C. Effects of NaCl on Antioxidant Enzymes Activity

The results revealed that salt stress led to a significant increase in activity of antioxidant enzymes of *C. capticum*. The SOD activity of NaCl-treated seedlings was different significantly as compared to control seedlings. NaCl induced a significant increase in the SOD activity of treated shoot and root at 100 mM (Fig. 3 (a)). NaCl also caused a significant increase in the SOD activity of calli of *C. capticum*. The highest activity was observed at 150 mM (Fig. 3 (b)). The mean comparison results indicated that salt stress caused a significant increase in the CAT activity of *C. capticum*. In NaCl-treated shoot, the CAT activity was increased significantly at severe stress conditions. However, the observed decreases at 150 mM NaCl in root tissue resulted with value which was still significantly higher than the control (Fig. 3 (c)). The same phenomenon was observed in callus (Fig. 3 (b)). The highest activity was observed at 150

mM (Fig. 3 (d)). APX activity severely increased during salt stress as compared to controls. The highest activity in seedlings and calli were observed at 150 mM (Fig. 3 (e), (f)).

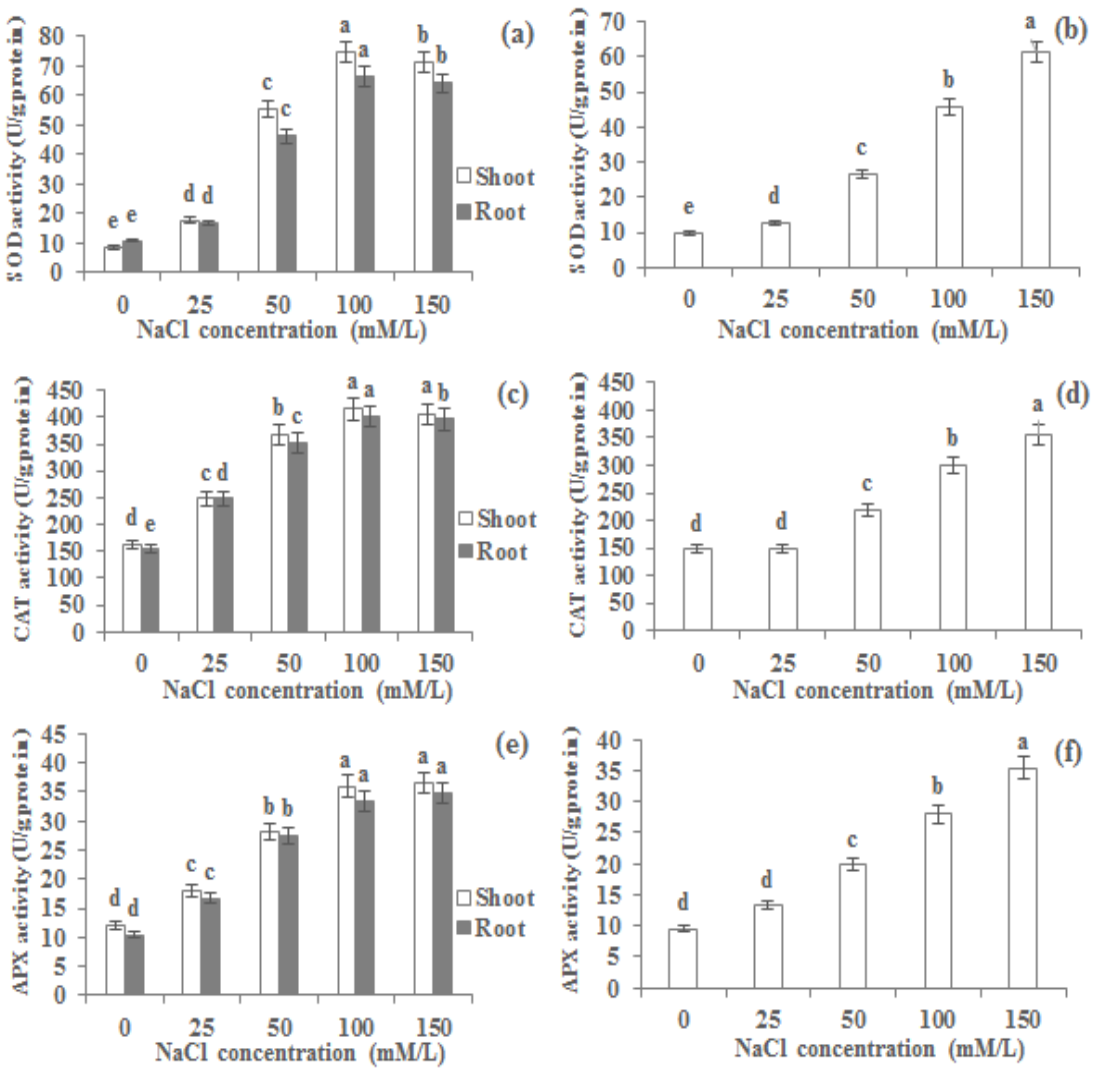

Fig. 3 The effect of NaCl on SOD activity of seedlings (a), callus (b), CAT activity of seedlings (c), callus (d), APX activity of seedlings (e) and callus (f) of *C. copticum*: Values are means of three replicates and dissimilar letters represent significantly differences according to Duncan's test ($P \leq 0.05$)

IV. Discussion

Salinity, as an important environmental factor causing osmotic stress, induces oxidative stress in plants. One of the characterized biochemical responses of plant cells to counteract this stress is the accumulation of compatible osmolyte such as proline and sugars. Under osmotic stress, proline mediates osmotic adjustment, stabilizes proteins, membranes, and subcellular structures and mitigate oxidative damage caused by free radicals produced in response to stress [8]. In this study, proline contents in seedlings and callus of *C. copticum* accumulated in large quantities in response to salt stress. Proline as an osmoprotectant is of particular interest for improving abiotic stress tolerance of plants. The accumulation of proline under abiotic stress conditions has been reported in numerous plants [17], [18]. Moreover, accumulation of reducing sugars in *C. copticum* seems to alleviate the salinity stress, either by osmotic regulation [19], or by conferring some desiccation resistance to plant cells [20].

Salt stress also caused oxidative damage to protein contents of seedlings and callus of *C. copticum*. The results showed a significant reduction of protein contents in relation to the severity of the salt stress, possibly due to the increased production of free radicals of oxygen, which inhibits protein synthesis or leads to protein denaturation [21]. It can also be due to reduced photosynthesis that caused to decrease materials required for protein synthesis under stress conditions [22]. Decreasing of solution protein in many plant species as a result of plant exposure to stress conditions was reported [23], [24].

One of the important strategies in plants for alleviation of oxidative damages and acquisition of stress tolerance is using

of antioxidant enzymes which scavenge ROS [25]. This study showed a positive relationship between activity of antioxidant enzymes and increase in stress levels. Compared to the control, the activity of antioxidant enzymes increased significantly under salt concentrations. The results suggest a possibility that oxidative stress produces reactive oxygen compounds such as superoxide radicals and hydrogen peroxide which function as intercellular signals and stimulate a number of genes and proteins involved in stress responses, such as SOD, CAT and APX [26]. Enhanced SOD activity represents the first line of defense in protection against the formation of superoxide radicals from the reduction of O_2 and offers protecting cells against superoxide-induced oxidative stress. *Vigna radiata* [27], potato [28] and cotton [29] had been reported similar to the present results. Moreover, the CAT and APX activity are the other protective enzymes against free oxygen radical, causing detoxification and decomposition of hydrogen peroxide and they prevent the breakdown of plant proteins. There are some investigations describing the increase of CAT and APX activities after salt stress in rice [30], pea [31] and *Thellungiella halophila* [32]. These results suggest that studied antioxidant enzymes play an important role in the physiological response to scavenge ROS and increase salt tolerance in seedlings and callus of *C. copticum*.

V. Conclusion

It can be concluded that proline and sugar accumulation in association with SOD, CAT and APX activities increase salt tolerance in ajwain plants via cooperation with different pathways.

Acknowledgment

The authors thank Payame Noor University, Iran for supporting this study.

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