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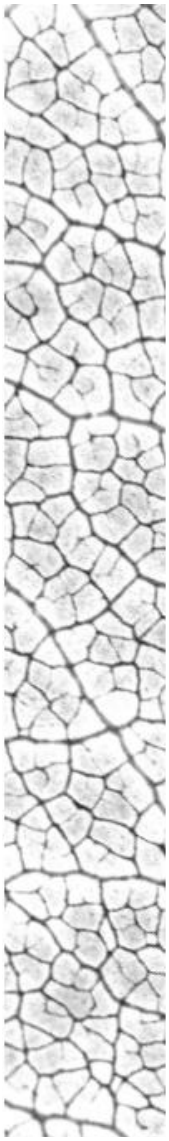

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Effects of salinity stress on biochemical and anatomical characteristics of *Ceratophyllum demersum* L.

Dunya A. Al-Abbawy [1*], Zaineb Al-Sweid [1], Sahar A. Al-Saady [2]

[1] University of Basrah, College of Science, Department of Ecology, IRAQ
[2] University of Basrah, College of Science, Department of Biology, IRAQ
*Corresponding author: dunya.hussain@uobasrah.edu.iq

Abstract
The effects of NaCl concentrations (2, 5, and 10 ppt) on submerged macrophyte coontail (*Ceratophyllum demersum* L.) were studied under laboratory conditions for three weeks. Control plant treatment was also chosen in this experiment. Every week, biochemical characteristics (chlorophyll contents, protein, and proline) were measured as well as anatomical changes in leaves, and stems at the beginning and the end of the experiment period. Experimental results showed significantly reduced in chlorophyll content (from 8.410 µg/g to 0.320 µg/g) while in control treatment was increased during the experiment period (from 8.912 µg/g to 13.090 µg/g). Protein also decreased (from 37% to 22%) at 10 ppt NaCl concentration in comparison with control treatment (from 38.4% to 50.3%). However, the praline level increased when the salinity increases (from.0.15 µg/g to 0.55 µg/g) in response to salt stress resistance. The anatomical study of the leaves and the stems of the exposure plants showed that the high concentrations of NaCl causes a reduction in thickness of leaves because of the accumulation of high NaCl levels in the tissues of the plant. The leaves thickness ware 53.30, 66.33 and 80.80 µm after exposure to 2, 5 and 10 ppt NaCl, respectively. The results stem thickness of *C. demersum* proved that the cortex thickness, aerenchyma tissue and xylem elements increased with increasing NaCl concentration, the thickness of the epidermis decreased with increased salt stress.

Keywords: salinity, submerged plant, anatomical, biochemical characteristics, coontail

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INTRODUCTION

Submerged aquatic macrophytes considered as the key of healthy water bodies, because they play a very important role in their ecosystems like providing dissolved oxygen, refuges for different kinds of aquatic organisms, removal of some heavy metals (e.g. arsenic, copper, nickel, and zinc) and also remediate organic pollutants (Ganjo and Khwakaram, 2010; Hammad, 2011; Rahman and Hasegawa, 2011). However, some recent ecological disturbances such as increasing salinity in the Shatt Al-Arab River due to low quantities of water supply have negative effects on the composition and diversity of submerged macrophytes communities in freshwater environments (Herbert et al. 2015; Tootoonchi and Gettys, 2019). Many studies have been conducted to prove the effectiveness of submerged plants in remediation and management of polluted aquatic ecosystems by choosing the most suitable aquatic plant species. Several aquatic plant characteristics, such as oxygen production, consumption, growth, photosynthesis pigment contents, and salinity tolerance, were used to test the effectiveness of aquatic plants. Salinity tolerance is important especially when the plant is used to remediate an aquatic ecosystem.

Salinity is one of the major abiotic stresses (Munns and Tester, 2008). The increasing of salinity depends on the amount of saltwater and type of plants (Vicente et al., 2004). At low salinity, the damages in plants are due to osmotic stress, nutritional imbalance, and ion toxicity. At low salt concentration, shoot dehydration is the primary response of plants; at moderate to high salt concentrations, nutritional imbalance and toxicity caused by accumulated ions, especially Na+ and Cl−, are the main effects of salinity on plant physiological and biochemical activities (De Pascale et al. 2003). Shatt Al-Arab River, which is a freshwater ecosystem, is increasingly becoming saline because of the loss of a large quantity of water from the Euphrates and Tigris and the influx of the salt content from the Persian Gulf. This study was performed to investigate the salt tolerance of *Ceratophyllum demersum* and to determine the effects of NaCl stress on this plant.

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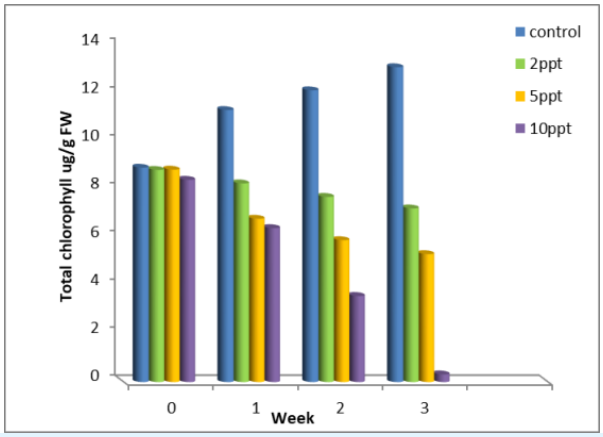

Fig. 1. Effect of NaCl concentrations on the total chlorophyll of *Ceratophyllum demersum*

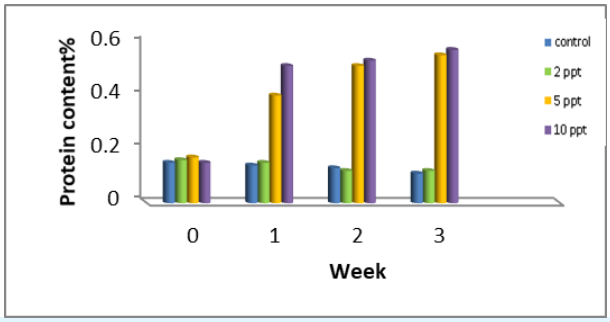

Fig. 2. Effect of NaCl concentrations on the protein content of *C. demersum*

MATERIALS AND METHODS

The salt tolerance of submerged aquatic macrophyte *Ceratophyllum demersum* L. was evaluated experimentally. The plant was collected from several areas in the Shatt Al-Arab River and brought to the laboratory. The sample (25 g) was weighted and put in aquaria (capacity, 3 L; 60 cm × 50 cm × 40 cm) filled with water. To enrich the nutrient content, each aquarium was supplemented with 0.1 g of mixed N, P, and K fertilizer weekly. Various amounts of NaCl crystals were used to reach the desired salinity level. The salinity of each medium was determined using a conductivity meter. The series of salinity were 0 ppt (control), 2, 5, and 10 ppt of NaCl. The growth rate was monitored weekly by identifying the plant's freshness. Biochemical characteristics were measured weekly, and an anatomical study was conducted at the beginning and end of the experiment.

Anatomical study

For sectioning, the fresh material of leaves and stems were fixed in FAA (Formalin- acetic acid- 70% ethyl alcohol, 5:5: 90 ml) for 48 hours, and preserved in 70% alcohol, then dehydrated in ethyl alcohol series (70, 80, 90, 95 and 100 %) and then paraffin. The samples were sectioned on a rotary microtome and stained by safranin (1 g from safranin in 100 ml distal water) and fast green, which was prepared by mixed 1 g from fast green melted with absolute alcohol, the samples clearing with xylene and then mounted by Canada balsam (Johansen,1968).

All data were analyzed statistically by SPSS v.20.

RESULTS AND DISCUSSION

Total Chlorophyll

Fig. 1 shows a summary of the total chlorophyll contents at different NaCl concentrations. The total chlorophyll declined with increasing salt concentration. While the chlorophyll contents were increased in the control treatment from 8.912 µg/g at the beginning of the experiment to 13.090 µg/g on the third week of treatment. The total chlorophyll declined to 7.220, 5.320, and 0.320 µg/g as the salt concentration increased to 2, 5, and 10 ppt, respectively.

Salinity is one of the abiotic stress factors restricting the growth and development of aquatic macrophytes and affecting various biochemical, metabolism, and physiological processes (Munns and Tester, 2008). NaCl inhibits many metabolic processes, such as the synthesis of the photosynthetic pigment in chlorophyll (Norhayati and Nurhuda, 2012) and inhibition of enzymatic activity, which is essential to build a chlorophyll molecule. The decrease in total chlorophyll with increasing NaCl may be due to changes in the lipid-protein ratio of pigment-protein complexes or increased chlorophyllase activity (Iyengar and Reddy, 1996). However, NaCl at high concentrations markedly depressed the photosynthetic pigments in *C. demersum*, consistent with the results of Agastian (2000).

Protein

Fig. 2 shows the changes in the protein of *C. demersum* exposed to three NaCl concentrations. The protein in the leaves of the control significantly increased gradually from 38.4 % to 50.3 % in the third week. The protein in the leaves irrigated with NaCl at different concentrations declined from 22 % to 10 % in the third week. The increased salt level may have reduced the total nitrogen in the green leaves, and this result was consistent with the results obtained by Pitzschke et al. (2006). Increasing salinity affected the cell stability and synthesis of proteins. This reduction could be attributed to the toxic effects of Na+ and Cl− on the physiologically active parts of the tissues; high salinity influenced the cell content of amino acids and reduced RNA and DNA in the plant (Izzati, 2016).

Proline

Proline concentrations in *C. demersum* increased significantly with increasing salinity. Treated plants showed higher proline content (0.55 µg/g) after three weeks of exposure to 10 ppt NaCl compared with the control (**Fig. 3**). This result agreed with the result obtained by Apel and Hirt (2004), whom found that

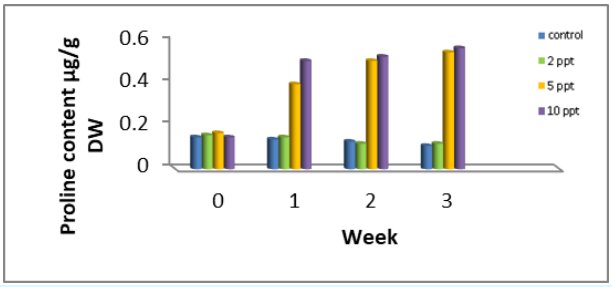

Fig. 3. Effect of NaCl concentrations on the proline content of *C. demersum*

proline contributed to the protection of *C. demersum* against high salinity. Furthermore, the accumulation of proline was related to the decrease in the gene encoding for proline dehydrogenase (Al-Bahrany, 1994).

Anatomical Study

Anatomical changes in the C. demersum leaves

Anatomical changes in the plant exposed to different NaCl concentrations are shown in **Table 1** and **Fig. 4**. Anatomical transverse sections of the *C. demersum* leaves showed several changes in the thickness, number, and shape of aerenchyma tissue and the shape of the epidermis cells. The results showed a reduction in the thickness of leaves because of the accumulation of high NaCl levels in the tissues of the plant. The leaves were 53.30, 66.33, and 80.80 µm thick after exposure to 2, 5, and 10 ppt NaCl, respectively. In the control treatment, the leaves of *C. demersum* consisted of the upper and lower epidermis, whilst the mesophyll consisted of aerenchyma tissue. The transverse sections of the leaf exposed to salt showed enlarged epidermis cells, sinuate to undulate surface of leaves

Table 1. Measurements of leaves in *C. demersum* (Mean and range in µm)

Concentration ppt	leaves thickness	Upper epidermis thickness	Lower epidermis thickness	Mesophyll thickness
Control	(7.5-55) 45.13	(12.5-25) 12.30	(2.5-25) 11.20	(7.5-22) 15.71
2	(7.5-67.5) 53.30	(12.5-22.5) 17.5	(2.5-10) 5.21	(12.50-47.5) 35.71
5	(15-75.5) 66.33	(5-15) 30.21	(2.5-35) 23.30	(42.5-50) 47.50
10	(40.8-91.5) 80.80	(17.5-32.5) 25.42	(2.5-37.5) 25.11	(42.5-50) 47.50

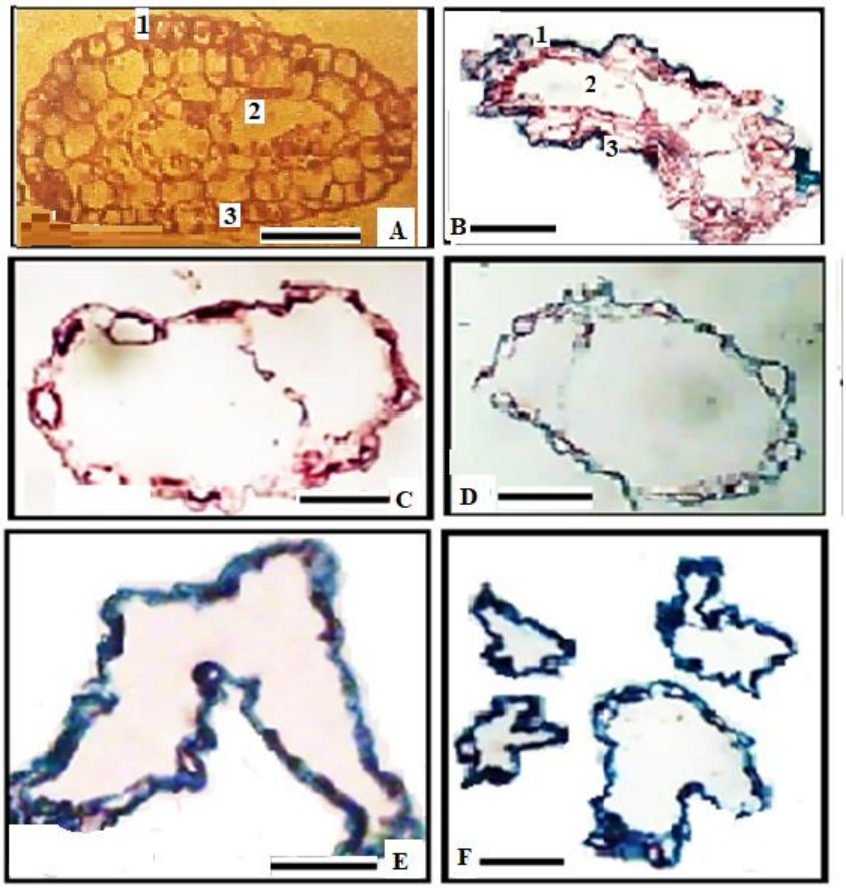

Fig. 4. Transverse sections of the leaves of *C. demersum*. (A) Control treatment; (B) 2 ppt NaCl; (C, D) 5 ppt NaCl; (E, F) 10 ppt NaCl; (1) upper epidermis; (2) aerenchyma tissue; and (3) lower epidermis (Scale bar: 50 µm)

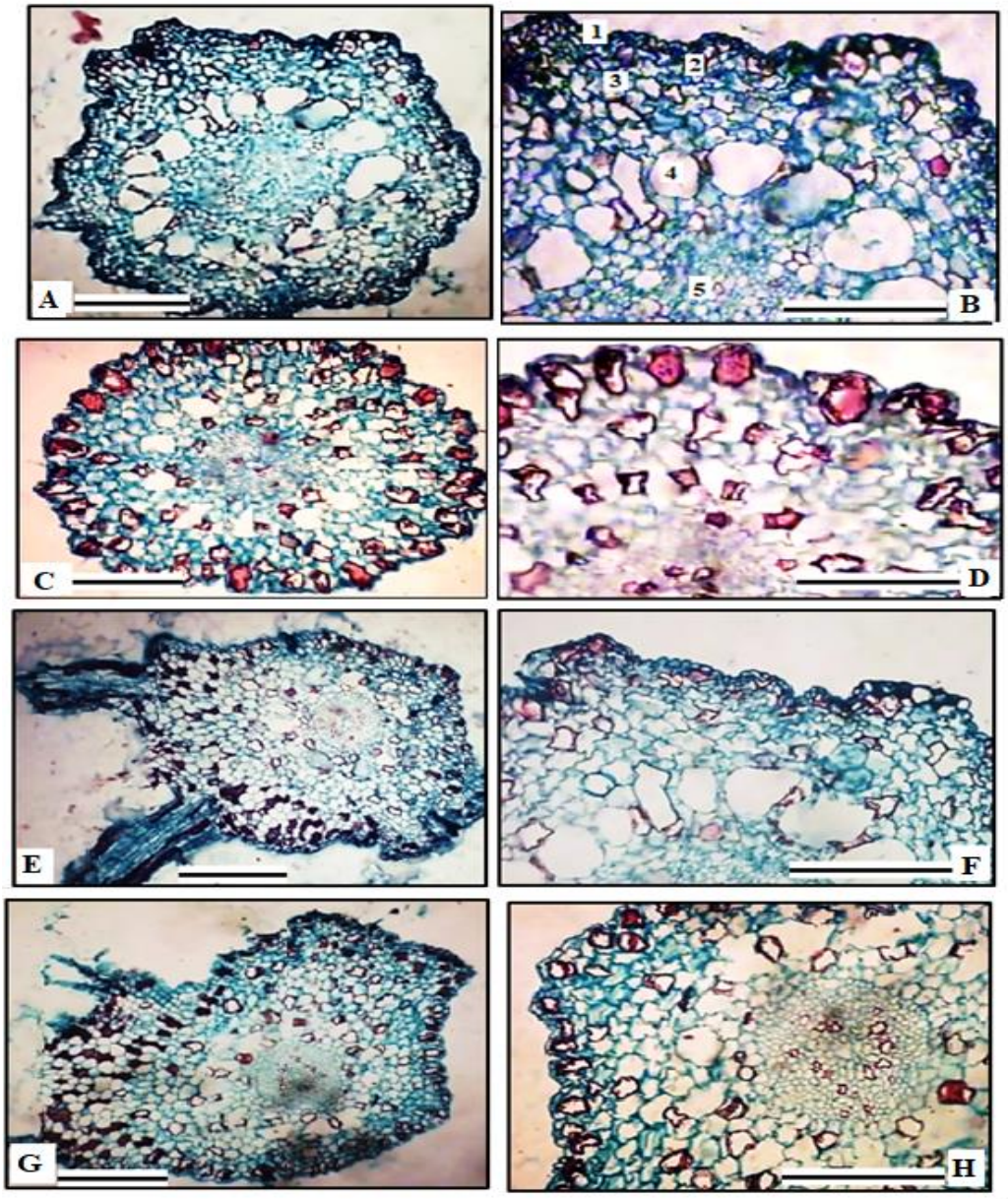

Fig. 5. Transverse sections of the stem of *C. demersum*. (A, B) Control treatment; (C, D) 2 ppt NaCl; (E, F) 5 ppt NaCl; (G, H) 10 ppt NaCl; (1) epidermis; (2) tannin in the cortex layer; (3) cortex; (4) aerenchyma tissue; (5) vascular cylinder; and (5) xylem elements (Scale bar: 100 µm)

(especially the upper and lower epidermis), and increased size of the aerenchyma chamber. The main change in the leaf tissue after exposure to 10 ppt NaCl was the mesophyll filled with one aerenchyma cell surrounded by one layer of the epidermis. The results showed irregular changes in the shape of cells may be due to the toxic effect of the salts or the ability of salt concentration to upset the hormonal balance of *C. demersum* (Aminirad and Sonboli, 2008).

Transverse sections of C. demersum stems

Anatomical changes in the *C. demersum* stems between the control and treated plants were observed (**Fig. 4**). In the control treatment, the stem contained a single epidermis layer enclosing several layers from the cortex. This layer was followed with one layer from the aerenchyma tissue. The center of the stem had pith (**Fig. 5A**). The results showed increases in the thickness of the stem of *C. demersum* with increased salt stress. The stem was 385.62 µm thick in the control treatment but 3340.77 µm at 10 ppt NaCl (**Table 2**). By contrast, the thickness of the epidermis decreased with increased salt stress. The epidermis was 30.93 µm in the control treatment and decreased to 13.68 µm at 10 ppt NaCl. The results proved that the cortex thickness, aerenchyma tissue, and xylem elements increased with increasing NaCl concentration (**Table 2**).

Table 2. Measurements of stems in *C. demersum* (Mean and range in µm)

Concentration ppt	Stem thickness	Epidermis thickness	Cortex thickness	Aerenchyma tissue thickness	Xylem thickness	Phloem thickness
Control	(367.5-402) 385.62	(5-55) 30.93	(10-52.5) 42.50	(12.5-52.5) 36.25	(23-47) 30.66	(25-150) 140.13
2	(651-1038) 848.12	(5-25.5) 16.12	(30-72.5) 58.75	(25-90) 47.81	(45-61) 50.14	(62-100) 81.25
5	(906-1281) 901	(2.5-22) 14.87	(27.5-87.5) 73.75	(27-177) 86.56	(55-73) 67.21	(25-50) 40.13
10	(3315-3375) 3340.77	(5-27.5) 13.68	(55-150) 94.22	(102.5-152.5) 140.50	(143-166) 150.11	(25-32.5) 28.75

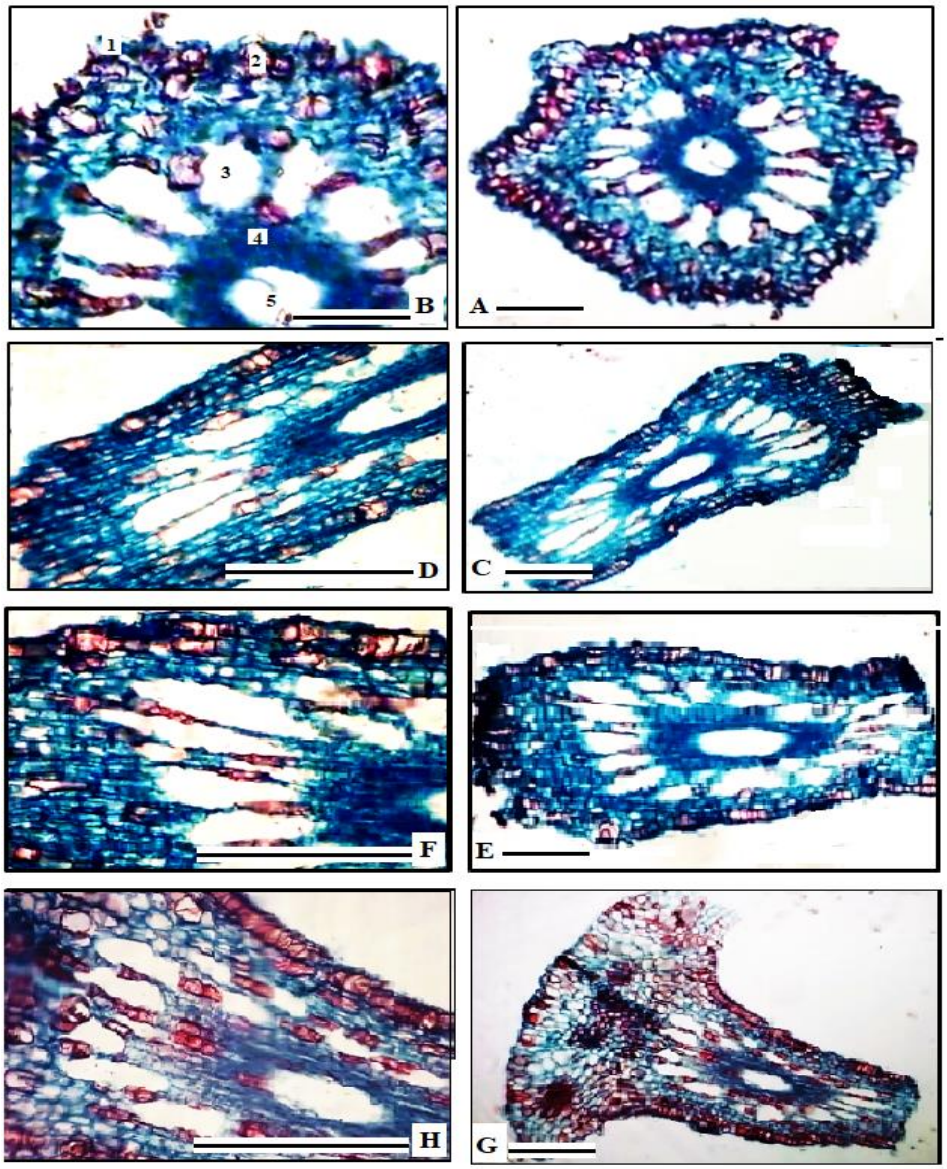

Fig. 6. Transverse sections of the stem of *C. demersum* at 10 g/L NaCl. (1) Epidermis; (2) tannin in the cortex layer; (3) aerenchyma tissue; (4) vascular cylinder; and (5) xylem elements (Scale bar: 100 µm)

The control treatment consisted of one layer of the epidermis, followed by 10–13 layers of cortex and one layer of aerenchyma tissue, and the center of the stem contained a vascular bundle (**Figs. 6A** and **B**). Salt stress treatment resulted in changes in the stem thickness, size, and a number of the air chambers, presence or absence of tannin cells, and size of xylem thickness. At 2 ppt NaCl, the shape of the stem changed, and the number of tannin cells in epidermis cells increased compared with the control treatment, but the size of aerenchyma tissue was reduced (**Fig. 4**). At 5 ppt NaCl, new growth appeared in the stem, which increased the stem size, changed the shape of the stems, and increased xylem thickness (**Fig. 4**). The main changes could be observed at 10 ppt NaCl, in which the stems were enlarged and changed in shape. The epidermis was filled with tannin cells, the size and

number of aerenchyma cells increased and the xylem vessels were enlarged (**Fig. 4**).

Salt damages on the plant cells changes the structures of the cells and tissues and decrease the growth rate (Aminirad and Sonboli, 2008). Pieter and Kuiper (1968) reported the cellular uptake of Na+ and Cl− at the plasma membrane of the epidermis or cortical cells and the plasma membrane of the xylem parenchyma cells. NaCl concentrations influence the cellular physiology and metabolic pathway of *C. demersum* plants and causes nutritional imbalance because of the high accumulation of Na+ and Cl^{-} in the leaves and shoots (Sandalio et al., 2001; Al-Saadi et al., 2013; Al-Zurfi et al., 2018).

CONCLUSION

We concluded that the salinity affected the growth of *Ceratophyllum demersum* and their metabolic and physiological processes and in its leaves content of chlorophyll and protein. The High concentration of salts increased the proline content in the plant to resist salt stress. The anatomical study showed that the high concentration of salt affected the external appearance and the internal structure of the plant.

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