

## Structural and biochemical responses of the seagrass *Halodule uninervis* to changes in salinity in Kuwait Bay, Doha area

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### Abstract

The effects of salinity in Doha bay, Kuwait, on the osmotic potential, amino acids, and proline accumulation as osmoprotectants in *Halodule uninervis* tissues from seagrass meadows were investigated. The relationships between osmotic potential, amino acids, proline levels in leaves and rhizome/root tissues of *H. uninervis* and a range of osmolality of seawater was determined in the laboratory. The mean salinity of the seawater in the intertidal zone of the bay was 38.03‰, and relative water content of mature leaves was less than 45%. There was a high correlation between salinity and the osmotic potential of cell sap of *H. uninervis* tissues. Total amino acids accumulated in backshore samples were higher than those in foreshore samples. Proline was the most abundant amino acid (41-51%), followed by glutamine and alanine in both leaves and rhizome/root tissues in mid-summer. Anatomical study showed ultrastructural adaptations including thicker tangential cell walls in leaf epidermal cells, highly invaginated plasmalemma, numerous chloroplasts, plastoglobuli and mitochondria in epidermal cells associated with increased salinity. Physiologically, salinity tolerance by *H. uninervis* was related to the accumulation of compatible solutes. The minimum threshold of salinity that caused tissue damage in *H. uninervis* was -6.36 MPa and -8.06 in rhizomes and leaves, respectively.

**Keywords:** *Halodule uninervis*; osmoregulation; proline; salinity; seagrass.

### 1. Introduction

Seagrasses are monocotyledonous flowering plants that are more closely related to the lily family than true grasses. Morphologically, these plants have shoots with 3-5 leaves above-ground, interconnected stems or rhizomes and a fibrous root system. Seagrasses play a prominent ecological role and support the productivity on which many communities of marine animals feed and reproduce (Natiq *et al.*, 2014). Seagrass habitats are important nurseries for commercially important fish and shellfish species (Orth *et al.*, 2006; Waycott *et al.*, 2009) and act as sediment traps, providing sediment stabilization (Costanza *et al.*, 1997; Wright & Jones, 2006). They are found submerged in marine or in shallow estuarine and coastal waters and are adapted to survive in saline water. To survive in marine environment, seagrasses must (1) be well anchored in sediment to withstand wave action and tidal currents, (2) adapted to saline medium, (3) grow when completely submerged, and (4) have a capacity for pollination in seawater (den Hartog, 1967).

Mean seagrass percentage cover and Biomass of *Halodule uninervis*, *Thalassia hemprichii*, and *Halophila*

*ovalis* were observed to decline (Kamil *et al.*, 2013). The survival of these plants in a saline environment is quite a challenge with respect to obtaining water from seawater with a negative osmotic potential and the high concentrations of potentially toxic sodium and chloride ions that can decrease metabolic functions of these plants.

Seagrasses must exhibit specific plant phenotypic plasticity to be able to acclimate to the changing environment (Nicotra *et al.*, 2010). When exposed to changes in salinity, most intertidal seagrasses would utilize a complex set of physiological and biochemical changes for acclimation to fluctuating salinities in their habitat. Grime (1977) suggested that seagrasses are stress-adapted, since they tolerate varying levels of salinity, light fluctuation, and other abiotic factors. Seagrasses exposed to changes in salinity can suffer osmotic stress, with consequent changes at the biochemical and physiological levels (Touchette, 2007). Adaptive mechanisms in response to osmotic stress include: developmental traits, structural traits, physiological mechanisms and alteration in photosynthetic metabolism and the accumulation of compatible osmolytes or solutes (McCue & Hanson, 1990;

Delauney & Verma, 1993; Griffin & Durako, 2012; Koch *et al.*, 2007(b); Marín-Guirao *et al.*, 2013a; Sandoval-Gil *et al.* 2012b).

Abiotic stresses that cause cell dehydration in many plants may result in the accumulation of one or more of several low molecular weight organic solutes termed compatible solutes, which include amino acids and their derivatives, sugars and polyols. Compatible solutes can lower the cell water potential without disrupting metabolism (Brown & Simpson, 1972). They are involved in normal cellular metabolism at high concentrations and may be accumulated up to 5-10% of plant dry weight. They facilitate water uptake and retention (McCue & Hanson, 1990), can directly protect enzymes from high salt concentration (Smirnoff & Stewart, 1985), prevent chemical denaturation (Yancy & Somero, 1980), retard thermal denaturation (Smirnoff & Stewart, 1985), and can stabilize membranes (Crowe *et al.*, 1984).

Proline appears to be the most widely distributed osmolyte accumulated under stress conditions in plants, eubacteria, protozoa, marine invertebrates and algae (McCue & Hanson, 1990; Measures, 1975; Singh *et al.*, 1972). Some seagrasses respond to increased salinities by utilizing some of the inorganic ions initially and then producing organic osmolites such as proline and glycine betaine (Tyerman, 1989). The osmotic roles of proline, alanine, and glutamine have been shown in *Ruppia* (Murphy *et al.*, 2003; Brock, 1981; Adams & Bates, 1994), *Zostera* (Van Diggelen *et al.*, 1987), *Posidonia* and *Cymodocea* (Sandoval-Gil *et al.* 2012a, Sandoval-Gil *et al.* 2014). Proline may be involved in minimizing the effects of a particular form of cell damage and not simply adjusting the intracellular osmotic potential (Delauney & Verma, 1993).

In Kuwait, meadows of two species of seagrass (*Halophila ovalis* and *H. uninervis*) have been documented (Al-Bader *et al.*, 2014; Al-Hasan & Jones, 1989). *H. uninervis* is the dominant species in Doha area of the Kuwait Bay. Seagrasses in Kuwait Bay are exposed to many environmental stresses including extremes of temperature and salinity that can potentially affect their growth, development and survival. The survival and adaptive processes of seagrasses in Kuwait Bay have not been studied, despite the fact that they contribute to coastal productivity and biodiversity.

The objectives of this study were to determine the salinity levels that *H. uninervis* can tolerate and the levels that could cause the decline of the seagrass population in

Kuwait Bay. Also, to determine the levels of proline and amino acids that accumulated as compatible solutes in *H. uninervis* as a possible response to different salinities of seawater.

## 2. Materials and methods

### 2.1. Plant material and sampling

*H. uninervis* samples were collected from Kuwait Bay at Doha in triplicates, every two weeks, from early summer to late summer, over a period of two years. Samples were taken from the backshore (closer to the shore) and the foreshore (far from the shore) of the intertidal zone. Samples were collected during the low tide in sealed plastic bags, transferred to the lab on ice and were immediately stored at -80 °C for further analysis.

Concurrently, sediment and seawater samples were collected from the seagrass meadow areas and stored at -80°C for analysis. The seawater temperature, pH and salinity were measured.

### 2.2. Determination of relative water content and osmotic potential of samples

#### Relative water content (RWC)

The RWC of fully expanded leaves was determined using ten rectangular pieces of tissue. The combined fresh weight of the leaf pieces was initially determined, and then hydrated in 5 ml of distilled and deionized water for 24 h. The leaf pieces were reweighed to obtain their weights after saturation and dried in an oven at 80°C for 72 h to determine the dry weights. Five replicates of leaf tissue were used. The relative water content (RWC) was calculated as follows:

$$\text{RWC} = (\text{fresh weight} - \text{dry weight}) \times 100 / (\text{saturated weight} - \text{dry weight})$$

### 2.3. Osmotic potential

Plants in each sample were separated into above ground tissues (i.e. shoot) and below ground tissues (rhizomes with roots), from each sample, 4 g of each tissue type was analyzed for osmotic potential. Cell sap was extracted from the separated tissues using a garlic press. The extract collected was centrifuged at 9000 rpm for 20 min at 4°C. The osmotic potential of the centrifuged cell sap was measured using a Wescor Vapor Pressure Osmometer 5520 (Wescor Inc. Logan, Utah) by transferring 0.1ml aliquot of the undiluted sap into the osmometer. Three measurements were taken for each sample, and the data

were expressed as MegaPascals (MPa) according to Van't Hoff's equation (Tyerman, 1982; Nobel, 2009).

Sediments samples were thawed and centrifuged at 5000 rpm for 30 min to collect the "pore water". The pore water (supernatant) was passed through a 0.22 µm Millipore filter, and the osmotic potential was measured as described above.

Osmotic potential of filtered seawater collected from shore pools of the sample area was also measured as described above.

#### 2.4. Determination of free proline and soluble amino acids by HPLC

The procedure used was Pico Tag method developed by Waters Inc. USA. Aliquots (10 µl) of leaf and rhizome samples were dried under vacuum for 1h at 105°C. The dried samples were hydrolyzed with 200 µl of 6N HCl with 1% phenol in stream of nitrogen and dried under vacuum. This step was repeated 2-3 times before drying at 110°C for 20-24 h. The samples were further dried after adding 10 µl of a (2:2:1) mixture of ethanol : water : triethylamine. Derivatization was done by the addition of 20 µl of ethanol : triethylamine : water : phenylisothiocyanate (7:1:1:1), vigorously mixed for a few seconds using a vortex and allowed to stand for 20 min at room temperature. Derivatization was followed by a 30-45 min drying under vacuum to remove all traces of phenylisothiocyanate.

The derivatized samples were analyzed for total soluble amino acids and proline using HPLC (Waters 680, USA) fitted with a photodiode array detector (Waters 996, USA). Separation of amino acids and proline was accomplished on Pico-Tag amino acid column (60 Å, 4 µm, 300 x 3.9 mm). Run time was 20.5 min, flow rate (1-1.5 ml/min), detector sensitivity was 0.1 AUFS at UV wavelength of 254 nm. Column temperature was 38°C, The mobile phase consists of two eluents labeled A (P/N 88108) and B (P/N 88112), a standard gradient elution program, was used. The amino acids and proline were quantified by comparing each sample with known standards using a photodiode array detector. Data were stored and analyzed using a PC equipped with Waters Millennium 32 software.

#### 2.5. Determination of inorganic ions in the tissues of *H. uninervis*

Leaves and rhizomes samples from Doha were thoroughly rinsed with sterile deionized water to remove salts, soil

particles and plant debris. Then 4g of each sample of leaves and rhizomes were dried in porcelain crucible, overnight in a furnace at 500°C. The plant residue was digested overnight in 10 ml of 0.1 mM HNO<sub>3</sub>. The solution was filtered and diluted to bring the concentration of sodium, magnesium, potassium and chlorine to a suitable range for analysis.

Sodium, magnesium and potassium concentrations were determined in flame atomic absorption spectrophotometer (Perkin-Elmer 5100 PC, Norwalk, USA), The chloride ion content was eluted with a mixture of 1.8 mM sodium carbonate and 1.7 mM sodium bicarbonate and its concentration was determined by ion chromatography using IonPac AS4A column (4 mm i.d. x 250 mm, P/N 043174) (Dionex, 4500i, USA), the sample injection volume was 10µL, the flow rate was 1.2 ml/min, the column temperature was 30°C. The run time was approximately 20min with an ion exchange capacity of 20 µeq/column and the backpressure between 1000-1400 psi. Analyzed samples were compared to a linear calibration curve with a correlation coefficient of >0.995.

#### 2.6. Effect of salinity on the leaves and rhizomes of *H. uninervis*

The effects of different salinities of seawater on leaf and rhizome tissues were studied under laboratory conditions. Commercially produced seawater (Sigma-Aldrich) was evaporated in a water bath at 60°C to different osmotic potentials. The osmotic potentials were as follows: (-2.04,-2.23, -2.45 MPa diluted seawater), (-3.64 MPa control), (-4.75, -6.36, -8.66 MPa evaporated seawater). Rhizomes with five shoots and four internodes of the same length were placed in 100 ml of the seawater that contains different solute concentrations. The set up was done in three replications. Leaf and rhizome tissues were evaluated histologically for the changes in tissue solute potential, proline and soluble amino acids after 3 days.

The osmotic potential, proline and amino acids concentrations in leaf and rhizome tissues were quantified as described above.

#### 2.7. Histological study

Tissue of leaves and rhizomes of plants subjected to four salinity treatments (-3.64, -3.82, -4.94, -8.07 MPa) were examined for anatomical and structural changes. The tissues from the different salinities of seawater were fixed under vacuum in cold 2.5% gluteraldehyde in Millionigs Phosphate buffer (pH 7.4) for 24 h, post-fixed in 2%

osmium tetroxide for 2 h, dehydrated in graded ethanol series, and embedded in epon resin. Sample blocks were trimmed and 1µm sections were cut out with an ultramicrotome (LEICA Ultracut-Uct LEICA, Austria) and mounted on microscopic glass slides. Sections were stained with 1% toluidine blue. The stained sections were finally examined using light microscope to locate the desired area. Ultra-thin sections (50 - 75 nm) were stained with uranyl acetate and lead citrate, viewed and photographed with JEOL'S JEM-1200 EX II, Japan electron microscope.

### 3. Statistical analysis

To determine the significant difference between samples or treatments, a t-test with equal variance, and one tail distribution was used (Microsoft Office Excel 2007 version). Significance was calculated at  $P \leq 0.05$ .

For HPLC and the osmotic potential data, a standard deviation was calculated for triplicates of each sample. For ion chromatography and flame atomic absorption spectrophotometer results, standard error of means was calculated by t-test statistical analysis using IBM-SPSS version 22.

## 4. Results

### 4.1. Growth conditions of meadows

The salinity of 38.68‰ for the backshore pools of seawater was slightly higher than the 37.38‰ of the foreshore in 2004. There was no significant difference in the monthly values of the entire summer of 2004 seawater with average temperature 34.0°C ( $P=0.45$ ), pH 7.9 ( $P=0.24$ ) and salinity 38.03‰ ( $P=0.21$ ) at the intertidal zone. The highest recorded salinity of the intertidal backshore area for the seawater was 41‰. The same was observed, when the experiment was repeated in 2005. There was no significant difference in the means of seawater temperature (30.62 °C,  $P=0.34$ ), pH (8.28,  $P=0.40$ ) and salinity (35.61 ‰,  $P=0.00$ ) at the intertidal zone.

*H. uninervis* was found mostly in the intertidal zone of Doha area. The larger meadows were at the foreshore, while smaller patches were at the backshore of the intertidal zone. The plants at the backshore were smaller in size with shorter leaf blades. From mid-July to August plants in the backshore zone had shoot tip burn, and the exposed rhizomes showed areas of reddish brown discoloration. The shoots of the foreshore plants were twice as long as those at the backshore with very little tip burn and healthier (whitish) rhizomes.

### 4.2. The relative water content and solute potential of leaves and rhizomes

The RWC of leaves obtained from the backshore of the intertidal zone was greater than that of plants sampled from the foreshore zone. The range of RWC was 20.1% to 41.0% and the mean values of RWC were 33.5% (SD=7.2) and 29.4% (SD=8.4) for backshore and foreshore leaf samples, respectively.

The osmotic potentials of tidal pools and sediment water, in addition to those of *H. uninervis* tissues, were measured for the two successive years (Table 1). The highest recorded osmotic potential was -6.37 MPa. The osmotic potential of backshore and foreshore seawater samples were similar during 2004 ( $P=0.15$ ) and 2005 ( $P=0.33$ ) (Table 1). The osmotic potentials of rhizome/root and leaf samples were greater than the osmotic potentials of tidal pools, and of sediment waters regardless of site.

The osmotic potential of sediment water levels did not differ significantly with respect to the of distance from the shore ( $P= 0.37$  in 2004 ,  $P=0.37$  in 2005 ) or different sampling dates ( $P=0.39$  in 2004,  $P= 0.38$  in 2005) (Table 1), although in the year 2004 the osmotic potential was slightly greater. Similarly, the osmotic potential of sediment water did not change significantly for both backshore and foreshore samples.

The highest recorded osmotic potential for the rhizome/root tissues from backshore pools was -5.27 MPa, while those offshore pools was -6.29 MPa. In 2004 the mean osmotic potential of rhizome/roots in foreshore samples was greater than that obtained for backshore samples, but in 2005 the rhizome/roots mean osmotic potentials of both sites were about the same (Table 1).

**Table 1.** The mean osmotic potentials (-MPa) of seawater and *H. uninervis* tissues sampled from Kuwait bay, Doha.

Year	Site ^	Seawater	Sediment water	Leaves	Rhizome/ roots
2004	Site A	2.81	3.08	4.53	4.99
	Site B	3.39	3.13	3.71	4.68
2005	Site A	3.31	2.78	4.30	4.00
	Site B	3.29	2.78	4.42	3.93
	Mean site A	3.06	2.93	4.42	4.50
	Mean site B	3.34	2.96	4.07	4.31

^:Represents sampling areas in the intertidal zone at Doha. Site A represent sampling areas at the backshore of intertidal zone and site B represents areas at the foreshore.

#### 4.3. Proline and soluble amino acids in field samples

Analysis of the leaf and rhizome/root samples by HPLC for amino acids showed the presence of proline, glutamine, threonine, alanine, aspartate, arginine, lysine, valine, phenylalanine, tyrosine, glycine, serine, and leucine. The amino acids that were produced in significant amounts ( $\geq 28\%$ ) in both leaves and rhizome/roots were proline, glutamine, threonine and alanine. Proline was the most abundant amino acid (41 – 51% of total amino acids) (Table 2), followed by glutamine (28 - 43% of total amino acids) in both leaf and rhizome/root tissues.

**Table 2.** Concentrations of proline and total amino acids measured in *Halodule uninervis* leaves and rhizome/roots tissues. Values represent the mean of triplicate samples.

Year	Site <sup>^</sup>	Leaves		Rhizome/roots	
		Proline	Total A. acids	Proline	Total A. acids
		$\mu\text{mol g}^{-1}$ fresh wt ( $\pm$ Standard error of means)			
2004	Site A	5.15 ( $\pm$ 1.26)	12.65 ( $\pm$ 4.3)	9.18 ( $\pm$ 1.5)	23.86 ( $\pm$ 5.6)
	Site B	5.90 ( $\pm$ 0.41)	10.19 ( $\pm$ 1.58)	8.15 ( $\pm$ 1.1)	16.25 ( $\pm$ 2.2)
2005	Site A	7.38 ( $\pm$ 1.1)	17.98 ( $\pm$ 3.0)	8.82 ( $\pm$ 1.8)	23.75 ( $\pm$ 7.1)
	Site B	7.43 ( $\pm$ 1.5)	15.90 ( $\pm$ 3.2)	6.31 ( $\pm$ 1.2)	16.26 ( $\pm$ 3.6)
	Mean site A	6.27	15.32	9.00	23.81
	Mean site B	6.67	13.05	7.23	16.26

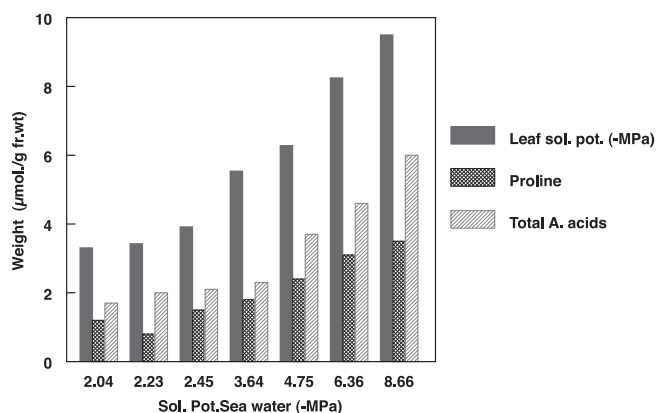
<sup>^</sup>:Represents sampling areas in the intertidal zone at Doha. Site A represent sampling areas at the backshore of intertidal zone and site B represents areas at the foreshore.

Compared to the foreshore, backshore leaf samples had significantly greater amounts of total amino acids ( $P=0.04$  in 2004 and  $P=0.03$  in 2005). Similarly, rhizome/root samples from the backshore had significantly higher concentration of proline ( $P=0.04$ ) and total amino acids ( $P=0.02$ ) than foreshore samples (Table 2). The accumulation of amino acids in the rhizome/roots was significantly higher than in the leaves ( $P=0.04$ ).

#### 4.4. The effect of salinity on the tissues of *H. uninervis*

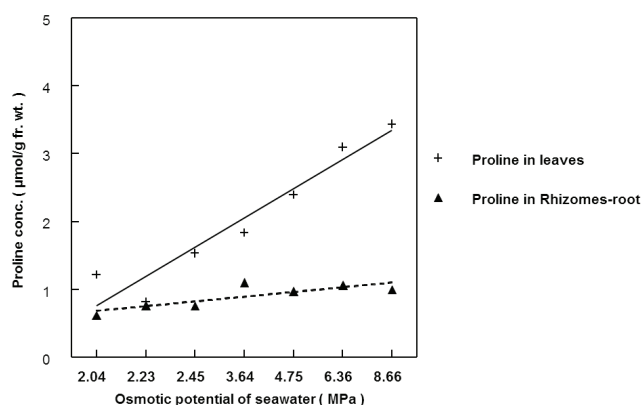
Laboratory experiments on the effect of different osmotic potentials on leaves and rhizome/roots indicated that the amino acids that were produced in significant amounts in both leaf and rhizome/root samples were proline, glutamine, threonine and alanine respectively. Leaf tissues in modified seawater of decreasing osmotic potential (-2.04 - -8.66 MPa) had a significant increase in

the proline concentration ( $r^2=0.94$ ), total amino acids and significant increase in leaf osmotic potential (Figure 1).



**Fig. 1.** The leaf solute potential, free proline and total amount of amino acids in leaves of *Halodule uninervis* in different salinities of seawater (-MPa), Exposed for 3 days.

In rhizomes, although proline was still the predominant amino acid, the concentration of proline was not significantly different ( $P=0.36$ ,  $r^2 = 0.67$ ) as shown in Figure (2) with increasing salinity or osmotic potential of seawater (Table. 3).

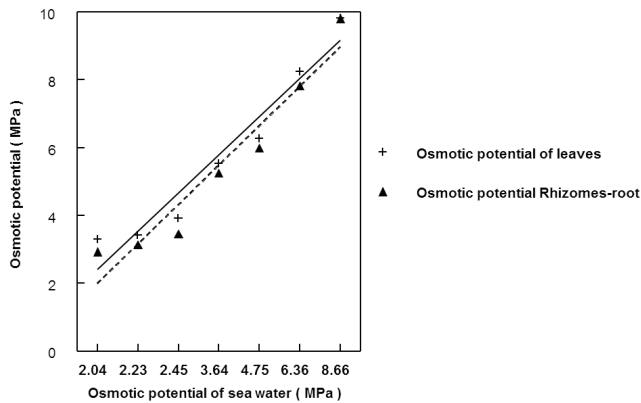


**Fig. 2.** Effect of salinity on the accumulation of free proline in *H. uninervis* leaf and rhizomes-root tissues. (MPa is negative)

**Table 3.** The production of proline and total amino acids in rhizomes/roots of *Halodule uninervis* in different salinities of seawater.

Osmotic Potential of Sea water	Osmotic Potential of rhizome/roots	Proline	Total amino acids
(-MPa) ( $\pm$ Standard deviation)		( $\mu\text{mol g}^{-1}$ fresh wt.) ( $\pm$ Standard error of means)	
2.04 ( $\pm$ 0.03)	2.94 ( $\pm$ 0.09)	0.621 ( $\pm$ 0.10)	1.465 ( $\pm$ 0.15)
2.23 ( $\pm$ 0.01)	3.15 ( $\pm$ 0.03)	0.764 ( $\pm$ 0.06)	1.674 ( $\pm$ 0.17)
2.45 ( $\pm$ 0.02)	3.46 ( $\pm$ 0.02)	0.761 ( $\pm$ 0.18)	2.330 ( $\pm$ 0.25)
3.64 ( $\pm$ 0.02)	5.26 ( $\pm$ 0.18)	1.105 ( $\pm$ 0.20)	2.391 ( $\pm$ 0.24)
4.75 ( $\pm$ 0.03)	6.00 ( $\pm$ 0.11)	0.974 ( $\pm$ 0.17)	1.345 ( $\pm$ 0.30)
6.36 ( $\pm$ 0.01)	7.83 ( $\pm$ 0.24)	1.066 ( $\pm$ 0.43)	1.428 ( $\pm$ 0.35)
8.66 ( $\pm$ 0.01)	> 9.33	0.999 ( $\pm$ 0.12)	1.308 ( $\pm$ 0.35)

There was a linear relationship between osmotic potentials of seawater and that of leaf and rhizome tissues ( $r^2=0.94$ ) (Figure 3).



**Fig. 3.** The effect of salinity on the osmotic potential of leaves and rhizomes-root tissues of *Halodule uninervis*. (MPa is negative)

Decreasing osmotic potential immersed in such waters resulted in significant increase in the osmotic potential in both the leaves and the rhizomes. The osmotic potential in both leaves and rhizomes was not significantly different, ( $P = 0.42$ ); (Fig. 3).

#### 4.5. Inorganic ions in the leaves and rhizome/roots of *H. uninervis*

The concentrations of sodium, chloride and potassium ions were relatively the same in leaf and in the rhizome/root tissues from backshore and foreshore plants (Table 4), but the total amount of inorganic ions accumulated in plant tissues was higher in leaf tissues than in the rhizome/root tissues.

**Table 4.** Concentrations of different inorganic ions in the leaves and root/rhizomes of *Halodule uninervis* tissues.

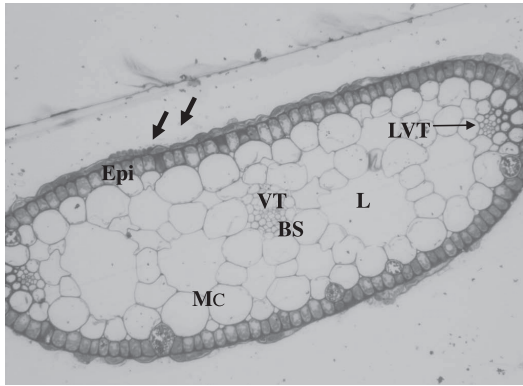
Year	Sample identification	Site of collection <sup>^</sup>	mmol g <sup>-1</sup> fresh wt. (±Standard error of means)				Total Inorganic ions
			Mg <sup>2+</sup>	Na+	K+	Cl	
2004	leaves	Site A	4.72(±0.6)	106.27(±12.8)	23.60(±3.0)	105.92(±16.7)	240.51
		Site B	3.74(±0.6)	70.55 (±8.3)	18.57(±4.8)	67.67(±8.2)	160.53
	Rhizome-roots	Site A	1.99(±0.7)	34.22 (±3.2)	8.81 (±1.2)	33.37(±6.2)	78.39
		Site B	2.92(±1.0)	39.21 (3.1)	11.83(±2.1)	37.77(±2.6)	91.73
2005	leaves	Site A	1.438(±1.3)	125.88(±11.3)	19.44(±1.8)	110.73 (±8.5)	257.48
		Site B	1.468(±0.6)	152.20(±38.0)	27.81(±3.9)	136.83 (±4.3)	318.32
	Rhizome-roots	Site A	0.510(±0.3)	43.21 (±3.2)	7.75 (±0.8)	39.15 (±2.1)	90.62
		Site B	0.638(±0.2)	47.45 (±3.7)	9.32 (±1.6)	44.09 (±3.9)	101.50

<sup>^</sup>:Represents sampling areas in the intertidal zone at Doha. Site A represent sampling areas at the backshore of intertidal zone and site B represents areas at the foreshore.

#### 4.6. Histological study

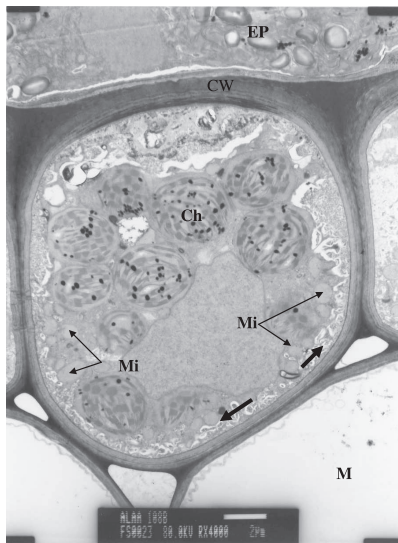
Leaf and rhizome tissues exposed to a range of salinities in modified seawater (-3.64 - -8.66 MPa) for 3 days were examined under a light microscope and TEM for changes in structural integrity. Both leaf and rhizome samples in control (-3.64 MPa) maintained the structural integrity of their tissues, with well defined epidermal, ground and vascular tissues (Figures 4), and had thick epidermal cell

walls and numerous chloroplasts. The larger thin-walled and undifferentiated mesophyll cells had very few or no chloroplasts and were highly vacuolated. The mesophyll layer also had a number of air canals (lacunae) and the cells at the leaf margins had relatively thicker walls than the other cells. The leaves have three longitudinal veins, a mid-vein with a lateral vein on either side of it.

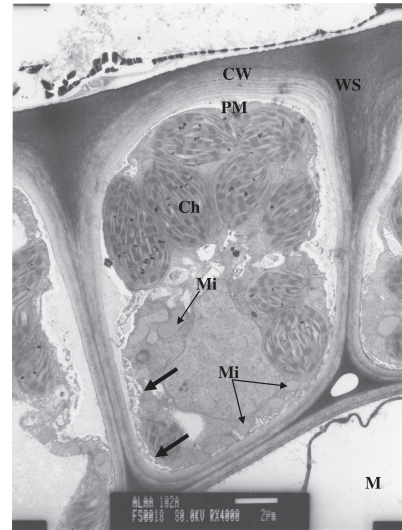


**Fig. 4.** Light micrograph of leaf blades of *Halodule uninervis* exposed to control seawater (-3.64 MPa) for 3 days. Section through entire leaf (20x). Epidermal (Epi), mesophyll cells (MC) and vascular tissues (VT) median vein surrounded by bundle sheath (BS); lateral vein (LVT), lacunae (L); short thick arrows point to epiphytes.

When treated with modified seawater (-3.82 MPa) of salinity treatment, the ultrastructure of the leaf blade epidermal cells showed a highly invaginated plasmalemma and an extracellular cytoplasmic zone with fibrillar material between it and the cell wall. Associated with the invaginated plasmalemma were numerous mitochondria (Figure 5). The chloroplasts, which were slightly circular in shape and had more plastoglobuli and well-developed grana, remained undamaged in the local seawater. With decreasing osmotic potential of the seawater, the epidermal cells walls still appeared intact. However, the chloroplast appeared spindle shaped with less plastoglobuli than in the control sample, and possessed deformed compressed grana (plate not shown). The plasmalemma towards the outer epidermal cell wall was less convoluted (Figure 6).

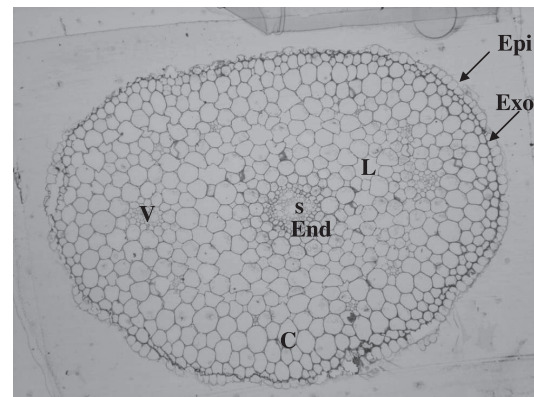


**Fig. 5.** Electron micrograph of *Halodule uninervis* leaf blade epidermal cells exposed to control seawater (-3.64 MPa) for 3 days. Cells with thick outer tangential wall covered by cell wall (CW), highly invaginated plasmalemma (thick arrows), chloroplasts (Ch), mitochondria (Mi) pointed out by thin arrows, mesophyll cell (M); epiphytes (EP).



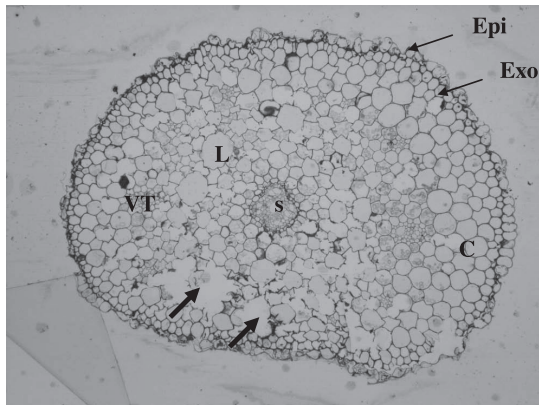
**Fig. 6.** Electron micrograph of leaf blade epidermal cells of *Halodule uninervis* exposed to seawater (-3.82 MPa) for 3 days. Epidermal cells are covered by thick outer tangential wall cell wall (CW), smooth or less invaginated plasmalemma (PM), highly invaginated plasmalemma (pointed out by thick arrows), chloroplasts (Ch), mitochondria (Mi), mesophyll (M), wall striations (WS).

Rhizomes in control seawater (-3.64 MPa) showed undamaged epidermal layer, exodermis, cortex, endodermis and vascular tissue (Figure 7).

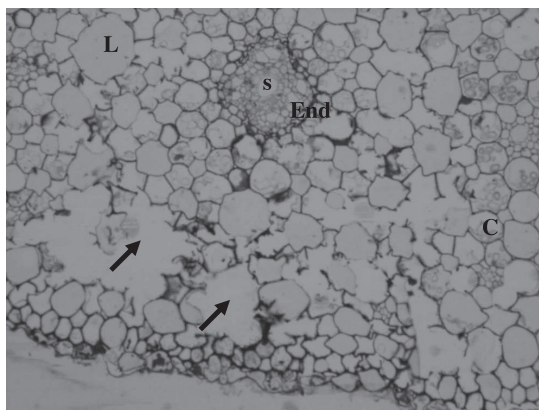


**Fig. 7.** A light micrograph of the rhizome of *Halodule uninervis* exposed to control sea water (-3.64 MPa) for 3 days. Cross section of an entire rhizome (10x) showing the epidermis (Epi), exodermis (Exo), endodermal (End), vascular stele (s), vascular tissue (V) and a rather uniform cortical tissue (C) and lacunae (L).

Rhizome tissues exposed to seawater with osmotic potential  $\leq -6.36$  MPa or 30% or more increase salinity showed tissue damage in four significant ways. Firstly, large segments of the epidermal layer appeared sloughed off. Secondly, the exodermis with its thicker outer walls stained more deeply, and segments of it were destroyed. Thirdly, the destruction of the cortical parenchyma cells resulted in the formation of disorganized and irregular shaped intercellular spaces, and fourthly, the endodermis, like the exodermis, lost its structural integrity (Figures 8a & b osmotic potential = -8.07 MPa).



(a)



(b)

**Fig. 8.** A light micrograph of the rhizome of *Halodule uninervis* exposed to seawater (-8.07 MPa) for 3 days. A cross section of an entire rhizome (10x) (a). A portion of the damaged epidermis and endodermis (b). The Epidermis is indicated by the letters (Epi), exodermis (Exo), endodermal (End) vascular stele (s), vascular tissue (VT) and cortical tissue (C) and lacunae (L). Thick arrows point to destruction of cortical cells.

## 5. Discussion

Meadows of *H. uninervis* in Doha, Kuwait Bay, experienced fluctuations in salinity at low tides and in the intertidal pools of water in summer on a daily basis. The highest seawater temperature and salinity were 39.5°C and 41.0‰, and the minimum values were 31.7°C and 33.0‰, respectively. Thus, *H. uninervis* in this area does not appear to be stenohaline or is not restricted to narrow limits of salinity (den Hartog, 1970) but may be euryhaline. The mean salinity of seawater at the intertidal zone was 38.03‰. The average salinity of seawater is about 34.3‰ with an osmotic potential of -2.54 MPa (Barnes, 1954). The seawater in Doha area, Kuwait Bay could be considered oceanic to hypersaline with the osmotic potential of the sea and sediment waters of -3.33 and -3.24 MPa, respectively. If *H. uninervis* is to survive in hypersaline water, it is apparent that the

plants must maintain water potentials equal to or greater than -3.24 MPa. The osmotic potentials of the leaf and rhizome/root tissues were greater than those of the sea and sediment waters in the meadows. It has been reported that *H. uninervis* has the broadest temperature and salinity tolerance among all seagrass (Masini *et al.*, 2002). Thus the plant can withstand very saline environment.

The relative water content of the mature leaves was also below 45%, which implies increase in respiration, proline and abscisic acid accumulation, since the water potential is less than -1.5 MPa (González & González, 2001). Analysis of leaf samples from meadows showed the highest accumulation of proline was between the end of June through July (the hottest months in Kuwait). There was a high correlation between proline accumulation and the progression of summer at both sampling sites.

The ability of *H. uninervis* to grow in seawater at Doha in water of salinity averaging 38.03‰ in this study showed its adaptive or osmoregulatory capacity in hypersaline environment. Osmoregulation to salinity in this study appeared to be related to the accumulation of amino acids, particularly proline that maintains osmotic balance in a number of plants including seagrasses. This is as reported by Stewart & Le, (1974), Brock (1981), Adams & Bates (1994). Generally, proline functions in osmotic adjustment as a stabilizer of enzymes and membranes (Yancy & Somero, 1980; Smirnov & Stewart, 1985; Smirnov, 1998), as a scavenger of radicals (Papageorgiou & Muarar, 1995) and as a sink for energy (Saradhi & Saradhi, 1991). Proline was the major component of total amino acids accumulated in both leaf and rhizome/root tissues from either meadows or the laboratory study samples. In both backshore and foreshore meadow samples, proline levels were lowest in May and highest at the end of June through July, when the biomass accumulation was optimum. In the laboratory study proline constituted 42 - 76% of total amino acids in leaves in a range of osmolalities of seawater. There was also a high correlation between proline concentration in the tissues of *H. uninervis* and the different osmolalities of seawater. The proline accumulated was not a pathological consequence of salinity stress, because no tissue damage symptoms were observed in osmolalities less than or equal to -6.36 MPa. All the samples used were healthy plants from meadows that were flourishing; thus the proline produced was not from impaired protein synthesis. The increase in proline in leaves with decreasing osmolality of seawater is related to an adaptive response to salinity (Taylor, 1996; Hasegawa *et al.*, 2000).



The concentration of proline in leaves was generally greater than that in rhizome/root samples from both meadows and laboratory study. This concurs with the observation of halophytes (Stewart & Lee, 1974; Briens & Larher, 1982).

In the local seawater (salinity 38.03‰), anatomical studies of *H. uninervis* leaves showed the presence of numerous chloroplasts, plastoglobuli and an invaginated plasmalemma with numerous mitochondria present in the epidermal cells. Barnabas & Kasavan (1983) documented similar ultrastructural features of *H. uninervis*. Ultrastructurally, the epidermal cells resemble transfer cells (Esau, 1977), and a number of researchers have referred to them as such (Iyer & Barnabas, 1993; Jagels, 1983). It appears that one of the functions of the epidermal cells is osmoregulation. The epidermal cells of other seagrasses with similar ultrastructure have been implicated or associated with osmotic adjustment (Iyer & Barnabas, 1993; Jagels 1983). In this study, decreasing osmotic potential of seawater (below 6.36 MPa) appeared to damage the grana, reduced the number of plastoglobuli and reduced the extent of invagination of the plasmalemma towards the outer epidermal wall; but the entire cells were still intact. This observation is not in agreement with that of Jagels & Barnabas (1989), who associated high salinity (24 ‰) with the development of more extensive invaginated plasmalemma-mitochondria system in *Ruppia maritima*. The difference in these observations may be due to the fact that the highest salinity used by Jagels & Barnabas (1989), was 24‰ on a different seagrass, whereas in this study salinities in excess of 38‰ were used.

The concentration of inorganic ions in the tissues of *H. uninervis* was higher in leaf tissues than in the rhizome/root tissues. *H. uninervis* may have the ability to accumulate these ions in its leaves to overcome the external hypersaline osmotic potential. On the other hand, uptake of ions without a mechanism to excrete them could prove deleterious to tissues.

This study showed that proline accumulation correlated with a range of seawater osmolalities in the laboratory study and field samples from meadows that experience a range of salinities. *H. uninervis* appeared to accumulate proline as an adaptive response to salinity stress. The role of the proline could probably be: (1) to protect against salinity stress by stabilizing enzymes and membranes i.e. maintaining membrane integrity and chloroplast structure (Van Rensburg *et al.*, 1993). In this study, we also observed

the preservation of the invagination of plasmalemma and the grana in the chloroplasts with decreasing osmolality  $\leq -6.36$  MPa and (2) In the mitochondria, proline pools supply a reducing potential for mitochondria and therefore contribute to energy supply for resumed growth. Analysis of meadow samples probably reflected this by the initial low levels of proline early in the growing season in May and the maximum accumulation in July and August, when growth had ceased.

The osmotic potential and proline accumulation in *H. uninervis* tissues depended on the osmolality of the seawater. This study showed that there was not only an increase in osmolytes (amino acids and proline) associated with increase in salinity but also a high correlation between amino acids, proline accumulation and the range of osmolalities of seawater. This suggests that one of the responses of *H. uninervis* to salinity stress involved amino acid synthesis that acts as an osmoprotectant, or in osmotic adjustment. These results are consistent with some previous studies which observed similar responses in seagrasses exposed to hypersaline conditions (Torquemada & Lizaso, 2006; Gacia *et al.*, 2007; Koch *et al.*, 2007a,b; Pagès *et al.*, 2010; Khalafallah *et al.*, 2013). Although, leaf tissues appeared well protected within the range of osmolalities used in this study, rhizome/root tissues were damaged at osmolalities  $\leq -6.36$  MPa. Leaves are probably better protected because they may have more than one mechanism to osmotically adjust to salinity (Barnabas & Kasavan, 1983; Iyer & Barnabas, 1993).

## 6. Conclusion

*H. uninervis* from the Kuwaiti coast can tolerate a wide range of salinity fluctuation through biochemical and anatomical responses. These responses include the production of higher proline concentration and higher inorganic ions (e.g.  $\text{Na}^+$ ,  $\text{Cl}^-$ ,  $\text{K}^+$ ), thicker tangential cell walls in epidermal cells and increased invagination of plasmalemma. There was a direct correlation between both the increase in the atmospheric temperature and salinity with the accumulation of proline and total amino acids. Leaf tissues were found to be more tolerant to salinity than root and rhizome tissues. The biochemical and structural responses exhibited by *H. uninervis* help in the protection against salinity stress and therefore, help these plants to survive under high salinity conditions such as found in the Doha coastal waters.

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## استجابة نجيل البحر *Halodule uninervis* التركيبية والكيميائية الحيوية للتغيرات في ملوحة مياه الدوحة في الكويت

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### خلاصة

تم دراسة العلاقة بين ملوحة مياه شاطئ الدوحة في جون الكويت وتأثيرها على الجهد التناضحي والأحماض الأمينية ومستوى تراكم البرولين كمحصن ضد التناضح في أنسجة نجيل البحر *Halodule uninervis* من خلال دراسة مختبرية. تم في الدراسة تحديد العلاقة بين الجهد التناضحي والأحماض الأمينية وزيادة تركيز البرولين في أنسجة أوراق وجذور هذا النجيل البحري ومدى علاقتها بمستوى تناضحية ماء البحر. وصل متوسط ملوحة ماء البحر في منطقة المد جزرية إلى 38.03‰ بينما وصل محتوى الماء النسبي في الأوراق المكتملة النمو إلى أقل من 45%. تبين وجود علاقة قوية بين الملوحة والجهد التناضحي لخلايا أنسجة *H. uninervis*. أظهرت النتائج أن محتوى الأحماض الأمينية في عينات الأوراق القريبة من الشاطئ هي أعلى من نظيراتها في العينات البعيدة عن الشاطئ. كما أظهرت النتائج أن البرولين كان الحمض الأميني الأكثر وفرة حيث وصلت نسبته في كل من أنسجة الأوراق والجذور في منتصف الصيف إلى ما بين 41-51% يتبعه كلا من الغلوتامين والألانين بنسب أقل. أظهرت الدراسة التشريحية تكيفا في التراكم الدقيقة للخلايا مثل تغلظ الجدران العرضية للخلايا في خلايا البشرة للأوراق وانبعاج كبير للغشاء السيتوبلازمي وتعدد البلاستيدات الخضراء والحبيبات الكروية، كذلك تعدد الميتوكوندريا الملازمه للغشاء السيتوبلازمي في خلايا البشرة وهو الامر الذي يرتبط بزيادة الملوحة. ومن الناحية الفسيولوجية، فإن مقاومة الملوحة في *H. uninervis* كانت مرتبطة بتراكم المواد المذابة المتوافقة واتضح ان الحد الأدنى لملوحة ماء البحر التي يبدأ عندها التلف يعادل 6.36- ميجا باسكال لأنسجة الأوراق و 8.06- للجذور.

كلمات البحث: نجيل البحر، الملوحة، تنظيم التناضح، البرولين.