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Effect of environmental parameters on the content and sugar composition of sulfated polysaccharides in some tropical seagrass

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ABSTRACT

Seagrasses are a paraphyletic group of marine angiosperms that evolved three to four times from land plants and returned to the sea. *Halophila ovalis, Thalassia hemprichii* and *Enhalus acoroides* (Hydrocharitaceae) are species that can occur in wide salinity ranges. Sulfated polysaccharides (SPs) comprise a complex group of macromolecules with many critical biological functions. We assume that SP may play a role in salt tolerance in seagrass. In this study, three seagrass species collected in both rainy and dry seasons from the fields were analyzed to determine the total SP contents and different functional groups of SP. Quantification of total SP was done by photometric assays. High-performance anion-exchange chromatography with Pulsed Electrochemical Detection (HPAEC) determined different functional groups of SPs. The results indicated higher total SP contents in seagrass are present in plants at higher salinities and environmental temperatures. The percent of functional groups of SPs are present in the following order: glucose > galactose > arabinose > mannose > rhamnose > fucose. The order is not different between the two seasons.

Keywords: Functional groups, salinity, seagrass, sulfated polysaccharides.

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INTRODUCTION

Seagrasses are higher plants that formed from a paraphyletic group of marine angiosperms. Les et al., [1] reported that they evolved more than two times from land plants back to the sea. Saline environments like estuaries and the coastal zone are the main habitats for seagrasses. Sulfur-containing compounds and proteins play a leading role in the adaptation to the marine environment. In marine plants, it is known that similar proteomic adaptation mechanisms and similar metabolism were formed [2]. The prominent roles, such as antimicrobial, or antimacrofouling activity of several sulfur-containing compounds, seagrass enzymes, and sulfur-containing secondary metabolites were reported [3, 4]. Based on the complete genome sequence from two seagrass species, including *Halophila ovalis* (R.Br.) Hook. f. [5], *Zostera marina* L. [6], and algae *Ectocarpus siliculosus* (Dillwyn) Lyngbye [7], comparative analysis became more meaningful.

Sulfated polysaccharides (SPs) are a complex group of macromolecules that reveal several esential biological functions [8, 9]. The monosaccharide composition of sulfated polysaccharides is glucose, galactose, arabinose, rhamnose, mannose, and fucose. These anionic polymers are widely found in nature, occurring in many organisms. SP is found in vertebrates, invertebrates [10, 11], and seaweeds [12]. So far, SPs were also found in some salt-tolerant plants, halophytic angiosperms [13], and freshwater plants [14]. Carragenans and agarans are SP in the red seaweed, fucan and fucoidan are SP in the brown seaweed, and heteropolysaccharides are usually found in green algae. The previous study of Aquino et al., [15] indicated that different halophytic aquatic plants such as marine angiosperms (*Halodule wrightii* Ascherson, *Halophila decipiens* Ostenfeld and *Ruppia maritima* L.), and other mangrove species *Rhizophora mangle* L., *Avicennia schaueriana* Stapf & Leechm. ex Moldenke) and *Acrostichum aureum* L. showed higher SP concentrations exposed to higher salinities. Therefore, SPs content is positively correlated with the salinity of the environment. This effect has yet to be found in terrestrial glycophyte angiosperms such as *Oryza sativa* L., *Zea mays* L., and *Phaseolus vulgaris* L. However, the concentration of carboxylate polysaccharides in *O. sativa* was increased, but induction of the biosynthesis of SPs was not observed [15].

SPs of examined plant species are built up differently. For example, galactose units are the main SP of seagrass species. SP of mangrove species mainly contains arabinose and galactose, and SPs of the pteridophyte contain glucose units [15]. Dantas-Santos et al., [14] revealed that *E. crassipes* contained the main monosaccharides, including galactose, glucose, and arabinose, and they can be comparable with the monosaccharide composition of green algae. Papenbrock [2] reported that the biosynthesis of SPs probably starts with a precursor of lower molecular weight and degree of sulfation, suggesting that both glycosyltransferases and sulfotransferases may function simultaneously during the biosynthesis of SPs, at least in *R. maritima*. Moreover, the green algae (ancestor of higher plants) showed almost units of SPs found in all investigated halophytic aquatic plants [16]. Aquino et al., [15] suggested that SPs production may be conserved throughout the plant kingdom from green algae. Therefore, activating and inhibiting some genes, such as glycosyltransferase, may alter the composition of SPs among the different phyla.

SPs were found in several aquatic species of invertebrates and halophytic aquatic plants, showing a positive correlation between SPs and water salinity [15, 17]. The production of SPs in aquatic species of invertebrates and halophytic aquatic plants is correlated with environmental salinity. Marine plants can adapt in wide salinity ranges may be suitable species to use to analyze the function of SPs. By comparing salt tolerance and adaptations to low salinities in a freshwater strain and a marine strain of *E. siliculosus*, Dittami et al., [18] reported that this alga showed profound but reversible transcriptomic, physiological and morphological changes when transferred to seawater. Hence, with the colonization of freshwater, genomic alterations of *E. siliculosus* have appeared that composed permanent changes in the metabolite profiles among SPs to stabilize the transition. In summary, the current knowledge suggests

evidence that the presence of SPs in plants is an adaptation to the high salt environments that have been conserved during evolution of plants from marine green algae. Because seagrasses have evolved from land plants and moved back to the sea, they are a unique group of plants to further test this role of SPs in salinity tolerance. Three seagrass species, including *Halophila ovalis*, *Enhalus acoroides*, and *Thalassia hemprichii* (Hydrocharitaceae family), have been shown to verify this finding further and examine the abundance of SPs as well as monosaccharide composition in these three tropical seagrass species of the Hydrocharitaceae, we collected *Halophila ovalis*, *Enhalus acoroides*, and *Thalassia hemprichii* along the coast of Vietnam at different habitats and seasons characterized by four main environmental parameters, including salinity, temperature, turbidity, and pH.

MATERIALS AND METHODS

Seagrass collection

Seagrass collections were carried out from three different sites along the coast of Khanh Hoa province, Vietnam (Figure 1). Site 1 is Thuy Trieu lagoon (TT: 109°11'E; 12°02'N), located in the southern part of the province and is characterized as a shallow lagoon. Site 2 is Cam Ranh bay (CR: 109°11'E; 11°56'N) and is connected to Thuy Trieu lagoon in the north part and the East Vietnam Sea in the east part. Site 3 is Song Lo (SL: 109°09'E; 11°53'N) and is in the open sea. Seagrass plants were collected in the dry (April 2020) and rainy (December 2021) seasons. Based on their natural distribution, *Enhalus acoroides* were collected at all sites, whereas *Halophila ovalis* and *Thalassia hemprichii* were collected at sites 1 and 2. Three different samples (0.5–1.0 kg/sample) of each species were collected at each sampling site. The distance among sampling points was 10 m. At each site, the *in situ* information of environmental parameters, including water temperature (°C), salinity (‰), and turbidity (NTU), were measured by using YSI ProDSS Multiparameter (Xylem Analytics, Weilheim, Germany). A total of 21 seagrass samples (six samples of *Halophila ovalis*, six samples of *Thalassia hemprichii*, and nine samples of *Enhalus acoroides*) were collected per season. The samples were washed with seawater in the field to remove epiphytes and debris attached to the plants and stored in stable and dark conditions in a cooler until brought to the laboratory within a day. In this study, we concentrated only on the leaf tissue from the three species. In the laboratory, leaf tissue was re-washed with de-ionized water to remove seawater and dried at room temperature. The tissue was ground in liquid nitrogen by mortar and pestle before the analysis.

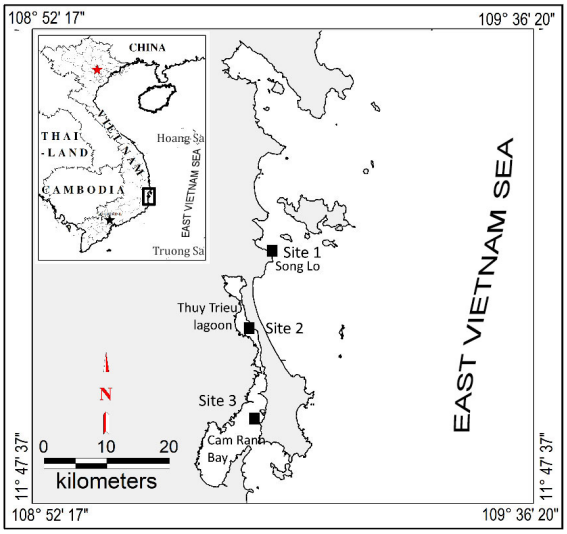

Figure 1. The map of Khanh Hoa coast showing sampling sites (■) at Cam Ranh bay (CPN), Thuy Trieu lagoon (TT), and Nha Trang bay (SL). *Halophila ovalis* was collected at CPN and TT, *T. hemprichii* was collected at CPN and SL, and *E. acoroides* were collected from all three sites

Extraction and quantification of SP from the field collections

Briefly, 10 g of pulverized sample were incubated two times with ethanol to remove pigments and lipids (50 mL per g of dry tissue) following the protocol of Aquino et al., [15]. The ethanol solution was removed and then the residue was used for protein digestion with 1 g

of papain (Merck, Darmstadt, Germany) in 400 mL, 0.1 M sodium acetate (pH 6.0) with 5 mM ethylenediamine tetra-acetic acid, and 5 mM cysteine. After incubating for 24 h at 60°C, the mixture was centrifuged (2,500 × g for 30 min), and the supernatant was subjected to ethanol precipitation. The pellet was used for a second extraction procedure, and the SP was pooled. The procedure was repeated a third time. The SP in solution (supernatant) was precipitated with 800 mL absolute ethanol (2 volumes) at 4°C for 24 h. The precipitate was collected by centrifugation (2,500 × g for 30 min at 4°C), dried in a vacuum, and re-suspended in distilled water.

Sulfated polysaccharides were quantified using the toluidine blue assay using fucoidan as a standard. Polysaccharide pellets obtained from each extraction were dissolved in distilled water and diluted until polysaccharide concentrations were in the calibration curve range and could be calculated by direct extrapolation. Fucoidan standard from *Undaria pinnatifida* solutions (Merck, Darmstadt, Germany) was used from 0 to 10 μg in aliquots of 200 μL. Diluted samples were mixed with 1 mL of a 0.01 mg/mL solution of toluidine blue, and absorbance readings were taken at 620 nm after 10 min using a spectrophotometer (U-2900, Hitachi, Tokyo, Japan). Polysaccharide determinations of both standards and samples were made in triplicate and median values were used for calculations.

Determination of different functional groups of SP

The monosaccharide compositions of SP were determined by high-performance anion-exchange chromatography with pulsed electrochemical detection (HPAEC). 50 mg pulverized samples were hydrolyzed with 2 M trifluoroacetic acid (TFA) at 100°C for 6 h. The hydrolysate was dried under vacuum to remove acidic excess prior to direct analysis by ion chromatography on the Dionex ICS-6000 system with a guard MA1 (50 mm × 4) and an analytical column MA1 (250 mm × 4), AS-DV module, and detection by a pulsed amperometric detector. The operating conditions included the mobile phase NaOH 1 M with a flow rate of 0.4 mL/min for 30 min. A standard curve was plotted for different concentrations of glucose, galactose, rhamnose, arabinose, mannose, and fucose (Monosaccharides Kit, Merck, Darmstadt, Germany) and used for the determination of SP in the collected seagrass tissues.

Statistical analyses

Within species, the differences in SP content of seagrass collected at different locations were analyzed using one-way ANOVA. The difference in SP content of each seagrass collected in the two seasons was also tested using a one-way ANOVA. Correlation (correlation coefficient, *r*) between SP contents in three seagrass species and the four environmental parameters recorded at the sites was performed by Pearson's correlation. The commercial statistics software package SPSS (version 26.0) for Windows was used for the statistical analysis. Redundancy analysis (RDA) was applied to find the relationships between SP content and environmental parameters using XLSTAT (Addinsoft, New York, USA).

RESULTS

Environmental parameters

The temperature values in three different dry season sites were not significantly different. They were 30.7, 29.8, and 30.4°C at sites 1, 2, and 3, respectively. In the rainy season, the temperatures at site 1, 2 and 3 were 26.4, 26.4, and 26.8°C, respectively. There were significant temperature differences between the two seasons. The pH values did not vary among sites or seasons; they ranged from 7.70–8.03. In the rainy season, the turbidity (NTU) at sites 1 and 2 (7.0 and 5.2 NTU, respectively) showed higher values than in the dry season (3.9 and 2.3 NTU, respectively). However, site 3 did not vary much between the two seasons (2.0 NTU in the dry season and 2.4 NTU in the rainy season). The values of salinities showed significant differences in all sites and ranged approximately 4‰ between the two seasons in the same site. Site 1 showed around 26‰ in the rainy season and around 30‰ in the dry season,

whereas site 2 showed around 28‰ in the rainy season and around 32‰ in the dry season. In the same way, 30 and 34‰ are the values of salinities at site 3, respectively (Table 1).

Table 1. Variation of average salinity (‰), temperature (°C), turbidity (NTU) and pH in dry and rainy seasons at sampling sites

	Dry season			Rainy season		
	Site 1	Site 2	Site 3	Site 1	Site 2	Site 3
Salinity	26	28	30	30	32	34
Temperature	30.7	29.8	30.1	26.4	26.3	26.8
Turbidity	3.9	2.3	2.1	7.0	5.2	2.4
pH	8.03	8	8.03	7.7	7.6	8

Sulfated polysaccharides contents in different species and sites

The analysis results revealed that SPs contents vary among species and sites. For *H. ovalis*, the average SPs content collected in the rainy season was 10.90 µg/mg DW at site 1 (S‰ = 26) and lower than at site 2 (S‰ = 28) with 12.01 µg/mgDW. There were no significant differences in SP contents from this species between the two sites. In the dry season, however, the SP content from this species was higher, with 21.70 and 23.03 µg/mg DW at sites 1 and 2, respectively, and showed no significant differences between the two sites (Figure 2A). SP contents between the two seasons were significantly different. The average SP contents of *T. hemprichii* also showed similar values between site 1 (10.01 µg/mgDW) and site 2 (10.43 µg/mgDW) in the rainy season. However, SP contents of *T. hemprichii* in site 2 (16.52 µg/mgDW) were much higher than in site 1 (10.69 µg/mgDW) in the dry season (Figure 2B). Significant differences in SP contents of *T. hemprichii* were also found between the two sites for *E. acoroides.* The average SP contents showed lower a concentration of 8.34 µg/mgDW at site 1 than at site 2 (9.53 µg/mgDW) and site 3 (9.48 µg/mgDW) in the rainy season. Statistical analyses also revealed no significant differences in SP contents of *E. acoroides* among sites. In the dry season, the average SP contents of *E. acoroides* were 12.11, 19.41, and 21.75 µg/mgDW at sites 1, 2, and 3, respectively, and were higher than those values in the rainy season for the three sites. There were no significant differences in SP contents between samples collected at sites 2 and 3 (Figure 2C). Significant differences were also found between the two seasons compared with data in the rainy season.

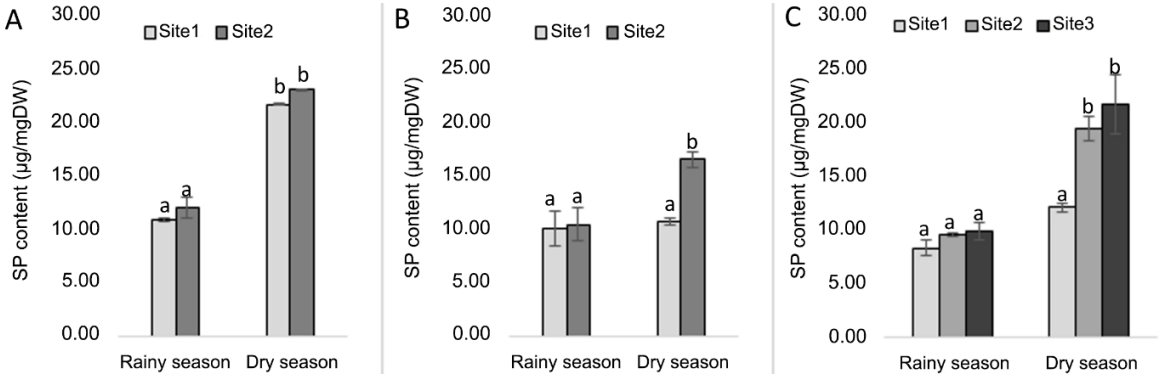

Figure 2. The average SPs contents in three species of the Hydrocharitaceae collected in different seasons and sites. (A) *Halophila ovalis*; (B) *Thalassia hemprichii*; (C) *Enhalus acoroides*. Different letters (a, b) printed within the same column show significantly different means of observed data ($p < 0.05$). Data are presented in mean ± SD ($n = 3$)

The correlation analyses showed that the SPs contents of both species *Halophila ovalis* and *Enhalus acoroides* were positively related to temperature and salinity but were negatively correlated with turbidity. The SPs contents of *T. hemprichii* were positively related to salinity and were negatively correlated to turbidity. However, the SPs contents of *T. hemprichii* did not correlate to temperature. There was no correlation between SP contents and pH for the three species (Table 2). Redundancy analysis shows that the SPs contents of *E. acoroides* and *T. hemprichii* were similar in the upper part of the graph as they were characterized by salinity and temperature. Turbidity in the lower part of the graph showed a negative factor to SPs content (Figure 3).

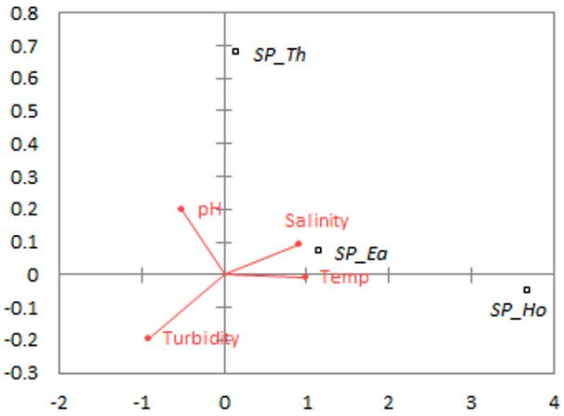

Figure 3. Two-dimensional plot displaying the first two axes of a redundancy analysis. SP: sulfated polysaccharide; Ea: *Enhalus acoroides*; Ho: *Halophila ovalis*; Th: *Thalassia hemprichii*

Table 2. Pearson's correlation between environmental parameters and SP content in three seagrass species

	Salinity (‰)	Temperature (^{o}C)	Turbidity (NTU)	pH
H. ovalis	+	+	-	0
T. hemprichii	+	0	-	0
E. acoroides	+	+	-	0

Notes: +/-: positive/negative correlations significant at $p = 0.001$; 0: no correlation.

Different functional groups of SPs

The monosaccharide composition showed a slight differed among the three seagrass species collected in different salinities. Glucose was the most abundant sugar (38 ± 2.9% in *H. ovalis*, 46 ± 2.9% in *E. acoroides*, and 52 ± 3.7% in *T. hemprichii*). Galactose in *T. hemprichii* (21.75 ± 1.3%) was slightly higher than in *H. ovalis* (18.5 ± 0.6%) and *E. acoroides* 17.67 ± 0.82%). However, arabinose in *T. hemprichii* (13%) was lower than in *E. acoroides* (24.2 ± 3.8%) and *H. ovalis* (20.75 ± 5.6%). The monosaccharide amounts of rhamnose (*T. hemprichii*: 6.75%; *H. ovalis*: 13.75%; *E. acoroides*: 5.35%), mannose (< 10%) and fucose were lower (< 3%) (Table 3, Figure 4).

Table 3. Monosaccharide composition of sulfated polysaccharides from three seagrass species collected in the fields

	Species growing in the different salinities (‰)						
	H. ovalis		*T. hemprichii*		*E. acoroides*		
Neutral sugar (%)	26‰	28‰	28‰	32‰	26‰	28‰	32‰
Glucose	35–41	36–40	51–52	48–57	44–50	42–52	44
Galactose	18	19	22	20–23	16	16–18	17
Arabinose	14–23	19–27	13	13	23–31	22–26	21–22
Rhamnose	12–18	10–15	6–9	6	4–5	3.5–9	7
Mannose	5–9	2–9	6–7	3-6	4.5–5.5	4.1–6	8–9
Fucose	1–6	1–3	1	1	0.5	0.5–1	2

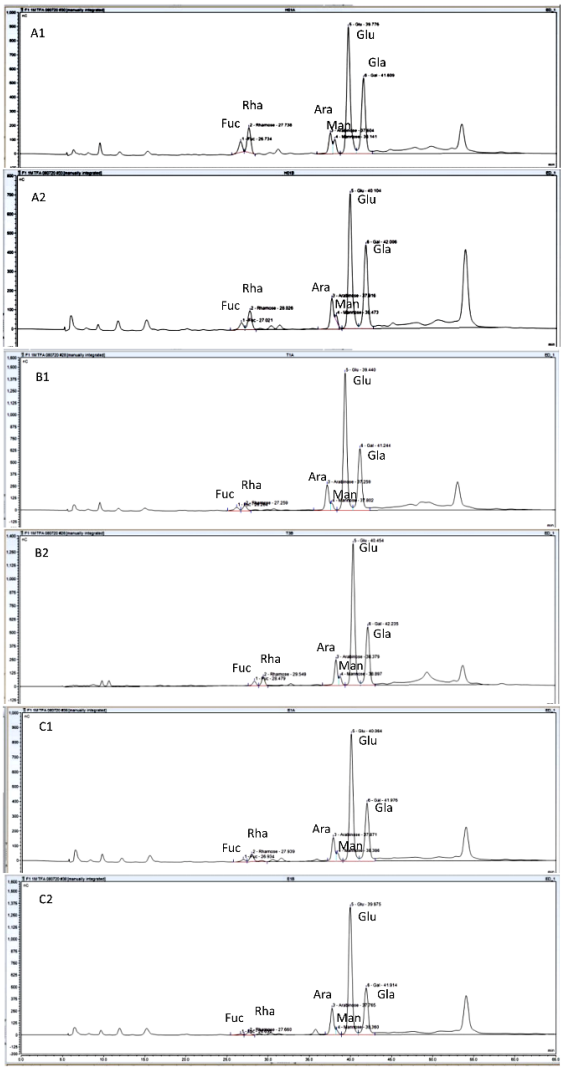

Figure 4. Chromatograms indicating peaks of each sugar in high- performance anion-exchange chromatography with pulsed electrochemical detection (HPAEC). A: *Halophila ovalis*; B: *Thalassia hemprichii*; C: *Enhalus acoroides*; 1: rainy season; 2: dry season

DISCUSSION

SP content varies among the species and salinity conditions. Our results showed that the SP contents in *H. ovalis* seemed slightly higher than in *T. hemprichii* and *E. acoroides* in both seasons. SP contents from all three species were higher at higher salinities than at lower salinities. Previously, Aquino et al., [13] proposed that the occurrence of SP in marine organisms may result from physiological adaptations of seagrass to the saline environment, and SP showed positively correlated with salinity. SP in the leaves of *Halodule wrightii* (Cymodoceaceae) collected from higher salinity (38‰) was 22.4 µg/mgDW, whereas this value was 12.2 µg/mgDW from the samples collected at 35‰. It was similar way from another species, *Ruppia maritima* (Ruppiaceae). Our results also reveal that the SP contents of *H. ovalis* (23.03 µg/mgDW at 32‰) were four times higher than was reported by Aquino et al., [15] from the sister species *H. decipiens* (5.15–7.69 µg/mgDW at 35‰). These results also reveal that SP from members of the Hydrocharitaeae was also positively correlated with a saline environment. For the red marine microalga, *Porphyridium purpureum* (Bory), Drew and Ross, Ferreira et al., [19] also found that higher amounts of SP were found at 32‰ than at 18‰. Bunson and Prathep [20] reported that salinity was the most relevant factor positively correlated to SP production in the red algae *Gracilaria tenuistipitata* Chang & Xia. In addition, salinity Eh, pH, and DO in the environment also positively correlated to SP production. However, other factors, including high nutrient availability, turbidity, and phytoplankton blooms, inhibited the production of SP in the red seaweed *Gracilaria* spp. in Venice lagoon (Italy) [21]. To better understand SP's biological function in salt tolerance, our following study will conduct more experiments on the correlation between SP content and salinity in the laboratory.

The monosaccharide composition showed a few variations in the different species. Glucose, galactose, and arabinose are the most abundant sugars, whereas rhamnose, mannose, and fucose showed much lower. Yuvaraj and Arul [22] reported that galactose contents in *Halophila ovalis* were the most abundant sugars, with 82.4%, and other sugars were lower than 8%. However, *Thalassodendron ciliatum* (Cymodoceaceae) collected from Egypt showed a high abundance of galacturonic acid (35%) and glucuronic acid (20%). Glucose and mannose were in equal percentages, 15%, while

arabinose was 10% [23]. The monosaccharide composition of another member of the Cymodoceaceae, *Cymodocea nodosa*, revealed that galactose (44.9%) and mannose (17.3%) were the main sugars [24]. The report of Silva et al., [25] indicated that galactose, glucose, and xylose were at the same molecular ratio as *Halodule wrightii* (Cymodoceaceae) from Brazil. Recently, in a study on *Zostera marina* (Zosteraceae), Pfeifer et al., [26] showed the roles of arabinogalactan proteins in adapting seagrasses to the marine environment. Galactose (43.1–50.8%) and arabinose (30.8–36.0%) were the main monosaccharides in three organs, including leaves, shoots, and roots. In the dead seagrass material of *Posidonia oceanica* (Posidoniaceae), most of the isolated polysaccharide moieties were composed of xylose-containing polysaccharides. Other plant cell wall polysaccharides and cell wall proteins were degraded and thus only present in very minor amounts. Concerning monosaccharide composition, glucose, xylose, and galactose were the main sugars [27]. The monosaccharide composition showed differences in different species and different organs within one species. On the other hand, there are several methods used for SP extraction. For example, papain-containing buffer at pH 6.0 [13], hot water after acetone and ethanol pre-extraction [24], sodium chloride solution (0.25 M) adjusted to pH 8.0 after depigmentation with acetone [25], 1% (w/v) aqueous ammonium oxalate with following pectinase treatment, aqueous extract and Yariv-precipitation after depigmentation with acetone [26], and others reviewed in Pfeifer and Classen [28]. Therefore, our subsequent studies will apply more protocols, including other members of Cymodoceaceae, such as *Cymodocea serrulata*, *C. rotundata*, and *Syringodium isoetifolium*.

Finally, this present study showed that SP content from three members of Hydrocharitaceae correlated positively with the concentration of salt and temperature in the environment, whereas turbidity correlated negatively with SP content. Glucose, arabinose, and galactose are the main sugars, supporting SP's role in salt tolerance from seagrasses. Our following study will investigate the gene expression of sulfotransferases involved in the sulfation of polysaccharides under different salinity and temperature treatments from some selected seagrass species.

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