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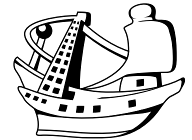

Seasonal variations of epiphytic flora, abscisic acid production and physiological response in the brown alga *Cystoseira foeniculacea* (Linnaeus) Greville

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Abstract: In seaweeds, growth and reproduction are affected by seasonal and environmental fluctuations during their life cycle and this requires physiological control. In order to provide insights into this questions, the influence of seasonal variations and epiphytic flora on abscisic acid production, proline biosynthesis, phenolic compounds accumulation, and activity of antioxidant enzymes in the brown alga *Cystoseira foeniculacea* were investigated. In summer, the highest eckol content was found in the upper and lower parts of the thallus. The highest catalase activity was measured during spring, whereas the lowest one was observed in summer. In contrast, the highest ascorbate peroxidase activity was significantly increased during spring and winter in lower parts of the thallus of *C. foeniculacea*. Similarly, the highest superoxide dismutase activity was measured during winter. Glutathion levels were 2- and 3-fold higher in spring compared to other seasons. The abscisic acid concentration in the lower and upper parts of the thallus showed differences between seasons. Overall, the results suggest that abscisic acid accumulation with catalase activity and proline production could be related to tolerance to increased seawater temperature during summer, while eckol accumulation might be related to epiphytic flora variations.

Résumé : *Variations saisonnières de la flore épiphyte, production d'acide abscissique et réponse physiologique chez l'algue brune* Cystoseira foeniculacea *(Linnaeus) Greville.* Chez les algues, la croissance et la reproduction sont affectées par les fluctuations saisonnières et environnementales au cours de leur cycle de vie, et ceci requiert un certain contrôle physiologique. Dans le but d'apporter des éléments de réponse à ces questions, nous avons étudié l'influence des variations saisonnières et de la flore épiphyte sur la production d'acide abscissique, la biosynthèse de la proline, l'accumulation de composés phénoliques et l'activité d'enzymes antioxydantes chez l'algue brune *Cystoseira foeniculacea*. Les plus hautes teneurs en eckols ont été observées dans les parties supérieures et inférieures du thalle. L'activité catalase la plus élevée a été mesurée au printemps dans les thalles supérieurs et inférieurs alors que la plus faible a été observée en été. En revanche, l'activité ascorbate peroxydase était significativement plus élevée au printemps et en hiver dans la partie inférieure des thalles de *C. foeniculacea*. De façon similaire, l'activité superoxyde dismutase plus élevée a été observée en hiver dans la partie inférieure du thalle. Le taux de glutathion était deux à trois fois plus élevé

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au printemps par rapport aux autres saisons de l'année. Les concentrations en acide abscissique dans les parties inférieures et supérieures du thalle ont présenté des différences selon les saisons. Nos résultats suggèrent que l'accumulation d'acide abscissique, l'activité catalase et la production de proline pourraient être liées à la tolérance aux hautes températures estivales, alors que l'accumulation d'eckols pourrait être liée aux variations de la flore épiphytique.

Keywords: Abscisic acid • Catalase • Superoxide dismutase • Ascorbate peroxidase • Proline • Phlorotannin • Brown algae

Introduction

Cystoseira C. Agardh, 1820 species constitute important elements of the Mediterranean coastal ecosystems. They are known to contain a large number of secondary metabolites (Amico et al., 1997), especially polyphenolic compounds. Phlorotannins, a type of polyphenols only found in brown algae, protect algae against herbivores and are involved in various biological activities (Kim et al., 2013). Previous studies reported that the phlorotannin production in brown algae varies spatially and temporally due to several biotic and abiotic factors (Pavia et al., 1997; Pavia & Brock, 2000). They have been associated with a variety of functions including preventing bacterial infections and protecting from the harmful effects of UV and high photosynthetically active radiations (Pavia et al*.,* 1997), grazing activities (Van Alstyne, 1988), and nutrient deficiency (Ilvessalo & Tuomi, 1989). However, there is little information known on the role of phlorotannins in the physiological response to seasonal variations.

Abscisic acid (ABA) is known to be synthetized by different algal groups, including Heterokontophyta, Chlorophyta, and Rhodophyta (Guajardo et al., 2016). Recent genomic analyses showed the presence in macroalgae of genes encoding zeaxanthin epimerase, 9-cis-epoxycarotenoid dioxygenase, xanthoxin dehydrogenase, ABA-insensitive 5-like proteins, one farnesylcysteine lyase, which are involved in ABA biosynthetic pathways and associated signal transduction mechanisms (Mikami et al., 2016; Sun et al., 2018). Some studies moreover suggested that in some algae, lunularic acid could play a role analogous of that of ABA (Tarakhovskaya et al., 2007).

In seedless plants, previous studies showed that the desiccation tolerance of the moss *Atrichum undulatum* (Hedw.) P. Beauv., 1805 by preventing the reduction of photosynthesis can be induced via exogenous ABA supply (Beckett et al., 2000). Dietz & Hartung (1999) suggested that ABA could have a positive effect on membrane function under extreme water regimes in lichens. Similarly, it has been suggested in macroalgae that ABA might play roles in algal development stage; such as the inhibition of the growth of sporophytes in Heterokontophyta (Nimura & Mizuta, 2002). Previous studies on Chlorophyta groups demonstrated that the endogenous ABA content is not only associated with growth stage, but can also be influenced by environmental stress (Guajardo et al., 2016). Some studies indeed showed that, like in vascular plants, ABA plays a role as a stress response hormone. Shimizu et al. (2018) demonstrated that ABA accumulation related with defence activities against oxidative burst in young sporophytes of *Saccharina japonica* (Areschoug) C.E.Lane, C.Mayes, Druehl & G.W.Saunders, 2006 (Phaeophyta, Laminariales). However, despite the growing number of studies on algal hormones, information on the role of ABA in response to seasonal variations of the environmental factors in macroalgae is still limited.

Reactive Oxygen species (ROS) are highly toxic and can greatly damage lipids, proteins, and nucleic acids and then inhibit plant and algae growth (Tang et al., 2007). Several enzymatic and non-enzymatic compounds are involved in responses to environmental stress in plants and macroalgae, such as the water-soluble ascorbate, glutathione, and the membrane-bound α-tocopherol (Asada, 1999). Glutathione is an important compound in photosynthetic organisms because it is a part of the ascorbate-glutathione cycle (Noctor & Foyer, 1998). The cycle of reduced (GSH) and oxidized (GSSG) glutathione has many important functions, such as the scavenging of ROS (Noctor & Foyer, 1998). In addition, enzymatic such as ascorbate peroxidase (APX), catalases (CAT) and superoxide dismutase (SOD) are well known for their involvement in ROS detoxification, notably in algae (Mallick & Mohn, 2000). Interestingly, it has been shown in Chlorophyta that their regulation by ABA can help coping with environmental stress (Yoshida et al., 2004).

The subject of the present work is to study the seasonal changes in the concentration of phlorotannins and ABA in *Cystoseira foeniculacea* (Linnaeus) Greville. We also assessed the activity of the ROS detoxifying enzymes CAT, APX, SOD, as well as the GSH and proline levels at the four seasons. This study thus aims at better understanding the relationship between seasonal environmental variations such as, salinity, temperature, dissolved oxygen levels, epiphyte covering and phlorotannin contents and antioxidative systems in the brown alga *C. foeniculacea*, as a representative species of near-shore Mediterranean ecosystems.

Materials and Methods

Sample collection and Water Quality

To limit possible habitat-derived differences, the study was carried out at one selected station. The sampling area was in the upper infralittoral zone (0-1 m depth) in Kusadasi on the Aegean Coast of Turkey, in a zone sheltered from high hydrodynamism and human activities (N 46°44.57-E 027°50.42). Three thalli were collected from the same canopy, stored in plastic ziplock bags and transfered to the laboratory in an styrobox. Samples were collected seasonally, in February, April, July, October 2013, from the same canopy to limit interindividual variability. The thalli were divided in two parts: the upper part of the thallus (“leaves”) and the lower part including the holdfast and the stipe. The epiphytes on the thalli were brushed with a toothbrush and analysed under a microscope. Epiphyte free thalli were then stored at -20˚C until further analysis.

For each sampling, the temperature, dissolved oxygen, conductivity, salinity and pH of the seawater were measured using a multi parameter probe (CyberScan 600, EUTECH Instruments). Probes were calibrated before each measurement.

Determination of the cell density in seawater

During each sampling session, 1 L of seawater was collected in a glass bottle and fixed with 4% formaldehyde solution. In the laboratory, the water samples were filtered through 15 μm filter to concentrate the cells. The filtrate was then resuspended in 1 mL filtered seawater and 10 μL were transferred to the Neubauer counting chamber for cell counting under a light microscope (Olympus CX31).

Phlorotannin extraction and HPLC analysis

The thalli of *C. foeniculacea* were freeze-dried at -60°C for 24 h in a lyophilizator (Edwards Modulyo, UK) equipped with a high vacuum pump (Edwards E2M2, UK). A volume of 500 μL hexane was added to 100 mg of dried thalli and centrifuged three times at 3 200 x *g* for 3 min. Each time the supernatant was discarded. After removing the hexane from the pellet, 5 mL of an acetone:water (7:3) mixture with acetic acid was added to the pellet and centrifuged at 3 200 x *g* for 6 min. This step was repeated three times. After each centrifugation the supernatant was collected in a tube. The acetone was removed by a rotary vacuum evaporation system at 40°C to yield the water fraction. The residual supernatant was dissolved in 1 mL distilled water and filtered with a PTFE filter (Merck) and used for HPLC analysis. HPLC analyses were performed with a HPLC system (Shimadzu UFLCXR) equipped with an auto-sampler (SIL-20A XR) and a diode array detector (SPD-M20A). A C_{18} (15 cm x 0.46 mm, 5 μm) column (Supelco Analytical, Sigma-Aldrich) was used for the separation. The mobile phase consisted of a 50%:50%, acetonitrile:0.1% formic acid aqueous solution. The flow rate was 0.2 mL min^{-1}, the column temperature was 25°C, and the pressure was 18 bar. The peaks were detected during the isocratic separation at 190 nm wavelength. Phloroglucinol and eckol (Merck, Germany) standarts were used for calibration of the HPLC analyses.

Reduced glutathione analysis

Glutathione quantitations were conducted according to Cakmak & Marschner (1992). A homogenate was prepared with 0.5 g of the thalli with 2.5 mL of 5% trichloroacetic acid (TCA). The precipitated protein were discarded by centrifugation and the supernatant (0.1 mL) was used for the estimation of GSH. The supernatant (0.1 mL) was made up to 1.0 mL with 0.2 M sodium phosphate buffer pH 8.0. Standard GSH solutions corresponding to concentrations ranging between 2 and 10 nM were also prepared. Then, a volume of 2 mL of freshly prepared 5,5-dithio-bis-2-nitrobenzoic acid (DTNB) solution was added and the optical density was measured in a spectrophotometer (Pharo 300, Merck) at 412 nm, after 10 min. The values were expressed as μmol GSH per g sample.

Proline analysis

The proline content was measured following the method of Bates et al. (1973) with some modification

in the extraction procedure. A mass of 0.5 mg of thalli was homogenized in 10 mL of 3% (v/v) sulfosalicylic acid and the homogenate was kept under laboratory conditions for 24 h. The homogenate was filtered through six layers of cheesecloth, and 2 ml of the extracts containing proline were pipetted and subsequently treated with 2 mL of acid-ninhydrin at 90˚C for 1 h. The reaction was then terminated on ice and the colored complex extracted in 4 mL toluene for 15-20 s. The absorbance was measured at 520 nm. A standard curve for proline was prepared by dissolving proline in 3% sulfosalicylic acid to cover a concentration range of 0.5-10 µg mL^{-1}. The absorbance values were converted to absolute quantities using the standard curve and statistical tests were applied to the converted data.

Antioxidants and antioxidant enzymes analysis

A mass of 20 mg of the thalli were homogenized with a mortar and pestle in liquid nitrogen. The pellet was resuspended with 4 mL of homogenization buffer (50 mM phosphate buffer, pH 7.5 containing 1 mM EDTA, 2% Triton X-100, 2% polyvinylpyrrolidine). The homogenates were centrifuged at 12 000 x *g* at 4°C for 20 min, transferred to microtubes and immediately used as enzyme extracts.

The CAT (EC 1.11.1.6) activity was assayed in the extract diluted in 100 mM phosphate buffer supplemented with 6 mM H_2O_2. The decomposition of H_2O_2 was monitored at 240 nm and the CAT activity was calculated using an extinction coefficient of 39.4 M cm^{-1} (Bergermeyer, 1970). The SOD (EC 1.15.1.1) activity was determined according to Beyer & Fridowich (1987). One unit of SOD activity was defined as the quantity of SOD that caused a 50% inhibition of nitro blue tetrazolium (NBT), and the specific enzyme activity was expressed as Units mg^{-1} protein. The antioxidant enzyme activities were expressed as the specific activity by measuring the protein content (Bradford, 1976). At last, the APX (EC 1.11.1.11) activity was measured from the ascorbate absorbance decrease at 290 nm, according to Nakano & Asada (1981). The concentration of the oxidized ascorbate was determined using an extinction coefficient of 2.8 mM^{-1} cm^{-1}. One unit of APX activity was defined as the concentration of ascorbate oxidized per min and APX content expressed in Unit mg^{-1} total soluble protein. Each measurement included three replicates.

Abscisic acid analysis by ELISA

The ABA content was measured using a plant hormone ELISA Kit (Cusabio, USA). For this, 20 mg of the thalli were ground in liquid nitrogen. The pellet was resuspended with the sample extraction buffer and was shaken overnight at 4°C in dark conditions. A volume of 50 µL of standards and the sample were added into each well. Then 50 µL of antibody was added to each well and incubated for 30 min at 37°C. Each well was washed with 200 µL wash buffer. Then 100 µL of the Horseradish Peroxidase conjugate was added to each well and incubated for 30 min at 37°C. A volume of 90 µL tetramethylbenzidine substrate was added to each well followed by an incubation at 37°C for 20 min. Finally, 50 µL of stop solution was added to each well and the optical density of each well was measured within 10 min, using a microplate reader set at 450 nm. All reads were repeated 3 times. The standard solutions were prepared according to manufacturer recommendations and a calibration curve was constructed and used to calculate absolute concentrations.

Epiphyte determination using Polymerase Chain Reaction

Epiphytes of the thallus were ground in liquid nitrogen with sterile mortar and pestle for DNA extraction. DNA was extracted using the Plant DNA extraction kit (Qiagen, USA) and quality and quantity were estimated using a Nanodrop spectrophotometer (ThermoScientific, USA). Polymerase Chain Reactions (PCR) were conducted with prokaryote (27F-1492R; Cya359F-Cya359Rb) and eukaryote (ITS1-ITS4) specific primers (Table 1). Both PCR reactions were performed in 25 µL total volume of

Table 1. Primer sequences used for polymerase chain reaction analyses.

Primer name	Primer Sequence (5'-3')	Gene Region	Reference
27F	GAGTTTGATCCTGGCTCAG	16S rDNA	Suzuki et al., 1998
1492R	TACGGCTACCTTTGTTACGACTT		
Cya359F	GGGGAATYTTCCGCAATCGG	16S rDNA	Nübel et al., 1997
Cya359Rb	GACTACAGGGGTATCTAATCCCTTT		
ITS1	TCCGTAGGTGAACCTGCGG	ITS (Internal Transcript Spacer)	White et al., 1990
ITS4	TCCTCCGCTTATTGATATGC		

reaction buffer containing 2.5 mM dNTPs (Thermo Scientific), 20 µM of each primer, 0.4 µL Dream Taq DNA polymerase, (Thermo Scientific), 10 × Buffer, and 5 ng template DNA. PCR analysis was performed in a thermal cycler (Biorad, USA). PCR conditions for both primer pairs were started with initial denaturation step at 95°C for 2 min, which was followed by 30 cycles of DNA denaturation at 95°C for 30 s, primer annealing for 1 min at 50°C, DNA strand extension at 72°C for 1 min, and a final extension step at 72°C for 10 min. The PCR products were separated on 1.5% agarose gel electrophoresis, stained with SafeView Stain (ABM) and visualized by fluorescence in an image analyzer.

Statistical analyses

Statistical analysis was performed with a one-way analysis of variance (ANOVA) or Student's t-test followed by a *post-hoc* Tukey test (SPSS for Windows version 11.0).

Results

Seawater parameters

Not surprisingly, the lowest seawater temperature was measured in winter (16.2˚C) and the highest in summer (24.9˚C). The temperature difference between summer and winter was thus of 8.7˚C. The highest conductivity value was measured in autumn as 58.62 mS and the lowest one in spring as 51.95 mS. The highest and lowest values for salinity were measured 37.92 on summer and 35.55 in winter. The pH of the seawater was 8.05 during autumn and winter, 7.98 on summer and 7.96 on spring. The dissolved oxygen levels were 7.9 mg L^{-1} in winter and 6.6 mg L^{-1} on summer.

Table 2. *Cystoseira foeniculacea*. Seasonal variations of phlorotannin (Eckol and Phloroglucinol) contents in thallus.

Seasons	Part of Thallus	Phlorotannin (mg.g dw^{-1})	
		Eckol	Phloroglucinol
Spring	Lower	60.30 ± 0.02	53.29 ± 0.03
	Upper	7.31 ± 0.13	34.17 ± 0.98
Summer	Lower	119.75 ± 0.09	113.91 ± 0.23
	Upper	148.88 ± 0.04	175.94 ± 1.12
Autumn	Lower	30.98 ± 0.15	25.58 ± 0.92
	Upper	3.42 ± 0.24	37.46 ± 0.03
Winter	Lower	77.89 ± 0.01	37.02 ± 0.02
	Upper	7.73 ± 0.05	33.33 ± 0.02

Biochemical quantitations

The eckol and phloroglucinol contents of the *C. foeniculacea* thalli (upper and lower parts), determined by HPLC, were much higher in summer compared to the other seasons, in both the upper and lower parts of the thalli (Table 2). The GSH content levels in the upper thallus were generally ~2-fold higher in summer than in winter (Fig. 1). However, in the lower part of the thallus, the GSH level was approximately two to three times higher in the spring when compared to the other seasons.

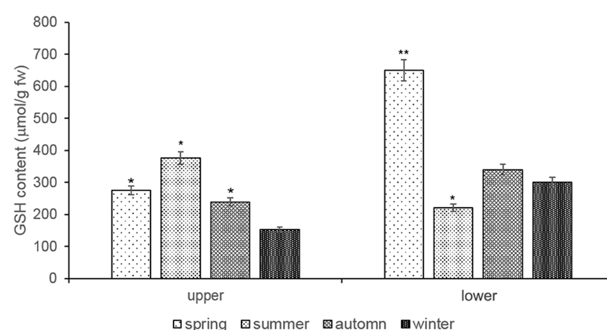

Figure 1. *Cystoseira foeniculacea.* Gluthatione concentration in thalli at different season. *Represents a statistically significant difference of $p < 0.05$ when compared with the winter both upper and bottom part of thalli, **represents a statistically significant difference of $p < 0.01$.

Antioxidant enzyme activities (SOD, CAT, and APX) showed differences according to the collection date and part of the thallus (Figs. 2-4). The highest SOD and APX activity was measured in spring and winter, while the highest CAT activity in spring and the lowest one in summer.

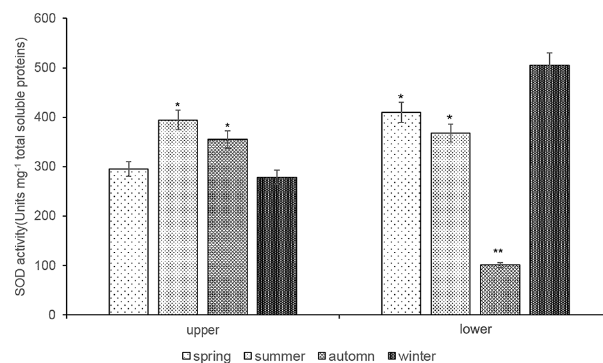

Figure 2. *Cystoseira foeniculacea.* Super Oxyde Dismutase activity in thalli at different season. *Represents a statistically significant difference of $p < 0.05$ when compared with the winter both upper and bottom part of thalli, **represents a statistically significant difference of $p < 0.01$.

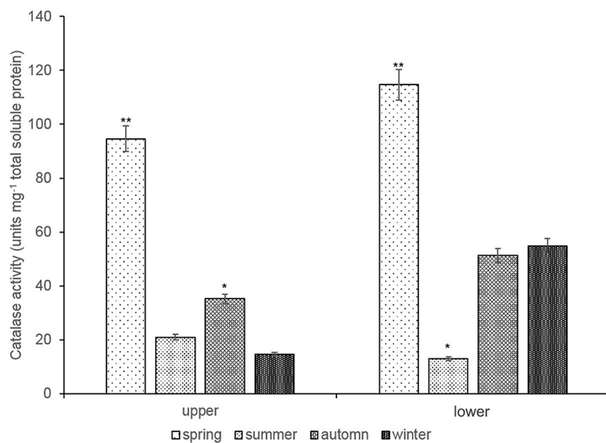

Figure 3. *Cystoseira foeniculacea.* Catalase activity of CAT in thalli at different season *Represents a statistically significant difference of $p < 0.05$ when compared with the winter both upper and bottom part of thalli, **represents a statistically significant difference of $p < 0.01$.

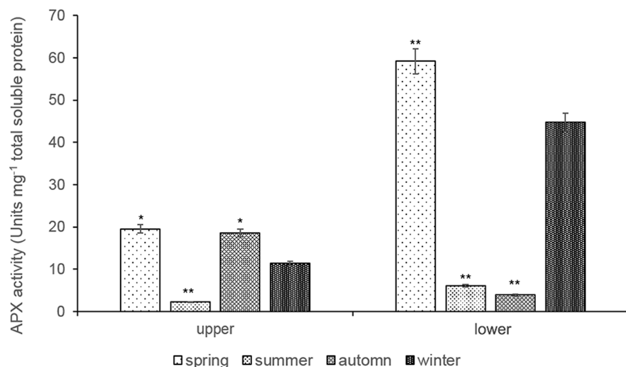

Figure 4. *Cystoseira foeniculacea.* Peroxidase activity in thalli at different season. *Represents a statistically significant difference of $p < 0.05$ when compared with the winter both upper and bottom part of thalli, **represents a statistically significant difference of $p < 0.01$.

Proline results showed differences depending on seasons and the considered part of the thallus (Fig. 5). The highest values were observed in summer in the upper part of the thalli while in the lower part, the highest proline content were measured in spring. In the lower part, the lowest proline was measured in winter but in the upper part spring and autumn samples gave the lowest values.

The ABA concentration in the lower and upper parts of the thallus of *C. foeniculacea* showed differences between seasons (Fig. 6). Although in the lower part of the thalli the ABA content decreased from spring to winter, the upper part showed a different pattern. The ABA concentration was higher on summer and winter and lower on autumn and spring. Statistical analyses

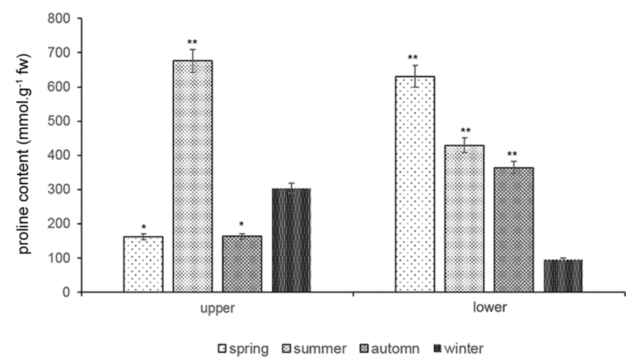

Figure 5. *Cystoseira foeniculacea.* Proline content in thalli at different season. *Represents a statistically significant difference of $p < 0.05$ when compared with the winter both upper and bottom part of thalli, **represents a statistically significant difference of $p < 0.01$.

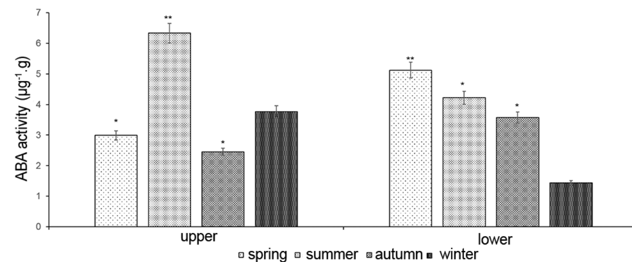

Figure 6. *Cystoseira foeniculacea.* Content of ABA in thalli at different season. *Represents a statistically significant difference of $p < 0.05$ when compared with the winter both upper and bottom part of thalli, **represents a statistically significant difference of $p < 0.01$.

showed that there was a positive correlation between proline content and ABA concentration in *C. foeniculacea* ($R^2 = 0.93$).

Epiphyte analyses by Polymerase Chain Reaction

Eukaryote specific primers only gave results with the samples collected in winter season from both the upper and lower parts of the thalli (Table 3). Light microscope observations supported these results since we could not observe any eukaryotic organisms on the thalli at any season except winter. By contrast, prokaryotic primers gave positive results for all seasons but not for both parts. Summer and winter samples gave positive amplifications with both lower and upper thallus parts. Spring samples were positive only for the lower part and autumn samples for the upper parts.

Estimation of phytoplanktonic cell density in seawater

The concentrated seawater samples observed under light microscope showed a phytoplankton community

Table 3. *Cystoseira foeniculacea*. Results of epiphyte presence tests using polymerase chain reaction analyses, on the lower or upper part of the thalli at different seasons.

Seasons	Part of thallus	Eukaryotes	Prokaryotes
Spring	Lower	-	+
	Upper	-	-
Summer	Lower	-	+
	Upper	-	+
Autumn	Lower	-	-
	Upper	-	+
Winter	Lower	+	+
	Upper	+	+

dominated by diatom species. The variation of cell density in seawater samples are shown in figure 7. In spring, the cell density was 76 x 10^4 cells mL^{-1} and in summer it increased to 330 x 10^4 cells mL^{-1}. Then in autumn the density decreased to 149 x 10^4 cells mL^{-1} and finally in winter it reached the lowest value of 43 x 10^4 cells mL^{-1}.

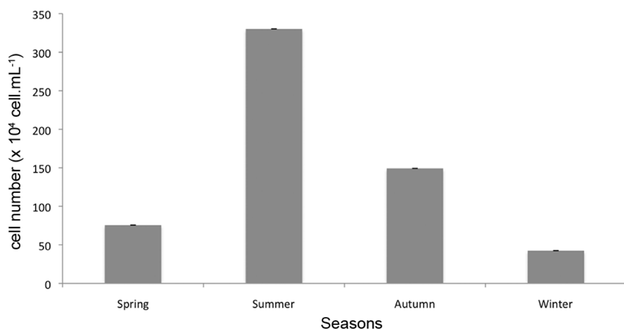

Figure 7. *Cystoseira foeniculacea.* Cell density (x 10^4 cell mL^{-1}) of microphytoplanktonic cells counted with Neubauer Chamber from concentrated seawater samples.

Discussion

Brown algal phlorotannins are essential components of the cell wall (Cruces et al., 2012) and many factors affect their production, including environmental stresses related to nutrients, salinity, UV radiations, and temperature (Abdala-Diaz et al., 2006). Concerning the effect of the season on the production of phlorotannins and phenolic compounds, previous studies reported maximum levels in summer or spring (Connan et al., 2004). Abdala-Diaz et al. (2006) also showed that the levels of phenolic compounds in *Cystoseira tamariscifolia* (Hudson) Papenfuss, 1950 increased during the summer, while they rapidly decreased in the fall/winter period. Our findings are thus in agreement with these studies, since we measured the highest and lowest eckol and phloroglucinol contents in summer and autumn, respectively. This seasonality of the phlorotannin content of *C. foeniculacea* is probably at least partly linked with high seawater temperature and UV/visible light exposure in summer. In addition, from epiphytic flora perspective, there is no significant difference of prokaryotic flora between seasons while winter season is the only time period we observed eukaryotic epiphytes. We hypothesize that phloroglucinol can be protective against light stress while eckol might have a role against epiphyte-related stress. However, the elucidation of the interaction between epiphytic flora and types of secondary compounds in *C. foeniculacea* certainly require additional studies.

The results regarding the phloroglucinol repartition in the thallus are comparable with the work of Abdala-Diaz et al. (2014), which showed that the apical regions of the thallus contained more phloroglucinol. However, the average eckol content was higher in the lower part of the thalli and when the total amount of phlorotannins (eckol + phloroglucinol) was considered, the lower parts of the thalli contained higher levels of these compounds. These findings are in contrast with previous studies in the green alga *Dasycladus vermicularis* (Scopoli) Krasser, 1898 (Perez-Rodriguez et al., 2001). Other studies pointed out that herbivore deterrent secondary metabolites like phlorotannins were higher in the older parts of the thallus (stipes or holdfasts) than in the upper ones (apical region, reproductive structures; Connan et al., 2006). In addition, there are works indicating no such differences between the thallus parts (Toth & Pavia, 2002) or even with the opposite variations (Van Alstyne et al., 1999; Amsler, 2008). Thus, many studies have discussed the intra-thallus variations of the phlorotannin contents and, since there is likely not only one reason for accumulation of phlorotannins in a given thallus zone, there is probably not a single explanation for differential phlorotannin concentrations in macroalgae. Interestingly, Taylor et al. (2002) showed that the browm alga *Sargassum filipendula* C. Agardh, 1824 induces chemical defenses against grazing in the upper thallus regions, which then become unpalatable, whereas the holdfast would be protected by virtue of its toughness. In this context, our results suggest that the holdfast and the bottom stipe of *C. foeniculacea* would be more protected by deterrents from grazing, since there were more

phlorotannins in the samples of the lower parts of the thalli.

Seaweeds cope with environmental stresses with synergistic physiological responses. Previous studies have shown that the seasonal differences in sun exposure and emersion are very important factors to understand the physiological response, although the results are often considerably complicated to interpret (Sampath-Wiley et al., 2008). However, as most stresses induce oxidative stress, antioxidant physiological processes allow monitoring the global stress level of algae. For instance, in the freshwater dinoflagellate *Peridinium gatunense* Nygaard 1925, the activity of SOD, APX, and other antioxidant enzymes was altered depending on seasonal changes (Butow et al., 1997). In addition, the study by Sampath-Wiley et al. (2008) suggests that antioxidant activity is a necessary attribute for species in the upper intertidal regions. In the present study, GSH pool increased during the summer period in *C. foeniculacea*. However, the lowest SOD activity was in autumn in the lower part of thallus, while the highest activity was in winter (Fig. 2). We hypothesize that this might be because of the colder water temperatures and sharper wave actions in winter. Aguilera et al. (2002) showed an increased SOD activity during the summer in microalgae from an Arctic fjord. However, in the present study, the SOD activity of the thallus mainly increased in the lower part during the spring and winter. There is no significant difference between upper and lower part of the thallus in summer although, in autumn, the lower parts of the thallus showed approximately 3.5 times reduced SOD activity than upper part (Fig. 2). It is known that the regulation of SOD activity in algae depends on environmental conditions such as visible light stress, UV radiation stress, nutrient stress and metal toxicity (Wolf-Simon et al., 2005). The cause of these differences may be a result of higher visible light irradiance on apical regions of the algae than on the lower parts. Also, according to epiphyte observations and PCR analysis, we could not evidence the presence of any eukaryotic epiphytes on the thallus except in winter samples, while prokaryotes were almost always present. Thus, High light penetration and epiphyte covering might cause a combined effect on upper parts of the thallus, which caused a stress increase inducing SOD activity.

Sampath-Wiley et al. (2008) showed that APX is very sensitive to the combined effects of sun exposure and emersion, and these responses changed seasonally. In contrast, the highest APX activity was significantly increased during the spring and winter in the lower part of the thallus of *C. foeniculacea* (Fig. 3). If this increase was related to sun exposure or emersion it would mainly be shown in the upper part of the thallus. The increasing antioxidant capacity in the lower part of the thallus of *C. foeniculacea* might thus be related to tolerance to the winter period, during which seawater temperature was low (16.2°C). Accordingly, Lohrmann et al. (2004) reported that tolerance to cold temperature in macroalgae rely on antioxidant capacities. However, the high APX activity in the spring in the lower part of thalli remains enigmatic.

In many photosynthetic organisms, proline can plays a cytosolute role, as a storage compound or a protective agent for cytoplasmic enzymes and cellular structures (Demir, 2000). In a previous study, it was reported that proline content increased during salt and drought stresses due to osmotic adjustments, and it could protect plant cells from oxidative damage (Hien et al., 2003). Other studies suggested that proline accumulation is rather an indicator of injury and would not confer tolerance to abiotic stresses (Mehta & Gaur, 1999). However, in the present study, the concentration of the proline content varied according to the season and thallus parts (Fig. 5). The proline levels in the upper part of the thallus of *C. foeniculacea* were approximately five times higher in the summer than in the spring and autumn, and two times higher than in the winter. In contrast, the lower part of the thallus showed the highest level of proline in the spring compared to in the summer, autumn, and winter (Fig. 5). The lowest proline level was observed in the lower part of the thallus of *C. foeniculacea* in the winter season when compared to all the other samples (Fig. 5). Highest proline content in the upper part in summer is expected if light and UV stresses are considered. But the decrease of proline level in upper part of the thallus during spring and autumn is not consistent with previous literature. Nevertheless, Mattioli et al. (2009) suggested that significant high proline contents could be related to reproduction or/and developmental purposes in non-stressed plants. Since growth and reproduction are higher in spring and autumn, this proline accumulation might be the reason of developmental issues rather than stress factors.

Macroalgae from the intertidal zone may be exposed to long emersion periods depending on the tide level and seasonal changes (Harker et al., 1999). Macroalgae do not have a cuticle and they cannot avoid desiccation. Therefore, they should use effective defense mechanisms for survival in their ecosystem. ABA is one important stress regulating hormone in vascular plants. There are many studies in plants on

the involvement of ABA in regulating a variety of processes, including adaptive responses to desiccation stress (Meurs et al., 1992; Vishwakarma et al., 2017); however, our knowledge about ABA metabolism in macro- and micro-algae is very limited. For example, Guajardo et al. (2016) demonstrated that during periods of desiccation, the concentration of ABA increased significantly in *Pyropia orbicularis*. Since ABA is known to be involved in the activation of desiccation tolerance mechanisms, it is possible that algae at the upper limit of the intertidal zone would have higher concentrations of ABA. In the present study, the highest ABA concentration in the upper part of the thalli was observed in the summer and increased approximately three times when compared to spring and autumn (Fig. 6). Similarly, the ABA concentration in the lower part of the thallus of *C. foeniculacea* was approximately four times higher in spring than in winter (Fig. 6). In general, the ABA concentrations and proline contents in the upper and lower parts of the thallus were three to five times higher in the spring/summer months than in the autumn/winter months. These results might suggest that ABA and proline metabolisms might help to protect against oxidative stress caused by sun exposure.

Increasing seawater temperatures are thought to be one of the reasons of the decrease of populations of *Cystoseira* species in Mediterranean Sea (Serio et al., 2006; Boreo et al., 2008). Since *Cystoseira* spp. are known as a key habitat-forming organism, an impact on other organism populations is expected. Some studies on the thermotolerance mechanisms of marine macroalgal forests (Cebrian et al., 2018) showed that *Cystoseira crinita* Duby, 1830 populations from warmer Eastern Mediterranean had a temperature tolerance threshold 2˚C higher than the colder Northwestern Mediterranean populations. In their study, they observed a strong correlation between phenotypic responses and the local temperature regimes. Their study was the first study indicating the evidence for the role of thermal history in shaping the thermotolerance responses in macroalgae under contrasted temperature environments. According to our physical water parameters there is no significant differences in environmental conditions between seasons for salinity, pH and dissolved oxygen. The main differences in seawater temperature were between summer (24.9°C) and winter (16.2°C), which was an expected result and included the survival temperature range of *Cystoseira* spp. (Baghdadli et al., 1990).

In conclusion, the antioxidant capacities and the levels of secondary compounds changed depending on seasonal variations in the thallus of *C. foeniculacea*. It is known that photosynthetic organisms have developed strategies for acclimation to environmental variations. These responses change depending on the type of the stress factors and other ecological circumstances such as the variations in the epiphytic community structure on the thalli. Epiphytic species and their population may induce biotic stress resulting in the synthesis of protective secondary metabolites in *C. foeniculata*. The results obtained in this study suggest a relationship between eckol content and eukaryotic epiphyte flora on *C. foeniculata*. Besides, it can be suggested that the ABA signal mechanism could play a role in response to higher temperatures, while some antioxidants such as CAT could be involved in response to low temperatures The data obtained in this study will contribute to seaweed ecology and possible molecular signal mechanisms that algae develop against temperature stress.

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References

Abdala-Diaz R.T., Cabello-Pasini A., Marquez-Garrido E. & Figueroa F.L . 2014. Intra-thallus variation of phenolic compounds, antioxidant activity, and phenolsulphatase activity in *Cystoseira tamariscifolia* (Phaeophyceae) from southern Spain. *Ciencias marinas,* 40: 1-10. Doi: 10.7773/cm.v40i1.2350

Abdala-Diaz R.T., Cabello-Pasini A., Pérez-Rodriguez E., Conde Àlvarez R.M. & Figueroa F.L. 2006. Daily and seasonal variations of optimum quantum yield and phenolic compounds in *Cystoseira tamariscifolia* (Phaeophyta). *Marine Biology,* 148: 459-465. Doi: 10.1007/s00227-005-0102-6

Aguilera J., Bischof K., Karsten U., Hanelt D. & Wiencke C. 2002. Seasonal variation in ecophysiological patterns in macroalgae from an Arctic fjord. II. Pigment accumulation and biochemical defence systems against high light stress. *Marine Biology,* 140: 087-1095. Doi: 10.1007/s00227-002-0792-y

Amico V., Piattelli M., Bizzini M. & Neri P. 1997. Absolute configuration of some marine metabolites from *Cystoseira* spp. *Journal of Natural Products*, 60: 1088-1093. Doi: 10.1021/np9700432

Amsler C.D. 2008. *Algal chemical ecology*. Springer-Verlag Berlin Heidelberg. 313 pp.

Asada K. 1999. The water-water cycle in chloroplast scavenging of active oxygens and dissipation of excess

photons. *Annual Review of Plant Physiology and Plant Molecular Biology*, 50: 601-639.
Doi: 10.1146/annurev.arplant.50.1.601

Baghdadli D., Tremblin G., Pellegrini M. & Coudret A. 1990. Effects of environmental parameters on net photosynthesis of a free-living brown seaweed, *Cystoseira barbata* forma *repens*: determination of optimal photosynthetic conditions. *Journal of Applied Phycology*, 2: 281-287.
Doi: 10.1007/BF02179786

Bates L., Waldren R.P. & Teare I.D. 1973. Rapid determination of free proline for water-stress studies. *Plant and Soil*, 39: 205-207. Doi: 10.1007/bf00018060

Beckett R.P., Csintalan Z. & Tuba Z. 2000. ABA treatment increases both the desiccation tolerance of photosynthesis, and nonphotochemical quencing in the moss *Atrichum undulatum*. *Plant Ecology*, 151: 65-71.
Doi: 10.1023/A:1026546707649

Bergmeyer H.U. (ed.) 1970. Methoden der Enzymatischen Analyse. vol. 1. pp. 636-647 pp. Akademie Verlag: Berlin.

Beyer W.F. & Fridowich I. 1987. Assaying for superoxide dismutase activity: Some large consequences of minor changes in conditions. *Analytical Biochemistry*, 161: 559-566. Doi: 10.1016/0003-2697(87)90489-1

Boreo F., Feral J.P., Azurro E., Cardin V., Riedel B., Despalatovic M., Munda I., Moschella P., Zaouali J., Fonda Umani S., Theocharis A., Wiltshire K. & Briand K. 2008. Climate warming and related changes in Mediterranean marine biota. Executive Summary of CIESM Workshop 35, Helgoland, 27-31 May 2008.

Bradford M.M. 1976. A rapid and sensitive method for the quantization of micro- gram quantities of protein utilizing the principle of the protein-dye binding. *Analitycal Biochemistry,* 72: 248-254. Doi: 10.1016/0003-2697(76)90527-3

Butow B.J., Wynne D. & Tel-Or E. 1997. Antioxidative protection of *Peridinium gatunense* in Lake Kinneret: seasonal and daily variation. *Journal of Phycology,* 33: 780-786.
Doi: 10.1111/j.0022-3646.1997.00780.x

Cakmak I. & Marschner H. 1992. Magnesium deficiency and high light intensity enhance activities of superoxide dismutase, ascorbate peroxidase, and glutathione reductase in bean leaves. *Plant Physiology*, 98: 1222-1227.
Doi: 10.1104/pp.98.4.1222

Cebrian E., Verdura J., De Caralt S., Santamaria J., Verges A., Joaquim G., Ballesteros E., Cefali M.E., Farina S., Fraschetti S., Ljiliana I., Mangialajo L. & Tüney I. 2018. Regional environmental conditions determine tolerance to future warming of a marine macroalgae forests. *MERCES 2nd Annual Meeting,* 17 pp.

Connan S., Delisle F., Deslandes E. & Gall E.A. 2006. Intra-thallus phlorotannin content and antioxidant activity in Phaeophyceae of temperate waters. *Botanica Marina*, 49: 39-46. Doi: 10.1515/BOT.2006.005

Connan S., Goulard F., Stiger V., Deslandes E. & Ar Gall E. 2004. Interspecific and temporal variation in phlorotannin levels in an assemblage of Brown algae. *Botanica Marina*, 47: 410-416. Doi: 10.1515/BOT.2004.057

Cruces E., Huovinen P. & Gómez I. 2012. Phlorotannin and antioxidant responses upon short-term exposure to UV radiation and elevated temperature in three South pacific kelps. *Photochemistry and Photobiology*, 88: 58-66.
Doi: 10.1111/j.1751-1097.2011.01013.x

Demir Y. 2000. Growth and proline content of germinating wheat genotypes under ultraviolet light. *Turkish Journal of Botany,* 24: 67-70.

Dietz S. & Hartung W. 1999. The effects of abscisic acid on chlorophyll fluorescense in lichens under extreme water regimes. *New Phytologist,* 143: 495-501.
Doi: 10.1046/j.1469-8137.1999.00473.x

Guajardo E., Correa J.A. & Contreras-Porcia L.I. 2016. Role of abscisic acid (ABA) in activating antioxidant tolerance responses to desiccation stress in intertidal seaweed species. *Planta*, 243: 767-781. Doi: 10.1007/s00425-015-2438-6

Harker M., Berkaloff C., Lemoine Y., Britton G., Young A.J., Duval J.C., Rmiki N.E. & Rousseau B. 1999. Effects of high light and desiccation on the operation of the xanthophyll cycle in two marine brown algae. *European Journal of Phycology*, 34: 35-42. Doi: 10.1017/S0967026299001924

Hien D.T., Jacobs M., Angenon G., Hermans C., Thu T.T., Son L.V. & Roosens N.H. 2003. Proline accumulation and D^1-pyrroline-5-carboxylate synthetase gene properties in three rice cultivars differing in salinity and drought tolerance. *Plant Science,* 165: 1059-1068.
Doi: 10.1016/S0168-9452(03)00301-7

Kim S.M., Kang W.S., Jeon J.S., Jung Y.J. Kim W.R., Kim C.Y. & Um B.H. 2013. Determination of major phlorotannins in *Eisenia bicyclis* using hydrophilic interaction chromatography: Seasonal variation and extraction characteristics. *Food Chemistry*, 138: 2399-2406.
Doi: 10.1016/j.foodchem.2012.11.057

Ilvessalo H. & Tuomi J. 1989. Nutrient availability and accumulation of phenolic compounds in the brown alga *Fucus vesiculosus*. *Marine Biology*, 101:115-119.
Doi: 10.1007/BF00393484

Lohrmann N.L., Logan B.A. & Johnson A.S. 2004. Seasonal acclimatization of antioxidants and photosynthesis in *Chondrus crispus* and *Mastocarpus stellatus*, two co-occurring red algae with differing stress tolerances. *The Biological Bulletin,* 207:325-232. Doi: 10.2307/1543211

Mallick N. & Mohn F.H. 2000. Reactive oxygen species: response of algal cells. *Journal of Plant Physiologyn* 157: 183-193. Doi: 10.1016/S0176-1617(00)80189-3

Mattioli R., Costantino P. & Trovato M. 2009. Proline accumulation in plants, not only stress. *Plant Signaling and Behavior,* 4: 1016-1018. Doi: 10.4161/psb.4.11.9797

Mehta S.K. & Gaur J.P. 1999. Heavy-metal-induced proline accumulation and its role in ameliorating metal toxicity in *Chlorella vulgaris. New Phytologist,* 143: 253-259.
Doi: 10.1046/j.1469-8137.1999.00447

Meurs C., Basra A.S., Karssen C.M. & Van Loon L.C. 1992. Role of abscisic acid in the induction of desiccation tolerance in developing seeds of *Arabidopsis thaliana*. *Plant Physiology*, 98: 1484-1493. Doi: 10.1104/pp.98.4.1484

Mikami K., Mori I.C., Matsuura T., Ikeda Y., Kojima M., Sakakibara H. & Hirayama T. 2016. Comprehensive quantification and genome survey reveal the presence of novel phytohormones action modes in red seaweeds. *Journal of Applied Phycology,* 28: 2539-2548.
Doi: 10.1007/s10811-015-0759-2

Nakano Y. & Asada K. 1981. Hydrogen peroxide is scavenged by ascorbate specific peroxidase in spinach chloroplasts. *Plant Cell Physiology*, 22: 867-880.
Doi: 10.1093/oxfordjournals.pcp.a076232

Nimura K. & Mizuta H. 2002. Inducible effects of abscisic acid on sporophyte discs from *Laminaria japonica* Areschoug

(Laminariales, Phaeophyceae). *Journal of Applied Phycology*, 14: 159-163. Doi: 10.1023/A:1019929712633

Noctor G. & Foyer C.H. 1998. Ascorbate and glutathione: keeping active oxygen under control. *Annual review of plant physiology and plant molecular biology*, 49: 249-279. Doi: 10.1146/annurev.arplant.49.1.249

Nübel U., Garcia-Pichel F., Clavero E. & Muyzer G. 2000. Matching molecular diversity and ecophysiology of benthic cyanobacteria and diatoms in communities along a salinity gradient. *Environmental Microbiology*, 2: 217-226. Doi: 10.1046/j.1462-2920.2000.00094

Pavia H. & Brock E. 2000. Extrinsic factors influencing, phlorotannin production in the brown alga *Ascophyllum nodosum*. *Marine Ecology Progress Series,* 193: 285-294. Doi: 10.3354/meps193285

Pavia H., Cervin G., Lindgren A. & Aberg P. 1997. Effects of UVB radiation and simulated herbivory on phlorotannis in the Brown alga *Ascophyllum nodosum. Marine Ecology Progress Series*, 157: 139-146. Doi: 10.3354/meps157139

Pérez-Rodríguez E., Aguilera J., Gómez I. & Figueroa F.L. 2001. Excretion of coumarins by the Mediterranean green alga *Dasycladus vermicularis* in response to environmental stress. *Marine Biology*, 139: 633-639. Doi: 10.1007/s002270100588

Sampath-Wiley P., Neefus C.D. & Jahnke L.S. 2008. Seasonal effects of sun exposure and emersion on intertidal seawed physiology:fluctuationns in antioxidant contents, photosynthetic pigments and photosynthetic efficiency in the red alga *Porphyra umbilicalis* Kützing (Rhodophyta, Bangiales). *Journal of Experimental Marine Biology and Ecology,* 361: 83-91. Doi: 10.1016/j.jembe.2008.05.001

Serio D., Alongi G., Catra M., Cormaci M. & Furnari G. 2006. Changes in the benthic algal flora of Linosa Island (Straits of Sicily, Mediterranean Sea). *Botanica Marina*, 49: 135-144. Doi: 10.1515/BOT.2006.018

Shimizu K., Uji T., Yasui H. & Mizuta H. 2018. Control of elicitor-induced oxidative burst by abscisic acid associated with growth of *Saccharina japonica* (Phaeophyta, Laminariales) sporophytes. *Journal of Applied Phycology,* 30: 1371-1379. Doi: 10.1007/s10811-017-1320-2

Sun X., Wu J., Wang G., Kang Y., Ooi H.S., Shen T., Wang F., Yang R., Xu N. & Zhao X. 2018. Genomic analyses of unique carbohydrate and phytohormone metabolisms in the macroalga *Gracilariopsis lemaneiformis* (Rhodophyta). *BMC Plant Biology*, 18: 94. Doi: 10.1186/s12870-018-1309-2

Suzuki M., Rappe M.S. & Giovannoni S.J. 1998. Kinetic bias in estimates of coastal picoplankton community structure obtained by measurements of small-subunit rRNA gene PCR amplicon length heterogeneity. *Applied Environmental Microbiology,* 64: 4522-4529.

Tang D., Shi D., Li C., Hu Y. & Liu S. 2007. Physiological and biochemical responses of *Scytonema javanicum* (cyanobacterium) to salt stress. *Journal of Arid Environments,* 71: 312-320. Doi: 10.1016/j.jaridenv.2007.05.004

Tarakhovskaya E.R., Maslov Y.I. & Shishova M.F. 2007. Phytohormones in Algae. *Russian Journal of Plant Physiology,* 54: 163-170. Doi: 10.1134/S1021443707020021

Taylor R.B., Sotka E. & Hay M.E. 2002. Tissue-specific induction of herbivore resistance: seaweed response to amphipod grazing. *Oecologia,* 132: 68-76. Doi: 10.1007/s00442-002-0944-2

Toth G.B. & Pavia H. 2002. Intraplant habitat and feeding preference of two gastropod herbivores inhabiting the kelp *Laminaria hyperborea*. *Journal of the Marine Biological Association of the United Kingdom,* 82: 243-247. Doi: 10.1017/S0025315402005416

Van Alstyne K.L. 1988. Herbivore grazing increases polyphenolic defenses in the intertidal brown alga *Fucus distichus*. *Ecology,* 69: 655-663. Doi: 10.2307/1941014

Van Alstyne K.L., McCarthy III J.J., Hustead C.L. & Kearns L.J. 1999. Phlorotannin allocation among tissues of Northeastern Pacific kelps and rockweeds. *Journal of Phycology*, 35: 483-492. Doi: 10.1046/j.1529-8817.1999.3530483

Vishwakarma K., Upadhyay N., Kumar N., Yadav G., Singh J., Mishra R.K., Verma R., Upadhyay R. G.,Pandey M. & Sharma S. 2017. Absisic acid signaling and abiotic stress tolerance in plants: a review on current knowledge and future prospects. *Frontiers in Plant Science,* 8: 161. Doi: 10.3389/fpls.2017.00161

White T.J., Bruns T., Lee S. & Taylor J.W. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols: A Guide to Methods and Applications* (M.A. Innis, D.H. Gelfand, J.J. Sninsky & T.J. White eds), pp. 315-322. Academic Press, Inc.: New York.

Wolf-Simon F., Grzebyk D., Schofield O. & Falkowski P.G. 2005. The role and evolution of superoxide dismutases in algae. *Journal of Phycology*, 41: 453-465. Doi: 10.1111/j.1529-8817.2005.00086

Yoshida K., Igaashi E., Waatsuki E., Miyamoto K. & Hirata K. 2004. Mitigation of osmotic and salt stresses by abscisic acid through reduction of stress-derived oxidative damage in *Chlamydomonas reinhardtii.* Plant Science, 167: 1335-1341. Doi: 10.1016/j.plantsci.2004.07.002