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Desiccation stress in intertidal seaweeds: Effects on morphology, antioxidant responses and photosynthetic performance

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ABSTRACT

Seaweeds are differentially distributed between the upper and lower limits of the intertidal zone of rocky coasts around the world. Daily changes in tide height cause water loss, triggering desiccation stress as a consequence. How this stress affected some of the morphological characteristics and physiological responses in representative intertidal seaweeds with contrasting vertical distributions was explored in the present work. The selected species were *Mazzaella laminarioides* (upper-middle distribution), *Scytosiphon lomentaria* and *Ulva compressa* (middle distribution), and *Lessonia spicata* and *Gelidium rex* (lower distribution). To assess tolerance response to desiccation, cellular and morphological alterations, ROS production, enzymatic activity of catalase (CAT) and ascorbate peroxidase (AP) and photosynthesis performance were measured after a simulated emersion stress experiment. Results show different tolerance responses to desiccation, with seaweeds having higher intertidal distributions displaying greater antioxidant enzymatic activity, suggesting a higher capacity to buffer ROS excess induced during desiccation. Contrarily, this capacity seems to be absent or deficient in low intertidal species (i.e. *L. spicata* and *G. rex*), where AP and CAT activities were below detection limits, ROS were higher than normal and caused an over-oxidation of bio-molecules and photosynthetic disarray, explaining from a functional stand point their low distribution in the intertidal zone.

1. Introduction

Marine macroalgae are pre-eminent producers occupying a basal position in aquatic food webs (Lobban and Harrison, 1994). Rocky intertidal zones are dynamic and stressful habitats for seaweeds as a result of the rapid changes in physical conditions associated with tides, in addition to the changes brought by seasonal variations (Kumar and Reddy, 2012). Seaweeds are distributed in bands parallel along the rocky intertidal zone, where distribution and relative abundance along the lower limits is mainly controlled by biotic factors such as predation and intra and interspecific competition, while the upper limit distribution is mostly determined by abiotic factors such as UV radiation, light, salinity, temperature, nutrient availability and air exposure (Zaneveld, 1969). Davison and Pearson (1996) and Ji and Tanaka (2002), while studying the photosynthetic and respiratory performance of various seaweeds collected from different intertidal regimes following 2 h of desiccation, suggested that the ability to withstand desiccation stress (fast recovery during rehydration) and not that to avoid desiccation (water retaining ability), is the key factor determining their vertical distribution.

Exposure of seaweeds during low tidal emersions demands the alga to prepare not only for desiccation but also for subsequent rehydration and eventual cellular damage (Burritt et al., 2002). Extended desiccation may cause a decline in photosynthesis rate while interrupting the electron flow between photosystem (PS) I and II (Heber et al., 2010; Gao et al., 2011). Additionally, fluctuating and dynamic environmental conditions in the intertidal zone trigger the accumulation of reactive oxygen species (ROS) (Collén and Davison, 1999a,b; Contreras et al., 2005, 2009; Kumar et al., 2010, 2011) that, if not buffered, result in an oxidative stress condition. Further, cellular dehydration resulting from desiccation increases

Abbreviations: AP, ascorbate peroxidase; CAT, catalase; DHAR, dehydroascorbate reductase; GR, glutathione reductase; PRX, peroxiredoxin; ROS, reactive oxygen species; RWC, relative water content.

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electrolyte concentration in the cell and brings changes to membrane structures, including thylakoids (Kim and Garbary, 2007). Acclimation to an adverse environment involves complex enzymatic and non-enzymatic antioxidant mechanisms functioning in an orchestrated manner to mitigate changes in cellular osmolarity, ion disequilibrium and ROS excess (Sampath-Wiley et al., 2008; Contreras et al., 2009; Kumar et al., 2011).

The effects of desiccation on the vertical distribution of macroalgae are poorly understood. Only few reports highlight the induction of ROS and activation of the antioxidant system in seaweeds in response to desiccation (Collén and Davison, 1999a,b; Burritt et al., 2002; Ross and Van Alstyne, 2007; Contreras-Porcia et al., 2011, 2013; Kumar et al., 2011; López-Cristoffanini et al., 2013). Independent studies investigating algal responses to fluctuating environmental conditions show alterations in photosynthetic performance (*Fv*/*Fm*), cellular morphology and ontogenetic development (e.g. Abe et al., 2001; Varela et al., 2006; Kumar et al., 2011; Contreras-Porcia et al., 2012; Gao and Wang, 2012). However, integrative studies addressing cellular responses to desiccation are lacking.

Along the Chilean coast, the upper-most part of the intertidal zone is characterized by a seasonal dominance of *Porphyra* and *Pyropia* species such as *Pyropia columbina* (Montagne) WA Nelson (formerly *Porphyra columbina* Montagne) (Bangiales, Rhodophyta). The upper-middle zone is dominated, among others, by *Mazzaella laminarioides* (Gigartinales, Rhodophyta) and the middle zone by *Ulva compressa* (Ulvales, Chlorophyta), *Scytosiphon lomentaria* (Ectocarpales, Heterokontophyta), *Ceramium* spp. and *Polysiphonia* spp. (Ceramiales, Rhodophyta). The lower intertidal zone is dominated by *Codium* spp. (Bryopsidales, Chlorophyta), *Lessonia* spp. (Laminariales, Heterokontophyta), *Gelidium* spp. (Gelidiales, Rhodophyta) and also several species of crustose calcareous red algae (Hoffmann and Santelices, 1997). Experimental studies on the littoral zone have been important in unraveling the factors regulating the vertical distribution of these species (Alveal, 1970; Moreno and Jaramillo, 1983; Buschmann, 1990). Recently, Contreras-Porcia et al. (2011) demonstrated that *P. columbina* exposed to natural desiccation during low tide loose 90–95% water and displays an excess of ROS, elevated activities of antioxidant enzymes and high concentration of photosynthetic pigments. Furthermore, desiccation results in over expression of tolerance genes, as those coding for ABC (ATP binding cassette) transporter proteins, antioxidant enzymes, heat shock proteins, cytochrome P450, cell wall proteins and specific transcriptional factors, among others (López, 2012; Contreras-Porcia et al., 2013). It is highly likely that these mechanisms, present in *P. columbina*, represent part of the functional tools available to the plants to tolerate desiccation and, at the end, may help to explain its ecological dominance in the higher part of the intertidal zone. In this context, we assessed the general validity of the responses in *P. columbina* by testing the hypothesis that the presence and adequacy of functional responses to desiccation stress in a selected group of common intertidal seaweeds defines their altitudinal position in the intertidal zone.

2. Materials and methods

The presence and adequacy of functional responses to desiccation in *M. laminarioides*, *S. lomentaria*, *U. compressa*, *Lessonia spicata* and *Gelidium rex* were determined by monitoring and recording (i) cellular alteration, (ii) attenuation of ROS over-production, (iii) oxidation of biomolecules, (iv) antioxidant enzymatic activity, and (v) photosynthetic response after desiccation. These responses were recorded in plants affected by desiccation in vitro, and compared with those in plants rehydrated in fresh seawater.

2.1. Sampling

Hydrated vegetative individuals of each species were collected along 250–300 m of coastline in Maitencillo beach (32°39.5′ S, 71°26.6′ W) during low tide. Plants were kept in plastic bags with seawater and transported to the laboratory in a cooler with ice packs at 5–7 °C. Once in the laboratory, these hydrated individuals were exhaustively rinsed with 0.45 μm-filtered seawater, cleaned using an ultrasonic bath (575T, Crest, NJ, USA) and acclimated to laboratory conditions in a growth chamber for 24 h at 14 ± 2 °C, 12:12 light:dark photoperiod and 30–40 μmol photon m^{-2} s^{-1} irradiance.

2.2. In vitro experiment of desiccation and recovery

In vitro experiments were conducted according to previous work in *P. columbina* (Contreras-Porcia et al., 2011), where responses to 4 h desiccation were similar in magnitude to those recorded after 4 h of natural desiccation. In vitro desiccation experiments included an initial blot drying of the hydrated plants followed by air exposure in a growth chamber at 16 °C and 70–80 μm photon m^{-2} s^{-1} irradiance for 4 h. In addition, a subset of dehydrated fronds was immediately re-hydrated in 0.45 μm-filtered seawater, during 4 h, to characterize the recovery from oxidative stress caused by desiccation. This timing was selected because plant tissues of all species under study have reached 95–100% relative water content (RWC). Control plants were obtained after acclimation, and frozen immediately in liquid nitrogen.

2.3. Level of desiccation

The level of desiccation experienced by different algal species during the in vitro trials was expressed as relative water content (RWC%) following the formula: $RWC\% = [(Wd - Wdo) \times (Wf - Wdo)^{-1}] \times 100$, where *Wf* is the wet weight of fully hydrated fronds, *Wd* is the dehydrated weight after desiccation, and *Wdo* is the dry weight determined after drying for 48 h at 80 °C. In this context, the RWC is the complement of desiccation status and thus, a fully hydrated thallus has RWC of 100% and a fully dehydrated thallus has RWC near to 0%; decreasing RWC means increasing desiccation.

2.4. Morphological effects of desiccation

Hand-made cross sections of naturally hydrated, dehydrated and rehydrated fronds (five of each category) were used to characterize morphological changes at light microscopy level. The hydrated sections were mounted in seawater and the dehydrated section in a synthetic, water-free resin (Permount™, Electron Microscopy Sciences, PA, USA) to prevent rehydration during analysis. Cell size in each category of fronds was defined as the mean value from 100 cell measurements. Images were captured with a Nikon Microscopy Unit (Nikon Corp., Tokyo, Japan) coupled to a digital recording system (CoolSNAP-Pro*cf*, Media Cybernetics, MD, USA) and analyzed using the Image Pro Plus Version 4.5 software (Media Cybernetics, MD, USA).

Changes in ultrastructure as a result of desiccation stress were analyzed by transmission electron microscopy (TEM) using triplicate samples of hydrated, dehydrated and rehydrated fronds. Tissue samples were fixed in 0.22 μm-filtered seawater containing 3% (v/v) glutaraldehyde and 1% (v/v) *p*-formaldehyde for 3 days at 5 °C (Correa and McLachlan, 1991). Post-fixation for 2 h at 5 °C in 0.05 M sodium cacodylate buffer (pH 7.8) with the addition of 2% (w/v) OsO_4 and 1% (w/v) potassium hexacyanoferrate, was followed by dehydration in ethanol series (10–100%, v/v) and embedding

in Spurr's resin (Spurr, 1969) for 1 week. Sections for TEM were stained with uranyl acetate-lead citrate and analyzed in an electron microscope (Tecnai 12, Philips, Holland), operated at 60 kV.

2.5. Quantification of hydrogen peroxide (H_2O_2)

H_2O_2 was determined according to Contreras et al. (2009) in hydrated, dehydrated and rehydrated fronds. Fresh tissue (1–2 g) were incubated in 100 mL of 5 μM 2′,7′ dichlorohydrofluorescein diacetate (DCHF-DA, Calbiochem, CA, USA) for 1 h at room temperature. After incubation, the tissue was ground in liquid nitrogen, suspended in 5 mL of 40 mM Tris–HCl buffer pH 7.0 and centrifuged at 20,600 × *g* for 15 min. Fluorescence was measured in a Perkin-Elmer LS-5 spectrofluorometer at an excitation wavelength of 488 nm and at an emission wavelength of 525 nm. Final fluorescence values were obtained using a standard curve prepared with 0–500 nM of DCF (Sigma, St. Louis, USA). The in situ production of H_2O_2 was detected in hydrated and desiccated plants immediately after incubation in DCHF-DA. After rinsing the plants in filtered seawater, hand-made cross-sections of the tissues were observed using a Nikon Optiphot II fluorescence microscope with an emission filter of 459–490 nm, coupled to a digital recording system (CoolSNAP-Pro*cf*, Media Cybernetics, MD, USA) and analyzed using the Image Pro Plus Version 4.5 software (Media Cybernetics, MD, USA).

2.6. Lipid peroxidation and protein oxidation

Lipid peroxidation levels were determined as the amount of thiobarbituric acid (T-BARS) reactive species (i.e. lipoperoxides) according to Ratkevicius et al. (2003). Protein oxidation levels were determined according to Contreras-Porcia et al. (2011) based on the reaction of carbonyls resulting from free radical modification of proteins and 2,4-dinitrophenyl hydrazine (DNPH). For that, 4–5 g fresh algal tissue were frozen in liquid nitrogen and homogenized in a pre-chilled mortar with a pestle. A total of 5 mL of 50 mM sodium buffer pH 7.0, containing 0.1 mM EDTA and 1% (w/v) polyvinyl-polypyrrolidone were added during the homogenization. The homogenate was centrifuged at 7400 × *g* for 15 min at 4 °C and the supernatant stored at −20 °C. Two aliquots, each containing 1 mg of proteins, were mixed with an equal volume of 20% (v/v) trichloroacetic acid (TCA) and centrifuged at 7400 × *g* for 15 min at 4 °C. Pellets were suspended with 300 μL of 2 N HCl with or without (blank) 10 mM DNPH and left for 1 h at room temperature. Samples were then precipitated with 500 μL of 20% (v/v) TCA for 10 min at −20 °C, centrifuged at 7400 × *g* for 15 min at 4 °C, and the supernatant discarded. After rinsing with 500 μL ethanol: ethyl acetate (1:1, v/v), pellets were dissolved in 3 mL 20 mM sodium phosphate buffer (pH 6.8) containing 6 M guanidinium hydrochloride and centrifuged at 7400 × *g* for 15 min at 4 °C. Finally, the carbonyl concentration was calculated from the difference in absorbance recorded at 380 nm for DNPH-treated and HCl-treated (blank) samples ($\varepsilon = 22\ mM^{-1}\ cm^{-1}$) and expressed in μmol of DNPH incorporated per mg of protein.

2.7. Antioxidant enzyme activities

Protein extracts and the activities of antioxidant enzymes were determined according to Contreras et al. (2005, 2009). Briefly, algal tissue (20 g fresh weight) was frozen in liquid nitrogen and homogenized in a pre-chilled mortar using a pestle. A total of 60 mL of 0.1 M phosphate buffer (pH 7.0), containing 5 mM β-mercaptoethanol was added during the homogenization. The homogenate was filtered through Miracloth paper (Calbiochem, San Diego, CA, USA) and centrifuged at 7400 × *g* for 15 min at 4 °C. Proteins were precipitated by addition of 0.5 g of ammonium sulfate per mL of extract during 2.5 h at 4 °C. The protein pellet was dissolved in 2 mL of 0.1 M phosphate buffer (pH 7.0), containing 2 mM β-mercaptoethanol and 20% (v/v) glycerol, and the protein concentration was determined using the bicinchoninic acid assay according to the protocols by Smith et al. (1985). Final protein extracts (2–4 mg mL^{-1}) were stored at −80 °C and the activities were determined using 50–200 μg of proteins. The specific activity of the antioxidant enzymes catalase (CAT) was measured in a reaction mixture containing 0.1 M phosphate buffer, pH 7.0, and 18 mM H_2O_2. After the addition of H_2O_2, its consumption was determined at 240 nm for 1 min and the activity was calculated using the extinction coefficient of H_2O_2 ($\varepsilon = 39.4\ mM^{-1}\ cm^{-1}$). For AP activity, the reaction mixture contained 0.1 M phosphate buffer pH 7.0, 400 μM ascorbate (ASC) and 20 mM H_2O_2. After the addition of ASC, its consumption was determined at 290 nm for 1 min and the activity was calculated using the extinction coefficient of ASC ($\varepsilon = 2.8\ mM^{-1}\ cm^{-1}$).

2.8. Photosynthetic efficiency activity

Maximum photosynthetic efficiency (*Fv*/*Fm*) was assessed in 5–10 plants, according to Contreras-Porcia et al. (2011). Before recording fluorescence, and to allow complete re-oxidation to photosystem (PSII) reaction centers, fronds were kept in plastic bags and dark-acclimated with leaf-clips for 30 min for *M. laminarioides* and *U. compressa*, 25 min for *G. rex*, and 20 min for *S. lomentaria* and *L. spicata*. The fluorescence emission rates were measured using a portable fluorometer (Plant Efficiency Analyser PEA, Hansatech Instruments Ltd., UK) with a maximum light intensity of 2000 μmol $m^{-2}\ s^{-1}$ for *M. laminarioides* and *U. compressa*, 2500 μmol $m^{-2}\ s^{-1}$ for *S. lomentaria* and *L. spicata*, and 3000 μmol $m^{-2}\ s^{-1}$ for *G. rex*. The different saturating intensities were chosen based on saturation kinetics of *Fv*/*Fm*.

2.9. Statistical analyses

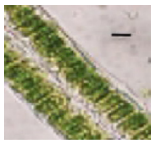
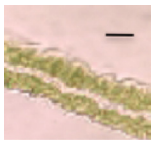
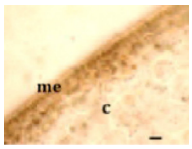
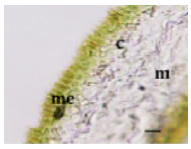
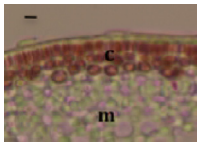
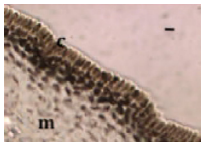
Significance of the differences were determined by two-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons tests (*T*), considering species and treatment as fixed effects factors with three levels in the latter (control or natural hydration, desiccation and rehydration). Prior to the statistical analyses, data were checked for variance homogeneity using Levene and Bartlett tests and for normal distribution using Kolmogorov–Smirnov and Anderson–Darling tests (Zar, 2010). Data were transformed using the Box–Cox method when necessary. Subsequently, the effect sizes in each dependent variable were determined estimating Cohen's *d* (Cohen, 1988), where an effect size of 0.2–0.4 is considered a small effect, 0.5–0.7 is considered a medium effect, and ≥0.8 is considered a large effect. Differences between mean values were considered to be significant at a probability of 5% ($P \leq 0.05$).

3. Results

3.1. Morphological changes

Plants exposed to desiccation displayed low relative water content (RWC %), ranging between 4 and 22%. For example, *M. laminarioides*, *U. compressa* and *S. lomentaria* lose more water (i.e. ca. 94%, 98% and 97%, respectively) than plants growing at the lower intertidal zone (i.e. *L. spicata* ca. 83% and *G. rex* ca. 78%). Moreover, cross-sections of dehydrated fronds showed a decrease in size of cortical cells and medullar zone in relation to hydrated fronds (Table 1). For example, the most evident alterations were observed in *U. compressa*. In their hydrated fronds, cells appeared polygonal, 19.1 ± 2 μm in length and 9 ± 2 μm in width, whereas in dehydrated individuals the size decreased by 53–56% (i.e. 9 ± 2 μm in

Table 1
Cellular dimensions (μm) of hand-cut cross sections of vegetative hydrated and dehydrated fronds of *Mazzaella laminarioides*, *Scytosiphon lomentaria*, *Ulva compressa*, *Lessonia spicata* and *Gelidium rex* under hydration and desiccation stress. Images show the major cellular changes observed during desiccation in *U. compressa*, *L. spicata* and *G. rex*. Scale bar = 10 μm. me, meristoderm; c, cortex; m, medulla. Each value is an average of 100 measurements ± SD.

Species	Zone tissue (μm)	Treatment	
		Hydration	Desiccation
Mazzaella laminarioides	Cortical cell (length)	7.3 ± 2	5.2 ± 2*
	Medulla (width)	133 ± 13	81 ± 19*
Scytosiphon lomentaria	Cortical cell (length)	6.3 ± 1	3.6 ± 1*
Ulva compressa	Poligonal (length × width)	19.1 ± 2 × 9 ± 2	9 ± 2* × 4 ± 2*
			
Lessonia spicata	Cortical cell (length)	4.4 ± 2	2.0 ± 1*
			
	Medulla (width)	79.1 ± 8	46.4 ± 3*
Gelidium rex	Cortical cell (length)	6.0 ± 1	4.5 ± 1*
			

* Significant ($P < 0.05$) differences among hydrated and dehydrated values.

length and 4 ± 2 μm in width). In *M. laminarioides* and *L. spicata* the medullar zone decreased by ca 41% and in *S. lomentaria* and *G. rex* the cortex in a 43–25%, respectively.

Major negative effects induced by desiccation were recorded in *L. spicata* and seemed to have an impact on the fine structure of the cells. In naturally hydrated fronds, cellular structure of the cortical cells was normal (Fig. 1a), with the parietal chloroplast (Fig. 1a) and characteristic distribution of thylakoids (Fig. 1b). In contrast, during desiccation there was a considerable retraction of the protoplast (Fig. 1c) and thylakoid disorganization (Fig. 1d). It is important to mention that the fine structure of *L. spicata* did not recover during rehydration (Fig. 1e and f).

3.2. Over-production of hydrogen peroxide (H_2O_2)

Desiccation had a different effect on H_2O_2 production according to the species (Table 2). Desiccation increased H_2O_2 in cortical cells (Fig. 2a), from 9.4 to 25 fold above levels recorded in hydrated plants. The smallest increase in H_2O_2 during desiccation was recorded in *M. laminarioides* ($T = 8.71$, $P < 0.001$, Cohen' $d = 5.78$) and *S. lomentaria* ($T = 10.18$, $P < 0.001$, Cohen' $d = 7.5$); 9.4 and 14 times the level of hydrated plants, respectively. Moreover, this increase was more significant in middle and low intertidal species (*U. compressa*, *L. spicata* and *G. rex*) than in upper-middle intertidal species, with *L. spicata* displaying the highest increase (25 times) ($T = 12.27$, $P < 0.001$, Cohen' $d = 9.42$). For example, in sections of *L. spicata* and *G. rex*, ROS-fluorescence was patchy and appeared in both meristoderm and cortex (Fig. 2b). During rehydration, the concentration of H_2O_2 declined in *M. laminarioides* ($T = 2.39$, $P = 0.540$, Cohen' $d = 1.93$) and *S. lomentaria* ($T = 3.16$, $P = 0.156$, Cohen' $d = 1.73$) to the basal levels. However, in lower intertidal species the levels of H_2O_2 persisted 7–11 times higher than those registered in hydrated plants (in all cases $P < 0.001$, Cohen' $d > 3.5$) (Fig. 2a).

3.3. Molecular oxidation

Desiccation had a significant effect on the oxidation of biomolecules (Table 2). Levels of lipoperoxides increased significantly ($P < 0.050$, Cohen' $d > 2$) during desiccation in tissues of all species, except *S. lomentaria* ($T = 1.63$, $P = 0.300$, Cohen' $d = 1.81$),

Table 2
Summary of 2-way-ANOVA analyses to compare hydrogen peroxide levels, lipid peroxidation, protein oxidation production, ascorbate peroxidase and catalase activities, and photosynthetic efficiency (*Fv*/*Fm*), in the species *M. laminarioides*, *S. lomentaria*, *L. spicata* and *G. rex* under hydration, desiccation and rehydration treatments. df (degrees of freedom) treatment = 2; df species = 4, df treatment × species = 8.

Sources of variation	*F*-ratio	*P*-value
Hydrogen peroxide		
Treatment (*T*)	256.51	<0.001
Species (*S*)	19.76	<0.001
T × *S*	3.69	0.004
Lipid oxidation		
Treatment (*T*)	192.77	<0.001
Species (*S*)	16.87	<0.001
T × *S*	5.18	<0.001
Protein oxidation		
Treatment (*T*)	66.35	<0.001
Species (*S*)	23.01	<0.001
T × *S*	4.73	0.001
Ascorbate peroxidase		
Treatment (*T*)	58.41	<0.001
Species (*S*)	96.28	<0.001
T × *S*	10.40	<0.001
Catalase		
Treatment (*T*)	144.17	<0.001
Species (*S*)	59.96	<0.001
T × *S*	17.00	<0.001
Fv/*Fm*		
Treatment (*T*)	653.01	<0.001
Species (*S*)	12.84	<0.001
T × *S*	14.16	<0.001

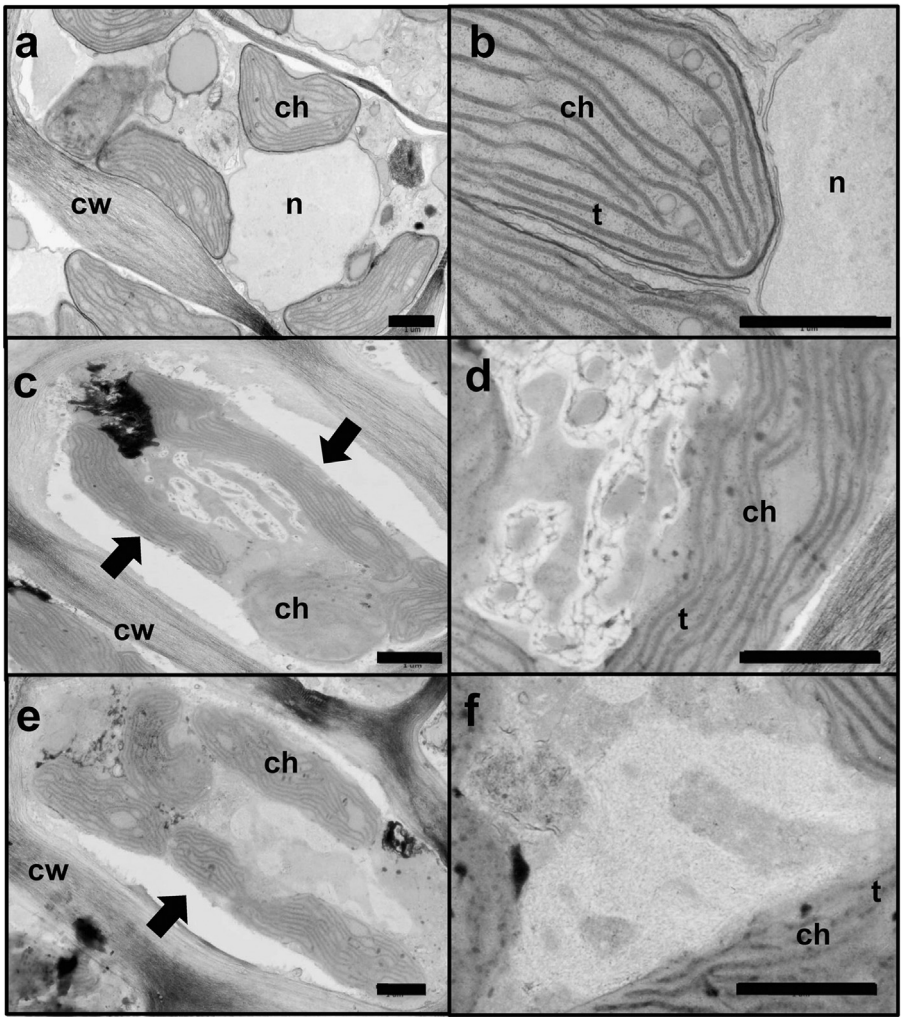


Fig. 1. Ultrastructure of *Lessonia spicata* during hydration (a and b), desiccation (c and d) and rehydration (e and f). (a) General view of cortical cells with parietal chloroplast (ch), central nucleus (n) and cell wall (cw) in contact with plasmatic membrane. (b) Chloroplast detail with the typical arrangement of thylakoids (t). Nuclear pore is also observed. (c) Cortical cell detail, with clear protoplast retraction (arrows) and separation between plasmatic membrane and cell wall. (d) Abnormal arrangement of thylakoids and diffuse cytoplasm. (e) Cortical cell detail of rehydrated plants, showing the same ultrastructural pattern than cells under desiccation. (f) Chloroplast and nucleus detail of rehydrated plants, where limited cellular structure is observed. Scale bar = 1 μm.

in comparison with hydrated plants (Fig. 3a). This increase varied from 2.4 to 8 fold higher than the values recorded in hydrated plants, and the effect was stronger in low intertidal species (Fig. 3a). During rehydration, lipoperoxide content in all species reached levels even higher than under desiccation, except for *M. laminarioides* ($T = 0.69$, $P = 1.0$, Cohen' $d = 1.38$) (Fig. 3a).

Protein oxidation (Fig. 3b) under desiccation stress increased 1.5–4.5 times in all species ($P < 0.050$), except in *M. laminarioides* ($T = 1.11$, $P = 0.997$, Cohen' $d = 1.01$) and *S. lomentaria* ($T = 2.12$, $P = 0.712$, Cohen' $d = 1.18$). Differences in tolerance to desiccation, however, were clear during rehydration. The results showed that carbonyl levels did not increased in *M. laminarioides* and *S. lomentaria* undergoing rehydration. Contrarily, in *U. compressa* it was higher than in hydrated plants ($T = 4.80$, $P = 0.003$, Cohen' $d = 5.69$), and in *L. spicata* ($T = 7.93$, $P = 0.0001$, Cohen' $d = 6.54$) and *G. rex* ($T = 6.47$, $P = 0.0001$, Cohen' $d = 6.32$), carbonyl level was higher even than under desiccation (Fig. 3b). *L. spicata* presented the highest levels of oxidized proteins.

3.4. Antioxidant responses

Desiccation and rehydration had significant effects on the antioxidant responses and it varied according to the species (Table 2). With the exception of the low intertidal *L. spicata*, CAT activity increased significantly (in all cases $P < 0.01$ and Cohen' $d > 2$) during desiccation compared to hydrated plants (Fig. 4a), ranging from 2.6 to 9.4 times the basal activity, with the highest values recorded in *M. laminarioides* and *S. lomentaria* (Fig. 4a). During rehydration, CAT activity returned to the basal levels except in *U. compressa*, where it remained elevated ($T = 5.64$, $P = 0.0004$, Cohen' $d = 5.98$). Whereas in *L. spicata* CAT activity was not detected during rehydration, in *G. rex* it displayed levels lower than basal activity (ca. 23%) ($T = 5.15$, $P = 0.0012$, Cohen' $d = 6.03$) (Fig. 4a).

During desiccation, AP activity increased significantly in *M. laminarioides* ($T = 3.64$, $P = 0.05$, Cohen' $d = 2.5$) and in *S. lomentaria* ($T = 3.71$, $P = 0.041$, Cohen' $d = 2.74$) (Fig. 4b). On the contrary, in *U. compressa* this activity decreased by 87% in comparison with the basal activity ($T = 4.67$, $P = 0.004$, Cohen' $d = 4.76$). In *L. spicata* ($T = 0.67$, $P = 1.0$, Cohen' $d = 0.8$) and *G. rex* ($T = 3.44$, $P = 0.086$, Cohen' $d = 1.97$) AP activity was the same as in hydrated plants (Fig. 4b). During rehydration, AP activity in *M. laminarioides* ($T = 1.68$, $P = 0.924$, Cohen' $d = 4.39$) and *S. lomentaria* ($T = 1.50$, $P = 0.967$, Cohen' $d = 1.74$) returned to basal levels, but in *U. compressa* it dropped below basal levels by 88% ($T = 5.73$, $P < 0.001$, Cohen' $d = 5.613$). Finally, whereas in *L. spicata* AP activity was not

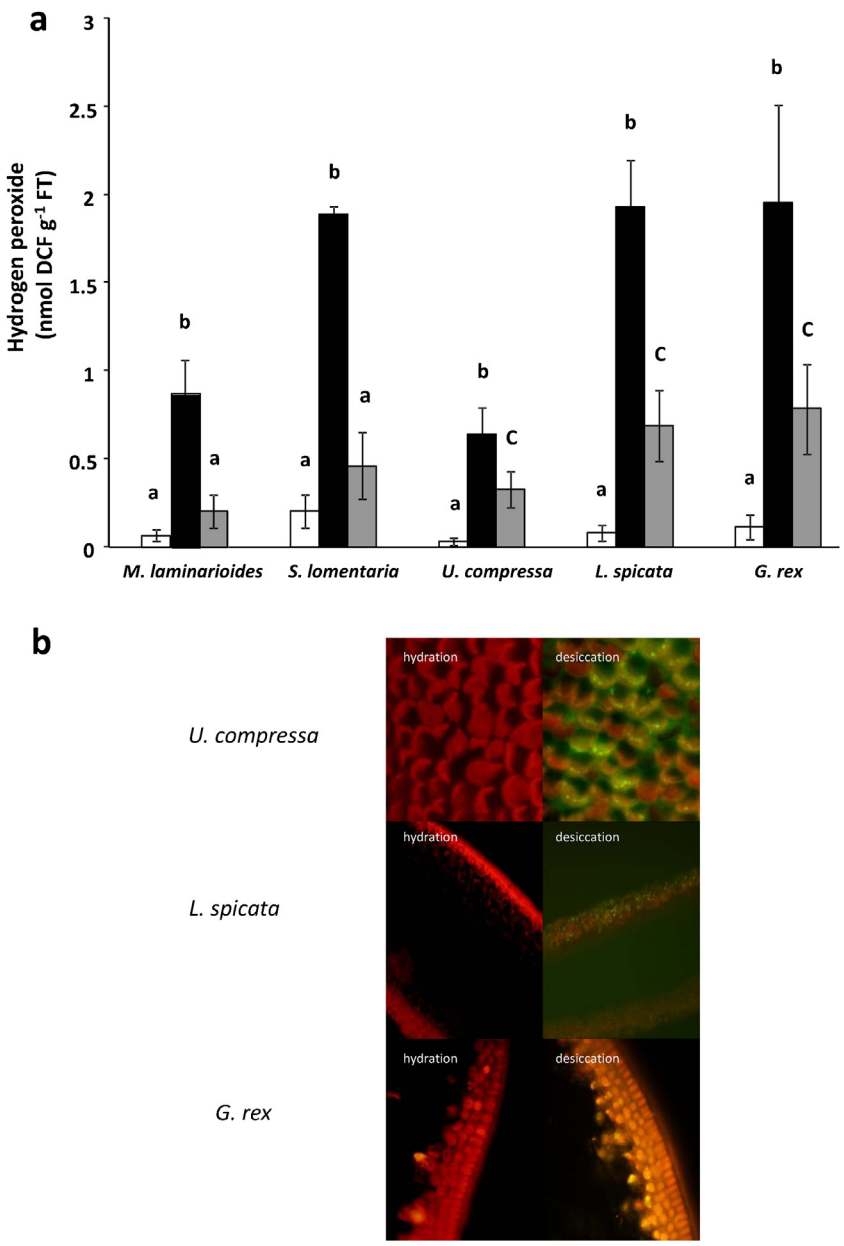


Fig. 2. Hydrogen peroxide (H_2O_2, nmol DCF (dichlorofluorescein) g^{-1} FT (fresh tissue)) levels (a) in *Mazzaella laminarioides*, *Scytosiphon lomentaria*, *Ulva compressa*, *Lessonia spicata* and *Gelidium rex* during hydration (white bars), desiccation (black bars) and rehydration (gray bars). Values represent the mean of 3–6 independent replicates ± SD. Letters indicate significant differences ($P < 0.05$) between treatments for each species. (b) Localization of hydrogen peroxide, visualized by fluorescence microscopy, in species with high H_2O_2 levels registered during desiccation stress (i.e. *U. compressa* surface view, and cross sections of *L. spicata* and *G. rex*).

recorded, in *G. rex* it was the same as in hydrated plants ($T = 0.39$, $P = 1.0$, Cohen' $d = 0.961$) (Fig. 4b).

3.5. Effects of desiccation on photosynthetic efficiency

Maximum basal photosynthetic efficiency (*Fv*/*Fm*) in hydrated plants varied from 0.470 to 0.740 (Fig. 5). Even though during desiccation *Fv*/*Fm* decreased by 92–98%, after rehydrated *M. laminarioides* and *S. lomentaria* *Fv*/*Fm* was restored to the maximum levels (Fig. 5). In *U. compressa*, however, *Fv*/*Fm* increased only by 30% with respect to the basal level ($T = 16.39$, $P < 0.001$, Cohen' $d = 5.04$). Finally, the low intertidal *L. spicata* and *G. rex* displayed an *Fv*/*Fm* below the maximum levels by 2% ($T = 13.08$, $P < 0.001$, Cohen' $d = 3.56$) and 3% ($T = 11.59$, $P < 0.001$, Cohen' $d = 6.69$), respectively (Fig. 5), demonstrating an irreversible inactivation of the photosystem.

4. Discussion

Our results support the hypothesis that the presence and adequacy of functional responses to desiccation in a selected group of common intertidal seaweeds defines their bathymetric position in the intertidal zone. In summary, our results show that representative intertidal seaweeds responded differently to desiccation, and those living higher in the intertidal regime display a better tolerance to this stress. Desiccation – triggered by in vitro air exposure – led to significant increase in ROS, inhibition of antioxidant enzymes, cellular alterations, and photosynthetic inactivation, mainly in the lower intertidal *L. spicata* and *G. rex*. Detrimental effects observed during rehydration were also stronger in the lower intertidal species. Hence, these results help to explain the bathymetric segregation of seaweed species within the intertidal zone.

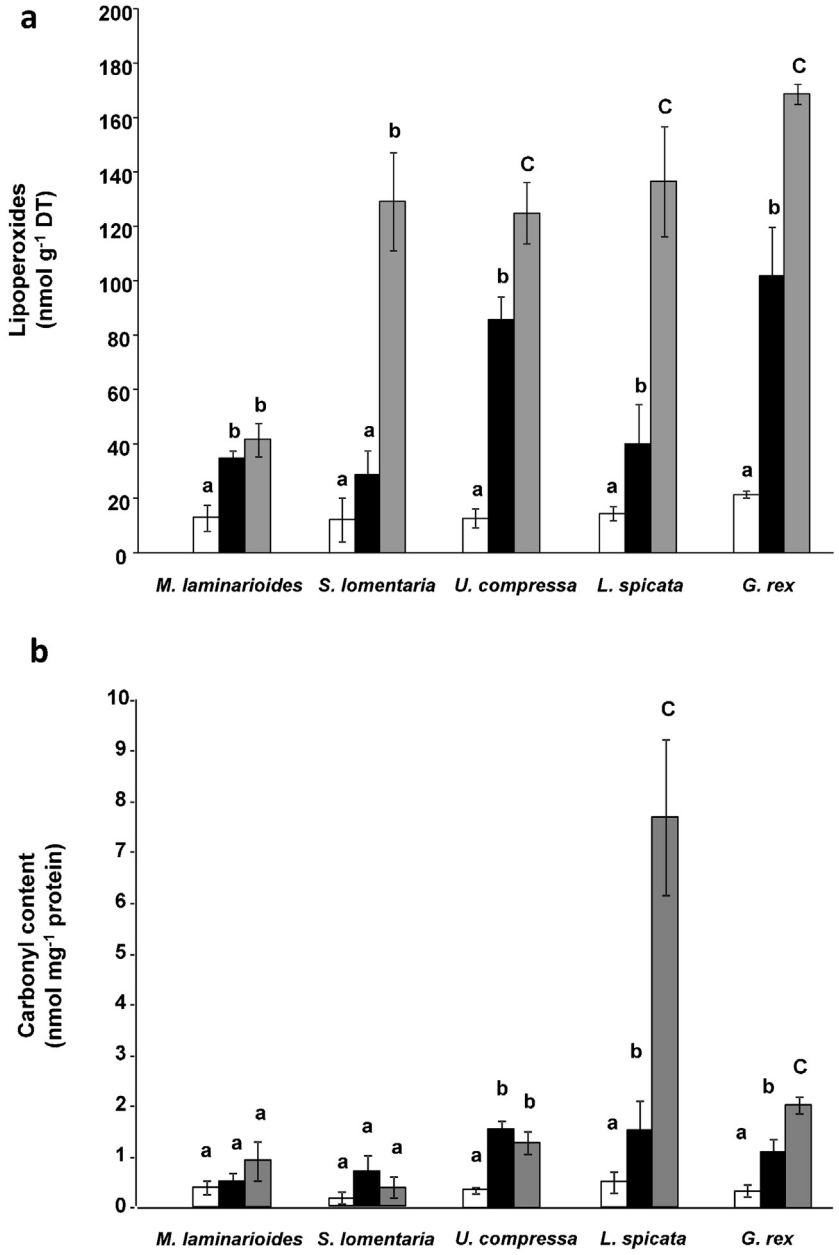


Fig. 3. Lipid peroxidation (a) and protein oxidation (b) in *Mazzaella laminarioides*, *Scytosiphon lomentaria*, *Ulva compressa*, *Lessonia spicata* and *Gelidium rex* during hydration (white bars), desiccation (black bars) and rehydration (gray bars). Values represent the mean of 3–6 independent replicates ± SD. Letters indicate significant differences ($P < 0.05$) between treatments for each species. DT, dry tissue.

4.1. Antioxidant mechanisms and desiccation tolerance

It was clear that different seaweed species displayed different desiccation tolerance and such a difference was reflected in their antioxidant metabolism. Control of ROS during desiccation by the antioxidant system has been demonstrated in intertidal seaweeds during tide-driven desiccation (Collén and Davison, 1999b; Contreras-Porcia et al., 2011). For example, the medium to high intertidal *Mastocarpus stellatus* (Gigartinales, Rhodophyta) (Collén and Davison, 1999b) and *P. columbina* (Contreras-Porcia et al., 2011) regulate ROS production during desiccation by activating their antioxidant metabolism more effectively than lower intertidal species. Some land plants have similar responses to desiccation. In the primitive *Selaginella bryopteris*, desiccation leads to ROS concentrations three times higher than basal levels; however this excess is efficiently buffered during rehydration by its antioxidant metabolism (Pandey et al., 2010). Antioxidant control of ROS has been also reported in resurrection plants – a heterogeneous group, mostly angiosperms – that can revive after extended periods of dehydration (Alpert, 2006), a feature used to explain their high tolerance to desiccation. Among ROS, hydrogen peroxide has been described as an important cellular signaling molecule involved in diverse physiological responses (Rhee, 2006). However, under conditions of high concentration and low buffering capacity of the stressed organisms, ROS can be extremely harmful. This is coincident with our current results, where middle to lower intertidal species (*U. compressa*, *L. spicata* and *G. rex*) displayed high levels of hydrogen peroxide accumulation even during rehydration and, consequently, they are less tolerant to desiccation, which is demonstrated by cellular alteration and antioxidant enzymatic inactivation. The high intertidal species *P. columbina*, on the other hand, rapidly buffered ROS excess after a brief rehydration period

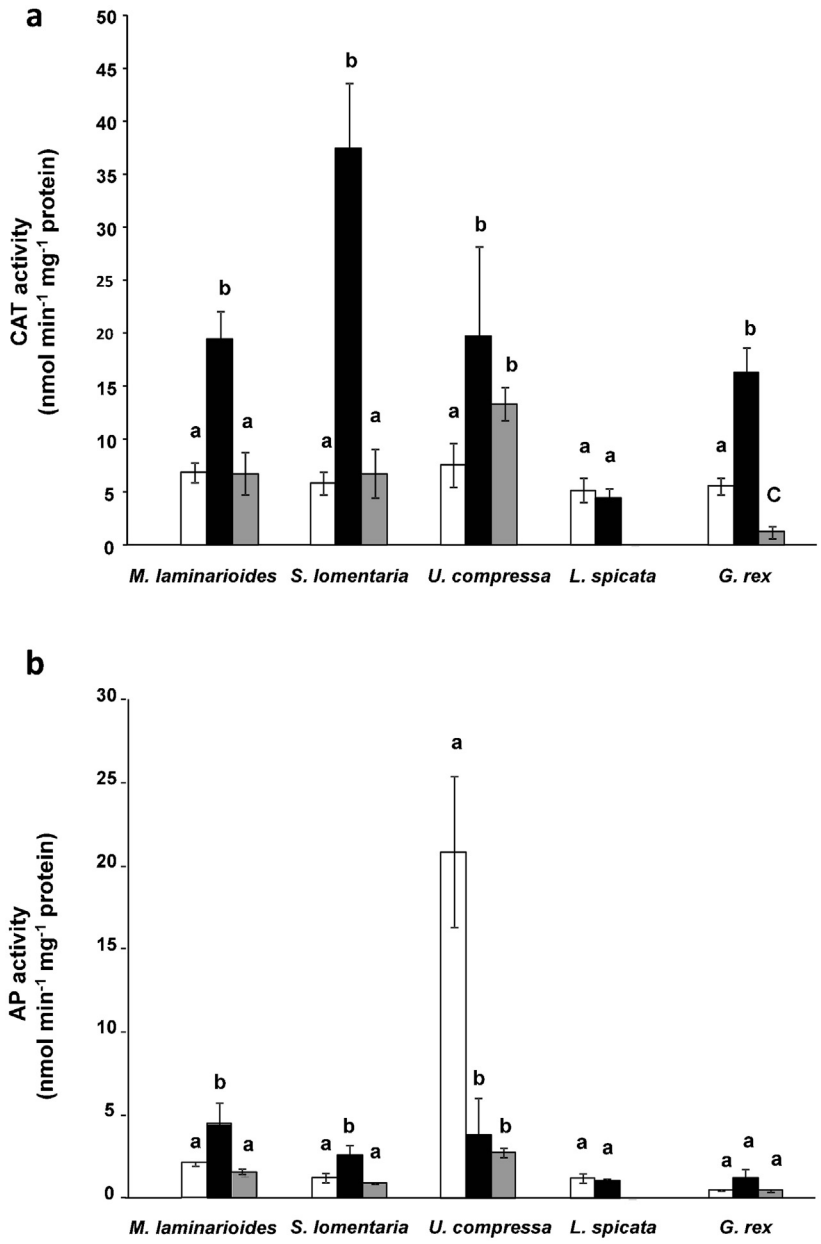


Fig. 4. Activity of the antioxidant enzymes CAT (a) and AP (b) in *Mazzaella laminarioides*, *Scytosiphon lomentaria*, *Ulva compressa*, *Lessonia spicata* and *Gelidium rex* during hydration (white bars), desiccation (black bars) and rehydration (gray bars). Values represent the mean of 3–6 independent replicates ± SD. Letters indicate significant differences ($P < 0.05$) between treatments for each species.

(Contreras-Porcia et al., 2011) and, as expected, this is a species highly tolerant to desiccation. Based on the above, it is suggested that low intertidal species, as those used in this study, do not have the appropriate capacity to attenuate ROS excess during desiccation. This physiological limitation, instead, could make them poorly suited to inhabit the higher intertidal. Contrarily, species well adapted to flourish in the higher intertidal, like *P. columbina*, keep ROS under control by activating their antioxidant system, minimizing or eliminating oxidative damage of the cells.

4.2. ROS excess and cell damage

The accumulation of lipoperoxides and carbonyls are a clear indication that ROS buffering mechanisms are not effective (Achary et al., 2008; Jackie et al., 2011). Our results demonstrated an overproduction of these oxidative markers in low intertidal species concurrent to high ROS production (i.e. H_2O_2). Exceptions to this pattern were evident in *M. laminarioides* and *S. lomentaria* where lipoperoxides remained high during rehydration even though ROS levels were drastically reduced. A similar situation was reported in *S. lomentaria* (Contreras et al., 2009), where exposure to copper stress induced arachidonic acid-dependent lipoxygenase activity (LOX), a defense enzyme involved in the production of derivatives of fatty acid oxylipins (Howe and Schilmiller, 2002). Thus, *S. lomentaria*, and probably *M. laminarioides*, might require the induction of additional tolerance pathways (e.g. oxylipins production) to buffer oxidative stress caused by desiccation. In contrast, in desiccation tolerant plants of *P. columbina* there was no evidence of excess lipoperoxides during the desiccation/rehydration cycle (Contreras-Porcia et al., 2011). This can be explained by the activation of peroxiredoxins (PRXs) (Contreras-Porcia et al., 2011, 2013) that use fatty acid hydroperoxides as substrate, inhibiting the accumulation of oxidized molecules under a stress condition (Lovazzano et al., 2013). In resurrection plant under desiccation stress the activation

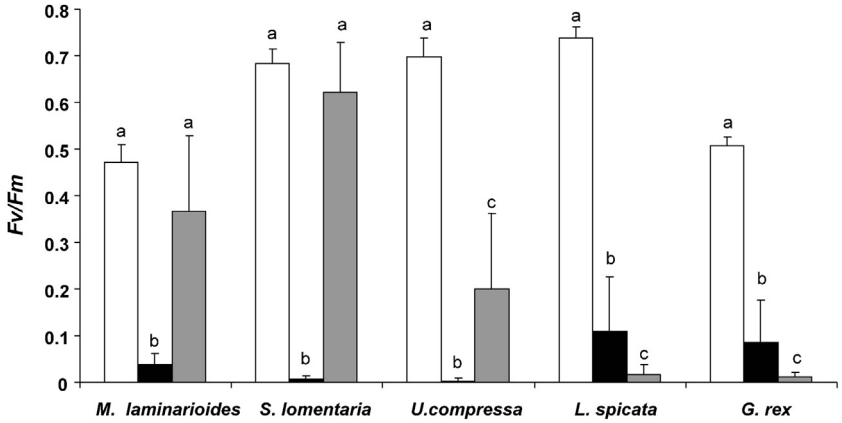


Fig. 5. Photosynthetic efficiency (*Fv*/*Fm*) in *Mazzaella laminarioides*, *Scytosiphon lomentaria*, *Ulva compressa*, *Lessonia spicata* and *Gelidium rex* during hydration (white bars), desiccation (black bars) and rehydration (gray bars). Values represent the mean of 5–10 independent replicates ± SD. Letters indicate significant differences ($P < 0.05$) between treatments for each species.

of PRX is also an important part of desiccation stress mechanisms (Leprince and Buitink, 2010). Thus, our results indicate that lower intertidal species are more exposed to oxidative damage caused by desiccation due to their limited antioxidant capabilities.

4.3. Ecological consequences of limited antioxidant capacity

In spite the scarcity of information, the occurrence and effectiveness of antioxidant mechanisms seems to be a requirement in plants inhabiting environments which cause desiccation. This is true in groups of plants phylogenetically quite distant, such as the resurrection plants and the high intertidal seaweeds (Collén and Davison, 1999b; Farrant, 2000; Hoekstra et al., 2001; Burritt et al., 2002; Alpert, 2006; López-Cristoffanini et al., 2013). For example, antioxidant activity in resurrection plants increases during desiccation (Le and McQueen-Mason, 2006; Moore et al., 2009), and this has been used as argument for explaining the high tolerance of these plants to their dry habitats. In seaweeds, two studies have reported the relationship between the abatement of ROS excess by enhanced antioxidant activity and tolerance to desiccation stress (*Stictosiphonia arbuscula* (Ceramiales, Rhodophyta), Burritt et al., 2002 and *P. columbina*, Contreras-Porcia et al., 2011). Consistent with the previous argument, desiccation intolerant species seem to have less effective or inefficient antioxidant systems. A possible role of the antioxidant enzymes, including AP, on desiccation tolerance become apparent from our findings where, in middle and lower intertidal species like *U. compressa*, *L. spicata* and *G. rex*, they were inactivated or had an activity below detection limits. We further believe that high levels of protein oxidation during desiccation and rehydration in these species may be an indication that enzymes, including AP, are inactivated. Sampath-Wiley et al. (2008) studied the seasonal effects of sun exposure and rehydration during tidal emersion in *Porphyra umbilicalis* (Bangiales, Rhodophyta) suggested that the elevated level of antioxidant enzymes and carotenoids during emersion contributes to its colonization in upper intertidal region. Similarly, López-Cristoffanini et al. (2013) suggested that consequences of limited antioxidant capacity in seaweeds may also affect the geographic distribution in species such as *L. spicata*, wherein desiccation was accompanied with inactivation of antioxidant enzymes (AP and CAT). Thus, the inability of middle-low intertidal species to grow in the high intertidal zone could be explained by an inefficient antioxidant system.

4.4. Photosynthetic performance and desiccation tolerance

The functional status of the photosynthetic system of various seaweeds and plant models has been widely used as an indicator of stress caused by desiccation (Bell, 1993; Skene, 2004; Varela et al., 2006; Gylle et al., 2009; Pandey et al., 2010; Schagerl and Möstl, 2011). For example, in the resurrection plant *S. bryopteris*, the rapid and complete recovery of *Fv*/*Fm* after rehydration clearly indicates the absence of marked photoinhibitory or thermal injury to PSII during desiccation. In intertidal and sublittoral ecotypes of *Fucus vesiculosus* (Fucales, Heterokontophyta) responded to desiccation with a different efficient photosynthetic intensity, and the intertidal ecotype appeared more resistant (Gylle et al., 2009). Similar responses were reported in individuals of *M. laminarioides*, where high intertidal individuals had ratios significantly higher than those plants from the lower intertidal when exposed to desiccation stress (Varela et al., 2006). In both resurrection plants and in *P. columbina* (Farrant et al., 2003; Contreras-Porcia et al., 2011), photoinhibition was suggested as the tolerance mechanism to cope with desiccation. This is the result of a reversible reduction of the photosynthetic quantum yield due to down-regulation of PSII, where energy is thermally dissipated. Even though carbon fixation could be inhibited during desiccation, electron flow might continue and thus form ROS (Dinakar et al., 2012). Given this, photoinhibition could prevent ROS formation and help to overcome desiccation (e.g. Figueroa et al., 1997; Korbee et al., 2005; Contreras-Porcia et al., 2011). However, the current results suggest that in species of lower intertidal distribution (*U. compressa*, *L. spicata*, and *G. rex*) the poorer photosynthetic performance under desiccation/rehydration cycle is generated by a disarray of the photosynthetic machinery under this stressful condition and not by means of a photoinhibition mechanism. Thus, diverse tolerance mechanisms need to be activated to cope with this environmental condition, which are deficient and/or absent in lower intertidal species.

5. Conclusion

The results of this study supported the hypothesis that the presence and adequacy of functional responses to desiccation in common seaweeds defines their altitudinal position in the intertidal zone. These differences seem to be independent of the algal division or the functional-form groups to which the plants belong. High intertidal species are tolerant to desiccation and that tolerance seems to be based on the various mechanisms that efficiently attenuate ROS excesses caused by desiccation (high antioxidant activity, biomolecule oxidation attenuation and photoinhibition tolerance). By the contrary, lower intertidal species are less tolerant to desiccation and this is likely due to their inefficiency in attenuating the ROS excess produced during desiccation and early recovery.

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