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Stress tolerance and reactive oxygen metabolism in the intertidal red seaweeds *Mastocarpus stellatus* and *Chondrus crispus*

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ABSTRACT

***Mastocarpus stellatus* and *Chondrus crispus* are morphologically similar red seaweeds that co-occur on rocky intertidal seashores in the Northern Atlantic. *Mastocarpus stellatus* grows higher on the shore and is more tolerant of environmental stress, caused by factors such as freezing and desiccation, than *C. crispus*. Here we report a correlation between reactive oxygen metabolism and stress tolerance, which suggests that reactive oxygen metabolism may play a role in stress tolerance of intertidal red seaweeds. *Mastocarpus stellatus* scavenged added H_2O_2 slightly faster, and was more resistant to oxidative stress induced by addition of H_2O_2 and Rose Bengal, than *C. crispus*. These data were consistent with higher levels of ascorbate and β-carotene and higher activities of catalase and glutathione reductase, in *M. stellatus*. Tocopherol content and activities of superoxide dismutase and ascorbate peroxidase were similar in both species. Activities of reactive oxygen scavenging enzymes generally increased with tidal height in *M. stellatus*; this was, however, not a consistent trend in *C. crispus*.**

Key-words: *Chondrus; Mastocarpus*; anti-oxidants; ascorbate peroxidase; catalase; intertidal; oxidative stress; reactive oxygen; superoxide dismutase; zonation

INTRODUCTION

A common denominator of many types of stress afflicting photosynthetic organisms, including freezing, high light, temperature, ozone and desiccation stress, is the increased formation of reactive oxygen (McKersie & Leshem 1994). Reactive oxygen includes a number of compounds; these differ in reactivity, but all are more reactive than ground state oxygen. Examples of reactive oxygen are singlet oxygen ($^1O_2$), superoxide ions (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH·). Formation of these compounds is a normal part of the metabolism of plants and animals but excessive production can cause damage to DNA, proteins and lipids (Halliwell & Gutteridge 1989). Reactive oxygen metabolism in plants has been reviewed by Alscher, Donahue & Cramer (1997), McKersie & Leshem (1994) and Asada & Takahashi (1987).

The defence system against reactive oxygen in plants includes anti-oxidants such as ascorbate, glutathione, β-carotene and α-tocopherol and reactive oxygen scavenging enzymes, such as catalase (EC 1·11·1·6), superoxide dismutase (SOD, EC 1·15·1·1), glutathione reductase (GR, EC 1·6·4·2) and ascorbate peroxidase (APX, EC 1·11·1·11). These mechanisms work in concert, or alone, to reduce the levels of reactive oxygen in the cells. Generally, stress-tolerant plants have a more effective defence system against reactive oxygen than stress-susceptible species; this may be constitutive or induced by exposure to stress. Higher content of anti-oxidants, and increased activities of reactive oxygen scavenging enzymes, have been documented after stress, or correlated with stress tolerance, in, for example, the dinoflagellate *Peridinium gatunense* (Butow, Wynne & Tel-Or 1994), brown algae of the genus *Fucus* (Collén & Davison 1999a,b), spinach (*Spinacia oleracea*) (Schöner & Krause 1990), alfalfa (*Medicago sativa*) (McKersie *et al*. 1993), maize (*Zea mays*) (Pastori & Trippi 1993), tobacco (*Nicotiana tabacum*) (Sen Gupta *et al*. 1993), cotton cells (*Gossypium hirsutum*) (Gossett *et al*. 1996), and pea leaves (*Pisum sativum*) (Donahue *et al*. 1997). In alpine environments, where light, ultraviolet and freezing stress increase with altitude, so do levels of anti-oxidants in alpine herbs and trees (Bermadinger-Stabentheiner 1996; Wildi & Lütz 1996). Overall, these findings suggest that more effective reactive oxygen metabolism may increase stress tolerance.

Intertidal seaweeds experience drastic environmental changes on a daily basis. During low tide they may be exposed to desiccation, hyper- or hypo-osmotic shock, high or freezing temperatures or high light, depending on season and latitude. The frequency and duration of these stresses increases with tidal elevation. The zonation patterns typical of rocky intertidal seashores, with different species occupying different levels, sometimes only centimetres apart, can partially be explained by differences in stress tolerance (Davison & Pearson 1996). Generally, stress tolerance against, for example, high light, freezing and desiccation increases with tidal elevation from stress-susceptible low-shore species to stress-tolerant high-shore forms. The mechanisms of environmental tolerance in intertidal seaweeds are not thoroughly known, but it has been suggested that

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reactive oxygen metabolism may play a key role (Davison & Pearson 1996; Collén & Davison 1999a,b).

We previously found a correlation between stress tolerance and reactive oxygen metabolism in three species of the brown algal genus *Fucus* (Collén & Davison 1999a,b). The least stress-tolerant species, *F. distichus*, which occupies tidal pools and thereby inhabits a less stressful environment, generally had the lowest activities of reactive oxygen scavenging enzymes and the lowest content of anti-oxidants and showed a drastic increase of reactive oxygen production after exposure to desiccation or freezing stress. The most stress-tolerant species, *F. spiralis,* which lives highest on the shore, had higher activities of SOD and APX than *F. evanescens,* which lives in the lower to middle intertidal. Here we describe a series of experiments designed to determine whether the stress tolerance of red algae could also be explained by reactive oxygen metabolism.

Mastocarpus stellatus Stackhouse and *Chondrus crispus* (Stackhouse) Guiry are two morphologically similar intertidal red macroalgae, belonging to the order Gigartinales, that occur on the western and eastern coasts of the northern to middle Atlantic (Guiry & West 1983). At the site of collection and at many other places along the Maine coast, *C. crispus* occurs in the low intertidal, with *M. stellatus* occurring higher on the shore (Dudgeon, Davison & Vadas 1989). Between the pure stands of two species is a mixed zone where both species occur at the same tidal elevation. *Mastocarpus stellatus* is more desiccation- and freezing-tolerant than *C. crispus* (Davison, Dudgeon & Ruan 1989; Dudgeon, Davison & Vadas 1989, 1990, Dudgeon *et al*. 1995). However, *C. crispus* has the ability to acclimatize to freezing stress; photosynthesis of *C. crispus* subjected to moderate repeated freezing was more tolerant to freezing than control plants (Dudgeon *et al*. 1990). This acclimatization to freezing stress was not found in *M. stellatus*. We wanted to determine whether part of the differences in stress tolerance between the two species could be explained by differences in reactive oxygen metabolism.

Here we test three hypotheses.

(1) *M. stellatus* is better able to tolerate reactive oxygen than *C. crispus,* which could partly explain the higher stress tolerance of *M. stellatus*.
(2) *Mastocarpus stellatus* scavenges reactive oxygen more efficiently than *C. crispus*, with higher contents of anti-oxidants and/or higher activities of reactive oxygen scavenging enzymes.
(3) *Chondrus crispus,* but not *M. stellatus,* exhibits changes in reactive oxygen metabolism with tidal height, caused by its ability to acclimatize.

MATERIALS AND METHODS

Plant material

Chondrus crispus Stackhouse (Gigartinales, Rhodophyta) and *Mastocarpus stellatus* (Stackhouse) Guiry (Gigartinales, Rhodophyta) were collected at low tide from the shore at Long Cove Point, Chamberlain, Maine (43°54′N, 69°28′W) in September and October 1997. Samples for analyses of anti-oxidants and reactive oxygen scavenging enzymes were collected immediately after being uncovered by the tide, and frozen and kept in liquid nitrogen until analyses were made. The seaweeds were collected both from the area of greatest abundance and also from the mixed area between the more or less uni-algal zones. The physiology and ecology of these populations have been studied earlier by Davison *et al*. (1989), Dudgeon *et al*. (1989, 1990, 1995) and Kübler & Davison (1993, 1995). Fronds used to determine *in vivo* scavenging of H_2O_2 and toxicities of H_2O_2, methyl viologen and Rose Bengal were collected from the areas of greatest abundance, cleaned from visible epiphytes, and kept in seawater in the laboratory at 12 °C at an irradiance of 50 μmol photons m^{-2} s^{-1} (provided by cool white fluorescent tubes) in a 16 h light:8 h dark cycle no longer than 36 h before experiments.

Resistance to oxidative stress

Oxidative stress was induced by incubating seaweeds in: (1) different concentrations of H_2O_2; (2) methyl viologen (MV), which induces formation of O_2^- (Asada & Takahashi 1987) which is subsequently disproportionated spontaneously or by the aid of SOD to H_2O_2 and oxygen; and (3) Rose Bengal, which increases the formation of $^1O_2$ (Foote 1976). The seaweeds were pre-incubated for 12 h in darkness at 12 °C in seawater with MV and Rose Bengal before experiments. Incubations in hydrogen peroxide were performed in a flow-through system that maintained constant concentrations of H_2O_2. Approximately 1 g FW of apices were incubated in transparent PVC tubes with a continuous flow (600 mm^3 s^{-1}) of seawater, with or without additions of H_2O_2.

Resistance against oxidative stress was measured by studying changes in F_v/F_m after exposure to different concentrations of H_2O_2 or reactive oxygen inducers after 4 h in the light (350 μmol photons m^{-2} s^{-1}) at 12 °C. Light was provided by a tungsten–halogen lamp in a slide projector and measured with a 4 π collector (QSL-100, Biospherical Instruments Inc., San Diego, California, USA). Fluorescence data were obtained with a modulated fluorometer (Opti-Sciences OS-500, Haverhill, Massachusetts, USA). Seaweeds were incubated in darkness 15 min before measurements of fluorescence.

In vivo H_2O_2 scavenging

To study *in vivo* scavenging of hydrogen peroxide, 1 g fresh weight (FW) of seaweed thalli were incubated in 20 cm^3 seawater with an initial addition of 100 mmol m^{-3} H_2O_2 at 12 °C. These experiments were performed under low light (40 μmol photons m^{-2} s^{-1}) with gentle air bubbling to achieve mixing. Samples (40 mm^3) were taken at different time points from the seawater surrounding the seaweeds, diluted 50 times with seawater, after which H_2O_2 was measured spectrofluorometrically according to Miller & Kester (1988). The concentrations used in the scavenging experi-

ments were lower than used in the experiments with toxicity of H_2O_2 to more closely mimic natural (chloroplastic) H_2O_2 concentrations.

Anti-oxidants

The content of ascorbate was measured according to Foyer, Rowell & Walker (1983). Apices of approximately 0·1 g FW were ground in liquid nitrogen, mixed with 1 cm^3 2·5 kmol m^{-3} $HClO_4$ and incubated on ice for 30 min with occasional mixing. The extracts were neutralized with 1·25 kmol m^{-3} K_2CO_3 and centrifuged. The ascorbate content was determined by following the decrease in absorbance at 265 nm after addition of 5 U cm^{-3} ascorbate oxidase to 700 mm^3 sodium phosphate buffer (100 mol m^{-3}, pH 5·6 with 1% polyvinylpyrrolidone, molecular weight 40 000 [PVP-40]) and 50 mm^3 sample. Amounts were quantified with a standard curve using authentic ascorbate treated as the samples.

Total glutathione (reduced and oxidized) was measured according to Anderson (1985). Samples were ground in liquid nitrogen and extracted with a volume five times the FW in 5% 5-sulphosalicylic acid with 1% PVP-40. The assay contained 700 mm^3 buffer (143 mol m^{-3} sodium phosphate pH 7·5 with 6·3 mol m^{-3} Na-EDTA and 300 mmol m^{-3} NADPH), 100 mm^3 of 6 mol m^{-3} 5,5′-dithio-bis(2-nitrobenzoic acid), 175 mm^3 water and 25 mm^3 sample, mixed, and 25 mm^3 glutathione reductase (266 U cm^{-3}) were then added. The rate of increase in absorbance at 412 nm at 30 °C was measured and quantified against a standard curve prepared from authentic GSH.

For the extraction of tocopherols, apices of approximately 0·1 g FW were ground in liquid nitrogen, mixed with 1 cm^3 methanol and 4 cm^3 hexane and vortexed for 5 min. The samples were centrifuged and the hexane phase collected. The extraction was repeated twice. The hexane phases were pooled and evaporated under a flow of nitrogen at room temperature. The residues were diluted by 20 times the fresh weight of methanol before analysing tocopherol content by HPLC. Separation was performed according to De Roeck-Holtzhauer *et al*. (1991) on a reversed phase C-18 column (Supplecosil LC-18) with a mixture of methanol and water (98:2, flow 2 cm^3 min^{-1}). Detection was performed fluorometrically with excitation at 296 nm and emission at 330 nm using a Shimadzu RF-551 detector. Amounts were quantified using authentic α-tocopherol (Sigma).

For measurements of β-carotene and chlorophyll, approximately 0·1 g FW was ground in liquid nitrogen and extracted twice with 5 times the fresh weight of acetone. β-Carotene content was measured spectrophotometrically after centrifugation and calculated using the equations of Seely, Duncan & Vidaver (1972).

Enzyme extraction and assays

Samples (0·1–0·2 g FW) for assays of soluble SOD were ground in liquid nitrogen and extracted with 1 cm^3 50 mol m^{-3} potassium phosphate buffer (pH 7·0) containing 0·25% Triton X-100 and 1% w/v PVP-40. Extracts were centrifuged for 5 min at 20 000 *g* at 4 °C. The supernatant was dialysed for 45 min at 0 °C against 50 mol m^{-3} potassium phosphate buffer (pH 7·0) using Slide-A-Lyzer dialysis cassettes (Pierce, Rockford, Illinois, USA). SOD activity was measured according to Mishra *et al*. (1993). The assay mixture (700 mm^3) contained: 50 mol m^{-3} potassium phosphate buffer pH 7·8, 100 mmol m^{-3} EDTA, 18 mmol m^{-3} cytochrome *c* and 0·1 mol m^{-3} xanthine. Xanthine oxidase (Sigma) was added to give an increase of absorbance at 550 nm of 0·025 ± 0·002 min^{-1} at 20 °C. Samples (10–90 mm^3) and H_2O were added to give a final volume of 800 mm^3, and the change of absorbance at 550 nm was measured for an additional minute. SOD activities were expressed according to McCord & Fridovich (1969).

Samples for assay of catalase were ground in liquid nitrogen and extracted with five times the fresh weight of the same buffer used for SOD extraction. Catalase activity was analysed according to Aebi (1984); 50 mm^3 extract was added to 700 mm^3 potassium phosphate buffer (50 mol m^{-3}, pH 7·0). The reaction was started by the addition of 11 mol m^{-3} H_2O_2, and followed by monitoring the decrease in absorbance at 240 nm at 20 °C for 1–3 min (this resulted in a decrease in absorbance from 0·45 to approximately 0·40).

Apices for assays of ascorbate peroxidase were ground in liquid nitrogen and extracted in five times the fresh weight of potassium phosphate buffer (pH 7·0, 50 mol m^{-3}) containing 1% PVP-40, 0·25% Triton X-100 and 0·5 mol m^{-3} ascorbate. Extracts were centrifuged for 5 min at 20 000 *g* at 4 °C. APX activity was assayed according to Nakano & Asada (1981). The decrease of absorbance at 290 nm was followed for 30 s after adding 0·1 mol m^{-3} H_2O_2 to a buffer containing 5–10% extract, potassium phosphate buffer (pH 7·0, 50 mol m^{-3}) containing 0·1 mol m^{-3} EDTA and 0·5 mol m^{-3} ascorbate. The assays were performed at 20 °C.

Glutathione reductase activity was assayed according to Sen Gupta *et al*. (1993). Apices were ground in liquid nitrogen and extracted in five times the fresh weight of potassium phosphate buffer (pH 7·0, 50 mol m^{-3}) containing 1% PVP-40, 0·25% Triton X-100 and 0·1 mol m^{-3} EDTA. Extracts were centrifuged for 5 min at 20 000 *g* at 4 °C. In the assay, 60 mm^3 extract was mixed with 600 mm^3 assay buffer (100 mol m^{-3} Tris–HCl pH 7·8, containing 2 mol m^{-3} EDTA, 50 mol m^{-3} NADPH and 0·5 mol m^{-3} oxidized glutathione) The change in absorbance at 340 nm was followed for 180 s at 20 °C.

Glutathione peroxidase (EC 1·11·1·9) activity was assayed according to Navari-Izzo & Izzo (1994). The tissue was ground in liquid nitrogen, extracted in three times the fresh weight of 20 mol m^{-3} potassium phosphate buffer (pH 6·8) containing 0·1 mol m^{-3} EDTA and 1% PVP-40. In the assay, 700 mm^3 of 20 mol m^{-3} potassium phosphate buffer (pH 6·8) containing 2 mol m^{-3} reduced glutathione, 2·5 mol m^{-3} KCN and 0·24 mol m^{-3} NADPH was mixed with 3·75 mm^3 0·3% H_2O_2 and 25 mm^3 glutathione reductase (200 U cm^{-3}, Sigma). Extract (15–25 mm^3) was added

and the change in absorbance at 340 nm caused by oxidation of NADPH was measured. Protein content of the enzyme extracts was measured according to Bradford (1976) using bovine serum albumin as standard.

Statistics

Data were analysed using one way analysis of variance (ANOVA), with differences between species and growth location determined using Duncan's multiple comparisons test. Differences were considered to be significant at a probability of 5% ($P < 0{\cdot}05$). Tests were performed using the StatMost32 statistical program (DataMost Corp.).

RESULTS

Resistance to oxidative stress

Both *M. stellatus* and *C. crispus* were highly resistant to reactive oxygen-inducing chemicals. Concentrations up to 20 mol m^{-3} of methyl viologen in the seawater did not cause increased photo-inhibition, i.e. reduce F_v/F_m values, after 4 h at 350 μmol photons m^{-2} s^{-1}, relative to controls without addition (Fig. 1). *Mastocarpus stellatus* also tolerated additions of Rose Bengal at concentrations up to 100 mmol m^{-3}, whereas at 100 mmol m^{-3} a decrease of F_v/F_m values could be seen in *C. crispus*. Addition of 1 mol m^{-3} H_2O_2 to the seawater caused no reduction in F_v/F_m values in *M. stellatus*, but a 14% decrease in *C. crispus*. Concentrations of 10 mol m^{-3} H_2O_2 caused a reduction of F_v/F_m values in both species; in *M. stellatus* the reduction was 73% and in *C. crispus* F_v/F_m values fell to almost zero.

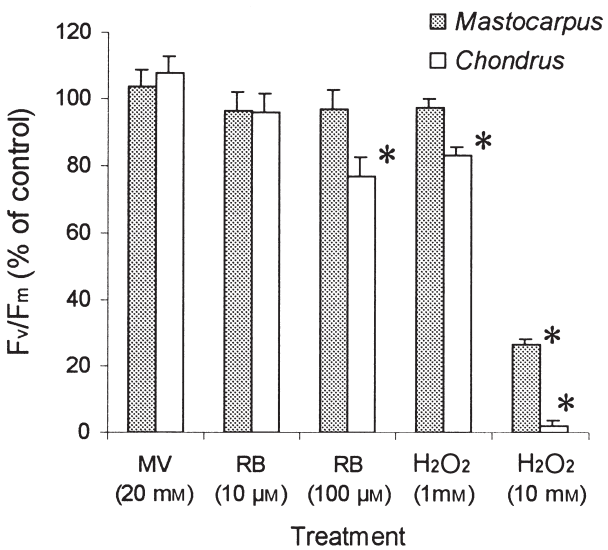

Figure 1. Effect of methyl viologen (MV), Rose Bengal (RB) and hydrogen peroxide (H_2O_2) on photo-inhibition, as F_v/F_m values, in *Mastocarpus stellatus* and *Chondrus crispus*. The seaweeds were pre-incubated for 12 h in darkness with different concentrations of MV and RB. Seawater with H_2O_2 was added to 1 g FW with a flow of 600 mm^{-3} s^{-1}. F_v/F_m values were measured after 4 h incubation at 12 °C in an irradiance of 350 μmol photons m^{-2} s^{-1}. Means and SE are shown, $n = 5$. Values were compared within species and treatment, and values significantly different ($P < 0{\cdot}05$) from controls are indicated by an asterisk (*).

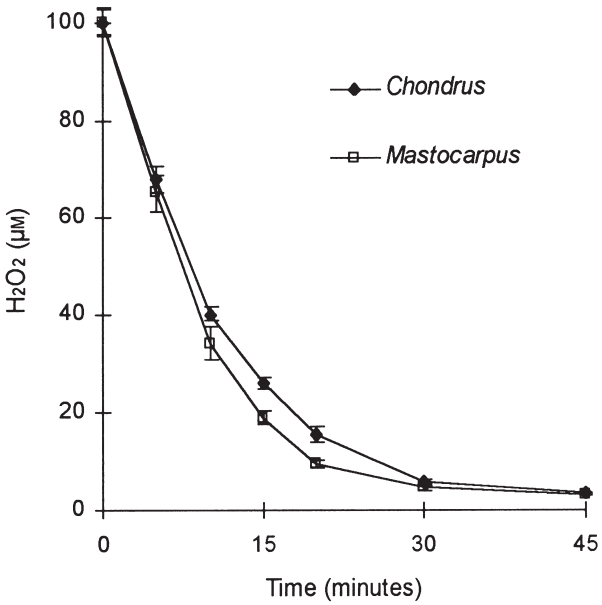

Figure 2. *In vivo* scavenging of added H_2O_2 by *Mastocarpus stellatus* and *Chondrus crispus*. Seaweeds (1 g FW in 20 cm^3 of seawater) were incubated in seawater with an initial addition of 100 mmol m^{-3} H_2O_2 at an irradiance of 40 μmol photons m^{-2} s^{-1} at 12 °C with gentle bubbling. Means and SE are shown, $n = 3$.

Scavenging of H_2O_2

When subjected to experimental additions of 100 mmol m^{-3} H_2O_2, *M. stellatus* scavenged hydrogen peroxide slightly faster *in vivo* than *C. crispus* (Fig. 2). After 30 min, the extracellular concentrations of H_2O_2 were less than 6 mmol m^{-3} in both species. The disappearance of H_2O_2 showed first-order kinetics for the first 30 min. There was minimal spontaneous decrease in H_2O_2 concentration in the absence of seaweeds (not shown).

Anti-oxidants and pigments

The content of ascorbate was significantly higher in *M. stellatus* than *C. crispus*. No difference was seen in content within one species at different tidal heights (Fig. 3A). The amounts of glutathione were approximately one-tenth those of ascorbate, with significantly higher levels in *C. crispus* than *M. stellatus*; again no difference was observed between growth locations within one species (Fig. 3B).

Levels of α-tocopherol were not significantly different between two species in the mixed zone, or between the two *M. stellatus* sites. However, levels were significantly lower in the lowest *C. crispus* zone (Fig. 4A). Contents of other

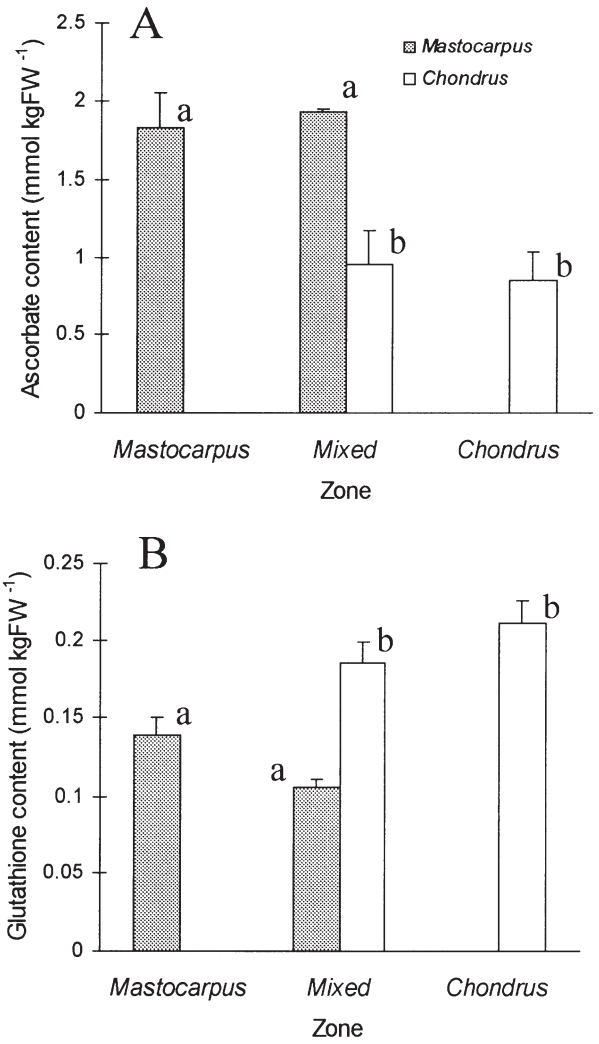

Figure 3. Content of ascorbate and total glutathione in *Mastocarpus stellatus* and *Chondrus crispus*. Means and SE are shown, $n = 5$. The mid-shore was dominated by a more or less homogenous zone of *M. stellatus* (*Mastocarpus*), below which was a mixed layer (*Mixed*), with the lowest part of the intertidal being an almost pure stand of *C. crispus* (*Chondrus*). Values were compared within species and zone, with values that were significantly different ($P < 0{\cdot}05$) being indicated by different letters.

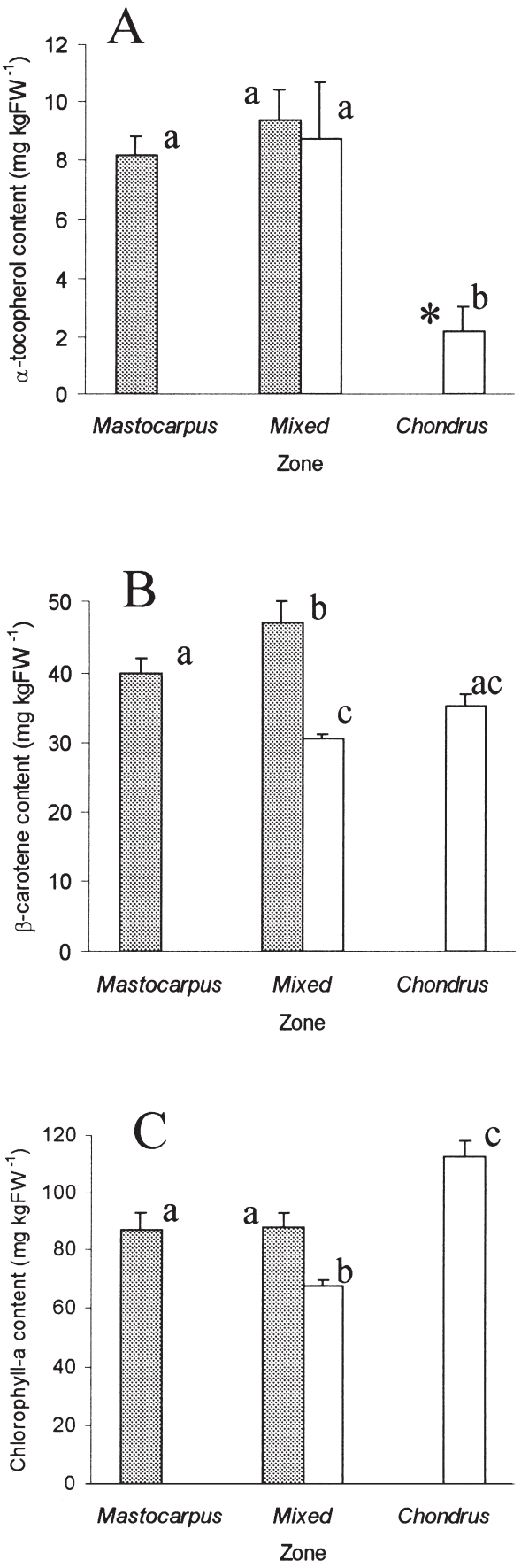

Figure 4. Content of α-tocopherol (A), β-carotene (B) and chlorophyll-a (C) in *Mastocarpus stellatus* and *Chondrus crispus*. Values were compared within species and zone, with values that were significantly different ($P < 0{\cdot}05$) being indicated by different letters. Means and SE are shown, $n = 5$. *Seaweeds from the lowest *C. crispus* zone contained a higher content of other tocopherols (β-, γ- and δ-tocopherol) than the higher zone and the difference in total tocopherol content is therefore less than shown.

tocopherols (β-, γ- and δ-tocopherol) were higher in the lowest *C. crispus* zone (not shown). Differences in total tocopherol content are therefore not as large as indicated in Fig. 4(A). The content of β-carotene was significantly higher in *M. stellatus* than in *C. crispus*; and in both species β-carotene increased in the lower growth location (Fig. 4B). The content of chlorophyll *a* was similar from both growth locations in *M. stellatus*, and was significantly higher than the chlorophyll *a* content of *C. crispus* in the mixed zone, but lower than that of *C. crispus* in the lowest zone (Fig. 4C).

Reactive oxygen scavenging enzymes

Activities of SOD were similar in all samples, except for *M. stellatus* from the mixed (lower) zone, which had lower activity than the pure (higher) zone (Fig. 5A). Catalase activity of *M. stellatus* was more than twice that of *C. crispus*. No difference was found between the activity of catalase in different growth locations of one species (Fig. 5B).

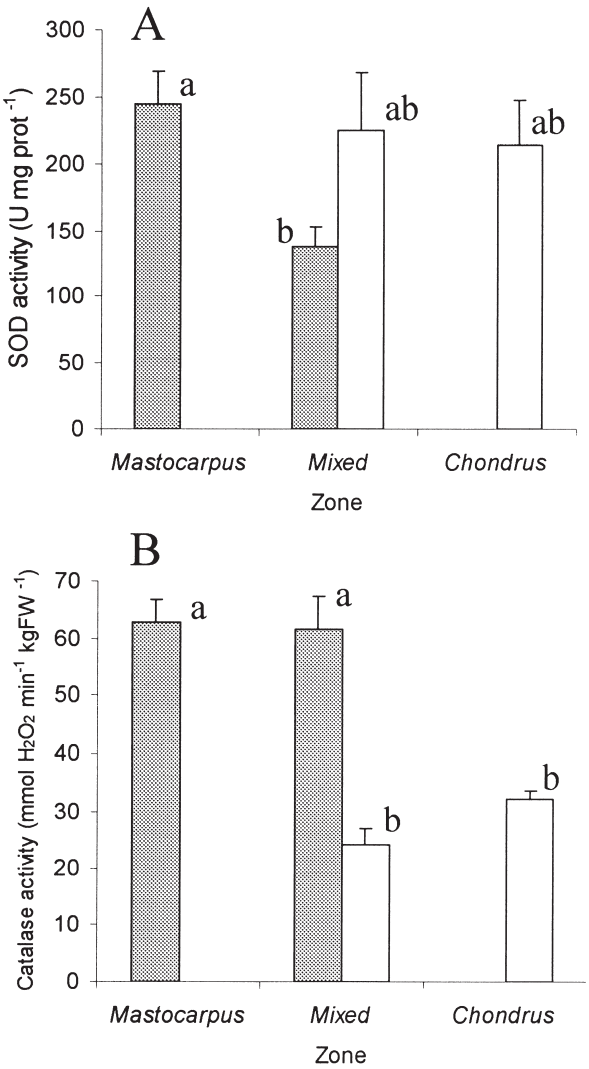

Figure 5. Activities of (A) superoxide dismutase (SOD) and (B) catalase in *Mastocarpus stellatus* and *Chondrus crispus*. Values were compared within species and zone, with values that were significantly different ($P < 0{\cdot}05$) being indicated by different letters. Means and SE are shown, $n = 5$.

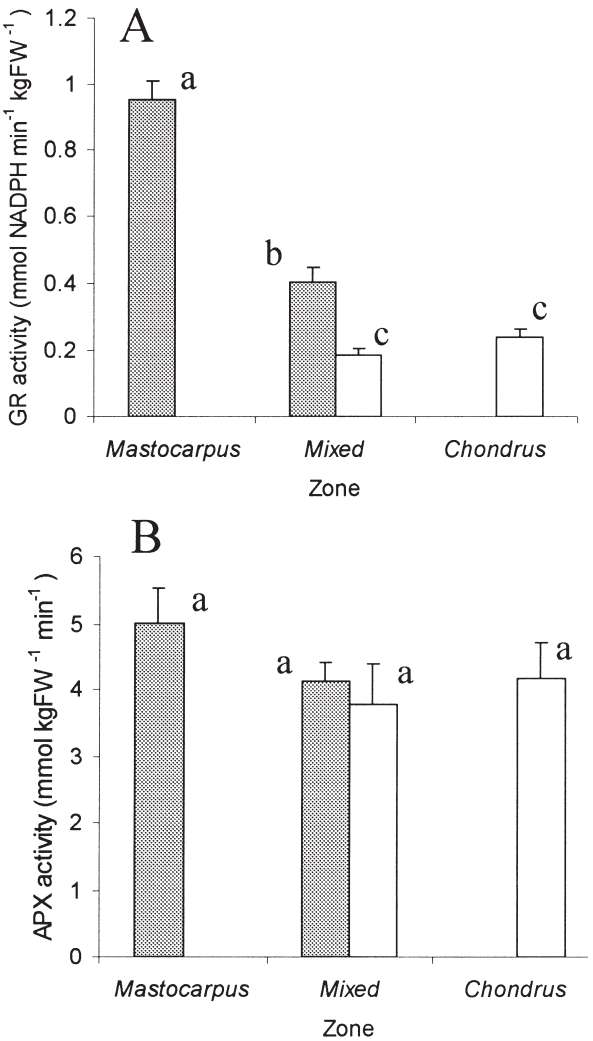

Figure 6. Activities of (A) glutathione reductase (GR) and (B) ascorbate peroxidase (APX) in *Mastocarpus stellatus* and *Chondrus crispus*. For explanation of zones see Fig. 3. Values were compared within species and zone, with values that were significantly different ($P < 0{\cdot}05$) being indicated by different letters. Means and SE are shown, $n = 5$.

M. stellatus had higher activities of GR than *C. crispus*, and the activity was higher in the homogenous *M. stellatus* zone than the mixed zone (Fig. 6A). There was no difference in GR activity between growth location in *C. crispus*. Activities of APX were similar for both species and between growth locations. No detectable glutathione peroxidase activity was found in any species. Despite that, GSH can be used to regenerate ascorbate from dehydroascorbate with the aid of the enzyme dehydroascorbate reductase (Asada & Takahashi 1987).

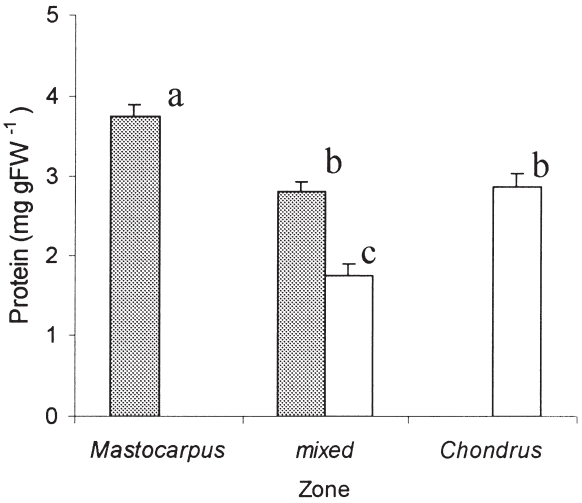

Figure 7. Protein content of *Mastocarpus stellatus* and *Chondrus crispus* extracts used for enzyme assays. For explanation of zones see Fig. 3. Values were compared within species and zone with values that were significantly different ($P < 0{\cdot}05$) being indicated by different letters. Means and SE are shown, $n = 15$.

The protein content was higher in the pure *M. stellatus* zone than in the mixed zone (Fig. 7); however, in *C. crispus* the trend was reversed. The content of protein was higher in *M. stellatus* than *C. crispus*. Protein levels were thus highest in the area of greatest abundance. If enzyme activities are compared on a protein rather than a fresh weight basis, the pattern is similar to that shown in Figs 5(B) and 6(A) for GR and catalase (i.e. higher in *M. stellatus* than *C. crispus*). However, the activity of APX in *C. crispus* was higher in the higher tidal elevation than the lower zone when expressed on a protein basis.

DISCUSSION

Resistance to oxidative stress

Our data support the first hypothesis that *Mastocarpus stellatus* would be more resistant to oxidative stress than *Chondrus crispus*, which could partly explain the inter-specific differences in stress tolerance. *Mastocarpus stellatus* was less sensitive than *C. crispus* to oxidative stress induced by added H_2O_2 or Rose Bengal, a compound that induces increased formation of singlet oxygen. The higher resistance of *M. stellatus* may be attributable to the higher levels of β-carotene and α-tocopherol, both of which are important in the scavenging of $^1O_2$ (Pallett & Young 1993; Hess 1993). Even though *C. crispus* had higher levels of β-, γ- and δ-tocopherol, these are less efficient at $^1O_2$ scavenging than α-tocopherol (Hess 1993). In longer term (1–3 days), experiments with the green microalga *Dunaliella bardawil* Jiménez & Pick (1993) showed that Rose Bengal concentrations higher than 0·5–4 mmol m^{-3} reduced growth, and that increased content of β-carotene decreased sensitivity to Rose Bengal.

We found that both species had a very high resistance to methyl viologen. MV is toxic because it channels electrons from PSI to oxygen, causing the formation of O_2^- (Asada & Takahashi 1987). High resistance to MV has also been found in the red alga *Porphyra yezoensis* (Imada & Abe 1982), which growth being 1000 times less MV-sensitive than diatoms. The sensitivity was reduced after cultivation with cysteine, suggesting that sulphite reductase was important in the resistance to MV, since sulphite reductase can accept electrons from MV and reduce sulphate (Saito, Tamura & Shinano 1969). The low toxicity of MV could also be partly due to low permeability through the cell or chloroplast membrane but since the seaweeds were incubated for 12 h in the different concentrations of MV this is less likely. An alternative explanation for the high resistance to MV of *M. stellatus* and *C. crispus* may be the comparatively high activity of SOD that could detoxify the O_2^- produced. SOD activity has been shown to be very important for MV resistance in, for example, the cyanobacterium *Synechococcus*, in which growth of a mutant that lacked Fe-SOD was sensitive to 0·1 μmol m^{-3} MV whereas the wild-type could tolerate 1 mmol m^{-3} (Samson *et al*. 1994). The activities of SOD in *M. stellatus* and *C. crispus* were 10 times higher than those of *Fucus* spp., which are more sensitive to MV than *M. stellatus* and *C. crispus* (Collén & Davison 1999a,b). Compared to other photosynthetic organisms, the two red algae studied here had 3–25 times higher SOD activity than mosses (Seel, Hendry & Lee 1992), maize (Pinhero *et al*. 1997), rice (Dai *et al*. 1997), *Arabidopsis thaliana* (Rao, Paliyath & Ormrod 1996), the dinoflagellate *Peridinium gatunense* (Butow, Wynne & Tel-Or 1997) and the unicellular red alga *Porphyridium cruentum* (Misra & Fridovich 1977).

Reactive oxygen metabolism

Our data supported the second hypothesis that *M. stellatus* would have a more efficient reactive oxygen metabolism than *C. crispus*. First, *M stellatus* exhibited a slightly faster decomposition of added H_2O_2 than *C. crispus*. Second, where there were inter-specific differences in levels of anti-oxidants and reactive oxygen scavenging enzymes, these were generally higher in *M. stellatus* than *C. crispus*. The difference between ascorbate and glutathione might reflect the higher nitrogen limitation that can occur in the upper intertidal zone (Schonbeck & Norton 1979), favouring the use of non-nitrogenous ascorbate instead of the nitrogen-containing glutathione. This might cause different species to adopt different strategies for anti-oxidants; with the lower shore, less nitrogen-limited *C. crispus* preferentially using glutathione, and *M. stellatus* using ascorbate. *M. stellatus* also showed higher activity of catalase and GR than *C. crispus*, while the SOD and APX activity were similar in both species. We suggest that the increase in reactive oxygen scavenging capability in the higher intertidal species is an adaptation to the increased environmental stress. Compared to brown macroalgae of the genus *Fucus*, which grow on the same shore as *M. stellatus* and *C. crispus*,

the activity of SOD and APX is higher in the rhodophytes than in *Fucus* spp., and the activity of catalase is lower (Collén & Davison 1999b).

The higher *in vivo* scavenging of H_2O_2 in *M. stellatus* correlates with a higher catalase activity, whereas the APX activity was similar in both species. This suggests either that catalase is the most important enzyme for the breakdown of H_2O_2, or that the higher content of ascorbate in *M. stellatus* increases the efficiency of the APX-mediated scavenging of H_2O_2. The potentially greater importance of catalase contrasts with the green macroalgae *Ulva rigida* and *Fucus* spp. in which APX has been suggested to be the most important enzyme for scavenging externally added H_2O_2 (Collén & Pedersén 1996; Collén & Davison 1999b).

Acclimatization and zonation within one species

The data do not support our third hypothesis that *C. crispus,* but not *M. stellatus,* would exhibit changes in reactive oxygen metabolism with tidal height. This hypothesis was based on the observation that *C. crispus* acclimates to moderate stress, whereas *M. stellatus* has a constant and higher stress tolerance (Dudgeon *et al*. 1990, 1995). Contrary to our expectation, *M stellatus* but not *C. crispus* exhibited changes in reactive oxygen metabolism with tidal height that were consistent with acclimatization to increased stress on the upper shore. Activities of GR and SOD were higher in *M. stellatus* from the upper zone, with a similar but non-significant increase in APX and glutathione. This increased enzyme activity was associated with an increase in soluble protein content in the higher *M. stellatus* zone, suggesting that one of the costs of stress tolerance is a requirement to increase the allocation of resources to enzymes. The pattern of higher levels of anti-oxidants with higher growth location was not found in *C. crispus*, which exhibited similar activities of reactive oxygen scavenging enzymes and antioxidants from both tidal heights. There are several possible explanations for the different acclimatization response of *M. stellatus* reported here and in previous laboratory studies (Dudgeon *et al*. 1990). For example, it is possible that the time required for acclimatization is longer than was used in the laboratory experiments. It is also possible that acclimatization does not occur in response to freezing stress, but does occur in response to high light or desiccation. It should be noted that our study was conducted in September and October which is a comparatively benign season compared to summer, which has a higher risk of desiccation and high light intensities, or winter, which has a risk of freezing. This may also explain the absence of acclimatization in *C. crispus*. In addition, the algae used in our studies are unlikely to have been nitrogen-limited, which is likely to influence the content of enzymes and certain antioxidants during summer months.

Overall, the results of this study support the hypothesis that reactive oxygen metabolism plays an important role in stress tolerance of intertidal seaweeds. This appears to apply to both red and brown algae, although the specific protective mechanisms used appear to differ both between and within these groups.

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