

Article

Oxidative Stress Response in the Seaweed *Padina pavonica* Associated with the Invasive *Halimeda incrassata* and *Penicillus capitatus*

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Abstract: The western Mediterranean Sea is one of the most affected areas by the introduction of alien seaweed. *Penicillus capitatus* Lam. and the recently introduced *Halimeda incrassata* (J.Ellis) J.V.Lamour are tropical algae that invade native algae assemblies in the shallow sandy beds of Mallorca (Balearic Islands, western Mediterranean) where they are already settled. The aim of this study was to evaluate the effect of a potential competition between the invasive *P. capitatus* and *H. incrassata* and the native *Padina pavonica* (L.) Thivy, by means of biomarkers. *P. pavonica* samples were collected in their habitat without invasive species and areas where *P. capitatus* and *H. incrassata* cohabitated. *P. pavonica* densities were measured in the three investigated areas. The coexistence of the invasive algae and *P. pavonica* was related to a significant decrease in the densities of native algae when compared to the area without the invaders. Antioxidant enzymes, catalase and superoxide dismutase, and the reduced glutathione and polyphenols levels were significantly increased in *P. pavonica* in the presence of both invasive algae. Malondialdehyde, a marker of oxidative damage, and the reactive oxygen species production tended to increase in the presence of the alien species, but without significant differences. The obtained results show that the presence of *P. capitatus* and *H. incrassata* altered the normal vegetative growth of *P. pavonica* and caused an antioxidant response that led to a context of stress, but without evidence of oxidative damage. In conclusion, the presence of the alien *H. incrassata* and *P. capitatus* can be considered a source of competitive stress for *P. pavonica*, although further research regarding the increased water temperature is required.

Keywords: alien species; antioxidant; biomarkers; competition; oxidative stress; western Mediterranean



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1. Introduction

The introduction of alien species in marine environments is recognized as one of the main threats to marine ecosystems [1,2]. Globalization and increased connectivity among different regions of the world have accelerated marine invasions in the last decades [3]. The Mediterranean Sea is one of the areas that are more strongly affected by species introductions, since it is a hotspot for maritime trade [4,5], not only by entries occurring through the Suez Canal and the strait of Gibraltar, but also by accidental releases from recreational aquaculture and aquariums [4,6]. Among different taxonomical groups, the Mediterranean Sea is the most invaded region of the world in terms of exotic seaweeds which are considered an environmental problem [6,7]. In the case of the western Mediterranean, many algae

adapted to tropical areas have found appropriate conditions for their development, such as *Halimeda incrassata* (J.Ellis) J.V.Lamour and *Penicillus capitatus* Lam. [6]. These two algae are chlorophytes from warm waters of the western Atlantic [8,9], which have settled in the Balearic Islands (western Mediterranean). *H. incrassata* was recorded in 2011 for the first time [10], while *P. capitatus* has been known for several decades [11]. Both species are known to mainly develop in shallow open sandy and sandy/rocky substrate around *Posidonia oceanica* (L.) Delile meadows, exerting pressure over native coastal communities [12].

The presence of invasive species can induce a stressful situation for the native species [13,14]. The organisms can respond to physiological stress through the activation of the metabolism, which results in an increase in the production of reactive oxygen species (ROS). ROS comprise several reactive chemical species mostly derived from the univalent reduction of O₂ as a consequence of oxidative metabolism [15]. The most common ROS are superoxide anion and hydrogen peroxide which, under physiological conditions, act as secondary messengers modulating redox-sensitive pathways to maintain cellular homeostasis [15]. However, under stress conditions, the increased production of ROS can result in an excessive accumulation of these reactive species that can interact with cellular biomolecules and lead to oxidative damage. To protect from oxidative damage, cells can trigger both enzymatic and non-enzymatic mechanisms in order to neutralize the excess of ROS production [16]. If antioxidant mechanisms are not capable of effectively counteracting the ROS levels, the resulting imbalance induces a state of oxidative stress [17]. The activation of antioxidant defences has been observed in algae subjected to several stressful abiotic factors such as pollutants, salinity, temperature, or physical damage [18], and also under biotic stress such as herbivory or competition [12,17,19]. Competition between algae communities and other sessile organisms is known to be mainly related to physical competitions for light, substrate, and nutrients [20]. Mechanisms of chemical competition among algae such as allelopathy have also been reported, involving in some cases ROS production and the activation of an antioxidant response [21,22].

Both *P. capitatus* and *H. incrassata* can form assemblages with high ecological flexibility [23], although with limited competitive advantage against healthy seagrass meadows [12,23]. This restricts the invasiveness in the surrounding assemblages, mainly on algae communities that develop in sandy and sedimentary areas. Among these assemblages, *Padina pavonica* (L.) Thivy is a brown alga from the *Dichtyophyceae* family known to develop extensive communities in infralittoral shallow clay and sandy soils [24]. *P. pavonica* distribution comprises the Atlantic coasts with the northern limit in the south British coasts, the Black Sea, and the Mediterranean coast where it is widespread throughout [25]. Studies on the competition between *P. pavonica* and other taxa are scarce and limited to pointing out its antibacterial [26] and antioxidant properties [27] for medical applications. Additionally, its use as a bioindicator has been proposed, at least for pollutant monitoring, since *P. pavonica* is abundant and easy to identify and develop in the infralittoral, where several alien species spread [28]. However, its use as a bioindicator for biotic stress or its competition with other algae have not been studied in the Mediterranean Sea. Given this, the aim of this study was to evaluate whether the presence of two invasive algae, *P. capitatus* and *H. incrassata*, can induce a stress situation in the native algae *P. pavonica* by means of antioxidant-related biomarkers.

2. Materials and Methods

2.1. Sampling Collection and Processing

The area of study was located in Cala Blava (south of Mallorca, Balearic Islands, 39°28' N 2°43' E) in the Marine Protected Area of Palma Bay. The study area was characterized by mild winds and few currents with small waves and almost imperceptible tides. This implies a low renewal of the water, especially in summer due to the rise in temperature [29]. Authorization for the collection of *P. pavonica* specimens was obtained from the Direcció General de Pesca (Balearic Government). Samples of *P. pavonica* were collected in three nearby areas: (1) control area, where *P. pavonica* grew isolated; (2) an area where *P. pavonica*

coexisted with *H. incrassata*; and (3) an area where *P. pavonica* coexisted with *P. capitatus* (Figure 1). The sampling was carried out in June 2022 given the annual cycle of *P. pavonica* and the fact that the thallus detaches every winter and regrows in spring, allowing us to study the period of maximum interaction between species [24]. The species used were identified according to their morphological characteristics [30].

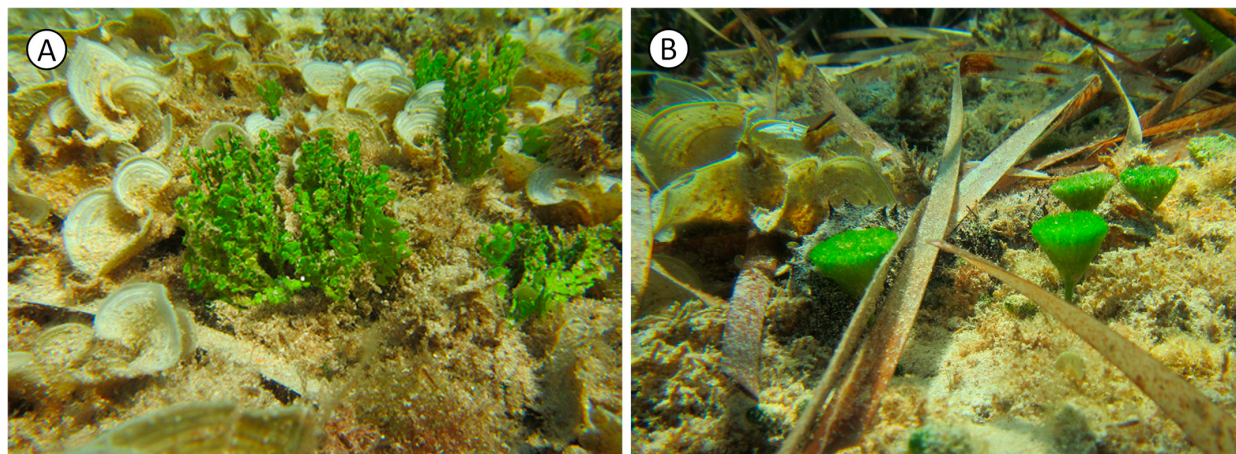


Figure 1. Representative images of the invasive algae *Halimeda incrassata* (A) and *Penicillus capitatus* (B) coexisting with *Padina pavonica*.

The physical and chemical properties of the three selected areas were obtained by measuring the values of temperature, pH, conductivity, salinity, dissolved oxygen, and oxygen saturation with multiparameter probes (YSI Pro30 and ProODO, Yellow Springs, OH, USA). Sediment grain size distribution was measured by drying 100 g of superficial sediment at 110 °C for 48 h. Samples were then sieved through sieves ranging from 2 mm to 56 µm. All samples were collected the same day by free diving at 2–2.5 m depth.

Twelve independent samples of *P. pavonica* were collected in each area and were immediately introduced in hermetic tubes with sea water and transported to the laboratory in less than 30 min. Samples were carefully dried, cleaned from epiphytes, and frozen at −80 °C until further processing. Algae specimens were homogenized with Tris-HCl buffer (50 mM, pH 7.5) using a dispersing system (Ultra-Turrax® Disperser, IKA, Staufen, Germany). Homogenized samples were centrifuged at 9000× g, 10 min, 4 °C and the supernatants collected for biochemical assays. All biochemical data were normalized per mg of protein using a commercial kit (Bio-rad®, Barcelona, Spain) with bovine serum albumin as a standard.

Densities of the three algae were estimated in the different stations employing a 50 × 50 cm quadrat sampling. The number of shoots was measured in three plots of *P. pavonica* alone and in three plots of *P. pavonica* cohabiting with both invasive species.

2.2. Biochemical Analysis

Catalase (CAT) and superoxide dismutase (SOD) activities were determined in homogenates using a Shimadzu UV-2100 spectrophotometer at 25 °C following methods described previously. Briefly, CAT activity ($\text{mK}(\text{s}^{-1})/\text{mg}$ protein) was monitored following the decomposition of H_2O_2 at 240 nm [31]. SOD activity was determined at 550 nm, monitoring the inhibition of the reduction of cytochrome C by superoxide anion produced by the xanthine oxidase enzyme [32].

Reduced glutathione (GSH, nmol GSH/mg protein) was determined in samples deproteinized with 30% trichloroacetic acid containing 2 mM EDTA after reaction with 5,5'-dithio-bis(2-nitrobenzoic acid) solution (DTNB) [33]. The resultant yellow derivative 5'-thio-2-nitrobenzoic acid was measured at 412 nm using a 96-well microplate reader (Epoch Microplate Spectrophotometer, Bio-Tek, Agilent Technologies, Madrid, Spain), using GSH as standard.

Total polyphenol levels were measured in *P. pavonica* homogenates following a modification of the Folin–Ciocalteu procedure using L-tyrosine as standard [34]. The results are presented as mg L-tyr/mg protein.

The levels of malondialdehyde (MDA), a lipid oxidation marker, were measured using a commercial assay kit (Merk Life Science S.L.U., Madrid, Spain) following the manufacturer's instructions. The assay was based on the reaction of MDA with a chromogenic reagent to form a colored product monitored at 586 nm in a 96-well microplate.

H₂O₂ production was determined in algae homogenates using 2,7-dichlorofluorescein diacetate (DCFH-DA) as fluorescent probe, following a previously described method [35]. Fluorescence (Ex. 480 nm; Em. 530 nm) was monitored at 25 °C in an FL9800 Microplate Fluorescence Reader (Bio-Tek, Agilent Technologies, Madrid, Spain) for 1 h.

2.3. Statistical Analyses

Statistical analyses were performed using the statistical package for social sciences (IBM PSS® v27.0, Armonk, NY, USA). The normal distribution of the data was assessed by the Kolmogorov–Smirnov test. The statistical significance was compared by one-way analysis of variance (ANOVA) with the absence or presence of the alien species as a comparison factor, followed by a Least Significant Difference (LSD) post hoc analysis. One-way multivariate analysis of variance (MANOVA) was performed to assess the possible association between the stress parameters and the physicochemical conditions in the three sampling sites. Results were expressed as mean $\pm$ standard error of the mean (S.E.M.) and $p < 0.05$ was considered statistically significant.

3. Results

Water characteristics—temperature, pH, conductivity, salinity, oxygen saturation, and dissolved oxygen—were similar among plots, showing a low variation between isolated *P. pavonica* and patches cohabiting with *H. incrassata* and *P. capitatus* (Table 1). Furthermore, sediments had a similar grain size distribution with a predominance of sand, constituting more than 98% of the sample. MANOVA analysis did not evidence statistically significant effects (Pillai's trace = 0.799, $F(10,6) = 0.399$, $p = 0.904$).

Table 1. General characteristics of the three sampling areas.

	<i>P. pavonica</i> Control	<i>P. pavonica</i> + <i>H. incrassata</i>	<i>P. pavonica</i> + <i>P. capitatus</i>
Temperature (°C)	24.67 $\pm$ 0.04	24.63 $\pm$ 0.03	24.63 $\pm$ 0.04
Conductivity (mS/cm)	57.93 $\pm$ 0.14	57.67 $\pm$ 0.09	57.90 $\pm$ 0.07
Salinity (ppt)	38.93 $\pm$ 0.08	38.77 $\pm$ 0.08	38.90 $\pm$ 0.07
Oxygen saturation (%)	89.20 $\pm$ 0.28	89.77 $\pm$ 0.15	89.43 $\pm$ 0.11
Dissolved oxygen (mg/L)	7.41 $\pm$ 0.02	7.46 $\pm$ 0.02	7.44 $\pm$ 0.02
Sediment grain size distribution (%)			
>2 mm	1.2 $\pm$ 0.1	1.1 $\pm$ 0.2	1.3 $\pm$ 0.2
<2 mm–>500 μ m	24.9 $\pm$ 1.9	24.6 $\pm$ 1.8	24.9 $\pm$ 1.4
<500 μ m–>125 μ m	73.8 $\pm$ 2.0	74.1 $\pm$ 2.0	73.7 $\pm$ 1.4
<125 μ m–>56 μ m	0.11 $\pm$ 0.01	0.1 $\pm$ 0.03	0.1 $\pm$ 0.02

The densities of the different algae species are presented in Figure 2. The densities of *P. pavonica* significantly decreased in the presence of alien species when compared to plots lacking them. *P. pavonica* densities varied from 233 $\pm$ 13 individuals/m² when living alone to 131 $\pm$ 12 individuals/m² in the presence of *H. incrassata* ($p < 0.001$) and 133 $\pm$ 13 individuals/m² in the presence of *P. capitatus* ($p < 0.001$). In the studied areas, the densities of the invasive algae were an average of 200 $\pm$ 18 individuals/m² for *H. incrassata* and 278 $\pm$ 23 individuals/m² for *P. capitatus*. The sampling areas were almost exclusively covered with the species selected for the research, except for some isolated specimens of *Dasycladus vermicularis* (Scop.) Krasser and *Halimeda tuna* (J.Ellis & Sol.) J.V.Lamour.

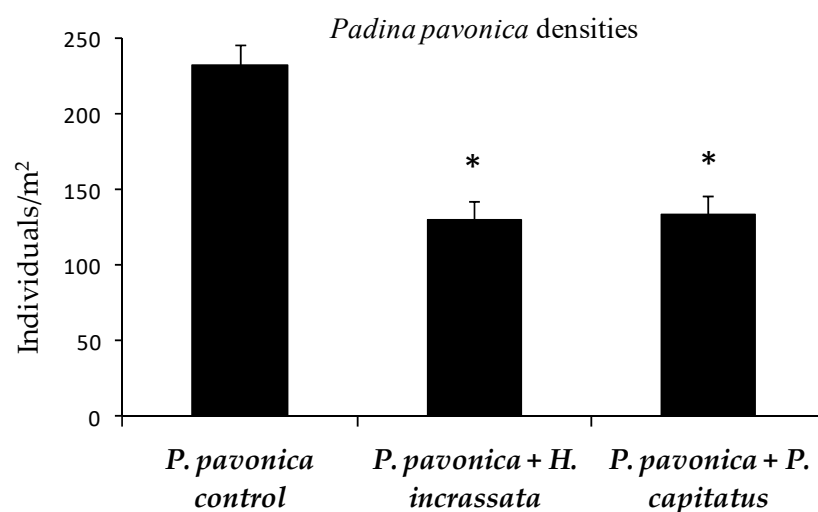


Figure 2. Density of *Padina pavonica* (Individuals/m²) in the three studied areas in absence and presence of the invasive species. * Indicates significant differences in *P. pavonica* densities compared to control (one-way ANOVA). Values are expressed as mean $\pm$ S.E.M.

The activities of antioxidant enzymes are shown in Figure 3. The activity of CAT was significantly increased when *P. pavonica* cohabited with both *H. incrassata* ($p < 0.001$) and *P. capitatus* ($p = 0.001$). A similar response was observed for SOD in the presence of *H. incrassata* ($p = 0.005$) and *P. capitatus* ($p = 0.007$). Non-enzymatic antioxidants showed the same response, with a significant increase in polyphenol and GSH levels in the presence of *H. incrassata* ($p = 0.009$ and $p = 0.039$, respectively) and *P. capitatus* ($p = 0.006$ and $p = 0.024$, respectively).

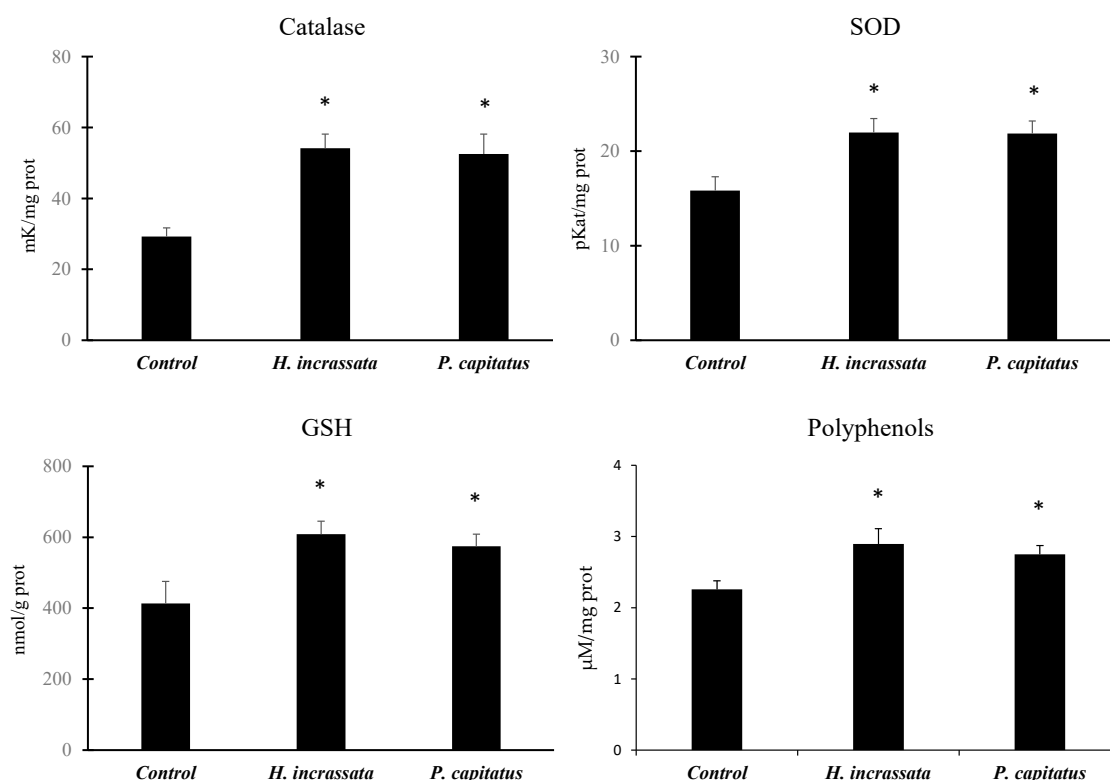


Figure 3. Antioxidant enzyme activities, catalase and superoxide dismutase (SOD), and non-enzymatic antioxidants reduced glutathione (GSH) and polyphenols in samples of *P. pavonica* (control) alone or coexisting with the invasive *H. incrassata* or *P. capitatus*. * Indicates significant differences compared to control (one-way ANOVA). Values are expressed as mean $\pm$ S.E.M.

The levels of MDA and the production of H_2O_2 are shown in Figure 4. The presence of the invasive algae did not increase the MDA levels of *P. pavonica*, and although there was a tendency to increase the production of H_2O_2 , the differences were not statistically significant.

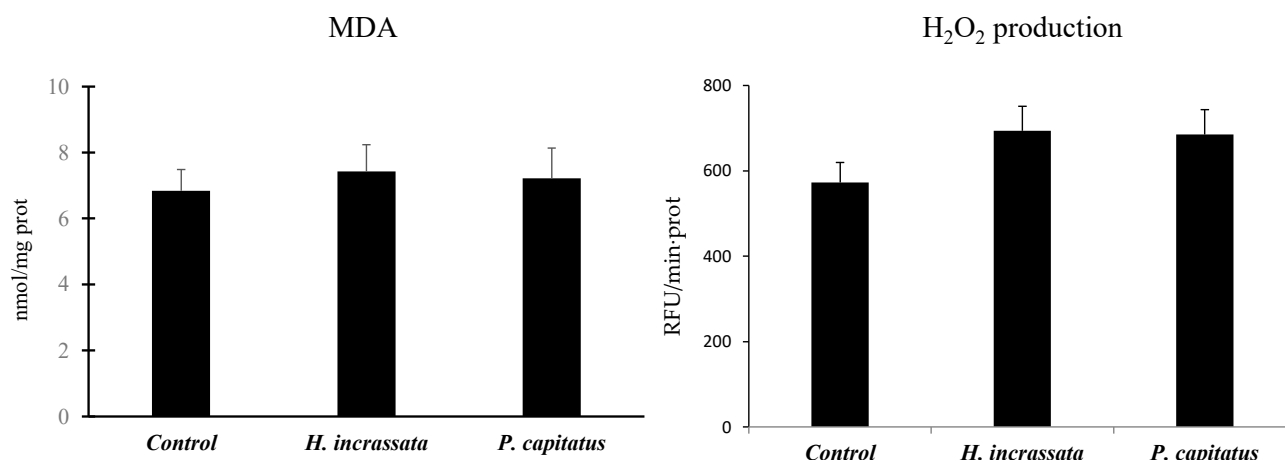


Figure 4. Levels of malondialdehyde (MDA) and production of H_2O_2 in samples of *P. pavonica* (control) alone or coexisting with the invasive *H. incrassata* or *P. capitatus*. No significant differences were found between groups (one-way ANOVA). Values are expressed as mean $\pm$ S.E.M.

4. Discussion

The arrival of alien species can threaten ecosystems through the competition and substitution of native taxa, causing both a disruption of normal ecosystem functionality and a loss of biodiversity [2]. Increasing temperatures are increasing the arrival and establishment of several seaweeds, in a process of tropicalization of the Mediterranean Sea [36]. In the Balearic Islands, *H. incrassata* and *P. capitatus* are examples of tropical algae capable of settling and expanding over infralittoral algae communities [10,12,37]. The current study was carried out in the Cala Blava locality (Palma Bay) where both species thrive with a patchy distribution near *P. oceanica* seagrass meadows [12]. In these areas, *H. incrassata* and *P. capitatus* can compete with native species such as *P. pavonica* occupying the shallow infralittoral zone, mainly in sandy and sedimentary habitats. This fact is evidenced by a substantial decrease in the number of *P. pavonica* specimens in the areas where the native algae coexists with the invaders, suggesting that their normal growth and development is affected. Density variations in other areas such as the Canary Islands evidenced that *H. incrassata* invasion in conjunction with *P. capitatus* caused strong transformations in previous algal assemblages [38]. Moreover, *P. capitatus* is known to form assemblages with other tropical taxa in the invaded Mediterranean areas [36]. From previous studies, the quick and high colonization potential of *P. capitatus* and *H. incrassata* seem to be due to their invasive capabilities through efficient nitrogen metabolism [39,40], fast growth, and clonal reproduction by rhizoidal extension and fragmentation [10,38]. Moreover, both species have calcareous thalli that, combined with chemical defences, substantially reduce the susceptibility to herbivory in the new occupied areas [41]. All these traits are enhanced by the increase in the temperature, which allows them to extend their presence during the year and overgrow algal assemblages.

Organisms when faced with a potentially stressful situation respond with metabolic activation that translates into an increase in ROS production and the induction of antioxidant defence mechanisms, which can be used as biomarkers [42]. The present results evidenced that the native *P. pavonica* assemblages had an active antioxidant response when cohabiting with the allochthonous algae *P. capitatus* and *H. incrassata* when compared to isolated assemblages. The activation of the antioxidant defences is an indicator of stressful conditions that have been reported in the biotic interaction between other seaweeds and seagrasses previously [12,17,43]. Regarding the mechanisms by which these invasive species induce a stress situation, it has been shown that tropical green algae of the order Bryopsidales,

which includes the genera *Halimeda* and *Penicillus*, synthesize sesqui- and diterpenoids with allelopathic properties [44]. Moreover, the establishment of a stressful condition can occur by nutrient competition as described for *Halimeda* spp. [39] or by overgrowth as it has been described for *P. capitatus* [38,39]. In the case of *P. pavonica*, enzymatic antioxidant activation (SOD and CAT) has been previously reported in stressful situations, mainly derived from a decreased photochemical efficiency under nutrient assimilation shortage and decreased temperature [45]. The activation of antioxidant enzymes has also been observed in diverse algae competing with alien species [12,17,19]. In addition to the enzymatic activation, the presence of the alien species also increased the levels of the non-enzymatic antioxidants (polyphenol and GSH) in *P. pavonica*. Polyphenols are relevant non-enzymatic antioxidants which are mainly produced for photoprotection but also under nutrient, light, or herbivory stress [46]. It is well established that brown algae show a high production of phenolic compounds that are also involved in ROS detoxification [47]. In this sense, high polyphenol levels in *P. pavonica* can be linked to the antioxidant response related to the competition with the alien species [27]. GSH is a low-molecular-weight compound which accumulates under stress conditions as part of the Ascorbate–Glutathione cycle and as a low-molecular reductant to protect against metal-induced stress and the excess of ROS [48]. Increased GSH levels have been reported in competitive interactions between other seaweeds and seagrasses [12,19]. For brown seaweeds, GSH levels are known to be regulated jointly with CAT activity by secondary metabolites, such as fucoids [49]. In this sense, both increases in GSH and in CAT in *P. pavonica* are in accordance with the expected ROS detoxification response. Regarding MDA levels, as an indicator of oxidative damage, no significant differences were observed when compared with isolated *P. pavonica*, whereas ROS production displayed a substantial but non-significant increase under both interactions. A similar pattern was previously observed in *D. vermicularis* coexisting with *H. incrassata*, showing increased antioxidant response without evidence of oxidative damage, and also in invasive seaweeds such as *Caulerpa cylindracea* Sonder for successful invasion [12,43]. An activation of the antioxidant response together with a lack of increased MDA levels is considered an adaptation to stressful conditions since it entails efficient ROS detoxification. Thus, the fact that ROS levels did not show differences between groups may derive from the enhanced capacity of the antioxidant systems to eliminate these reactive species, reducing their interaction with the DCFH-DA fluorophore. Altogether, *P. pavonica* displays efficient ROS detoxification mechanisms that prevent the damage related to the oxidation, allowing this seaweed to cope with the stress conditions derived from competition with *P. capitatus* and *H. incrassata*.

5. Conclusions

We studied the competitive interaction between the tropical allochthonous chlorophytes *H. incrassata* and *P. capitatus* and the native brown algae *P. pavonica* by means of antioxidant/oxidative biomarkers. Antioxidant, enzymatic, and non-enzymatic responses were activated when *P. pavonica* cohabited with *H. incrassata* and *P. capitatus*, indicating a condition of stress. However, a moderate increase in ROS production and the absence of MDA variations indicated the capability of resilience under the competitive interaction. However, the decreased algae densities indicated a complex interaction which could vary regarding the health status of native algae assemblies and future scenarios of increasing temperature. In the future, it would be interesting to evaluate if *P. pavonica* and the two invasive *H. incrassata* and *P. capitatus* occur together and the possible effects it may have. Moreover, further research is required to monitor whether the Mediterranean Sea tropicalization translates to the substitution of alien algae assemblies, especially in sandy disturbed areas prone to rapid colonization.

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Data Availability Statement: Researchers wishing to access the data used in this study can make a request to the corresponding author: antoni.sureda@uib.es.

Conflicts of Interest: The authors declare no conflict of interest.

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