ENDOGENOUS POLYAMINE RESPONSE IN EPIPHYTIC *Bonnemaisonia hamifera* (BONNEMAISONIALES: RHODOPHYTA) DUE TO INTERACTION WITH ITS HOST

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ABSTRACT.-The endogenous polyamine (Pas) content of free and bound-acid soluble fractions was assessed in the epiphyte *Bonnemaisonia hamifera* under the presence of its host *Gelidium arbuscula* and of extracts obtained from *G. arbuscula* and from *G. robustum*. In the presence of fresh thalli of *G. arbuscula* the free putrescine (PUT) content decreased ($P < 0.05$), while spermine and spermidine (SPD) showed a slightly increased concentration. The content of free PUT also decreased in all treatments with the different extracts and the dose. Bound-soluble PAs showed a different trend, particularly with the highest dose of ethanolic extract of *G. robustum,* PUT and SPD bound-soluble PA content increased significantly. Based on our results we infer that PAs are involved in the epiphytic relationship between macroalgae, and that PUT produces the greatest response of *B. hamifera* in the interaction with host and its extracts. The potential causes of its variation and its use for the production of secondary metabolites are discussed.

Keywords: Polyamine, seaweed, extracts, biotic relationships, epiphyte.

Respuesta de una poliamina endógena en el alga epifita *Bonnemaisonia hamifera* (Bonnemaisoniales: Rhodophyta) debido a interacciones con su hospedero

RESUMEN.- Se evaluó el contenido endógeno de poliaminas, tanto de la fracción libre como de la conjugada-ácido soluble en la macroalga epifita *Bonnemaisonia hamifera* en presencia de su hospedero *Gelidium arbuscula* y de extractos obtenidos de *G. arbuscula* y *G. robustum*. En presencia de talos vivos de *G. arbuscula,* el contenido de putrescina libre disminuyó ($P < 0.05$) y la espermina y espermidina tuvieron un ligero incremento sin diferencia significativa. En los tratamientos con los distintos extractos y dosis, el contenido de putrescina libre también disminuyó. La fracción de poliaminas conjugadas mostró una tendencia diferente, particularmente con la dosis más alta del extracto etanólico de *G. robustum* donde putrescina y espermidina conjugadas-solubles se incrementaron significativamente. Los resultados obtenidos sugieren que las poliaminas juegan un papel en el establecimiento de las relaciones epifito-hospedero, y que la putrescina es la que presenta la mayor respuesta en *B. hamifera* en la interacción con su hospedero y en presencia de los extractos. Se discute la variación de las poliaminas y las posibles causas

Palabras clave: Poliamina, macroalga, extractos, relacion biótica, epifita.

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INTRODUCTION

Seaweeds and many other marine organisms are under strong biotic and abiotic interactions due to the changing nature of their environment. The effects of environmental factors as light and nutrients availability on the secondary metabolism of seaweeds have been extensively studied, particularly in the production of polyphenols (Paul & Van Alstyne, 1992; Cronin & Hay, 1996a; 1996b; Puglisi & Paul, 1996; Pavia *et al.,* 1997; Pavia & Toth, 2000; Swanson & Druehl, 2002; Pansch *et al.,* 2009). Biotic relationships are as important as the abiotic ones. Herbivorism and epiphytism can be considered natural enemies of terrestrial plants and seaweeds. These interactions create stressful conditions that probably have played a key role in the natural selection of these organisms (Bakus 1971). There are metabolic and proteomic markers that are used to measure said stress (Shulaev & Oliver, 2006). Endogenous polyamines (PAs) have been used for many years as stress indicators related to ecological relationships. This is due to the diverse functions that PAs play in plant metabolism, where free putrescine (PUT), spermidine (SPD), and spermine SPM are the most important PAs found in living beings (Groppa & Benavides, 2008). In free form the PAs are positive charged, and they react with negatively charged molecules such as DNA, RNA, proteins and phospholipids. Therefore PAs are involved in the regulation of the physical and chemical properties of membranes and the structure of nucleic acids, and enzymatic activity (Galston & Sawhney, 1990; Tiburcio *et al*., 1993; Bachrach, 2010). Currently in terrestrial plants there are a many investigations where the correlation between the change in the endogenous PA content under different environmental abiotic stress sources is reported, such as osmotic stress and drought, heat, chilling, oxidative stress, hypoxia, ozone, UV radiation, nutrients, mechanical wounding, heavy metal toxicity and herbicides (Alcazar *et al*., 2006; 2010; Liu *et al*., 2007; Groppa & Benavides 2008; Kusano *et al*., 2008). Also, in bi-

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otic relationships, PAs are involved in the establishment of simbiotic relationships with fungus (Bais *et al*. 2000; Niemi *et al*., 2007; Cheng *et al*. 2012), in response to viral infections (Belles *et al*., 1993; Yoda *et al*., 2009; Sagor *et al*., 2013) and with other plants in cell cultures (Cvikrova *et al*. 2008).

Polyamine related research on macroalgae is far from what it has been achieved with terrestrial plants. There are reports of their physiological role as growth regulators (García-Jiménez *et al*., 1998; Cohen *et al*., 1984; Marián *et al*., 2000), the reproductive implications in the development and maturation of the cystocarp and sporulation (Guzmán-Urióstegui *et al*., 2002; Sacramento *et al*., 2004), and the stress response caused by abiotic factors such as salinity in *Grateloupia doryphora* (García-Jiménez *et al*., 2007) and *Ulva fasciata* (Lee 1998;). More recently it was reported that PAs in *Ecklonia maxima* have a seasonal variation, with maximum values during periods of stress caused by high wave activity (Papenfus *et al*., 2012). In addition to the direct participation of PAs in the stress response, the PAs are precursors of a wide variety of alkaloids and are related with other metabolic pathways. For example, PUT is the precursor of pyrrolidine, tropane and calystegines alkaloids (Ghosh 2000; Kumar *et al*., 2006; Bhattacharya & Rajam,2007). Spermidine and PUT are also important precursors for the production of lunarine and pyrrolizidine alkaloids (Graser & Hartmann, 2000; Bhattacharya & Rajam, 2007). These types of PAs derivatives have medical applications, and have increased the interest on biotechnological investigation.

Epiphytism is a common phenomenon in macroalgal communities. However, the chemical response in biotic interactions between host and epiphyte has been scarcely investigated. We are also interested in determining if PAs are involved in biotic relationships in macroalgae, in order to get a better understanding of the factors that affect their endogenous content. Thus, we also expect that PAs can be used as stress markers in epiphytic relationships. This is the case of the association between the epiphytic red algae *Bonnemaisonia hamifera* P. Hariot (Bonnemaisoniales: Rhodophyta) a small filamentous alga in sporophytic phase that can be found living in most of the samples of its host *Gelidium arbuscula* (Bory de Saint-Vincent ex Børgesen). In this research, we studied the alteration in PAs levels of *B. hamifera* when cultivated in the presence of fresh thalli of *G. arbuscula,* and with extracts from this alga and from *Gelidium robustum*.

MATERIALS AND METHODS

*Seaweed Collection and Culture***.** Both *G. arbuscula* and *B. hamifera* (Fig. 1) were collected from a rocky, intertidal shore during low tide in Las Palmas de Gran Canaria, Spain. Samples were stored in plastic bags and immediately transported to the laboratory where the host *G. arbuscula* and its epiphyte *B. hamifera* were separated. Samples of *G. robustum* for the extraction were collected in the Pacific coast of the Baja California peninsula, México. Samples were sun dried and transported in plastic bags.

The ethanolic extracts were obtained from the alga *G. arbuscula* and the non-native macroalgae *G. robustum*. The algae were dried and milled and soaked with ethanol 100%. The ethanol was then filtrated and replaced with new ethanol every 3 days three times. The extracts were concentrated to dryness at 40° C and low pressure in a rota-evaporator (Yamato re500)

Sporophyte stock cultures of *B. hamifer* were established from filaments excised from the thallus of collected *G. arbuscula*. The epiphyte *B. hamifera* was cultivated in the laboratory in IMR culture medium (Paasche *et al*., 1996). The stock cultures were maintained in the laboratory at 22±2ºC, and 18:6 light:dark photoperiod for up to two months, until enough samples were obtained to carry out the experiments. Treatments designed for this assay are described in table 1. The assays had a duration of 48 h, 3 plates per treatment, and were maintained in the conditions described above. However, *G. arbuscula* could not be cultivated; thus, fresh thalli were collected one day before the beginning of the experiment.

Polyamine Analysis. The extraction method used was the same as in Sacramento *et al*. (2004), but with a modification in the weight of the sample, because of the small amount available of *B. hamifera*. The extract was dissolved again in 0.5 mL of acetonitrile.

In short, for the extraction, samples (25-30 mg) were powdered in a mortar with liquid nitrogen and then added 1.5 mL of 5% percloric acid (PCA), and pounded in the mortar until a homogeneous paste was obtained. This was centrifuged at 9000 g for 20 minutes at 6° C. The supernatant was divided into two parts: one to measure free acid-soluble PAs (260 µL), which was frozen for further dansylation, while the other portion was used to determine the bound acid-soluble PAs (300 µL) after it was digested in sealed vials, using 300 µL of HCl (12 M, HPLC grade) overnight at 100° C. The samples were then filtered, vacuum dried, and re-dissolved in 260 µL of 5% PCA.

Both free and bound-soluble PAs samples were dansylated following the following method. Briefly, 40 µL of diamino heptane (HTD, 0.05 mM) was added as internal control of dansylation, plus 200 µL of a saturated solution of sodium carbonate (Na_2CO_3), and 400 µL of dansyl chloride (5 mg mL^{-1}, in acetone). It was then incubated overnight in the dark and at room temperature (24° C ±3). Then, 100 µL of proline (100 mg mL^{-1}, in water) were added to re-

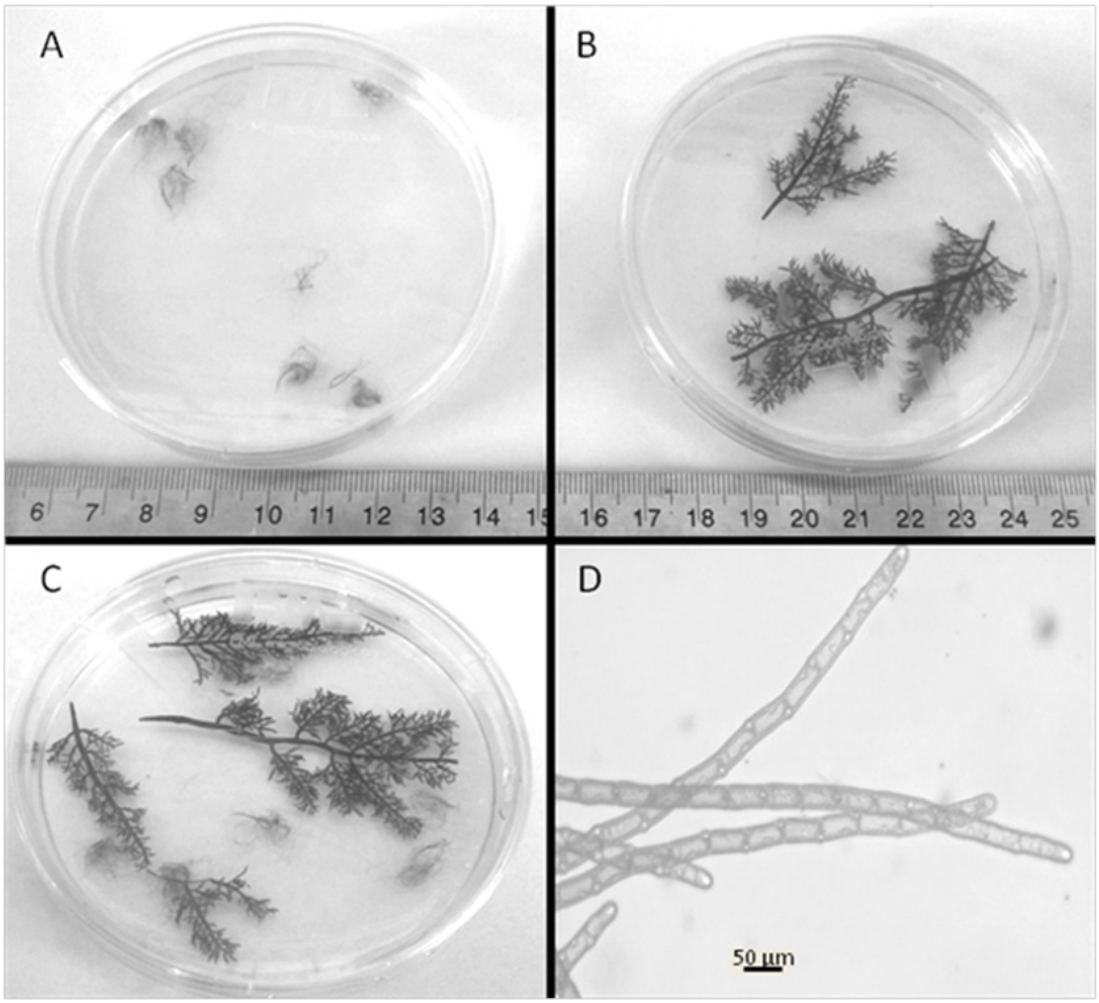

Figure 1.- *Bonnemaisonia hamifera* and *Gelidium arbuscula* in culture plates. A) Amount of thalli of *B. hamifera* used for all treatments (25-30 mg); B). Amount of fronds of *G. arbuscula* (around 300 mg) used for the treatments with *B. hamifera*; C) Both algae together for the treatment; D) Morphology of *B. hamifera* showing filament cells and vesicles.

move the dansyl chloride excess. Finally, 500 µL of toluene was added and mixed in a vortex. Then 400 µL of the upper decanted phase were vacuum dried and re-dissolved in acetonitrile. All samples were filtered with syringe filters of 0.45 µm membrane before HPLC analysis.

Chromatographic analysis was carried out in a Varian system integrated by a fluorescence detector (Varian ProStar 363) at 365 (excitation) and 510 nm (emission), a 5 µm reverse phase column (Varian C-18), auto sampler (Varian ProStar 410) and pump (Varian 9002). Solvents used were (A) acetonitrile and (B) water. The elution protocol was as follows: 0-3 min, 70% of A; 3-12 min, 100% of A; 12-20 min, 70% of A. The flow rate was 1 mL min^{-1} and the injection volume was 20 µL. The approximate retention times for the polyamines under these con-

Table 1. Treatments and controls for the experiment

Treatments	Description
IMR	*B. hamifera* in IMR medium (control)
LGA	*B. hamifera* in IMR with live *G. arbuscula*
GR1	*B. hamifera* in IMR with *G. robustum* ethanolic extract [0.01 mg mL^{-1}]
GR0.5	*B. hamifera* in IMR with *G. robustum* ethanolic extract [0.005 mg mL^{-1}]
GA1	*B. hamifera* in IMR with *G. arbuscula* ethanolic extract [0.01 mg mL^{-1}]
GA0.5	*B. hamifera* in IMR with *G. arbuscula* ethanolic extract [0.005 mg mL^{-1}]

ditions were as follows: PUT-7.1 min, SPD-8.1 min, SPM-9.4, and the internal control HTD-7.8 min.

Statistical Analysis. Values were subjected to one way ANOVA or Kruskal-Wallis test, depending on whether the data showed normality and homoscedasticity. Post-hoc analysis was applied in case of a significant difference among treatments; a Tukey test was carried out with ANOVA, and with a one to one comparison using the Mann-Whitney U for Kruskal-Wallis.

RESULTS

In the presence of live *G. arbuscula* (LGA; Fig. 2), the free-soluble PUT in *B. hamifera* showed a significant decrease ($P < 0.05$), while SPM and SPD had a slight increase compared with the control (IMR). —Statistical significance was observed for SPM ($P < 0.05$)—. Bound-soluble PAs showed no significant differences.

Significant differences between treatments with extracts and control were found. Free PUT had a decrease of over 90% in treatments GR0.5, GA1 and GA0.5 ($P < 0.05$), and SPD of about 80% in these same treatments. While in GR1 treatment SPD increased three times its content ($P < 0.05$). Figure 3A shows the difference in PUT and SPD ($P < 0.05$) between GR1 treatment and the others.

In the bound-soluble fraction (Fig 3B) only the GR1 treatment had a significant increase ($P < 0.05$) in PUT and SPD in relation to all other treatments and control.

DISCUSSION

Endogenous content of PAs varies greatly among species. In vascular plants PA content is high, ranging from 0.01 to 9 µmol g^{-1} fresh weight (fw) for PUT, from 0.006 to 1.72 µmol g^{-1} fw for SPD, and from 0.005 to 0.74 µmol g^{-1} fw for SPM (Altman, 1989). In macroalgae there is a large variation among species (Table 2). Endogenous content of PAs in *B. hamifera* was in this order PUT>SPD>SPM which coincide with the overall trend observed for macroalgae and vascular plants and its values were within the range described above.

The results suggest that *B. hamifera* "senses" the presence of living *Gelidium* or even its extracts. In this regard, in terrestrial plants the typical response to external factors, mainly abiotic, is by increasing the endogenous content of PAs, which is considered a resistance characteristic (Alcázar *et al*., 2010; Groppa & Benavides, 2008). The importance of PAs in this process is such that in stressed plants the PUT content may represent up to 1.2% dry weight, which is equivalent to 20% of the total nitrogen content (Galston & Sawhney, 1990). While in the opposite case, the decrease in the PAs content suggests sensitivity to the stress factor.

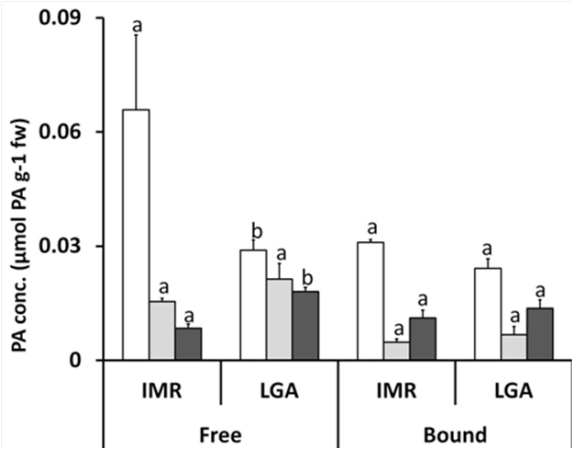

Figure 2.- Concentrations of free and bound-soluble PAs of *B onnemaisonia hamifera* (control, IMR) ad cultured in the presence of live *Gelidium arbuscula* (LGA). Data correspond to three replicates and are represented as mean (±SE). PUT- white, SPD- gray, SPM-dark. Different letters show significant differences between treatments in each PA according to the statistical and post-hoc tests ($P < 0.05$). Treatments are described in Table 1.

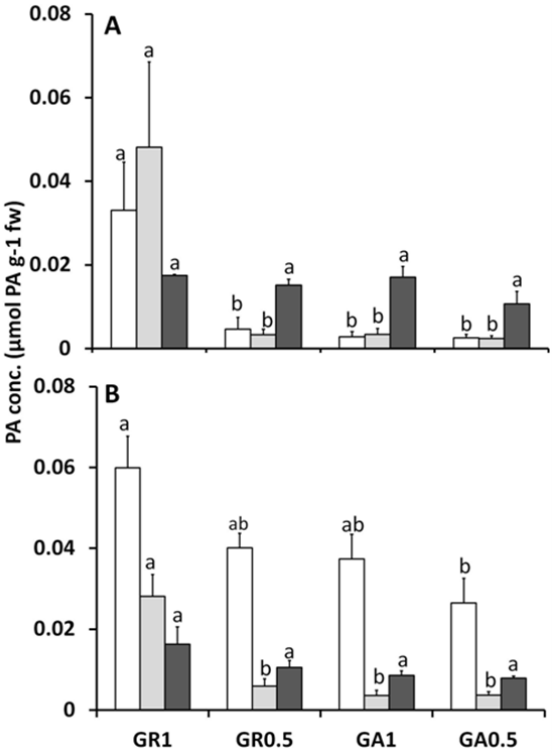

Figure 3.- Concentrations of endogenous PAs of *B onnemaisonia hamifera* in the treatments with extracts. Mean values (+SE). A) Free-soluble PAs content; B) Bound-soluble fraction; PUT- white, SPD- gray; SPM-dark. Different letters show significant differences between treatments in each PA by the statistical and post-hoc tests ($P < 0.05$).

For macroalgae it has been reported that PA response under saline conditions is based on the accumulation of the free fraction of PUT, SPD and SPM (Lee, 1998; García-Jiménez *et al*., 2007) which correlates with a decrease in transglutaminase activity and the increase of arginine decarboxylase activity in a salt-tolerant macroalgae (García-Jiménez *et al*., 2007). While in terrestrial plants, this topic was studied extensively considering mainly environmental factors, such as drought, salinity, temperature, mineral nutrition, wounding, UV treatment, metal, and oxidative and osmotic stress. All these are shown in tolerant plants in which the induced over expression and silencing of genes for PA biosynthetic enzymes are common (Basu & Ghosh, 1991; Groppa & Benavides, 2008; Alcázar *et al*., 2010). The plant response will depend on the particular species, therefore it is not possible to generalize or predict the PAs variation, but what it can be expected is that PUT are responsible of short term stress responses and that may occur in a lapse of hours. However, the action mechanism of the stress response is still unclear (Lefèvre *et al*., 2001; Zacchini & de Agazio, 2004; Bassard *et al*., 2010).

The epiphyte *B. hamifera* senses the presence of live *Gelidium* and reacts by decreasing significantly the PUT content after 48 h. For vascular plants it was reported that a decrease in PUT levels is regulated by elevated levels of ethylene (Kumar *et al*., 1996), and osmotic stress due to saline conditions (El-Shintinawy, 2000; Zapata *et al*., 2004; Tang *et al*., 2007). The decrease in PUT content may be caused by: a) an increase in the catabolic degradation or excretion; b) the use of PUT for the production of secondary metabolites; c) an increase in the synthesis of SPD; d) conjugation of free PUT with other cellular compounds (bound-soluble); e) a decrease in *di novo* synthesis. This raises the question, where is the free PUT going?

Regarding option c, only the GR1 treatment showed an increase of SPD, although this was not significant ($P > 0.05$). Regarding option d, four treatments showed values close to the control without a significant difference ($P > 0.05$). Therefore, these two options are unlikely. Moreover, in terrestrial plants it is reported that PAs derivatives are involved in the establishment of biotic relationships with fungus (Bais *et al*., 2000; Niemi *et al*., 2007; Nogales *et al*., 2009; Cheng *et al*., 2012), viral infections (Belles *et al*., 1993; Yoda *et al*., 2009; Sagor *et al*., 2013), and with other plants in cell suspension cultures (Cvikrova *et al*., 2008). For example, with viral infections, PAs can be used as a source of hydrogen peroxide, catalyzed by diamine and PA oxidases (Yoda *et al*., 2003; 2009). It has also been reported that the addition of fungal elicitors (culture filtrates) promotes the production of coumarines, by modifying the endogenous content of PAs (Bais *et al*., 2000), with PUT closely involved in this process (Bais *et al*., 1999). Phenolamides are other PA derivatives that form a large class of secondary metabolites in plants, and they are considered as a link between phenolic and nitrogen metabolism (Morant *et al*., 2007; Bassard *et al*., 2010).

Regarding the ecological role of phenolamides, they are part of a defensive strategy against plant pathogens (Martin-Tanguy, 1985; von Röpenack *et al*., 1998), or an insect deterrence activity (Tebayashi *et al*., 2007), and are involved in the plant response to abiotic stress, because of their antioxidant and radical scavenging activity due to the nature of its phenolic and PA constituents (Edreva *et al*., 2007).

The differences between extracts suggest that

Table 2. Endogenous PA content (µmol g-1 fresh weight) reported in other studies for marine macroalgae and in the present study (modified from Guzmán-Uriostegui *et al*., 2003)

	PUT		SPD		SPM	
	Free	BS	Free	BS	Free	BS
Ulva reticulata	1.36	0.13	0.10	0.05	0.014	0.006
Ulva lactuca	1.36	0.51	0.15	0.01	0.014	0.001
Ulva fasciata	0.25	0.25	0.03	0.09	0.019	0.001
Chaetomorpha crassa	0.47	0.68	0.04	0.017	0.029	0.008
Valoniopsis pachynema	0.56	0.22	0.11	0.13	0.004	0.003
Dictyota dichotoma	10.43	-	0.015	-	0.005	-
Gelidium canariensis	17	-	0.02	-	0.044	-
Grateloupia doryphora	7.94	-	0.03	-	0.014	-
Gracilaria cornea	4.42	3	0.01	0.008	0.018	0.006
Ecklonia maxima	0.68	-	-	-	0.148	-
Bonnemaisonia hamifera	0.06 ± 0.03	0.03 ± 0.001	0.01 ± 0.001	0.004 ± 0.001	0.008 ± 0.002	0.01 ± 0.003

the addition of the nonnative *G. robustum* extract induces a singular response. The higher dose was significantly different from all others, suggesting that *B. hamifera* response to this extract is dose-dependent. The research on the chemistry of *Gelidium* species resulted in only one report on secondary metabolites, the gelidene, a cyclic polychlorinated monoterpen isolated from *G. sesquipedale* (Aazizi *et al*., 1989) but nothing is known about its biological activity. Additional and recent information obtained by our research group with different chromatographic methods (unpublished data), suggests that terpenoids are an important part of *G. robustum* extracts.

Our study indicates that the epiphytic relationship between macroalgae affect the production of PAs and mostly free PUT. This biotic interaction could be considered as an important source of stress, according to studies conducted on the endogenous content of PAs. As reported for terrestrial plants, we also found that in macroalgae PUT is the responsible for the short term stress response. These results generate many other questions and show the need of further investigation. For example, studying the activation of PA biosynthetic enzymes and genes in response to the stress, could help to explain the mechanisms in which the endogenous PAs fluctuate. We also suggest doing research on the possible effect of *Gelidium* extracts in the production of PA derivatives by *B. hamifera.*

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