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Within-thallus variation on phlorotannin contents and physodes amount in *Stypopodium zonale* (Phaeophyceae)

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Abstract. Phlorotannins are defensive compounds produced by brown macroalgae and stored in vesicles called physodes. However, few studies have been performed to determine the relationship between the number of these cellular structures and the total amount of phlorotannins in different regions of the thalli of seaweeds. In order to verify this relationship, we quantified the number of physodes and the amount of phlorotannins in apical and basal portions of *Stypopodium zonale* thallus. The results showed that *S. zonale* apical regions exhibited significantly lower phlorotannin concentrations (0.49% DW $\pm$ 0.04) than corresponding basal ones (0.78% DW $\pm$ 0.21; p = 0.007). In addition, apical and basal regions of *S. zonale* also differed in the density of physodes, since apical fragments exhibited less physodes per cell (10.8 $\pm$ 5.85) than basal fragments (42.73 $\pm$ 16.16; p = 0.0035). Due to the defensive property of phlorotannins, a low amount of phlorotannins and physodes can become young or apical regions of *S. zonale* more susceptible to herbivory.

Keywords: phlorotannins, physodes, tropical macroalga, *Stypopodium zonale*

Resumo. Variação intra-talo da concentração de polifenóis e da quantidade de fisóides em *Stypopodium zonale* (Phaeophyceae). Florotaninos são conhecidos como substâncias defensivas produzidas por macroalgas pardas e armazenados em fisóides. Entretanto, poucos estudos foram realizados para determinar a relação entre a quantidade destas estruturas celulares e a concentração total de florotaninos em diferentes regiões do talo de macroalgas. Para verificar esta relação, foram quantificados o número de fisóides e a concentração de florotaninos nas porções apical e basal do talo de *Stypopodium zonale*. Na porção apical de *S. zonale* foi encontrada concentração significativamente menor de florotaninos (0.49% peso seco $\pm$ 0.04) do que a porção basal (0.78% peso seco $\pm$ 0.21; p = 0.007). Além disso, regiões apical e basal de *S. zonale* também diferiram na densidade de fisóides, uma vez que fragmentos apicais possuem menos fisóides por célula (10.8 $\pm$ 5.85) do que os fragmentos basais (42.73 $\pm$ 16.16; p = 0.0035). Devido à propriedade defensiva dos florotaninos, o baixo teor destas substâncias e o menor número de fisóides podem tornar regiões jovens ou apicais de S. zonale mais suscetíveis à herbivoria.

Palavras chave: polifenóis, fisóides, macroalga tropical, *Stypopodium zonale*

Introduction

Brown macroalgal phlorotannins, also known as polyphenols, are typical chemical constituents of these macroalgae and are crucial for many important aspects of their biology (Schoenwaelder 2008). In fact, phlorotannins may be multifunctional or multiecological chemicals with putative roles in herbivore deterrence (Pereira & Yoneshigue-Valentin 1999, Amsler & Fairhead 2006), anti-larval settlement, anti-bacterial and anti-fungal growth (Wikström & Pavia 2004, Plouguerné *et al.* 2012), antioxidant (Cruces *et al.* 2012), protection against harmful UV-B radiation (Pavia *et al.* 1997, Maschek & Baker 2008), and chelating of

toxic heavy metal ions (Karez & Pereira 1995). Besides, they have also been identified as having primary metabolic roles in wound healing (Lüder & Clayton 2004) and cell-wall construction (Schoenwaelder & Clayton 1999).

In plants, the production of biologically active metabolites is related to a capability to partition compounds into specialized storage structures in order to avoid autotoxicity (McKey 1979). In macroalgae, secondary metabolites are also located at usually specialized structures that can be single cells or cell components possessing varied ultrastructures. For example, as a rule, lipophilic compounds are accumulated in specialized cells such as *corps en cerise* (Salgado *et al.* 2008) or gland cells (or vesicle cells), in which the vesicle occupies practically the entire cellular space (Young & West 1979, Dworjanyn *et al.* 1999). Hydrophilic chemicals are stored in an unknown but probably aqueous environment, the physodes (Schoenwaelder 2002).

Physodes are membrane-bound, spherical bodies containing phlorotannins, specifically phloroglucinol and its derivatives or polymers (Ragan & Craigie 1976, Schoenwaelder 2008). These organelles may occur in most tissues of brown macroalgae, but most commonly occur in the outermost layer, such as the epidermis and cortical cell layers (Ragan & Glombitza 1986, Tugwell & Branch 1989). In addition, they are known to be transferred through the cytosol and incorporated into the cell wall, playing a structural or primary role (Pelletreau & Targett 2008).

Secondary metabolites are usually not distributed uniformly within the intra-whole of macroalgae and do not show a clear pattern or local of higher abundance. For example, in *Laminaria digitata*, phlorotannin content is maximized in the holdfast, whereas in *L. hyperborean* the highest value is known for the basal part of the old blades (Connan *et al.* 2006). Concentration of phlorotannins may be significantly higher in blades than in the stipes of *Durvillaea antarctica* (Duarte *et al.* 2011) or may be low and similar in all parts of *Sargassum furcatum* (Pereira & Yoneshigue-Valentin 1999).

Different tissues may also exhibit distinct phlorotannins content. For example, about 90% of the total phlorotannin contents in *Ecklonia radiata* was found in the epidermal layer (Lüder & Clayton 2004), similar to that observed in other *Ecklonia* species (Shibata *et al.* 2004). Phlorotannins were found to be almost entirely restricted to the thin outer meristoderm of *Ecklonia maxima*, *Laminaria pallida*, and *Macrocystis angustifolia*, as predicted, since they serve as a defense against grazers (Tugwell & Branch 1989).

Other interesting fact related to the distribution, abundance, and function of these metabolites is the differences along latitudinal temperate/tropical gradients (Steinberg 1989, Van Alstyne & Paul 1990). Species from high latitudes show high concentrations of these compounds, ranging from 2% up to 25% dry weight, as has been previously observed (Ragan & Glombitza 1986, Van Alstyne & Paul 1990, Van Alstyne *et al.* 1999). In contrast, the most common species in temperate Australasia exhibit more than 10% of total polyphenols (Steinberg 1989).

The fact that physodes contain phlorotannins is widely accepted (see Ragan & Glombitza 1986). In addition, studies on the tissue localization and quantitative variability of phenolic compounds provide a fundamental prerequisite for understanding the ecological and physiological functions of these compounds (*e.g*. Pereira & Yoneshigue-Valentin 1999, Schoenwaelder 2008). Then, in the brown macroalgae, the content of phlorotannins in different parts of the thallus may be associated with presence and number of physodes, but this aspect has not been previously investigated. Here, we show evidence on the relationship within the number of physodes and the phlorotannin content in apical and basal regions of the brown macroalga *Stypopodium zonale*.

Material & Methods

Study site and organisms

Stypopodium zonale is a tropical brown macroalga found abundantly on the Brazilian coast (Oliveira Filho 1977). Specimens of this macroalga were collected at Praia do Forno, Armação de Búzios (22°45'S, 41°52'W, Southeastern Brazil, Rio de Janeiro State) in shallow waters (about 3m depth). In February 2011, 9 individuals of *S. zonale* were collected and transported to the laboratory at Universidade Federal Fluminense, where they were cleaned and fragments from the apical (n= 9) and basal (n= 9) parts of each individual were separated and frozen for later lyophilization and extraction for the determination of phlorotannin content. Apical and basal parts of 5 of these individuals were submitted to chemical fixation for further microscopic analysis. The top 2 cm of each thallus of *S. zonale*, which contains the marginal row of apical cells, was cut to correspond to apical region, while the tissue 6-10 cm below was considered base.

Extraction

After lyophilization, apical and basal regions of *S. zonale* were ground to powder. Prior to

the extraction, to maximize phlorotannin extraction, lipids were removed from these algal parts with the addition of 1mL hexane 3 times, removed after 3 min as previously described (Koivikko *et al.* 2007). Extraction was made using 10mL of a mixture of acetone:water (7:3) for 100mg alga (dry weight - DW) for 2h under magnetic agitation, followed by centrifugation for 10min at 3500 rpm and filtration. After acetone was completely evaporated at room temperature to obtain a water-extract that was centrifuged for 10min at 3500 rpm, the volume was measured and the sample was frozen for later quantification of phlorotannins.

Quantification of phlorotannins

In order to quantify the amount of phlorotannins, the Folin-Ciocalteau (FC) method (Folin & Ciocalteau 1921) was utilized. FC reagent 1N (Sigma-Aldrich) was added to an aliquot of diluted extract, and, after 3 min, sodium carbonate 20% and water were added to the solution and the reaction was kept in dark for 45min, after which phlorotannins were quantified in spectrophotometer Schimadzu UV1800 at 750nm, using as standard a calibration curve obtained with phloroglucinol (r^2= 0.99). Three aliquots or replicates of each extract were prepared and analyzed. Phlorotannin concentration is represented as % DW.

Optical microscopy analysis

The ImageJ program (Abràmoff 2004) was used to count the number of physodes inside cells within parts of *S. zonale* thallus (n= 60). The cells were chosen from basal and apical portions of *S. zonale* thallus in different individuals (n= 5). Samples were fixed in a solution of 4% formaldehyde, 5% glutaraldehyde, and 1% caffeine diluted in 0.05 M cacodylate buffer, pH 7.3, prepared with seawater. The fixed samples were post-fixed in osmium tetroxide 1% for 2h at room temperature. Algal samples were then dehydrated in a crescent acetone series (until 100%) and embedded in Spurr resin at room temperature. The polymerization process was performed at 70 ^{o}C. The semi-thin sections (300 nm) were obtained in a Reichert ultramicrotome. Samples were stained with toluidine blue and the images were performed by a Zeiss Axioplan microscope coupled with an XC 30 camera.

Statistical analysis

Total content of phlorotannins from the different parts of the thallus of *S. zonale* was compared by Wilcoxon Matched Pairs, as data were not normally distributed. Number of physodes found in both apical and basal fragments of *S. zonale* was compared using a dependent *t*-test. As apical and basal fragments studied were from the same individual of *S. zonale*, data from both approaches were tested with specific tests for dependent samples.

Results

Spectrophotometric analysis revealed that apical regions of *S. zonale* presented significantly lower concentrations of phlorotannins (0.49% DW ± 0.04) than the corresponding basal ones (0.78% DW ± 0.21; p = 0.007; Figure 1).

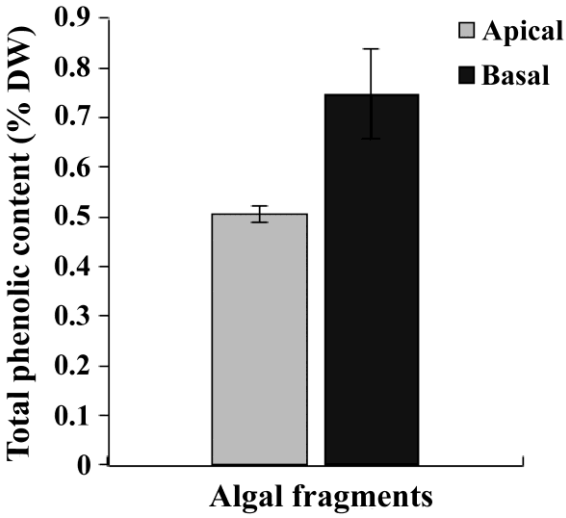

Figure 1. Total phlorotannin content (% DW) in apical and basal fragments of *Stypopodium zonale*. Data expressed represent mean values ± standard error.

In this study, we also verified that cells of apical and basal fragments of *S. zonale* differed in the number of physodes, since in apical fragments significantly less physodes per cell were verified (10.8 ± 5.85) than in basal fragments (42.73 ± 16.16; p = 0.0035; Figure 2). This clear difference was also observed by microscopic analysis, with a higher number of physodes in basal cells (Figure 3 a and b) compared to apical ones (Figure 3 c and d).

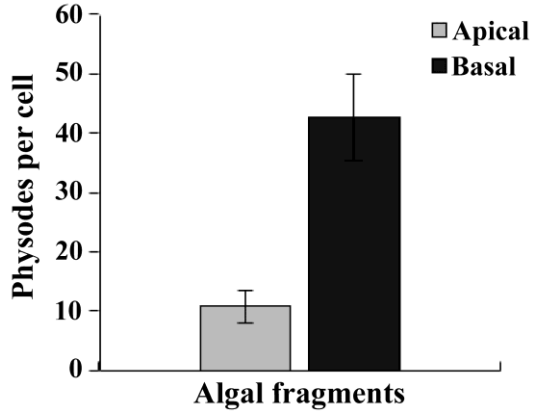

Figure 2. Number of physodes per cell in apical and basal fragments of *Stypopodium zonale*. Data expressed represent mean values ± standard error.

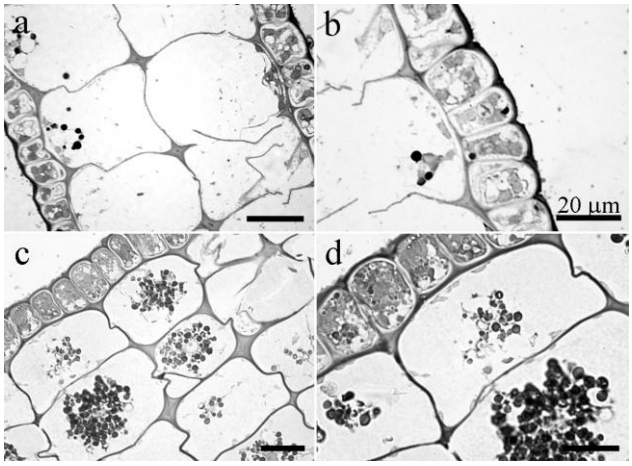

Figure 3. Microscopic images of *Stypopodium zonale* fragments (**a** and **b** – apical; **c** and **d** – basal portion of the thallus).

Discussion

Within-thallus variation of phlorotannin content in macroalgae was previously investigated but did not reveal a clear pattern. For example, higher concentrations of phlorotannins were found in the stipes of *Ascophylum nodosum* compared to the annual shoots and reproductive tissue of this macroalga (Pavia *et al.* 2002). Meristematic regions of some species of Laminariales and Fucales possess higher levels of phlorotannins than other non-meristematic tissues (Van Alstyne *et al.* 1999). Sometimes, even differences in levels of phlorotannins were not verified within distinct parts of some macroalgae (*e.g*. Fairhead *et al.* 2005).

Other defensive chemicals distinct from phlorotannins also did not exhibit a clear pattern of abundance in parts of the macroalgae. While apical region of the green seaweeds *Avrainvillea elliottii* (Lima *et al.* 2008) and *Halimeda* (Paul & Van Alstyne 1988) are richer in defensive metabolites, the brown macroalga *Dictyota ciliolata* shows older portions of the thallus as more defended against consumers (Cronin & Hay 1996).

The brown macroalga *Zonaria angustata* presents very low density of physodes in the subapical cells but higher density in apical cells, and this distribution was negatively correlated with grazing by amphipods (Poore 1994). A high density of physodes in apical cells was also observed in macroalgae belonging to the order Sphaceraliales (Clayton 1990). Tissues of brown macroalgae rich in physodes clearly have higher phlorotannin concentrations than those with less of these subcellular structures (Ragan & Glombitza 1986).

According to our results, lower concentrations of phlorotannins and a smaller number of physodes observed in the apical region when compared to the basal region could make the apical region of *S. zonale* more susceptible to herbivory. For example, mesograzers are known to prefer new algal tissues, including the brown macroalgae *Cystoseira baccata* (Arrontes 1990) and *Zonaria angustata* (Poore 1994) or the red species *Hypnea spinella* (Brawley & Adey 1981) and *Chondrus crispus* (Shacklock & Croft 1981). In *S. zonale*, the growth initiates by a marginal row of apical cells, and this region would be more vulnerable to consumption.

The present data are in accord with the growth-differentiation balance hypothesis (GDBH), which predicts that apices, which are actively involved in growth processes, would have low levels of secondary metabolites and would be less tough than older tissue because cell walls have not yet matured (Cronin 2001). This accordance was also described before for brown macroalgae from the Northeast Pacific (Van Alstyne *et al.* 1999). Thus, the highest levels of phlorotannins and the highest number of physodes found in the basal regions of *S. zonale* thallus could be related strictly to the role of these substances in primary metabolism, like cell wall strengthening and improvement of the holdfast structure.

At low concentrations, as the found in apical and basal regions of *S. zonale* (0.49% DW and 0.78% DW, respectively), phlorotannins would probably be insufficient to inhibit herbivory. Similar low concentrations of these chemicals were also found in Phaeophyceae from Guam, the tropical Pacific region, and neighboring areas (Steinberg & Paul 1990, Van Alstyne & Paul 1990), and these quantities were considered too low to deter herbivores, mainly fishes (Steinberg & Paul 1990). However, *S. zonale* is known to be chemically defended tropical seaweed due to its terpenoids of mixed biogenesis (Pereira *et al.* 2004). Due to the possibility of producing both classes of chemical compounds, which are potentially defensive against consumers, a trade-off between biogenetic routes to produced mixed terpenoids and phlorotannins should be investigated. In addition, future studies would be necessary to verify the correlation between physodes densities, amount of phlorotannins, and within-thallus susceptibility of *S. zonale* to consumption, mainly by mesograzers that exhibit small size and ability to feed selectively on a small scale.

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