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Effects of extracts and isolated molecules of two species of *Gracilaria* (Gracilariales, Rhodophyta) on early growth of lettuce

Priscila Torres[a,*], Paula Novaes[a], Luciana Garcia Ferreira[b], Janaína Pires Santos[c], Ester Mazepa[b], Maria Eugênia R. Duarte[b], Miguel D. Noseda[b], Fungyi Chow[c], Deborah Y.A.C. dos Santos[a]

[a] Phytochemical Laboratory, Department of Botany, Institute of Biosciences, University of São Paulo, São Paulo, SP, Brazil
[b] Carbohydrate Chemistry Division, Department of Biochemistry and Molecular Biology, Federal University of Paraná, Curitiba, PR, Brazil
[c] Laboratory of Marine Algae Édison José de Paula, Department of Botany, Institute of Biosciences, University of São Paulo, São Paulo, SP, Brazil

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ABSTRACT

Crude extracts using hexane, dichloromethane, methanol, 80% methanol or water of two agarophytes (*Gracilaria caudata* and *Gracilaria domingensis*) and their phase partitions were evaluated on early growth of lettuce. Hexane, methanol, 80% methanol and aqueous extracts of *G. caudata* and dichloromethane and aqueous extracts of *G. domingensis* were biostimulants. Palmitic acid, the major compound of non-polar extracts and phase partition, showed a significant stimulant activity in the concentrations tested (ranging from 0.49 mM to 1.95 mM), increasing 83% of lettuce root length, comparing to control, at the highest concentration. The promoting effect of the aqueous extracts is probably related to the presence of agaran, a typical hydrocolloid polysaccharide from red algae, which is mostly an exclusive constituent of these extracts. The agarans of *G. domingensis* and *G. caudata* promoted an increase in lettuce root length of 60% and 40%, respectively (both at 1 mg·mL^{-1} of extract). Differences in the effects promoted by these two agarans are probably related to the different content of sulfate groups (higher for polysaccharide from *G. domingensis*). These results suggest that structural features of agaran-type polysaccharides can elicit distinct responses. To the best of our knowledge, this is the first report on the identification of compounds with biostimulating potential from *Gracilaria*.

1. Introduction

Marine algae have been used by mankind since prehistoric times from around 16,000 BCE [1], mainly as food, but also as fertilizers by farmers located on the coast all over the world. Nowadays, seaweeds or their derivative products are commercially available for vegetable crops. *Ascophyllum nodosum* (L.) Le Jolis and *Ecklonia maxima* (Osbeck) Papenfuss are two important examples of brown algae used as fertilizers. Green and red seaweeds, in a lower scale, can also be used for this purpose. Species of *Lithothamnion* Heydrich, a genus of calcareous red seaweed, are sold not only as fertilizers but also for soil pH correction [2,3]. Seaweeds can be marketed as a dry powder or, more commonly, as concentrated extracts [4]. These extracts are ordinarily prepared by alkaline hydrolyzation, but also by fermentation and high-pressure cooking [5].

The use of seaweeds for crop cultivation is quite attractive due to the positive effects observed on crop plant resistance to biotic and abiotic stresses, and the increase in soil moisture retention and mineral availability [3]. Notwithstanding, a higher crop growth is the most important factor for seaweed trade in this scenario. This increased growth has been attributed to seaweed macronutrients, such as nitrogen and potassium [2], as well as to micronutrients, such as zinc, magnesium, and sulfur, also found in the extracts [6]. However, the use of seaweed ashes does not result in the same effects on growth. Therefore, the organic material present in the algal preparation has a fundamental role in stimulating plant growth [7,8].

The elucidation of the plant stimulant properties coming from algal extracts has been extensively discussed in the literature, but there is no consensus about what substances promote it [9]. Growth regulators, such as cytokinin, auxin, ethylene, gibberellin, abscisic acid and brassinosteroids; osmolytes, such as proline and betaines; polysaccharides, such as agarans, carrageenans and alginates; and other secondary metabolites, such as phlorotannins, are some examples of compounds that could be responsible for crop improvement [4,9]. Thereby, extract complexity and the possible synergism between the algal compounds could be responsible for the stimulating effect.

Nowadays, the decrease on crop productivity and food availability, related to global climate changes and higher food demand [10],

* Corresponding author.
E-mail address: priscila.torres@usp.br (P. Torres).

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increased the search for strategic actions that could ameliorate soil use, increase crop productivity and resistance to abiotic and biotic stresses. They are an important topics of worldwide research. In this context, seaweeds have a large potential to help world agriculture. Species of the red algae *Gracilaria* Greville show some interesting characteristics for use in agriculture. For example, Singh et al. [11] observed that application of seaweed sap of *Gracilaria edulis* (S.G. Gmelin) P.C. Silva led to an increase in corn (*Zea mays* L.) productivity. Despite that, there are few studies about the elucidation of the compounds responsible for the biostimulating power. Therefore, in the present work, we evaluated the possible effects of the extracts and/or isolated compounds of two macroalgae, *Gracilaria caudata* J. Agardh and *Gracilaria domingensis* (Kützing) Sonder ex Dickie, on germination and early development of *Lactuca sativa* L., which has been an important plant model commonly used for laboratory assays.

2. Material and methods

2.1. Seaweed biomass

Two seaweed species were collected on Northeast Brazilian coast in 2013: *G. caudata* (Mãe Luiza Beach, Natal, Rio Grande do Norte State - 5°47′39.1″S, 35°10′55.8″W) and *G. domingensis* (Morro de Pernambuco, Ilheus, Bahia State - 14°48′21.6″S, 39°01′25.6″W). *Gracilaria caudata* was identified by Dr. Eliane Marinho Soriano (Federal University of Rio Grande do Norte – Brazil), based on morphological characteristics. The identity of *G. domingensis* was confirmed by Dr. Beatriz Nogueira Torrano da Silva using COI-5P marker based on barcoding technique [12], and the sequences were compared with public reference database. The material was washed in tap water to remove sand and epibionts and then air-dried for transportation to the laboratory to be completely dried in an oven at 40 °C for seven days.

2.2. Extraction and partition of active extracts

The dried biomass of *G. caudata* and *G. domingensis* was used separately, powdered in knife mill (sieve of 30 mesh) (Fortinox® STAR FT 80, Piracicaba, SP, Brazil) and submitted to successive maceration at 50 ± 5 °C for 8 h with different solvents of increasing order of polarity: hexane, dichloromethane, methanol, 80% methanol and ultrapure water. The solvent ratio used during extraction was of 1:10 (w/v) except for the aqueous extract that was 1:20 (w/v). After filtration, the resulting extracts were concentrated in a rotary evaporator (t < 40 °C) and lyophilized until complete dryness. Salt-rich extracts (methanol and 80% methanol) were desalted adding methanol as an anti-solvent. The process was repeated several times until all salt was removed, and no precipitation was observed. The presence of halogenated salts was verified by the Mohr's method and then, the supernatant was collected and concentrated as described above. Since methanolic and 80% methanolic extracts of *G. caudata* showed a significant stimulant activity on early growth of lettuce, these extracts were further resuspended in 50% methanol and partitioned with dichloromethane. The dichloromethane phases of both extracts were pooled together due to their similar chemical composition.

Thereby, the extraction procedure resulted in five crude extracts for both *G. caudata* and *G. domingensis*, named hexane (HX), dichloromethane (DCM), desalted methanol (MeOH), desalted 80% methanol (80% MeOH) and aqueous. In addition, three-phase partition was also obtained only for *G. caudata*: 50% methanol originated from methanolic extract, 50% methanol originated from 80% methanolic extract and dichloromethane.

2.3. Analysis by gas chromatography–mass spectrometry (GC–MS)

Extracts and phase partitions (1 mg·mL^{-1}) were derivatized using 50 μL of BSTFA [*N*,*O*-Bis(trimethylsilyl)trifluoroacetamide] in 50 μL of pyridine for 30 min at 80 °C, and submitted to analysis in GC–MS (Agilent 6890N/5975, Santa Clara, CA, USA). The chromatographic conditions were: HP-5MS column (30 m × 0.25 mm × 0.25 μm), the initial temperature at 100 °C for 6 min, then increasing at 15 °C·min^{-1} until 225 °C, and at 5 °C·min^{-1} until 300 °C, remaining at this temperature for 9 min. Helium was used as the carrier gas at a flow rate of 1 mL·min^{-1}. The injector, ions source, and quadrupole temperatures were at 300, 230 and 150 °C, respectively. MS detection was performed with electron multiplier voltage (EM) at 70 eV, operating in the full-scan acquisition mode with a range of 10–1000 units of atomic mass and 2.64 scans·s^{-1}. The relative content of each component was calculated based on total peak area. Identification of the components was carried out using the NIST/NBS database (accepting similarity index higher than 900) and data on the literature.

2.4. Protein, carbon and sulfur content of aqueous extracts

Carbon (C %) and nitrogen (N %) from the water extracts were obtained by elemental microanalysis in Perkin-Elmer® 2400 Series II analyzer (Waltham, MA, USA). The protein content was estimated by multiplying the percentage of nitrogen by the correction factor (N % × 6.25) [13,14].

The sulfur content was determined by turbidimetry using barium chloride-gelatin method with modifications for analysis in 96-well microplate readers (BioTek® Synergy™ H1, Winooski, VT, USA). Total and non-esterified sulfur content was measured and used to estimate the esterified sulfur content. Aliquots of 500 μL of aqueous extract (20 mg·mL^{-1}) added with 500 μL of 1 mol·L^{-1} hydrochloric acid were heated in a dry bath at 100 °C for 2 h. After cooling, the solution was centrifuged at 12,000*g* for 5 min. The residues were discarded and the supernatants (hydrolyzed samples) were used to analyze total sulfur content. Non-hydrolyzed samples were obtained as described, without heat. The turbidimetric reagent was prepared based on Dodgson and Price [15], with modifications. 50 mg of gelatin of animal origin (sulfur free) was dissolved in 10 mL of boiling ultrapure water. This solution was added to 500 mg of barium chloride ($BaCl_2$) and 500 mg of sodium chloride (NaCl). The final volume was adjusted to 50 mL. In each microplate well, 25 μL of hydrolyzed or non-hydrolyzed samples, 50 μL of the turbidimetric reagent and 125 μL of ultrapure water were added. Microplates were shaken for 5 min, incubated for 15 min at room temperature, and the absorbance measured at 405 nm. For negative control, the sample volume was substituted by 0.5 mol·L^{-1} HCl. Sulfur content was calculated using a standard curve, which was performed with sodium sulfate (Na_2SO_4) diluted in 0.5 mol·L^{-1} HCl at different concentrations (0 to 2000 μg·mL^{-1}). Esterified sulfur content (ES) was calculated using the following formula: ES (%) = H (%) − NH (%), where, H is the hydrolyzed value and NH is the non-hydrolyzed value. The sulfur substitution rate in sulfated polysaccharides (SR) was estimated using ES and C percentages, by SR (%) = 4,5 × ES (%) × [C (%)]$^{-1}$ [13].

2.5. Nuclear magnetic resonance (NMR) spectroscopy of aqueous extracts

Aqueous extracts were submitted to NMR analyses using an NMR spectrometer (BRUKER, Advance DRX400, Billerica, MA, USA) equipped with a 5-mm multinuclear inverse detection probe. The base frequency was 100.63 MHz for ^{13}C and 400.13 MHz for ^{1}H nuclei. Analyses were recorded at 70 °C and acetone was used as an internal standard at 30.2 and 2.224 ppm for ^{13}C and ^{1}H NMR spectra, respectively. For ^{13}C NMR spectra, the samples (40 mg·mL^{-1}) were dissolved in D_2O:H_2O (1:9). For ^{1}H and 2D NMR experiments, the samples were deuterium exchanged by successive freeze-drying steps in D_2O (99.9%) and then dissolved in D_2O (20–25 mg·mL^{-1}). ^{1}H, ^{13}C and ^{13}C DEPT acquisition parameters were previously reported [16]. 2D ^{1}H, ^{13}C HSQC experiment was carried out using the pulse program supplied with the Bruker manual.