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A laser-scanning confocal microscopy study of carrageenan in red algae from seaweed farms near the Caribbean entrance of the Panama Canal

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Abstract *Kappaphycus alvarezii* (Doty) Doty ex P.C. Silva, a red macroalga, is a commercial source of carrageenan, a widely used polysaccharide compound important in the food and pharmaceutical industries, in nanotechnology, and in pharmacological applications. Carrageenan is found mainly in the cell wall and in the intercellular matrix. This is the first study to propose the characterization of carrageenans in vitro, using the auto-fluorescence properties of the alga treated with different polyamines: putrescine, spermidine, and spermine. This study suggests a four-phase cultivation sequence for seaweed farmers to enhance and assess the potential carrageenan yield of their crops. In phase 1, seedlings were treated with each of the polyamines. Explants were subsequently transferred through two additional culture phases before being planted on the sea farms in phase 4 and then harvested after 60 days for analysis. Images from transverse sections of 11 representative cultured *K. alvarezii* samples were obtained at 561 nm excitation wavelength for both the cell center and the cell wall of each sample. Spectral data were also analyzed using the spectral phasor algorithm of SimFCS developed at the Laboratory for Fluorescence Dynamics (www.lfd.uci.edu). We report on the identification of several spectral fluorescence emission fingerprints from different auto-fluorescence compounds spatially mapped using this technique. These fingerprints have the potential to improve strain selection of explants for enhanced carrageenan yield in seaweed farming operations as well as to enable wholesale pricing to correspond with crop quality.

Keywords Carrageenan · *Kappaphycus alvarezii* · In vitro · Seaweed farms · Polyamines · Confocal microscopy · Fluorescence emission · Fingerprints

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Introduction

Carrageenan is a general name for polysaccharides extracted from certain species of red algae (Rhodophyta), mainly *Gigartina stellata* (Stackhouse) Batters, *Chondrus crispus* Stackhouse, *Eucheuma* spp., and *Kappaphycus* spp. Carrageenans are high molecular weight (MW = 200,000 to 800,000 Da) polysaccharides made up of repeating galactose units and 3,6-anhydrogalactose with varying numbers of sulfate groups connected by α-1,3 and β-1,4 glycosidic linkages. They are biocompatible compounds with specific gelling properties useful in a multitude of ways, including food and biotechnology programs and controlled drug release applications. Their reported anticancer, antioxidant, and antiviral activities have potential in the pharmaceutical industry, and the high metal binding capacities of carrageenans make them excellent candidates for nanoparticle synthesis and encapsulation processes (Raman et al. 2015). There are three primary

classifications of carrageenan, and the genus *Kappaphycus* was segregated from *Eucheuma* primarily on the basis of carrageenan type (Doty et al. 1986). DNA re-association kinetics were used to determine nuclear genomic organization and complexity in the carrageenophyte *Kappaphycus alvarezii* (Doty) Doty ex P.C. Silva (Kapraun and Lopez-Bautista 1997).

Carrageenan occurs naturally in the cell walls and in the intercellular matrix of several red algae, and it has been studied using diverse methodologies and measures (Gordon-Mills et al. 1978; Kapraun and Lopez-Bautista 1997; Lechat et al., 2000). Biochemical analysis and Fourier transformed infrared spectroscopy (FTIR) are the most commonly used tools for evaluation of carrageenan quality (Chiovitti et al. 1996; Chopin et al. 1990; Pereira et al. 2009; Pérez-Gonzáleza et al. 2009).

Kappaphycus alvarezii (Solieriaceae Rhodophyta) has been actively farmed in many countries as a biomass source for carrageenan production since the early 1970s (Doty and Alvarez 1974). However, the present-day carrageenan industry faces numerous challenges related to the quality and quantity of raw materials. As a result, in 2000, of the first-recorded occurrence of *Polysiphonia* sp. epiphytic filamentous algae (EFA), annual *K. alvarezii* production and the number of seaweed farmers declined rapidly (Critchley et al. 2004; Hurtado et al. 2006; Vairappan et al. 2007). This raised serious concerns about the future stability of the algal resource that persists to the present time. The outbreaks of EFA caused by *Polysiphonia* sp. in the Philippines (Hurtado et al. 2006) and the epiphyte *Neosiphonia savatieri* (Hariot) M.S. Kim & I.K. Lee in the Philippines and Malaysia (Hurtado et al. 2006; Vairappan 2006) caused a significant decrease in global *K. alvarezii* production. This decrease is reportedly correlated with drastic environmental changes in areas with low water motion in both of these locations. Morphological studies performed in China revealed that the main epiphyte on *K. alvarezii* was *N. savatieri* and reduced photosynthetic activity caused by extensive shading could be one of the primary reasons for the decreased productivity of *K. alvarezii* infected by *N. savatieri* (Pang et al. 2011).

A sustainable seaweed farms project was established in the Caribbean Sea near the northern entrance of the Panamá Canal by Gracilarias de Panamá S. A. The objective was to cultivate species of red algae used by the communities of the Panamanian Caribbean areas and to provide an important livelihood activity for residents. The first seaweed farms were developed with the species *Gracilaria domingensis* (Kützing) Sonder ex Dickie and *Eucheuma* sp. (Vega 2004a, b). These two genera were reported to already inhabit this area (Abbott and Norris 1985; Doty et al. 1986). In 2006, in vitro culture methods were initiated at the Galeta Point Marine Laboratory of the Smithsonian Tropical Research Institute, which is located close to the areas where the seaweeds are cultivated (Perez 2008; Soler and Batista 2007). Panama is among the few countries in the Americas with commercial farming of *Kappaphycus* sp. that has exported production to Europe and Asia (Hurtado et al. 2014).

Expertise obtained through the development and operation of the seaweed farms in the Caribbean of Panama revealed a need to improve and standardize various aspects of cultivation. In addition, it was hypothesized that improved selection processes for quality seedstock would result in a crop with higher carrageenan content. This study presents a procedure to identify and broaden knowledge of carrageenan content located in the cell wall and in the cell centers of the red alga *K. alvarezii.* Processes for enhancing carrageenan content were explored through in vitro techniques with treatment by three polyamines administered at different concentrations. Carrageenan was imaged in the treated explants at the Laboratory for Fluorescence Dynamics at the Department of Biomedical Engineering, University of California, Irvine, USA. A fit-free approach for the analysis of fluorescence spectral components in the microscopy images which does not require an a priori knowledge of the basis spectra was used. This technique is non-invasive and could be utilized to quantitatively identify different molecular species in live samples (Wright et al. 2012).

In this work, we present a biometric tool developed for identifying the presence and the concentration of carrageenans that occur naturally in the wall cell and in the intercellular matrix of red algae. This tool also has the potential to be useful for the identification of other seaweed-derived hydrocolloids. The tool detects spectral emission from explants using confocal microscopy, and it has been found to be a powerful method for identifying specific emission fingerprints of auto-fluorescence of compounds present in seaweeds. This study reports on the identification of several spectral fluorescence emission fingerprints from different auto-fluorescence compounds spatially mapped in the commercially important red algal species *K. alvarezii*. The explants imaged were cultivated in vitro and treated with the polyamines spermidine, putrescine, and spermine.

Materials and methods

Study area The culture system was developed in four phases using three cultivation areas. Phase 1: in vitro culture, grown in seawater in the laboratory. Phase 2: explants from phase 1 transferred to indoor culture tanks in the laboratory. Phase 3: explants from phase 2 transferred to outdoor cultivation tanks on the mainland. Phase 4: explants from phase 3 transferred to ropes anchored in the sea. In situ sea culture (phase 4) was carried out within the Maritime Concessions of the company "Gracilarias de Panamá, S.A." in the Cativa area of the Province of Colon near the Caribbean entrance of the Panama Canal. One of the fourteen farms designated by polygons within the Maritime Concessions was selected for in situ

sea culture. It was identified as polygon 11. The selected polygon was located in the northern section of Largo Remo within a lagoon open to the sea and surrounded by mangrove trees; substratum was predominantly sandy bottom with patches formed by small communities of sea grasses (Vega 2009; Vega et al. 2004). Seaweed cultivation activities were originally established in this area to involve local community members with limited livelihood options in the development of a type of aquaculture considered "eco-friendly" (Vega 2009; Vega et al. 2004). The physical infrastructures of the farms were thought to potentially buffer mangroves and coral reefs from the negative impacts of extreme weather events (Contreras and Rosenthal 2003). In addition, it was hoped that the long-term allocation of the marine common spaces for sustainable seaweed farming in the Concessions might shield this sensitive area from mega-scale development projects in the rapidly industrializing region (Brooks 2015).

The Polygon 11 used for this study in phase 4 is located within one of the most closely monitored tropical coastal zones in the world (Cubit et al. 1988). Environmental information has been collected throughout the area since the early 1900s, with an archive of historical in situ data recorded by the Smithsonian Tropical Research Institute (Cubit et al. 1988) and 80 years of data collected by the Panama Canal Authority. In 2000, the Coastal Research Institute of GKSS, Germany, installed an in situ hightech monitoring system to integrate environmental data at the site. The seaweed farm sites were selected based upon environmental parameter averages identified as ideal measures for successful seaweed cultivation. These were determined by geographical area and degree of variation by season (Vega et al. 2004). Data indicated an annual temperature range of 28 to 30 °C, pH range, 7.0–9.0, and salinity of 32.8–34.8 ‰. All these data ranges have remained relatively stable over the last several years (Contreras and Rosenthal 2003).

Growth of *K. alvarezii* is favored when salinity is about 32 to 35 ‰ and it is inhibited below 28 ‰ (Paula et al. 2001). *Kappaphycus alvarezii* growth rates seem to be optimal in a pH range from 7.0 to 9.0 with explants exposed to solar light at 30 cm depth and solar light intensity 845–1837 μmol photons m^{-2} s^{-1}. (Vega 2009; Vega et al. 2004). In all phases of this study, explants were planted in the sea on ropes similar to those used in commercial seaweed farms.

To observe carrageenan in the cell walls and within the center cellular matrix (Fig. 1) of the cultured algae by confocal microscopy, it was necessary to develop a model of in vitro culture. This was done in phase 1 at the laboratory level using incubators containing cultivated *K. alvarezii* seed stock obtained from the seaweed farms. The incubator was designed at the Galeta Point Marine Laboratory of the Smithsonian Tropical Research Institute constructed with ATT double glazed window glass consisting of two panels with an intermediate air chamber and double sealed with butyl and polysulfide or silicone. This insulation maintained a controlled temperature between 30 and 25 °C and constant humidity between 60 and 75 %. The incubator maintains a irradiation with two fluorescent light tubes at 40 W and two 20 W (TLT W/54RS) tubes, with a photoperiod of 12 h light and 12 h dark. The spectral emissions and confocal microscopy were explored as alternative technologies for measurement of carrageenan quality of seaweed. This histological analysis was performed at the Laboratory for Fluorescence Dynamics, University of California, Irvine, USA.

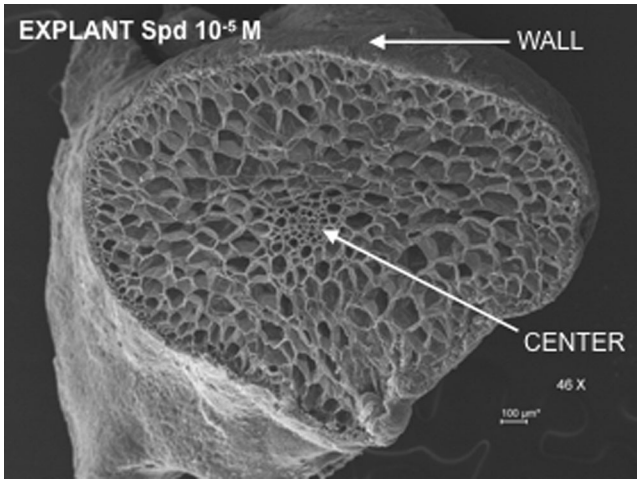

Fig. 1 Cell walls and intercellular matrix of *Kappaphycus alvarezii* SEM images obtained with Zeiss Model Evo40 VP. Images of explant treated with spermidine 10^{-5} M

Experimental phases

Selection of explants *Kappaphycus alvarezii* seed stock was collected from seaweed farm, polygon 11, over a 3-year period from 2012 through 2014. From the collected seed stock, explants were selected to be planted for phase 1 and those that survived were transferred to phases 2, 3, and 4 until the 60th day in each phase. For each phase, the explants treated with the different polyamines in phase 1 were transferred to different sites according to experimental design. The weight in grams and length in centimeters of the explants were measured every 15 days from the day of planting. Beginning in phase 2, width in centimeters was also measured for each explant. Explants were selected by size in phase 1 to maintain consistency with previously published reports from *K. alvarezii* (Soler and Batista 2007; Perez 2008; Robaina et al. 2008). All explants selected for the study appeared clean, green, and healthy. The existence of color strains of *K. alvarezii* with varied pigmentation (green, red, yellow, and brown) has been reported in the literature for decades, with differences in their physiological characteristics

including growth performance and photosynthesis reported from laboratory studies (Dawes and Koch 1991; Aguiree-Lipperheide et al. 1995; Hurtado et al. 2014; Pang et al. 2011).

Phase 1 began with the selection of explants of *K. alvarezii* that measured 1.0 cm long and weighed 0.02 g and which were seeded in Berlin packaging jars Wm Pp of 500 mL. All explants selected had a single shaft axis at the end without tangible protuberances to ensure consistency and proper counting of shoots and calli on the first day of measurement. The explants were placed in incubators after having been seeded into one of three jars. Each jar was seeded with four explants and the experimental culture medium added. The culture medium had been prepared previously and contained the polyamines, putrescine (Put), spermidine (Spd), and spermine (Spm) at the following rates: 0 M (control); Put 10^{-5} M, Put 10^{-7} M, and Put 10^{-9} M; Spd 10^{-5} M, Spd 10^{-7} M, and Spd 10^{-9} M; and Spm 10^{-5} M, Spm 10^{-7} M, and Spm 10^{-9} M. Standard culture media sodium metasilicate, Na_2SiO_3 was added. The pH was measured to be between 7.5 and 7.8. The polyamines described above were added to the medium after being autoclaved, and the solution further treated by filtration. Representative explants were measured and weighed every 15 days until completion on the 60th day of planting.

In phase 2, the surviving treated explants from phase 1 were transferred into each of two aquaria inside the laboratory designated ponds 2A and 2B in a semi-protected area, with a wall and a ceiling. Three explants were planted on each of three ropes of 50 cm long with a space of 12 cm between each seedling. Each culture tank had 12 algae in a total area of 12.5 cm^2. Representative explants were collected and measured every 15 days. In addition, new apices and calli that grew on each individual explant were counted and recorded.

For phase 3, explants obtained from phase 2 that appeared healthy and felt solid to the touch were transferred to the tanks. Each tank has an area of 1.0 m^2 at a depth of 0.50 m. We planted four ropes of 0.80 cm long. In each rope, we planted four seedlings spaced apart by 20 cm. Each tank contained 16 seedlings m^{-2}.

In phase 4, all surviving explants from phase 3 were transferred to the seaweed farm in three lines with 16 explants attached to each line, which were attached to structures anchored to the sea floor. Two sets of structures were anchored one beside the other, spanning an area of about 40 m^2. We randomly selected four explants to measure and observe every 15 days until the 60th day.

Daily growth rate Thirty-six explants were randomly selected from a population of approximately 96. Each explant was weighed in grams and measured in centimeters at 15-day intervals in order to determine the daily growth rate (DGR = % day^{-1}. The Sokal and Rohlf (1995) model was applied.

$$W_f = W_o e^{\mu t}$$
$$\mu = \left((\ln W_f - \ln W_o) t^{-1}\right) \times 100$$

where, W_f = final weight, W_o = initial weight, μ = growth rate, and t = planted day, until reaching the last date of the research. This variable was then used as the dependent variable. If there were more than two classifications, a Fisher's analysis of variance test was performed for completely randomized designs with Tukey's HDS test multiple comparison methods. All tests were with a 5 % significance level.

Statistical analysis SYSTAT statistical software package was used to run repeated measure analysis of variance on each of the variables, length, weight, and number of apices and calli were tested for normality and homogeneity of variance. Analysis of variance (ANOVA) and multifactorial analysis of variance were also conducted to determine the differences in growth rate (alpha = 0.05). Out of the nine replicates that were treated, 1 and 3 were selected randomly to be used in the analyses for a balanced design.

Confocal microscopy Two samples were used to detect fluorescence of carrageenan powder from two manufacturing companies known to produce good-quality carrageenan powder. The first sample was from Malaysia and the second from Japan Marine Science. A small portion of each powder was mounted on a slide with a drop of water. Observations and image acquisition were made with a confocal microscope (Olympus FV 1000) at the laboratory of electronic microscopy of the Smithsonian Tropical Research Institute in Panama to determine the emission spectra of carrageenan. Autofluorescence was detected using the Lambda acquisition mode of the Olympus microscope with laser excitation of 546 nm and photo-counting method. The emission was scanned every 3 nm from 560 to 660 nm.

Approximately 117 samples of explants from the cultivation phases were selected at random and recorded in a database by seed number, seed type, days of growth, and treatment. All samples were stored between 10 and 20 °C. They were later transported to the Laboratory for Fluorescence Dynamics at the University of California in Irvine for imaging and corresponding analysis. Upon detecting emission, we proceeded to check the fluorochrome in the library of the microscope to identify the carrageenan within the cells of the cultivated algae with auto-fluorescence. We then proceeded to make cross-sections of several samples of algae to observe auto-fluorescence from within the walls and throughout the centers of the cells.

Transverse sections of calli and apexes from explants of *K. alvarezii* were prepared from 11 explants in total,

representing samples cultured in the treatments. Observations of the spectra of carrageenan in the cell walls and within the intercellular matrix were made at the Laboratory for Fluorescence Dynamics, UCI, using the confocal microscope Zeiss LSM710, ×40 NA 1.2 water immersion objective.

Samples of *K. alvarezii* were as a follows: Callus samples 15, 17, and 86 were treated with 10^{-5} M spermidine. Apex samples 16 and 85 were treated with 10^{-5} M spermidine. Callus sample 91 and apex samples 88 and 107 treated with 10^{-7} M spermidine. Callus samples 83 and apex 87 were given control treatment. Sample 114 was a dried powder from the untreated parent alga.

For each of the samples described, cross-sections were made in order for the cell walls and intercellular matrices to be observed using confocal microscopy Zeiss LSM710, ×40 NA1.2 water immersion objective. Images of the cell wall and the cellular matrix of each sample were obtained at 561 nm excitation wavelength. Emission data was collected with the spectral mode of the Zeiss 710 LSM in the range 562–722 nm about every 3 nm for a total of 51 spectra. The spectral image of the Zeiss Zen software was used to display the images color coded according to the spectral color palette. Spectral data were also analyzed using the spectral phasor algorithm of SimFCS (Laboratory for Fluorescence Dynamics, www.lfd.uci.edu).

The spectral phasor approach The spectral phasor consists of a mathematical transformation of the emission spectrum into the Fourier domain as described by the following equations (Cutrale et al. 2013).

$$x\,\text{coordinate} = \text{g} = \frac{\sum_{\lambda} I(\lambda)\cos\left(2\pi n\lambda \Big/ L\right)}{\sum_{\lambda} I(\lambda)} \quad (1)$$

$$y\,\text{coordinate} = \text{s} = \frac{\sum_{\lambda} (\lambda)\sin\left(2\pi n\lambda \Big/ L\right)}{\sum_{\lambda} I(\lambda)} \quad (2)$$

Where *I(λ)* is the emission spectrum measured over a wavelength range *L* at each pixel of the image and *n* is 1 in our case. A fundamental property of the phasor transformation achieved by Eqs. (1) and (2) is that if the spectrum at each pixel of the image is due to the sum of two spectral components, then the phasors must align along a straight line joining the phasors of the pure components. Instead, if the spectrum at each pixel contains molecular species form more than two components, the phasor values cannot be aligned. Therefore, the spectral phasor analysis is a very simple way to obtain a specific fingerprint of a sample of unknown composition. This property is used in this manuscript to show that there are more than two spectral components in each sample and that each sample can be classified in terms of percent of pixels corresponding to at least three spectral components.

Results

Results of phase 1 showed statistical significance in length and weight with *p* value = 0.001 among treatments cultivated in vitro for 60 days. The explants treated with Spd 10^{-5} M had the longest length at 1.2–2.0 cm and the greatest weight increase at 0.05–0.90 g. They differed from the other treatments which obtained lengths ranging between 0.5 and 1.1 cm) and weights ranging between 0.013 and 0.040 g (Fig. 2a, b).

The growth rate in centimeters per day showed significant differences among treatments *p* value = 0.005 while growth rate in grams showed no significant differences *p* value = 0.11 (Fig. 2c, d). The explants treated with Spd 10^{-5} M obtained the greatest rate in centimeters (1.05–1.4 % cm day^{-1}) and also in grams (2.72–4.5 % g day^{-1}), although not significant (Fig. 2c, d).

Statistical significance was also found in new formations of callus among treatments *p* value = 0.025 and apexes *p* value = 0.046, Fig. 2e, f. The greatest number of new callus formation was six, in explants treated with Put 10^{-5} and the most apexes, four in Spd 10^{-7}.

In phase 2, selected explants from phase1 treated with Put 10^{-7} M and Put 10^{-9} M; Spd 10^{-5} M, Spd 10^{-7} M, and Spd 10^{-9} M; and control were cultivated in outdoor pond sites for 60 days. Differences in length and weight showed statistical significance with *p* value = 0.0001 and 0.001 respectively among treatments. The explants treated with spermidine 10^{-7} M presented greater length (3.0 cm) and weight (0.11 g) while explants treated with putrescine 10^{-9} M showed the shortest length (0.94 cm) and lowest weight (0.02 g) Fig. 3a, b.

The growth rate (% day^{-1}) in grams also showed statistical significance between treatments *p* value = 0.0001. Even though the explants treated with Put 10^{-7} M increased 1.86 (% day^{-1}) and Spd 10^{-7} M 2.77 (% day^{-1}) the most significant difference occurred in explants treated with Spd 10^{-5}, which presented the highest growth rate in grams 4.26 (% day^{-1}) (Fig. 3c). Statistical significance was not found in new callus formation or apex development among treatments *p* value = 0.902 and apexes *p* value = 0.646 (Fig. 2d, e).

In phase 3, selected explants from phase 2 treated with Put 10^{-5} M, Put 10^{-9} M, Spd 10^{-5} M, and control were cultivated in outdoor tanks for 60 days. The weight and length of the explants were not statistically significant between treatment means. *p* values = 0.515 and 0.257, respectively (Fig. 4a, b). Growth rates of explants in cm and in g (% day^{-1}), showed statistical significance between treatments *p* value = 0.001 and 0.033, respectively. The explants treated with Spd 10^{-5} M

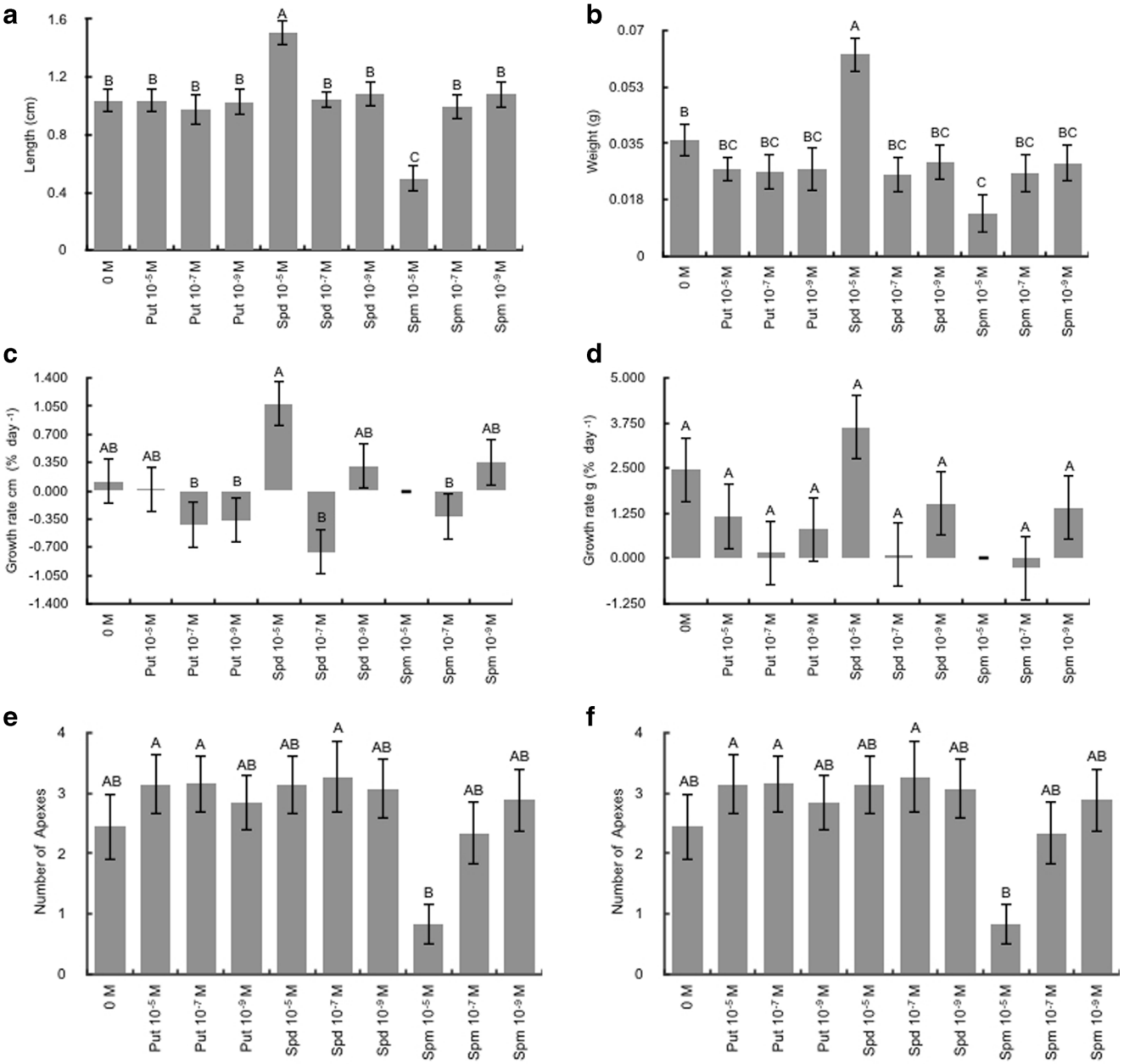

Fig. 2 **a** Length (cm), **b** weight (g), **c** growth rate (% day^{-1}) length (cm), **d** growth rate (% day^{-1}) weight (g), **e** number of callus, and **f** number of apexes of *Kappaphycus alvarezii* explants cultivated in vitro, phase 1 under different molar concentrations of polyamines; 0 M (control); Put 10^{-5} M, Put 10^{-7} M, and Put 10^{-9} M; Spd 10^{-5} M, Spd 10^{-7} M, and Spd 10^{-9} M; and Spm 10^{-5} M, Spm 10^{-7} M, and Spm 10^{-9} M until 60 days. Values presented are averages (n = 4). *Bars* show polyamines with different concentrations. The *vertical bars* show the standard error. *Uppercase letters* represent significant differences among the treatments according to the repeated measures analysis of variance using Tukey's HSD test under the hypothesis that all population means are equal considering $p < 0.05$

obtained the greatest range increase in centimeters, 4.37–5.17 (% day^{-1}) and in grams, 11.74–15.23 (% day^{-1}) (Fig. 4c, d).

Statistical significance was also found with respect to new formations of callus among treatments p value = 0.025, while the number of apexes presented no statistical significance p value = 0.98 (Fig. 4e, f). The explants treated with Spd 10^{-5} M had the greatest number of callus (24), and the number of apices (7) was statistically similar to the control.

In phase 4, explants selected from phase 3 treated with Put 10^{-5}, Put 10^{-9}, Spd 10^{-5}, Spm 10^{-7}, Spm 10^{-9}, and the control were transferred to seaweed farms for a duration of 60 days. In this phase, all explants planted on the farms increased in weight, length, and number of apices and calluses. Statistical significance was determined among all treatments for length (cm) with a p value = 0.0001, weight (g) p value = 0.0001. A wide centimeter variable was added which also showed statistical significance among all treatments with p value = 0.0001.

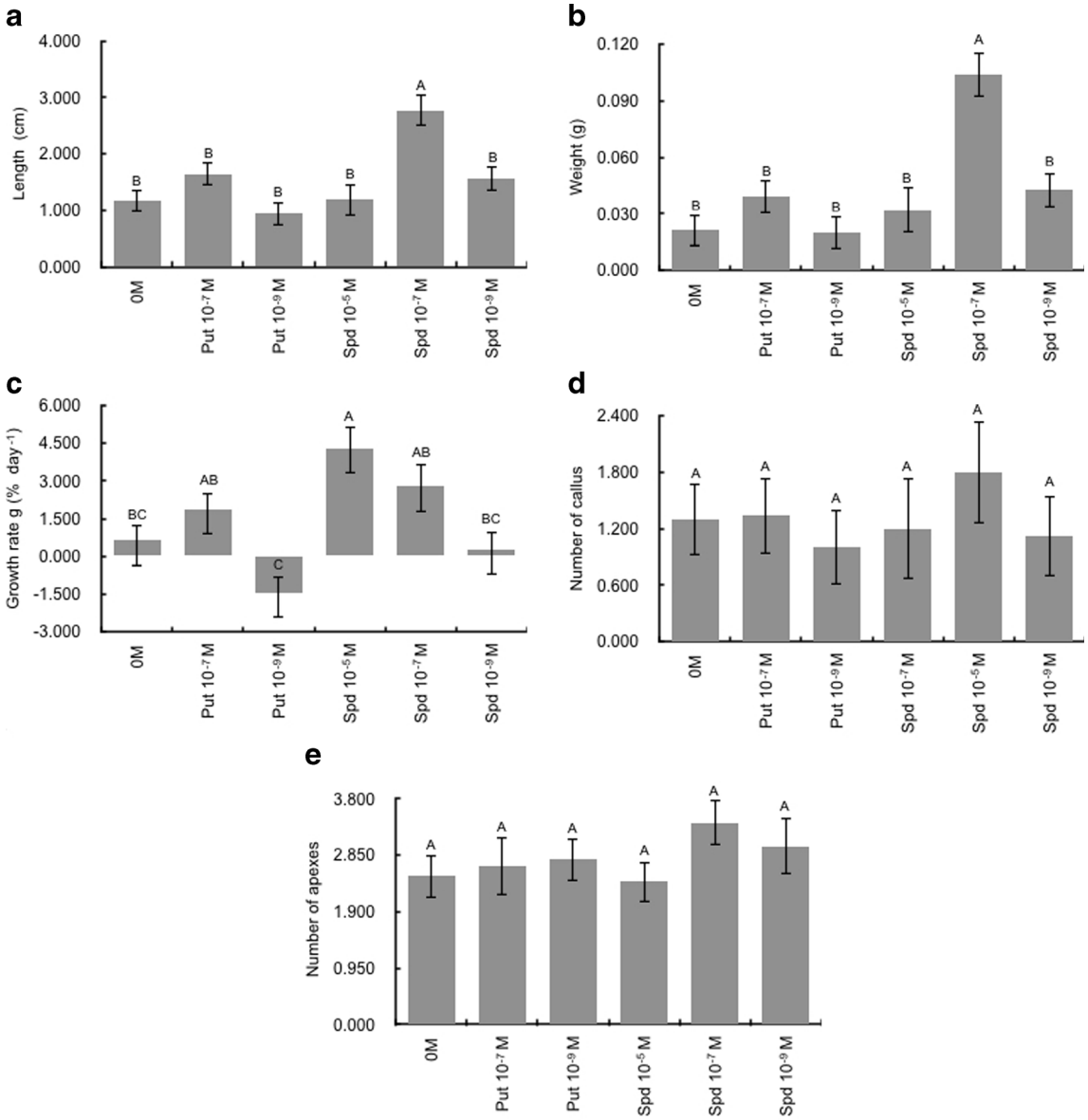

Fig. 3 **a** Length (cm), **b** weight (g), **c** growth rate (% day^{-1}) weight (g), **d** number of calli, and **e** number of apexes of *Kappaphycus alvarezii* in phase 2. The explants from phase 1 were transferred into ponds outside the laboratory in a semi-protected area under different molar concentrations of polyamines: 0 M (control); Put 10^{-7} M and Put 10^{-9} M; and Spd 10^{-5} M, Spd 10^{-7} M, and Spd 10^{-9} M until 60 days. Values presented are averages (n = 4). *Bars* show polyamines with different concentrations. The *vertical bars* show the standard error. *Uppercase letters* represent significant differences among the treatments according to the repeated measures analysis of variance using Tukey's HSD test under the hypothesis that all population means are equal considering $p < 0.05$

Number of calluses and apexes were also significant among all treatments p value = 0.017 and 0.019, respectively.

The mean length at 11.23 cm, weight at 34.0 g, width at 14.8 cm, number of calluses (20), and number of apexes of explants treated with spermidine 10^{-5} M were statistically different with higher values from the other explants (Fig. 5a–f). In that treatment, growth rate in grams also had a higher range of 2–11.0 % day^{-1} (Fig. 5d). The growth rate presented no statistical significance in grams (% day^{-1}) with p value = 0.19. The explants treated with spermina 10^{-9} presented the lowest values in number of both callus formation (2) and apices (3), as well as in length (0.43 cm) and weight (0.067 g). The lowest width (2.88 cm) was observed in Spm 10^{-7} M (Fig. 5a–c, e, f).

Confocal microscopy

For the confocal microscopy study, the following samples of *K. alvarezii* were analyzed: *15*, 16, 17, *83*, *85*, 86, *87*, 88, 91, 107, and 114 from the same mother alga no. 28. Images were obtained at 561 nm excitation wavelength of both the cell

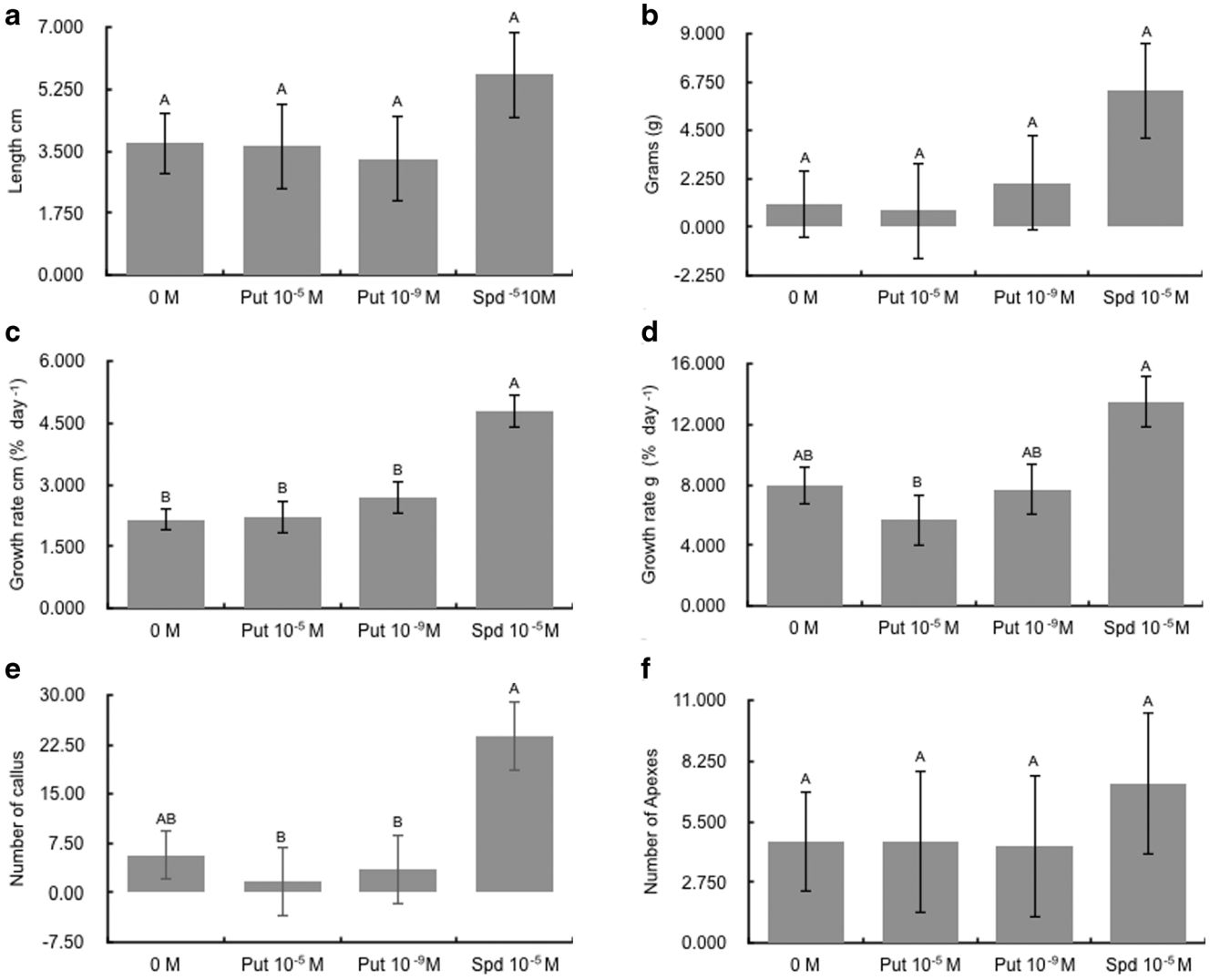

Fig. 4 **a** Length (cm), **b** weight (g), **c** growth rate (% day^{-1}) length (cm), **d** growth rate (% day^{-1}) weight (g), **e** number of calli, and **f** number of apexes of *Kappaphycus alvarezii* in phase 3. The explants from phase 2 0 M, Put 10^{-5} M, Put 10^{-9} M, and Spd 10^{-5} M, were transferred into outside tanks until 60 days of being planted. Values presented are averages (n = 4). *Bars* show polyamines with different concentrations. The *vertical bars* show the standard error. *Uppercase letters* represent significant differences among the treatments according to the repeated measures analysis of variance using Tukey's HSD test under the hypothesis that all population means are equal considering $p < 0.05$

center and the cell wall of each sample, except for sample no 114, the algae powder, for which only a single image was obtained. The samples with numbers in bold are reported in the figures of the manuscript.

Kappaphycus alvarezii samples are shown in Figs. 6, 7, 8, and 9, with the image colored according to the spectral scale and the intensity as provided by the ZEN software. These are shown together with the spectral phasor and the image colored according to the three-cursor selections as shown in the spectral phasor plot. Both cell center and cell wall are shown for each sample.

Each of the panel of figures shows the image colored according to the spectral palette, the spectral phasor image, and the image according to the colors blue-green-red as shown by the cursor selection in the respective spectral phasor. These cursors correspond to pixels in the spectral region 580 nm (blue), 586 nm (green), and 591 nm (red). The pie graphs in Figs. 6, 7, 8, and 9 show the percentage of pixels in the three-cursor selections for each image. In the phasor plot, if the pixels are aligned along a line, this implies that a pixel contains the linear combination of the spectra at the extreme of that line. We note that the pixels in the phasor plot are not aligned, indicating that we need more than two basic spectra to describe the emission of these samples. Since the emission spectrum is mainly from carrageenan, this indicates that there are more than two species of carrageenan present in these samples. The color maps in Figs. 6, 7, 8, and 9 reveal the region of the sample where these species are most abundant.

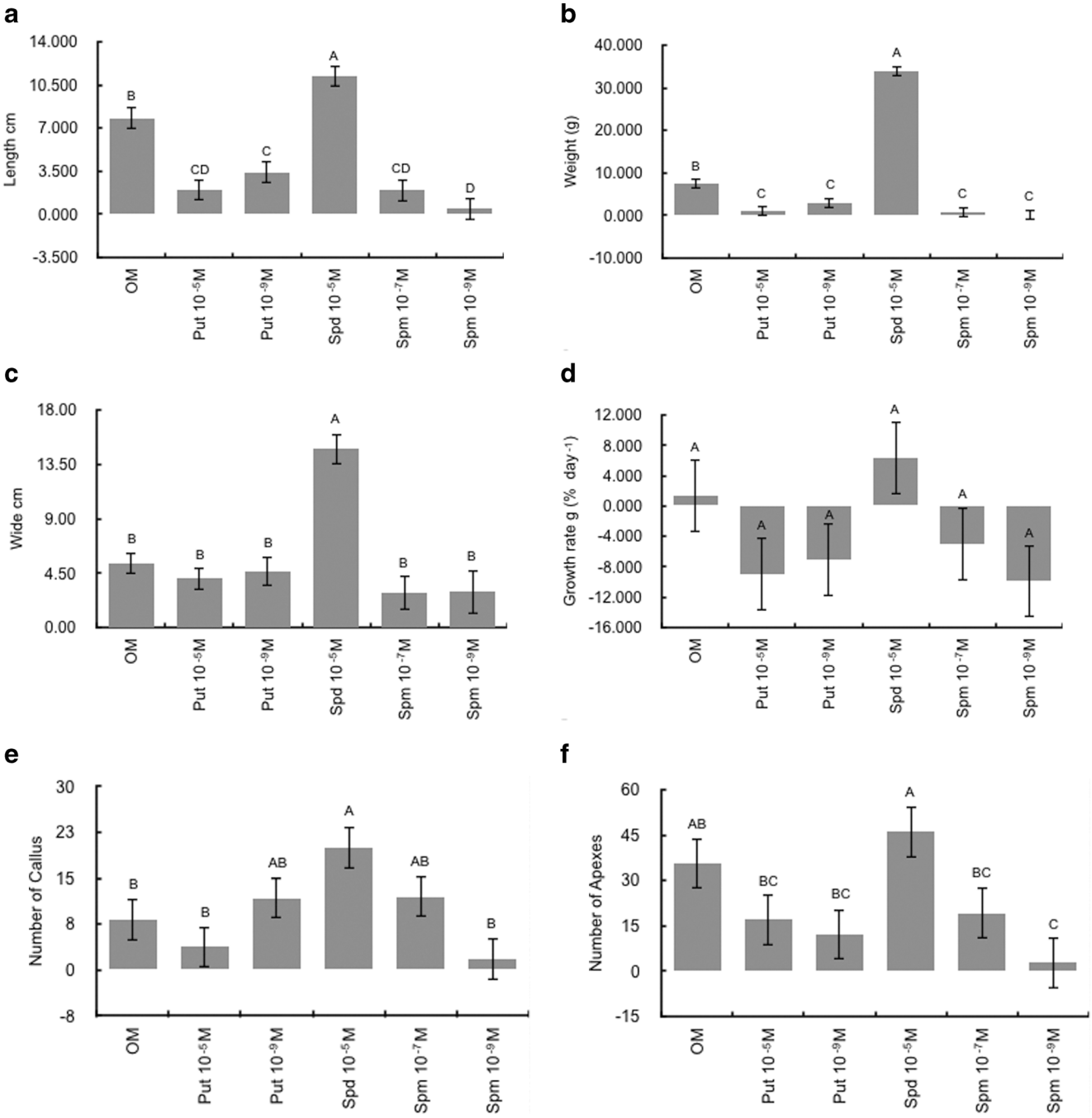

Fig. 5 **a** Length (cm), **b** weight (g), **c** width (cm), **d** growth rate (% day^{-1}) weight (g), **e** number of calli, and **f** number of apexes of *Kappaphycus alvarezii* in phase 4 from phase 3. The explants 0 M (control); Put 10^{-5} M and Put 10^{-9} M; Spd 10^{-5} M; and Spm 10^{-7} M and Spm 10^{-9} M cultivated in seaweed farms until 60 days of being planted. Values presented are averages (n = 4). *Bars* show polyamines with different concentrations. The *vertical bars* show the standard error. *Uppercase letters* represent significant differences among the treatments according to the repeated measures analysis of variance using Tukey's HSD test under the hypothesis that all population means are equal considering $p < 0.05$

Taken together, the analysis of all samples shows that different samples can be characterized by at least three emission components that seem to be representative of the carrageenan content and treatment. The exact source of this emission is unknown at present time, but we show that we can use an unsupervised classification and obtain a quantification of the abundance of different species based on the spectral phasor plot.

Control sample no. 83, callus from an explant of *K. alvarezii*, shown in the pie graph (Fig. 6), has a wider distribution of the green cluster presented both at the cell center and at the cell wall. This suggests a

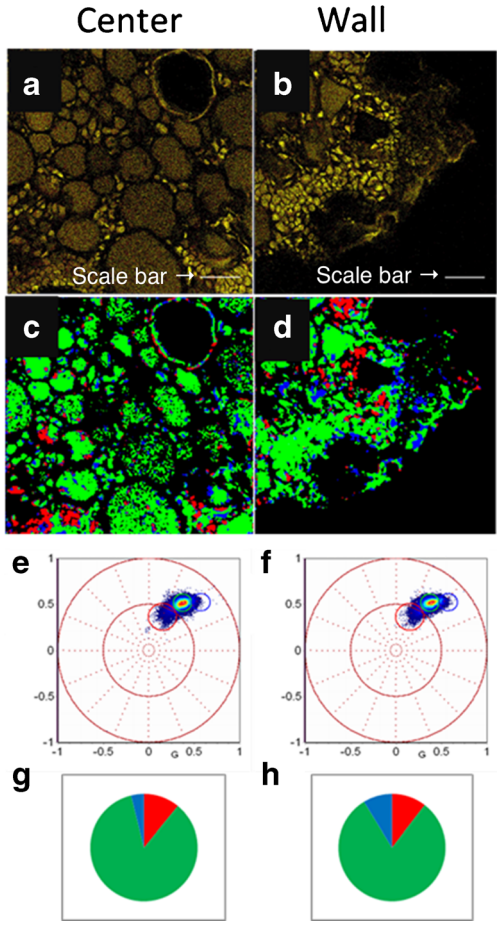

Fig. 6 Images obtained from a callus of an explant of *Kappaphycus alvarezii* cultivated in vitro treated as a control by confocal microscopy Zeiss LSM710, ×40 NA1.2 at both center and cell wall. **a**, **b** Images shown here are colored according to the spectra palette. *Scale bar* is 5 μm. **c**, **d** Images shown here are painted according to the spectral scale and intensity as provided by the ZEN software of (**a**) and (**b**) images. **e**, **f** Phasor plot histogram that corresponds to pixels in the spectral region: 580 nm (*blue*), 586 nm (*green*), and 591 nm (*red*) of (**c**) and (**d**) images, respectively. **g**, **h** Pie graphs show the percentage of pixels in the three cursors for each image

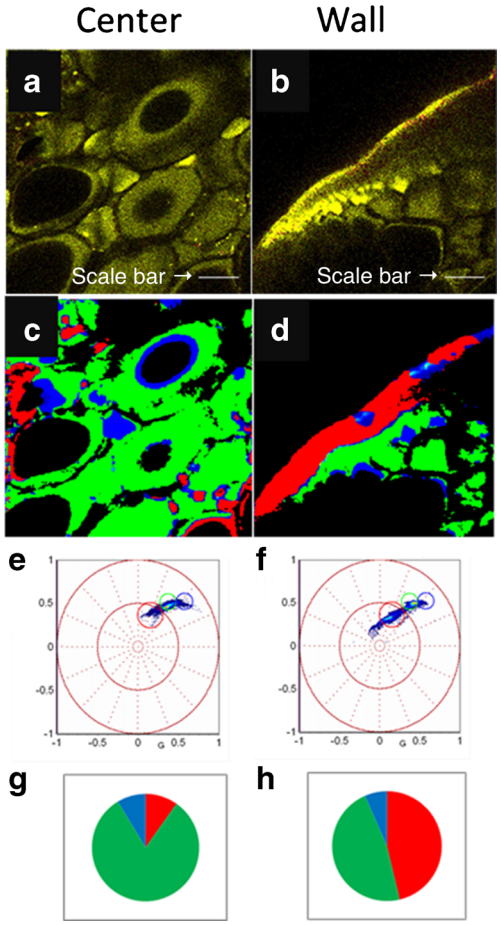

Fig. 7 Images obtained from an apex from explant treated as a control of *Kappaphycus alvarezii* cultivated in vitro by confocal microscopy Zeiss LSM710, ×40 NA1.2 at both center and cell wall. **a**, **b** Images shown here are colored according to the spectra palette. *Scale bar* is 5 μm. **c**, **d** Images painted according to the spectral scale and intensity as provided by the ZEN software of (**a**) and (**b**) images. **e**, **f** Phasor plot histogram that corresponds to pixels in the spectral region: 580 nm (*blue*) 586 nm (green) and 591 nm (red) of (**c**) and (**d**) images, respectively. **g**, **h** Pie graphs show the percentage of pixels in the three cursors for each image

distinct characteristic, compared with the callus treated with spermidine 10^{-5} M. Control sample no. 87, apex from explant (Fig. 7), shows a wider distribution of the green cluster in the center of the cell, and the red cluster at the cell wall also suggests a distinct characteristic compared with the apex treated with spermidine 10^{-5} M.

Callus and apex from sample no. 15 (Fig. 8) and sample no. 85 (Fig. 9), respectively, treated with spermidine 10^{-5} M showed that the callus had a much larger contribution of the red cluster in the cell wall. A wider distribution of green cluster at the center of the cell showed distinct color characteristics compared with the apex that was treated similarly to sample no. 15. This sample demonstrated a different morphology than the sample in Fig. 6. This type of morphology seems to result in a large contribution of the green component present both at the center and at the cell wall.

Discussion

This study combines an experimental approach to enhance commercial carrageenan productivity with the novel application of confocal laser-scanning microscopy, a technique capable of measuring and characterizing carrageenan content in vitro. We tuned the wavelengths of excitation and emission to record the emission fluorescence of carrageenans, and each color visually represents a different type of carrageenan although in this paper we have not identified the specific types.

The identification of several spectral fluorescence emission fingerprints from different auto-fluorescence compounds from

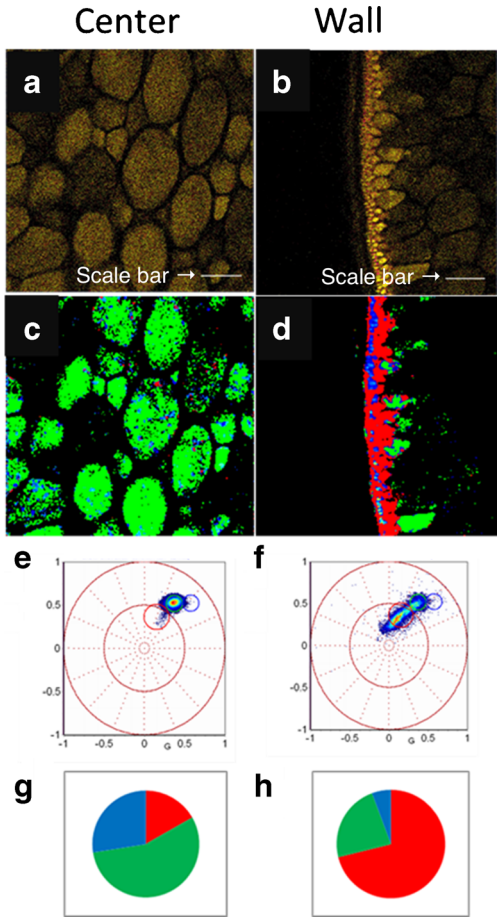

Fig. 8 Images obtained from a callus of explant treated with spermidine 10^{-5} M from *Kappaphycus alvarezii* cultivated in vitro by confocal microscopy Zeiss LSM710, ×40 NA1.2 at both center and cell wall. **a**, **b** Images shown here are colored according to the spectra palette. *Scale bar* is 5 μm. **c**, **d** Images shown here are painted according to the spectral scale and intensity as provided by the ZEN software of (**a**) and (**b**) images. **e**, **f** Phasor plot histogram that corresponds to pixels in the spectral region: 580 nm (*blue*) 586 nm (*green*), and 591 nm (*red*) of (**c**) and (**d**) images, respectively. **g**, **h** Pie graphs show the percentage of pixels in the three cursors for each image

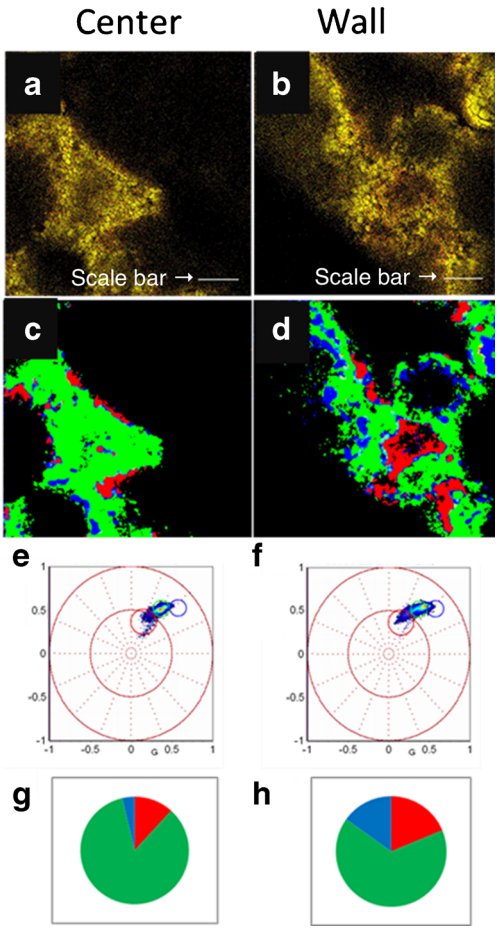

Fig. 9 Images obtained from an apex of explant treated with spermidine 10^{-5} M from of *Kappaphycus alvarezii* cultivated in vitro by confocal microscopy Zeiss LSM710, ×40 NA1.2 at both center and cell wall. **a**, **b** Images shown here are colored according to the spectra palette. *Scale bar* is 5 μm. **c**, **d** Images shown here are painted according to the spectral scale and intensity as provided by the ZEN software of (**a**) and (**b**) images. **e**, **f** Phasor plot histogram that corresponds to pixels in the spectral region: 580 nm (*blue*) 586 nm (*green*), and 591 nm (*red*) of (**c**) and (**d**) images, respectively. **g**, **h** Pie graphs show the percentage of pixels in the three cursor for each image

explants of *K. alvarezii* are reported and spatially mapped. These fingerprints have the potential to improve strain selection of explants with the aim to increase the carrageenan yield of seaweed farming operations and to potentially enable wholesale pricing to correspond with crop quality.

Carrageenans were characterized using auto-fluorescence properties of the species of *K. alvarezii* subjected to treatment with different polyamines: putrescine, spermidine, and spermine. A four-phase cultivation pipeline is presented for enhancing and assessing the carrageenan content of seaweed crops encompassing in vitro culture techniques.

Measurements of carrageenan fluorescence using bright field microscopy detected carrageenan in both the tetrasporophyte and in the female tissues of *Eucheuma isiforme* (C. Agardh) J. Agardh, and the calculation of metachromatic indices gave a higher value for the medullary cell walls in the tetrasporophyte than in the female gametophyte (Gordon-Mills et al. 1978).

During culture in the laboratory, we followed the explants growth by measuring size, weight, and number of new apices and calluses every 15 days after seeding. We used polyamines Put, Spd, and Spm as plant growth regulator (PGR) treatments that were expected to favor the development of cystocarps. Particularly, Spm promoted sporulation in several Rhodophytes such as *Hydropuntia cornea* (formerly *Gracilaria cornea*) and *Grateloupia imbricate* (García-Jiménez and Robaina 2015).

Investigations into the effects of PGR on algal growth and development are necessary to understand the physiological basis of algal growth, callus formation, and regeneration. This information can then lead to improved seaweed cultivation techniques by establishing and adopting viable methods

of enhancing strains of commercially important species (Yokoya et al. 2004). Zitta et al. (2013) found that the thick wall of the cells, which are composed in part of callus filaments, showed the presence of acidic polysaccharides, suggesting a large content of carrageenan and neutral polysaccharides.

In this study, calli and new apices were counted from day 15. Calli observed gave rise to irregularly branched uniseriate filaments, and we presume callus formation was initiated in the first week of seeding in phase 1. The new apical structures were elongated and we assumed that cell elongation occurs in the presence of large amounts of disorganized mitochondria and chloroplasts. The increased number of these organelles could be related to an increase in the process of cellular respiration and thus energy metabolism (Schmidt 2009), supporting subsequent cell divisions and the formation of lateral branching. According to Doty (1988), *K. cottonii* thalli are compressed to flatten above the basal segment; prostate, irregular in form, or with linear segments with irregular occurrences of protuberances or branches.

The images obtained in this study by the confocal microscopy of the control callus and apices of an explant of *K. alvarezii* indicated a broader distribution of the green cluster at the cell center and at the cell wall of the callus. The apices showed a higher percentage of red color at the cell wall while color at the center of the cell was very similar to the callus. This suggests a distinct characteristic compared with the callus and apex treated with spermidine 10^{-5} M (Figs. 8 and 9). These samples demonstrated a different morphology than the sample shown in Figs. 6 and 7. This type of morphology seems to result in a large contribution of the green component present both at the center and at the cell wall of the apex and more red color in the cell wall of the callus.

It is possible that the content and type of carrageenan may also correspond to specific parts of the algal structure, and these could possibly be impacted by various environmental conditions. Therefore, the spectral phasor analysis is a very simple, time-sensitive way to obtain a specific fingerprint of a sample of uncertain carrageenan composition. The fluorescence properties of carrageenan are used in this study to show that there are more than two spectral components in each sample and that each sample can be classified in terms of percent of pixels corresponding to at least three spectral components.

While the primary focus of this research was carried out utilizing different laboratory culture techniques, the success of the approach also depends on the efficacy of polyamine treated seedstocks in the natural marine habitat. We found in our study that improvements in seedlings produced in the laboratory also held in the natural marine habitat. If this success is shown to be repeatable, then our approach could be utilized to improve the quality and quantity of the product from the farms with obvious benefits to the industry.

This study suggests a novel technique for seaweed cultivators to assess the potential carrageenan yield of their crops in order to better select in vitro cultured explants prior to planting out on farms at sea. Consideration must also be given to these commercial possibilities beyond the usage of carrageenan in the food processing industries, such as in pharmacology, where high-quality parameters are required.

Food products for human consumption, mainly associated with the Asian market, account for 83 to 90 % of the total value of macroalgae. Chemical products extracted from macroalgae account for most of the remaining value. The various species possess high levels of secondary metabolites and structural polysaccharides of commercial value (Roesijadi et al. 2010). These include alginates from brown algae and agar and carrageenans from red algae. Today's growing demand for carrageenan and the need for more sustainable farming practices is a large challenge. There is an urgent requirement to understand and detect differences among types and qualities of carrageenan as a means of managing higher yielding, economically viable, long-term farming practices (Vairappan et al. 2007).

According to FAO statistics, world carrageenan seaweed farming production increased from less than 1 million wet tonnes in 2000 to 5.6 million wet tonnes in 2010, with the corresponding farmgate value increasing from US$72 million to US$1.4 billion. Major carrageenan seaweed farming countries include Indonesia, the Philippines, the United Republic of Tanzania, Malaysia, and China (Valderrama et al. 2013).

Despite the high demand for carrageenans, seaweed scientists, farmers, buyers, and processors still lack accessible tools to accurately and inexpensively assess the carrageenan content of seaweed crops and associated products throughout all stages of growth and refinement.

We continue to hone our studies related to the application of in vitro culture techniques of red seaweeds of commercial importance. Our efforts will focus on developing new plant biotechnology applications for the prevention of diseases of genera such as *Kappaphycus*, *Gracilaria*, and others of commercial importance.

Finally, carrageenan seaweed farming in particular, has evolved into a successful commercial endeavor in a number of tropical countries endowed with clear, unpolluted intertidal environments and protected beach locations (Valderrama et al. 2013). The area of farming at the entrance of the Caribbean side of the Panama Canal has a very specific annual weather pattern with a rainy season that lasts 9 months with 3 months of dry season. During our studies, we have not found a correlation between the seasonal weather patterns and the quality of the product (Vega 2009). We note that this area of the Caribbean seems to be protected from the adverse impacts of hurricanes (Contreras and Rosenthal 2003; Cubit 1994). This

unique condition is an important consideration regarding the importance of commercially viable seaweed farms in this region.

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