JÔNATAS MARTINEZ CANUTO DE SOUZA

Bioprocessos e potencial biotecnológico em espécies de *Gracilaria* (Rhodophyta, Gracilariales): uma abordagem fisiológica e bioquímica

Tese apresentada ao Instituto de Botânica da Secretaria da Infraestrutura e Meio Ambiente, como parte dos requisitos exigidos para obtenção do título de DOUTOR em BIODIVERSIDADE E MEIO AMBIENTE, na área de Concentração de Plantas Avasculares e Fungos em Análises Ambientais.

São Paulo

2020

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Ficha Catalográfica elaborada pelo **NÚCLEO DE BIBLIOTECA E MEMÓRIA**

Souza, Jônatas Martinez Canuto de
S729b Bioprocessos e potencial biotecnológico em espécies de *Gracilaria* (Rhodophyta, Gracilariales): uma abordagem fisiológica e bioquímica / Jônatas Martinez Canuto de Souza – São Paulo, 2020.
119p.; il.

Tese (Doutorado) -- Instituto de Botânica da Secretaria de Infraestrutura e Meio Ambiente, 2020.
Bibliografia.

1. *Gracilaria*. 2. Biofiltro. 3.Biotecnologia. I. Título.

CDU: 582.26

Aos meus pais, ao meu filho
e à minha querida esposa,
com muito amor e carinho!

Agradecimentos

Agradeço...

À Deus, pela força que me deu, pelo conhecimento e pelas conquistas que tive durante toda minha vida. Agradeço pelo conforto durante as dificuldades e momentos de solidão.

À minha família. Se não fosse por todo apoio e estrutura familiar que tive, nada disso seria possível. Pai Moacir, Mãe Rita que sempre me incentivaram e me ajudaram nos momentos mais difíceis que enfrentei durante toda minha vida;

À minha querida esposa Nátali. A pessoa com quem mais compartilhei, amor, alegrias, tristezas, conquistas. A pessoa que sempre me incentivou a seguir na carreira e sempre me estimulou a produzir, pensar e estudar. Sempre me alegrou e sempre esteve ao meu lado durante o mestrado e o doutorado.

Ao meu querido filho Luigi. A pessoinha que mais me alegrou desde que nasceu. Muitas saudades senti dele durante as viagens que fiz. Te amo Filhão.

À minha incrível orientadora, Dra. Nair Sumie Yokoya. Desde a iniciação cientifica até a defesa do doutorado, foram 14 anos de convivência que valeram a cada momento. Não tenho palavras que agradeçam as oportunidades, ensinamentos, conselhos e amizade que você me proporcionou, serei eternamente grato por tudo.

Aos pesquisadores e funcionários do Núcleo de Pesquisa em Ficologia, Dra. Mutue, Dra. Silvia, Dra. Célia, Dra. Luciana, Dra. Andrea, Valdilene e Neide, pelos conselhos, suportes e ótimas conversas;

Aos alunos do Núcleo de Pesquisa em Ficologia, Cesar, Andrea Dias, Patrícia Guimarães, Henrique Borborema, Angélica, Fábio Nauer, Júlia Zardi, Dani Chen, Tiago, Thais, Beatriz, Helena Iris e Patrícia Sanchéz, pela amizade, festas, viagens e ótima convivência ao longo desses anos;

Ao Cesar Bertagia Pasqualleti, que além de ser fiel companheiro de trabalho, é uma pessoa que não mede esforços para ajudar quem está ao seu lado. É um grande amigo que ganhei da pós-graduação e para a vida toda;

Ao Instituto de Botânica de São Paulo, por toda a infraestrutura e apoio oferecido;

Ao Dr. Pio Colepicolo Neto, Dr. Leonardo Vilella, Ednailson e Dr. Cícero por sempre me receber bem em seu laboratório, pelo apoio necessário para o desenvolvimento do trabalho e pela amizade;

À Fundação de Amparo à Pesquisa do Estado de São Paulo, pelo auxílio financeiro sob a forma de concessão de bolsa de doutorado (Processo FAPESP n° 2017/13259-3 e bolsa BEPE (Processo FAPESP n° 2019/16398-0);

Ao Conselho Nacional de Desenvolvimento Científico, pelo auxílio financeiro de concessão da bolsa de doutorado (Processo CNPq 145023/2017-6);

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior pelos recursos concedidos para a execução do projeto (CAPES/AUXPE-CIMAR 1991/2014/23038.001431/2014-75)

Ao professor Dr. Ondrej Novák e toda sua equipe pela oportunidade de estágio e por todo conhecimento adquirido no laboratório e na Universidade Palacky.

....Muito Obrigado!!!

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RESUMO

As macroalgas marinhas são fontes de compostos de interesse comercial e biotecnológico e podem ser biofiltradoras, uma vez que utilizam os nutrientes do meio no seu metabolismo. As espécies de *Gracilaria* Greville são exploradas como matéria prima para a produção de ágar e estudos sobre o seu desenvolvimento são necessários para a produção sustentável de biomassa, de ágar, biofertilizantes e de novos compostos bioativos. O presente estudo teve como objetivos avaliar os efeitos da disponibilidade de nutrientes (nitrogênio e fósforo) no metabolismo e no potencial biotecnológico de *Gracilaria caudata* J. Agardh e *Gracilaria domingensis* (Kützing) Sonder ex Dickie.). Foram analisadas as seguintes variáveis: taxas de crescimento, morfologia, fotossíntese (fluorescência da clorofila), proteínas solúveis totais, pigmentos (clorofila *a*, ficoeritrina, ficocianina e aloficocianina), carboidratos, conteúdo de elementos do talo (carbono, hidrogênio, nitrogênio e fósforo) e análise dos nutrientes disponíveis na agua do mar. Para avaliar o potencial biotecnológico, foram analisadas as características do ágar (rendimento, quantificação dos teores de sulfato e de 3,6-anidrogalactose) e testes de atividade inibitória da enzima acetilcolinesterase em extratos oriundos da biomassa de algas cultivadas em biorreatores e coletadas em campo. Foi realizado o perfil dos fitorreguladores em 11 espécies: cinco espécies coletadas no Brasil (quatro espécies de *Gracilaria* e uma alga marrom, *Sargassum vulgare*) e seis espécies coletadas nas Ilhas Shetlands do Sul, Antártica (cinco algas marrons e uma alga verde). *G. caudata*, apesar de não apresentar altas taxas de crescimento e não apresentar boa eficiência de remoção de nitrato e fosfato, apresentou altas concentrações de pigmentos e de proteínas solúveis totais. *G. domingensis* apresentou alta eficiência na remoção de excesso de nutrientes na água, indicando que esta espécie tem potencial como biofiltro. O cultivo nos biorreatores mostrou que *G. domingensis* apresentou maior crescimento, maior concentração de proteínas e pigmentos em tratamentos com adição de diferentes concentrações de N: P, mas *G. caudata* apresentou maior qualidade de ágar em tratamentos com adição de N: P do que as amostras coletadas em campo. Podemos concluir que *G. caudata* apresentou menor potencial de absorver os nutrientes disponíveis na água do mar, não sendo uma boa opção para uso como biofiltros em cultivos multitróficos integrados e no mar. Por outro lado, *G. domingensis* apresentou maior potencial de biofiltro, podendo ser uma boa opção para ser cultivada em sistemas multitróficos integrados ou no mar, reduzindo os nutrientes de ambientes marinhos

eutrofizados. Entretanto, *G. caudata* pode ser cultivada em sistema de aquicultura multitróficos sob níveis mais altos de nutrientes para obtenção de um ágar de maior qualidade. Os resultados mostraram que as espécies de *Gracilaria* apresentam a presença de auxinas, jasmonatos e ácido abscísico, sendo úteis para a agricultura como fertilizante natural. Estudos adicionais sobre a identificação de fitorreguladores em algas marinhas devem ser feitos não apenas para selecionar espécies com potencial de aplicação na agricultura, mas também para entender o papel desempenhado pelos fitorreguladores no desenvolvimento das algas marinhas.

Palavras–chave: Ágar, biofiltro, biotecnologia, Fitorreguladores, *Gracilaria*, nutrientes.

ABSTRACT

Marine macroalgae are sources of compounds of commercial and biotechnological interest and can be biofilters, since they use the nutrients of the environment in their metabolism. *Gracilaria* Greville species are exploited as raw material for agar production and studies on their development are necessary for the sustainable production of biomass as sources of agar, biofertilizers and new bioactive compounds. The present study aimed to evaluate the effects of nutrient availability (nitrogen and phosphorus) on the metabolism and biotechnological potential of *Gracilaria caudata* J. Agardh and *Gracilaria domingensis* (Kützing) Sonder ex Dickie.). The following variables were analyzed: growth rates, morphology, photosynthesis (chlorophyll fluorescence), total soluble proteins, pigments (chlorophyll *a*, phycoerythrin, phycocyanin and allophicocyanin), carbohydrates, thallus content of elements (carbon, hydrogen, nitrogen and phosphorus) and remotion of nutrients available in the seawater. To evaluate the biotechnological potential, agar characteristics (yield, quantification of sulfate and 3,6 anhydrogalactose contents) and tests of acetylcholinesterase inhibitory activity of seaweed extracts from biomass cultivated in bioreactors and from biomass of field collected specimens were analyzed. The characterization of Plant Growth Regulators (PGRs) was performed in 11 species: five species collected in Brazil (four species of *Gracilaria* and the brown alga *Sargassum vulgare*) and six species collected from South Shetland Islands, Antarctica (five brown algae and one green alga). *G. caudata* did not present higher growth rates and higher nitrate and phosphate removal efficiencies, but presented higher concentrations of pigments and total soluble proteins. On the other hand, *G. domingensis* showed higher efficiency in removing nutrients in seawater, indicating that this species has potential as a biofilter. Cultivation in bioreactors showed that *G. domingensis* showed higher

growth rates, higher protein and pigment concentrations in treatments with the addition of different N: P concentrations, but *G. caudata* showed higher quality of agar in treatments with addition of N: P than samples collected in the field. We can conclude that *G. caudata* does not have a high potential to absorb the nutrients available in seawater, not being a good option for use as biofilters in multitrophic cultures and in the sea, on the other hand *G. domingensis* showed potential for biofilter and can be a good option to be grown in integrated multitrophic systems or in the sea, reducing nutrients from eutrophic marine environments. *G. caudata* can be grown in a multi-trophic aquaculture system under higher levels of nutrients to obtain a higher quality agar. Results on PGRs showed that *Gracilaria* spp. have some presence of endogenous concentrations of auxins, jasmonates and abscisic acid, what could be useful for agriculture as a natural fertilizer. Additional studies on the identification of PGRs in seaweeds should be done not only to select species with potential for application in agriculture, but also to understand the role played by PGRs in seaweed development.

Keywords: Agar, biofilter, biotechnology, *Gracilaria*, nutrients, Plant Growth Regulators.

Introdução Geral

Importância econômica de *Gracilaria*

O gênero *Gracilaria* Greville (Gracilariaceae, Rhodophyta) compreende mais de 172 espécies, algumas com distribuição cosmopolita em águas tropicais e temperadas (Guiry & Guiry 2020). Este gênero apresenta um histórico de vida trifásico, compreendendo a fase carposporofitica, gametofítica e tretrasporofítica, sendo as duas últimas isomórficas e independentes. (Oliveira & Plastino 1994).

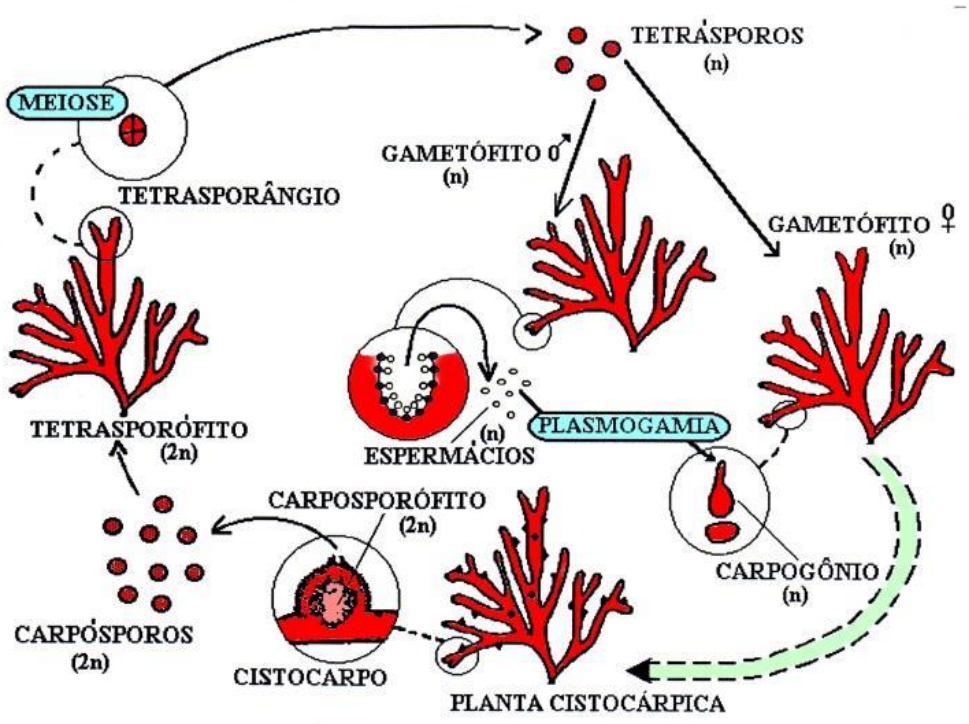

Fig. 1. Histórico de vida de *Gracilaria* spp. (Oliveira & Plastino 1994).

O gênero *Gracilaria* apresenta ampla distribuição geográfica e está entre os mais cultivados no mundo para a extração de ágar, sendo que a China e a Indonésia são os principais países produtores, e o Chile é o maior produtor na América do Sul (Zemke-White & Ohno 1999; Hayashi et al. 2014; Porse & Rudolph 2017).

A aquicultura é o setor de produção de alimentos com maior crescimento global (Ottinger et al. 2016; Troell et al. 2017; Liu et al. 2018), gerando aproximadamente 160

milhões de toneladas de organismos marinhos em 2015 (FAO 2018). A produção global de plantas aquáticas cultivadas, dominada por algas marinhas, cresceu de 13,5 milhões de toneladas em 1995 para pouco mais de 30 milhões de toneladas em 2016, gerando um aumento de USD 60 milhões para mais de USD 1 bilhão em 2016 (FAO 2018). Indonésia, Chile e República da Coreia são os principais exportadores, e China, Japão e Estados Unidos da América são os principais importadores (FAO 2018). As espécies cultivadas de maior importância são: *Eucheuma* (10,2 milhões de toneladas), seguida de *Laminaria japonica* (8 milhões de toneladas), *Gracilaria* spp. (3,9 milhões de toneladas), *Undaria pinnatifida* (2,3 milhões de toneladas), *Kappaphycus* (1,8 milhões de toneladas) e *Porphyra* spp. (1,2 milhões de toneladas) (FAO 2018).

Gracilaria domingensis (Kützing) Sonder ex Dickie ocorre ao longo do litoral brasileiro e é uma das poucas espécies de *Gracilaria* que ocorrem na região sul do país (Oliveira & Plastino 1994). *G. domingensis* é comercializada "in natura" para a alimentação humana, e há registros de sua exportação para o mercado japonês (Plastino et al. 1999). *Gracilaria caudata* J. Agardh apresenta uma ampla distribuição ao longo do litoral brasileiro e é explorada comercialmente para a produção de ágar (Plastino & Oliveira 1997). As atividades extrativistas em populações naturais de *G. caudata* da região nordeste do Brasil expandiram-se a partir da década de 1970 (Oliveira 1998).

No Brasil, a exploração de macroalgas marinhas iniciou-se por volta da década de 1940 e o impacto sócio-econômico desta atividade era reduzido e estava restrito à região nordeste (Oliveira 1998). O cultivo artesanal de *G. birdiae* tornou-se uma atividade econômica para os produtores locais nos estados da Paraíba, Rio Grande do Norte, e Ceará; entretanto, o uso de "mudas" extraídas das populações naturais causou uma redução desse recurso (Hayashi et al. 2014). Uma alternativa para evitar o extrativismo das populações de *G. birdiae* seria o cultivo multitrófico associado a camarões (Marinho-Soriano et al. 2009).

A comercialização do ágar cresceu de 9.600 toneladas, em 2009, até 14.500 toneladas em 2015 (Porse & Rudolph, 2017). Dentre os componentes do ágar, os teores de 3,6-anidrogalactose e de sulfato estão relacionados à força que o gel apresentará, sendo esta diretamente proporcional à quantidade de 3,6-anidrogalactose e inversamente proporcional ao teor de sulfato (Cosson et al. 1995). Estes polissacarídeos, além de apresentarem vasta aplicação na indústria alimentícia e biotecnológica (Pengzhan et al. 2003), são de extrema importância para algumas funções biológicas e imprescindíveis para a adaptação das algas ao ambiente marinho, pois mantêm a umidade quando expostas ao dessecamento em marés baixas, mantêm a concentração celular interna em ocasiões de chuva, além de fortalecerem e oferecerem flexibilidade ao talo, tornando-o resistente à ação das ondas (Usov, 2011). As galactanas sulfatadas possuem estrutura constituída de moléculas alternadas de 3-β-D-galactopiranose e 4-α-galactopiranose. Quando sintetizados na conformação L são denominadas agaranas e na conformação D, carragenanas (Piriz and Cerezo 1991; McHugh 2003; Usov 2011). A quantificação dos teores de sulfato na molécula de ágar fornece um dos parâmetros da qualidade deste ficocolóide, e a sua substituição por 3,6 anidrogalactose aumenta a qualidade do gel (Mathieson et al. 1984; Lahaye & Rochas 1991; Murano et al. 1995; Rebello et al. 1997). Além disso, os tratamentos alcalinos possibilitam o melhoramento da qualidade do gel em *Gracilaria* spp., favorecendo a sua utilização industrial (Yoshimura, 2006).

Espécies de *Gracilaria* como biofiltradoras

A eutrofização está associada a uma variedade de problemas ambientais, incluindo baixo teor de oxigênio dissolvido, deterioração na qualidade da água, redução da biodiversidade de rios e baías, perda de habitats críticos e crescente proliferação de algas tóxicas (Rose et al. 2015). O estado trófico da água do mar pode ser classificado de acordo com as variações nas concentrações de nitrogênio e fósforo, e as macroalgas são eficientes na

remoção desses nutrientes, principalmente em águas eutrofizadas (Nunes 2006; Carneiro 2007).

O nitrogênio é o principal elemento que limita o crescimento das algas marinhas bentônicas e controla a produtividade primária dos oceanos, sendo o nitrato e o amônio importantes para o crescimento desses organismos e responsáveis pela síntese de aminoácidos, purinas, pirimidinas, açúcares e aminas (Lobban & Harrison 2004).

No ambiente marinho, o nitrogênio ocorre nas formas de nitrato (NO_3^-, 0-40 μmol N Kg^{-1}), nitrito (NO_2^-, 0-1 μmol N Kg^{-1}), amônio (NH_4^+), 0-1 μmol N Kg^{-1}), dinitrogênio (N_2, 1 μmol N Kg^{-1}) e nitrogênio orgânico dissolvido (Tyrrel 1999). O mais abundante das formas disponíveis é o nitrato e o dinitrogênio não está disponível para as macroalgas (Tyrrel 1999). As etapas envolvidas no metabolismo do nitrogênio nas macroalgas marinhas são: Captação do nutriente, assimilação, armazenamento e "turnover" (Lobban & Harrison 2004). A captação do nitrato é administrada por uma ATPase, que está localizada na membrana plasmática. No Citoplasma, o nitrato pode ser armazenado no vacúolo ou reduzido a nitrito por uma reação catalisada pela enzima Nitrato Redutase. O nitrito é transportado pra o cloroplasto, onde é reduzido a amônio pela enzima Nitrito Redutase. O amônio será incorporado ao glutamato, numa reação dependente de ATP, formando uma glutamina. O grupo amino da glutamina será transferido a um alfacetoglutarato, formando dois glutamatos. Esta reação é catalisada pela Glutamato Sintase. O grupo amino do glutamato será transferido a um alfacetoácido, produzindo um novo aminoácido e um novo alfacetoglutarato. Essa reação é catalisada por uma aminotransferase (Figura 1). Por fim os aminoácidos serão incorporados a proteínas ((DeBoer 1998, Lobban & Harrison 2004).

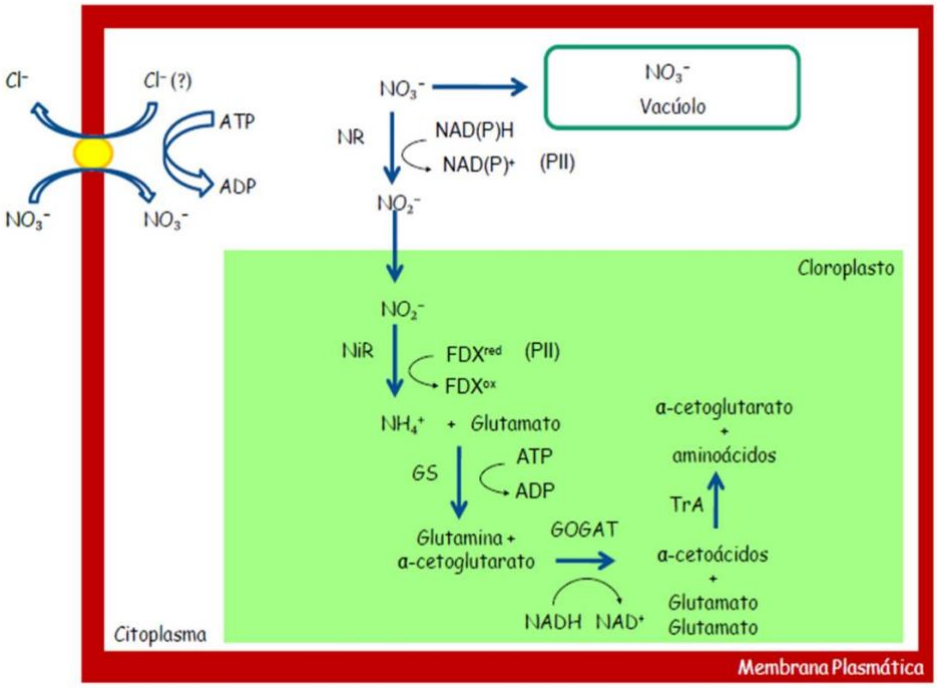

Fig. 2. Esquema do metabolismo do nitrogênio em macroalgas marinhas bentônicas segundo Deboer (1981), com modificações. NR = nitrato redutase, NiR = nitrito redutase, FDX = ferrodoxina, GS = glutamina sintetase, GOGAT = glutamato sintase, NAD = nicotinamid.

Assim como o nitrogênio, o fósforo é um nutriente limitante no desenvolvimento das algas marinhas, responsável por funções estruturais e transferência de energia (como formação de ATP, por exemplo) e está disponível principalmente na forma de ortofosfato ($HPO4^{-2}$), que corresponde a 97% do fosfato inorgânico presente na água do mar (DeBoer 1981, Lüning 1990, Tyrrel 1999). O fósforo também está disponível na forma de outros íons fosfato, como 1% de íon fosfato ($PO4^{-3}$) e 2% como fosfato dihidrogênio ($H2PO4^{-}$) (Lobban & Harrison 2004).

O fosfato inorgânico presente na água do mar é captado pela alga por transporte ativo, havendo gasto de energia na forma de ATP. Esse fosfato inorgânico pode ser armazenado no citoplasma em quatro formas de polifosfatos (Poly-A, B, C e D) que podem formar moléculas de DNA, RNA, ou podem ser utilizados na formação de fosfolipídeos e como fonte de energia na forma de ATP nos processos de respiração e fotossíntese. O fosfato inorgânico pode ser armazenado no vacúolo e, em excesso, pode retornar ao meio marinho na forma de fosfato orgânico dissolvido (Figura 2).

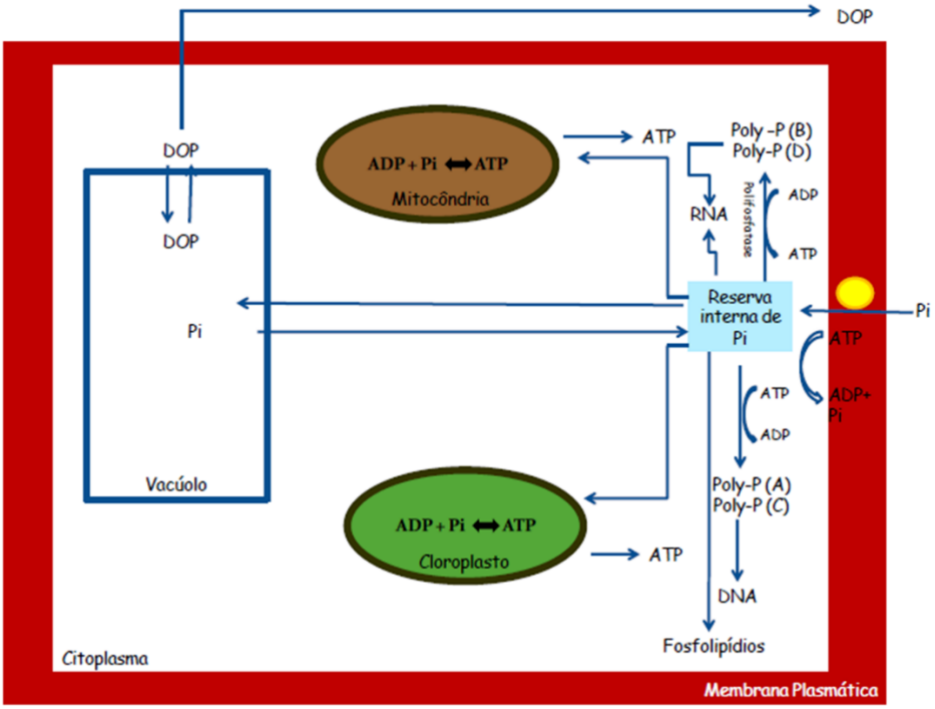

Fig. 3. Esquema do metabolismo do fósforo em macroalgas marinhas bentônicas, modificado segundo Lobban & Harrison (2004). Pi = fosfato inorgânico, DOP = fosfato orgânico dissolvido, Poly – P = polifosfato.

Alguns estudos evidenciaram que *Gracilaria* spp. pode remover os nutrientes através da utilização dos nutrientes em excesso (por exemplo, N e P) em sistemas de cultivos multitróficos integrados com peixes, vieiras ou camarões em áreas eutróficas (Buschmann et al. 1994; Buschmann et al. 1996; Troell et al. 1997; Neori et al. 1998; Jones et al. 2001; Hernández et al. 2006; Yang et al. 2006; Zhou et al. 2006; Huo et al. 2012).

Estudos em laboratório também avaliaram a capacidade de absorção de nitrogênio e/ou fósforo em diferentes espécies de *Gracilaria*, como *G. gracilis* (Martínez-Aragón et al. 2002), *G. lemaneiformis*. (Mao et al. 2009), e *G. cornea* (Navarro-Angulo & Robledo 1999), mas também em outras espécies de algas vermelhas, como *Gelidium coulteri* (Bracken & Stachowick 2006), *Pyropia* (Carmona et al. 2006) e *Palmaria palmata* (Corey et al. 2013; Grote 2016).

Potencial biotecnológico de *Gracilaria*

As macroalgas contêm substâncias bioquímicas que possuem atividades biológicas, como, por exemplo, antibacterianas, antifúngicas, antienvelhecimento, antimalárica, dietéticas, antiinflamatórias, anticoagulantes, antimitóticas, antibióticas, anticancerígenas, antioxidantes e hipolipidemias que são amplamente estudadas em diferentes países (Smit 2004, Chan et al. 2015, Agregan et al. 2017).

O oxigênio, nitrogênio e enxofre produzem ânion superóxido, radical hidroxila, peróxido de hidrogênio e óxido nítrico, e estes compostos podem causar estresses oxidativos e danos celulares, levando ao aparecimento de doenças tão diversas quanto o câncer, diabetes, doenças de Alzheimer e de Parkinson (Yangthong 2009; Lü et al. 2010). Antioxidantes são compostos que retardam ou inibem as reações oxidativas, e estes compostos podem ser usados para prevenir uma ampla gama de doenças e atuam como conservantes em alimentos e em cosméticos (Zhang 2007). O potencial antioxidante foi demonstrado em estudos com *G. tenuistipitata* (Yang 2012), *G. bursa-pastoris* (Piao 2014), e *G. changii* (Chan et al. 2014; 2016).

O dano causado por espécies reativas de oxigênio é considerado um fator contribuinte de diversas doenças, incluindo a Doença de Alzheimer (Houghton et al. 2007), que é caracterizada pela diminuição da função colinérgica no cérebro (Perry et al. 2003). Estudos desenvolvidos com várias espécies de *Gracilaria* tem mostrado o seu potencial na inibição da enzima acetilcolinesterase, como por exemplo *G. gracilis* (Natarajan et al. 2009), *G.lancifolia* (Nguyen 2012), *G. corticata*, *G.salicornia* (Ghannadi 2013), e *Gracilaria* sp. (Bianco et al. 2015). Além disso, a crescente demanda por suplementos alimentares funcionais exigiu mais estudos sobre as propriedades nutricionais das algas vermelhas, sendo estudadas as espécies *G. edulis* e *G.changii* (Sakthivel and Pandima Devi 2015; Chan & Mantanjun 2017).

As macroalgas também são utilizadas na agricultura devido aos seus efeitos benéficos nas plantas, conferindo maior produtividade e resistência aos fatores bióticos e abióticos (Battacharya 2015). As espécies de *Gracilaria* também apresentaram efeitos positivos em plantas terrestres. Por exemplo, Chitra et al. (2013) observaram que o uso do extrato aquoso de *G. corticata* e *Vigna radiata* (Fabaceae) promoveram o aumento no número de folhas e no comprimento de raízes e caules. Torres et al. (2018) analisaram extratos de *G. caudata* e de *G. domingensis* como bioestimulantes para o cultivo de alface. Jiménez et al. (2011) observaram que extratos aquosos e etanólicos de *G. chilensis* inibiram o fungo fitopatógeno *Phytophthora cinnamoni.*

Potencial biotecnológico das macroalgas marinhas na agricultura

As algas desenvolveram um papel importante nas comunidades costeiras ao longo dos séculos, seu cultivo está crescendo em todo o mundo e uma produção econômica de macroalgas tem sido praticamente a mesma desde o início dos anos 80 (Gachon et al. 2010). Hoje, as algas marinhas são usadas em uma infinidade de aplicações, expandindo indústrias globais baseadas em hidrocolóides, cosméticos e suplementos alimentares, e também como uma fonte potencial de biocombustível, com 32 países cultivando ativamente algas para diversos fins (Monagail et al. 2017).

Parte da produção de algas é usada para suplementos nutricionais, como bioestimulantes ou biofertilizantes, para aumentar o crescimento e o rendimento das plantas e estão disponíveis para uso na agricultura e horticultura. Os produtos de algas marinhas exibem atividades que estimulam o crescimento, e o uso de formulações de algas como bioestimulantes na produção agrícola está bem estabelecido (Khan et al. 2009). O uso de bioestimulantes cresceu dramaticamente na última década e espera chegar a US $ 2 bilhões até 2018 (Saa-Silva et al. 2013, Calvo et al. 2014).

Estudos com o extrato comercial da alga *Ascophyllum nodosum* (Linnaeus) Le Jolis (Phaeophyceae), denominado "AMPEP" (Extrato de alga em pó), verificou-se que o AMPEP estimula o crescimento de *Kappaphycus* e a produção de carragenina (Hurtado et al. 2009; Loureiro et al. 2014) e melhora a produção de novos e aprimorados explantes de *Kappaphycus* para um viveiro comercial (Yunque et al. 2011; Tibubos et al. 2017, Ali et al. 2018a). O AMPEP também pode ter um efeito "semelhante a uma vacina" em mudas de Kappaphycus alvarezii (Loureiro et al. 2012), enquanto reduz epim e cargas endofíticas (Borlongan et al., 2011; Ali et al. 2018b).

A detecção de citocininas endógenas tornou-se mais sensível, permitindo a detecção em ambas as macroalgas (Stirk et al. 2003), com base na técnica de quantificação de citocininas descrita por Nóvak et al. (2003). Stirk et al. (2003) estudaram 31 espécies de macroalgas marinhas da costa da África do Sul e determinaram a concentração de 10 compostos diferentes derivados de zeatina, 3 compostos diferentes derivados de isopenteniladenina e 6 tipos de citocininas aromáticas, incluindo benzilaminopurina. Yokoya et al. (2010) estudaram as citocininas endógenas e foram quantificadas em 11 algas vermelhas coletadas no litoral brasileiro. Entre as citocininas identificadas estão as isoprenóides (2-isopenteniladenina, cis-Zeatina e trans-Zeatina) e citocininas aromáticas (benzilaminopurina, orto e meta-topolinas).

Exemplos de citocininas que ocorrem em algas marinhas incluem zeatina detectada em *Macrocystis pyrifera* (De Nys et al. 1990) e *Valoniopsis pachynema* (Farooqi et al. 1990), cZ e isopenteniladenosina (iPR) encontradas em *Sargassum muticum* (Zhang et al. 1991) e *Laminaria japonica* (Duan et al. 1995) e iPR em *Porphyra perforata* (Zhang et al. 1993). Dezenove citocininas, incluindo grupos isoprenóides e aromáticos, foram identificadas em cinco espécies de Chlorophyta, sete Phaeophyta e 19 Rhodophyta da África do Sul (Stirk et al. 2003).

O ácido indolil-3-acético (AIA) foi identificado em várias algas, incluindo *Ascophyllum nodosum* (Kingman e Moore 1982, Sanderson et al. 1987), *Caulerpa paspaloides* (Jacobs et al. 1985), *Ecklonia maxima* (Crouch et al. 1992), *Pyrgulopsis perforata* (Zhang et al. 1993) e *Undaria pinnatifida* (Abe et al. 1972). Outros derivados do indol, como o ácido indol-3-carboxílico (AIC), foram identificados em *Botryocladia leptopoda* (Bano et al. 1987), *Prionitis lanceolata* (Bernart e Gerwick 1990), *Ecklonia maxima* (Crouch et al. 1992) e *Undaria pinnatifida* (Abe et al. 1972).

JUSTIFICATIVAS

O presente trabalho visou o conhecimento de bioprocessos para aumentar o potencial biotecnológico de produção de biomassa de espécies do gênero *Gracilaria*. Considerando a importância econômica de *Gracilaria caudata* e de *G. domingensis*, como matéria prima para a produção de ágar e de bioativos, e a necessidade da produção de biomassa para o seu uso sustentável, o presente trabalho visa avaliar a capacidade de remoção e assimilação de nutrientes (nitrogênio e fósforo) e avaliar a atividade inibitória da enzima acetilcolinesterase dos extratos dessas espécies, assim como caracterizar os fitorreguladores em espécies tropicais (*Gracilaria* spp. e *Sargassum vulgare*) e em algas marinhas da Antártica.

OBJETIVOS

Objetivo geral

O principal objetivo do presente trabalho foi avaliar os efeitos da disponibilidade de nutrientes (nitrogênio e fósforo) no metabolismo e no potencial biotecnológico de *Gracilaria caudata* J.Agardh e *G. domingensis* (Kützing) Sonder ex Dickie.

Objetivos específicos

a. Avaliar os efeitos da disponibilidade de nutrientes (nitrogênio e fósforo) no crescimento, na morfologia, na fotossíntese, e no conteúdo de pigmentos, proteínas, carboidratos e de C, N e P em *Gracilaria caudata*;
b. Avaliar os efeitos da disponibilidade de nutrientes (nitrogênio e fósforo) no crescimento, na morfologia, na fotossíntese, e no conteúdo de pigmentos, proteínas, carboidratos e de C, N e P em *Gracilaria domingensis*;
c. Comparar os efeitos da disponibilidade de nutrientes (nitrogênio e fósforo) no crescimento, proteínas, pigmentos e características do ágar (rendimento, quantificação dos teores de sulfato e de 3,6-anidrogalactose) extraído de amostras de *Gracilaria caudata e G. domingensis* cultivadas em biorreatores e coletadas na natureza;
d. Comparar as atividades biológicas (atividade inibitória da enzima acetilcolinesterase) de extratos de *Gracilaria caudata e G. domingensis* cultivadas em biorreatores e coletadas na natureza.
e. Caracterizar os fitorreguladores em espécies tropicais (*Gracilaria* spp. e *Sargassum vulgare*) e em algas marinhas da Antártica.

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Capítulo 1

EFFECTS OF DIFFERENT CONCENTRATIONS OF NITRATE AND PHOSPHATE ON PHYSIOLOGICAL RESPONSES OF *GRACILARIA CAUDATA* (GRACILARIALES, RHODOPHYTA)

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ABSTRACT

Benthic marine algae can be biofilters by assimilation of nutrients of the medium in their metabolism. Species of *Gracilaria* Greville are exploited as raw material for the agar production and studies on their development are necessary for the sustainable production of biomass. The present study aims to evaluate the effects of nitrogen and phosphorus availabilities in the metabolism of *Gracilaria caudata* J. Agardh. Experiments was performed with 50% von Stosch solution without N and P, which were added to the medium as nitrate (in concentrations from 0.0 to 500 μM), and phosphate, in the N:P ratio of 10:1 to 100:1. Each treatment was tested with three replicates (n=3). Growth rates, number of primary and secondary branches, pigments (chlorophyll *a*, phycoerythrin, phycocyanin and allophycocyanin), total soluble proteins, total soluble carbohydrates, photosynthetic parameters (Pmax, yield, Ik and α), and removal efficiency were analyzed. Experiments were conducted under temperature of 23±2°C, photoperiod of 14h, salinity 32 psu, and 80±10 μmol photons.m^{-2} s^{-1}. Data were analysed by two-way of variance (ANOVA) followed by "a posteriori" test of Student-Newman-Keuls. The highest growth rate was 5.72±0.24% d^{-1} in high N:P concentration (500.0 μM, N:P of 10:1). The higher numbers of primary branches (9.00±0.40) and secondary branches (4.00±0.81) were observed in the treatment of 500.0

µM, N:P of 10:1. Total soluble sugars of ethanolic and aqueous extracts were higher, respectively, in treatment of 125.0 µM (N:P of 100:1), and control (without N:P addition). The higher values of Pmax was 5.4±1.2 ETR in treatment of 500.0µM (N:P of 10:1), yield was 0.325±0.07 in 250.0µM (N:P of 10:1), Ik was 5.40±1.20 in 500.0 µM (N:P of 10:1), and α was 0.04±0.01 in 125.0 µM (N:P of 100:1). *G. caudata* cultured with 250.0µM, N:P of 10:1 showed the highest concentrations of phycoerythrin (337.56±15.89 $mg.g^{-1}$ FM), and phycocyanin (84.48±18.61 mg g^{-1} FM), while treatment of 500.0µM, N:P of 10:1, induced higher chlorophyll *a* concentration (41.64±2.17 mg g^{-1} FM). On the other hand, allophycocyanin concentration (135.33±9.64 mg g^{-1} FM) was higher in 250.0µM (N:P of 100:1), and protein content (4.60±0.50 mg g^{-1} FM) was higher in 125.0µM (N:P of 100:1). The removal efficiency of nitrate was higher in treatment control (98.76±0.05%) while phosphate removal (66.82±5.83%) was higher in treatment of 50.0 µM (N:P of 100:1). Our results show that *G. caudada* did not uptake high values of nutrients, and it is not indicated as a biofilter.

Keywords: Biofilter, *Gracilaria*, Nitrate, phosphate

INTRODUCTION

Among seaweeds, species of Rhodophyta are the most harvested and/or cultivated in Brazil. The genera that have been most exploited (on and off) on the Brazilian coast are *Gracilaria*, *Gracilariopsis*, *Hypnea*, *Pterocladiella* and the introduced *Kappaphycus* (Simioni et al. 2019).

The genus *Gracilaria* Greville (Gracilariaceae, Rhodophyta) comprises more than 172 species, some with cosmopolitan distribution in tropical and temperate waters (Guiry and Guiry 2020). The species *G. caudata* is being classified as *Crassiphycus caudatus* (J. Agardh) Gurgel, J.N. Norris & Fredericq, however we are analyzing the studies and later we will make the change if necessary. This genus has a three-phase life history, comprising the carposporophytic, gametophytic and tretrasporophytic phase, the last two being isomorphic

and independent. (Oliveira and Plastino 1994). On the Brazilian coast, *Gracilaria caudata* J. Agardh has a wide distribution, and it is one of the main species exploited for agar production (Oliveira and Miranda 1998; Oliveira et al. 2000). Common on bedrock, it occurs mostly in protected bays and turbid waters, extending from the mesolittoral to the infralittoral fringe (Plastino and Oliveira 1997).

The commercialization of agar increased from 9,600 tons in 2009 to 14,500 tons in 2015 (Porse and Rudolph 2017). The main species exploited from natural beds or cultivated in artisanal farms for agar production are *Gracilaria birdiae* E.M. Plastino & E.C. Oliveira, *Gracilaria caudata* J. Agardh, *G. cornea* J. Agardh, *G. cervicornis* (Turner) J. Agardh and *G. domingensis* (Kützing) Sonder ex Dickie (Marinho-Soriano 2017). *Gracilaria caudata* has higher quality agar because it has higher concentration of 3,6 anhydrogalactose, when compared to other species of *Gracilaria* from Brazil (Yoshimura 2006).

Some studies have shown that *Gracilaria* spp. can remove nutrients through the assimilation of nutrients (N and P) in multitrophic crop systems integrated with fish, scallops or shrimp in eutrophic areas (Buschmann et al. 1998, Troell et al. 1998, Carneiro et al. 2011, Marinho-Soriano 2009, 2017, Huo et al. 2012). Laboratory studies also evaluated the nitrogen and/or phosphorus absorption capacity of different *Gracilaria* species, such as *G. gracilis* (Martínez-Aragón et al. 2002), *Gracilaria lemaneiformis* (Bracken and Stachowick 2006), and *G. cornea* (Navarro-Angulo & Robledo 1999).

The biotechnological and maricultural potential of *G. caudata*, have demonstrated in some studies: Bermúdez et al. (2011) observed a good nickel absorption by the species; Sales et al. (2020) obtained positive results in treatment with polysaccharide in the erosion in the esophagus; The structural characterization of the polysaccharide showed a structure close to ideal for commercialization (Barros et al. 2013); Chaves et al. (2013) obtained positive results in the anti-inflammatory test with agar; Significant reduction of the nutrients NH4 +, $NO3^-$ and PO4- in the integrated cultivation with shrimp (Marinho-Soriano et al. 2009);

Ascophylum nodosum seaweed extract (AMPEP) stimulated the growth of *G. caudata* (Souza et al. 2018); Miranda et al. (2012) presented ideal results for salinity, temperature and irradiance.

The main objective of the present work is to evaluate the effects of nutrient (nitrogen and phosphorus) availability on growth, morphology, proteins, contents of pigments, carbohydrates, nutrients uptake and C, N, H and P in *Gracilaria caudata.*

MATERIAL AND METHODS

Collection

Female Gametophytes of *Gracilaria caudata* were collected at Boa Viagem Beach, Recife, Pernambuco state, northeastern Brazil (8º 08' S and 34º 54' W) in October 2006. Voucher specimens were deposited in the Herbarium SP (accession number SP 365602) and isolates are deposited in the Culture Collection of Algae, Fungi and Cyanobacteria of Institute of Botany (CCIBt).

Biomass necessary for the experiments was obtained by vegetative propagation of gametophytes thallus segments (10 mm). The culture medium selected comprised sterilized seawater (salinity of 30 - 31) enriched with von Stosch's solution at half strength (VSES/2) following Oliveira et al. (1995), modified with vitamin concentrations reduced to a 50 % concentration (thiamin (100 µg L^{-1}), biotin (100 µg L^{-1}) and cyanocobalamin (1.0 µg L^{-1}) as proposed by Yokoya 2000). Culture media was renewed every two weeks. Cultures were incubated at: 23 ± 1 ºC, photon flux densities of 60.0 - 70.0 µmol photons m^{-2} s^{-1} (provided by cool-white fluorescent lamps), with a 14:10h light:dark cycle, without aeration. Irradiance was measured with a quantum photometer (LI-250, Li-Cor, Lincoln®, Nebraska, USA) equipped with underwater quantum sensor (LI-192 SA, Li-Cor).

Experimental design

Experiments were performed in Erlenmeyer flasks (500 mL) with 200 mg algal biomass per 400 mL culture medium. Treatments were composed of sterilized seawater enriched with VSES/4 modified (von Stosch's solution prepared without nitrate and phosphate, but with salts of iron, manganese, ethylenediaminetetraacetic acid (EDTA), and three vitamins, following Oliveira et al. (1995) and Yokoya (2000). Nitrate ($NaNO_3$) and phosphate ($Na_2HPO_4.12H_2O$) were added to the VSES/4 modified medium in order to obtain nitrogen/phosphorus (N/P) ratios of 10:1 and 100:1. Nitrate concentrations varied from 0.0 to 500 μmol, and phosphate concentrations varied from 0.0 to 50 μmol L^{-1} or 0.0 to 5 μmol L^{-1} in the N:P ratio of 10:1or 100:1, respectively. Each treatment was tested with four replicates (n = 4). Other laboratory conditions were the same as those described for unialgal cultures.

For biochemical analyses, after four weeks, algae were weighed for growth analysis, and the culture medium was renewed and, after 4 days, samples of each replicate were weighed, frozen in liquid nitrogen and stored at -20 °C.

Growth rates

Fresh biomass was recorded at weekly intervals before renewal of the media during four weeks. Daily growth rates were calculated using the following equation: $GR=[(Wt/Wi)^{1/t} - 1]\times100\%$, where Wt is the weight after t days, Wi is the initial weight, and t is the experimental period (Yong et al. 2013).

Morphology

The number of lateral branches per explant was determined by analyses of photos of three apical segments per replicate taken in digital camera (Panasonic, Lumix DC Vario model).

In vivo chlorophyll fluorescence

The in vivo fluorescence of chlorophyll was measured using an underwater Walz Diving-PAM flow meter. Apical segments of *G. caudata* (n = 3) were placed on a magnetic sample holder to avoid overlap. Eight levels of irradiance were used to construct photosynthesis × irradiance curves of photons m^{-2} s^{-1}. The apical segments were exposed for 20 s in each irradiance, interspersed with a 0.8 s saturation pulse. The effective quantum yield (ΔF/Fm′) was provided by Diving-PAM after an initial saturation pulse, when the samples had a pulse of very low intensity of blue light (approximately 0 μmol of photons m^{-2} s^{-1}). The following parameters were calculated and analyzed: photosynthetic efficiency (α ETR), maximum electron transport rate (ETR max), light saturation (Ik) and effective quantum yield (ΔF/Fm′).

Total soluble protein contents

For total soluble protein analysis, 80 mg of algal fresh biomass for each replicate (n = 3 for each treatment) were ground with liquid nitrogen, and extractions were carried out at 4 °C using 0.2 M phosphate buffer (pH 8) containing 5 mM of ethylenediaminetetraacetic acid (EDTA) and 1 mM of dithiotreitol (DTT). Buffer was added in the proportion of 10 mL g^{-1} fresh biomass, and the homogenates were centrifuged at 12,000×g for 15 min. Total soluble protein contents were determined according to Bradford (1976), using a Bio-Rad® protein assay kit and BSA as standard.

Pigment analyses

The algal mass (75 mg fresh mass for each replicate, n=3) was ground with liquid nitrogen and 1 ml of 50 mM (pH 5.5) phosphate buffer was added. Pigment extractions were carried out at 4ºC, in the dark. Crude extracts were centrifuged at 14,000×g for 20 min in order to obtain the phycobiliproteins, and chlorophyll-a was extracted after dissolving the

pellet in 90% acetone, followed by centrifugation at 10,000×g for 15 min. Pigments were quantified using a spectrophotometer (Shimadzu®, UV 1800), and concentrations were calculated according to Kursar et al. (1983) for phycobiliproteins, and Jeffrey and Humphrey (1975) for chlorophyll *a*.

Carbohydrate extraction and quantitative analysis

The extraction of soluble carbohydrates was performed according to Carvalho et al. (1998) with modifications. For each replicate (n=3), 60 mg of fresh biomass was tritured with liquid nitrogen, suspended in 1 mL of 80% ethanol, kept in a 80 °C water bath for 1 h and then centrifuged at 1082 g for 15 min. The supernatant was stored and the precipitate was subjected to the same procedure for two more times. The final residue was resuspended in 1 mL of distilled water, kept in a water bath at 60 °C for 1 h. The supernatant was stored and the precipitate was subjected to the same procedure for two more times. Quantification of total soluble carbohydrates of ethanolic and aqueous extracts was carried out using the phenol-sulfuric colorimetric method (490 nm) of phenol-sulfuric acid according Dubois et al. (1956).

Thallus contents of C, H, N, and P

Analyses of endogenous contents of C, H, and N were determined in samples of each replicate (n=3) according to the PreglDumas' method using PerkinElmer® 2400 Series II equipment. Endogoenous contents of P were determined by inductively coupled plasma optical emission spectrometry (ICP-OES), using a SPECTRO ARCOS® high-resolution analyzer. Analyses were performed by Analytical Center of Instituto de Química, Universidade de São Paulo, São Paulo, Brazil.

Analysis of seawater nutrients

Nitrate (NO_3^-) and phosphate (PO_4^{3-}) analysis was performed according to Grasshoff et al. (1999) using a segmented flow analyser (Bran Luebbe®, Auto Analyzer II) and a spectrophotometer (Thermo®, Evolution 201), respectively. Removal efficiency (percentage) was calculated as $((C_i\text{-}C_7)/C_0) \times 100$ for each nutrient, where C_i is the initial concentration before the incubation of seaweed into seawater, C_0 is the nutrient concentration right after the incubation of seaweed into seawater, and C_7 is the nutrient concentration after the incubation period (7 days). Nutrient uptake was calculated according to Kregting et al. (2008) as $((C_i - C_7)/(FW \times time)) \times volume$, where FW is the seaweed fresh biomass after 7 days of incubation, time is 7 days, and volume is the volume of culture medium in each erlenmeyer (400 mL). These samples were filtered through a cellulose acetate membrane with pore size of 0.45 μm (Millipore®) and stored at −20 °C until nutrient analyses. After 7 days, the algal biomass was measured for growth rate analysis, and the culture medium (with the same nitrogen and phosphorus concentrations) was renewed.

Statistical analyses

Data were analyzed by two-way of variance (ANOVA) followed by the Student-Newman-Keuls *a posteriori* test for multiple comparisons to distinguish significant differences ($p < 0.05$). Statistica 10.0 software was used to perform all statistical analyses.

RESULTS

The growth rates of *G. caudata* were higher in all treatments when compared to control, with the exception of treatment with N: P of 500.0 / 50.0 µM (Fig. 1) (F = 136.93 *p* = 0.00), and treatments with N : P of 100: 1 were greater than with N: P of 10: 1 (F = 78.99 *p* = 0.00).

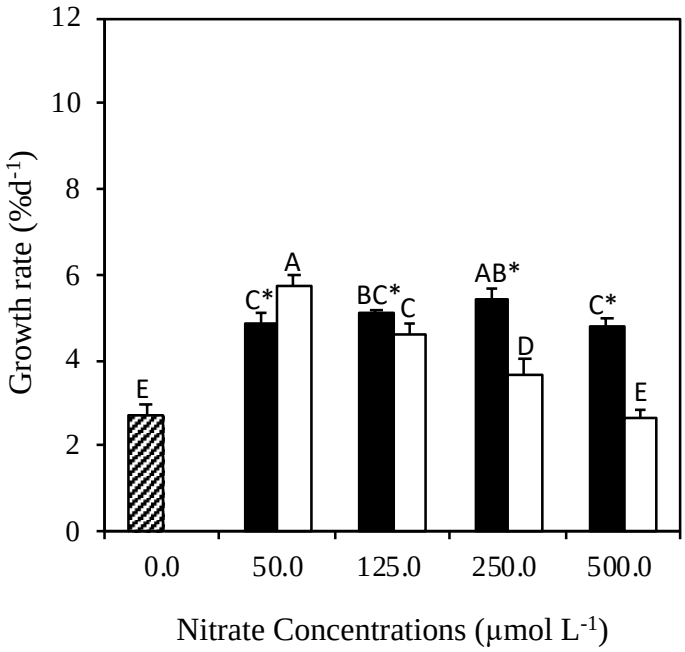

Fig. 1. Growth rates of *Gracilaria caudata* cultured in different concentrations of nitrate and phosphate, in the ratio of 100:1 (black columns) and 10:1 (white columns) for 28 days. Mean ± SD (n = 4). Treatments with distinct letters were significantly different from each other, according to the Student-Newman-Keuls comparison test (p <0.05). Asterisks indicate significant differences between N: P ratios.

The number of primary branches of *G. caudata* was higher in the treatment with N: P of 500.0 / 50.0 µM when compared to the control (F = 4.52 *p* = 0.00) (Figures 12 and Figure 13 A) and there were no differences between the ratios (F = 4.11 p = 0.06). As for the number of secondary branches, the highest value was observed in the treatment with N: P of 500.0 / 50.0 when compared to the control (F = 14.70 p = 0.00) (Fig. 2A and Fig. 2 B) and there were no differences between the reasons (F = 0.314 *p* = 0.58).

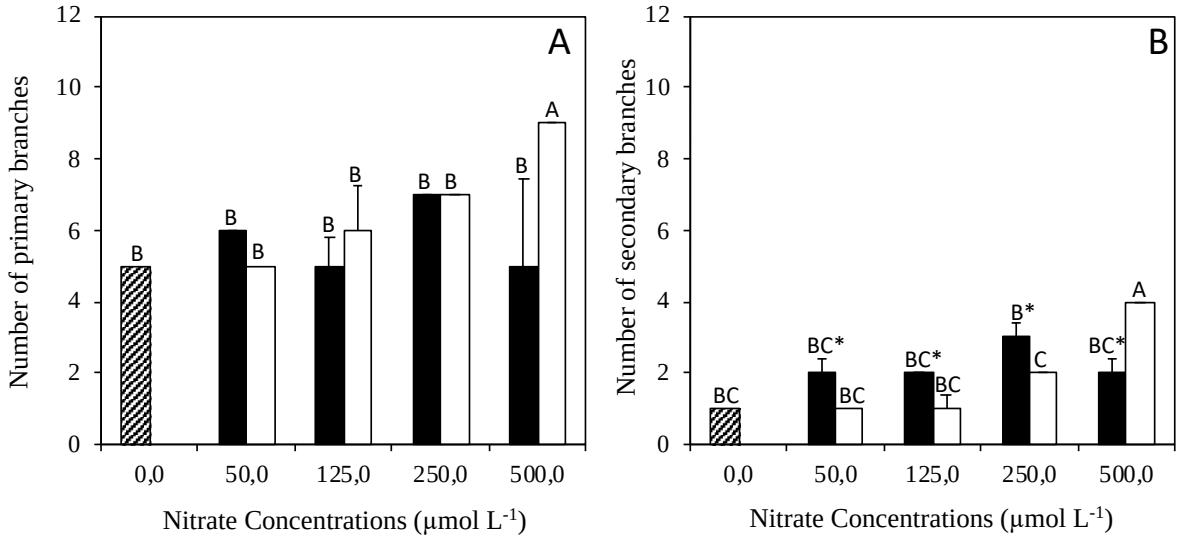

Fig. 2. **N**umber of primary branches (A) and number of secondary branches (B) of *Gracilaria caudata* cultured in different concentrations of nitrate and phosphate, in the ratio of 100:1 (black columns) and 10:1 (white columns) for 28 days. Mean ± SD (n = 3). Treatments with distinct letters were significantly different from each other, according to the Student-Newman-Keuls comparison test ($p < 0.05$). Asterisks indicate significant differences between N: P ratios.

As for photosynthesis of *G. caudata*, the highest value of ETR was observed in the treatment with N: P of 500.0 / 50.0 µM and the lowest was observed in the treatment with N: P of 125.0 / 1.25 µM of N: P (Table 1.). The quantum yield did not differ between the treatments tested ($F = 0.763$, $p = 0.56$), and the same occurred between the ratios ($F = 1.18$ $p = 0.28$) (Table 1.). The maximum photosynthesis of *G. caudata* did not vary between the treatments tested ($F = 1.5$ $p = 0.23$), and among the ratios, 10: 1 was greater than 100: 1 ($F = 12.63$, $p = 0.00$). The photosynthetic efficiency did not vary between the different treatments tested ($F = 0.48$ $p = 0.74$), the same occurred between the reasons ($F = 0.08$ $p = 0.77$).

Table. 1. Effective quantum yield (EQY), maximum photosynthesis (Pmax), photosynthetic efficiency (α) and irradiance of saturation (Ik) in *Gracilaria domingensis* cultured in different concentrations of nitrate and phosphate at a ratio of 100: 1 and 10: 1 of N: P for 28 days. Mean ± SD (n = 3). Treatments with different letters indicate significant differences between concentrations, according to the Student-Newman-Keuls comparison test ($p < 0.05$). Asterisks indicate significant differences between the reasons.

TREATMENTS N/P Concentrations (micromol.L^{-1})	PHOTOSYNTHETIC PARAMETERS			
	EQY	Pmax	α	Ik
0.0	0.190±0.01	4.3±1.0	0.03±0.02	143.0±19.1 AB
50.0 / 0.5	0.305±0.14	2.8±0.7	0.03±0.00	93.3±14.4 BC
125.0 / 1.25	0.178±0.07	2.4±0.6	0.04±0.00	60.0±8.24 C
250.0 /2.5	0.109±0.00	2.1±0.6	0.04±0.01	52.5±7,1 C
500.0 / 5.0	0.222±0.07	3.1±1.0 *	0.03±0.01	103.3±6,2 BC
50.0 / 5.0	0.233±0.07	4.3±0.7 *	0.04±0.02	107.5±20,1 BC*
125.0 / 12.5	0.200±0.42	3.6±0.9 *	0.03±0.01	120.0±9.5 AB*
250.0 /25.0	0.325±0.07	4.3±1.2 *	0.03±0.00	143.3±7.2 AB*
500.0 / 50.0	0.240±0.12	5.4±1.2 *	0.03±0.00	180.0±37.5 A*

The total soluble protein content was higher in all treatments with addition of N:P when compared to the control, and the treatment with 125.0 / 1.25 µM showed the highest concentration in relation to the other treatments (Fig. 3) ($F = 156.87$, $p = 0.00$) and the 100: 1 ratio was higher than the 10: 1 ratio ($F = 12.57$, $p = 0.00$).

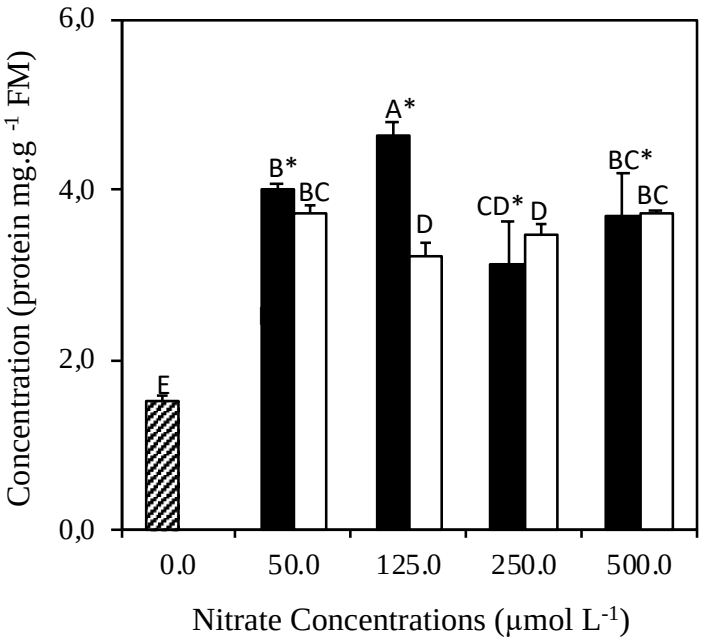

Fig. 3. Content of total soluble protein in *Gracilaria caudata* cultured indifferent concentrations of nitrate and phosphate, in the ratio of 100:1 (black columns) and 10:1 (white columns) for 32 days. Mean ± SD (n = 3). Treatments with distinct letters were significantly different from each other, according to the Student-Newman-Keuls comparison test ($p < 0.05$).

The phycoerythrin content in *G. caudata* was higher in all treatments with the addition of N: P when compared to the control ($F = 32.52$ $p = 0.00$) (Fig. 4 A) and there were no differences between the ratios ($F = 0.41$ $p = 0.52$). Phycocyanin content did not vary between treatments tested ($F = 0.36$ $p = 0.83$) (Fig. 4 B) and between ratios ($F = 0.01$ $p = 0.90$). The same result was observed for the content of allophicocyanin ($F = 1.43$ $p = 0.25$) (Fig. 4 C) and among the reasons ($F = 0.67$ $p = 0.42$). The chlorophyll a content was higher in all treatments with the addition of N: P when compared to the control ($F = 94.54$ $p = 0.00$) (Fig. 4-D) but there were no differences between the ratios ($F = 2.82$ $p = 0.10$).

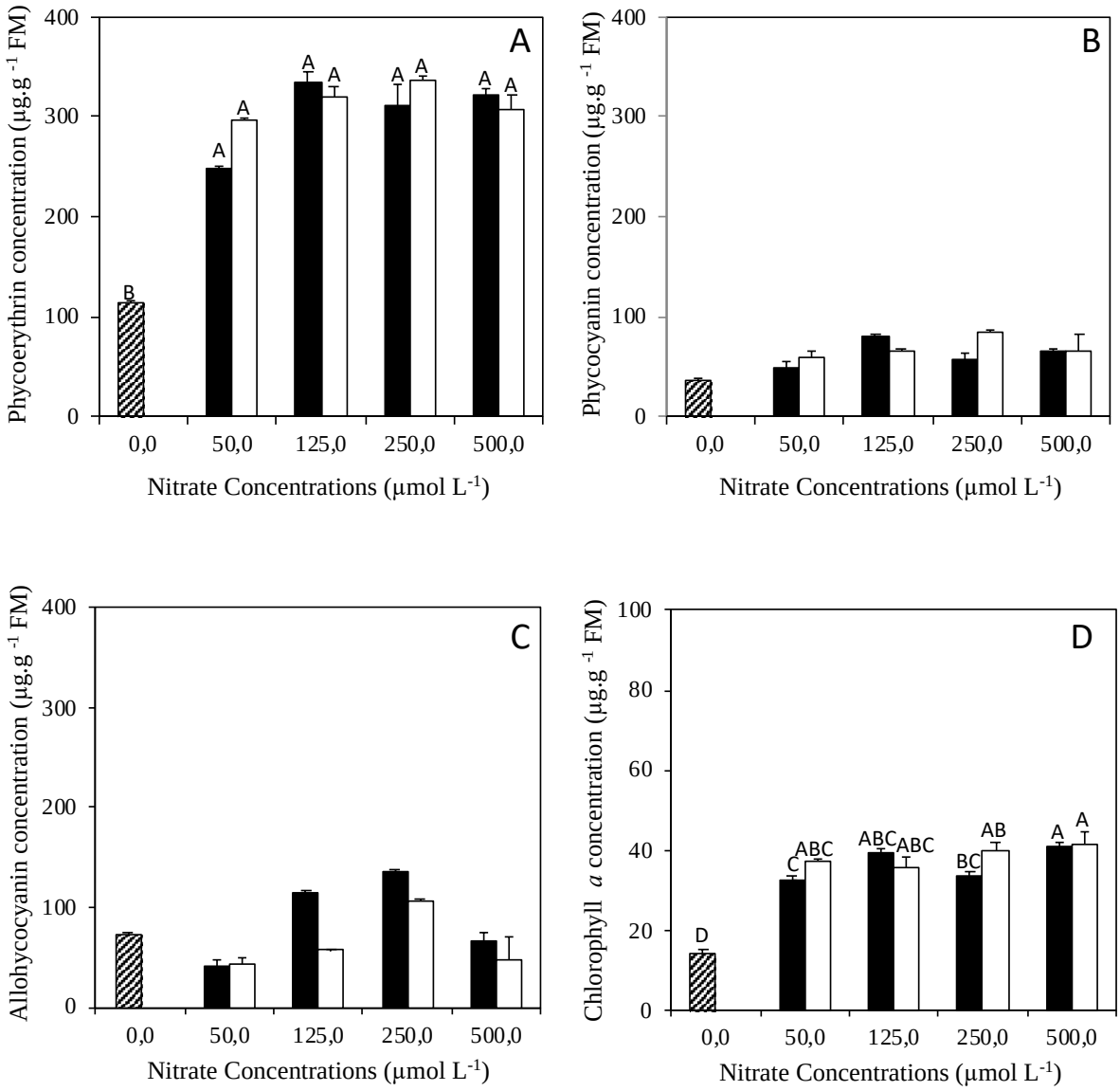

Fig. 4. Concentrations of phycoerythrin (A), phycocyanin (B), allophycocyanin (C) and chlorophyll a (D) in *Gracilaria caudata* cultured in different concentrations of nitrate and phosphate, in the ratio of 100:1 (black columns) and 10:1 (white columns) for 32 days. Mean ± SD (n = 3). Treatments with distinct letters were significantly different from each other, according to the Student-Newman-Keuls comparison test ($p < 0.05$). Asterisks indicate significant differences between N: P ratios.

The concentrations of low molecular weight carbohydrates in *G. caudata* did not vary among all treatments tested ($F = 0.44$ $p = 0.77$) (Fig. 5 A), and the same was observed between the reasons ($F = 0.06$ $p = 0.80$). The polysaccharide concentrations decreased in the treatments with N: P of 50.0 / 5.0 µM and 500.0 / 5.0 µM, when compared to the control (F =

6.82 p = 0.00) (Fig. 5 B) and there were no differences between the ratios (F = 0.65 p = 0.42) (Fig.8 B).

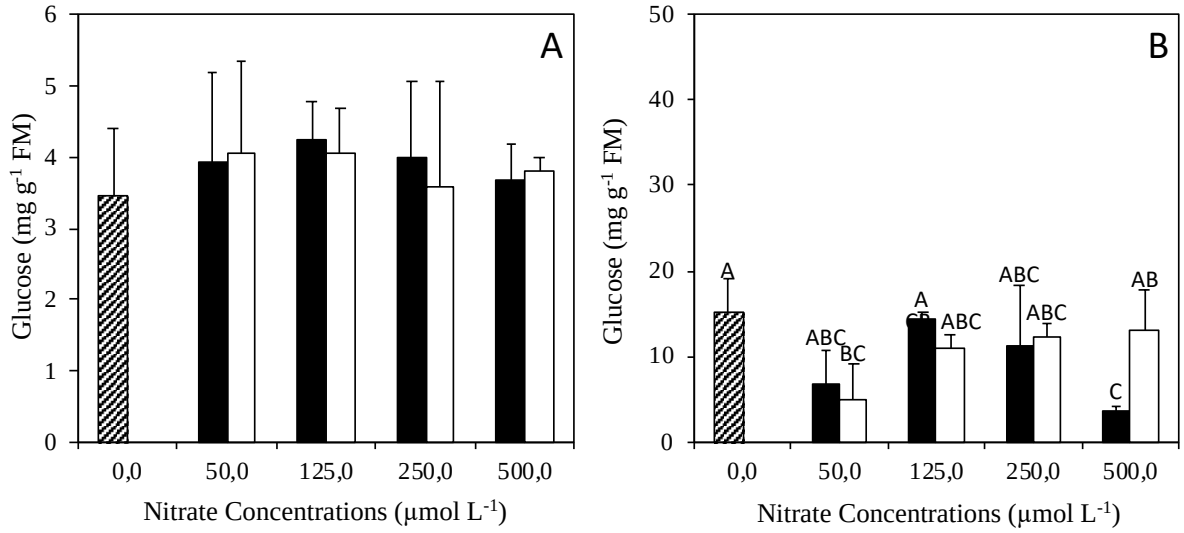

Fig. 5. Concentrations of total soluble carbohydrates in the ethanolic (A) and aqueous (B) extracts of *Gracilaria caudata* cultured in different concentrations of nitrate and phosphate at the ratio of 100:1 (black columns) and 10:1 (white columns) of N: P for 32 days. Mean ± SD (n = 3). Treatments with distinct letters were significantly different from each other, according to the Student-Newman-Keuls comparison test (p <0.05). Asterisks indicate significant differences between N: P ratios.

The results of the *G. caudata* tissue analysis showed that the concentrations of tissue carbon were lower in the 10: 1 ratio (Fig. 6 A) (F = 18.87 p = 0.00), when compared to the control. Among the reasons, N: P 100: 1 was greater than 10: 1 (F = 15.69 p = 0.00). Tissue hydrogen was lower in all concentrations of N: P 10: 1 when compared to the control (Fig. 6 B) (F = 8.09 p = 0.00) and among the reasons, N: P 100: 1 was greater than 10: 1 (F = 19.89 p = 0.00). The concentrations of tissue nitrogen increased in all treatments with different concentrations of N: P when compared with the control 0.0 µmol L^{-1} (F = 2.43 p = 0.05) (Fig. 6 C) and between the ratios, N: P 100: 1 and 10: 1 had no differences (F = 6.78 p = 0.00). Phosphorus concentrations increased by 125.0 / 12.5, 250.0 / 2.5, 250.0 / 25.0, 500.0 / 50.0 µmol L^{-1} (F = 30.86 p = 0.00). The 100: 1 ratio was greater than 10: 1 (Fig. 6 D) (F = 79.17 p = 0.00).

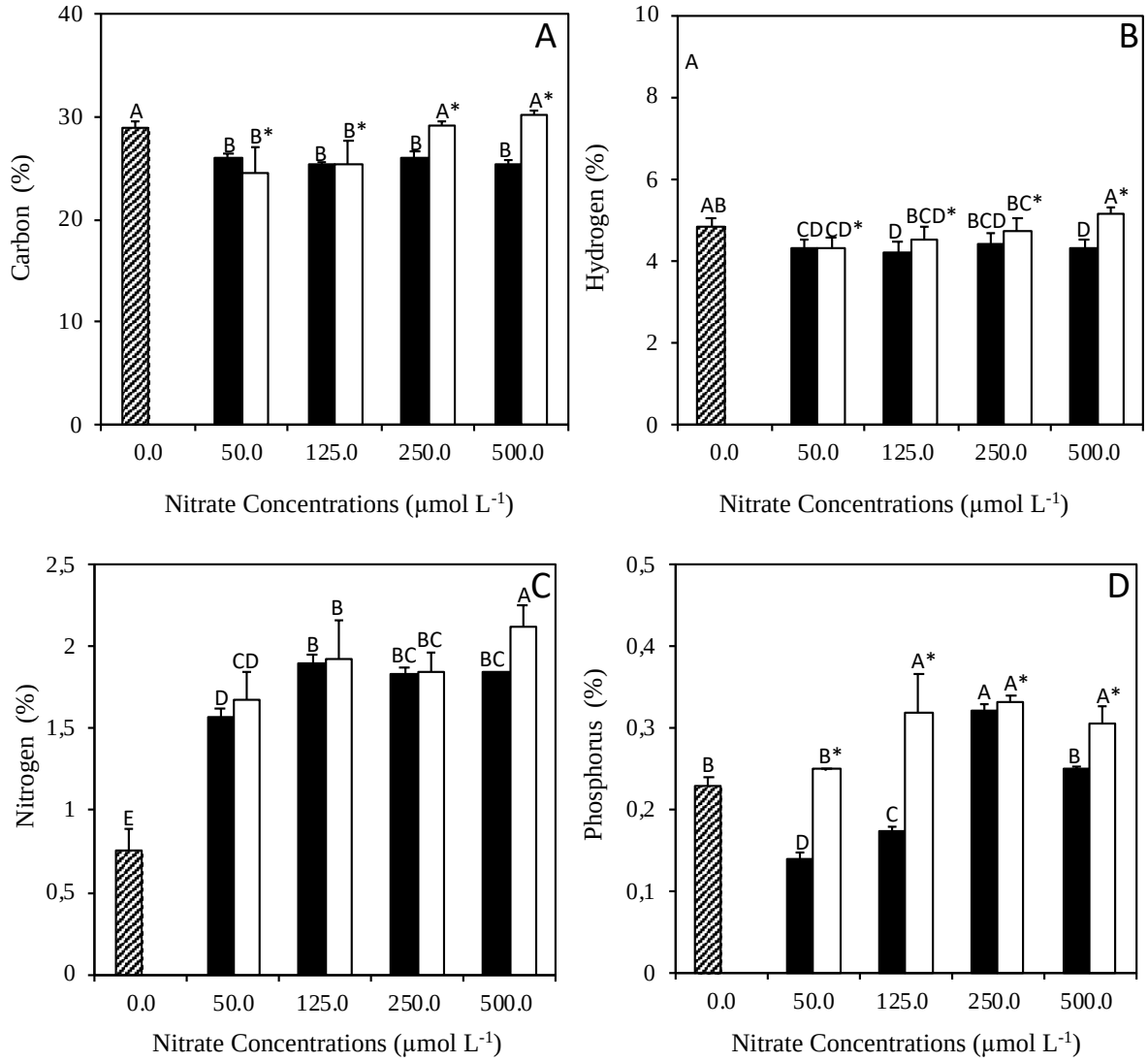

Fig. 6. Thallus contents of Carbon (A), Hydrogen (B), Nitrogen (C) and Phosphorus (D) in *Gracilaria caudata* cultured in different concentrations of nitrate and phosphate at the ratio of 100:1 (columns in black) and 10:1 (columns in white) 32 days. Mean ± SD (n = 3). Treatments with distinct letters were significantly different from each other, according to the Student-Newman-Keuls comparison test ($p < 0.05$). Asterisks indicate significant differences between N: P ratios.

The efficiency of *G. caudata* in removing nitrate showed an inverse relationship with the increase in the concentration of N: P ($F = 12316.55$ $p = 0.00$) (Fig. 7 A) and among the ratios, 100: 1 showed greater values than 10: 1 ($F = 182.5$ $p = 0.00$). Regarding the efficiency of phosphate removal, the highest values observed for treatments with 50.0 / 5.0 µM N: P, 125.0 / 1.25 µM and 250.0 / 2.5 µM ($F = 15.01$ $p = 0.00$) (Fig. 7 B) and among the reasons , 100: 1 was greater than 10: 1 ($F = 319.04$ $p = 0.00$).

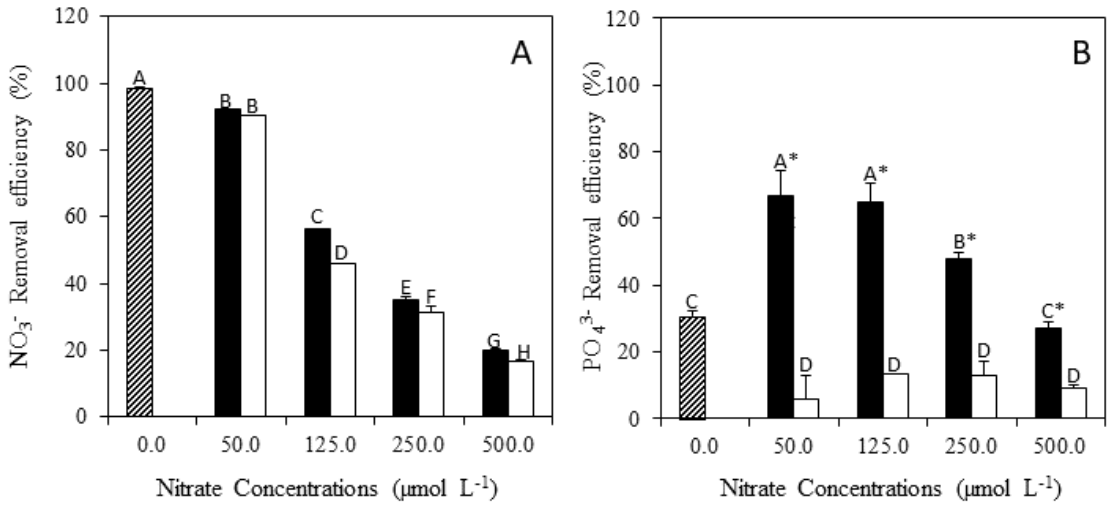

Fig. 7. Efficiency of removal (%) of nitrate ($NO3^-$) (A) and phosphate (PO_4^{3-}) (B) in *Gracilaria caudata* cultured in different concentrations of nitrate and phosphate at the ratio of 100:1 (columns in black) and 10:1 (columns in white) for 7 days. Mean ± SD (n = 3). Treatments with distinct letters were significantly different from each other, according to the Student-Newman-Keuls comparison test ($p < 0.05$). Asterisks indicate significant differences between N:P ratios.

NO_3^- uptake increased with the addition of N:P in the ratios of 100:1 and 10:1 (Fig. 8 A) ($F = 143.61$ $p = 0.00$), and the highest values were observed in treatments with N:P 10:1 than 100:1 ($F = 5.68$ $p = 0.03$). PO_4^{3-} uptake increased with the addition of N:P in the ratios of 10:1 and 100:1 (Fig. 8 B) ($F = 14.79$ $p = 0.00$), and the highest values were observed in treatments with N:P 10:1 than 100:1 ($F = 9.81$ $p = 0.00$).

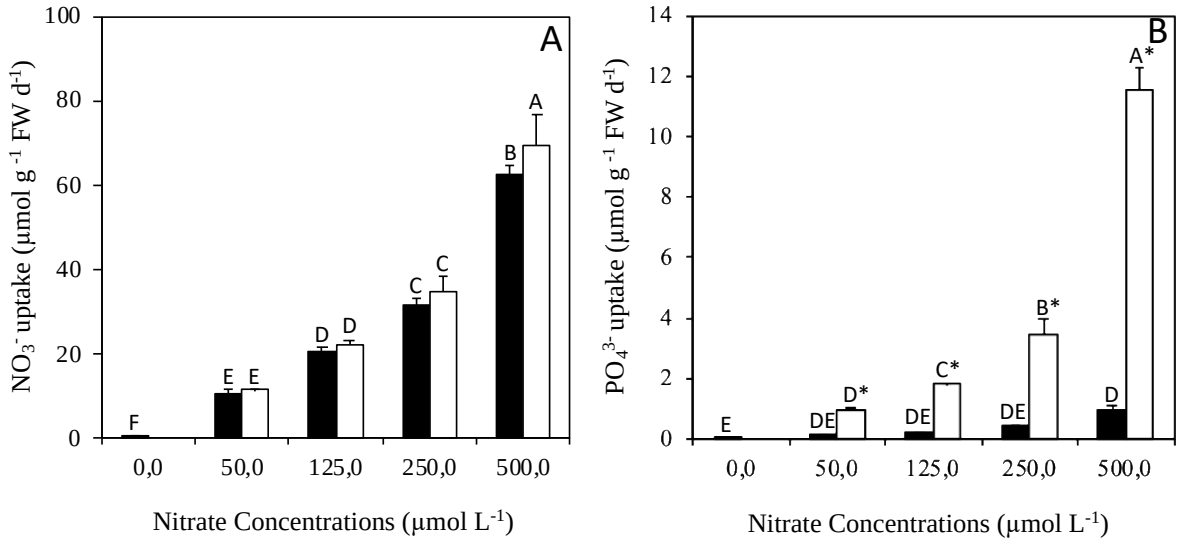

Fig. 8. Uptake of nitrate ($NO3^-$) (A) and phosphate (PO_4^{3-}) (B) in *Gracilaria caudata* cultured in different concentrations of nitrate and phosphate at the ratio of 100:1 (columns in black) and 10:1 (columns in white) for 7 days. Mean ± SD (n = 3). Treatments with distinct letters were significantly different from each other, according to the Student-Newman-Keuls comparison test ($p < 0.05$). Asterisks indicate significant differences between N: P ratios.

DISCUSSION

The growth of *G. caudata* was limited in treatments with high concentrations of N: P (500.0 / 50.0 µM) when compared to the control, therefore these results show that the species does not tolerate high levels of nitrogen and phosphorus. These results are similar to those described for the wild and green strains of *G. cornea*, which showed higher growth rates in VSES 12.5% (8.4% day^{-1}) than in VSES 25% (7.1% day^{-1}) (Ferreira et al. 2006) and G. *lemaneiformis* which showed a decrease in the growth rate with the increase in the concentration of nitrogen (Xu & Kaihong 2006). The lowest growth rates were observed in treatments grown only in sea water and the highest with the addition of 150, 250, 500 and 700 µM nitrate (Wanderley 2009).

The increase of nitrate and phosphate decreased the concentration of primary and secondary branches in *G. caudata* these results differ with the addition of 150, 250, 500 and 700 µM nitrate showed greater ramifications (Wanderley 2009).

The addition of N: P did not influence the effective quantum yield, the maximum photosynthesis and the photosynthetic efficiency of *G. caudata*, and these responses differed from the other species of *Gracilaria* previously described. High rates of photosynthesis in G. *verrucosa* and *G. tikvahiae* were observed in treatments with high concentrations of nutrients (Dawes and Koch, 1991) and in *G. lemaneiformis* (Xu et al. 2010). Barufi et al. (2011) observed that the nitrogen limitation caused a decrease in the effective quantum yield of *G. tenuistipitata*.

The levels of total soluble proteins of *G. caudata* increased with the greater availability of nitrogen, indicating the role of these compounds as a nitrogen reserve and their allocation for growth under conditions of nutrient limitation (Andria et al., 1999). Similar results were observed in *G. domingensis*, where the interaction between high availability of nutrients (50% VSES) and high irradiance stimulated the accumulation of total soluble protein (Ramlov 2011). According to Naldi & Wheeler (2002), the increase in the nitrate concentration in *G. pacifica* increased the concentration of total soluble proteins. The increase in nitrate concentrations in growth medium led to increased protein / carbohydrate ratios in the apical segments of *G. verrucosa* and it can be suggested that the constituents of the stem, protein and carbohydrate, can be used to assess the state of nutrient deficiency. *G. verrucosa* (Bird, 1984). García-Sánchez (1993) showed that total soluble proteins decreased in *G. tenuistipitata* with the reduction of nitrate in the culture medium.

The greater availability of nitrate and phosphate increased the pigment concentrations in *G. caudata* brought about an increase in the concentrations of phycoerythrin and chlorophyll a. Similar responses were observed in *G. tikvahiae* McLachlan (Bird et al. 1984) and in color variants of *G.birdiae* described by Costa (2005), who observed an increase in pigment content, with the exception of allophicocyanin, in response to the increase in nitrate. In addition, enrichment with a high nitrate content in *G. tenuistipitata*, increased the content of photosynthetic pigments compared to low nitrate concentrations (Barufi et al. 2011). *G.*

tenuifrons grown in seawater without the addition of nitrate or with the addition of 50% nitrate, showed depigmentation, that results are in agreement with the results of *G. caudata* (Wanderley 2009).

The concentration of carbohydrates in *G. caudata* didn't differ significantly between treatments with addition of N: P in the ratios of 100: 1 and 10: 1. Similar results were observed by Navarro and Robledo (1999) in *G. cornea*. Bird et al. (1982) observed that the content of total soluble carbohydrates increased with higher concentrations of nitrate in *G. tikvahiae*. In relation to polysaccharides, the study with *G. lemaneiformis* showed that its cultivation must be done for two weeks in a low proportion of N∶P and a high concentration of phosphorus to increase the quality of the polysaccharides (Xu & Kaihong, 2006). In *G. bursa-pastoris*, the levels of starch and carbohydrates increased with the highest levels of nitrate (Korsen, 2016).

The high availability of nitrogen increased the tissue nitrogen concentration in *G. caudata*, the results are similar to those presented by Ribeiro et al. (2016) with *Hypnea aspera*, in *Ulva fasciata* (Lapointee and Tenore 1981), *G. tikvahiae* (Friedlander and Dawes 1985), *Ulva clathrata* (Copertino et al. 2009) and *Palmaria palmata* (Corey et al. 2013). Studies with *Chondrus crispus* (Chopin and Wagey 1999) and *Chaetomorpha linum* (Menéndez et al. 2002) found high levels of phosphorus in high concentrations of nitrate and phosphate. Peckol (1994) showed that by adding higher concentrations of nitrate and phosphate, *G. tikvahiae* showed higher concentrations of tissue nitrate than those cultivated with lower concentrations of these nutrients. The results obtained with the cultivation of *G. birdiae* in aquaculture systems as a biofilter showed the species' ability to remove these excess nutrients from the environment (Marinho-Soriano 2009).

The results of efficient removing nutrients in *G. caudata* showed that the species is not efficient in removing high concentrations of nitrate and phosphate. Abreu et al. (2011) studied the nitrogen absorption responses of *G. vermiculophylla* and the results showed that high

concentrations of nutrients also inhibited the absorption of nutrients, the same was reported for *G. verrucosa* and *G. tikvahiae*, where the greatest efficiencies were in lower concentrations of nutrients (Du et al. 2013). The species *G. lemaneiformis*, decreased the phosphate level in aquariums (Yang et al 2006). The removal efficiency observed in *G. verrucosa* and *G. tikvahiae* was higher in lower nutrient concentrations (Du et al. 2013). However, the uptake values of the nutrients increased in the concentrations of nitrate and phosphate in *G. caudata*.

The species *G. caudata*, despite not being a species with high growth rates and not showing good removal efficiency nitrate and phosphate, presented high concentrations of pigments and total soluble proteins, however we can conclude that this species doesn't have a high potential for uptake nutrients available in seawater, not being a good option for use as biofilters in multi-trophic crops and in the sea.

ACKNOWLEDGMENTS

This research was supported by a scholarship provided by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, Brazil, proc. no. 2017/13259-3) to the first author. This study is part of the thesis presented by the first author to the Graduate Program in Plant Biodiversity and Environment, Institute of Botany, São Paulo, Brazil.

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Capítulo 2

PHYSIOLOGICAL RESPONSES AND BIOFILTER POTENTIAL OF *GRACILARIA DOMINGENSIS* (GRACILARIALES, RHODOPHYTA) IN DIFFERENT CONCENTRATIONS ON NITRATE AND PHOSPHATE

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ABSTRACT

Benthic marine algae can be biofilters by assimilation and metabolism of nutrients. Species of *Gracilaria* Greville are exploited as raw material for agar production, and studies on their development are necessary for the sustainable production of biomass. The present study aimed to evaluate the physiological responses of *Gracilaria domingensis* (Kützing) Sonder ex Dickie cultivated in different concentrations of nitrogen and phosphorus. Treatments were composed of von Stosch solution (VSES/2) prepared without nitrate and phosphate. Nitrate concentrations varied from 0.0 to 500 μmol, and phosphate concentrations varied from 0.0 to 50 μmol L^{-1} or 0.0 to 5 μmol L^{-1} in the N:P ratio of 10:1or 100:1, respectively. Growth rates of *G. domingensis* increased with addition of N:P, and higher values were observed in treatments with 100:1. Pigment contents increased with N:P addition, and treatment with 125:1.25 μmol L^{-1} stimulated the highest contents of phycoerythrin, phycocyanin and allophycocyanin. Chlorophyll *a* did not vary significantly. Similar effects were observed for protein contents. The highest efficiency of nitrate removal was observed in 50:5 μmol L^{-1} of N:P. On the other hand, efficiency of phosphate removal increased with N:P addition. Uptakes were increase on nitrate and phosphate treatments. Phosphorus internal showed different results with nutrients addition. Our results showed that *G. domingensis* has a high

potential as biofilter and could be cultivated in integrated multitrophic aquaculture, due to its high efficiency in nutrient removal from seawater.

Keywords: Biofilter, *Gracilaria*, Nitrate, nutrient removal, phosphate

INTRODUCTION

The genus *Gracilaria* Greville (Gracilariaceae, Rhodophyta) comprises more than 172 species, some with cosmopolitan distribution in tropical and temperate waters (Guiry and Guiry 2020). The genus *Gracilaria* has a wide geographical distribution and is among the most cultivated in the world for agar extraction, with China and Indonesia being the main producing countries, and Chile is the largest producer in South America (Zemke-White and Ohno 1999; Hayashi et al., 2014; Porse and Rudolph 2017).

Aquaculture is the food production activity with the highest global growth (Ottinger et al. 2016, Troell et al. 2017, Liu et al. 2018), generating approximately 160 million tons of marine organisms in 2015 (FAO 2018). Global production of aquatic plants dominated by seaweed grew from 13.5 million tons in 1995 to just over 30 million tons in 2016, generating an increase of USD 60 million to over USD 1 billion in 2016 (FAO 2018). Indonesia, Chile and the Republic of Korea are the main exporters, and China, Japan and the United States of America are the main importers (FAO 2018). The most important cultivated species are *Eucheuma* (10.2 million tonnes), followed by *Laminaria japonica* (8 million tonnes), *Gracilaria* spp. (3.9 million tonnes), *Undaria pinnatifida* (2. million tonnes), *Kappaphycus* (1.8 million tonnes) and *Porphyra* spp. (1.2 million tonnes) (FAO 2018).

Gracilaria domingensis (Kützing) Sonder ex Dickie occurs along the Brazilian coast and is one of the few *Gracilaria* species that occur in the southern region of the country (Oliveira and Plastino 1994). *G. domingensis* is commercialized in natura for human consumption, and there are records of its export to the Japanese market (Plastino et al. 1999).

Nitrogen is the main element that limits the growth of benthic marine algae and controls the primary productivity of the oceans. Nitrate and ammonium are important for the

growth of these organisms and are responsible for the synthesis of amino acids, purines, pyrimidines, sugars and amines (Lobban and Harrison 2004). Like nitrogen, phosphorus is a limiting nutrient in the development of marine algae, and is related to molecules with structural and energy transfer functions, such as ATP formation (DeBoer 1981).

Some studies have shown that *Gracilaria* spp. can remove nutrients through the assimilation of nutrients (N and P) in multitrophic crop systems integrated with fish, scallops or shrimp in eutrophic areas (Buschmann et al. 1996, Troell et al. 1998, Huo et al. 2012).

Laboratory studies also evaluated the nitrogen and / or phosphorus absorption capacity of different *Gracilaria* species, such as *Gracilaria gracilis* (Martínez-Aragón et al. 2002), *Gracilaria lemaneiformis* (Bracken & Stachowick 2006), and *G. cornea* (Navarro-Angulo & Robledo 1999), but also in other red algae species such as *Gelidium coulteri* (Corey et al. 2013, Grote 2016), *Pyropia* (Carmona et al. 2006) and *Palmaria* (Mao et al. 2009).

G. domingensis is a commercial importance in the use in the human food (in natura), and there are records of having been sporadically exported to the Japanese market (Plastino et al. 1999). The cultivation of *G. domingensis* was feasible in the same long line system used in *Perna perna* cultivation (Yoshimura et al. 2004).

The main objective of the present work is to evaluate the effects of nutrient (nitrogen and phosphorus) availability on growth, morphology, contents of pigments, proteins, carbohydrates and thallus endogenous endogenous contents of C, N, H and P in *Gracilaria domingensis*.

MATERIAL AND METHODS

Species studied and unialgal cultures

Unialgal cultures of female gametophyte of *Gracilaria domingensis* collected at Lagoinha Beach, Florianópolis, Santa Catarina Satate, southern Brazil (27º 35’ S e 48º 33’ W) in October 14, 2008. Voucher specimens were deposited in the Herbarium SP (accession

number SP 400837), and isolates are deposited in the Culture Collection of Algae, Fungi and Cyanobacteria of Institute of Botany (CCIBt)

Biomass necessary for the experiments was obtained by vegetative propagation of gametophytes thallus segments (10 mm). The culture medium selected comprised sterilized seawater (salinity of 30 - 31) enriched with von Stosch's solution at half strength (VSES/2) following Oliveira et al. (1995), modified with vitamin concentrations reduced to a 50 % concentration (thiamin (100 µg L^{-1}), biotin (100 µg L^{-1}) and cyanocobalamin (1.0 µg L^{-1}) as proposed by Yokoya 2000). Culture media was renewed every two weeks. Cultures were incubated at: 23 ± 1 ºC, photon flux densities of 60.0 - 70.0 µmol photons m^{-2} s^{-1} (provided by cool-white fluorescent lamps), with a 14:10h light:dark cycle, without aeration. Irradiance was measured with a quantum photometer (LI-250, Li-Cor, Lincoln®, Nebraska, USA) equipped with underwater quantum sensor (LI-192 SA, Li-Cor).

Experimental design

Experiments were performed in Erlenmeyer flasks (500 mL) with 200 mg algal biomass per 400 mL culture medium. Treatments were composed of sterilized seawater enriched with VSES/4 modified (von Stosch's solution prepared without nitrate and phosphate, but with salts of iron, manganese, ethylenediaminetetraacetic acid (EDTA), and three vitamins, following Oliveira et al. (1995) and Yokoya (2000). Nitrate ($NaNO_3$) and phosphate ($Na_2HPO_4.12H_2O$) were added to the VSES/4 modified medium in order to obtain nitrogen/phosphorus (N/P) ratios of 10:1 and 100:1. Nitrate concentrations varied from 0.0 to 500 µmol, and phosphate concentrations varied from 0.0 to 50 µmol L^{-1} or 0.0 to 5 µmol L^{-1} in the N:P ratio of 10:1or 100:1, respectively. Each treatment was tested with four replicates (n = 4). Other laboratory conditions were the same as those described for unialgal cultures.

For biochemical analyses, after four weeks, algae were weighed for growth analysis, and the culture medium was renewed and, after 4 days, samples of each replicate were weighed, frozen in liquid nitrogen and stored at -20 °C.

Growth rates

Fresh biomass was recorded at weekly intervals before renewal of the media during four weeks. Daily growth rates were calculated using the following equation: $GR=[(Wt/Wi)^{1/t} - 1]\times100\%$, where Wt is the weight after t days, Wi is the initial weight, and t is the experimental period (Yong et al. 2013).

Morphology

The number of lateral branches per explant was determined by analyses of photos of three apical segments per replicate taken in digital camera (Panasonic, Lumix DC Vario model).

In vivo chlorophyll fluorescence

The in vivo fluorescence of chlorophyll was measured using an underwater Walz Diving-PAM flow meter. Apical segments of G. domingensis (n = 3) were placed on a magnetic sample holder to avoid overlap. Eight levels of irradiance were used to construct photosynthesis × irradiance curves of photons m^{-2} s^{-1}. The apical segments were exposed for 20 s in each irradiance, interspersed with a 0.8 s saturation pulse. The effective quantum yield (ΔF/Fm′) was provided by Diving-PAM after an initial saturation pulse, when the samples had a pulse of very low intensity of blue light (approximately 0 μmol of photons m^{-2} s^{-1}). The following parameters were calculated and analyzed: photosynthetic efficiency (α ETR), maximum electron transport rate (ETR max), light saturation (Ik) and effective quantum yield (ΔF/Fm′).

Total soluble protein contents

For total soluble protein analysis, 80 mg of algal fresh biomass for each replicate (n = 3 for each treatment) were ground with liquid nitrogen, and extractions were carried out at 4 °C using 0.2 M phosphate buffer (pH 8) containing 5 mM of ethylenediaminetetraacetic acid (EDTA) and 1 mM of dithiotreitol (DTT). Buffer was added in the proportion of 10 mL g^{-1} fresh biomass, and the homogenates were centrifuged at 12,000×g for 15 min. Total soluble protein contents were determined according to Bradford (1976), using a Bio-Rad® protein assay kit and BSA as standard.

Pigment analyses

The algal mass (75 mg fresh mass for each replicate, n=3) was ground with liquid nitrogen and 1 ml of 50 mM (pH 5.5) phosphate buffer was added. Pigment extractions were carried out at 4ºC, in the dark. Crude extracts were centrifuged at 14,000×g for 20 min in order to obtain the phycobiliproteins, and chlorophyll-a was extracted after dissolving the pellet in 90% acetone, followed by centrifugation at 10,000×g for 15 min. Pigments were quantified using a spectrophotometer (Shimadzu®, UV 1800), and concentrations were calculated according to Kursar et al. (1983) for phycobiliproteins, and Jeffrey and Humphrey (1975) for chlorophyll *a*.

Carbohydrate extraction and quantitative analysis

The extraction of soluble carbohydrates was performed according to Carvalho et al. (1998) with modifications. For each replicate (n=3), 60 mg of fresh biomass was tritured with liquid nitrogen, suspended in 1 mL of 80% ethanol, kept in a 80 °C water bath for 1 h and then centrifuged at 1082 g for 15 min. The supernatant was stored and the precipitate was subjected to the same procedure for two more times. The final residue was resuspended in 1 mL of distilled water, kept in a water bath at 60 °C for 1 h. The supernatant was stored and

the precipitate was subjected to the same procedure for two more times. Quantification of total soluble carbohydrates of ethanolic and aqueous extracts was carried out using the phenol-sulfuric colorimetric method (490 nm) of phenol-sulfuric acid according Dubois et al. (1956).

Thallus contents of C, H, N, and P

Analyses of endogenous contents of C, H, and N were determined in samples of each replicate (n=3) according to the PreglDumas' method using PerkinElmer® 2400 Series II equipment. Endogoenous contents of P were determined by inductively coupled plasma optical emission spectrometry (ICP-OES), using a SPECTRO ARCOS® high-resolution analyzer. Analyses were performed by Analytical Center of Instituto de Química, Universidade de São Paulo, São Paulo, Brazil.

Analysis of seawater nutrients

Nitrate (NO_3^-) and phosphate (PO_4^{3-}) analysis was performed according to Grasshoff et al. (1999) using a segmented flow analyser (Bran Luebbe®, Auto Analyzer II) and a spectrophotometer (Thermo®, Evolution 201), respectively. Removal efficiency (percentage) was calculated as $((C_i\text{-}C_7)/C_0) \times 100$ for each nutrient, where C_i is the initial concentration before the incubation of seaweed into seawater, C_0 is the nutrient concentration right after the incubation of seaweed into seawater, and C_7 is the nutrient concentration after the incubation period (7 days). Nutrient uptake was calculated according to Kregting et al. (2008) as $((C_i - C_7)/(FW \times time)) \times volume$, where FW is the seaweed fresh biomass after 7 days of incubation, time is 7 days, and volume is the volume of culture medium in each erlenmeyer (400 mL). These samples were filtered through a cellulose acetate membrane with pore size of 0.45 μm (Millipore®) and stored at −20 °C until nutrient analyses. After 7 days, the algal

biomass was measured for growth rate analysis, and the culture medium (with the same nitrogen and phosphorus concentrations) was renewed.

Statistical analyses

Data were analyzed by two-way of variance (ANOVA) followed by the Student-Newman-Keuls *a posteriori* test for multiple comparisons to distinguish significant differences ($p < 0.05$). Statistica 10.0 software was used to perform all statistical analyses.

RESULTS

Growth rates increased with the addition of N:P in the ratios of 100:1 and 10:1 when compared to control (Fig. 1) ($F = 144.25$ $p = 0.00$), and higher values were observed in treatments with concentrations higher than 125 μmol L^{-1} of N:P of 100:1 than 10:1 ($F = 81.99$ $p = 0.00$).

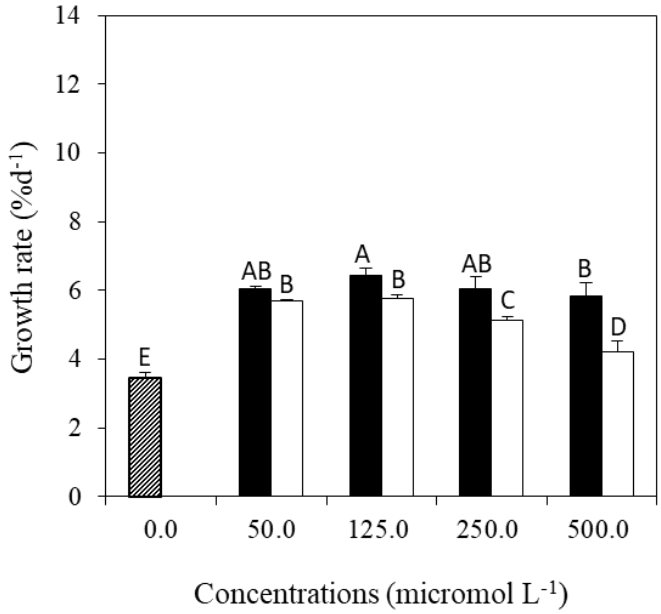

Fig. 1. Growth rates of *Gracilaria domingensis* cultured in different concentrations of nitrate and phosphate, in the ratio of 100:1 (black columns) and 10:1 (white columns) for 28 days. Mean ± SD (n = 4). Treatments with distinct letters were significantly different from each other, according to the Student-Newman-Keuls comparison test ($p < 0.05$).

The number of primary branches of *G. domingensis* was higher in treatments with N:P of 10:1 at concentrations of 125.0/12.5 and 250.0/25.0 μmol L^{-1}, and in treatments with N:P of 100:1 at concentrations of 250.0/2.5 and 500/5 μmol L^{-1} when compared to control (F = 15.03 p = 0.00) (Fig. 2A). Regarding the number of secondary branches, the highest value was observed in the treatment with 125.0/12.5 μmol L^{-1} N:P when compared to the control (F = 14.97 p = 0.00) (Fig. 2B). The treatments with N:P of 10:1 presented higher averages when compared to treatments with N:P of 100:1 (F = 5.23 p = 0.03).

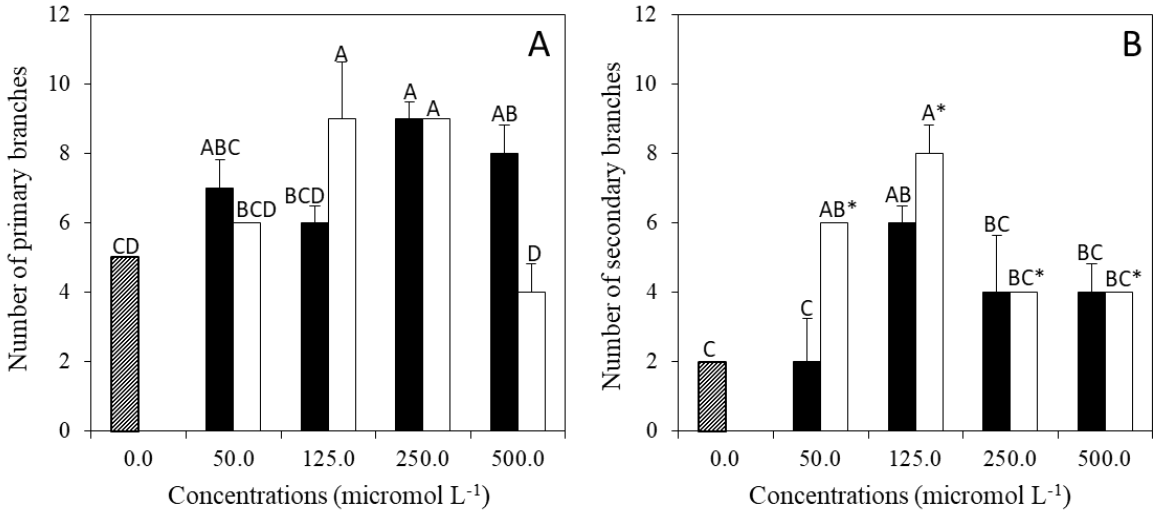

Fig. 2. **N**umber of primary branches (A) and number of secondary branches (B) of *Gracilaria domingensis* cultured in different concentrations of nitrate and phosphate, in the ratio of 100:1 (black columns) and 10:1 (white columns) for 28 days. Mean ± SD (n = 3). Treatments with distinct letters were significantly different from each other, according to the Student-Newman-Keuls comparison test ($p < 0.05$). Asterisks indicate significant differences between N: P ratios.

The Highest ETRs were found from the treatment of 125.0 / 1.25 μM N: P and the smallest in 250.0 / 25.0 μM N:P. The effective quantum yield was lower in treatments with the addition of nitrate and phosphate. In nutrient treatments, the highest values were 125.0 / 1.25 μM and 250.0 / 2.5 N:P μM and the lowest were 50.0 / 5.0 μM and 250.0 / 25.0 μM of N: P (F = 434.54 p = 0.00) (Table 1), among the reasons there were also differences between

the means, with 10: 1 greater than 100: 1 (F = 126.99 p = 0.00). The maximum photosynthesis of *G. domingensis* showed differences, being higher in the treatments 125.0 / 1.25 µM, 250.0 / 2.5 µM, 125.0 / 12.5 µM and 500.0 / 50.0 µM of N : P (F = 23.80 p = 0.00), between the reasons there were no differences between the means (F = 1.50 *p* = 0.23). The photosynthetic efficiency did not vary significantly between the different treatments tested (F = 2.39 *p* = 0.08), the same occurred between the means of the ratios (F = 0.50 *p* = 0.48). Irradiance of saturation was higher in 125.0 / 1.25 µM (F = 132.71 *p* = 0.00) and the 100: 1 ratio showed a higher average in relation to the 10: 1 ratio (F = 9.925 *p* = 0.00).

Table. 1. Effective quantum yield (EQY), maximum photosynthesis (Pmax), photosynthetic efficiency (α) and irradiance of saturation (Ik) in *Gracilaria domingensis* cultured in different concentrations of nitrate and phosphate at a ratio of 100: 1 and 10: 1 of N: P for 28 days. Mean ± SD (n = 3). Treatments with different letters indicate significant differences between concentrations, according to the Student-Newman-Keuls comparison test (p <0.05). Asterisks indicate significant differences between the reasons.

TREATMENTS N/P Concentrations (micromol.L^{-1})	PHOTOSYNTHETIC PARAMETERS			
	EQY	Pmax	α	Ik
0.0	0.325±0.00 A	3.2±0.3 CD	0.03±0.01	140±30 C
50.0 / 0.5	0.201±0.07 E*	2.9±0.2 D	0.03±0.00	148.3±11.7 C*
125.0 / 1.25	0.274±0.08 B*	7.6±0.3 A	0.01±0.00	776.3±30.9 A*
250.0 /2.5	0.281±0.06 B*	6.4±0.6 AB	0.02±0.01	321±38.8 B*
500.0 / 5.0	0.204±0.06 E*	4.8±0.1 BC	0.03±0.01	154.4±4.1 C*
50.0 / 5.0	0.189±0.06 F	4.2±0.0 CD	0.03±0.02	141.1±23.14 C
125.0 / 12.5	0.252±0.07 C	5.8±0.0 B	0.02±0.00	291.6±23.5 B
250.0 /25.0	0.179±0.09 F	3.9±0.7 CD	0.03±0.01	132.2±23.4 C
500.0 / 50.0	0.219±0.04 D	6.4±2.4 AB	0.03±0.01	213.3±82.7 C

The total soluble protein content of *G. domingensis* was higher in all treatments with N:P addition (Fig. 3) (F = 35.67 *p* = 0.00) but no differences were observed between the N:P ratios (F = 4.05 *p* = 0.06).

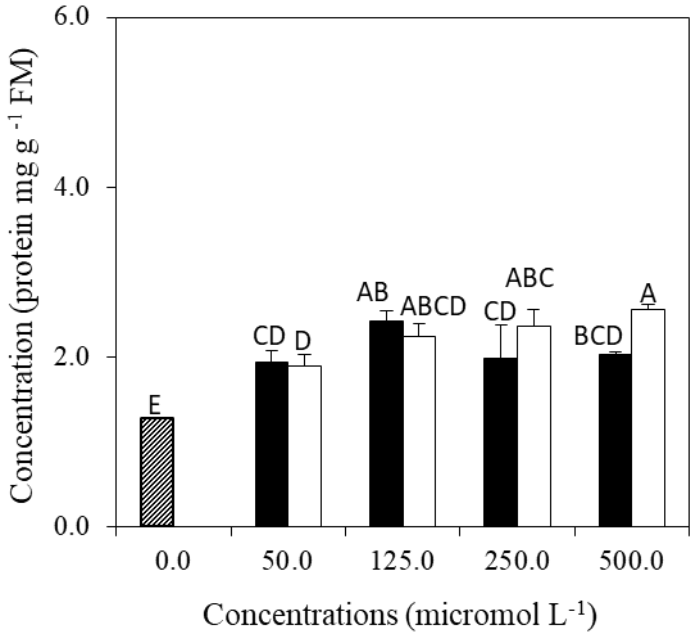

Fig. 3. Content of total soluble protein in *Gracilaria domingensis* cultured indifferent concentrations of nitrate and phosphate, in the ratio of 100:1 (black columns) and 10:1 (white columns) for 32 days. Mean ± SD (n = 3). Treatments with distinct letters were significantly different from each other, according to the Student-Newman-Keuls comparison test (p <0.05).

The phycoerythrin content of *G. domingensis* was higher in all treatments with N:P addition when compared to control (F = 28.16 p = 0.00) (Fig. 4A), and no differences were observed between N:P of 100:1 and 10:1 (F = 2.11 p = 0.16). Phycocyanin concentrations were higher in N:P treatments of 100:1 (50.0/0.5 μmol L^{-1} and 125.0/ .25 μmol L^{-1}) and N:P 10:1 (125.0/12, 5 μmol L^{-1}, 250.0/25.0 μmol L^{-1} and 500.0/50.0 μmol L^{-1}) (F = 19.73 p = 0.00) (Fig. 4B), and no differences were observed between the two N:P ratios (F = 0.314 p = 0.58). Higher concentrations of allophycocyanin were observed in the treatments with 50.0/0.5 and 125.0/1.25 μmol L^{-1} (F = 10.26 p = 0.00) (Fig. 4C) and treatments with N:P of 100:1 presented higher values than those with N:P of 10:1 (F = 10.26 p = 0.00). Chlorophyll *a* content was higher in all treatments with N:P addition when compared to control (Fig. 4D) (F = 42.68 p = 0.00), but no differences were observed between treatments (F = 1.12 p = 0.30).

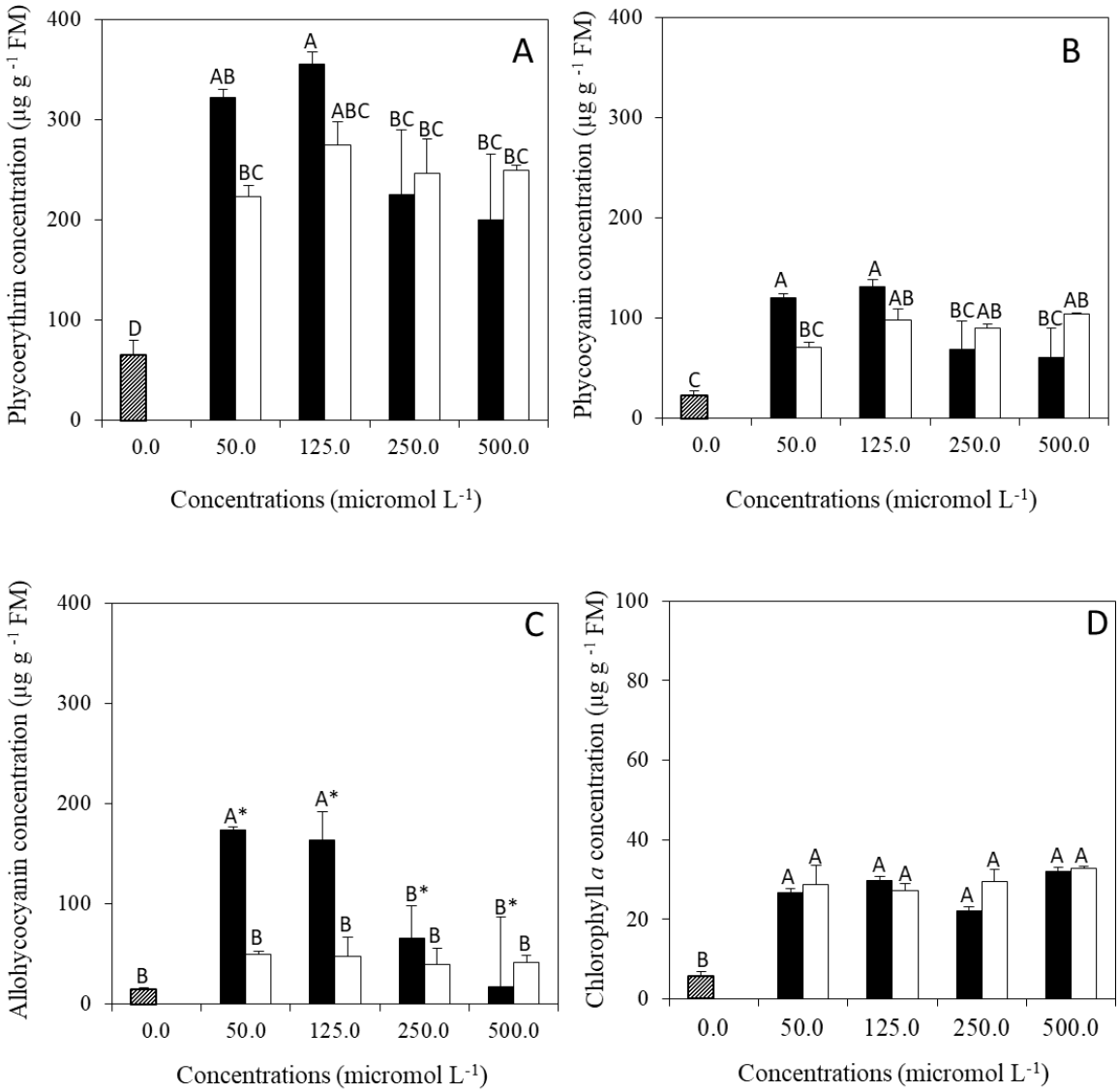

Fig. 4. Concentrations of phycoerythrin (A), phycocyanin (B), allophycocyanin (C) and chlorophyll a (D) in *Gracilaria domingensis* cultured in different concentrations of nitrate and phosphate, in the ratio of 100:1 (black columns) and 10:1 (white columns) for 32 days. Mean ± SD (n = 3). Treatments with distinct letters were significantly different from each other, according to the Student-Newman-Keuls comparison test ($p < 0.05$). Asterisks indicate significant differences between N: P ratios.

The concentrations of the low molecular weight carbohydrates of *G. domingensis* did not vary in the different treatments tested ($F = 0.37$ $p = 0.82$) (Fig. 5A), as well as among the N:P ratios of 100:1 and 10: 1 ($F = 0.031$, $p = 0.86$). However, the concentrations of polysaccharides were lower in treatments 125.0 / 1.25 and 250.0 / 2.5 µmol L^{-1}. (Fig. 5B) (F =

3.98 p = 0.01) and the treatments with N:P of 10: 1 had higher values than 100:1 (F = 8.61 p = 0.00).

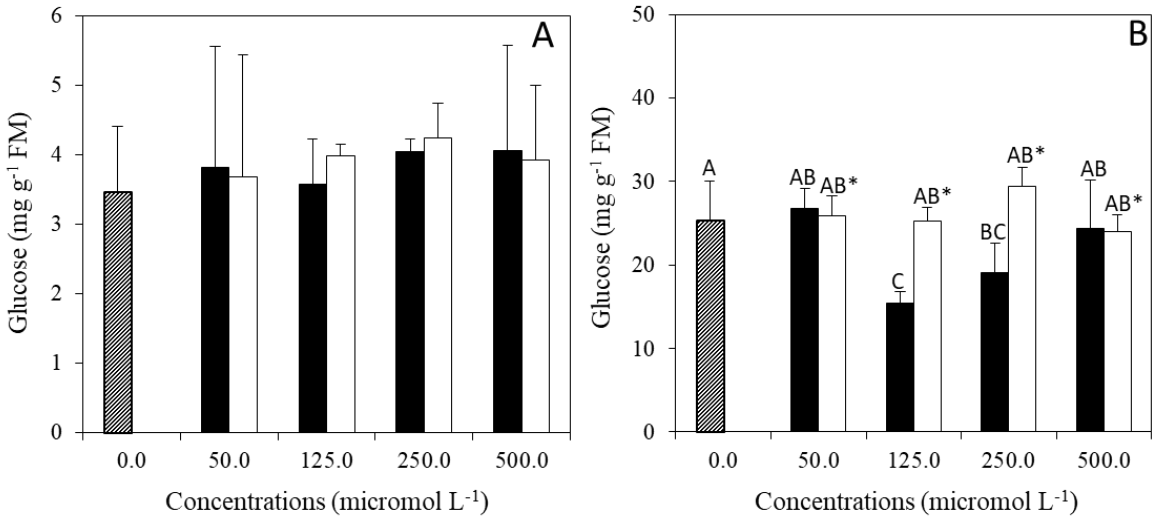

Fig. 5. Concentrations of total soluble carbohydrates in the ethanolic (A) and aqueous (B) extracts of *Gracilaria domingensis* cultured in different concentrations of nitrate and phosphate at the ratio of 100:1 (black columns) and 10:1 (white columns) of N: P for 32 days. Mean ± SD (n = 3). Treatments with distinct letters were significantly different from each other, according to the Student-Newman-Keuls comparison test (p <0.05). Asterisks indicate significant differences between N: P ratios.

The results showed that thallus carbon concentration decreased in the ratio 10:1 (Fig. 6A) (F = 7325.80 p = 0.00), between the ratios there were no differences N:P 10:1 than 100:1 (F = 2.56 p = 0.13). The hydrogen contents varied as carbon contents (Fig. 6B) (F = 6.09 p = 0.00) and between the ratios (F = 0.01 p = 0.94). Nitrogen contents increases with different concentrations of N:P when compared to control (F = 26.55 p = 0.00) (Fig. 6C). The phosphorus concentration was higher with increasing concentrations of N:P (F = 334.01 p = 0.00) and the ratio 100:1 was higher than 10:1 (F = 21.68 p = 0.00) (Fig. 6D).

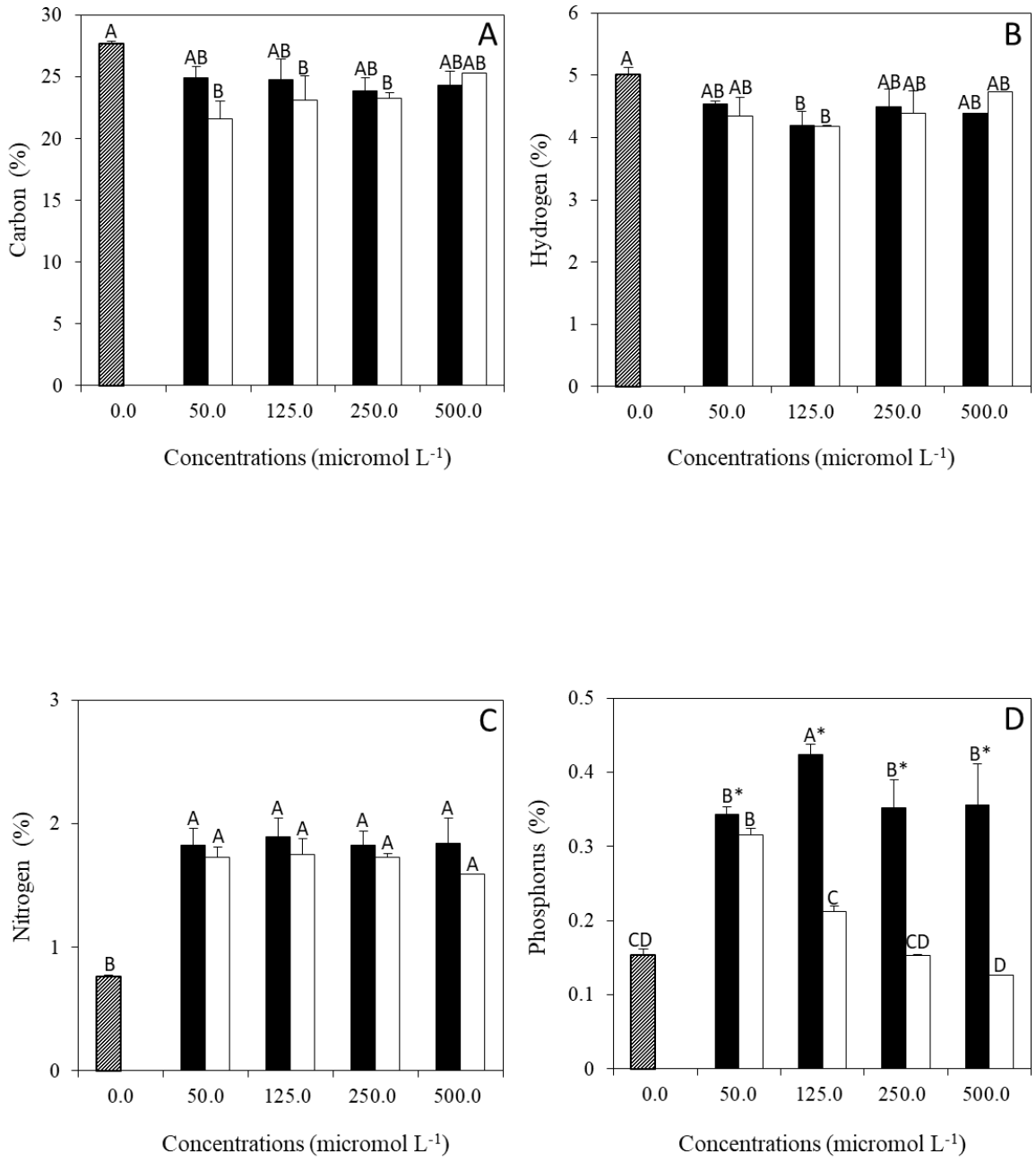

Fig. 6. Thallus contents of Carbon (A), Hydrogen (B), Nitrogen (C) and Phosphorus (D) in *Gracilaria domingensis* cultured in different concentrations of nitrate and phosphate at the ratio of 100:1 (columns in black) and 10:1 (columns in white) 32 days. Mean ± SD (n = 3). Treatments with distinct letters were significantly different from each other, according to the Student-Newman-Keuls comparison test ($p < 0.05$). Asterisks indicate significant differences between N: P ratios.

The efficiency of nitrate removal in *G. domingensis* was inversely proportional to the increase of N:P concentrations of 100:1 and 10:1, being lower in the highest concentration tested 500.0 / 50.0 and 500.0 / 5.0 μmol L^{-1} ($F = 501.74$ $p = 0.00$) (Fig. 7A) and but no

differences were observed between the ratios (F = 1.86 p = 0.18). Phosphate removal efficiency in all treatments with N:P addition was higher than control and the highest percentages of phosphate removal were observed in treatments with 50.0/5.0 μmol L^{-1}, 250.0/2.5 μmol L^{-1}, 500.0/5.0 μmol L^{-1}, and 500/50.0 μmol L^{-1} (F = 208.55 p = 0.00) (Fig. 7B) and the mean N:P treatments of 10:1 were higher than treatments with N:P of 100:1 (F = 5.11 p = 0.03).

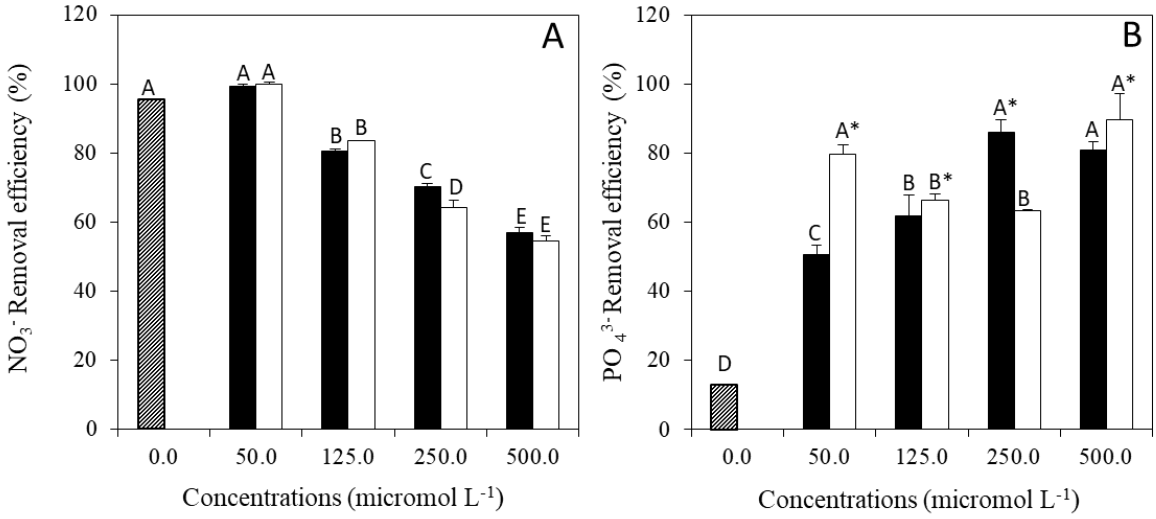

Fig. 7. Efficiency of removal (%) of nitrate ($NO3^-$) (A) and phosphate (PO_4^{3-}) (B) in *Gracilaria domingensis* cultured in different concentrations of nitrate and phosphate at the ratio of 100:1 (columns in black) and 10:1 (columns in white) for 7 days. Mean ± SD (n = 3). Treatments with distinct letters were significantly different from each other, according to the Student-Newman-Keuls comparison test (p <0.05). Asterisks indicate significant differences between N:P ratios.

NO_3^- uptake increased with the addition of N:P in the ratios of 100:1 and 10:1 (Fig. 8A) (F = 143.61 p = 0.00), and the highest values were observed in treatments with N:P 10:1 than 100:1 (F = 5.68 p = 0.03). PO_4^{3-} uptake increased with the addition of N:P in the ratios of 100:1 and 10:1 (Fig. 8B) (F = 14.79 p = 0.00), and the highest values were observed in treatments with N:P 10:1 than 100:1 (F = 9.81 p = 0.00).

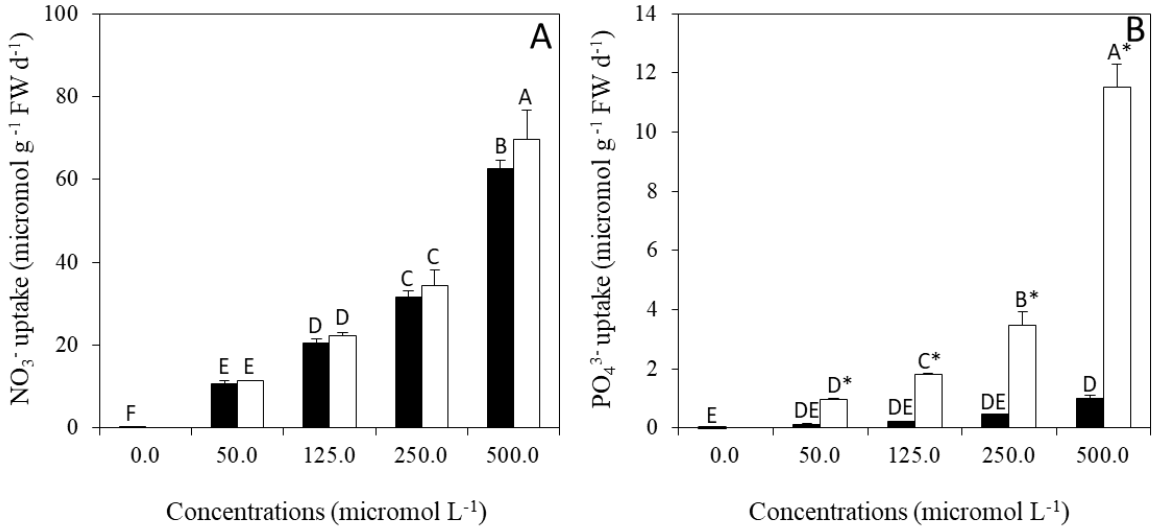

Fig. 8. Uptake of nitrate ($NO3^-$) (A) and phosphate (PO_4^{3-}) (B) in *Gracilaria domingensis* cultured in different concentrations of nitrate and phosphate at the ratio of 100:1 (columns in black) and 10:1 (columns in white) for 7 days. Mean ± SD (n = 3). Treatments with distinct letters were significantly different from each other, according to the Student-Newman-Keuls comparison test ($p < 0.05$). Asterisks indicate significant differences between N: P ratios.

DISCUSSION

Growth rates of *G. domingensis* were higher in all treatments with N:P addition when compared to the control, evidencing its tolerance to high concentrations of nitrate and phosphate and its potential as biofilter in integrated multitrophic cultivation. Similar results were observed. in the first weeks of the experiment with *G. parvispora* (Nelson et al. 2001), *G. lemaneiformis* (Yang et al. 2006), and *G. tikvahiae* (Samocha et al. 2015). Growth rates of *G. lemaneiformis* increased when the N:P ratios also increased from 50.0 / 3.13 μmol L^{-1} to 400.0 / 25.0 μmol L^{-1}, but decreased significantly when N:P concentrations exceeded 400.0 / 25.0 μmol L^{-1}, indicating that this species does not tolerate high concentrations of N:P (Yang et al. 2006). Similar results were observed in *G. tenuistipitata*, which had the highest growth rate at low nitrate concentrations (4 μmol. L^{-1}) (Wu et al. 1994).

The growth rates of *G. domingensis* were higher in treatments with N:P ratio of 100: 1 than 10: 1. These results differ from the data obtained by Mendes et al. (2012), which obtained the highest growth rate (6.38% d^{-1}) of the same species in treatments with 80 μmol

of nitrate and 8 μmol of phosphate, but the culture medium was synthetic differing from our experiments that were performed with seawater. Navarro & Robledo (1999) observed that the highest growth rates of *G. cornea* J. Agardh were at nitrate concentrations of 50 μmol L^{-1} in the N:P ratio of 10: 1. These results are similar to those described for wild-type and green stranis of *G. cornea* that presented higher growth rates in VSES/8 medium (8.4% day^{-1}) than in VSES/4 medium (7.1% d^{-1}) (Ferreira et al. 2006) and *G. lemaneiformis*, which showed a decrease in the growth rates with increasing nitrogen concentrations (Xu and Kaihong 2006).

The addition of nitrogen and phosphorus produced an increase in maximum photosynthesis and in photosynthesis efficiency in *Gracilaria domingensis,* however the effective quantum yield showed lower values with the addition of nutrients. Barufi et al. (2011) showed that in *G. tenuistipitata*, nitrate was necessary for growth and photosynthesis, and nitrogen limitation caused a decrease in effective quantum yield. Photosynthesis rates of *G. verrucosa* and *G. tikvahiae* were high in higher concentration of nutrients and therefore promoted absorption of nutrients by algae (Dawes and Koch, 1990), the same occurring in the maximum photosynthesis of *G. domingensis* in the high concentration of N: P 500.0 / 50.0 μM. High concentrations of nitrate and phosphate favored the increase in photosynthetic capacity in *G. lemaneiformis*, which improved markedly (Xu et al. 2010).

The total soluble protein contents of *G. domingensis* increased with higher nitrogen availability, indicating the role of these compounds as a nitrogen reserve and their allocation to growth under conditions of nutrient limitation (Andria et al. 1999). Similar results were observed in *G. domingensis*, where the interaction between high nutrient availability (VSES/2) and high irradiance stimulated the accumulation of total soluble protein (Ramlov et al. 2011). According to Naldi & Wheeler (2002), the increase of nitrate availability increased the concentration of total soluble proteins in *G. pacifica*. García-Sánchez (1993) showed that the total soluble proteins decreased in *G. tenuistipitata* with the reduction of nitrate in the culture medium. The increase of nitrate concentrations in growth medium has led to increased

protein / carbohydrate ratios in the apical segments of *G. verrucosa* and it may be suggested that the constituents of thallus, protein and carbohydrate can be used to evaluate the nutrient deficiency status of *G. verrucosa* (Bird, 1984).

Our results showed that the higher availability of nitrate and phosphate increased the concentrations of phycoerythrin and chlorophyll a in *G. domingensis*. Similar responses were observed in *G. tikvahiae* (Bird et al. 1982) and in color variants of *Gracilaria birdiae* (Costa 2005), who observed an increase in pigment contents, with the exception of allophycocyanin, in response to the increase of nitrate. In addition, high nitrate enrichment in *G. tenuistipitata* increased the content of photosynthetic pigments compared to low nitrate concentrations (Barufi et al. 2011).

Our results showed that *G. domingensis* presented high nitrate and phosphate uptake with the addition of these nutrients. Xu et al. (2010) showed that high phosphorus concentrations stimulated nitrogen uptake in *G. lemaneiformis.* Nitrate uptake by *G. domingensis* was higher than nutrient uptake by *G. foliifera* and *Agardhiella subulata* (Haines and Wheeler 1978). In the color strains of *Hypnea pseudomusciformis*, nitrate uptake increased with high nitrate concentrations, with uptake rate of 9 μmol g^{-1} FW d^{-1} in treatment with addition of 100 μmol nitrate (Martins 2007).

The carbohydrate concentration in *G. domingensis* did not differ significantly among treatments with addition of N:P in the ratios of 100: 1 and 10: 1. Similar results were observed by Navarro and Robledo (1999) in *G. cornea*, however, Bird et al. (1982) observed that the total soluble carbohydrate content increased with higher nitrate concentrations in *G. tikvahiae*. In relation to polysaccharides, the study with *G. lemaneiformis* showed that its cultivation should be done for two weeks in low proportion of N:P and high concentration of phosphorus to increase the quality of the polysaccharides (Xu and Kaihong 2006). In *G. bursa-pastoris*, starch and carbohydrate levels increased with higher nitrate levels (Korsen 2016).

Our study showed that nutrient concentrations did not interfere with thallus concentrations of carbon, and hydrogen, only increasing the concentration of nitrogen in the thallus. Peckol (1994) showed that in the area with higher concentration of nitrate and phosphate, *Gracilaria tikvahiae* had higher thallus nitrate concentrations than in the area with lower concentrations of these nutrients.

The high percentages of nitrogen and phosphorus removal reported by *G. domingensis* indicate their potential in removing nutrients from the marine environment as observed in *G. gracilis* (Martínez-Aragón et al. 2002) and *G. lemaneiformis* (Mao et al. 2009). Abreu et al. (2011) studied the nitrogen uptake responses of *G. vermiculophylla* and the results showed that high concentrations of nutrients also inhibited the absorption of nutrients. The same was reported for *G. verrucosa* and *G. tikvahiae*, where the highest efficiencies were in lower concentrations of nutrients (Du et al., 2013). *G. lemaneiformis* decreased the phosphate level in aquariums (Yang et al 2006). On the other hand, *G. domingensis* presented a high percentage of phosphate removal in the N:P treatment of 500.0 / 50.0 μmol L^{-1}. T However, the nutrient uptake tends to increase with the increase of nutrient concentrations, but this uptake depends on the thallus concentrations of these nutrients before the start of the experiment (Harrison and Hurd 2001, Pedersen et al. 2004, Runcie et al. 2004).

The high efficiency of *G. domingensis* in the removal of excess nutrients in the water indicates that this species has potential as a biofilter and could be cultivated in integrated multitrophic systems or at sea, reducing the nutrients of eutrophic marine environments.

ACKNOWLEDGMENTS

This research was supported by a scholarship provided by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, Brazil, proc. no. 2017/13259-3) to the first

author. This study is part of the thesis presented by the first author to the Graduate Program in Plant Biodiversity and Environment, Institute of Botany, São Paulo, Brazil.

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Capítulo 3

BIOCHEMICAL CHARACTERISTCS AND ACETYLCHORINESTERASE INHIBITORY ACTIVITY OF *GRACILARIA CAUDATA* AND *GRACILARIA DOMINGENSIS* (GRACILARIALES, RHODOPHYTA) FROM FIELD AND CULTURED IN BIOREACTORS

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ABSTRACT

Marine macroalgae are sources of compounds of commercial and biotechnological interests, and can be biofilters, since they assimilate nutrients from the medium in their metabolism. Species of *Gracilaria* Greville are exploited as raw materials for agar production, and studies on their development are required for the agar and new bioactive compounds. The present study aimed to evaluate the biochemical characteristcs and acetylchorinesterase inhibitory activity of extracts of *Gracilaria caudata* J. Agardh and *Gracilaria domingensis* (Kützing) Sonder ex Dickie from field in comparison to samples cultured in bioreactors. Bioreactor experiments were performed with the addition of nitrate and phosphate in seawater. The following variables were analyzed: growth rates, contents of total soluble proteins and pigments (chlorophyll *a*, phycoerythrin, phycocyanin and allophycocyanin), agar characteristics (yield, quantification of sulfate and 3,6-anhydrogalactose contents) and acetylcholinesterase enzyme inhibitory in extracts of seaweeds cultured in bioreactors and collected from field. *G. caudata* and *G. domingensis* showed an increase in the growth rate, in the contents of total soluble proteins and pigments with the addition of N:P, however *G. domingensis* had more efficiency in these parameters than *G. caudata*. The agar yield showed

higher values in the species collected in the field. Sulfate concentration decreased with the addition different concentration N:P in *G. caudata* and *G. domingensis* species comparing collected in the field, However *G. caudata* increased the concentration of 3,6 anhydrogalactose. The inhibiting activity of the enzyme acetylcholinesterase was observed only in methanolic extract in both species, collected in the field and cultured in bioreactors. *Gracilaria domingensis* had higher growth, higher concentration of proteins and pigments in treatments with addition of different N:P concentrations, but *G. caudata* showed higher agar quality in treatments with addition of N:P than the specimens collected in field, what indicated that *G. caudata* can be cultivated in multi-trophic aquaculture system under higher nutrient levels and produced high agar quality.

Keywords: Acetylcholinesterase inhibitory activity, agar yield, bioreactor cultivation, nitrate, phosphate

INTRODUCTION

Seaweeds contain biochemical substances that have biological activities, such as antibacterial, antifungal, anti-aging, antimalarial, anti-inflammatory, anticoagulants, antimitotic, antibiotic, anticancer, antioxidant and hypolipidemias, that are widely studied in different countries (Smit 2004, Chan et al. 2015, Agregan et al. 2017).

Oxygen, nitrogen and sulfur produce superoxide anion, hydroxyl radical, hydrogen peroxide and nitric oxide, and these compounds can cause oxidative stress and cell damage, leading to the appearance of diseases as diverse as cancer, diabetes, Alzheimer's and Parkinson's diseases (Yangthong 2009; Lü et al. 2010). The damage caused by reactive oxygen species is considered to be a contributing factor to several diseases, including Alzheimer's Disease (Houghton 2007), which is characterized by decreasing cholinergic function in the brain (Perry et al. 2003). This is a neurodegenerative disease associated with neurotransmitter deficiency in the brain, and the treatment is the restoration of cholinergic

function obtained by inhibiting the acetylcholinesterase enzyme (Francis et al., 1999; Trevisan et al., 2003; Nair & Hunter, 2004).

Studies developed with some species of *Gracilaria* have shown the potential in inhibiting the enzyme acetylcholinesterase, such as observed in *G. gracilis* (Stackhouse) Steentoft, L.M. Irvine & Farnham (Natarajan et al. 2009), *G. corticate* (J. Agardh) J. Agardh, *G. salicornia* (C. Agardh) E. Y. Dawson (Ghannadi 2013), and *Gracilaria* sp. (Bianco et al. 2015). In addition, the growing demand for functional food supplements required further studies on the nutritional properties of red algae, what were studied in *G. edulis* (S.G.Gemelin) P.C. Silva and *G.changii* (B.M. Xia & I.A. Abbott) I.A. Abbott, J.Zhang & B.M.Xia (Sakthivel & Pandima Devi 2015; Chan & Mantanjun 2017). The inhibitory action on the enzyme acetylcholinesterase was also observed in other seaweeds as *Gelidiella acerosa* (Forsskål) Feldmann & Hamel (Syad et al., 2012), *Laminaria japonica* Areschoug (Sevevirathne et al., 2011) and *Ochtodes secundiramea* (Montagne) M. Howe (Machado et al., 2015).

The objective of this study was to compare biochemical differences and acetylchorinesterase inhibitory activity in *Gracilaria caudata* and *G. domingensis* collected in the field and cultured in bioreactors with different nitrate and phosphate availabilities.

MATERIALS AND METHODS

Collection

Algal samples were collected during periods of low tides according data described in the Table 1. The collected material was cleaned and identified under microscope and stereomicroscope. Voucher specimens were deposited in the Herbarium of the Institute of Botany (SP), São Paulo, Brazil.

Table. 1. Collecting data of specimens of *Gracilaria caudata* and *G. domingensis*. Bioareactor = biomass produced by isolates cultured in bioreactors; Field = biomass collected directly from field conditions.

Species	Source of Biomass	Life History Phase	Collecting date and site
Gracilaria caudata J. Agardh	Bioreactor	Female Gametophyte	9 October 2006, Boa Viagem Beach, Recife 8° 5' 44.84'' S and 34° 53' 9.1'' W
	Field	Female Gametophyte	20 November 2018, Boa Viagem Beach, Recife 8° 5' 44.84'' S and 34° 53' 9.1'' W
Gracilaria domingensis (Kützing) Sonder ex Dickie	Bioreactor	Female Gametophyte	14 October 2008, Lagoinha Beach, Florianópolis 27° 23'21''S and 48° 25' 29''W
	Field	Tetrasporophytes	24 September 2018, Armação do Itapocoroi, Penha 26º 47' 20.6" S and 48º 37' 13.4" W

Unialgal cultures

Unialgal cultures were obtained by vegetative propagation of 10 mm of thallus segments. The culture medium selected comprised sterilized seawater (salinity of 30 - 31) enriched with von Stosch's solution at half strength (VSES/2) following Oliveira et al. (1995), modified with vitamin concentrations and reduced to a 50 % concentration (Yokoya 2000). The von Stosch´s solution had the following vitamins and concentrations: thiamin (100 µg L^{-1}), biotin (100 µg L^{-1}) and cyanocobalamin (1.0 µg L^{-1}). Renewal of the culture media was carried out every two weeks. Cultures were incubated at: 23 ± 1 ºC, photon flux densities of 60.0 - 70.0 µmol photons m^{-2} s^{-1} (as provided by cool-white fluorescent lamps), with a 14:10h light:dark cycle, without aeration. Irradiance was measured with a quantum photometer (LI-250, Li-Cor, Lincoln®, Nebraska, USA) equipped with underwater quantum sensor (LI-192 SA, Li-Cor).

Experimental design

The experiments were carried out in a bioreactor system (model TE-Bit-E3, Tecnal, Brazil) composed of 15 reaction vessels (volume 2.5 L) with temperature sensor (Tecnal), pH

(Metter Toledo) and oxygen (Metter Toledo). The vessels were connected to a control and data acquisition system (Tecbio-soft, Tecnal) of temperature (24 ºC ± 3.0 ºC, pH (7.9 ± 0.4) and oxygen dissolved in the culture medium (8.7 ± 0.6 mg. L^{-1}). The bioreactors and sensors were sterilized by autoclaving at 121ºC for 30 min before the beginning of the experiments. The experiments were carried out with a culture medium composed of sterile sea water enriched with 50% of the Von Stosch nutrient solution, which was prepared without the addition of $NaNO_3$ and $Na_2HPO_4.12H_2O$. The N:P concentrations tested were: 0.0, 125.0/1.25 and 250.0/2.5 µmol L^{-1}. The initial biomass provided by unialgal cultures was 1 g for 2 L of culture medium. Each treatment was tested with 3 simultaneous replicates (n=3). The medium was changed weekly and the experiment lasted 28 days. Aeration was 30 min on and 30 min off. The air was filtered and moistened before reaching the vases. The air outlet was made through a manifold with 15 outlets and the regulation of the air flow of each vessel was made with a rotameter.

Growth rates

Fresh biomass was recorded at weekly intervals before renewal of the media during four weeks. Daily growth rates were calculated using the following equation: $GR=[(Wt/Wi)^{1/t} - 1]\times100\%$, where Wt is the weight after t days, Wi is the initial weight, and t is the experimental period (Yong et al. 2013).

Total soluble protein contents

For total soluble protein analysis, 80 mg of algal fresh biomass for each replicate (n = 3) were ground with liquid nitrogen, and extractions were carried out at 4 °C using 0.2 M phosphate buffer (pH 8) containing 5 mM of ethylenediaminetetraacetic acid (EDTA) and 1 mM of dithiotreitol (DTT). Buffer was added in the proportion of 10 mL g^{-1} fresh biomass, and the homogenates were centrifuged at 12,000×g for 15 min. Total soluble protein contents

were determined according to Bradford (1976), using a Bio-Rad® protein assay kit and BSA as standard.

Pigment analyses

After four week culture period, the algal thallus were weighed (as above), and their culture medium was renewed. On the 32st day of experiment, samples from each treatment were weighed, frozen in liquid nitrogen and stored at -20^{o}C. The algal mass (75 mg fresh mass for each triplicate) was ground with liquid nitrogen and 1 ml of 50 mM (pH 5.5) phosphate buffer was added. Pigment extractions were carried out at 4^{o}C, in the dark. Crude extracts were centrifuged at 14,000×g for 20 min in order to obtain the phycobiliproteins, and chlorophyll-a was extracted after dissolving the pellet in 90% acetone, followed by centrifugation at 10,000×g for 15 min. Pigments were quantified using a spectrophotometer (Shimadzu®, UV 1800), and concentrations were calculated according to Kursar et al. (1983) for phycobiliproteins, and Jeffrey and Humphrey (1975) for chlorophyll *a*.

Agar extraction

The agar extractions were carried out with algae cultured in the bioreactors and collected in the field. The extraction of native agar was performed according to the method described by Marinho-Soriano et al. (1999). Samples were dried at 60 ° C for 48 hours. Then, they were washed in running water to remove salts and with 1% hypochlorite. Immediately after sanitization, an imbibition was made with distilled water in the proportion of 1 part of seaweed (approximately 1 g) and 30 of water, for 2 hours; the excess water was removed, and part of the seaweed was crushed. The seaweed was placed in a water bath at 100 ° C, for 2 hours, after the end of this time the seaweed was hot filtered, in nylon fabric, to facilitate the filtration; the filtrate was left to stand to gel for 24 hours; after this time, the filtrate was placed in a domestic freezer to freeze for another 24 hours; at the end of this time, the filtrate

was removed from the freezer and after thawing, the filtrate was placed in an air circulation oven, for 72 hours at 65 ° C, for the water to be removed.

Agar yield was determined using the equation: Agar yield % = agar dry weight (g) x 100 / seaweed dry weight (g).

Quantification of sulfate content

Sulfate content was quantified according to Saito (1997). Agar samples of approximately 30 ± 1 mg were moistened in 100 µL of 0.5 N HCl. The samples were hydrolyzed in boiling water for 2 h. The volume was made up with Milli-Q water to 10 ml. The samples were centrifuged at 12,000 rpm for 15 min at room temperature to remove suspended materials. 2 ml of the supernatant was dissolved in 18 ml of Milli-Q water and 2 ml of 0.5 N HCl. After adding HCl, the samples were slightly agitated, followed by the addition of 1 ml of barium chloride solution. gelatine. The samples were again stirred, leaving them at room temperature for 30 min. After this period, the absorbances of the samples were obtained in a spectrophotometer at 550 nm. From the absorbances, the amount of sulfate (%) was calculated using the calibration curve.

Quantification of 3,6-anhydrogalactose content

Content of 3,6-anhydrogalactose was quantified according to modified methods of Matsuhiro (1995) and Saito (1997). Approximately 24 ± 1 mg of agar (dry weight) was dissolved in 40 ml of Milli-Q water in an 80- water bath. 85ºC until complete dissolution (90 to 120 min). Samples were cooled in an ice-water bath and the volume was made up to 50 ml with Milli-Q water (working solution). Next, 10 mL of the working solution and 5 mL of Milli-Q water (sample dissolution) were mixed. In a Falcon tube, 2 ml of Milli-Q water were pipetted and added: 0.5 ml of 5% thymol, 5 ml of 0.5% ferric chloride and 2 ml of the sample dissolution. Samples were homogenized, heated in a water bath for 13 min at 80 ºC and

cooled in an ice bath with water. 10 mL of 98% ethanol was added to the samples. After these procedures, the samples were analyzed in a spectrophotometer at 635 nm and their absorbances were recorded to calculate the amount of 3,6-anhydrogalactose (%) using the calibration curve.

Acetylcholinesterase inhibitory activity of (AChEI)

Algal material and extraction

80 g (dry weight) of field collected samples and 15 g (dry weight of all treatments mixed, (0.0, 125.0/1.25 and 250/2.5 µmol L^{-1})) of bioreactor cultured samples were used for extractions in hexane, dichloromethane, ethyl acetate and methanol (20 ml of solvent per 1 g of seaweed, Machado et al. 2014). The material was maintained in a dark room at 20 °C for one day, and then the extracts were filtered through a Whatman Nº. 5 filter paper and concentrated by under low pressure and re-extract for 4 times. All chemicals used were of analytical grade (Merck, Darmstadt, Germany).

Qualitative evaluation of AChEI by autographic assay

The AChEI activities of hexane, dichloromethane, ethyl acetate and methanol crude extracts were detected by using a thin-layer chromatography (TLC) autographic assay as previously described (Marston et al. 2002). Aliquots of 100 µg of each dried seaweed extract and 0.3 µg of physostigmine (Sigma–Aldrich, used as positive control) were dissolved, spotted on TLC layers (Silica gel 60 F254, 10×10cm, layer thickness 0.2mm, E. Merck, Germany), which were developed with mobile phase hexane:ethyl acetate:methanol (2:7:1 v/v/v), and then dried. Next, the plates were sprayed with the enzyme solution (6.66 U/ml) (Electric eel AChE type V, product no. C 2888, 1000 U – Sigma–Aldrich), thoroughly dried, and incubated in a humid atmosphere at 37 °C for 20 min. Afterwards, the plates were sprayed with 0.25% of 1- naphthylacetate in ethanol (5 ml) plus 0.25% of aqueous Fast Blue

B salt solution (20 ml). The spots corresponding to potential of acetylcholinesterase inhibitors were identified as clear zones against a purple background.

Statistical analyses

Results were analyzed by two-way of variance (ANOVA) followed by the Student-Newman-Keuls *a posteriori* test for multiple comparisons so as to distinguish significant differences (p <0.05). Statistica 10.0 software was used to perform all statistical analyses.

RESULTS

Growth rates increased with the addition of N:P in *G. caudata* and *G. domingensis* when compared to control (Fig. 1) (F = 411.73 p = 0.00), and higher values were observed in treatments with concentrations 250.0/2.5 μmol L^{-1} of N:P in *G. caudata* and *G. domingensis*. The higher growth rates were observed in the *G. domingenis* (F = 75.20 p = 0.00).

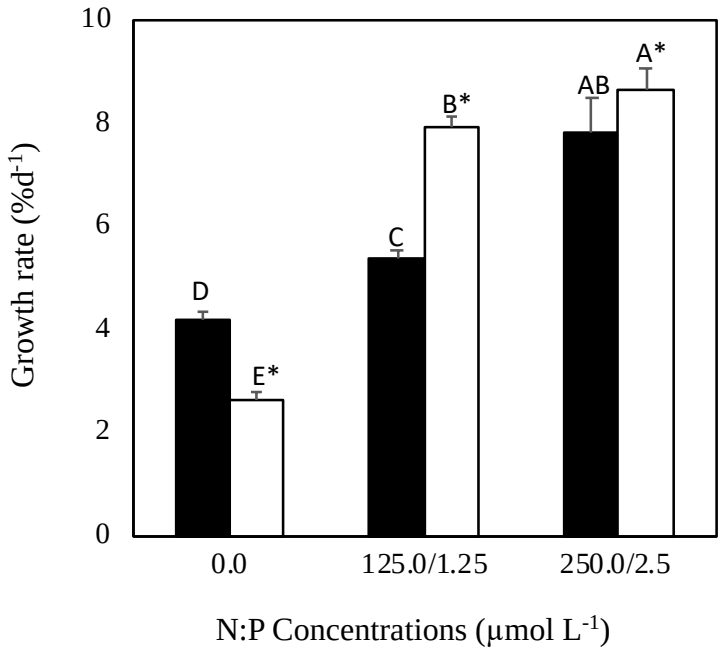


Fig. 1. Growth rates of *Gracilaria caudata* (black columns) and *Gracilaria domingensis* (white columns) cultured in bioreactor vessels with different concentrations of nitrate and phosphate, for 28 days. Mean ± SD (n = 4). Treatments with distinct letters were significantly different from each other, according to the Student-Newman-Keuls comparison test (p <0.05). Asterisks indicate significant differences between species.

The content of total soluble proteins of *G. domingensis* and *G. caudata* were higher in all treatments with the addition of N:P (Fig. 2) (F = 58.23 *p* = 0.00). The higher concentrations were observed in treatments with concentrations 250.0/2.5 μmol L^{-1} of N:P in *G. caudata* and 125.1.25 and 250.0/2.5 μmol L^{-1} of N:P in *G. domingensis*. The highest content of total soluble proteins were observed in the *G. caudta* (F = 14.16 *p* = 0.00).

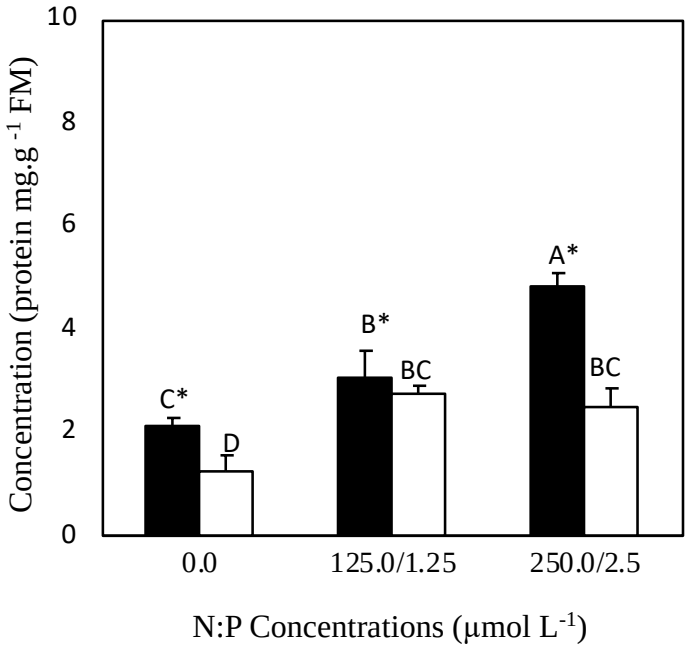


Fig. 2. Content of total soluble protein in *Gracilaria caudata* (black columns) and Gracilaria domingensis (white columns) cultured in bioreactor vessels with different concentrations of nitrate and phosphate, for 32 days. Mean ± SD (n = 3). Treatments with distinct letters were significantly different from each other, according to the Student-Newman-Keuls comparison test (p <0.05).

The phycoerythrin contents of *G. caudata* and *G. domingensis* were higher for all treatments with the addition of N:P when compared to the control (F = 108.45 *p* = 0.00) (Fig. 3. A), but there were not differences between species (F = 3.43 *p* = 0.054). Phycocyanin and allophycocyanin concentrations were also higher in concentrations with the addition of N:P when compared to controls (F = 155.02, *p* = 0.00 and F = 48.04 *p* = 0.00, respectively), and the concentrations of phycocyanin and allophycocyanin were higher in *G. domingensis* when compared to *G. caudata* (F =11.57 *p* = 0.00) and (F =26.14 *p* = 0.00) (Fig. 3 B and C).

Chlorophyll *a* contents were higher in all treatments with the addition of N:P when compared to the control in *G. caudata* and *G. domingensis* (Fig. 3 D) (F = 18.93 p = 0.00), but there were not differences between species (F = 15.70 p = 0.79).

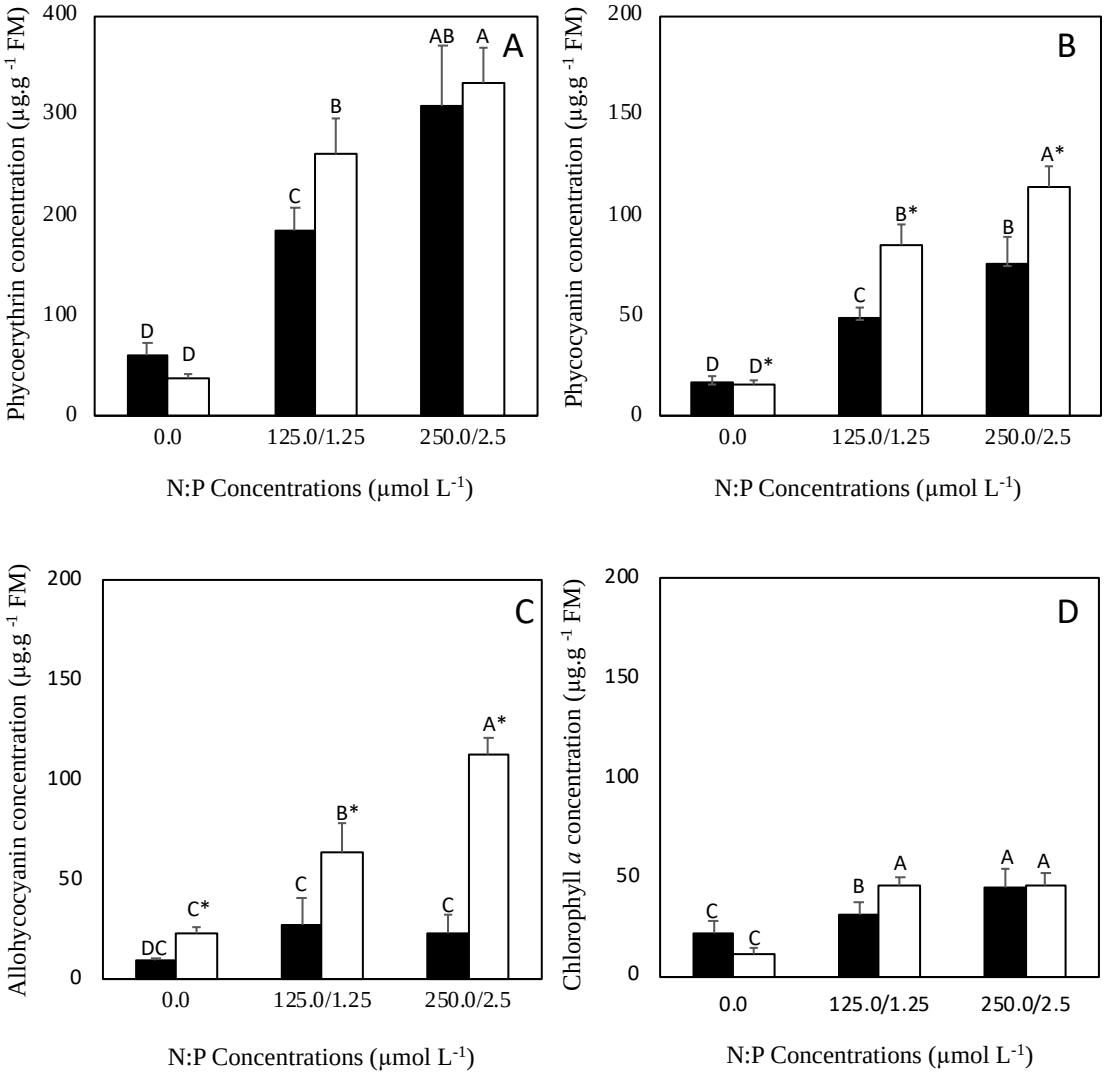


Fig. 3. Concentration of phycoerythrin (A), phycocyanin (B), allophicocyanin (C) and chlorophyll a (D) of *Gracilaria caudata* (black columns) and *Gracilaria domingensis* (white columns) cultured in bioreactor vessels with different concentrations of nitrate and phosphate, for 32 days. Mean ± SD (n = 3). Treatments with distinct letters were significantly different from each other, according to the Student-Newman-Keuls comparison test (p <0.05). Asterisks indicate significant differences between N: P ratios.

Agar yield did not increase in the tested N:P concentrations (F = 9.33 p = 0.00, Fig. 4). Treatments without addition of N:P and 125.0 / 1.25 µmol L^{-1} were lower in treatments when

compared to the agar yield of *G. domingensis* and *G. caudata* collected in the field and *G. domingensis* cultivated in bioreactor at N:P concentration of 250.0/2.5 μmol L^{-1} (Fig. 4). Agar yields did not differ between species (F = 0.06 *p* = 0.93).

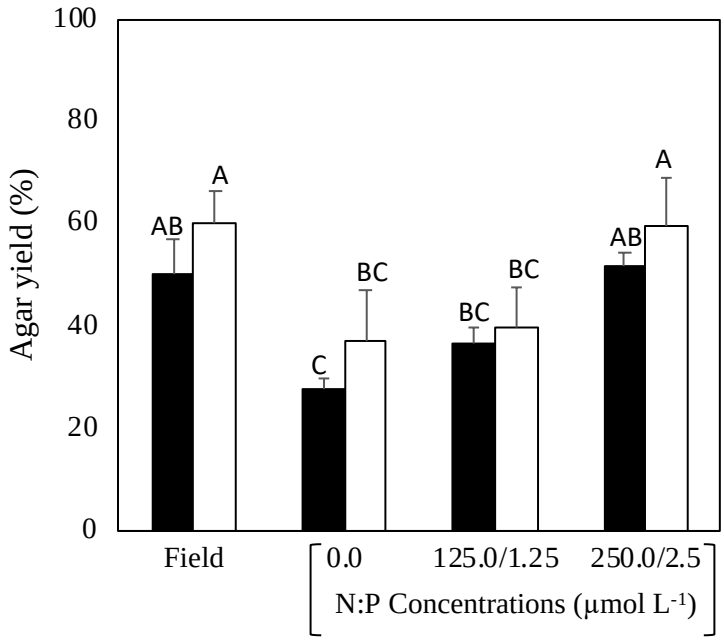


Fig. 4. Agar yields of *Gracilaria caudata* (black columns) and *Gracilaria domingensis* (white columns) in field collected specimens and cultured in bioreactor vessels with different concentrations of nitrate and phosphate, for 32 days. Mean ± SD (n = 3). Treatments with distinct letters were significantly different from each other, according to the Student-Newman-Keuls comparison test (*p* <0.05).

Contents of sulfate in *G. domingensis* and *G. caudata* were higher in agar of specimens collected in the field than those cultured in bioreactors (Fig. 5 A) (F = 3.86 *p* = 0.00). *G. domingensis* showed higher sulfate concentrations than *G. caudata* (F = 28.51 *p* = 0.00). The amount of 3,6 anhydrogalactose on the agar was higher in *G. domingensis* collected in the field than those cultured in the bioreactors, but the addition of N:P increased the concentration in *G. caudata* and decreased in *G. domingensis* (F = 53.41 *p* = 0.00). *G. caudata* showed highest concentrations of 3,6 anhydrogalatose when compared with *G. domingensis* (F = 30.81 *p* = 0.00, Fig. 5B).

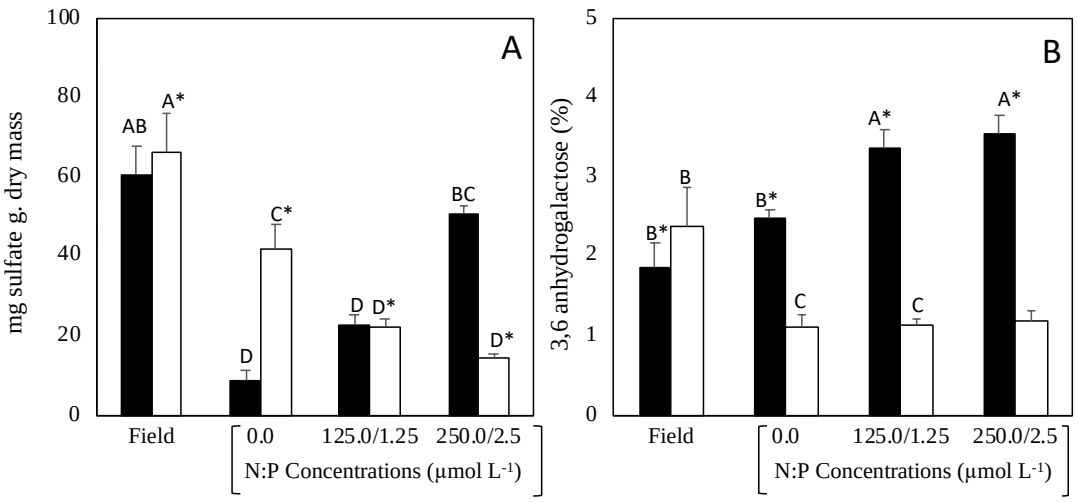
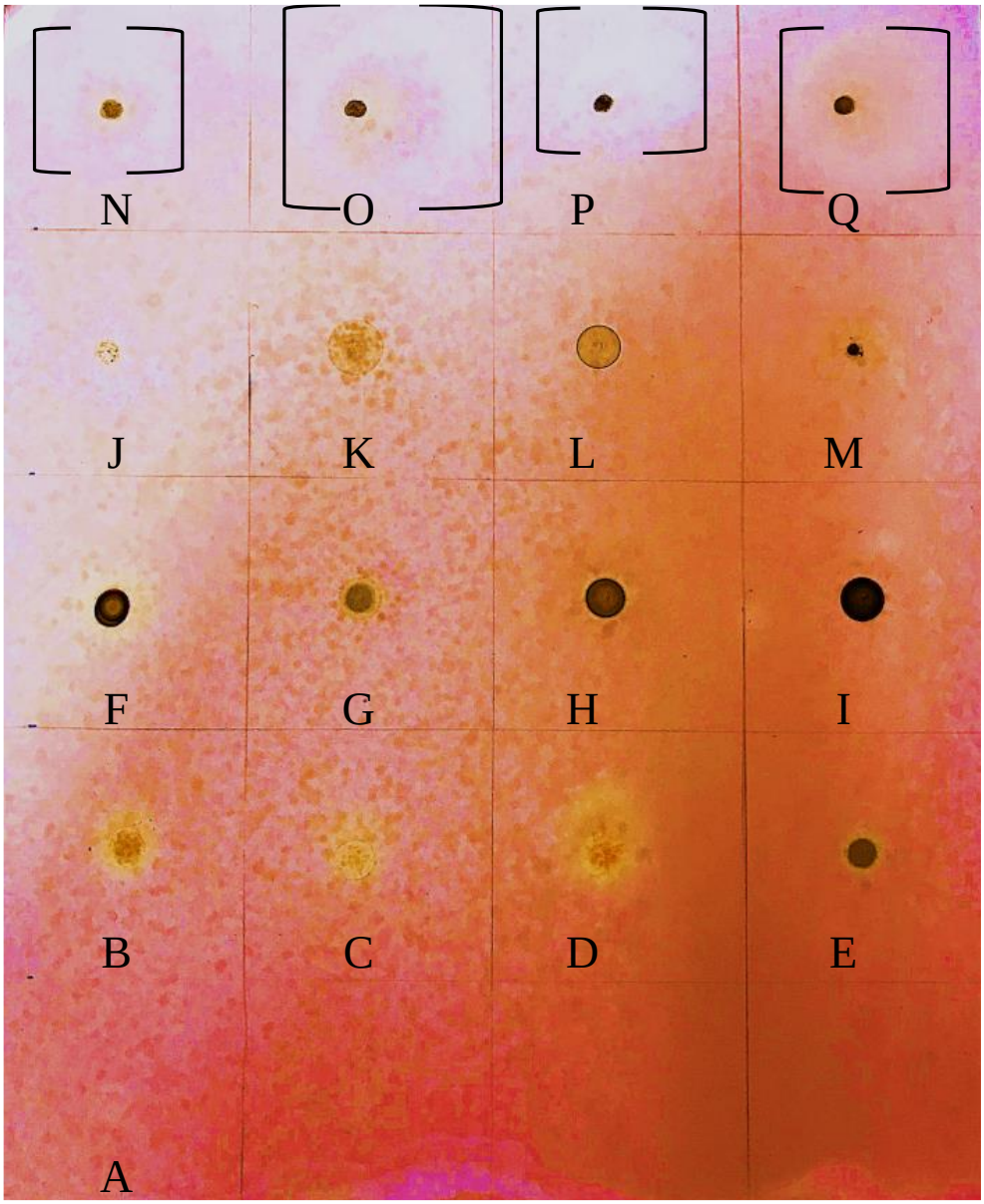


Fig. 5. Sulfate content (A) and 3,6 anhydrogalactose (B) of *Gracilaria caudata* (black columns) and *Gracilaria domingensis* (white columns) in field collected specimens and cultured in bioreactor vessels with different concentrations of nitrate and phosphate, for 32 days. Mean ± SD (n = 3). Treatments with distinct letters were significantly different from each other, according to the Student-Newman-Keuls comparison test (p <0.05).

The Inhibitory action on the enzyme acetylcholinesterase were observed only in methanol extracts, that cholinesterase inhibitors were identified as clear zones against a white background indicated in the Fig. 6. N, O, P and Q in the species of *G caudata* and *G domingensis*, either for field or cultivated in bioreactors (Fig. 6).



Solvents	Hexane		Dichloromethane		Ethyl acetate		Methanol	
Source of biomass	1	2	1	2	1	2	1	2
G. caudata	B	C	F	G	J	K	N	O
G. domingensis	D	E	H	I	L	M	P	Q

Fig. 6. Acetylcholinesterase inhibitory activity of extracts of *Gracilaria caudata* and *G. domingensis*. Source of biomass: 1. Biomass cultured in bioreactor in the treatment with N: P 0.0, 125.0/1.25 and 250.0/2.5; 2. Biomass collected in field conditions.

DISCUSSION

The growth rates of *Gracilaria caudata* and *G. domingensis* increased with the addition of N:P and showed values of approximately 8% d^{-1} for both species, and these values are higher than those of Mendes et al. (2012), where after optimizing the conditions of temperature, light and nutrients for cultivation in synthetic seawater of G. *domingensis*, observed that the highest growth rate was 6.4% d^{-1}. According to Martins et al. (2016), the growth rates of *Dictyota menstrualis* grown in bioreactors with VSES/2 and with added nutrients reached 16% d^{-1}, a higher value than those obtained in the present study. When compared to the growth rates among other species of *Gracilaria*, these results are similar to those described for the wild and green strains of *G. cornea* that showed higher growth rates in VSES 12.5% (8.4% day^{-1}) and VSES 25% (7.1% day^{-1}) (Ferreira et al. 2006).

The high availability of nitrate and phosphate increased the pigment concentrations in *Gracilaria domingensis* and *G. caudata*. There was an increase in the concentrations of phycoerythrin, phycocyanin, allophycocyanin and chlorophyll *a*. Similar responses were observed in *G. tikvahiae* (Bird et al. 1982) and in color variants of *G. birdiae* (Costa 2005), who observed an increase in pigment content, with the exception of allophycocyanin in response to the nitrate increase. In addition, enrichment with a high nitrate content in *G. tenuistipitata*, increased the content of photosynthetic pigments compared to the low nitrate concentrations (Barufi et al. 2011).

The levels of total soluble proteins of *Gracilaria domingensis* and *G. caudata* increased with the high availability of nitrogen, indicating the role of these compounds as a nitrogen reserve and their allocation for growth under conditions of nutrient limitation (Andria et al., 1999). Similar results were observed in *G. domingensis*, where the interaction between high availability of nutrients (50% VSES) and high irradiance stimulated the accumulation of total soluble protein (Ramlov 2011). According to Naldi & Wheeler (2002), the increase of nitrate concentration in *G. pacifica* increased the concentration of total soluble proteins. The increase in nitrate concentrations in growth medium led to increased protein /

carbohydrate ratios in the apical segments of *G. verrucosa* and it can be suggested that the constituents of the stem, protein and carbohydrate, can be used to assess the state of nutrient deficiency. *G. verrucosa* (Bird, 1984). García-Sánchez (1993) showed that total soluble proteins decreased in *G. tenuistipitata* with the reduction of nitrate in the culture medium.

The agar yields of *Gracilaria domingensis* and *G.caudata* were higher in specimens collected in the field and in the highest N:P concentration tested in bioreactors (250.0 / 2.5 μmol L^{-1}). On the other hand, the agar yield of *G. domingensis* was higher than the agar yield of *G. caudata*. Souza (2015) showed that at different concentrations of 2-isopentenyladenine and benzylaminopurine in *G. caudata* grown in bioreactors, they had no effect on the agar yield and quality. The present study also showed higher values of agar yield. Similar results were observed by Yoshimura (2006), who observed that the agar yield of *G. caudata* is low (20%), even when the extractions were made with different alkaline treatments. However, the agar yield of *G. domingensis* was higher (35%), and this value increased to 50% when extractions were made with alkaline treatments (Yoshimura, 2006). The agar yields of *G. caudata* and *G. domingensis* cultivated in different concentrations of N: P were higher than those found in different treatments with phytoregulators in bioreactors that varied from 38 to 40% (Souza, 2015) and higher than those obtained by Yohimura (2006). Marinho-Soriano et al. (2001) observed that the yield of *G. cervicornis* agar was 11 to 20%, lower than the values obtained in the present study with *G. caudata*. The agar yields of *G. caudata* were higher than those obtained with alkaline treatments in other species of *Gracilaria* (Levy et al., 1990; Hurtado-Ponce, 1992, Orosco et al., 1992; Rebello et al., 1997). High values of agar yield, although lower than those found in the present study, were obtained in species of *Gracilaria* by Durairatnam et al. (1990), Santos (2011) for *G. domingensis*, and Yenigul (1993) for *G. verrucosa*.

The different concentrations of N:P caused a decrease in the 3,6 anhydrogalactose and sulfate contents in agar produced by *Gracilaria domingensis* when compared with those of

field collected specimens of *G. caudata*, which showed higher sulfate concentration when cultivated with different N:P concentrations in the bioreactor and higher 3,6 anhydrogalactose concentration in field collected specimens or cultured in higher N:P concentration. According to Yoshimura (2006), the commercially accepted 3,6 anhydrogalactose levels are above 15%. The high values of 3,6 anhydrogalactose obtained in the present study show that the agar quality of *G. caudata* is much higher than that determined for commercial use.

Yoshimra (2006) obtained agar with 3,6 anhydrogalactose levels above 14% in *G. caudata* collected in Enseada da Armação Itapocoroy (Penha, Santa Catarina). The levels presented in the present study were above 50% of the agar yield. The higher content of 3.6 anhydrogalactose gives higher gelling property and better quality to the agar (Matulewicz, 1996) increasing its market value. However, Yoshimura (2006) observed that the alkaline treatments produced percentages of 3,6 anhydrogalactose from 9 to 15%, values that did not make them marketable.

The results of inhibition of anticholinesterase enzyme proved efficient in the methanol extracts, however there were no differences between the species *Gracilaria caudata* and *G. domigensis*, the same occurred between species harvested in the field and grown in bioreactors. Gracilaria edulis showed inhibitory activity for the anticholinesterase enzyme, with low values (Suganthy et al. 2010). Bianco et al. 2015 studied several species with anti-cholinesterase enzyme inhibiting activity, *Gracilaria sp*. showed low activity in this study. *Gracilaria gracilis* and *Gracilaria edulis* showed no inhibitory activity to Anticholinesterase enzyme (Natarajan et al. 2009). The species *G. caudata* and *G. domingensis* despite showing activity in the inhibition of the anticholinesterase enzyme, as well as other studies with other species of *Gracilaria* did not show a strong activity when compared to other drugs.

Considering the our experimental conditions, *Gracilaria domingensis* higher growth, higher concentration of proteins and pigments in treatments with addition of different N:P

concentrations, but *G. caudata* showed higher agar quality in treatments with addition of N:P than the specimens collected in field, what indicated that *G. caudata* can be cultivated in multi-trophic aquaculture system under higher nutrient levels and produced high agar quality.

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Capítulo 4

CHARACTERIZATION OF PLANT GROWTH REGULATORS IN TROPICAL AND ANTARCTIC SEAWEEDS: BIOTECHNOLOGICAL POTENTIAL FOR AGRICULTURE

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ABSTRACT

Seaweed products exhibit activities that stimulate growth and the use of seaweed formulations as biostimulants have been used in agricultural production. Phytohormones are physiologically important small molecules that play essential roles in intricate signaling networks that regulate diverse processes in plants. Recent results suggest that measuring phytohormone levels in various seaweed products could be a very potent tool for plant biotechnology. The aim of this study was simultaneous targeted profiling of phytohormone-related analyses from minute amounts of dry seaweed material characterizing plant growth regulators (PGRs) of a total of 11 species: five species collected from Brazil (four *Gracilaria* species and the brown alga *Sargassum vulgare*), and six species collected from Antarctica (five brown algae and one green alga). The methodologies used for identification and quantification were rapid and nonselective extraction, fast sample purification, and extremely sensitive ultra-high-performance liquid chromatography-tandem mass spectrometry enable

concurrent quantification of uxins, Abscisic acid, and Jasmonates. In the present study, six auxins (free and conjugated forms of IAA) were analyzed, and three forms were detected: free IAA, 2-oxindole-3-acetic acid (oxIAA) and indole-3-acetyl glutamic acid (IAAGlu). Higher free IAA concentrations were observed in *Gracilaria caudata*, *G. domingensis* and *Phaeurus antarcticus*. Abscisic acid (ABA) was detected in the five seaweeds from Brazil, and the highest concentration was detected in *G. caudata*. However, all samples of Antarctic species showed concentrations below the limit of detection. Thirteen jasmonates derivatives were analyzed, but only three were detected in seaweed samples: *cis*OPDA, 9,10-dihydrojasmonic acid D6-JA and Jasmonic acid, and higher concentrations were observed in *Sargassum vulgare, Gracilaria sp.* and *S. vulgare*, respectively. Jasmonates are lipid-derived compounds that act as signals in plant stress responses and developmental processes, and the present study is the first to report Jasmonates in Rhodophyta. Moreover, this is the first study to describe the endogenous phytohormone profile of Antarctic seaweeds. A comparison among tropical and Antarctic seaweeds reveals that the occurrence of ABA in tropical seaweeds and not in Antarctic seaweeds may be related to photoperiod responses. Keywords: Antarctic Seaweeds, Biostimulant, Biotechnology, Plant Growth Regulators, Tropical seaweeds.

INTRODUCTION

Seaweed have developed an important role in coastal communities over the centuries, their cultivation is growing worldwide, and an economic production of macroalgae has been practically the same since the early 1980s (Gachon et al. 2010). Today, seaweeds are used in a multitude of applications expanding global industries based on hydrocolloids, cosmetics and food supplements, and also as a potential source of biofuel, with 32 countries actively cultivating seaweed for various purposes (Monagail et al. 2017).

Part of seaweed production is used for nutritional supplements such as biostimulants or biofertilizers to increase plant growth and yield and are available for use in agriculture and

horticulture. Seaweed products exhibit activities that stimulate growth, and the use of seaweed formulations as biostimulants in agricultural production is well established (Khan et al. 2009). The use of biostimulants has grown dramatically in the last decade and expects to reach $ 2 billion by 2018 (Saa-Silva et al. 2013, Calvo et al. 2014).

A commercial extract of the North Atlantic seaweed *Ascophyllum nodosum* (Linnaeus) Le Jolis (Phaeophyceae), called "AMPEP" (*Ascophyllum* / Acadian powdered seaweed extract) was applied to the red algae carrying carrageenan *Kappaphycus alvarezii* (Doty) Doty ex PC Silva. AMPEP was found to stimulate *Kappaphycus* growth and carrageenan production (Hurtado et al. 2009; Loureiro et al. 2014) and to improve the production of new and improved *Kappaphycus* explants for a commercial nursery (Yunque et al. 2011; Tibubos et al. 2017; Ali et al. 2018a). AMPEP may also have a "vaccine-like" effect on *Kappaphycus alvarezii* seedlings (Loureiro et al. 2012), while reducing epim and endophytic loads (Borlongan et al. 2011; Ali et al. 2018b).

The detection of endogenous cytokinins has become more sensitive, allowing detection in both macroalgae (Stirk et al. 2003), based on the technique of quantification of cytokinins described by Nóvak et al. (2003). Stirk et al. (2003) studied 31 species of marine macroalgae from the coast of South Africa and determined the concentration of 10 different compounds derived from zeatin, 3 different compounds derived from isopentenyladenine and 6 types of aromatic cytokinins, including benzylaminopurine. Yokoya et al. (2010) studied the endogenous cytokinins and were quantified in 11 red algae collected in the Brazilian coast. Among the identified cytokinins are the isoprenoid (2-isopentenyladenine, cis-Zeatin, and trans-Zeatin) and aromatic cytokinins (benzylaminopurine, ortho and meta-Topolines).

Examples of cytokinins occurring in seaweeds include zeatin detected in *Macrocystis pyrifera* (De Nys et al. 1990) and *Valoniopsis pachynema* (Farooqi et al. 1990), cZ and isopentenyladenosine (iPR) found in *Sargassum muticum* (Zhang et al. 1991) and *Laminaria japonica* (Duan et al. 1995), and iPR in *Porphyra perforata* (Zhang et al. 1993). Nineteen

cytokinins, including both isoprenoid and aromatic groups, were identified in five Chlorophyta, seven Phaeophyta, and 19 Rhodophyta species from South Africa (Stirk et al. 2003).

IAA has been identified in numerous seaweeds, including *Ascophyllum nodosum* (Kingman and Moore 1982, Sanderson et al. 1987), *Caulerpa paspaloides* (Jacobs et al. 1985), *Ecklonia maxima* (Crouch et al. 1992), *Porphyra perforata* (Zhang et al. 1993), and *Undaria pinnatifida* (Abe et al. 1972). Other indole derivatives, such as indole 3 carboxylic acid (ICA), have been identified in *Botryocladia leptopoda* (Bano et al. 1987), *Prionitis lanceolata* (Bernart and Gerwick 1990), *Ecklonia maxima* (Crouch et al. 1992), and *Undaria pinnatifida* (Abe et al. 1972).

The objective of the present study was characterizing the phyohormone profile of seaweed species from Brazil and Antarctica.

MATERIALS AND METHODS

Collection and processing of algal samples

The algal samples were collected during periods of low tides. In the Antarctic region, samples were collected South Shetland Islands. From Brazil, the species were collected at Mãe Luiza beach in Rio Grande do Norte state, Brazil. The collected material was cleaned and identified under microscope and stereomicroscope. Voucher specimens were deposited in the Herbarium of the Institute of Botany (SP), São Paulo, Brazil.

Table. 1. Brazilian and Antarctic seaweeds collected in this study.

Species	Collecting date and site
Brazilian seaweeds	
Sargassum vulgare C. Agardh *Gracilaira birdiae* E.M. Plastino & E.C. Oliveira *Graciaria caudata* J. Agardh *Gracilaria domingensis* (Kützing) Sonder ex Dickie *Gracilaria* sp.	07 December 2018, Praia Mãe Luiza, Natal, Rio Grande do Norte state,5°47'53.9" S 35°10'50.3" W
Antarctic Seaweeds	
Prasiola crispa (Lightfoot) Kützing	07 January 2017, Esperanza Base, Antarctic, 63°23'47.0" S 56°59'38.2" W
Adenocystis utricularis (Bory) Skottsberg	11 December 2017, Deception Island, Antarctic, 62°56'00.9"S 60°32'31.0"W
Ascoseira mirabilis Skottsberg *Desmarestia antarctica* R.L. Moe & P.C Silva *Phaeurus antarticus* Skottsberg	26 January 2017, Half Moon Island, Antarctic, 62°35'13.5" S 59°55'12.4" W
Cystophaera jacquinotii (Montagne) Skottsberg	30 January 2017, Nelson Island, Antarctic, 62°16'03.7"S 59°04'07.0"W

Identification and quantification cytokinins

The procedure used for cytokinins purification was a modification of the method described by Šimura et al. (2018). Samples (2 mg dry weight, n = 4) weight into 2 ml plastic micro tubes (Eppendorf, Germany). The samples were extracted in 0.5 mL modified Bielski buffer (187.5 mL MeOH, 50 mL water, 12.5 mL HCOOH) and 0.2 μl Internal Standards Olchemim Ltd., Olomouc, Czech Republic), after using a MM 301 vibration mill (Retsch GmbH & Co. KG, Haan, Germany) operating at a frequency of 27 Hz for 5 min. Samples were sonicated for 3 min at 4°C using a Transsonic T310 ultrasonicator with an ice block-filled bathtub (Elma GmbH & Co KG, Singen, Germany) and subsequently extracted using a Stuart SB3 benchtop laboratory rotator (Bibby Scientific Ltd., Staffordshire, UK) for 30 min at 15 rpm and 4°C. After centrifugation (10 min, 20,000 rpm, 4°C; Beckman Avanti™ 30), the supernatant was transferred to clean plastic microtubes and added Add 2.5 mL 1M HCOOH. The samples were purified with cartridges Oasis®MCX, 30 mg/1mL (obtained from Waters Co. (Milford, MA, USA) that was be washed with 1 ml of 100% MeOH and 1 ml of

deionized water, then equilibrated with 50% aqueous ACN (vol/vol). After loading a sample (supernatant obtained following the procedure described above), the flow-through fraction was collected in a glass tube (Fisherbrand™). The cartridge was rinsed with 1 ml of 80% MeOH and this fraction was collected in the same glass tube as the flow-through fraction. After this single step SPE, the samples were evaporated to dryness under a gentle stream of nitrogen using a TurboVap® LV evaporation system (Caliper Life Sciences, Hopkinton, MA, USA) and were stored at -20°C until analysis. For UHPLC–ESI–MS/MS analysis, the samples were dissolved in 40 µl of 30% ACN (vol/vol) and transferred to insert-equipped vials, then 20 µl portions of each sample was injected (in two 10-µl injections) into the UHPLC-ESI-MS/MS system.

Identification and quantification of auxins

The procedure used for IAA and conjugates purification consisted put 2 mg dry weight, (n = 5) into 2 ml plastic micro tubes (Eppendorf, Germany). were extracted in cold phosphate buffer (50 mM PBS; pH 7.0) containing 0.02% sodium diethyldithiocarbamate and a mixture of 15N／2 H5-labeled internal standards (Olchemim Ltd.). After centrifugation (10 min, 20,000 rpm, 4°C; Beckman Avanti™ 30), the supernatants were acidified with 1M HCl (pH 2.7) and purified using Oasis HBL 30 mg (Waters Oasis HBL 30 mg/1cc). The metabolites were eluted from the with 2 mL of 80% acidified methanol and evaporated to dryness in vacuum. The eluents were dried and reconstituted in 30 mL of 25% MeOH (10 mM HCOOH) for HPLC–MS quantification. Analysis was performed by tandem mass spectrometry (MS／MS) after HPLC separation (Acquity, Waters) as described by Pěnčík et al. (2009).

Identification and quantification of Jasmonates and ABA

Samples (2 mg dry weight of each sample, n = 4) were homogenized and extracted with Add 1 mL of extraction solution pre-cooled to 20 ºC, 10% MeOH with 10 µL IS (0.1

pmol D2-JAILe, 20 pmol D4-SA, 10 pmol D6-JA, D5-OPDA, D6-ABA, D5-AIA). Sonicated the samples for 5 minutes in laboratory ultrasonic bath. after using a MM 301 vibration mill (Retsch GmbH & Co. KG, Haan, Germany) operating at a frequency of 27 Hz for 5 min. Samples were sonicated for 3 min at 4°C using a Transsonic T310 ultrasonicator with an ice block-filled bathtub (Elma GmbH & Co KG, Singen, Germany) and subsequently extracted using a Stuart SB3 benchtop laboratory rotator (Bibby Scientific Ltd., Staffordshire, UK) for 30 minutes at 15 rpm and 4°C. After centrifugation (10 min, 20,000 rpm, 4°C; Beckman Avanti™ 30) supernatant were transferred to glass tubes and evaporate crude extract to dryness *in vacuo*.

The samples were purified with cartridges OASIS® HBL, cartridge 30 mg/1mL (obtained from Waters Co. (Milford, MA, USA) that was be washed with 2 mL of 80% MeOH and washed with distillated water. After loading a sample (supernatant obtained following the procedure described above), the flow-through fraction was collected in a glass tube (Fisherbrand™). The cartridge was rinsed with 3 ml of 80% MeOH and this fraction was collected in the same glass tube as the flow-through fraction. After this single step SPE, the samples were evaporated to dryness under a gentle stream of nitrogen using a TurboVap® LV evaporation system (Caliper Life Sciences, Hopkinton, MA, USA) and were stored at -20°C until analysis.

Identification and quantification of Gibberellins

Samples (10 mg dry weight of each sample, n = 5) were homogenized and extracted with Add 1 mL of extraction solution pre-cooled to -20 ^{o}C, 80% acetonitrile (ACN) + 5% formic acid and 45 μl of IS of internal standard mixture. Sonicate the samples for 5 minutes in laboratory ultrasonic bath. after using a MM 301 vibration mill (Retsch GmbH & Co. KG, Haan, Germany) operating at a frequency of 27 Hz for 5 min. Samples were sonicated for 3 min at 4°C using a Transsonic T310 ultrasonicator with an ice block-filled bathtub (Elma

GmbH & Co KG, Singen, Germany) and subsequently extracted using a Stuart SB3 benchtop laboratory rotator (Bibby Scientific Ltd., Staffordshire, UK) for 24 hours at 15 rpm and 4°C. After centrifugation (10 min, 20,000 rpm, 4°C; Beckman Avanti™ 30) re-extracted samples and the supernatant were transferred to glass tubes and evaporate crude extract to dryness *in vacuo*.

The samples were purified with cartridges 60 mg OASIS® MAX anion exchanger (obtained from Waters Co. (Milford, MA, USA) that was be washed with 3 mL of 100% MeOH and equilibrated with 25 mM-NH_4HCO_3. After loading a sample (supernatant obtained following the procedure described above), the flow-through fraction was collected in a glass tube (Fisherbrand™). The cartridge was rinsed with 3 ml of acetonitrile (ACN) and this fraction was collected in the same glass tube as the flow-through fraction. After this single step SPE, the samples were evaporated to dryness under a gentle stream of nitrogen using a TurboVap® LV evaporation system (Caliper Life Sciences, Hopkinton, MA, USA) and were stored at -20°C until analysis.

UHPLC-ESI-MS/MS

Targeted compounds were analyzed using an Acquity UPLC® I-Class System equipped with a Binary Solvent Manager, a Sample Manager with Flow-Through Needle, and an Acquity UPLC® CSHTM C18 RP column (150 x 2.1 mm, particle size of 1.7 µm) coupled to a triple quadrupole mass spectrometer Xevo® TQ-S MS, all from Waters (Manchester, UK). The mobile UPLC phase was consisted of binary gradients of ACN with 0.01% (vol/vol) FA (A) and 0.01% (vol/vol) aqueous FA (B), flowing at 0.5 ml min^{-1}, which depended on the ESI mode. MassLynxTM software (version 4.1, Waters, Milford, MA, USA) was used to control the instrument and to acquire and process the MS data.

RESULTS

Endogenous Jasmonates

The main Jasmonates present in some species analyzed in the present study were *cis*OPDA, ranging from 734.1 to 2,430.7 pmol g^{-1} DW in *Sargassum vulgare, Prasiola crispa, Desmarestia antarctica, Phaeurus antarcticus* from Antarctica (Table 2). The highest value was detected in *Sargassum vulgare* and the lower was in *Prasiola crispa*. The compound *cis*OPDA was not detected in the four species of *Gracilaria*, *Adenocystis utricularis* and *Ascoseira mirabilis*.

9,10-dihydrojasmonic acid D6-JA, ranging from 209.8 to 1,150.4 pmol g^{-1} DW, only in *Gracilaria caudata*, *Gracilaria domingenis* and *Gracilaria* sp., and the highest value was observed in *Gracilaria* sp. and the lower was in *Gracilaria caudata*, *Gracilaria birdiae*, *Sargassum vulgare* and concentrations below the detection limit were observed in all of species collected in Antarctica (Table 2).

Jasmonic acid concentrations varied from 11.5 to 32.3 pmol g^{-1} DW in *Gracilaria domingensis*, *Gracilaria* sp., *Sargassum vulgare*, *Cystosphaera jacquinotii* and *Desmarestia antarctica* (Table 2). *Sargassum vulgare* showed the highest value and *Gracilaria* sp. the lower. Jasmonic acid concentrations were below the detection limit in*Gracilaria birdiae, Gracilaria caudata*, *Prasiola crispa*, *Adenocystis utricularis*, *Ascoseira mirabilis* and *Phaeurus antarcticus* (Table 2).

Concentrations of the others derivatives of Jasmonates (OPC8, Jasmonoyl-isoleucine, Jasmonoyl-valine, jasmonoyl-phenylalanine, jasmonoyl-tryptophan, 12-hydroxyjasmonic acid, dihydrophaseic acid, phaseic acid, neophaseic acid, were below the limit of detection in all species studied.

Table. 2. Endogenous Jasmonates concentration (pmol g^{-1} dry weight) detected in some species from Brazil and Antarctica.

Species	*cis*OPDA	9,10-dihydrojasmonic acid D6-JA	Jasmonic acid
Gracilaira birdiae	< LOD	< LOD	< LOD
Graciaria caudata	< LOD	209.8 ± 66.22	< LOD
Gracilaria domingensis	< LOD	721.2 ± 48.34	22.1 ± 5.7
Gracilaria sp.	< LOD	1,150.4 ± 264.4	11.5 ± 3.7
Sargassum vulgare	2,430.7 ± 430.8	< LOD	32.3 ± 10.3
Prasiola crispa	734.1± 564.2	< LOD	< LOD
Desmarestia antarctica	2,254.3 ± 320.2	< LOD	11.7 ± 1.5
Adenocystis utricularis	< LOD	< LOD	< LOD
Ascoseira mirabilis	< LOD	< LOD	< LOD
Phaeurus antarticus	979.1 ± 99.2	< LOD	< LOD
Cystophaera jacquinotii	< LOD	< LOD	16.3 ± 1.6

Results are presented as mean ± SE (n = 4); <LOD indicates that Jasmonates concentrations were below the limit of detection.

Endogenous Auxins

Auxins were detected in all species analyzed in the present study, and IAA concentrations varied from 133.9 to 7,574.9 pmol g^{-1} DW, the highest value was observed in *Phaeurus antarcticus* and the lower was detected in *Sargassum vulgare*. Higher concentrations of free IAA were detected in *Gracilaria* species (Table 3).

The oxIAA concentrations ranged from 57.6 to 2,083.9 pmol g^{-1} DW, and it was detected in few species. The highest concentrations of oxIAA were detected in *Gracilaria caudata* and the lower was in *Gracialaria birdiae* (Table 3).

The IAGlu concentrations varied from 2.1 to 108.0 pmol g^{-1} DW in *Gracilaria birdiae*, *Gracilaria caudata*, *Gracilaria domingensis*, *Sargassum vulgare* and *Cystosphaera jacquinotti*. The highest IAGlu concentration was detected in *Gracilaria domingensis* and the lower was in *Cystosphaera jacquinotti* (Table 3).

Other auxin derivatives (IAAsp - indole-3-acetylaspartic acid, IAA-Glc - 1-O-indole-3-acetyl-β-D-glucose, oxIAA-Glc -1-O-(2-oxoindol-3-ylacetyl)-β-D-glucopyranose) were also analyzed but all species studied presented concentrations below the limit of detection.

Table. 3. Endogenous Auxin concentration (pmol g^{-1} dry weight) detected in some species from Brazil and Antarctica.

Species	IAA	oxIAA	IAGlu
Gracilaria birdiae	395.8 ± 125.2	57.6 ± 20.8	2.7 ± 0.6
Gracilaria caudata	7,411.7 ± 428.6	2,083.9 ± 91.7	107.7 ± 8.9
Gracilaria domingensis	5,242.0 ± 633.3	942.7 ± 57.2	108.0 ± 3.4
Gracilaria sp.	2,469.6 ± 206.5	1,086.3 ± 84.0	< LOD
Sargassum vulgare	133.9 ± 9.5	231.5 ± 20.4	45.5 ± 3.6
Prasiola crispa	1,053.4 ± 51.7	101.6 ± 15.3	< LOD
Desmarestia antarctica	738.3 ± 226.9	121.1 ± 32.9	< LOD
Adenocystis utricularis	286.2 ± 86.9	150.0 ± 146.5	< LOD
Ascoseira mirabilis	505.8 ± 46.7	744.3 ± 59.6	< LOD
Phaeurus antarticus	7,574.9 ± 578.3	733.4 ± 107.0	< LOD
Cystophaera jacquinotii	287.0 ± 17.7	283.4 ± 134.1	2.1 ± 0.5

Results are presented as mean ± SE (n = 5); <LOD indicates that Auxin concentrations were below the limit of detection, IAA - indole-3-acetic acid; oxIAA - 2-oxindole-3-acetic acid; IAGlu - indole-3-acetyl glutamic acid.

Endogenous Abscisic acid

ABA concentrations ranged from 4.0 to 44.7 pmol g^{-1} DW in *Gracilaria caudata*, *Gracilaria domingensis*, *Gracilaria* sp., *Sargassum vulgare* and *Prasiola crispa*. The highest ABA concentration was detected in *Gracilaria domingensis* and the lower was in *Prasiola crispa* (Fig. 1).

Gracilaria birdiae and all species from Antarctica presented ABA concentrations below the limit detection (Fig. 1). No conjugated ABA forms or ABA metabolites were detected in any of the species studied.

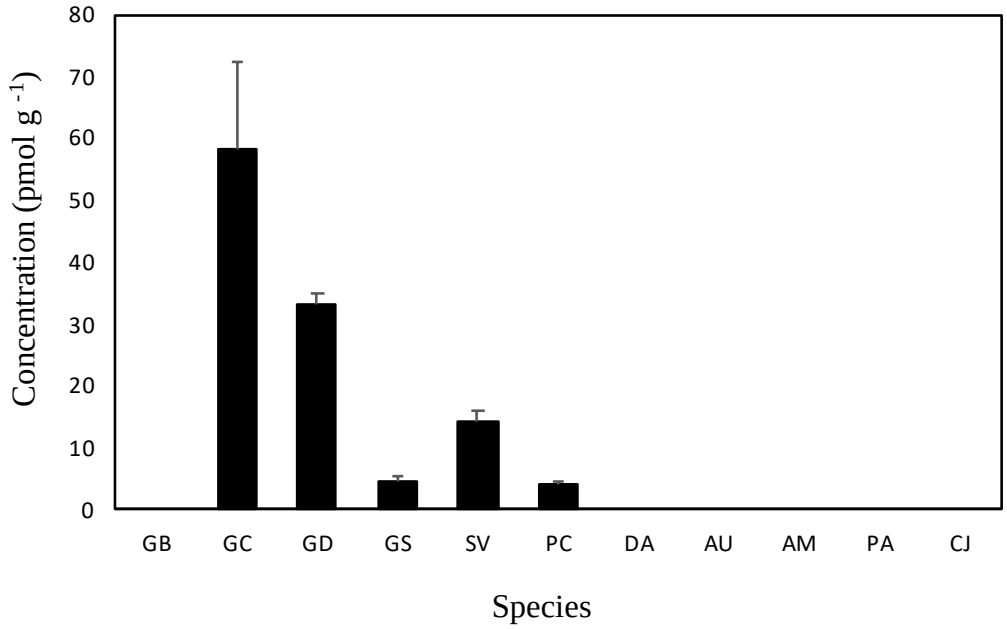

Fig. 1. Endogenous Abscisic acid concentrations (pmol g^{-1} dry weight) detected in some species from Brazil and Antarctica. GB – *Gracilaria birdiae*; GC – *Gracilaria caudata*; GD – *Gracilaria domingensis*; GS – *Gracilaria* sp.; PC – *Prasiola crispa*; DA – *Desmarestia antarctica*; AU – *Adenocystis utricularis*; AM – *Ascoseira mirabilis*; PA – *Phaeurus antarcticus*; CJ - *Cystosphaera Jacqinotii*.

DISCUSSION

Endogenous Jasmonates

The main Jasmonates detected in a few species analyzed in the present study were *cis*OPDA, 9,10-dihydrojasmonic acid D6-JA and Jasmonic acid. cisOPDA were not detected in any species of *Gracilaria,* and 9,10-dihydrojasmonic acid D6-JA was present only in *Gracilaria* species. JA and the JA precursor oxo-phytodienoic acid (OPDA) were detected in *Dictyota dichotoma*, a brown alga which produces terpenoids as a chemical defence mechanism (Wiesemeier et al. 2008). There are few studies with jasmonates in seaweeds. Jasmonic acid treatments did not change the profile of metabolites such as terpenes, phenolics, lipids and fatty acids in seven Phaeophyceae (*Dictyota dichotoma*, *Colpomenia peregrine*, *Ectocarpus fasciculatus*, *Fucus vesiculosus*, *Himanthalia elongate*, *Saccharina latissima* and *Sargassum muticum*) suggesting that jasmonates do not play a role in inducing

chemical defence responses in these species (Wiesemeier et al. 2008). Jasmonates are involved in developmental processes such as seed germination, root growth, tendril coiling, flower development, fruit ripening and senescence (Cheong & Choi 2003; Kombrink 2012). In addition, they are also involved in defense mechanisms of plants, increasing in concentration in response to biotic wounding by pathogens and herbivore as well as to abiotic stresses such as drought, salinity and UV radiation (Cheong & Choi, 2003; Kombrink 2012). One of the primary functions of jasmonates in vascular plants is inducing chemical defence mechanisms against grazers and activating defence related genes. There are similar responses in seaweeds. Seaweeds increase their secondary metabolites such as terpenoids and polyphenols as a chemical defence mechanism against herbivores such as littorine snails and various pathogens (Arnold et al. 2001; Küpper et al. 2009).

Auxins (IAA and oxIAA) were present in all of the seaweeds analyzed in the present study, and IAGlu were detected in a few species. Endogenous IAA and various conjugates are present in several seaweeds. For example, IAA was detected in *Kappaphycus alvarezii* (Rhodophyceae) and *Sargassum tenerrimum* (Phaeophyceae), and indole-3-pyruvic acid (IPA) in *Gracilaria edulis* (Rhodophyceae; Prasad et al. 2010). IAA and indole-3-butyric acid (IBA) were present in four wild harvested *Ulva* species (Gupta et al. 2011). *Gracilaria* species studied in the present work showed higher concentrations of endogenous auxin than the values detected to *Gracilaria birdiae* by Yokoya et al. (2010). They found IAA in 11 Rhodophyceae species and other conjugates including indole-3-ethanol (IEt) in eight species, indole-3-acetyl glutamic acid (IAGlu) in three species and indole-3-acetyl leucine (IALeu). Auxin concentrations were lower during the cooler months and increased during the warmer months in *Ulva fasciata* (Stirk et al., 2009).

Auxins are involved in seaweed physiology and exogenous auxin application eliciting physiological responses in seaweeds. IAA application to sporophyte discs of *Laminaria japonica*, *Undaria pinnatifida* and *Alaria crassifolia* (Phaeophyceae) had no effect on the

growth rate apart from being inhibitory at higher concentrations and delaying sorus formation (Kai et al. 2006). IAA stimulated embryo growth and apical formation in *Fucus vesiculosus* embryos, but had an inhibitory effect on photosynthesis, decreasing the maximum quantum yield (Fv/Fm), the rate of electron transfer under saturation irradiances (rETRmax) and photosynthetic efficiency (αETR) (Tarakhovskaya 2013).

ABA was detected in *Gracilaria caudata*, *Gracilaria domingensis*, *Gracilaria* sp. and *Sargassum vulgare*. ABA is found in all classes of seaweeds. ABA and lower concentrations of 2-trans-methyl ABA were identified in *Ascophyllum nodosum* (Phaeophyceae; Boyer & Dougherty, 1988), in *Ulva fasciata* (Stirk et al., 2009), in 10 species of Rhodophyta (Yokoya et al., 2010), and in five *Ulva* species and *Monostroma oxyspermum* (Gupta et al., 2011). Physiological responses to ABA application in *Laminaria hyperborea* sporophytes were influenced by daylength: ABA inhibiting growth in short daylength treatments and having no effect on long daylength treatments (Schaffelke, 1995). This result could explain the ABA concentrations below the limit of detection found in Antarctic seaweeds, which were collected during the summer, a period with long photoperiod.

In conclusion, results showed that *Gracilaria* species have some PGRs in high endogenous concentrations, which are useful for agriculture as a natural fertilizer. Further studies on identification of PGRs in seaweeds should be done not only to select species with potential for application in agriculture but also to understand the role played by PGRs in the seaweed development.

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Conclusões e Considerações finais

Avaliando os resultados obtidos nos capítulos anteriores, pode-se concluir que:

- *Gracialaria caudata*, apesar de não ser uma espécie com altas taxas de crescimento e não apresentar boa eficiência de remoção de nitrato e fosfato, apresentou altas concentrações de pigmentos e proteínas solúveis totais, no entanto, podemos concluir que essa espécie não apresenta um alto potencial de absorção dos nutrientes disponíveis na água do mar, não sendo uma boa opção para uso como biofiltros em cultivos multitróficos integrados e no mar.

- A alta eficiência de *Gracilaria domingensis* na remoção do excesso de nutrientes na água, indica que esta espécie tem potencial como biofiltro e pode ser cultivada em sistemas multitróficos integrados ou no mar, reduzindo os nutrientes dos ambientes marinhos eutrofizados.

- Considerando as condições experimentais, *Gracilaria domingensis* apresentou maior crescimento, maior concentração de proteínas e de pigmentos em tratamentos com adição de diferentes concentrações de N:P. Por outro lado, *Gracilaria caudata* apresentou maior qualidade de ágar em tratamentos com adição de N:P do que as amostras coletadas em campo, o que indicou que *Gracilaria caudata* pode ser cultivada em sistema de aquicultura multi-trófica sob níveis mais altos de nutrientes e produzindo ágar de melhor qualidade.

- Em conclusão, os resultados mostraram que as espécies de *Gracilaria* apresentaram auxinas, ácido abscísico e jasmonatos em altas concentrações endógenas, podendo ser úteis para a agricultura como fertilizante natural. Estudos adicionais sobre a identificação de fitorreguladores em algas marinhas devem ser realizados não apenas para selecionar espécies com potencial de aplicação na agricultura, mas também para entender o papel desempenhado pelos fitorreguladores no desenvolvimento de algas marinhas.

- Os estudos fisiológicos em *Gracilaria* devem ser continuados a fim de obter resultados que possibilitam seu cultivo no mar, reduzindo o extrativivismo a partir de banco naturais e contribuindo para sua conservação, assim como também a sua utilização como biofertilizantes ou biofiltro.