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# Physiological and anatomical assessments of tetrasporophytes with epiphyte gametophytes of wild and green variant strains of *Gracilaria caudata* (Gracilariales, Rhodophyta)

São Paulo, SP - Brasil

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# Physiological and anatomical assessments of tetrasporophytes with epiphyte gametophytes of wild and green variant strains of *Gracilaria caudata* (Gracilariales, Rhodophyta)

# Parâmetros fisiológicos e anatômicos de tetrasporófitos com gametófitos epífitos de linhagens selvagens e verdes de *Gracilaria caudata* (Gracilariales, Rhodophyta)

Dissertação apresentada ao Instituto de Biociências da Universidade de São Paulo, para obtenção de Título de Mestre em Ciências, na Área de Botânica

Orientadora: Profª. Drª. Estela Maria Plastino
Colaborador: Prof. Dr. Diego Demarco

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I - Universidade de São Paulo, Instituto de Biociências. Departamento de Botânica.

**Judging committee:**

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Professor Dr.

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Professor Dr.

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Professor Dr. Estela Maria Plastino (Supervisor)

This work is dedicated to my family, friends, and coworkers that were crucial to this project. Thank you all for your help and support!

*I can't compact their existence into 26 letters and call it a description*
*I tried once but the adjectives needed to describe them don't even exist*
*so I ended up with pages and pages full of words*
*followed with commas and more words and more comas*
*only to realize that there are some things in the world*
*so infinite that they can never use a full stop.*

“Broken English” - Rupi Kaur

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**Abstract**

*Gracilaria caudata* J. Agardh is one of the naturally occurring species in Brazil that produce good quality agar. In Rio do Fogo (RN), Brazil, a green variant was discovered in a *G. caudata* population of predominantly red (wild phenotype) tetrasporophytes. Epiphytes gametophytes on tetrasporophytes have been observed for both strains. Considering the wild and green variant strains in laboratory control conditions, this study: (i) assessed the impacts of epiphyte gametophytes on tetrasporophytes by evaluating the growth rates, the pigment content, and the photosynthetic potential of the tetrasporophytes; (ii) evaluated the amount of time required for the differentiation of cystocarps in free-living gametophytes and epiphyte gametophytes on tetrasporophytes; (iii) evaluated the number of epiphyte gametophytes produced by tetrasporophyte; and (iv) analyzed anatomical aspects of the intersection between the tetrasporophytes and the tetraspores that germinated on the tetrasporophytes. Samples were cultivated for 12 weeks on laboratory conditions. Control samples had epiphyte gametophytes growing on them, while treatment samples had their epiphyte gametophytes removed weekly. Physiological analyses compared control and treatment samples, while anatomical analysis used the control samples only. Considering the tetrasporophyte growth rates before fertility (2$^{nd}$ week), wild strain showed higher values than green variant; however, after 12 weeks, no differences were found between strains or between samples without epiphyte gametophytes. The wild strain produced tetraspores more frequently than the green variant and showed deficiency in phycoerythrin and allophycocyanin. Green variant treatment samples had higher content of allophycocyanin, phycocyanin and phycoerythrin than the control samples. Chlorophyll *a* was higher in the wild strain, and treatment samples had lower values in comparison to control samples. Epiphyte gametophytes possibly promoted reduction in pigment content of the tetrasporophytes on both strains. Wild strain samples without epiphyte gametophytes had higher photosynthetic efficiency. Epiphyte gametophytes produced cystocarps one week before free-living gametophytes. The amount of epiphyte gametophytes on tetrasporophytes is not different between strains. Epiphyte gametophytes were visible one week before on the wild strain than in the green variant strain. It was found that tetraspores give rise to epiphyte gametophytes through two distinct germination methods: outside or inside the thallus of the tetrasporophyte. Germination outside the tetrasporophyte thallus was more common, produced visible holdfasts and promoted cortex thickening in the cortical region of the tetrasporophyte. Germination inside the tetrasporophyte thallus did not show evidences of holdfasts nor cortex thickening on the tetrasporophytes. Epiphyte gametophytes holdfasts had adjacent cells to the cortex of the tetrasporophytes when they germinate outside the thallus; however, when germination occurred inside the thallus, the connection interface was closer to the medulla of the tetrasporophyte. Epiphyte gametophytes might represent a new life strategy that promotes sexual variability in a population mostly composed of tetrasporophytes. For cultivation purposes, wild tetrasporophytes with epiphyte gametophytes yielded more mass than the variant tetrasporophytes, being the best option for production. However, the green variant without epiphyte gametophytes produced more phycobiliproteins, making it a better option to harvest those substances.

**Resumo**

*Gracilaria caudata* J. Agardh é uma das espécies encontradas na costa brasileira que produzem ágar de boa qualidade. Em Rio do Fogo (RN), Brasil, uma linhagem variante verde de *G. caudata* foi descoberta em uma população composta principalmente por indivíduos de coloração vermelha (fenótipo selvagem). Gametófitos epífitos em tetrasporófitos foram observados para as duas linhagens. Considerando-se as linhagens selvagem e verde em condições controladas de laboratório, este estudo analisou: (i) os impactos dos gametófitos epífitos nos tetrasporófitos por meio de taxas de crescimento, conteúdo pigmentar e potencial fotossintetizante dos tetrasporófitos; (ii) o tempo necessário para a diferenciação de cistocarpos em gametófitos de vida livre e gametófitos epífitos; (iii) o número de gametófitos epífitos produzidos por tetrasporófito; e (iv) aspectos anatômicos da interseção entre tetrasporófitos e gametófitos epífitos. O experimento teve duração de 12 semanas. Amostras controle foram mantidas com seus gametófitos epífitos durante o experimento, enquanto as amostras tratamento tiveram seus gametófitos epífitos removidos semanalmente. Análises fisiológicas compararam amostras controle e tratamento, enquanto que para os estudos anatômicos utilizou-se apenas as amostras controle. A linhagem selvagem apresentou maiores taxas de crescimento que a linhagem verde nas duas primeiras semanas do experimento, quando ainda não estavam férteis; entretanto, após as doze semanas de cultivo, não foram observadas diferenças entre as linhagens tetrasporofíticas. A linhagem vermelha produziu tetrásporos com mais frequência do que a linhagem verde e apresentou deficiência em ficoeritrina e aloficocianina. As amostras tratamento da linhagem verde apresentaram valores maiores de aloficocianina e ficoeritrina do que às do controle. A linhagem selvagem apresentou menores teores de ficoeritrina e aloficocianina quando comparada à linhagem verde. As amostras tratamento da linhagem verde apresentaram maiores valores de aloficocianina, ficocianina e ficoeritrina que as amostras controle da mesma linhagem. A quantidade de clorofila *a* foi maior na linhagem selvagem, e as amostras tratamento desta linhagem apresentaram valores maiores que às do controle. A presença de gametófitos epífitos promoveu a redução no conteúdo pigmentar nas duas linhagens. Amostras tratamento da linhagem selvagem apresentaram potencial fotossintetizante maior do que amostras controle. A quantidade de gametófitos epífitos não foi diferente entre as linhagens. Gametófitos epífitos produziram cistocarpos uma semana antes do que gametófitos de vida livre. A linhagem selvagem produziu gametófitos epífitas uma semana antes da linhagem verde. A germinação de tetrásporos em tetrasporófitos ocorreu de duas maneiras: antes e após a liberação de tetrásporos. A liberação de tetrásporos seguida da germinação sobre o tetrasporófito foi mais frequente, produziu apressórios e promoveu aumento do número de células no córtex do tetrasporófito. A germinação de tetrásporos dentro do talo do tetrasporófito não apresentou evidências de apressórios ou espessamento da região cortical do tetrasporófito. A porção basal de apressórios de gametófitos epífitos permaneceu adjacente ao córtex do tetrasporófito. Quando a germinação do tetrásporo ocorreu ainda dentro do talo, a interface de conexão das células foi mais próxima da medula do tetrasporófito. Gametófitos epífitos podem representar uma nova estratégia de vida para a espécie por possibilitar a variabilidade por reprodução sexual em uma população predominantemente composta por tetrasporófitos. Para um possível cultivo em larga escala, tetrasporófitos com gametófitos epífitos da linhagem selvagem seriam mais adequados por produzirem mais massa que os da variante verde. Entretanto, se o objetivo for a produção de ficobiliproteínas, a linhagem verde seria a mais indicada.

## 1. General introduction

### 1.1. Economic relevance: *Gracilaria*

Aquaculture accounts for a significant portion of the exports of many countries, notably China, where algae farming accounts for almost a quarter of total exports for consumption and industry. In 2012, the amount of money generated by seaweed was around 6.4 billion dollars (FAO, 2013). The genera of economic interest most cultivated are: *Porphyra* spp., *Euchema* spp., *Kappaphycus* spp., *Undaria* spp., *Saccharina* spp. and *Gracilaria* spp. (FAO, 2016).

Among the phycolloids, substances derived from seaweeds, agar has the greatest economic relevance in the world. Historically, in Japan, *Gelidium* spp. was the main source of agar until the beginning of the 20th century when the demand for the substance began to exceed production. After agar was introduced in 1959 to the Paris Academy of Science, Western countries began to use it on a large-scale (Armisen, 1995). Among the genera currently used for agar production, *Gracilaria* is the most economically relevant, mainly because of its rapid growth (Kain & Destombe, 1995). It is the third most cultivated genera in the world, accounting for 3,752 thousand tons in 2014 (FAO, 2016). In the Americas, Chile stands out for the mass production of agar (McHugh, 1991, FAO, 2016).

In Brazil, commercial exploitation of phycolloids began in the 1960s, using the genus *Gracilaria* and *Hypnea musciformis (*Hayashi et al., 2014). The cultivation of algae occurs mostly in the Northeastern region of the country and is still not well established. Consequently, the cultivation of seaweed encounters problems with regulation and over-exploitation in some regions (Marinho-Soriano et al., 2009; Hayashi et al., 2014). The integrated cultivation of crustaceans with macroalgae (including those of *Gracilaria* spp.) has been presented as an efficient solution and alternative to improve water quality, since the algae absorb nitrogenous compounds derived from crustacean cultivation (Jones et al. 2001), minimizing and mitigating damages to the environment.

### 1.2. *Gracilaria*: life history and differences between generations

The life history found in *Gracilaria* spp. consists of three phases (**Figure 1**): a tetrasporophyte (diploid) followed by a gametophyte (haploid) and then a carposporophyte (diploid). This history is similar to the one on the genus *Polysiphonia*, in which tetrasporophytes and gametophytes are morphologically similar and the

offspring ratio of male and female individuals in the gametophyte generation is the same (Kain & Destombe, 1995). Fertilization occurs in the female gametophyte's thallus jn specialized cells (carpogonia). A new generation is formed in the fertilization spot due to numerous mitotic divisions: the carposporophytes. Under normal conditions, carposporophytes only develop when fertilization occurs in the female gametophyte (Ogata et al., 1972). These carposporophytes are protected by a pericarp, a protective structure composed of multiple layers of cells. The combination of the carposporophyte and pericarp form a structure called cystocarp. Carposporophytes can produce carpospores, that germinate into free-living tetrasporophytes. These tetrasporophytes have specialized reproduction cells called tetrasporangia. Through a meiotic process, tetrasporangia form haploid tetraspores, that germinate male and female gametophytes in a 1:1 ratio (Destombe et al., 1989).

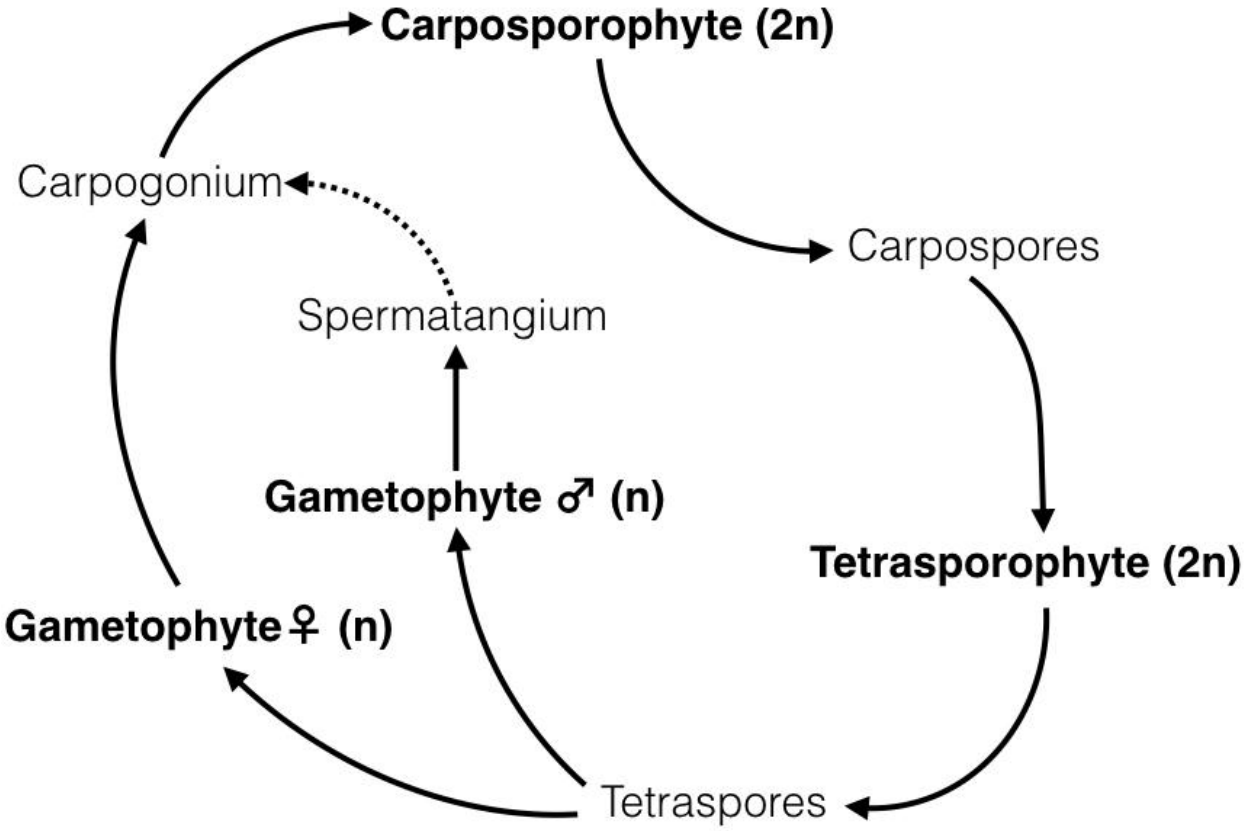


**Figure 1.** Representation of the three stages life history found on *Gracilaria* spp. Generations are represented in bold, while aspects representative of the reproduction are in regular letters. Isomorphism is found between tetrasporophytes and gametophytes. Cystocarps can be found growing on female gametophytes. Adapted from Destombe et al. (1989).

There is no consensus on the implications of a diplobiontic life history (Valero et al., 1992; Richerd et al., 1993; Hughes & Otto, 1999), although one of the possible

interpretations is an adaption to the most favorable environmental condition (Stebbins & Hill, 1980). Furthermore, there are evidences that isomorphic phases are not ecologically identical (Hughes & Otto, 1999, Guillemin et al., 2013, Faria et al., 2016). Diplobiontic isomorphic histories have a reduced cost related to sex, considering that sexual reproduction only occurs during the gametophyte phase (Hughes & Otto, 1999). Tetrasporophytes can show greater plasticity when compared to gametophytes, enhancing their ability to adapt to different environmental conditions and to mask deleterious mutations, considering that mutations are rare and that they might be paired with a normal allele (Crow & Kimura, 1965). Tetrasporophytes are also likely to accumulate more beneficial mutations since they have twice the amount of DNA than gametophytes (Paquin & Adams, 1983). Deleterious mutations on gametophytes are more evident in natural populations, which make them easier to be removed, since they are not masked by another allele (Hughes & Otto, 1999). Sexual reproduction between male and female gametophytes can help fix beneficial mutations of individuals that were fit enough to reach sexual maturity (Richerd et al.,1993). Once fertilization occurs, the carposporophyte phase can increase the reproductive yield per fertilization event, but it also reduces genetic variability among the progeny (Hughes & Otto, 1999). It was observed that tetrasporophytes are more frequent than gametophytes for *Gracilaria caudata* (Ayres-Ostrock et al., 2016). The same occurs for other species of *Gracilaria,* such as *G. mammillaris, G. cervicornis* (Plastino, 1985), *G. cornea* (Orduña-Rojas et al., 2002), *G. chilensis* (Guillemin et al., 2008), *G. vermiculophylla* (Terada et al., 2010), *G. gracilis* (Martín et al., 2011), and *G. birdiae* (Ayres-Ostrock et al., 2016).

### 1.3. Epiphyte gametophytes in *Gracilaria*

There are evidences of variations on the life history of *Gracilaria* species caused by spontaneous mutations, such as the bisexual trait (Bird et al., 1977; Santos, 2017) or gametophytes growing epiphytically and reaching sexual maturity on the tetrasporophytes (Hughes & Otto, 1999). It is suggested that those individuals have diplontic life history, and the reproductive haploid phase is contained in the diploid phase (Hughes & Otto, 1999). In some *Gracilaria* species, small gametophytes may develop as epiphytes in the tetrasporophytes. This phenomenon was observed in *G. tikvahiae* (Bird et al*.,* 1977), *G. caudata* (as *Gracilaria* sp.) and *G. cornea* (as *G. debilis*) (Oliveira & Plastino, 1984), and *G. gracilis (*Destombe et al., 1989). Germination of tetraspores in the thallus of the tetrasporophyte may represent a life strategy that

promotes propagation and distribution of the species (Kain & Destombe 1995). Hughes and Otto (1999) suggested the name “skipping diploid” for this life history in which the epiphyte gametophytes growing on the tetraspophytes can provide genetic variability during their reproduction. In addition, germination of tetraspores on the thallus of tetrasporophytes may suggest a greater adaptation of the tetrasporophyte phase to the environment in comparison to the gametophyte phase (Oliveira & Plastino, 1984), especially in environmental circumstances where one is favored in spite of the other (Hughes & Otto, 1999), although there’s no hard evidence of this hypothesis yet.

The life history of *Gracilaria caudata* (as *Gracilaria* sp.) was completed in 9 months under laboratory conditions (Oliveira & Plastino, 1984). These authors report the occurrence of tetraspore germination on the tetrasporophyte under laboratory conditions. There is also evidence of germination of epiphyte gametophytes on tetrasporophytes for the species in tetrasporophytes in nature (Plastino & Oliveira, 1988; Ayres-Ostrock, 2014).

### 1.4. Intraspecific diversity: color variants in *Gracilaria*

Adaptive processes are defined as consecutive mutations in an organism’s genome accumulated over the generations (Gantt, 1990; Plastino & Guimarães, 2001). Species variants and morphotypes are outcomes of these processes. Color variants are considered common, usually ranging from dark red to green phenotypes in Rhodophyta (Plastino, 2004).

Morphotypes are detected when two or more discontinuous forms of the same species occur concomitantly in the same habitat, and can be described as polymorphism when the frequency of a mutation in the same population is greater than 1% (Sheppard, 1975). The genetic variability resulting from these mutations can promote different responses to environmental stimuli, which can favor species that are in a stressed environment by increasing their chances of survival (Plastino & Guimarães, 2001; Plastino, 2008). Acclimatization is another process that might increase a particular organism’s fit by promoting adjustments to different environmental conditions. It can be expressed in an organism’s genome, while adaptation, in opposition, requires successive mutations in an organism’s genome over time. The recognition of chromatic variants is established by frequent observations in natural populations and confirmed by cultivating selected strains in a laboratorial condition, that allows the distinction between adaptive and acclimatization processes (Plastino, 2008).

Mutations associated with color have been used in different studies to better understand: genetics, life history, and pigment characterization (van der Meer, 1979; Kursar et al., 1983; Ramus & van der Meer, 1983). Color variants may have a distinct pigment composition compared to the most abundant (wild) lineage, so comparing wild and color variants under controlled conditions can reveal physiological distinctions (Costa & Plastino, 2011). Out of the species of *Gracilaria* that occur on the Brazilian coast, spontaneous chromatic variants were described for *G. domingensis* (Plastino et al., 1999), *G. cornea* (Ferreira et al., 2006), *G. birdiae* (Costa & Plastino, 2011), and *G. caudata* (Faria & Plastino, 2016; Santos, 2017). Out of these, inheritance patterns have been established for light green and greenish-brown variants of *G. birdiae* (Plastino, 2004; Costa & Plastino, 2011), and for a greenish-brown variant of *G. caudata* (Santos, 2017). Cultivating successive generations of strains from the field in laboratory conditions can assure the stability of these mutations (Plastino et al., 1999, Santos, 2017).

### 1.5. *Gracilaria caudata*

The genus *Gracilaria* (Gracilaraceae, Rhodophyta) was described in 1830 by Greville. This genus has 184 confirmed species (Lyra et al., 2015; Algaebase, 2017). *G. caudata* J. Agardh was first described in the Caribbean Sea. It can be found in tropical waters, from Central America (Barbados, Puerto Rico and Cuba) to South America. There are records of *G. caudata* for Venezuela and for most of the Brazilian coast, near the Equator, to its southern limit at 28ºS, in the state of Santa Catarina (Plastino & Oliveira, 1997; Nunes, 2005). Aside from the wild red phenotype, a stable and inheritable greenish-brown mutant was described for *G. caudata* in a population from the State of Ceará, Brazil (Santos, 2017). Furthermore, a green variant strain of *G. caudata* was identified and collected in Rio do Fogo, Rio Grande do Norte, Brazil in 2011 and has been cultivated in laboratory conditions, alongside samples of the wild strain.

*Gracilaria caudata* produces good quality and economically viable agar (Yoshimura, 2006). Their optimal growth rates are coincident with abiotic factors found in the Brazilian coast, such as temperatures between 26 and 30°C, (as *G. verrucosa*, Yokoya & Oliveira, 1992b) and salinity of 35, although this species has been tolerant to variations of these factors under laboratory conditions (as *G. verrucosa*, Yokoya & Oliveira, 1992a).

*Gracilaria caudata* has an erect and cylindrical thallus up to 34 cm in length by 1.7 mm in thickness. It also shows up to fifth order branches (Plastino & Oliveira, 1997). It has a pseudoparenchymal thallus and microcystidiate cell organization. Furthermore, its cortex consists of two heavily pigmented layers, with slightly elongated cells. The male gametophyte generation shows spermatangia scattered through the thallus in deep subcortical conceptacles, mainly the *verrucosa*-type. Tetrasporophytes produce scattered tetrasporangia in its thallus adjacent to anticlinally elongated cortical cells. Tetrasporangium is cruciately divided and measure on average 21.8 by 39.5 µm. Female gametophytes present carpogonia that are conical cells with a short trichogine in the apical portion. Cystocarps are found scattered in the thallus of the female gametophyte after fertilization occurs. Carposporophytes are located externally in relation to the medullary cells of the fertilized female gametophyte. This contact region is made up of layers of conspicuous cells. *Gonimoblasts are formed radially from a branched fusion cell*. Therefore, connective tubular cells are formed from the gonimoblasts and merge into the pericarp cells at the basal part of the cystocarp. The pericarp is formed from nine to eleven rows of cells. The diameter of the mature carposporangia is on average 20,9 µm (Plastino & Oliveira, 1997).

Although germination of epiphyte gametophytes on tetrasporophytes has already been observed in the laboratory and in natural populations, an analysis of the physiological impacts of the germination of tetraspores on tetrasporophytes of wild and green variant strains of *Gracilaria caudata* tetrasporophytes have never been done. Therefore, this analysis can contribute to a better interpretation of the three-phase life history and its possible evolutionary implications. Discoveries about epiphyte gametophytes and color variants raised questions related to: the possible implications of their occurrence in nature, their physiological performance, and their possible use for aquaculture. Consequently, the study of the germination of tetraspores on tetrasporophytes can clarify possible distinctions between the green variant and the wild strains. It can also provide information regarding the physiology of different strains, which is relevant to optimize putative large-scale cultivation of *G. caudata*.

## 2. Hypotheses and objectives

The following hypotheses were formulated considering both wild (red) and green variant *Gracilaria caudata* strains: (i) tetraspores that germinated on tetrasporophytes develop into epiphyte gametophytes that reach reproductive maturity in a shorter amount of time than gametophytes that germinated freely; (ii) tetrasporophytes without epiphyte gametophytes show superior vegetative performance when compared to tetrasporophytes with epiphyte gametophytes; (iii) wild strain tetrasporophytes have higher pigment content, higher growth rates, and higher photosynthetic potential than individuals of the green variant strain; (iv) the amount of epiphyte gametophytes on tetrasporophytes is different between the wild and the green variant strains; and (v) epiphyte gametophytes have a cellular connection to the tetrasporophytes.

To test these hypotheses, we: (i) assessed the impacts of epiphyte gametophytes on tetrasporophytes by evaluating the growth rates, the pigment content, and the photosynthetic potential of the tetrasporophytes; (ii) evaluated the amount of time required for the differentiation of cystocarps in free-living gametophytes and epiphyte gametophytes on tetrasporophytes; (iii) evaluated the number of epiphyte gametophytes produced by tetrasporophyte; and (iv) analyzed anatomical aspects of the intersection between the tetrasporophytes and the tetraspores that germinated over the tetrasporophytes. Hypotheses 1, 2, and 3 are discussed in Chapter I, while hypotheses 4 and 5 are examined in Chapter II.

## 3. Chapter I: Physiological impact of epiphyte gametophytes on tetrasporophytes of the wild and green variant strains of *Gracilaria caudata* (Gracilariales, Rhodophyta)


### Abstract

A green variant was discovered in a *Gracilaria caudata* population of predominantly red (wild) tetrasporophytes in Rio do Fogo (RN), Brazil. Epiphyte gametophytes on tetrasporophytes have been reported for both strains. This study aimed to access the impacts of epiphyte gametophytes on tetrasporophytes by evaluating for both strains: growth rates, pigment content, and photosynthetic capacity of the tetrasporophytes, and the amount of time required for the differentiation of cystocarps in free living gametophytes and epiphyte gametophytes. Samples were cultivated for 12 weeks. Control samples had epiphyte gametophytes growing on them, while treatment samples had their gametophytes removed weekly. Considering the tetrasporophyte growth rates before fertility (2$^{nd}$ week), wild strain showed higher values than green variant; however, after 12 weeks, no differences were found between strains or between samples without epiphyte gametophytes. The wild strain produced tetraspores more frequently than the green variant and showed deficiency in phycoerythrin and allophycocyanin. Green variant treatment samples had higher content of allophycocyanin and phycocyanin than the its respective control samples. Chlorophyll *a* was higher in the wild strain, and treatment samples had lower values in comparison to control samples. Wild strain samples without epiphyte gametophytes had higher photosynthetic efficiency. Epiphyte gametophytes produced cystocarps one week before free-living gametophytes. For cultivation purposes, wild tetrasporophytes with epiphyte gametophytes yielded more mass than the variant tetrasporophytes, while the green variant without epiphyte gametophytes produced more phycobiliproteins, making it a better option to harvest those substances.


### 3.1. Introduction

*Gracilaria caudata* J. Agardh is a common seaweed found on the Brazilian coast (Plastino & Oliveira, 1997; Nunes, 2005). This species produces good quality and economically viable agar (Yoshimura, 2006). The optimal growth rates for *G. cautata* are coincident with abiotic factors found in the Brazilian coast, such as temperatures between 26 and 30°C (as *G. verrucosa*, Yokoya & Oliveira, 1992b) and salinity of 35

ups, although this species has been tolerant to variations of these factors under laboratory conditions (as *G. verrucosa*, Yokoya & Oliveira, 1992a). Recent research has investigated color variants, color inheritance, inheritance sexual characters, and pigment content of *G. caudata* (Faria & Plastino, 2016; Santos, 2017).

The life history found in *G. caudata* consists of three phases: a tetrasporophyte (diploid) followed by a gametophyte (haploid) and a carposporophyte (diploid) (Plastino & Oliveira, 1997). Tetrasporophytes and gametophytes are morphologically similar and the offspring ratio of male and female individuals in the gametophyte generation is the same (Kain & Destombe, 1995). Tetrasporophytes are the dominant phase of many *Gracilaria* spp. in nature (Destombe et al., 1989), including *G. caudata*. In Brazil, *G. caudata* populations are mainly composed of tetrasporophytes, particularly in the Northeastern region (Ayres-Ostrock, 2014).

There is evidence of variations on the life history of *Gracilaria* species, such as gametophytes growing epiphytically and reaching sexual maturity on the tetrasporophytes due to tetraspore retention (Plastino & Oliveira, 1988; Destombe et al., 1989; Hughes & Otto, 1999, Costa & Plastino, 2001). Therefore, the life history of these individuals has the reproductive haploid phase contained in the diploid phase (Hughes & Otto, 1999). *G. caudata* populations described in Brazil had epiphyte gametophytes on the tetrasporophytes, being more frequent in populations from the Northeastern region (Ayres-Ostrock, 2014). Furthermore, epiphyte gametophytes on tetrasporophytes were observed in *G. tikvahiae* (Bird et al., 1977), *G. cornea* (as *G. debilis*) (Oliveira & Plastino, 1984), and *G. gracilis (*Destombe et al., 1989).

Germination of gametophytes on tetrasporophytes may represent a life strategy for propagation and distribution of the species (Kain & Destombe 1995). Epiphyte gametophytes are usually smaller and become fertile faster than those that germinate freely (Plastino, 1985; Destombe et al., 1989). The terminology “skipping diploid” was used to describe this life history, in which the epiphyte gametophytes growing on tetrasporophytes can promote greater genetic variability during their reproduction (Hughes and Otto, 1999). In addition, germination of tetraspores on tetrasporophytes may suggest a greater adaptation of the tetrasporophyte phase to the environment in relation to the gametophyte phase (Oliveira & Plastino, 1984), especially in environmental circumstances where one is favored in spite of the other (Hughes & Otto, 1999), although there is no hard evidence of this hypothesis yet.

Mutations associated with thallus color have been used in different studies, to better understand: genetics, life history, pigment characterization and photosynthetic rate estimation in *Gracilaria* spp. (van der Meer, 1979; Kursar et al., 1983; Ramus & van der Meer, 1983, Faria & Plastino, 2016; Santos, 2017). Aside from the wild phenotype, a stable and inheritable greenish-brown mutant was described for *G. caudata* in a population from Northeastern Brazil (Faria & Plastino, 2016; Santos, 2017). On the Brazilian coast, chromatic variants were also described for *G. domingensis* (Plastino et al., 1999), *G. birdiae* (Costa & Plastino, 2001), and *G. cornea* (Ferreira et al., 2006). Recently, a new green variant was found for *G. caudata.* It was identified and collected in Rio do Fogo, Rio Grande do Norte, Brazil, and raised questions about differences in physiology when compared to the wild strain.

Pulse amplitude modulation can be used to estimate the photosynthetic rate (Baker, 2008). Color variants may have a distinct pigment composition when compared to the most abundant (wild) lineage (Costa & Plastino, 2011), what might cause differences in photosynthetic potential. This approach was used to differentiate a greenish-brown variant strain of *G. caudata* from the wild strain (Faria & Plastino, 2016; Santos, 2017).

Epiphyte gametophytes growing on tetrasporophytes is a phenomenon that happens in nature and during laboratorial cultivation for both wild and green variant strains of *G. caudata*. It is hypothesized that: (i) tetraspores that germinated on tetrasporophytes develop into epiphyte gametophytes that reach reproductive maturity in a shorter amount of time than gametophytes that germinated freely; (ii) tetrasporophytes without epiphyte gametophytes show superior vegetative performance when compared to tetrasporophytes with epiphyte gametophytes; (iii) wild strain tetrasporophytes have higher pigment content, higher growth rates, and higher photosynthetic potential than individuals of the green variant strain. Thus, understanding the physiological implications of epiphyte gametophytes growing on tetrasporophytes on the wild and color variant strains of *G. caudata* can unveil the extent of the process associated with this phenomenon that has not been studied yet. To test these hypotheses in both strains, the amount of time required for the differentiation of cystocarps in free living gametophytes and epiphyte gametophytes on tetrasporophytes was evaluated, and the impacts of epiphyte gametophytes on tetrasporophytes generation were assessed by evaluating: growth rates, pigment content, and photosynthetic capacity of the tetrasporophytes. The study of the tetraspore germination

on tetrasporophytes can clarify possible distinctions between the green variant and the wild strains. It can also provide information regarding the physiology of different strains, which is relevant to optimize large-scale cultivation of *G. caudata*.

### 3.2. Material and methods

#### 3.2.1. Biologic material

The biological material used in the experiments was selected from non-axenic unialgaceous *Gracilaria caudata* J. Agardh cultures. Wild (red) and green variant cystocarpic plants were obtained from algae collected on a beach in the municipality of Rio do Fogo (RN), Brazil (05º15'41"S 35º23'11"W) on September 30$^{th}$, 2011 (SPF 57390 and 57391). Those samples were included and kept in a germoplasm bank located at the Laboratório de Algas Marinhas da Universidade de São Paulo, (Costa et al., 2012). Tetrasporophytes of *G. caudata* were obtained from the germination of carpospores from cystocarps of fertile female gametophytes. Wild (red) and color variant (green) strains produced samples that preserved their respective phenotype. Branches of female gametophytes with cystocarps were deposited in different Petri dishes filled with growth medium for approximately 4 hours. This period was enough for the cystocarp to release carpospores. Once released, carpospores were captured using a Paster pipette and then transferred to new Petri dishes filled with growth medium. They were kept under the conditions described above and had growth medium renewed weekly, until it could be confirmed that the sample is indeed a tetrasporophyte. These plantlets were then transferred to Erlenmeyer flasks with growth medium and aeration for mass increase. Penicillin G (Sigma) at 50mg/L concentration was added, when necessary, for 24 hours to eliminate bacterial contamination as described by Hoshaw and Rosowski (1973).

#### 3.2.2. Growth medium

Growth medium consisted of sterilized seawater (salinity of 32) and nutrient solution. Seawater was collected from the municipality of São Sebastião (SP), double filtered in a pressure filter (Cuno), with porosities of 5 and 1 µm and then sterilized by ionizing radiation (UV-C lamp, QUIMIS Q884-21 - 3.8L/min) (Petti & Plastino, 2012). Modified Von Stosch solution (nutrient solution) at ¼ recommended proportion was added to the sterile seawater, as described in Ursi & Plastino (2001). The medium was renewed weekly.

### 3.2.3. Temperature, irradiance, photoperiod and aeration parameters

Biological samples were maintained in a culture chamber set to the following conditions: temperature of 25±1°C, and irradiance of 70±5 μmol.m$^{-2}$.s$^{-1}$, provided by 2 "daylight" fluorescent lamps (Philips 40 W). Temperature was kept constant by a thermostat connected to an air conditioning unit. Irradiance was measured by a spherical sensor (Li-COR, model L1-193), connected to a meter (Li-COR, model L1-185). Photoperiod consisted of 14 hours of light. Constant aeration was provided by an oil-free diaphragm radial compressor (IBRAM, model C03).

### 3.2.4. Experimental design

The experiment consisted of two tests (**Figure 2**): Test 1 assessed the tetrasporophytes, and Test 2, evaluated the epiphyte gametophytes. All tests were made with both the wild and green variant, in order to compare the results of both strains. Test 1 evaluated the growth rates, fertility ratio, *in vivo* chlorophyll *a* fluorescence, phycobiliproteins and chlorophyll *a* of the tetrasporophytes, while Test 2 evaluated the fertility of the epiphyte gametophytes growing on the tetrasporophytes on the main test.

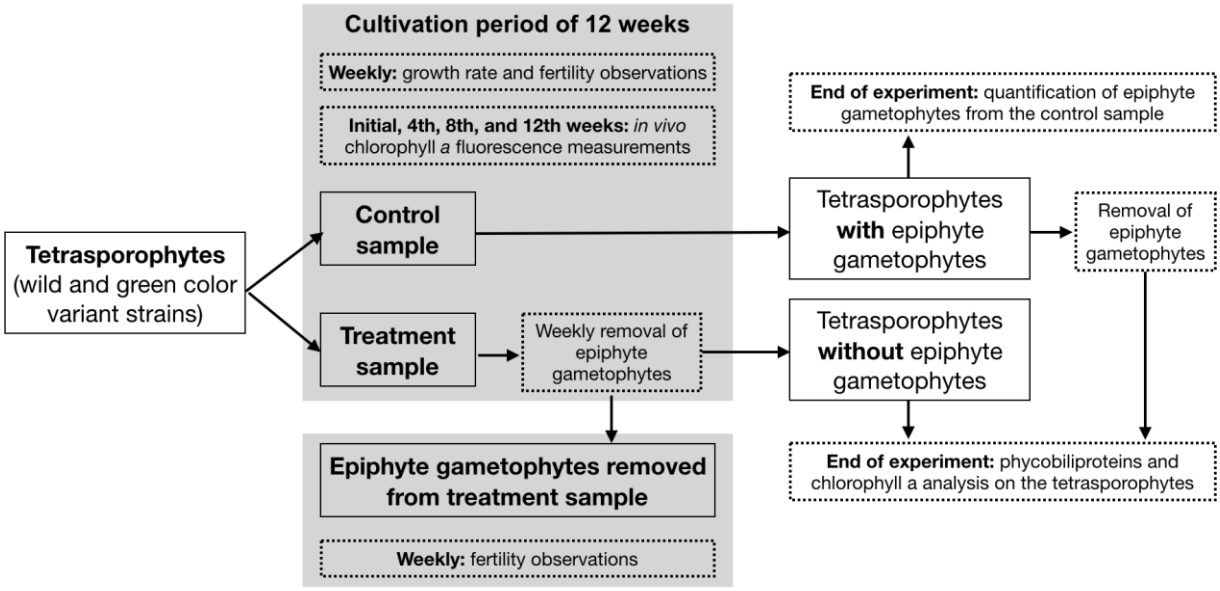


**Figure 2.** Flow chart of the experimental analysis. Solid line boxes indicate biological material, and dotted line boxes indicate tests or procedures. The experiment used wild and green variant strains of *G. caudata* (n=4). Epiphyte gametophyte test started on the 4$^{th}$ week.

For Test 1, four wild and four green variant tetrasporophytes were used. Tetrasporophytes were divided into two groups: Control and Treatment (n=4). Each sample consisted of three tetrasporophyte apical segments (approximately 1cm) that were cultivated in a 1000mL Erlenmeyer flask containing 800mL of growth medium for 12 weeks. Control samples kept their epiphyte gametophytes, while the experimental treatment samples had their epiphyte gametophytes removed weekly. Throughout the test, mass and fertility of tetrasporophytes of all groups of both strains were assessed weekly. The removal of epiphyte gametophytes growing on tetrasporophytes happened prior to the weekly mass assessment. Every 4 weeks, including the initial, *in vivo* photosynthetic rates were measured. On the $8^{th}$ and $12^{th}$ week, photosynthesis light curves were also assessed. At the end of the $12^{th}$ week, epiphyte gametophytes that grew on the control group were removed, and had their mass measured. The epiphyte gametophytes mass subtracted from the total control mass will result in the mass of the tetrasporophytes. Tetrasporophytes from control and treatment groups were frozen in nitrogen for a phycobiliproteins and chlorophyll *a* assessment. Test 2 consisted of cultivating epiphyte gametophytes removed from the treatment samples from Test 1 (n=2). They were cultivated for 8 weeks, from the $4^{th}$ until the end of the $12^{th}$ week. Samples in this test had their fertility status evaluated weekly. Additionally, fertility of epiphyte gametophytes was analyzed for control samples of Test 1 in order to make comparisons between them.

### 3.2.5. Growth rates and fertility ratio

The growth rates were assessed weekly for 12 weeks by mass measurements using a Mettler A200 analytical balance. The growth rates used in this experiment were determined by the formula [1]: where GR = growth rate; Mf = final mass; Mi = inicial mass; T = time, as described by Lignell & Pedersén (1989).

[1] GR = [(Mf / Mi) 1 / t - 1] .100%

Every week signs of tetraspore liberations by the samples in each flask were checked by observing the samples in a Leica stereomicroscope (up to 40 times magnification) (n=4). The results were plotted weekly in a spreadsheet. It was assigned 0 for absence and 1 for presence of tetraspores in the bottom of the flask. Ratio values were represented in percentage.

### 3.2.6. *In vivo* chlorophyll *a* fluorescence measurement

*In vivo* fluorescence measurements of chlorophyll were performed using Pulse Amplitude Modulation sub-aquatic fluorometer (Walz Diving-PAM). The apical part of the tetrasporophyte thallus samples were arranged over the edge of the optical fiber (Diving-F) by a magnetic sample holder clip, side by side, to prevent overlapping. All the samples were kept in the dark (<1µmol fotons.$m^{-2}$.$s^{-1}$) for 20 minutes for maximum quantum efficiency assessment, where saturation pulses of 6,100µmol fotons.$m^{-2}$.$s^{-1}$ were used. The light curve used 8 levels of irradiance, from 0 to 300µmol fotons.$m^{-2}$.$s^{-1}$, that varied slightly due to equipment calibration. The exposure time at each irradiance level was of 20s, interposed by saturation pulse with duration of 0.8s of 6.100µmol fotons.$m^{-2}$.$s^{-1}$. Maximum quantum efficiency (Y II) assessment was made using data provided by the Diving-PAM equipment. Curves estimating the photosynthetic potential were created plotting ETRr values (corrected electron transfer rate) with PAR (photosynthetic active radiation) in a scatter graph with values provided by the Diving-PAM equipment. Light curve parameters such as photosynthetic efficiency (αETR), maximum photosynthesis (Fmax) and light saturation (IK) were determined using the equations proposed by Webb et al. (1974) [2] and Jassby & Platt (1976) [3], as described and using the same terminology as Suggett et al. (2011), using a *Statistica* software module.

[2] $P = P_{(max)}\left[1 - \exp\left(\frac{-\alpha I}{P_{(max)}}\right)\right]$

[3] $P = P_{(max)} \tanh\left(\frac{\alpha I}{P_{(max)}}\right)$

### 3.2.7. Phycobiliproteins and chlorophyll *a*

Samples weighting 100mg were used to estimate the pigment content of phycobiliproteins and chlorophyll *a* for control an treatment samples of both strains (n=4). Samples were washed in distilled water and gently dried with absorbent paper and stored in a plastic 2mL Eppendorf tube at -80°C. The extraction was performed following an adaptation of the method described by Kursar et al. (1983), adapted by Plastino & Guimarães (2001). The samples were macerated in liquid nitrogen until obtaining fine powder. A frozen mortar was used in a dark environment to avoid the disintegration of the pigments by light radiation. Resulting powder from the maceration was diluted into a total of 4mL of 50mM phosphate buffer pH 5.5, followed by

centrifugation at 44,000g for 20 minutes. Supernatant containing the phycobiliproteins was removed and kept in test tubes in the dark at 4 °C until they had their absorbance measured in a spectrophotometer. To estimate the chlorophyll *a* content, 3mL of 90% acetone was added to the leftover sediment. The content was thoroughly mixed and then centrifuged at 12,000g and 4 °C for 15 minutes. The supernatant containing chlorophyll *a* was transferred to test tubes kept in the dark at 4°C until spectrophotometer reading. The absorbance measurements were made by an Epoch II spectrophotometer using 10nm optical path quartz cuvettes with a total volume of 1mL. The analyzed spectrum ranges from 400 to 700nm. The spectrophotometer was calibrated using phosphate buffer for phycobiliproteins, and 90% acetone for Chlorophyll *a.* The equations [4, 5, 6 and 7] proposed by Kursar et al. (1983) were used to determine the concentrations of phycobiliproteins and chlorophyll *a*, where *A* indicates a specific point in the absorbance spectrum.

[4] Allophycocyanin = $181,3 \times A_{651} - 22,3 \times A_{614}$

[5] Phycocyanin = $151,1 \times A_{614} - 99,1 \times A_{651}$

[6] Phycoerythrin = $155,8 \times A_{498,5} - 40 \times A_{614} - 10,5 \times A_{651}$

[7] Chlorophyll *a* = $11,85 \times A_{664} - 1,54 \times A_{647} - 0,08 \times A_{630}$

### 3.2.8. Statistics

Most physiological parameters such as growth rates, light curves and its parameters (Fmax, αETR and Ik), as well as the content of phycobiliproteins and chlorophyll *a*, were submitted to analysis of variance (one-way or repeated measures ANOVA), with a 95% confidence interval and SNK residual tests. A homoscedasticity and normality tests were performed to validate the results. Samples were considered different if $p<0.05$. Statistical determinations were made using the software Statistica (version 10).

### 3.3. Results

We observed differences in growth rates, fertility ratio, fertility of epiphyte gametophytes, light curve parameters and pigment content after the cultivation period of 12 weeks when comparing control and treatment samples from both wild and green variant strains of *G. caudata*. At the end of experiment, control samples of both wild and green variant strains were lighter in color than its counterparts.

#### 3.3.1. Growth rates, fertility ratio of tetrasporophytes and fertility in epiphyte gametophytes

There were differences among the tetrasporophytes samples mass ($F=60.0$, $p<0.01$) throughout the experiment, and control samples from wild and green variant strains had different mass measurements from the 9$^{th}$ week onwards. The average masses of the tetrasporophytes samples at the beginning and end (12$^{th}$ week) of the experiment and the mass of epiphyte gametophytes removed from the control samples were compiled in **Table 1**. Epiphyte gametophytes accounted on average for 76% of the control sample's final mass weight and 42% for the green variant. When the epiphyte gametophytes were removed from the tetrasporophytes on the control samples at the end of the experiment, the average mass of the tetrasporophytes was 0.89g for the wild strain and 1.55g for the green variant.

**Table 1.** Average mass and standard deviations of tetrasporophyte samples in the beginning and at the end (12$^{th}$ week) of the experiment (n=4). Control samples had epiphyte gametophytes growing on them during the experiment, while treatment samples had their gametophytes removed weekly from the third week onwards. EG stands for epiphyte gametophytes removed from the control samples at the end of the experiment. Different letters indicate significant differences according to Student Newman-Keuls post-hoc test ($p<0.05$). Regular and italic letters represent distinct Student Newman-Keuls post-hoc tests.

| | Initial mass | Final mass | EG mass |
|---|---|---|---|
| Wild - Control | 0.0051±0.0002g | 3.667±0.41g$^{a}$ | 2.775g±0.6g$^{a}$ |
| Wild - Treatment | 0.0050±0.0002g | 0.843±0.08g$^{c}$ | n/a |
| Variant - Control | 0.0051±0.0002g | 2.670±0.61g$^{b}$ | 1.120g±0.34g$^{b}$ |
| Variant - Treatment | 0.0052±0.0002g | 0.797±0.42g$^{c}$ | n/a |

Wild and variant tetrasporophyte strains showed differences in growth rates ($F=135.9$, $p<0.01$) (**Figure 3**). In the 2nd week, the growth rates of control and treatment samples differed for wild ($F=135.9$, $p<0.01$) and green variant samples ($F=135.9$, $p<0.01$), in which treatment samples showed higher growth rates than control samples for both strains. Wild strain control samples had higher growth rates than the wild strain treatment samples on the 8th week (16.1±1.4% and 10.0±0.7%, respectively) ($F=135.9$, $p<0.01$) and the 10th week (22.1±1.0% and 12.7±1.0%, respectively) ($F=135.9$, $p<0.01$).

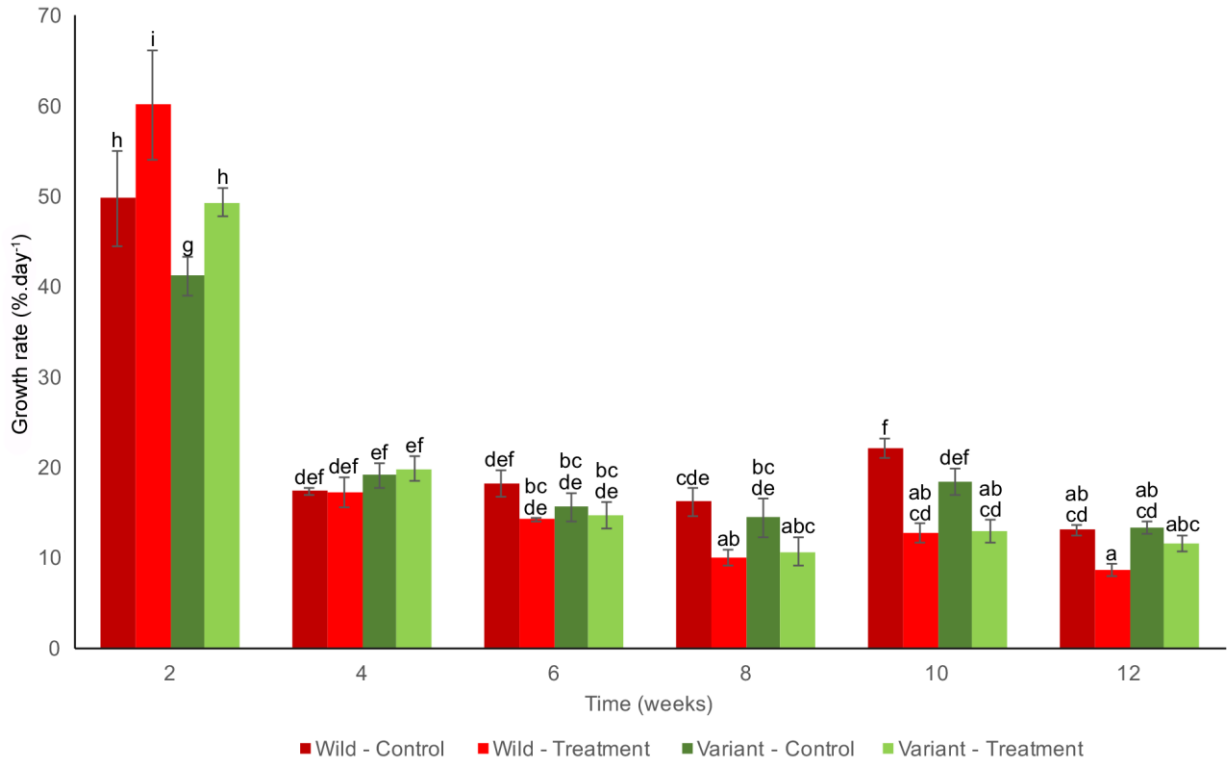


**Figure 3.** Growth rates of wild and green variant of *Gracilaria caudata* tetrasporophytes in percentage by time, biweekly for 12 weeks. Control samples had epiphyte gametophytes growing on them during the experiment, while treatment samples had their gametophytes removed weekly. Bars represent averages and standard deviations (n=4). Different letters indicate significant differences according to Student Newman-Keuls post-hoc test ($p<0.05$).

The relative growth rates were made using the initial and final (12th week) data in two scenarios (**Figure 4**). Two relative growth rates were made, one that included the epiphyte gametophytes growing on the tetrasporophytes mass for the control samples (**Figure 4.1**), and another that deducted the the epiphyte gametophytes mass, considering only the tetrasporophyte mass (**Figure 4.2**). When the epiphyte gametophytes mass of the control samples was used in the final measurement, differences were found between control and treatment growth rates for both strains

(F=28.4, p<0.01). The wild control sample exhibited higher growth rates (806.7±128.9%) than the green variant control sample (619.0±137.8%) (F=28.4, p=0.04), increasing on average 8.06 times the initial mass compared to the green variant's 6.19 times. For the tetrasporophyte growth rates without the epiphyte gametophytes mass, the wild and variant samples showed no differences between them (F=4.1, p=0.03). While the wild strain control sample tetrasporophytes without the epiphyte gametophytes increased on average 1.68 times, the green variant increased 2.89 times.

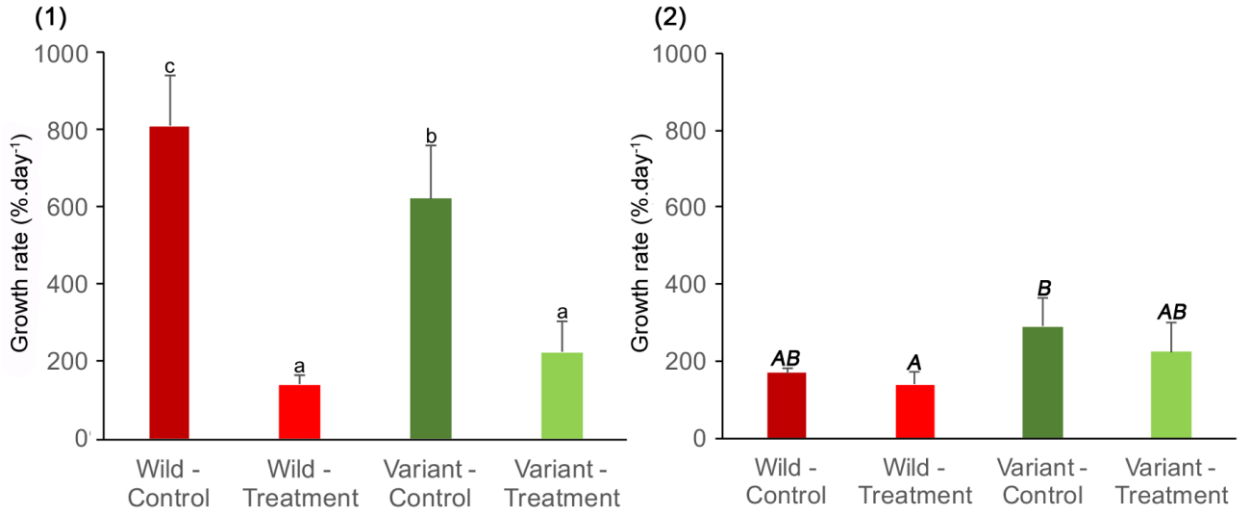


**Figure 4.** Relative growth rates of wild and green variant *Gracilaria caudata* tetrasporophyte strains in percentage by time, obtained using initial and final (12th week) mass measurements. **(1)** Control samples with epiphyte gametophytes growing on them during the experiment, while treatment samples had their gametophytes removed weekly. **(2)** Control samples had the respective epiphyte gametophyte weight deducted, leaving the tetrasporophyte mass only (removal of the epiphyte gametophytes growing on them happened at the 12th week), while treatment samples had their gametophytes removed weekly. Bars represent averages and standard deviations (n=4). Different letters indicate significant differences according to Student Newman-Keuls post-hoc test (p<0.05). Lowercase and uppercase letters indicate different post-hoc tests.

Tetrasporophytes of the wild strain have shown evidence of fertility throughout most of the experiment (**Figure 5**). There was no evident difference between control and treatment samples on this strain. The variant strain tetrasporophytes showed reduced fertility ratio up to the 6th week when compared to the wild strain. After this period, the fertility ratio was similar to the wild strain. After the 6th week, the green variant treatment samples showed a greater fertility ratio than the control samples of the same strain, and the ratio shifted on the 10th week, returning the previous trend on the

final week. Both wild and green variant strain epiphyte gametophytes had cystocarps on the 6th week. In general, epiphyte gametophytes growing on the tetrasporophytes reach fertility one or two weeks before (4 to 5 weeks) the ones that were removed and cultivated in separate (5 to 6 weeks).

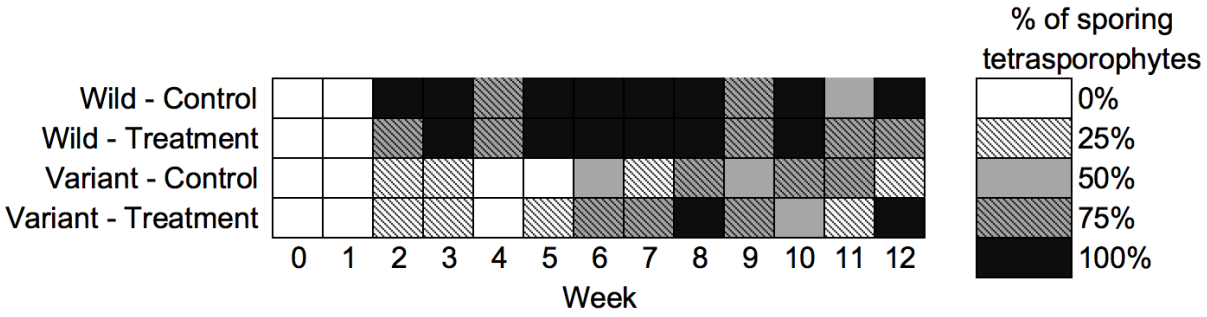


**Figure 5.** Fertility ratio of wild and green variant tetrasporophytes of *Gracilaria caudata* samples in the 12-week cultivation period, measured by observations of tetraspore liberation in the samples' cultivation flasks. Control samples had epiphyte gametophytes growing on them during the experiment, while treatment samples had their gametophytes removed weekly from the third week onwards (n=4).

### 3.3.2. Quantification of pigments: allophycocyanin, phycocyanin, phycoerythrin and chlorophyll *a*

After the cultivation period of 12 weeks, control (at this moment without the epiphyte gametophytes that grew on the tetrasporophyte, in order to evaluate exclusively the tetrasporophyte's pigment content) and treatment samples of the wild and green variant strains had their pigment content analyzed (**Figure 6**, **Figure 7**).

Allophycocyanin content was smaller in the control wild strain (29.94±8.94µg/g) when compared to the control green strain (84.81±10.69 µg/g) ($F=72.4$, $p<0.01$), and the treatment samples had higher allophycocyanin content than their respective control samples: wild strain (158.42±39.70µg/g) ($F=72.4$, $p<0.01$) and green variant (281.06±13.95µg/g) ($F=72.4$, $p<0.01$), making all the samples distinct among each other ($F=72.4$, $p<0.01$).

Phycocyanin content of the wild control (74.70±30.38µg/g) was smaller than of the treatment samples (200.92±34.25µg/g) ($F=11.7$, $p<0.01$). The same happened on the variant control samples (94.75±28.35µg/g) that were smaller than the treatment samples of the same strain (186.21±9.40µg/g) ($F=11.7$, $p<0.01$), rendering control samples on both cases different from the treatment samples, but not different between strains ($F=11.7$, $p<0.01$).

Phycoerythrin content of the wild control (124.34±48.29µg/g) and the wild treatment samples (181.36±31.38µg/g) were both smaller than the variant control samples (379.58±86.05µg/g) ($F=52.6$, $p<0.01$). The variant treatment samples had the highest phycoerythrin content (635.50±37.90µg/g) ($F=52.6$, $p<0.01$). It was possible to separate these differences in three groups: wild control and treatment samples, variant control samples, and variant treatment samples ($F=52.6$, $p<0.01$).

Chlorophyll *a* content was greater in the wild samples for the control (105±19.19µg/g) and treatment samples (151.81±9.70µg/g) ($F=25.5$, $p<0.01$), than for the green variant control (62.65±4.15µg/g) ($F=25.5$, $p<0.01$) and treatment samples (76.42±15.78µg/g) ($F=25.5$, $p=0.02$). These results pointed out differences between the wild control and treatment samples, wild control and green variant samples, and wild treatment and green variant samples ($F=25.5$, $p<0.01$).

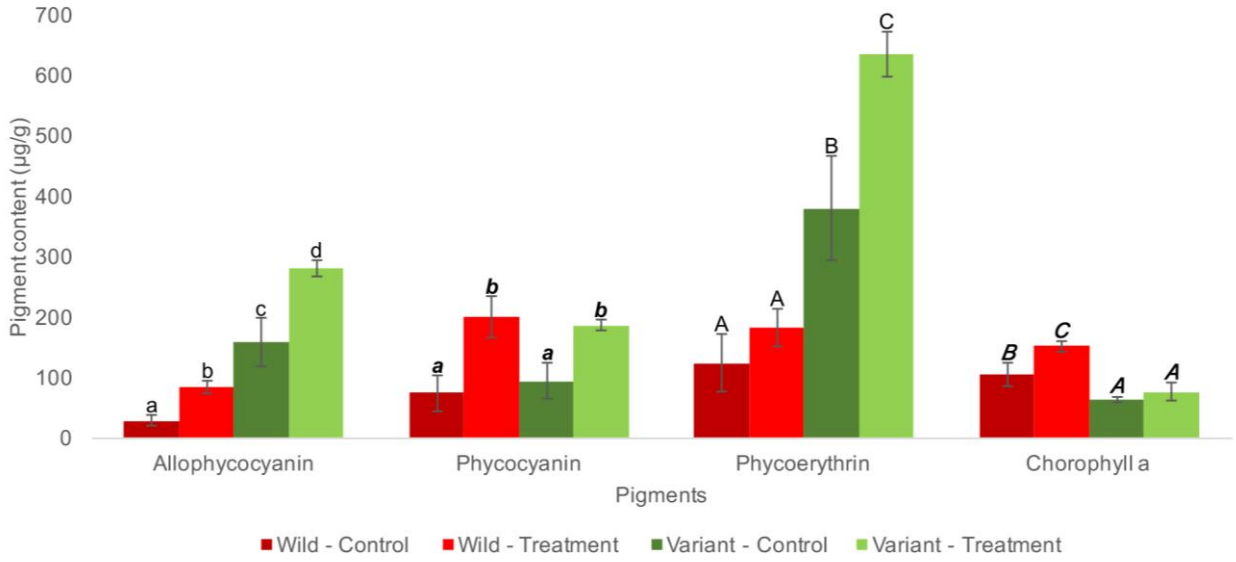


**Figure 6.** Pigment content in micrograms per gram of mass of wild and green variant of *Gracilaria caudata* tetrasporophytes on the 12th week. Control samples had epiphyte gametophytes growing on them during the experiment, while treatment samples had their gametophytes removed weekly. Only the tetrasporophyte portions were used in this analysis. Bars represent averages and standard deviations (n=4). Different letters indicate significant differences according to Student Newman-Keuls post-hoc test ($p<0.05$). Regular lowercase, bold and italic lowercase, regular uppercase, and uppercase bold and italic represent different post-hoc tests.

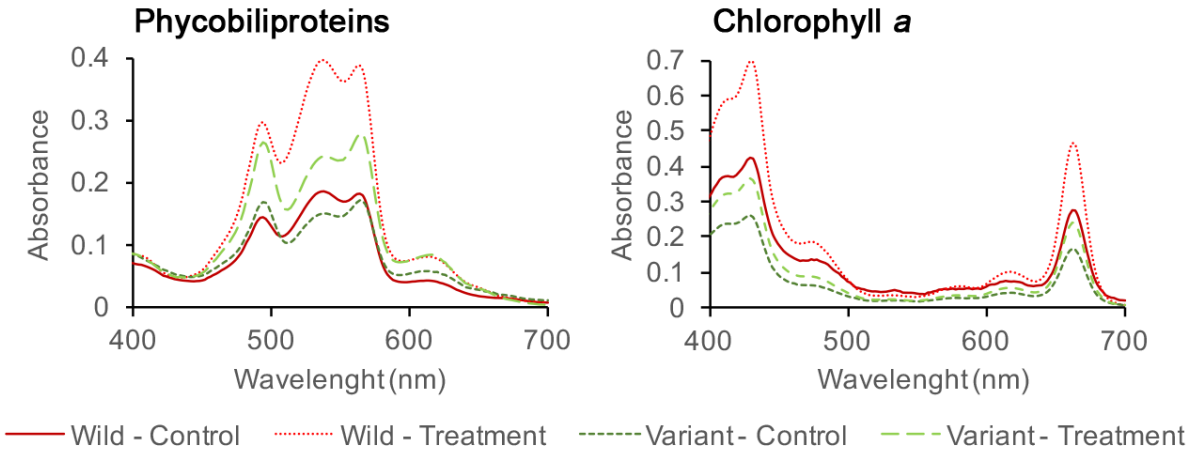


**Figure 7.** Absorption spectra of aqueous (Phycobiliproteins) and acetone (Chlorophyll *a*) of a wild and a green variant strains tetrasporophytes of *G. caudata* on the 12th week. Each line represents the strain average (n=4)

### 3.3.3. *In vivo* chlorophyll *a* fluorescence assessment

The maximum quantum efficiency (Y II) assessment of wild and green variant strains was plotted on a graph (**Figure 8**). There were differences between the wild and variant strains on the day the experiment started, in which the wild control (0.36±0.01) and treatment values (0.40±0.06) were greater than the green variant control (0.29±0.03) ($F=30.4$, $p<0.01$) and treatment values (0.32±0.02) ($F=30.4$, $p<0.01$). Another difference was found between the wild treatment sample (0.60±0.00) and the green variant treatment sample (0.54±0.02) on the 8th week, in which the wild treatment showed a greater Y(II) value ($F=30.4$, $p=0.04$). For each individual sample, it was possible to notice a build up in the maximum quantum efficiency values from the initial to 4th week and following the 8th week, when it reaches a peak, followed by a decrease on the 12th week.

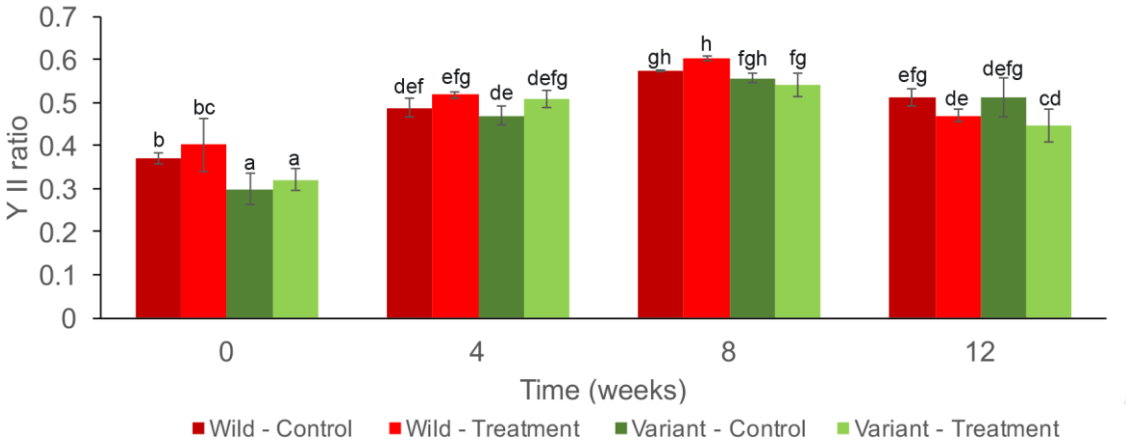

**Figure 8.** Maximum quantum efficiency assessment of wild and green variant tetrasporophytes of *Gracilaria caudata*. Control samples had epiphyte gametophytes growing on them during the experiment, while treatment samples had their gametophytes removed weekly. Bars represent averages and standard deviations (n=4). Different letters indicate significant differences according to Student Newman-Keuls post-hoc test ($p<0.05$).

Light curves were compiled in **Figure 9**. The 8th week wild strain light curve showed differences at 17 ($F=97.5$, $p<0.01$), 35 ($F=97.5$, $p<0.01$) and 55µmol fotons.$m^{-2}$.$s^{-1}$ irradiance levels ($F=97.5$, $p<0.01$) between control and the higher values of treatment samples, and reached a plateau after 100 µmol fotons.$m^{-2}$.$s^{-1}$. On the other hand, the 8th week variant strain light curve showed no differences between control and treatment samples at each individual level ($F=81.3$, $p<0.01$), and reached a plateau after 150 µmol fotons.$m^{-2}$.$s^{-1}$. Light parameters determined from the 8th week light curves were compiled in **Table 2**.The maximum electron transfer rate (mETR) values did not show differences among them for any of the analysed samples ($F=2.0$, $p=0.15$), while the α values showed differences between the control (0.03±0.00) and treatment (0.048±0.01) samples of the wild strain ($F=8.5$, $p=<0.01$), and between the wild treatment samples (0.048±0.01) and the variant treatment samples (1.431±0.23) ($F=8.5$, $p=<0.01$). The light saturation (IK) values showed differences between the lower values of treatment samples of the wild strain (28.41±.9.99) when compared to the variant strain (49.36±12.24) ($F=5.8$, $p=0.03$). The wild strain 12th week light curve showed no differences between control and treatment samples ($F=25.2$, $p<0.01$), and reached a plateau shortly after 100 µmol fotons.$m^{-2}$.$s^{-1}$. The same was observed on the 12th week variant strain light curve that showed no differences between control and treatment samples at each individual level ($F=65.0$, $p<0.01$), and displayed a plateau shortly before 150 µmol fotons.$m^{-2}$.$s^{-1}$. Light parameters determined from the 12th week light curves were compiled in **Table 3**. The mETR values did not show differences between control and variant treatments ($F=4.1$, $p=0.03$). There was a difference for α values between the lower wild strain value of control samples (0.02±0.00) when compared the higher treatment value (0.04±0.01) ($F=4.0$, $p=0.02$). All of the IK values showed no differences between them ($F=1.0$, $p=0.39$).

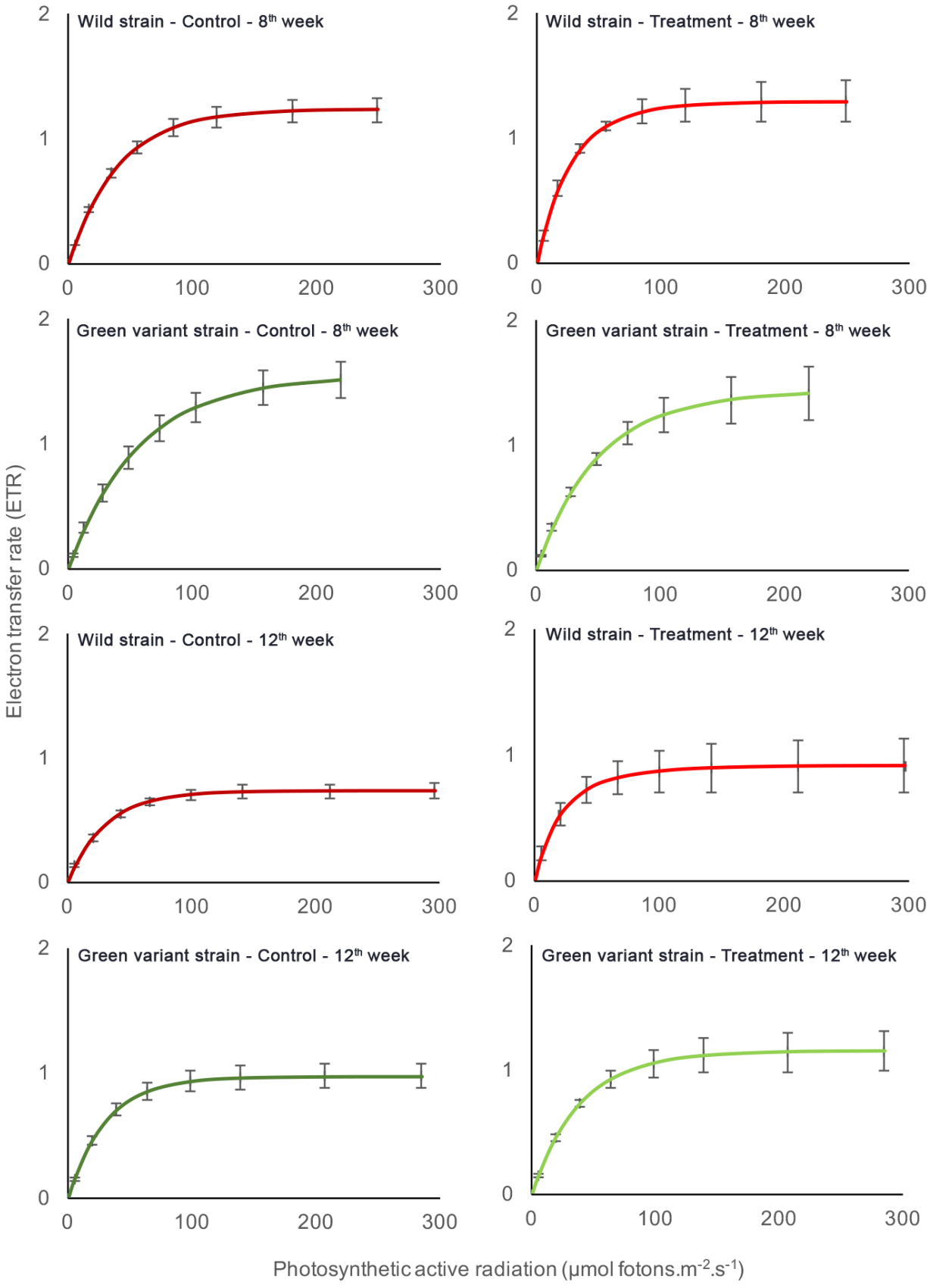


**Figure 9.** Electron transfer rate per photosynthetic active radiation of wild and green variant tetrasporophytes of *Gracilaria caudata* at the 8th and 12th week. Individual points plotted in the curve represent the sample's average and standard deviations at a

determined photosynthetic active radiation level (n=4). Control samples had epiphyte gametophytes growing on them during the experiment, while treatment samples had their gametophytes removed weekly.

**Table 2.** Average and standard deviations of light curve parameters of the 8th week light curve of wild and green variant tetrasporophytes of *G. caudata* (n=4). Control samples had epiphyte gametophytes growing on them during the experiment, while treatment samples had their gametophytes removed week. Different letters indicate significant differences according to Student Newman-Keuls post-hoc test ($p<0.05$). Regular, italic and bold letters represent distinct Student Newman-Keuls post-hoc tests.

| | **mETR** | **α** | **IK** |
|---|---|---|---|
| **Wild - Control** | 1.232±0.097 $^{a}$ | 0.031±0,001 $^{a}$ | 39.614±3.284 $^{\mathbf{ab}}$ |
| **Wild - Treatment** | 1.290±0.166 $^{a}$ | 0.048±0.01 $^{b}$ | 28.416±9.994 $^{\mathbf{a}}$ |
| **Variant - Control** | 1.544±0.155 $^{a}$ | 0.028±0.004 $^{a}$ | 56.841±8.271 $^{\mathbf{a}}$ |
| **Variant - Treatment** | 1.431±0.232 $^{a}$ | 0.030±0.003 $^{a}$ | 49.369±12.346 $^{\mathbf{a}}$ |

**Table 3.** Average and standard deviations of light curve parameters of the 12th week light curve of wild and green variant tetrasporophytes of *G. caudata*(n=4). Control samples had epiphyte gametophytes growing on them during the experiment, while treatment samples had their gametophytes removed weekly. Different letters indicate significant differences according to Student Newman-Keuls post-hoc test ($p<0.05$). Regular, italic and bold letters represent distinct Student Newman-Keuls post-hoc tests.

| | **mETR** | **α** | **IK** |
|---|---|---|---|
| **Wild - Control** | 0.729±0.062 $^{a}$ | 0.024±0.002 $^{a}$ | 30.249±4.972 $^{\mathbf{a}}$ |
| **Wild - Treatment** | 0.913±0.213 $^{ab}$ | 0.043±0.012 $^{b}$ | 25.228±14.9 $^{\mathbf{a}}$ |
| **Variant - Control** | 0.972±0.1 $^{ab}$ | 0.033±0.003 $^{ab}$ | 30.065±4.086 $^{\mathbf{a}}$ |
| **Variant - Treatment** | 1.148±0.165 $^{b}$ | 0.031±0.004 $^{ab}$ | 38.402±8.453 $^{\mathbf{a}}$ |

### 3.4. Discussion

Higher growth rates of *Gracilaria caudata* were found for the wild strain in comparison to the green variant strain only on the 2nd week of the experiment. Reproduction affected negatively the growth rates (Faria & Plastino, 2016) and differences between wild and color strains were less pronounced as cultivation time increased. It was recommended to evaluate growth rates of the wild and color variant strains before tetrasporophytes become fertile, as previously done when comparing a wild and a greenish-brown variant of *G. caudata* (Faria & Plastino 2016; Santos 2017). In fact, presence or absence of epiphyte gametophytes on tetrasporophytes showed no differences in relative growth rates, that used initial and final mass (12th week) of the tetrasporophytes only, for both wild and green variant strains. Color variants of other *Gracilaria* spp. also showed no differences in growth rates to their respective wild strains, such as *G. birdiae* (Ursi & Plastino, 2001) and *G. cornea* (Ferreira et al., 2006), despite having differences in pigment content between the wild and the color variant strains.

Wild tetrasporophytes contributed less for the final growth rates than for the green variant strain, therefore the greater final mass of the wild strain when compared to the green variant of *G. caudata* is attributed to the epiphyte gametophytes mass. The green variant took longer to develop epiphyte gametophytes in all the samples, so their tetrasporophytes had more time to grow without fertility interference. Furthermore, the wild strain had more ramifications than the green variant. Another variant of *G. caudata* (greenish-brown) had also fewer ramifications than their wild counterpart (Santos 2017), and similar results were found to *G. birdiae*, when comparing a wild and a light green variant (Ursi, 2005).

Throughout the experiment, the wild strain of *Gracilaria caudata* showed higher fertility ratio than the green variant, with all tetrasporophyte samples being fertile on the 3rd week for both control and treatment samples. The green strain control samples did not reach fertility on all samples at the same time, while the treatment samples reached fertility on all the flasks only on the 8th and 12th week. In our experiment, the fertility ratio of the tetrasporophytes did not seem to be the only driving factor that reduced the growth rates, as the green variant does not necessarily show greater growth rate after the 2nd week of cultivation. Therefore, it could have reduced the growth rates of the wild strain, that was fertile through most of the experiment.

Female epiphyte gametophytes growing on *Gracilaria caudata* tetrasporophytes produced cystocarps one week before gametophytes that were removed from the treatment sample and cultivated in separate flasks. We suggest similar that a similar pattern should happen in nature. Furthermore, the proximity between male and female epiphyte gametophytes in the tetrasporophyte could increase the mating chances, as the spermatia produced by the male gametophytes do not have flagella. Tetrasporophytes are more frequent than gametophytes in natural populations of *G. caudata* (Ayres-Ostrock, 2014). This way, epiphyte gametophytes are able to promote variability by sexual reproduction while still attached to the more environmentally fit tetrasporophytes. Tetrasporophytes with epiphyte gametophytes represent a distinct life strategy when compared to free tetrasporophytes and gametophytes (Otto & Hughes, 1999).

Tetrasporophytes of the green variant strain of *Gracilaria caudata* produced a higher ratio of allophycocyanin and phycoerythrin per gram of tetrasporophyte than the wild strain; otherwise, the wild strain produced more chlorophyll *a* than the green strain. Treatment samples yielded a greater ratio of allophycocyanin and phycocyanin per gram of tetrasporophyte when compared to their respective control samples. However, we have to consider that this analysis took place at the end of 12 weeks of cultivation, and fertility, alongside the amount of epiphyte gametophytes per tetrasporophyte, could have impacted pigment content.

At the 12$^{th}$ week, the control group wild tetrasporophytes were lighter in color, what indicated stress associated to gametophytes growing on them when compared to the treatment samples. In contrast, the control samples of the green variant strain were not lighter in color when compared to the treatment samples. Faria & Plastino (2016) and Santos (2017) also compared phycobiliproteins of wild and greenish-brown variant *G. caudata* tetrasporophytes in similar abiotic conditions. Santos (2017) cultivated tetrasporophytes for 2 weeks, while Faria & Plastino (2016) for 28 days in similar irradiance, but with double the amount of modified Von Stosch solution. Comparing our wild strain results with both papers, we found lower values of phycoerythrin, probably due to the length of our experiment.

The highest chlorophyll *a* content observed in wild strain of *Gracilaria caudata* might have compensated the lower phycobiliprotein content, particularly phycoerythrin. Our results were different from the ones that analyzed *G. caudata* population from the state of Ceará, as they found no differences between the wild and greenish-brown

variant for chlorophyll *a* content (Faria & Plastino 2016; Santos 2017). Depletion of the phycoerythrin in our wild strain tetrasporophyte samples could indicate that the wild strain was using part of its phycoerythrin content as source of nitrogen, as previously suggested for other Rhodophyta (García-Sanchez et al., 1993). We also hypothesized that the increased amount of epiphyte gametophyte holdfasts attached to the tetrasporophytes might have casted permanent shadow on the tetrasporophyte thallus, decreasing photosynthetic activity in the more basal parts of the tetrasporophytes. There is evidence of reduced activity on the most external layer of the cortex of the tetrasporophyte, under the epiphyte gametophyte's holdfast anchoring region, using fluorescence microscopy, discussed in Chapter II.

The presence of epiphyte gametophytes did not result in differences, with the exception of three photosynthetic active radiation levels (17, 35 and 55µmol fotons.$m^{-2}$.$s^{-1}$) in the 8$^{th}$ week for the wild tetrasporophytes of *Gracilaria caudata*. The light curve parameters were also unable to further explain differences between the wild and green variant strains nor control and treatment samples, with the exception of the α values, that were higher for the wild treatment samples in comparison to the control samples in the 8$^{th}$ and 12$^{th}$ week. The α values are associated to photosynthetic efficiency in are derived from the initial angle of the ETR curves. The treatment samples of the wild strain reached higher ETR than the control samples at the same irradiance level, suggesting greater efficiency at low irradiance levels. When the same analysis was done to another population of *G. caudata* from the state of Ceará, no differences in light curves between wild and greenish-brown variant strains were found (Faria & Plastino 2016). *G. birdiae* also did not show differences in light curves between the wild and green variant strains (Ayres-Ostrock 2014). In contrast, Santos (2017) found different α and Ik values between wild and greenish-brown variant tetrasporophytes of *G. caudata* after 14 days of cultivation. The control samples of both wild and green variant strains in our experiment showed a decrease in light saturation between the 8$^{th}$ and 12$^{th}$ weeks, as the number of epiphyte gametophytes increased in the samples. Light curve parameters were expected to lower as cultivation time progresses, as Santos (2017) observed for *G. caudata*. Our results for light curve parameters were higher in the 8$^{th}$ than in the 12$^{th}$ week, following the expected pattern.

The extended length of our experiment (12 weeks) contributed to mask differences between tetrasporophytes of the wild and green variant strain of *Gracilaria caudata*. Furthermore, the biomass per liter ratio was higher in the control samples than

the treatment samples, which could have limited the amount of nutrients available for the control samples, particularly for the wild control group at the end of experiment. Plasticity of the photosynthetic units might also have contributed to an acclimation process (Faria & Plastino, 2016). The energy costs associated with reproduction might have impacted the growth rates, as suggested for *G. verrucosa* (Kain & Destombe, 1995). Control sample growth rates were higher than treatment samples, suggesting the part of energy produced by tetrasporophytes was destined to promote or maintain the growth of epiphyte gametophytes attached to their thallus, at least in the beginning of development. This becomes evident when comparing the final mass and growth rates of the control and the treatment samples. Tetrasporophytes were supposed to have reduced growth rates once the fertilization periods start, as suggested by Kain & Destombe (1995). For cultivation purposes, tetrasporophytes of the wild strain of *G. caudata* yielded greater mass with epiphyte gametophytes than the variant strain, making it a more attractive option. Otherwise, the green variant concentrated a greater amount of phycobiliproteins in comparison to the wild strain, particularly phycoerythrin, making it a better choice for harvesting those substances.

## 4. Chapter II: Germination of tetraspores on tetrasporophytes on wild and green variant strains of *Gracilaria caudata* (Gracilariales, Rhodophyta): a morphological and anatomical assessment


### Abstract

*Gracilaria caudata* J. Agardh is one of the species in Brazil that produce good quality agar. A green variant was discovered in a *G. caudata* population of predominantly red (wild) tetrasporophytes in Rio do Fogo (RN), Brazil. Epiphyte gametophytes on tetrasporophytes have been reported for both strains. Considering both strains, this study assessed morphological and anatomical aspects of epiphyte gametophytes by: quantifying epiphyte gametophytes produced by tetrasporophyte, and analyzing anatomical aspects of the intersection between the tetrasporophytes and the tetraspores germinating on tetrasporophytes. A cultivation period of 12 weeks allowed documentation of the development. Anatomy analysis used light microscopy and auto-fluorescence analyses. The amount of epiphyte gametophytes on tetrasporophytes is not different between strains. Epiphyte gametophytes were visible one week before on the wild strain than in the green variant strain. It was found that tetraspores give rise to epiphyte gametophytes through two distinct germination methods: outside or inside the thallus of the tetrasporophyte. Germination outside the tetrasporophyte thallus, more common, produced visible holdfasts and promoted the increase of cell rows in the cortical region of the tetrasporophyte. Germination inside the tetrasporophyte thallus did not show evidences of holdfasts nor cortex thickening on the tetrasporophytes. Epiphyte gametophytes holdfasts showed adjacent cells to the cortex of the tetrasporophytes when they germinate outside the thallus; however, when germination occurs inside the thallus, the connection interface was closer to the medulla of the tetrasporophyte. Epiphyte gametophytes might represent a new life strategy that promotes sexual variability in a population mostly composed of tetrasporophytes.


### 4.1. Introduction

Cultivation and exploitation of seaweeds are relevant to the economy of countries, particularly the ones that have a coast (FAO, 2016). Agar is one of the most relevant substances that can be harvested from seaweeds, and *Gracilaria* spp. is a great source due to its rapid growth (Kain & Destombe, 1995). This genus has 184 confirmed species (Lyra et al., 2015; Algaebase, 2017). Records for *G. caudata* can be

found for most of the Brazilian coast (Plastino & Oliveira, 1997). Agar production using *G. caudata* is economically feasible, and similar to the one imported by Brazil (Yoshimura, 2006).

*Gracilaria caudata* has an erect and cylindrical thallus up to 34 cm in length by 1.7 mm in thickness. It also shows up to fifth order branches, pseudoparenchymal thallus, and microcystidiate cell organization. Its cortex is formed by two pigmented layers, with slightly elongated cells. Tetrasporophytes produce scattered tetrasporangia in its thallus adjacent to anticlinally elongated cortical cells. Tetrasporangia are cruciately divided and measure 21.8 by 39.5 µm (Plastino & Oliveira, 1997). *G. caudata* life history has three phases: a diploid tetrasporophyte, a haploid gametophyte and a diploid carposporophyte (Plastino & Oliveira, 1997), and the free generations (gametophyte and tetrasporophyte) are isomorphic (Kain & Destombe, 1995).

Tetrasporophytes are the dominant phase of many *Gracilaria* spp. in nature (Destombe et al., 1989), as they might have a greater adaptation to the environment than gametophytes (Oliveira & Plastino, 1984). The *G. caudata* population used in this study is from Rio do Fogo, Rio Grande do Norte (RN), Brazil, and is composed of 87.62% of tetrasporophytes (Ayres-Ostrock, 2014). Tetraspore retention on tetrasporophytes can allow gametophytes to grow as epiphytes and reach sexual maturity earlier than gametophytes that germinate freely (Plastino & Oliveira, 1988; Destombe et al., 1989; Hughes & Otto, 1999). Gametophytes developing as epiphytes on tetrasporophytes were observed in *G. tikvahiae* (Bird et al*.,* 1977), *G. cornea* (as *G. debilis*) (Oliveira & Plastino, 1984), *G. gracilis (*Destombe et al*.,* 1989) and *G. caudata* (Ayres-Ostrock, 2014). A population analysis of *G. caudata* of the Brazilian coast described the occurrence of epiphyte gametophytes on the tetrasporophytes, being more frequent in populations from the States of Ceará, Rio Grande do Norte, Paraiba, Pernambuco and Bahia, when compared to populations of the States of Espírito Santo and São Paulo (Ayres-Ostrock, 2014). Gametophytes that germinate freely are larger than epiphyte gametophytes growing on tetrasporophytes (Plastino, 1985; Destombe et al., 1989). Epiphyte gametophytes growing on tetrasporophytes can represent a change in the life strategy for propagation and distribution of the species (Kain & Destombe, 1995), and “skipping diploid” was the term suggested by Hughes and Otto (1999) for this chimera stage.

Although epiphyte gametophytes in tetrasporophytes were described before to *Gracilaria* sp. (Kain & Destombe, 1995), no detailing and understanding some key

components of the process could be found, such as number of epiphyte gametophytes and whether or not they had a connection to their host tetrasporophytes. Destombe et al. (1989) described the process on *G. verrucosa* as an aberration often observed on diploid individuals, particularly on the base portion of the individual. Costa & Plastino (2001) reported the difficulty of understanding the *in situ* germination in *G. birdiae*, referring to the process in which a tetraspore germinates *in situ*, in that case, inside the tetrasporangium.

Color variants have been the subject of genetics, life history, pigment quantification and photosynthetic analysis studies in the past (van der Meer, 1979; Kursar et al., 1983; Ramus & van der Meer, 1983). Considering *Gracilaria* species that occur on the Brazilian coast, stable chromatic variants were described for *G. domingensis* (Plastino et al., 1999), *G. cornea* (Ferreira et al., 2006), *G. birdiae* (Costa & Plastino, 2011), and *G. caudata* (Faria & Plastino, 2016). Besides the color change, the greenish-brown variant strain of *G. caudata* produces fewer ramifications than their wild counterparts (Santos, 2017).

A green variant of *Gracilaria caudata* was recently found in Rio do Fogo (RN). It is hypothesized that epiphyte gametophytes of wild and green color variant strains establish cellular connections with the tetrasporophytes, and the amount of epiphyte gametophytes that grow on the tetrasporophytes is greater in the wild strain than the color variant strain. In order to test these hypothesis, the anatomical aspects of the intersection between germinating tetraspores or epiphyte gametophytes and the host tetrasporophytes was analyzed, and the number of epiphyte gametophytes produced by tetrasporophytes of the wild and green color variant strain was quantified.

## 4.2. Material and methods

### 4.2.1. Biologic material

The biological material used in the experiments was selected from non-axenic unialgaceous *Gracilaria caudata* J. Agardh cultures. Wild (red) and green variant cystocarpic plants were obtained from algae collected on a beach in the municipality of Rio do Fogo (RN), Brazil (05º15'41"S 35º23'11"W) on September 30$^{th}$, 2011 (SPF 57390 and 57391). Those samples were included and kept in a germoplasm bank located at the Laboratório de Algas Marinhas da Universidade de São Paulo, (Costa et al., 2012). Tetrasporophytes of *G. caudata* were obtained from the germination of carpospores from cystocarps of fertile female gametophytes. Wild (red) and color variant (green) strains produced samples that preserved their respective phenotype. Branches of female gametophytes with cystocarps were deposited in different Petri dishes filled with growth medium for approximately 4 hours. This period was enough for the cystocarp to release carpospores. Once released, carpospores were captured using a Paster pipette and then transferred to new Petri dishes filled with growth medium. They were kept under the conditions described above and had growth medium renewed weekly, until it could be confirmed that the sample is indeed a tetrasporophyte. These plantlets were then transferred to Erlenmeyer flasks with growth medium and aeration for mass increase. Penicillin G (Sigma) at 50mg/L concentration was added, when necessary, for 24 hours to eliminate bacterial contamination as described by Hoshaw and Rosowski (1973).

### 4.2.2. Growth medium

Growth medium consisted of sterilized seawater (salinity of 32) and nutrient solution. Seawater was collected from the municipality of São Sebastião (SP), double filtered in a pressure filter (Cuno), with porosities of 5 and 1 µm and then sterilized by ionizing radiation (UV-C lamp, QUIMIS Q884-21 - 3.8L/min) (Petti & Plastino 2012). Modified Von Stosch solution (nutrient solution) at ¼ recommended proportion was added to the sterile seawater, as described in Ursi & Plastino (2001). The medium was renewed weekly.

### 4.2.3. Test design

Wild and green color variant tetrasporophyte strains of *Gracilaria caudata* were used in this test, totalizing 8 samples (n=4). The initial samples had the same weight at the beginning of the experiment (0.005g), but slightly different thallus sizes: 1.2 cm to 1.6 cm, regardless strains. Each sample grew individually in 1000mL Erlenmeyer flasks containing 800mL of growth medium for 12 weeks. All samples were photographed weekly to keep track of development. At the end of the cultivation period, the number of epiphyte gametophytes growing on the tetrasporophytes was quantified for both wild and green color variant strains (n=4). The stereomicroscopy analysis took place, before the light microscopy analysis, focusing on the areas where epiphyte gametophytes occurred. Microscopy analysis focused on epiphyte gametophytes growing on the tetrasporophytes in different development stages of epiphyte gametophytes. Younger epiphyte gametophytes were found on the medium portion of the thallus, while mature epiphyte gametophytes from the base portion.

### 4.2.3. Growth medium

Growth medium consisted of sterilized seawater (32 PSU salinity) and nutrient solution. Seawater was collected from the municipality of São Sebastião – SP, double filtered in a pressure filter (Cuno), with porosities of 5 and 1 µm and then sterilized by ionizing radiation (UV-C lamp, QUIMIS Q884-21 - 3.8L/min) (Petti & Plastino, 2012). Modified Von Stosch solution (nutrient solution) at ¼ recommended proportion was added to the sterile sea water, as described in Ursi & Plastino (2001). The medium was renewed weekly.

### 4.2.4. Temperature, irradiance, photoperiod and aeration parameters

Biological samples were maintained in a culture chamber set to the following conditions: temperature of 25±1°C, and irradiance of 70±5 $\mu mol.m^{-2}.s^{-1}$, provided by 2 "daylight" fluorescent lamps (Philips 40 W). Temperature was kept constant by a thermostat connected to an air conditioning unit. Irradiance was measured by a spherical sensor (Li-COR, model L1-193), connected to a meter (Li-COR, model L1-185). Photoperiod consisted of 14 hours of light. Constant aeration was provided by an oil-free diaphragm radial compressor (IBRAM, model C03).

### 4.2.5. Photography and stereomicroscopy

All samples were photographed weekly using a point-and-shoot Sony camera, attached to a tripod. The samples were positioned in a radiography viewer that used fluorescent lights. The camera was attached to a tripod, 15 cm away from the samples. At the end of the experiment, samples were photographed in a stereomicroscope Zeiss. The stereomicroscope's camera was attached to a computer that processed and captured the figures. Light was provided by Zeiss LED lights.

### 4.2.6. Light microscopy

Portions of tetrasporophytes of 10 to 20 mm showing gametophytes germinating on the tetrasporophytes were fixed in 2.5% glutaraldehyde in 0.1M sodium phosphate buffer pH 7.2 for 24 hours at 4°C. After this, the material was washed in the same buffer, dehydrated in a graded series of ethanol and embedded in glycolmethacrylate (GMA) (Leica Microsystems, Wetzlar, Germany). Transverse sections were performed in a Leica RM2145 rotary microtome with thicknesses of 5 to 10µm. The slides were stained with toluidine blue (O'Brien et al., 1964), mounted in tapper water and analyzed in a Leica DMLB light microscope.

### 4.2.7. Fluorescence microscopy

Fresh samples from both red and green strains were finely sectioned transversally using a free-hand razor blade. The cuts were mounted in a temporary slide using seawater. The samples were analyzed under ultraviolet light in a Leica DMLB fluorescence microscope (Gouveia et al., 2013).

### 4.2.8. Statistics

The number of epiphyte gametophytes removed from the samples of the wild and the green color variant strain were submitted to analysis of variance (one-way ANOVA), with a 95% confidence interval and SNK residual tests. A homoscedasticity and normality tests were performed to validate the results. The terminology "different" implies that $p < 0.05$. Statistical determinations were made using Statistica (version 10).

### 4.3. Results

#### 4.3.1. Development of epiphyte gametophytes on tetrasporophytes

Weekly photos were taken so the germination of epiphyte gametophytes on tetrasporophytes could be registered. Sequential photos show the development of epiphyte gametophytes growing on the tetrasporophytes of wild and green color variant strains of *G. caudata* during 12 weeks of the experimental period (**Figure 10**,**Figure 11**). The initial samples had the same weight at the beginning of the experiment, but slightly different thallus sizes: 1.2 cm to 1.6 cm (**Figures 10.1**, **11.1**). Ramifications could be observed on the 2nd week (**Figures 10.2**, **10.3**, **11.2**, **11.3**) throughout the thallus, but epiphyte gametophytes were only macroscopically visible on the 3rd week (**Figure 10.3**) for the wild strain, and 4th week for the green color variant strain (**Figure 10.4**). Epiphyte gametophytes developed and increased in number on the 6th week (**Figures 10.5**, **11.5**), especially in the base and intermediate portions of the thallus. Beyond the 8th week (**Figures 10.6**, **11.6**), epiphyte gametophyte fertility was macroscopically noticed as female epiphyte gametophytes produced cystocarps; moreover, constant aeration promoted fragmentation on some of the tetrasporophytes and epiphyte gametophytes. By the end of the experiment week (**Figures 10.7**, **11.7**), the number of epiphyte gametophytes and fragmented thallus increased on both strains. At the end of the 12th week, tetrasporophytes got up to 6.2cm, and the number of epiphyte gametophytes and fragmented thallus increased on both strains (**Figures 10.7**, **11.7**).

After the 12 weeks of cultivation, the wild strain samples had on average 69.7±15.1 epiphyte gametophytes, while the variant strain had 46±18.6. There is no difference between the amount of epiphyte gametophytes produced by each strain ($F=2.93$, $p=0.13$). Epiphyte gametophytes were usually thinner in diameter in comparison to the tetrasporophytes and shorter in total size, reaching up to 4 cm when still attached (**Figures 10.4**, **10.5**, **10.6**, **10.7**, **11.4**, **11.5**, **11.6**, **11.7**).

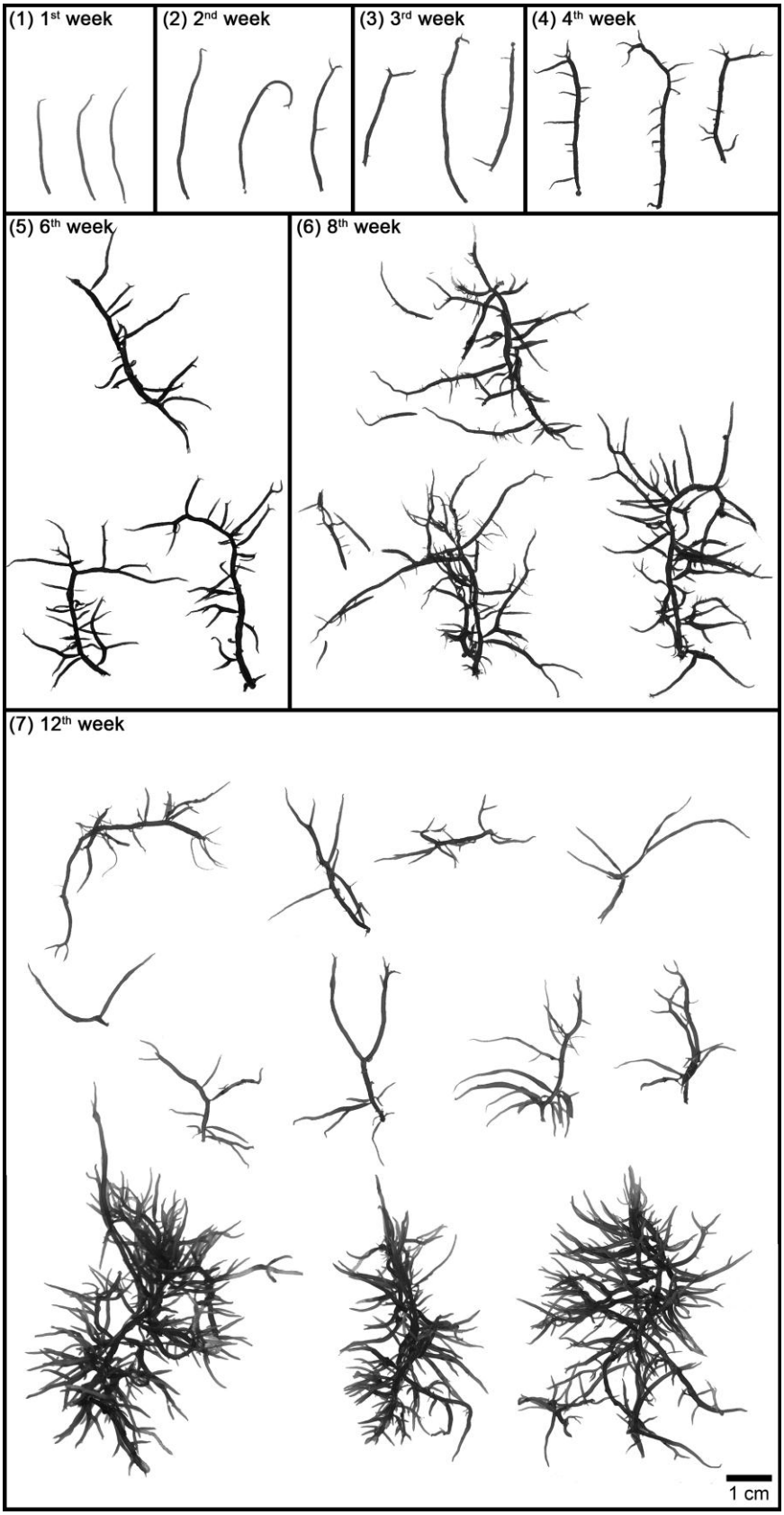


**Figure 10.** Apical segments of the wild strain of *G. caudata* during cultivation for 12 weeks, photographed at the end of each week.

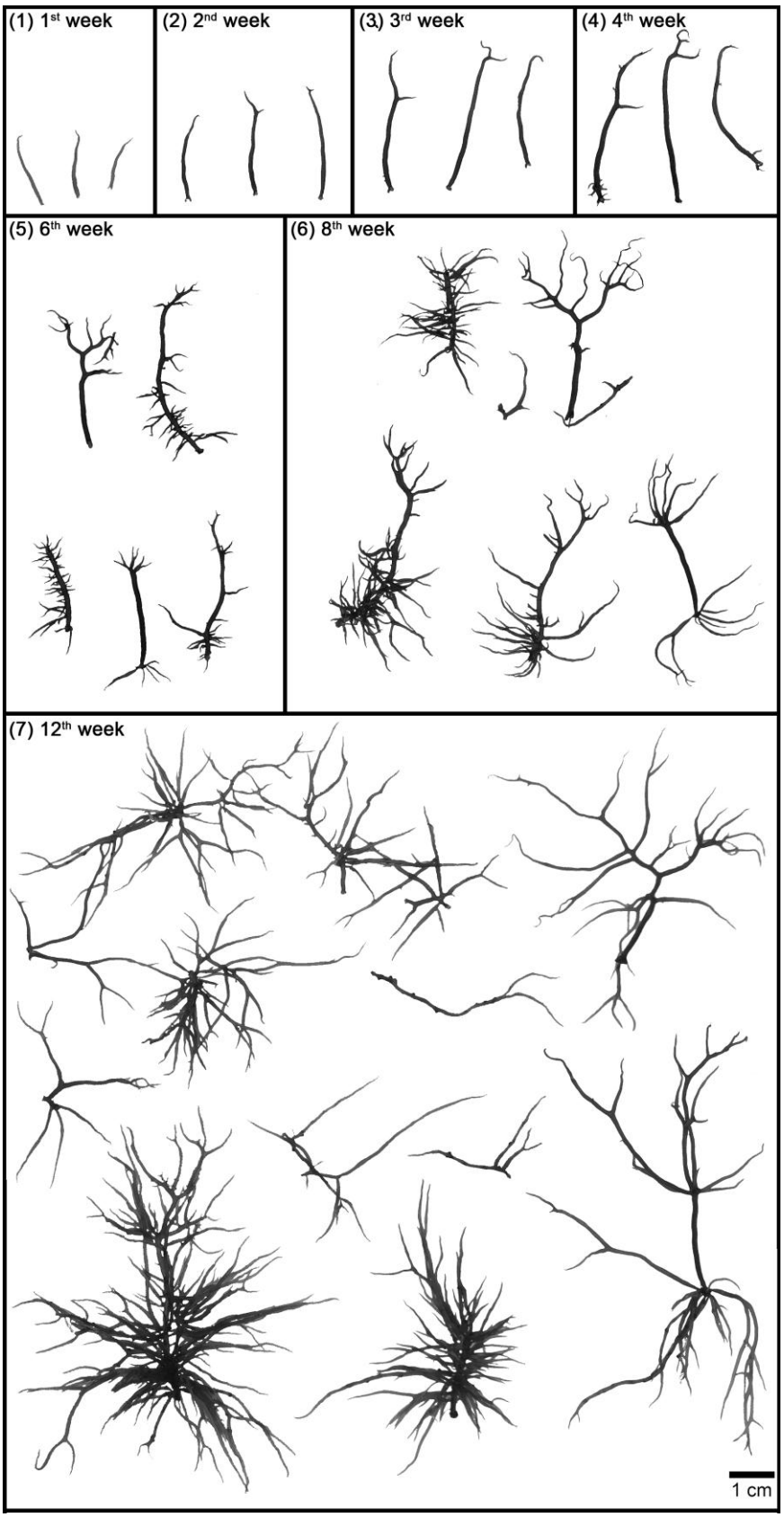


**Figure 11.** Apical segments of the green color variant strain of *G. caudata* during cultivation for 12 weeks, photographed at the end of each week.

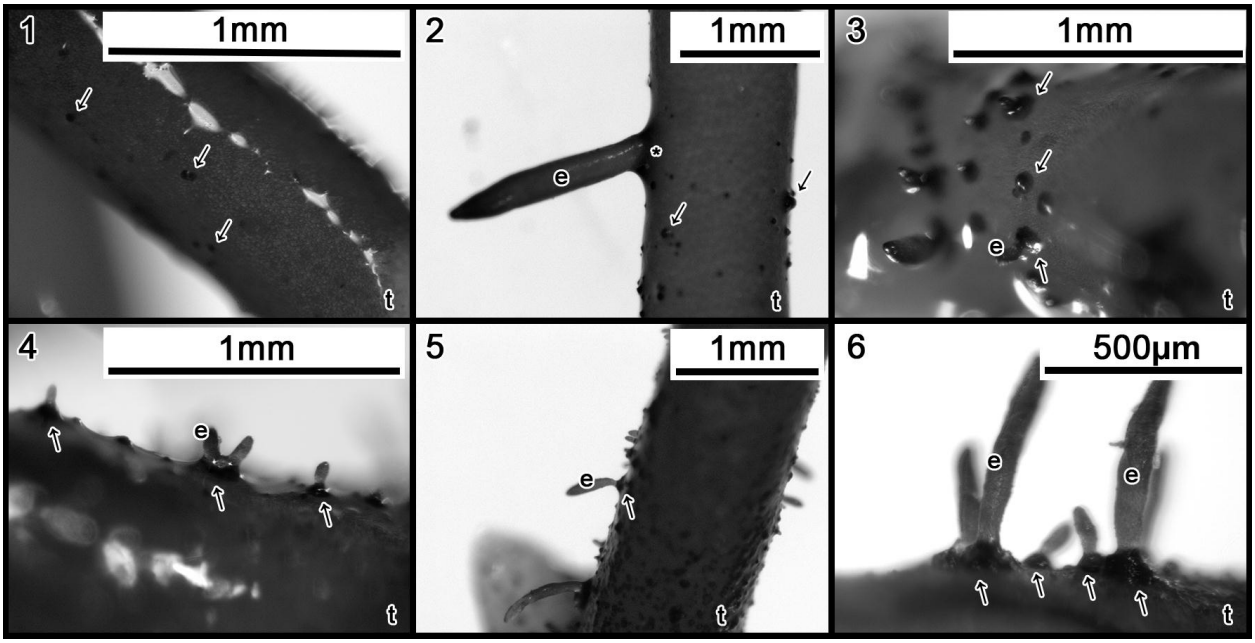


**Figure 12.** *G. caudata* tetrasporophyte showing with epiphyte gametophytes. **(1-2)** Medium thallus portion of the tetrasporophyte **(1)** Tetrasporophyte with germinating tetraspores (arrow). **(2)** Small epiphyte gametophytes developing on the thallus of the tetrasporophyte (arrow). Epiphyte gametophyte that germinated inside the tetrasporangium without an evident holdfast (asterisk). **(3-6)** Basal thallus portion of the tetrasporophyte **(3)** Different stages of development of holdfasts attaching epiphyte gametophytes to the tetrasporophyte (arrow). **(4)** Holdfasts of tetraspores that germinated outside the tetrasporophyte thallus (arrow). **(5)** Different stages of epiphyte gametophytes in the basal portion. Epiphyte gametophyte developing a branch (arrow). **(6)** Epiphyte gametophyte's holdfasts forming a cluster (arrow). Subtitles: **e** epiphyte gametophyte; **t** tetrasporophyte.

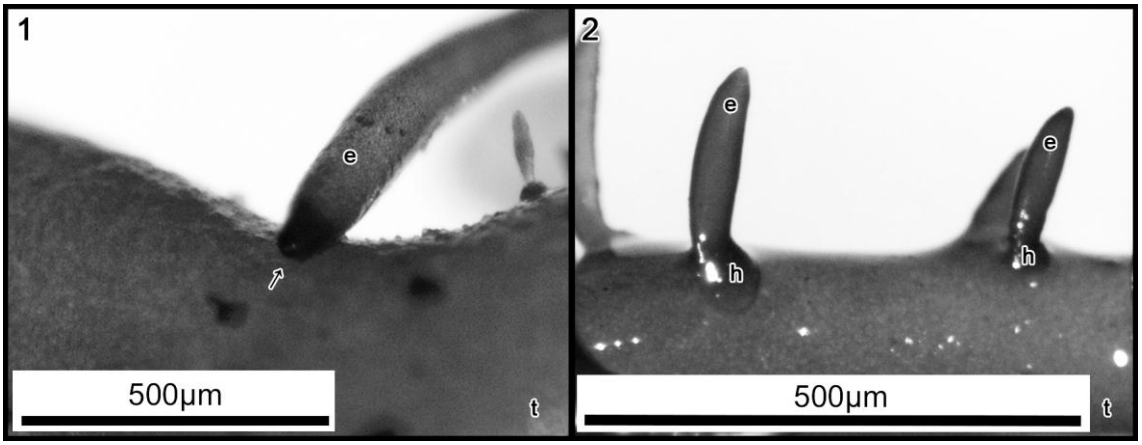


**Figure 13.** *Gracilaria caudata* tetrasporophyte medium portion thallus with epiphyte gametophytes. **(1)** Epiphyte gametophyte on a tetrasporophyte that has germinated inside the thallus. Absence of the epiphyte gametophyte's holdfast (arrow). **(2)** Epiphyte gametophyte that has developed from a tetraspore that germinated outside

the thallus of the tetrasporophyte. Subtitles: **t** tetrasporophyte, **e** epiphyte gametophyte, **h** epiphyte gametophyte's holdfast.

### 4.3.2. Anatomical aspects of the contact interface between epiphyte gametophytes and tetrasporophytes

Fertile tetrasporophytes showed abundant tetrasporangia on both strains (**Figure 12.1**). We observed that there are two different processes that originate epiphyte gametophytes on tetrasporophytes: germination inside the tetrasporophyte thallus, usually inside the tetrasporangia, and germination outside the tetrasporophyte thallus. Tetraspores that germinated outside the tetrasporangia, attached to the tetrasporophyte thallus were more frequent than inside the thallus (**Figure 13.1**, **13.2**). These processes were observed in both strains.

Both wild and green strains of *Gracilaria caudata* showed microcystidiate organization with small cortical cells on the periphery of the thallus that gradually increased in size as they get closer to the medulla (**Figure 14.1**). Tetrasporangia were found on the cortex layers on fertile tetrasporophytes auto fluorescing in blue, while most of the chlorophyll on the cortex emitted red fluorescence (**Figure 15.1**). Chloroplasts were observed in cortex cells and in the extremities of the medulla cells, while a thin layer of polysaccharide substance occurred on the external layer (**Figure 15.2**). Tetrasporophyte cortex has two or three cells rows on the cortex with tetrasporangia inserted in the cortex layers and the cruciated aspect of the tetrasporangium was evident, fluorescing in blue (**Figure 15.3**). Spores that were ready to be released, but still within tetrasporangium were observed above the polysaccharide layer (**Figure 15.4**).

#### 4.3.2.1. Germination of tetraspores outside the tetrasporophyte thallus

Some tetraspores were released and stayed attached to the exterior of the tetrasporophytes by the polysaccharide layer. The tetraspore underwent mitotic divisions, developing a young plantlet (**Figure 15.5**). As epiphyte gametophytes grew, they increase in size above this polysaccharide layer (**Figure 15.6**, **15.7**). Once the tetraspores started germinating, a holdfast was observed on some of the epiphyte gametophytes (**Figure 12.2**, **12.3**, **12.4**, **12.5**, **12.6**). However, there was an interruption on the external layer of the polysaccharide in the area where the epiphyte gametophyte's holdfast occurs (**Figure 14.2**, **14.3**, **14.4**, **14.8**, **14.9**). As epiphyte developed, it was possible to notice a small constrain on the epiphyte gametophyte's

thallus above the holdfast (**Figure 12.5**, **12.6**). Thickening under the epiphyte gametophyte on the tetrasporophyte's cortex layer is irregular, although they always showed three or more layers of cortical cells (**Figure 14.3**). Later stages of epiphyte gametophyte development promoted the widening of the holdfast region and the increased in number of cortical cells in the tetrasporophyte from 2 or 3 rows to up to 10. This tetrasporophyte cortex thickening process appeared to increase associated with the age of the epiphyte gametophyte, as more developed epiphyte gametophytes had more tetrasporophyte cortex layers below them (**Figure 14.4**, **14.5**, **14.6**).

Later development stages of epiphyte gametophytes allowed better distinction between a regular tetrasporophyte ramification and a tetraspore that germinated on the tetrasporophytes. Regular ramifications did not promote an increase in tetrasporophyte cortex layers and there is a regular continuum between the medulla cells, in contrast to the epiphyte gametophyte (**Figure 14.7**). A point of connection between the epiphyte gametophyte and the tetrasporophytes was also registered, with thickening in the tetrasporophyte's cortex and the epiphyte gametophyte's holdfast semi circular shape (**Figure 14.8**, **14.11**, **15.8**). The epiphyte gametophyte cellular connections disrupt the polysaccharide layer above the tetrasporophyte (**Figure 15.9**).

There was some plasticity observed in the epiphyte gametophyte's holdfast (**Figure 14.8**, **14.9**, **14.11**, **14.12**). Clusters of developed epiphyte gametophytes can merge holdfasts. As the holdfast increased, there was also a change in activity on the most external cortex layer (**Figure 15.10**, **15.11**), as the holdfast could cast a shadow, decreasing the activity on the cells under them. Moreover, there was an accumulation of polysaccharide between the epiphyte gametophyte and the tetrasporophyte thallus (**Figure 15.12**). Basal portions of the tetrasporophytes thallus hosted more epiphyte gametophytes and the epiphyte gametophyte's holdfasts can form a cluster around the tetrasporophyte's thallus (**Figure 14.12**).

#### 4.3.2.1. Germination of tetraspores inside the tetrasporophyte thallus

Epiphyte gametophytes that germinate inside the tetrasporophyte thallus showed a suppression of the holdfast anchoring them to the tetrasporophyte (**Figure 13.1**), in comparison to most of the epiphyte gametophytes that germinate outside the tetrasporophyte thallus and have a conspicuous holdfast anchoring them to the tetrasporophytes (**Figure 13.2**). Epiphyte gametophytes that germinate inside the tetrasporophyte thallus have their holdfast periphery cells closer to the tetrasporophyte's

medulla, indicating that the germination might have occurred inside the tetrasporophytes thallus (**Figure 14.10**). When tetraspores germinated outside the tetrasporophyte, the epiphyte gametophyte cells were adjacent to the external layers of the tetrasporophyte cortex (**Figure 14.8**, **14.9**) There were also no evidences of cortex thickening on the tetrasporophytes that illustrates the major proposed difference of germinations that happen inside the tetrasporophyte thallus, in the tetrasporangium (**Figure 14.11**). When epiphyte gametophytes germinated on the thallus of the tetrasporophytes, cellular connections were observed between the central portion of the epiphyte gametophyte's holdfast and the external layers of the thickened cortex of the tetrasporophytes (**Figure 14.4**, **14.5**, **14.9**, **14.10**). On the other hand, when the germination occurred inside the tetrasporophyte thallus, a connection was observed between the epiphyte gametophyte and more internal layers of the tetrasporophyte's cortex (**Figure 14.10**, **14.11**).

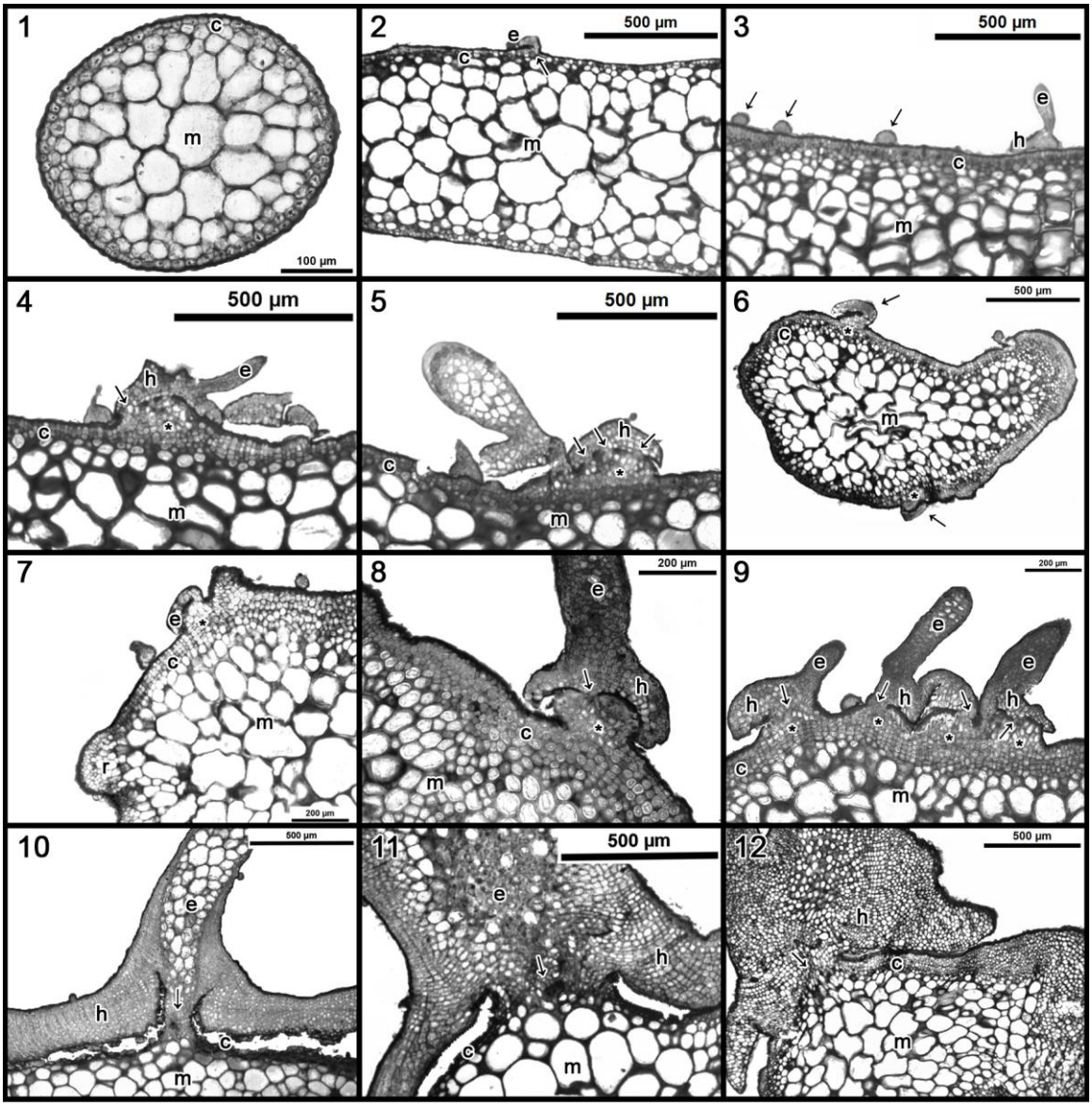


**Figure 14.** *Gracilaria caudata*: resin cuts dyed with toluidine blue on light microscopy. **(1)** Transversal section of tetrasporophyte. There is a gradual shrink in size of the medulla cells as they approach the cortex (two layers of small and pigmented cells). 10nm cut. **(2-5)** Longitudinal sections of tetrasporophyte. **(2)** Plantlet derived from tetraspore germinating just outside the cortex (arrow). 10nm cut. **(3)** Thickening in the tetrasporophyte cortex happens below the epiphyte gametophyte holdfasts. Epiphyte gametophyte in early stage of development (arrow). 10nm cut. **(4-5)** Epiphyte gametophytes growing outside the cortex. **(4)** Intersection between the tetrasporophyte's cortex cells and the gametophyte's holdfast (arrow). 10nm cut. **(5)** Intersection between the tetrasporophyte's cortex cells and the gametophyte's holdfast in three spots (arrows). 10nm cut. **(6-12)** Transversal sections of tetrasporophytes. **(6)** Epiphyte gametophytes growing on the tetrasporophyte (arrow). 10nm cut. **(7)** Regular ramification and an epiphyte gametophyte. Cortex layer did not increase in row size on the regular ramification. 10nm cut. **(8-12)** Intersection between tetrasporophytes and epiphyte gametophytes. **(8)** Epiphyte gametophyte that germinated on the tetrasporophyte (arrow). Thickened cortical extruding beyond the expected cortex

shape. 10nm cut. **(9)** Epiphyte gametophytes holdfasts forming a cluster (arrows). 10nm cut. **(10)** Epiphyte gametophyte with no thickening of the tetrasporophyte's cortex under the epiphyte gametophyte (arrow). This is associated to a tetraspore that germinated in the tetrasporophyte thallus (inside the tetrasporangia). 5nm cut. **(11)** Epiphyte gametophyte that germinated inside the tetrasporangium with large cellular connection region (arrow). 5nm cut. **(12)** Epiphyte gametophyte with apical branch on a tetrasporophyte showing an intersection between the tetrasporophyte and the epiphyte gametophyte (arrow). 10nm cut. Abbreviations: **c** cortex; **e** epiphyte gametophyte; **h** epiphyte gametophyte's holdfast; **m** medulla; **r** regular tetrasporophyte ramification; ***** thickened tetrasporophyte cortex region.

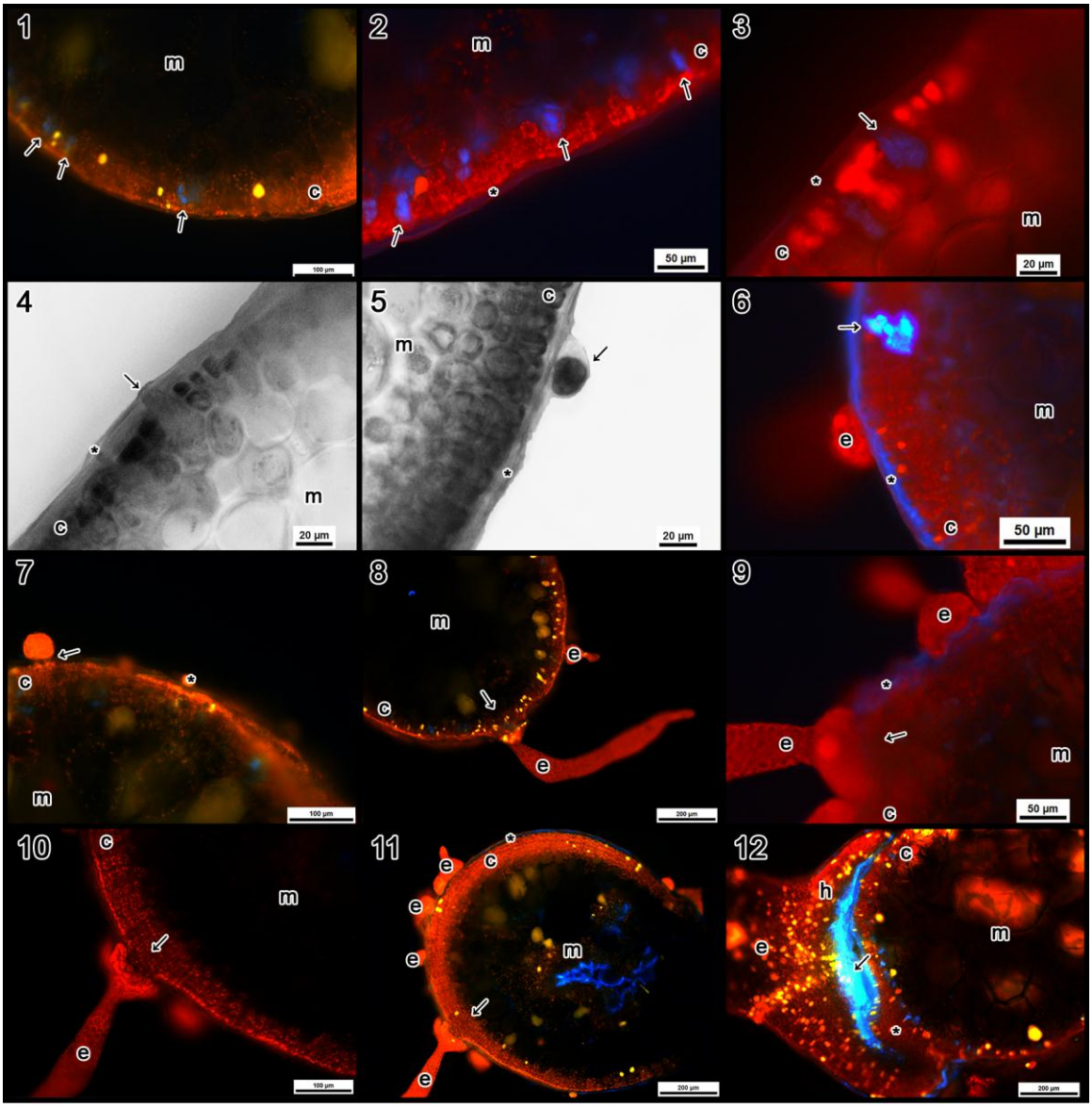


**Figure 15.** *Gracilaria caudata*: transversal fresh cuts on fluorescence and light microscopy. **(1-3, 6-12)** Auto-fluorescence. **(1-10)** middle portion of the tetrasporophyte thallus. **(1)** Tetrasporangia in the tetrasporophyte (in blue, arrow). **(2)** Chloroplasts in the tetrasporophyte (in red) around the medulla cells. Tetrasporangia (blue, arrow). Thin

layer of a polysaccharide above the cortex (asterisk). **(3)** Tetrasporophyte with a cruciate divided tetrasporangium in blue (arrow). **(4-5)** Light microscopy. **(4)** Tetrasporophyte with a tetrasporangium (arrow) and a thin layer of a polysaccharide above the cortex (asterisk). **(5-7)** early stages of epiphyte gametophyte development. **(5)** Tetrasporophyte with a developing tetraspore (arrow) surrounded by a polysaccharide layer (asterisk) above the cortex. **(6)** Epiphyte gametophyte developing above the polysaccharide layer (asterisk). Tetrasporangium in blue (arrow). **(7)** Epiphyte gametophyte with a connection between the epiphyte gametophytes and the tetrasporophyte, going through the polysaccharide layer (arrow). Germinating tetraspore (asterisk). **(8-10)** Epiphyte gametophytes with apical branches. **(8)** Cortex thickening under the epiphyte gametophyte (arrow). **(9)** Intersection between the epiphyte gametophyte and the tetrasporophyte (arrow). Thin layer of a polysaccharide above the cortex (asterisk), with an interruption on the region connecting the epiphyte gametophyte to the tetrasporophyte. Note cortex thickening under the epiphyte gametophytes. **(10)** Epiphyte gametophyte on the tetrasporophyte thallus showing a connection region between the epiphyte gametophyte and the tetrasporophyte. There is a change in the fluorescence intensity below the epiphyte gametophyte's holdfast, on the tetrasporophyte's cortex (arrow). **(11-12)** Basal portion of the tetrasporophyte. **(11)** Several epiphyte gametophytes on tetrasporophyte. Note interruption of the cortex external layer under the epiphyte gametophyte (arrow). Layer of a polysaccharide (asterisk) above the cortex absent around epiphyte gametophytes. Cells accumulating reserve substances in the medulla. **(12)** Polysaccharide pocket between the epiphyte gametophyte and the tetrasporophyte (arrow). Thickened cortex area on the region connecting the epiphyte gametophyte to the tetrasporophytes (asterisk). Subtitles: **c** tetrasporophyte's cortex; **e** epiphyte gametophyte; **h** epiphyte gametophyte's holdfast; **m** tetrasporophyte's medulla.

### 4.4. Discussion

The present study was able to produce epiphyte gametophytes in controlled conditions that provided detailed morphological and anatomical information regarding: the germination of tetraspores on tetrasporophytes, cellular relationships between epiphyte gametophytes and tetrasporophytes, and the amount of epiphyte gametophytes produced by wild and color variant strains of *Gracilaria caudata* that have not yet been analyzed. Gametophytes growing as epiphytes have already been reported for *Gracilaria* spp., but in previous studies, authors did not focus on the morphological and anatomical aspects of the germination, development and number of epiphyte gametophytes (Bird et al*.,* 1977; Oliveira & Plastino, 1984; Plastino, 1985; Destombe et al*.,* 1989; Kain & Destombe, 1995; Costa & Plastino, 2001; Ayres-Ostrock, 2014).

There were no morphological and anatomical differences between the wild and green color variant strains of *Gracilaria caudata*. Vegetative thallus of both strains were similar to the description of the species (Plastino & Oliveira, 1997). Auto-fluorescence of the cloroplasts observed in our samples are similar to *G. domingensis* (Gouveia et al., 2013). There was also no reference established for auto-fluorescence in *Gracilaria* spp. that would allow further interpretation of these results, as most studies with seaweed used stains to differentaite structures (Diannelids & Kristen, 1988; Gouveia et al, 2013; Rover et al., 2015). Furthermore, handcuts using a razorblade created angled edges on the samples that reflected some of the auto-fluorescence, what possibly caused some blue fluorescence reflection of the polysaccharide layer outside the tetrasporophyte thallus.

Slightly smaller, the green color variant of *Gracilaria caudata* showed a very similar habit to the wild strain. Wild and color variant strains of *G. birdiae* had displayed the same habit, while the main thallus showed a distinct size (Costa & Plastino, 2001). Epiphyte gametophytes were observed after 3 weeks for the wild strain and 4 for the green color variant strain, longer than what was described for *G. verrucosa* (Destombe et al., 1989). The epiphyte gametophytes found in our experiment grew more in size after 12 weeks, surpassing the 6 to 10mm described to *G. verrucosa*, reaching up to 4cm*.* Epiphyte gametophytes of *G. caudata* were found throughout the thallus with similar frequency in both wild and green variant strains.

Observations in this study allowed the distinction between two germination processes of tetraspores on the tetrasporophyte thallus of wild and green variant strains

of *Gracilaria caudata*. One of them was previously described as "*in situ* germination", since epiphyte gametophytes of *G. birdiae* originated from tetraspores that were not liberated (Costa & Plastino, 2001). Evidence presented in our study showed that epiphyte gametophytes could be originated from the germination of tetraspores inside or outside of the tetrasporophyte thallus. In fact, epiphyte gametophytes that germinated after tetraspore liberation are more frequent than the ones that originated from tetraspores that were retained inside the tetrasporangia of the tetrasporophyte. Epiphyte gametophytes that germinated inside the tetrasporangia promoted no or less pronounced thickening of the cortex layer of the tetrasporophyte and show a suppression of the holdfast that fastens the gametophyte to the tetrasporophyte. Hence, germination inside the tetrasporangia might have suppressed the development of the holdfast. In contrast, epiphyte gametophytes that develop from tetraspores that germinated outside the tetrasporophyte thallus had evidences of cortex thickening and the epiphyte gametophyte's holdfasts are conspicuous. Considering our discoveries, the terminology epiphyte gametophyte to *G. caudata* was preferred over *in situ* germination, following what was suggested by Destombe et al. (1989) and also used by Otto & Hughes (1999).

There was a change in the cortex anatomy of the tetrasporophytes of *Gracilaria caudata* caused by the attachment of the holdfast. This process had not been observed before, not even to other species. We suggested that the contact between the holdfasts of epiphyte gametophytes and the tetrasporophyte triggered a response from the tetrasporophyte, increasing the number of cell rows expected in cortex when compared to the *G. caudata* description (Plastino and Oliveira, 1997), thickening the region. The existence of adjacent cells between the epiphyte gametophytes and the tetrasporophytes could indicate substance exchange between both. We hypothesized that the substance flow is primarily from the tetrasporophyte to the epiphyte, mainly in young gametophytes, but it could not be proven at this point. This is suggested because fertility of epiphyte gametophytes happens in a smaller amount of time than fertility in free living gametophytes. However, tetrasporophyte cortex cells under developed holdfasts of epiphyte gametophyte showed decreased fluorescence activity, probably due to the shading caused by the holdfast.

Tetrasporophytes are more frequent than gametophytes for populations of *Gracilaria caudata* (Ayres-Ostrock et al. 2015) and for other *Gracilaria* species (Plastino, 1985; Orduña-Rojas et al. 2002; Guillemin et al. 2008, Terada et al. Martín et al. 2011;

Ayres-Ostrock et al. 2015). As presented in this study, epiphyte gametophytes of *G. caudata* combined the advantages of the tetrasporophyte and gametophyte stages, as they allowed a reduction of the time necessary for sexual reproduction, permitting greater genetic variability while still attached to the more abundant generation. Epiphyte gametophytes, regardless of germination method, increased the proximity between male and female gametophytes in the tetrasporophyte, which could have enhanced the mating chances, as the spermatia present in *Gracilaria* do not have flagella. Sexual reproduction is uses more energy than asexual reproduction, therefore changes in the life history probably represented a distinct strategy when compared to free tetrasporophytes and gametophytes (Otto & Hughes, 1999).

## 5. Final considerations

This study presented information about the physiological impacts of epiphyte gametophytes on tetrasporophytes in wild and green color variant strains of *Gracilaria caudata*. For the first time, the germination of tetraspores on the tetrasporophytes was analyzed from morphological and anatomical perspectives.

We could confirm the hypothesis that tetraspores that germinated on tetrasporophytes gave rise to gametophytes that reached reproductive maturity in a shorter amount of time than gametophytes that germinated freely. This could also suggest that the relationship between epiphyte gametophyte and tetrasporophyte was beneficial to the epiphyte gametophytes, as they reached fertility in less time than free-living gametophytes.

Tetrasporophytes without epiphyte gametophytes did not show superior vegetative performance when compared to tetrasporophytes with epiphyte gametophytes, rejecting our initial hypothesis, since the growth rates were similar. Higher growth rates were found for the wild strain, when compared to the green variant strains on the 2$^{nd}$ week of the experiment, before the tetrasporophytes became fertile. However, wild tetrasporophytes did not have higher growth rates, pigment content, and photosynthesis than individuals with green coloration at the end of the experiment, contrary to what our hypothesis suggested. The wild strain had lower allophycocyanin and phycoerythrin content than the green variant strain. On the other hand, no differences were found in phycocyanin levels. Chlorophyll *a* content was higher in the wild strain than in the green variant, what could compensate the lower phycobiliprotein content.

The presence of epiphyte gametophytes on the tetrasporophytes (in the control samples) have impacted the amount of pigments produced by the strains. The treatment samples yielded more allophycocyanin and phycocyanin than the control samples, indicating that the presence of epiphyte gametophytes changed the production or consumption of those pigments. Treatment samples of the green variant strain produced more phycocyanin than control samples. Control samples of the wild strain produce less chlorophyll *a* than the treatment samples, adding one more factor that is modulated by the epiphyte gametophytes.

The wild strain started developing epiphyte gametophytes before the variant strain, however the amount of epiphyte gametophytes that grow in tetrasporophytes was not different between the wild and color variant strains during cultivation.

Initially it was believed that germination occurred only inside the thallus in *Gracilaria* spp. (Costa & Plastino, 2001). This study presented that germination can happen inside or outside the tetrasporophyte. In culture conditions, tetraspores that germinated outside the tetrasporophyte thallus are more frequent than tetraspores that germinated inside. Morphological and anatomical evidences showed differences between these methods. Tetraspores that germinate outside develop a holdfast, while tetraspores that germinate inside the thallus do not. Germination outside the tetrasporophyte thallus caused the tetrasporophyte cortical layer to increase in number, and the connection interface between them happened between the holdfast of the epiphyte gametophyte and the external layers of the tetrasporophyte cortex. In contrast, germination of tetraspores inside the tetrasporophyte did not promote cortical thickening, and the connection point between the epiphyte gametophyte and the tetrasporophyte happened closer to the medulla cells in the tetrasporophyte. We suggested that the substance flow is primarily from the tetrasporophytes to the epiphyte gametophytes, mainly in young gametophytes. An evidence was the reduced amount of some phycobiliproteins when comparing control and treatment samples of both strains.

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