

Mario Celso Machado Yeh

---

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

São Paulo, SP - Brasil

2017



**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: Prof<sup>a</sup>. Dr<sup>a</sup>. Estela Maria Plastino

Colaborador: Prof. Dr. Diego Demarco

São Paulo, SP - Brasil

2017

Yeh, Mario Celso Machado (2017) Physiological and anatomical assessments of tetrasporophytes with epiphyte gametophytes of wild and green variant strains of *Gracilaria caudata* (Gracilariales, Rhodophyta). Msc Dissertation. Universidade de São Paulo. Sao Paulo, Brazil.

Dissertação de mestrado – Instituto de Biociências da Universidade de São Paulo. Departamento de Botânica. 68pp.

1 - *Gracilaria caudata*; epiphyte gametophytes; tetrasporophytes; color variants; Rhodophyta; growth rates; pigments; photosynthetic potential; anatomy; light microscopy; auto-fluorescence.

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

**Judging committee:**

---

Professor Dr.

---

Professor Dr.

---

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 commas  
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

Special thanks to my advisor for this opportunity, to professor Diego Demarco for the important instructions in the anatomical section, to the staff for their hard work behind the scenes, and to all the colleagues and coworkers for the valuable input presented in this piece of work. Most importantly, thanks to CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) for the grant that allowed this project to be executed. I appreciate all of your support and guidance.

## Table of Contents

<b>Abstract</b> .....	<b>1</b>
<b>Resumo</b> .....	<b>2</b>
<b>1. General introduction</b> .....	<b>3</b>
<b>1.1. Economic relevance: <i>Gracilaria</i></b> .....	<b>3</b>
<b>1.2. <i>Gracilaria</i>: life history and differences between generations</b> .....	<b>3</b>
<b>1.3. Epiphyte gametophytes in <i>Gracilaria</i></b> .....	<b>5</b>
<b>1.4. Intraspecific diversity: color variants in <i>Gracilaria</i></b> .....	<b>6</b>
<b>1.5. <i>Gracilaria caudata</i></b> .....	<b>7</b>
<b>2. Hypotheses and objectives</b> .....	<b>9</b>
<b>5. Final considerations</b> .....	<b>10</b>
<b>6. Bibliography and electronic references</b> .....	<b>12</b>



## 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. Epiphyte 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<sup>nd</sup> 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 tetrásporófitos 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 tetrásporófitos por meio de taxas de crescimento, conteúdo pigmentar e potencial fotossintetizante dos tetrásporófitos; (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 tetrásporófito; e (iv) aspectos anatômicos da interseção entre tetrásporófitos 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 tetrásporófitas. 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 tetrásporófitos ocorreu de duas maneiras: antes e após a liberação de tetrásporos. A liberação de tetrásporos seguida da germinação sobre o tetrásporófito foi mais frequente, produziu apressórios e promoveu aumento do número de células no córtex do tetrásporófito. A germinação de tetrásporos dentro do talo do tetrásporófito não apresentou evidências de apressórios ou espessamento da região cortical do tetrásporófito. A porção basal de apressórios de gametófitos epífitos permaneceu adjacente ao córtex do tetrásporófito. Quando a germinação do tetrásporo ocorreu ainda dentro do talo, a interface de conexão das células foi mais próxima da medula do tetrásporófito. 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 tetrásporófitos. Para um possível cultivo em larga escala, tetrásporófitos 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 fibiliproteínas, 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., *Eucheama* spp., *Kappaphycus* spp., *Undaria* spp., *Saccharina* spp. and *Gracilaria* spp. (FAO, 2016).

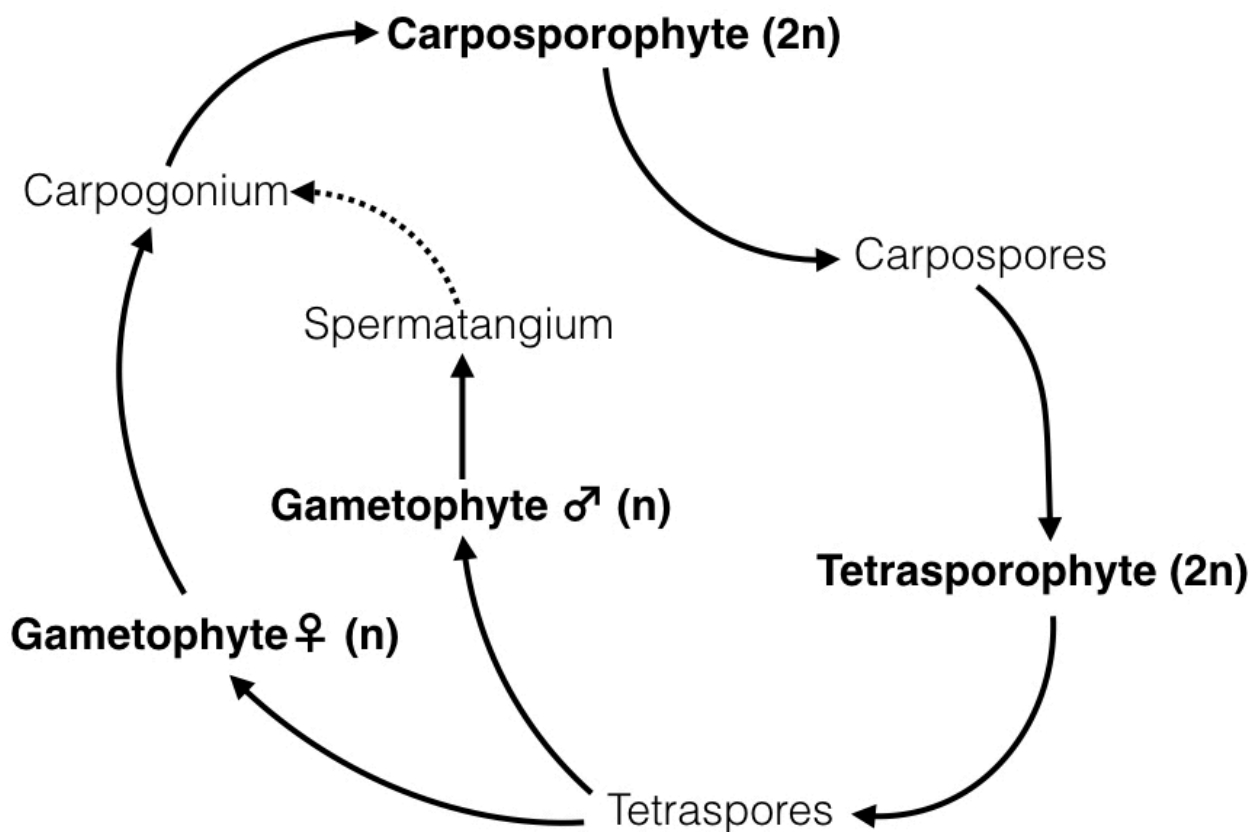
Among the phycocolloids, substances derived from seaweeds, agar has the greatest economic relevance in the world. Historically, in Japan, *Gelidium* spp. was the main source of agar until the beginning of the 20th century when the demand for the substance began to exceed production. After agar was introduced 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 phycocolloids 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 in 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 adaptation to the most favorable environmental condition (Stebbins & Hill, 1980). Furthermore, there are evidences that isomorphic phases are not ecologically identical (Hughes & Otto, 1999, Guillemín 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* (Guillemín 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 28°S, 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  $\mu\text{m}$ . 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  $\mu\text{m}$  (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.

## 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<sup>nd</sup> 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.

## 6. Bibliography and electronic references

- Algaebase. (2017) M.D. Guiry in Guiry, M.D. & Guiry, G.M. 2017. *AlgaeBase*. World-wide electronic publication, National University of Ireland, Galway. [http://www.algaebase.org/search/species/detail/?species\\_id=V37553e43c86cac7a](http://www.algaebase.org/search/species/detail/?species_id=V37553e43c86cac7a); accessed on 17 July 2017.
- Armisen, R. (1995) World-wide use and importance of *Gracilaria*. *Journal of Applied Phycology*, 7, 231-243.
- Ayres-Ostrock, L. M. (2014) Estudos populacionais em *Gracilaria birdiae* e *G. caudata* (Gracilariales, Rhodophyta): aspectos fenológicos, fisiológicos e moleculares. PhD thesis. Universidade de São Paulo. São Paulo, Brazil.
- Ayres-Ostrock, L. M., Mauger, S., Plastino, E. M., Oliveira, M. C., Valero, M., & Destombe, C. (2016) Development and characterization of microsatellite markers in two agarophyte species, *Gracilaria birdiae* and *Gracilaria caudata* (Gracilariaceae, Rhodophyta), using next-generation sequencing. *Journal of applied phycology*, 28, 653-662.
- Baker, N. R. (2008) Chlorophyll fluorescence: a probe of photosynthesis *in vivo*. *Annu. Rev. Plant Biol.*, 59, 89-113.
- Bird C. J., Edelstein T., McLachlan J. (1977) Studies on *Gracilaria*. Occurrence in Atlantic Canada, with particular reference to Pomquet Harbour, Nova Scotia. *Naturaliste can.* 104, 257-266.
- Costa, J. C., Gonçalves, P. R., Nobre, A., Alves, M. M. (2012) Biomethanation potential of macroalgae *Ulva* spp. and *Gracilaria* spp. and in co-digestion with waste activated sludge. *Bioresource technology*, 114, 320-326.
- Costa, V. L., Plastino, E. M. (2011) Color inheritance and pigment characterization of red (wild-type), greenish-brown, and green strains of *Gracilaria birdiae* (Gracilariales, Rhodophyta). *Journal of Applied Phycology*, 23, 599-605.
- Crow, J. F., & Kimura, M. (1965) Evolution in sexual and asexual populations. *The American Naturalist*, 99, 439-450.
- Destombe C, Valero M, Vernet P, Couvet D. (1989) What controls haploid-diploid ratio in the red alga, *Gracilaria verrucosa*? *J. evol. Biol.* 2, 317-338.
- Diannelidis, B. E., & Kristen, U. (1988) Comparative histochemical studies of reproductive and gametophytic tissue of marine red algae by means of fluorescent and light microscopy. *Botanica marina*, 31, 163-170.
- F.A.O. (Food and Agriculture Organization of the United Nations) (2014) The state of world fisheries and aquaculture. *Food and Agriculture Organization of the United Nations, Rome*.
- F.A.O. (Food and Agriculture Organization of the United Nations) (2016) The state of world fisheries and aquaculture. *Food and Agriculture Organization of the United Nations, Rome*.
- Faria, A. V., & Plastino, E. M. (2016) Physiological assessment of the mariculture potential of a *Gracilaria caudata*. *Journal of applied phycology*, 28, 2445-2452.
- Faria A. V. F., Barufi J. B., Plastino E. M. (2016) Ecotypes of *Gracilaria caudata* (Gracilariales, Rhodophyta): physiological and morphological approaches considering life history phases. *Journal of Applied Phycology*, 29, 707-719.

- Ferreira, L. B., Barufi, J. B., Plastino, E. M. (2006) Growth of red and green strains of the tropical agarophyte *Gracilaria cornea* J. Agardh (Gracilariales, Rhodophyta) in laboratory. *Revista Brasileira de Botânica*, 29, 187-192.
- García-Sánchez, M. J., Fernández, J. A., & Niell, F. X. (1993) Biochemical and physiological responses of *Gracilaria tenuistipitata* under two different nitrogen treatments. *Physiologia Plantarum*, 88, 631-637.
- Gouveia, C., Kreuzsch, M., Schmidt, É. C., Marthiellen, R. D. L., Osorio, L. K., Pereira, D. T., Santos, R., Ouriques, L. C., Martins, R. P., Latini, A., Ramlov, F., Carvalho, T. J. G., Chow, F., Maraschin, M., Bouzon, Z. L. (2013) The effects of lead and copper on the cellular architecture and metabolism of the red alga *Gracilaria domingensis*. *Microscopy and Microanalysis*, 19, 513-524.
- Guillemin, M. L., Faugeron, S., Destombe, C., Viard, F., Correa, J. A., & Valero, M. (2008) Genetic variation in wild and cultivated populations of the haploid–diploid red alga *Gracilaria chilensis*: how farming practices favor asexual reproduction and heterozygosity. *Evolution*, 62, 1500-1519.
- Guillemin M. L., Sepúlveda R. D., Correa J. A., Destombe C. (2013) Differential ecological responses to environmental stress in the life history phases of the isomorphic red alga *Gracilaria chilensis* (Rhodophyta). *Journal of Applied Phycology*, 25, 215-224.
- Hoshaw, R. W., Rosowski, J. R. (1973) Methods for microscopic algae. *Handbook of Phycological Methods*. Cambridge University Press, New York, 53-67.
- Hayashi, L., Bulboa, C., Kradolfer, P., Soriano, G., Robledo, D. (2014) Cultivation of red seaweeds: a Latin American perspective. *Journal of applied phycology*, 26, 719-727.
- Hughes, J. S., & Otto, S. P. (1999) Ecology and the evolution of biphasic life cycles. *The American Naturalist*, 154, 306-320.
- Jones, A. B., Dennison, W. C., Preston, N. P. (2001) Integrated treatment of shrimp effluent by sedimentation, oyster filtration and macroalgal absorption: a laboratory scale study. *Aquaculture*, 193, 155-178.
- Kain, J. M., & Destombe, C. (1995) A review of the life history, reproduction and phenology of *Gracilaria*. *Journal of Applied Phycology*, 7, 269-281.
- Kursar, T. A., van der Meer, J., Alberte, R. S. (1983) Light-harvesting system of the red alga *Gracilaria tikvahiae* I. Biochemical analyses of pigment mutations. *Plant Physiology*, 73, 353-360.
- Lignell, Å., Pedersén, M. (1989) Agar composition as a function of morphology and growth rate. Studies on some morphological strains of *Gracilaria secundata* and *Gracilaria verrucosa* (Rhodophyta). *Botanica marina*, 32, 219-228.
- Lyra, G. D. M., Costa, E. D. S., Jesus, P. B., Matos, J. C. G., Caires, T. A., Oliveira, M. C., Oliveira, E. C., Xi, Z., Nunes, J. M. C., Davis, C. C. (2015) Phylogeny of Gracilariaceae (Rhodophyta): evidence from plastid and mitochondrial nucleotide sequences. *Journal of phycology*, 51, 356-366.
- Marinho-Soriano, E., Panucci, R. A., Carneiro, M. A. A., Pereira, D. C. (2009) Evaluation of *Gracilaria caudata* J. Agardh for bioremediation of nutrients from shrimp farming wastewater. *Bioresource technology*, 100, 6192-6198.

- Martín, L. A., de Zaixso, A. L. B., & Leonardi, P. I. (2011) Biomass variation and reproductive phenology of *Gracilaria gracilis* in a Patagonian natural bed (Chubut, Argentina). *Journal of Applied Phycology*, 23, 643-654.
- McHugh D. J. (1991) Worldwide distribution of commercial resources of seaweeds including *Gelidium*. In Juanes JA, Santelices B, McLachlan JL (eds), International Workshop on *Gelidium*. Kluwer Academic Publishers, Dordrecht: 19–29.
- Nunes, J. M. C. (2005) Rodofíceas marinhas bentônicas do Estado da Bahia, Brasil. PhD thesis. Universidade de São Paulo, São Paulo, 410.
- O'brien TP, Feder N, Mccully ME (1964) Polychromatic staining of plant cell walls by toluidine blue O. *Protoplasma*, 59, 368–373
- Ogata, E., Matsui, T., & Nakamura, H. (1972) The life cycle of *Gracilaria verrucosa* (Rhodophyceae, Gigartinales) in vitro. *Phycologia*, 11, 75-80.
- Oliveira E. C., Plastino E.M. (1984) The life history of some species of *Gracilaria* (Rhodophyta) from Brazil. *Jap. J. Phycol.* 32, 203-208.
- Oliveira E. C., Plastino E. M. (1994) Gracilariaceae. In: Akatsuka I (ed). *Biology of economic algae*. SSB Academic Publishing, The Hague, 185-226.
- Orduña-Rojas, J., & Robledo, D. (2002) Studies on the tropical agarophyte *Gracilaria cornea* J. Agardh (Rhodophyta, Gracilariales) from Yucatán, México. II. Biomass assessment and reproductive phenology. *Botanica Marina*, 45, 459-464.
- Paquin, C., & Adams, J. (1983) Frequency of fixation of adaptive mutations is higher in evolving diploid than haploid yeast populations. *Nature*, 302, 495-500.
- Petti, R., Plastino, E. M. (2012) Estudo comparativo de dois processos de esterilização de água do mar para preparação de meios de cultura de algas em laboratório. In: XIV Congresso Brasileiro de Ficologia.
- Plastino, E. M. (1985) As Espécies de *Gracilaria* (Rhodophyta, Gigartinales) da Praia Dura, Ubatuba, SP. - Aspectos biológicos e fenologia. MsC dissertation. Universidade de São Paulo. São Paulo.
- Plastino, E. M. (2004) Diversidade intraespecífica em algas gracilarioides (Gracilariales, Rhodophyta). Tese de livre docência. Universidade de São Paulo. São Paulo, Brazil
- Plastino, E. M., Guimarães, M., Matioli, S. R., Oliveira, E. C. (1999) Codominant inheritance of polymorphic color variants of *Gracilaria domingensis* (Gracilariales, Rhodophyta). *Genetics and molecular biology*, 22, 105-108.
- Plastino, E. M., Guimarães, M. (2001) Diversidad Intraespecífica. In: Alveal, K.; Antezana, T.. (Org.). Sustentabilidad de la biodiversidad, un problema actual. Bases científico-tecnicas, teorizaciones y proyecciones. Concepcion: Universidad de Concepcion, 19-27.
- Plastino, E. M., Oliveira, E. C. (1988) Deviations in the life-history of *Gracilaria* sp. (Rhodophyta, Gigartinales), from Coquimbo, Chile, under different culture conditions. *Hydrobiologia*, 164, 67-74.
- Plastino, E. M., Oliveira, E. C. (1997) *Gracilaria caudata* J. Agardh (Gracilariales, Rhodophyta)-restoring an old name for a common western Atlantic alga. *Phycologia*, 36, 225-232.

- Ramus, J., & Meer, J. P. (1983) A physiological test of the theory of complementary chromatic adaptation. I. Color mutants of a red seaweed. *Journal of Phycology*, 19, 86-91.
- Richerd, S., Couvet, D., & Valéro, M. (1993) Evolution of the alternation of haploid and diploid phases in life cycles. II. Maintenance of the haplo-diplontic cycle. *Journal of Evolutionary Biology*, 6, 263-280.
- Rover, T., Simioni, C., Ouriques, L. C., & Bouzon, Z. L. (2015) Cytochemical, Structural and Ultrastructural Characterization of Tetrasporogenesis in *Bostrychia radicans* (Ceramiales, Rhodophyta) from the Mangroves of Itacorubi and Rio Ratones, Santa Catarina, Brazil. *American Journal of Plant Sciences*, 6, 2393.
- Santos, F. M. (2017) Intraspecific diversity in *Gracilaria caudata* (Gracilariales, Rhodophyta): genetic and physiological studies in the interpretation of the thallus color. MSc dissertation. Universidade de São Paulo. São Paulo, Brazil.
- Sheppard, Philip M. (1975) *Natural Selection and Heredity* (4th ed.) London: Hutchinson.
- Stebbins, G. L., & Hill, G. J. C. (1980) Did multicellular plants invade the land?. *The American Naturalist*, 115, 342-353.
- Suggett, D.J., Prásil O., Borowitzka, M.A. (2011) Chlorophyll a Fluorescence in Aquatic Sciences. Springer, Dordrecht.
- Terada, R., Abe, T., & Kawaguchi, S. (2010) Reproductive phenology of three species of *Gracilaria*: *G. blodgettii* Harvey, *G. vermiculophylla* (Ohmi) Papenfuss and *G. salicornia* (C. Agardh) Dawson (Gracilariales, Rhodophyta) from Okinawa, Ryukyu Islands, Japan. University of Tokyo. Tokyo, Japan.
- Ursi, S. (2005) Diversidade intraespecífica de *Gracilaria birdiae* (Gracilariales, Rhodophyta): crescimento, fotossíntese, pigmentos, polissacarídeos e genes da ficoeritrina de linhagens selvagens e variants. PhD thesis. Universidade de São Paulo. São Paulo, Brazil.
- Ursi, S., Plastino, E. M. (2001) Crescimento in vitro de linhagens de coloração vermelha e verde clara de *Gracilaria birdiae* (Gracilariales, Rhodophyta) em dois meios de cultura: análise de diferentes estádios reprodutivos. *Rev Bras Bot*, 24, 587-594.
- Van der Meer, J. P. (1979) Genetics of *Gracilaria* sp. (Rhodophyceae, Gigartinales). V. Isolation and characterization of mutant strains. *Phycologia*, 18, 47-54.
- Valero, M., Richerd, S., Perrot, V., & Destombe, C. (1992). Evolution of alternation of haploid and diploid phases in life cycles. *Trends in Ecology & Evolution*, 7, 25-29.
- Yokoya, N. S., Oliveira, E.C. (1992a) Effects of salinity on the growth rate, morphology and water content of some Brazilian red algae of economic importance. *Ciência Marina*, 18, 49-64.
- Yokoya, N. S., Oliveira, E. C. (1992b) Temperature response of economically important red algae and their potential for mariculture in Brazilian waters. *J. Appl. Phycol* 4, 339-345.
- Yoshimura, C. Y. (2006) Avaliação do potencial de cultivo e produção de ágar de *Gracilaria domingensis* e de *Gracilaria caudata* (Rhodophyta, Gracilariales) na Enseada de Armação do Itapocoroy (Penha, Santa Catarina). PhD thesis. Universidade de São Paulo. São Paulo, Brazil.