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Reproduction and cultivation of *Asparagopsis taxiformis* (Delile) Trevisan

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Declaração de autoria de trabalho

Declaro ser o autor deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam na listagem de referências incluída.

Universidade do Algarve, 15 Setembro de 2020

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ABSTRACT

Seaweeds have been receiving increased interest from the fisheries and aquaculture industries in recent years, with red algae continuing to be one of the more important components of seaweed aquaculture representing 54% of the harvested biomass (around US$ 6.3 billion). These seaweeds are important for both food and diverse of biotechnology usages. *Asparagopsis taxiformis*, a characteristic red alga, has been receiving increased attention for its biotechnological applications, the most interesting one being its ability to reduce methane emissions on the cattle. *A. taxiformis* presents a triphasic life history, passing from gametophyte to carposporophyte and finally to tetrasporophyte. The conditions for the lab manipulation and survival of this species are still not know, taking in consideration the difficulty of maintaining gametophyte cultures.

In this work, some abiotic factors affecting gametophyte maintenance and reproduction were experimentally tested. *A. taxiformis* gametophyte did not survive any common pretreatment used, only surviving in culture if cleaned of epizoa in sterilized filtered seawater and by selecting the less epiphyted individuals. *A. taxiformis* showed the highest growth rate when cultivated at 15 ºC, not growing at 20 ºC and dying at 25 ºC. An industrial fertilizer used showed similar growth rates compared to the Von Stosch medium. Photoperiod manipulation did not result in any sexual differentiation on the gametophytes. A low stock density (1 g/L) was required since at higher densities there was reduced (2 g/L) or no (4g/L) growth rate. *A. taxiformis* was able to grow at both 20 and 40 µmol photon m^{-2} s^{-1}, showing slightly higher (but not significant) growth rates at the latter. Attempts at inducing carposporophyte formation were unsuccessful. Temperature and light intensity were the two most important factors to maintain gametophyte culture.

Keywords: pretreatments, temperature, stock density, photoperiod, light intensity, Azores

RESUMO

Os setores de aquicultura e pescas são importantes para a economia global. Nos últimos 30 anos, a aquicultura tem sido o principal responsável pelo aumento no fornecimento de peixe e algas para consumo. Cerca de 40 espécies de macroalgas são cultivadas no mundo, com um aumento substancial da sua produção registado nos últimos 20 anos. As algas marinhas têm várias utilizações desde alimentação, rações e a aplicações biotecnológicas. As algas vermelhas (Rhodophytas) têm, na sua maioria, um ciclo de vida trifásico, que consiste na alternância de fases entre gametófito, carposporófito e tetrasporófito. A *Asparagopsis taxiformis*, uma alga marinha vermelha, apresenta diversas utilidades culinárias e biotecnológicas e tem recebido um interesse especial a nível global pela sua capacidade de redução das emissões de metanos em gado bovino através do seu uso em rações. *A. taxiformis* apresenta o ciclo típico das algas vermelhas, sendo, no entanto ainda desconhecido os fatores bióticos e abióticos que levam às alternâncias de fase em laboratório. É sabido que as manipulações de fatores como a temperatura e fotoperíodo são comumente os principais fatores para indução de reprodução, enquanto que outros fatores abióticos como a qualidade da luz, a intensidade desta e a suplementação com nutrientes, são pontos importantes para a manutenção e possível manipulação dos ciclos de vida. Nos Açores, *Asparagopsis* spp. está presente anualmente, ocorrendo em maior biomassa durante os meses de primavera/inicio de verão. Tanto o gametófito como o tetraesporófito são encontrados no meio natural ao logo do ano. Com o interesse comercial crescente deste género, é necessário controlar o seu ciclo de vida *in vitro* para facilitar a sua produção. Este trabalho teve como objetivo definir os requerimentos para a manutenção do gametófito de *A. taxiformis*, incluindo vários fatores abióticos promotores de crescimento (pré-tratamentos; qualidade e intensidade de luz; temperatura; suplementação de nutrientes; fotoperíodo; e densidade de cultivo) e determinar os fatores que permitem a carposporogénese e a respectiva libertação de carpósporos *in vitro*. Os pré-tratamentos que são normalmente utilizados para a limpeza de espécimes de algas marinhas vermelhas, tiveram, na sua generalidade, efeitos adversos no cultivo desta espécie. Apenas a limpeza com pinças em banhos de água do mar filtrada e esterilizada, e a seleção de indivíduos com baixa presença de epífitos foram eficientes na manutenção do cultivo. Cultivos a temperaturas superiores a 20 ºC foram deletérios para o gametófito, que apenas registou taxas de crescimento positivas quando cultivada a 15 ºC. A *A.taxiformis* quando cultivada juntamente com a adição de um fertilizante habitualmente utilizado em cultivos de laboratório (Von Stosch)

apresentou taxas de crescimento semelhantes aqueles registados quando cultivada com um fertilizante comercial, o que facilita o processo de cultivo. A exposição a diferentes fotoperíodos não resultou em diferenças nas características sexuais do gametófito durante o tempo de cultivo, mas a experiência foi interrompida por questões alheias ao cultivo, não tendo, por isto, os resultados sido conclusivos. Densidades de cultivo superiores a 1 g/L apresentaram taxas de crescimento mais reduzido, sendo que, à densidade de cultivo de 4 g/L as taxas de crescimento foram mesmo negativas para o gametófito. Por fim, o gametófito apresentou crescimento quando cultivado a 20 e a 40 µmol m^{-2} s^{-1}, com taxas de crescimento ligeiramente maiores (embora não significativas) na última condição. Todas as tentativas de indução da formação de carposporófito e da sua maturação não foram bem-sucedidas. As duas variáveis testadas que mostraram maior importância na manutenção do cultivo do gametófito foram a temperatura e a intensidade de luz. Este trabalho conclui que é possível a manutenção em laboratório de *A. taxiformis,* tendo as condições ótimas observadas, dentro do leque de opções testadas, sido o seu cultivo a uma temperatura de 15 ºC, com uma intensidade de luz menor ou igual a 40 µmol m^{-2} s^{-1} e uma densidade de cultivo de 1 g/L. São necessários mais estudos para determinar as condições necessárias para a indução e maturação do carpoesporófito.

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1. INTRODUCTION

1.1. Overview

Aquaculture and fisheries are important economic drivers accounting for more than 60% of the economic value of the global biosphere (FAO, 2020). Aquaculture has been responsible for the increasing supply of marine biotic resources in the last 30 years, representing 46% of total fish production and 97% of total seaweed production (FAO, 2020). Global aquaculture production in 2018 included 82.1 million tons of food fish (USD 250.1 billion) and 32.4 million tons of aquatic plants (USD 13.3 billion, mostly seaweed), with China being, by far, the largest producer of both (fish and macroalgae) sectors (FAO, 2020).

Wild fish stocks have long stabilized in the last century, with aquaculture accounting as the sole driver for the increasing supply of fish for the consumers (FAO, 2020). Yet, much remains to be done in order to foster a better use of the resources available and to mitigate the impact and optimize the cultivation of the existing resources, while, at the same time, trying to find new and unexplored sources of food.

1.2. Seaweeds

Seaweeds are the common name given to commercially available varieties of marine macroalgae (Dawczynski *et al.*, 2007). Seaweeds are described as extractive species for they can benefit the environment by removing waste materials, including organic waste produced by animals, and thus lowering the nutrient load in the water (FAO, 2020). Seaweeds are a diverse group of organisms that traditionally includes macroscopic, multicellular species belonging to three main Phyla (Rhodophyta, Chlorophyta and Ochrophyta), which are normally divided based on their main pigment color (Red: Rhodophyta; Green: Chlorophyta; Brown: Phaeophyceae), but can also include microscopic stages of their life cycles (Lee, 2008).

Around 40 algae are cultivated worldwide. Global production of farmed aquatic plants (overwhelmingly dominated by seaweeds) more than tripled in volume from 10.6 million tonnes (2000) to 32.4 million tonnes (2018) (FAO, 2020). Seaweeds have been used by humans for food (nutritional properties) and medicine (healing properties) for centuries, with many seaweed species being consumed as “sea vegetables” in Japan, China, and Korea (Pereira, 2011). They were also traditionally used as fertilizers, feed, and as thickening agents (McHugh, 2003; Lee, 2008).

Fish and fish products have a crucial role in nutrition and global food security, as they represent a valuable source of nutrients and micronutrients of fundamental importance for diversified and healthy diets. Seaweeds have a nutritional contribute, mainly, of micronutrient minerals (e.g. iron, calcium, iodine, potassium, selenium), vitamins (A, C, B-12), and also one of the only non-fish sources of natural omega-3 long-chain fatty acids (Angell *et al.*, 2016; FAO, 2020).

1.3. Rhodophyta (Red Algae)

The Rhodophyta is a well-characterized and morphologically diverse lineage of photosynthetic protists, being one of the oldest groups of eukaryotic algae. They range from unicells and uni- or multiseriate (arranged in rows) filaments to large (up to 3 m) pseudoparenchymatous, branched or unbranched, terete (cylindrical) to foliose (blade-like) thalli, including crustose and erect forms, some of which are calcified. More than 7,300 species are currently reported (Guiry & Guiry, 2020). They comprise the largest phyla of algae, currently consisting of two subphyla and seven classes with the majority of species being found in the Florideophyceae (6,751 spp.; 95% of all taxa), appearing to be a monophyletic group characterized by the presence of tetrasporangia and a filamentous gonimoblast in most species; mostly consisting of multicellular, marine algae, including many notable seaweed (e.g. *Asparagopsis* spp.) (Cole & Sheath, 1990; Fritsch, 1945; Lewis, 1964; Dixon & Irvine, 1977; Lee, 2008; Yoon *et al.*, 2016).

The Rhodophyta form a distinct group characterized by having eukaryotic cells without flagella and centrioles, chloroplasts that lack external endoplasmic reticulum and contain unstacked (stroma) thylakoids, and use phycobiliproteins as accessory pigments, which give them their red color. Most red algae are also multicellular, macroscopic, marine and reproduce sexually. Additional traits of some red algae include a complex life history composed of an alternation of two free-living and independent generations (gametophyte and tetrasporophyte) and a third generation, the carposporophyte, that occurs on the female gametophyte (Cole & Sheath, 1990; Fritsch, 1945; Lewis, 1964; Dixon & Irvine, 1977; Lee, 2008; Yoon *et al.*, 2016).

Most rhodophytes are marine with a worldwide distribution, playing key roles in nearshore ecosystems. Species of red alga range from the upper reaches of intertidal shores (e.g. members of Bangiales) to hundreds of meters in depth in clear tropical waters, providing habitat for a wide variety of organisms. Some marine species are found on sandy shores, however most are found attached to rocky substrata. Red algae are

represented by free-living macroalgal forms and smaller endo/epiphytic/zoic forms, meaning they live in or on other algae, plants, and animals. In addition, some marine species have adopted a parasitic lifestyle and may be found on closely or more distantly related algal hosts (Cole & Sheath, 1990; Fritsch, 1945; Lewis, 1964; Dixon & Irvine, 1977; Lee, 2008; Yoon *et al.*, 2016).

1.3.1 Life Histories

The red algal life history is unique in having an additional third phase (i.e. a triphasic life history) in most Florideophyceae (e.g. *Asparagopsis*). The triphasic life history is an alternation of generations of three phases, the gametophyte, carposporophyte, and tetrasporophyte. The triphasic life history is composed of haploid gametophytes (thalli that produce gametes), diploid carposporophytes, and diploid tetrasporophytes (thalli that typically produce four spores by meiotic division). Gametophytes and tetrasporophytes are generally independent photosynthetic thalli, whereas the carposporophyte is diploid tissue that occurs on or within the haploid female gametophyte as a result of the fertilization of the egg cell and subsequent development of the zygote (Cole & Sheath, 1990; Fritsch, 1945; Lewis, 1964; Dixon & Irvine, 1977; Lee, 2008; Yoon *et al.*, 2016). Male gametophytic plants produce spermatia (= nonmotile sperm) from spermatangial initial cells. Female gametophytic plants produce carpogonial branches that are composed of a terminal carpogonium (= egg cell) with a trichogyne (a hair-like extension) and differing numbers of subtending cells depending on the taxonomic group. Fertilization starts with attachment of spermatia to the trichogyne. Fusion of the gametic nuclei occurs in the carpogonium. The resulting diploid nucleus is either transferred, via an outgrowth from the carpogonium, to another cell (called the auxiliary cell), or remains in the carpogonium. In both cases, mitotic divisions of the diploid nucleus within a filamentous outgrowth (the gonimoblast) eventually result in the production of diploid carposporangia. Carpospores are released from the carposporangia and germinate to give rise to free-living diploid tetrasporophytes. Meiosis then occurs in specialized cells (tetrasporangial initial cells) in the tetrasporophyte, and the resulting tetrads of haploid spores are shed from the thallus. Individual spores germinate to give rise to gametophytes, completing the cycle (Yoon *et al.*, 2016). The typical life history includes isomorphic gametophytes and tetrasporophytes; however, in some red algae, heteromorphic generations, in which the tetrasporophyte is morphologically distinct from the gametophyte, also occur (e.g. Bonnemaisoniales) (Yoon *et al.*, 2016).

1.3.2 Commercial Importance

Red algae continue to be an important component of seaweed aquaculture, representing 54% of the harvested biomass and nearly 50% of the value (US $6.3 billion in 2018) (FAO, 2020). Most red seaweeds, either collected from the wild or farmed, are used in the production of human food (Gaspar *et al.*, 2019). Direct consumption as sea vegetables is important in the Asia Pacific region, and red algal hydrocolloids are used widely in the food and other industries. New applications are being developed for marine algal products (e.g. functional foods, medicine (as anti-inflammatory, antiviral, anticancer uses), cosmetics and cosmeceuticals, and as biomaterials in skeletal replacement or regeneration (Pereira, 2018; Gaspar *et al.*, 2019)). Some red algae are known to produce secondary metabolites, which appear to play a key defensive role against both herbivory and fouling (Paul *et al*., 2006; Persson *et al.*, 2011). Paul *et al*. (2006) examined the possible connection between the chemical composition of *Asparagopsis armata* and its ecological roles, which showed antifouling properties and therefore can be a protection against harmful bacterial colonization.

Recently, there has been an effort to understand the utility of the metabolites produced by the genus *Asparagopsis,* which has resulted in the discovery of interesting activities as antifouling (Paul *et al.*, 2006), antimicrobial (Paul *et al.*, 2006; Blunt *et al*., 2009; Genovese *et al*., 2009, 2012; Pinteus *et al*., 2015), antiviral (Haslin *et al*., 2001; Alburquerque *et al.*, 2019), antioxidant and cytotoxic activity (Neethu *et al*., 2017), cosmetical applications (Pereira, 2018); in agriculture (Alburquerque *et al.*, 2019) and in food biotechnology, for both humans (Nunes *et al*., 2018; Shannon & Abu-Ghannam, 2019) and farmed animals (Machado *et al*., 2014, 2015; Li *et al*., 2016; Vucko *et al*., 2017; Roque *et al*., 2019).

1.4. *Asparagopsis*

Asparagopsis spp. (Figure 1.1) are characterized by creeping prostrate bases (stolons) anchored by rhizoids; bases giving rise to cylindrical erect axes radially branched to several orders, divisions becoming progressively finer so plants are soft and silky, ultimate filaments consisting of few cells; erect axes unbranched below, densely branched above. Tetrasporangial phase occurs as diminutive tufts of terete branched filaments with polysiphonia-like structure, each central axial cell surrounded by 3 uncorticated pericentral cells; gland-like vesicles between cells of filaments, highly refractive. Tetrasporangia cruciate divided, the result of meiosis in pericentral cells.

Spermatangia on short, club-like branchletes. Auxiliary cells lacking; carposporophyte large, usually with broadened base, pedicellate (Abbott, 1999).

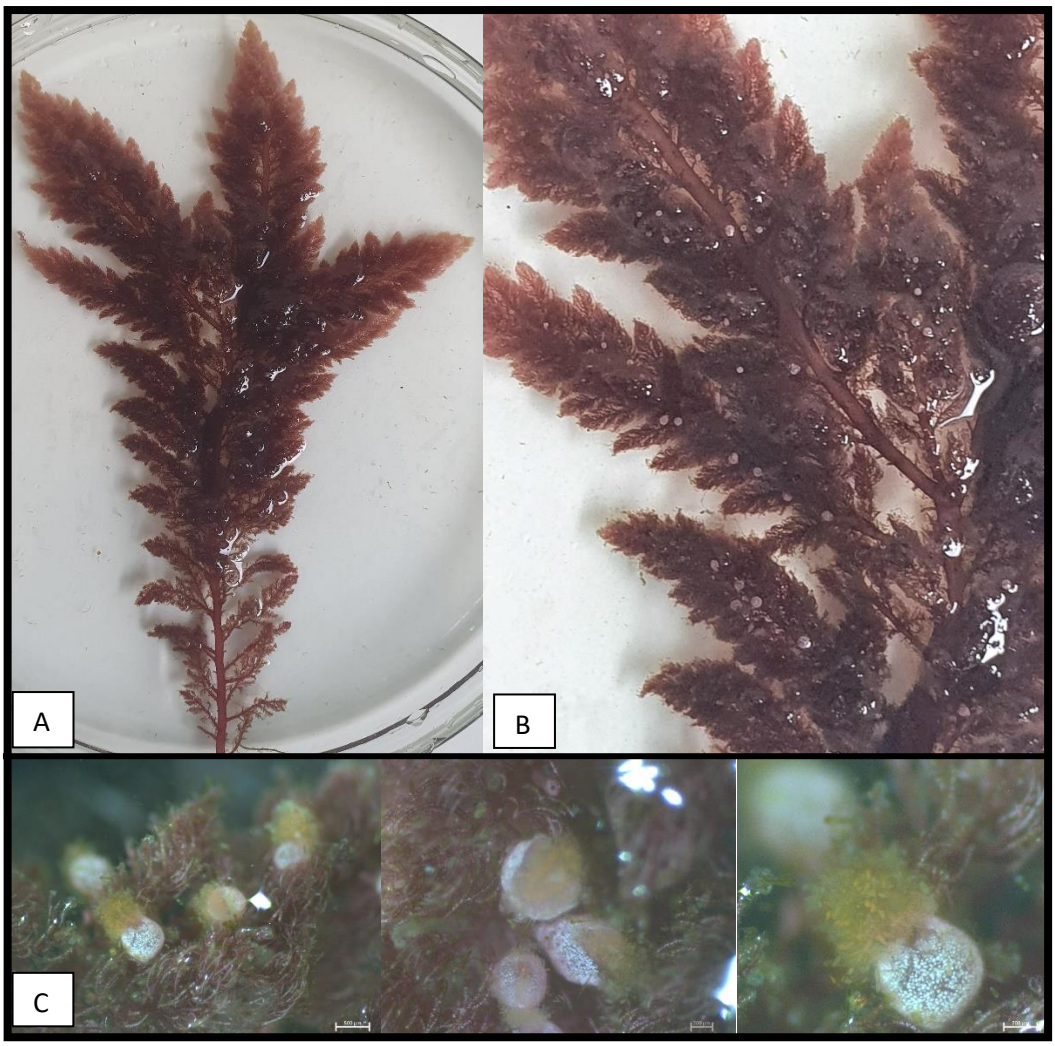

Figure 1.1. *Asparagopsis taxiformis* without rhizoids (A), collected from the wild in Pranchinha, São Miguel, Açores. Mature carposporophytes present (B) and carpospores being released (C).

Recent studies (Zanolla *et al.*, 2014; Zanolla *et al.*, 2017) have reported *Asparagopsis taxiformis* to be monoecious, having both male and female gametes on the same thallus.

Only two species have been taxonomically recognized *Asparagopsis armata* (Harvey) and *Asparagopsis taxiformis* (Delile) Trevisan (Guiry & Guiry, 2020) for this genus. The genus has been holistically studied in the last years, with a focus in areas such as ecology, physiology and ecophysiology (Andreakis *et al*., 2004; Andreakis *et al.*, 2016; Chualáin *et al.*, 2004; Cardigos *et al.*, 2006; Paul *et al.*, 2006; Padilla-Gamiño &

Carpenter, 2007; Altamirano *et al*., 2008; Zanolla *et al*., 2015, 2018, 2019; Martins *et al*., 2019); aquaculture (Oza, 1989; Guiry & Dawes, 1992; Schuenhoff *et al*., 2006; Figueroa *et al*., 2008; Mata *et al*., 2006, 2007, 2010, 2012, 2017) and in its biotechnological potential (Woolard *et al*., 1979; McConnell & Fenical, 1976; Paul *et al*., 2006; Mata, 2008; Genovese *et al*., 2012; Kinley *et al*., 2016; Castanho *et al*., 2017; Vucko *et al*., 2017; Clark *et al*., 2018; Pinteus *et al*., 2018; Nunes *et al*., 2018, 2020).

Asparagopsis taxiformis (Delile) Trevisan has been getting increased interest in recent years (e.g. Greener Grazing™, 2020; Blue Ocean Barns, 2020; Symbrosia, 2020). It is characterized by gametophytes of 10 to 20 cm tall, with several to many erect, generally plumose fronds, plumose portions to 3 cm diameter, lower axes usually naked, to 2 mm diameter, branching starting at lower fourth or third of each axis; dark rose to dark red unless subjected to intertidal exposure. Tetrasporangial phase known as "*Falkenbergia hillenbrandii*", uncommon in intertidal or shallow subtidal habitats but common as epiphyte on large number of other algae from 4 to 60 m depths.

1.5. Fertility Induction on Algae

Defining optimal culture conditions for cultivated algae species can increase yield and protect against undesired invasions by other algae. It is rather important the available knowledge of the ecological and molecular factors controlling the growth and reproduction, since the complex life cycles of algae, which often involves free-living haploid and diploid life stages, makes understanding and controlling life cycles challenging (Liu *et al*., 2017). Many species of algae have been studied in the last decades, with fundamental studies finding solutions for controlled laboratory fertility induction (e.g. blue light on *Laminaria saccharina* (Lüning & Dring, 1972), or photoperiod and temperature manipulation (Breeman *et al*., 1988)). Light quality and intensity, temperature, photoperiod and nutrient composition have been some of the major abiotic factors accounted for the successful life cycle manipulation in algae (Oza, 1989; Nash *et al*., 2005; Agrawal, 2009). Each phase of the life cycle of red seaweeds is dependent on several environmental and biotic factors. The reproductive behavior of algae may be triggered by one or more conditions, usually associated with seasonal changes (Hansen & Doyle, 1976). Many red seaweeds show strong seasonal fluctuations, with cystocarp formation and carpospore release peaking during spring (Pacheco-Ruiz *et al*., 2011). Florideophyceae gametophytes are known to develop reproductive structures during the

summer and early fall months, and most overwinter in their tetrasporophytic stage (Agrawal, 2009).

The *in vitro* reproductive behavior of red algae may be manipulated by changing culture conditions. The release of carpospores in some red seaweeds can be induced by immersing them in sterile seawater (Mantri *et al*, 2009, Avila *et al*., 2011). Nash *et al*. (2005) showed that all *Bonnemaisonia hamifera* tetrasporophytes grown in reduced nitrogen and phosphorous conditions became reproductive, developing tetrasporangia after 21 days, and later releasing viable tetraspores. *Asparagopsis armata* tetrasporophytes have been shown to develop tetrasporangia only when grown under short day conditions (8L:16D) at 15 ºC, when cultured in a medium with reduced N and P (Oza, 1977). Guiry & Dawes (1992) later confirmed that daylight and temperature were the primary factors to control tetrasporogenesis in this species. Therefore, laboratory cultivation conditions can help define life cycle events, opening the possibility of manipulating the abiotic factors to characterize the conditions needed to trigger vegetative or reproductive growth.

Algal life history is largely dependent on the germination of spores which allows for the transition between different life stages. *Asparagopsis taxiformis* has a complex life cycle, having both a gametophyte and tetrasporophyte stage, which produce carpospores and tetraspores, respectively (Yoon *et al*, 2016). Many environmental factors (e.g. light, temperature, nutrients, pH and water movement) affect algal spore germination (Agrawal, 2009). Agrawal (2009) found that the presence of inorganic nutrients, the presence of light, the presence of organic carbon and the need for a substrate where the spore can adhere to, were some of the most important factors for spore germination.

Asexual reproduction is mostly used in cultivation since it is an easier way to increase the biomass by area, avoiding the need of the reproductive effort in laboratory (e.g. fragmentation: *Ulva* spp. and some brown seaweeds). *Asparagopsis armata* gametophyte has long slender barbs which, when detached, are capable of regenerating into new thalli. However, fragments of fronds and stolons in this genus have no regenerative capacity (Haslin & Pellegrini, 2001). In contrast, the gametophytes of the sister species (*Asparagopsis taxiformis*) are reported to reproduce vegetatively by means of propagules formed at the tips of ultimate branches, which break off from the original frond and develop into young stolons (Haslin & Pellegrini, 2001).

1.6. Rationale

The genus *Asparagopsis* has a long history of being considered invasive, with two cryptic lineages of *A. armata* (both invasive) and six lineages of *A. taxiformis*, two of them considered to be invasive (L2 and L3) (Andreakis *et al*., 2004, 2016; Dijoux *et al*., 2014). This is an ecological problem but a profitable possibility for human use of new available resources (Andreakis & Schaffelke, 2012).

In the Azores, populations of *Asparagopsis* spp. are present yearly with greater mass occurring in late spring-beginning of summer (Neto *et al*., 2000; Neto, 2000, 2001). *A. armata* presents a cyclic exchange between gametophyte (fall/winter) and tetrasporophyte stages (winter/spring), with most of the gametophyte population disappearing in late summer (Tittley & Neto, 2005). *A. taxiformis* is a subtropical/tropical species (Womersley, 1996), which the gametophyte stage is present all year-round in the Azores (Tittley & Neto, 2005). Recently the abundance of *A. armata* has increased relative to the abundance of *A. taxiformis*, being highly present outside the marine protected areas (MPAs) while *A. taxiformis* is mostly present inside those areas (Cacabelos *et al*., 2019).

A. taxiformis is known for many different bioactivities (Pinteus *et al*., 2015; Neethu *et al*., 2017; Nunes *et al*., 2018; Roque *et al*., 2019) and is traditionally used in its native range (Hawaii) for human consumption (McDermid *et al*., 2019). The biomass available in the Azorean shores is already used for both food and biotechnological purposes (SeaExpert, 2019), but with increasing demand for the feed in the cattle industry (McDermid *et al*., 2019), new sources of this seaweed are needed.

The tetrasporophyte stage is already well studied for the sister species, *A. armata* (see works by Mata *et al*.), and there are on-going trials for massive supply of off-shore ropes with 'seeds' (Greener Grazing™). Therefore, there is interest in closing the *Asparagopsis* life cycle in *in vitro* laboratory conditions, to reduce the pressure to wild populations of *Asparagopsis.*

In the Azores, *Asparagopsis armata* is considered an invasive species, competing for space with *Asparagopsis taxiformis* (Cardigos *et al*., 2006), which is regarded as native. Considering the Portuguese legislation, which prohibits the cultivation of marine invasive species (Decreto-Lei n.º 92/2019), and considering the fact that this work was developed under an entrepreneurship scope (AQUAZOR, SA), this thesis focused on the

study of *Asparagopsis taxiformis* (non-invasive) life cycle in order to be able to produce it at a wider scale.

1.7. Objectives

The objective of this thesis was to define culture requirements for *Asparagopsis taxiformis*, including the abiotic factors that promote the growth and reproduction of each stage of its life cycle: (1) via the optimization of maintenance and growing conditions of the gametophyte stage (testing the effects of (a) pretreatment requirements; (b) light quality; (c) temperature; (d) nutrient requirements; (e) photoperiod; (f) stock density; (g) light intensity), and (2) to determine the factors that allow for the carposporogenesis to occur and promote its successful liberation *in vitro*.

2. MATERIAL & METHODS

To understand the cultivation requirements of *Asparagopsis taxiformis* several experiments were made. For the gametophyte stage, experimental trials tested (1) pretreatment requirements; (2) the effects of light quality and intensity; (3) temperature; (3) photoperiod; (4) nutrient requirements; and (5) stock density. In addition to this, the potential effects of variations in photoperiod and temperature on the carposporogenesis were also tested.

2.1. Material Collection

Fresh *Asparagopsis taxiformis* gametophytes were collected by snorkelling at different rocky shores around the south coast of São Miguel island (e.g. São Roque; Caloura) depending on weather and sea conditions, from October 2019 to May 2020. All the *Asparagopsis taxiformis* caught was brought to the laboratory in seawater to prevent air exposure in a thermic box at reduced temperature (freeze boards).

2.2. Pretreatment/Routine Cleaning

Fresh material was rinsed in seawater, cleaned of epiphytes and epifauna, and checked at a magnifier microscope (ZEISS Stemi 508). The presence or absence of reproductive structures and carpospores was observed to select for carposporophyte studies. The remaining non-fertile material was treated accordingly to each respective trial.

2.3. Material Preparation

All laboratory material was hand-washed with fresh water and deionized water, and monthly washed with soap or bleach to prevent the possible build-up of experimental residues. All glass material was sterilized in an autoclave (2 atm, 121ºC, 20 min., model Uniclave) and other non-glass material was passed in UV light for 10 min.

2.4. Medium Preparations

All seawater obtained from the nearby shore was filtered through two filters (1.2 and 0.2 µm pore-size membrane filters) using vacuum filtering to remove any sediments and phytoplankton. The filtered seawater was sterilized in an autoclave (2 atm, 40 min., 121ºC, model Uniclave) and left at room temperature to cool. When cold, the salinity of this Sterilized Filtered Seawater (SFS) was checked and corrected with deionized water

to have ca. 35 PSU. Von Stosch Medium (VS) was prepared with SFS following Guiry and Cunningham protocol (Guiry & Cunningham, 1984). Commercial Fertilizer (F) is a liquid fertilizer (Nutrea 12-4-6, Genyen®) with a known formula. To make the medium with this fertilizer a ratio of 250 µL (fertilizer) to 1 L (SFS) is used, giving a similar macronutrient content to Von Stosch Medium.

2.5. Trials

Most trials were done in the Cultivation Room of the AQUAZOR facilities under controlled temperature, photoperiod, and light intensity. For the trials which used different temperatures, cultivation chambers were used (Climatic Chamber, FITOCLIMA 600PL, SANYO, model MLR-351).

Trials were performed using plastic Petri Dishes (40 mL) and Erlenmeyers (500 mL). The latter had bottom aeration by aquaria tubes with attached sterilized points (Figure 2.1).

Figure 2.1. Example of *Asparagopsis taxiformis* in cultivation conditions inside a FITOCLIMA chamber.

2.5.1 Gametophyte

2.5.1.1 Pretreatments

This experiment aimed at seeing the gametophyte response to the different cleaning solutions normally used in seaweed to remove epiphytes/epizoa.

Collected gametophytes were sliced in their apical parts into 5 mm small segments maintaining a bush-like appearance. These segments were treated with different cleaning solutions, which are normally used in seaweeds: Freshwater (FW); Betadine 10%; Agar plates and Germanium Dioxide (GeO_2) (Mata *et al.*, 2017; Patarra *et al.* 2014, 2017, 2019; Shea & Chopin, 2007).

These segments were submitted to 9 different treatments (Control, A to H) to test the effects of cleaning in *Asparagopsis taxiformis*, corresponding to (i) Freshwater (A); (ii) Freshwater and Agar Plate (B); (iii) Freshwater and Betadine 10% (C); (iv) Freshwater and Germanium Dioxide (D); (v) Freshwater, Agar Plate and Betadine 10% (E); (vi) Freshwater, Agar Plate and Germanium Dioxide (F); (vii) Freshwater, Betadine 10% and Germanium Dioxide (G); (viii) Freshwater, Agar Plate, Betadine 10% and Germanium Dioxide (H); (ix) SFS (Control).

Each treated segment was transferred in triplicated to Petri Dishes (40 mL) with SFS, in a 12L:12D (Light:Dark) photoperiod, at 20 ºC and 20 µmol photon m^{-2} s^{-1}. Mass of the tips was recorded at the beginning and end of the experiment. Pigmentation status was scored every day for first three days of cultivation (survivability) and weekly thereafter.

2.5.1.2 Light Quality, Intensity and Medium

The effects of medium (SFS, VS, F), light intensity (10, 20 and 40 µmol photon m^{-2} s^{-1}) and quality (blue, red, white) was tested in an orthogonal experiment with triplicated Petri Dishes per combination of treatments totaling 81 Petri Dishes. Triplets of segments totaling 0.1 g of *A. taxiformis* tips were added in combination with 40 mL of selected medium to each Petri Dish. All replicates were cultivated under a 12L:12D photoperiod and a temperature of 20ºC. Each week, surviving tips (noticed mainly by pigment coloration) were accounted and weighted. Media was refreshed weekly.

2.5.1.3 Temperature

This experiment aimed at understanding the response of the *A. taxiformis* gametophyte to the different temperatures experienced in the wild throughout the year.

The effect of temperature (15, 20, and 25 ºC) on the growth of *A. taxiformis* was tested using 0.5 L Erlenmeyers. The selected temperatures correspond to the average range of temperatures experienced by this species during Winter, Spring and Summer in the Azores archipelago (Amorim *et al.*, 2017). All replicates were cultivated under a 12L:12D photoperiod and at 20 µmol photon m^{-2} s^{-1}. Prior to the start of the experiment, all seaweed stayed an initial week in SFS to acclimatize to culture conditions. Afterwards, cultivation was done using a commercial fertilizer (F) with weekly exchanges of medium. Algal mass was recorded weekly.

2.5.1.4 Media

This experiment aimed at understanding the gametophyte response to different media to understand if *Asparagopsis* was nutrient-limited and to find an easier solution for nutrient supplementation.

The effect of the media, (i) VS, (ii) F, (iii) 2F (double of Genyen®) on the growth of *A. taxiformis* was tested using 0.5 L Erlenmeyers. The selected media were chosen based on preliminary trials that showed a possibility of switching Von Stosch Medium based cultivation to this industrial based solution. All replicates were cultivated under a 12L:12D photoperiod, at 15 ºC and at 20 µmol photon m^{-2} s^{-1}. Prior to the start of the experiment, all seaweed stayed an initial week in SFS to acclimatize to culture conditions. Afterwards, cultivation was done with weekly exchanges of medium. Algal mass was recorded weekly.

2.5.1.5 Photoperiod

This experiment aimed at understanding the response of the gametophyte to the different photoperiods that *A. taxiformis* naturally experiences throughout the year in the Azores.

The effect of photoperiod (14L:10D, 12L:12D, 10L,14D) on the growth of *A. taxiformis* was tested using 0.5 L Erlenmeyers. The selected photoperiods correspond to the average range of photoperiods experienced by this species during Summer, Autumn/Spring and Winter in the Azores (Amorim *et al.*, 2017). All replicates were cultivated at 15 ºC and 20 µmol photon m^{-2} s^{-1}. Prior to the start of the experiment, all algae stayed an initial week to acclimatize to culture conditions in SFS. Afterwards, cultivation was done using a commercial fertilizer (F) with weekly exchanges of medium. Algal mass was recorded weekly.

2.5.1.6 Stock Density

This experiment aimed at understanding the gametophyte response to the different stock densities at which it can be cultivated.

The effects of different stock densities (1, 2, and 4 g/L of fresh algal mass) on the growth of *A. taxiformis* was tested using 0.5 L Erlenmeyers under a 12L:12D photoperiod, at 15 ºC and 20 µmol photon m^{-2} s^{-1}. Prior to the start of the experiment, all algae stayed an initial week to acclimatize to culture conditions in SFS. Afterwards, cultivation was

done using a commercial fertilizer (F) with weekly exchanges of medium. Algal mass was recorded weekly and the density was reduced to the initial stock density.

2.5.1.7 Light Intensity

This experiment aimed at understanding the gametophyte response to the different light intensity at which it can be cultivated.

The effects of two different light intensities (20 and 40 µmol photon m^{-2} s^{-1}) on the growth of *A. taxiformis* were tested using 0.5 L Erlenmeyers under a 12L:12D photoperiod, at 15 ºC. Prior to the start of the experiment, all algae stayed an initial week to acclimatize to culture conditions in SFS. Afterwards, cultivation was done using a commercial fertilizer (F) with weekly exchanges of medium. Algal mass was recorded weekly.

2.5.2 Carposporophyte

This experiment aimed at understanding the nutritional needs to promote carposporophyte growth and maturation.

The effect of cultivation medium (SFS and VS) on the onset of reproductive traits of *A. taxiformis* was tested using 0.5 L Erlenmeyers. All replicates were cultivated in a 16L:8D photoperiod, at 22 ºC and 20 µmol photon m^{-2} s^{-1} and were followed daily for changes in reproductive traits. Prior to the start of the experiment, all algae stayed an initial week to acclimatize to culture conditions in SFS, and afterwards cultivated in their respective treatments with weekly exchanges of medium. Algal mass was recorded weekly. Photos were taken to assess visual changes in reproductive traits.

2.6. Growth Parameters

To define growth, sample material was weighted using known practices (e.g. blotting) to access fresh mass (fm). Consisting of drying the fresh material in paper to remove excess water. All samples were weighted at beginning (w0) and end (wf) of each week of their respective trial.

An experiment to access dry mass (dm) was performed, in which *A. taxiformis* samples (n = 15) were dried at 60 ºC for 72h, and final dry mass was compared with initial fresh mass.

Following Hung *et al.* (2009), daily growth rates (DGR) and Productivity were used to quantify growth:

(i) **DGR** (%fm day^{-1}) = $[(w_f) / (w_0)] \wedge (1/\Delta t) - 1) * 100$;

(ii) **Productivity** (g dm m^{-3} day^{-1}) = dm x $[((w_f - w_0)/\Delta t)\ v]$

Where w_0 and w_f are initial and final fresh masses (fm) in grams, Δt is time in days, dm is the proportion of dry mass to fresh mass (mean of 15 samples = 0.114 ± 0.005), and v is the volume (0.0005) in m^3 of the Erlenmeyers.

2.7. Data Analyses

Generally, in each experiment, the effects of treatments were tested using a 2-way Analysis of Variance (ANOVA) with treatment and time as factors. Due to lack of independence of samples through time, most analyses were done using a Repeated Measures Analysis ANOVA (rmANOVA). Prior to analysis, data were checked for sphericity and transformation was applied where necessary (Underwood, 1997). Where data failed the sphericity prerequisite, data were analysed using a 1-way ANOVA for each time. In this case, heteroscedasticity was tested using Cochran's C test and transformations were applied where necessary (Underwood, 1997). A posteriori comparison, where needed, were tested using Bonferroni in the case of rmANOVA or Student-Newman-Keuls (SNK) in the case of ANOVA. rmANOVAs were done using the package rstatix in R (RStudio Version 1.2.5033), whilst ANOVAs were done using GAD package in R (RStudio Version 1.2.5033). Results were plotted using the graphical software Sigmaplot (12th edition, Systat Software, Inc).

3. RESULTS

3.1. Gametophyte

3.1.1 Pretreatments

The use of pretreatments (Fresh water, Agar Plate, Germanium Dioxide and Betadine) to clean *A. taxiformis* had a negative effect in the survivability of the individuals (Figure 3.1). Only non-treated seaweed (Control) survived more than a week in cultivation without showing depigmentation. Single pretreatments (A to D) had more survivability in the first day of cultivation but nevertheless most individuals in the different treatments died by day 2.

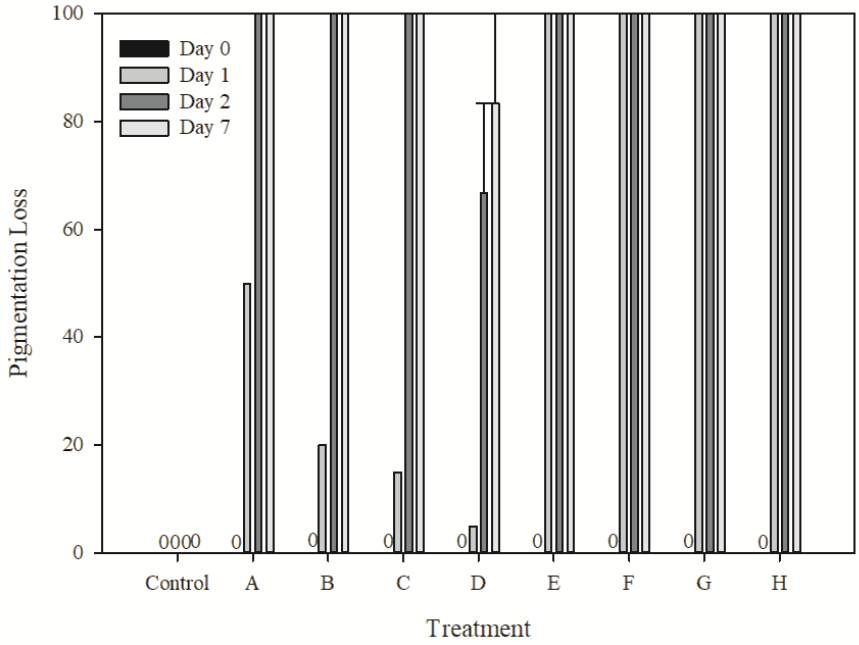

Figure 3.1. Pigmentation Loss (%) by Treatment in subsequent days of *Asparagopsis taxiformis* submitted to nine treatments (Control – Sterilized Filtered Seawater; A – Fresh Water; B – Fresh Water & Agar Plate; C – Fresh Water & Betadine 10%; D – Fresh Water & Germanium Dioxide; E – Fresh Water, Agar Plate and Betadine 10%; F – Fresh Water, Agar Plate and Germanium Dioxide; G – Fresh Water, Betadine and Germanium Dioxide; H – Fresh Water, Agar Plate, Betadine 10% and Germanium Dioxide). Values are mean ± SE (n = 3).

3.1.2 Light Quality, Intensity and Medium

This experiment aimed to test the best possible cultivation conditions of *Asparagopsis taxiformis*, since the bibliography showed difficulty in cultivating it. Although the seaweed survived for four weeks (most died after this, so the trial ended) the results were not conclusive for any of the treatments involved, with variable rates of growth during the four-week trial, with most replicates decreasing in mass weekly under

all the tested cultivation conditions (Figure 3.2). Some of the observed growth spikes may be due to experimental error owing to the great number of variables tested simultaneously plus the cultivation in Petri Dishes, which offers some limitations. Under no treatments was there an alteration of the reproductive characteristics.

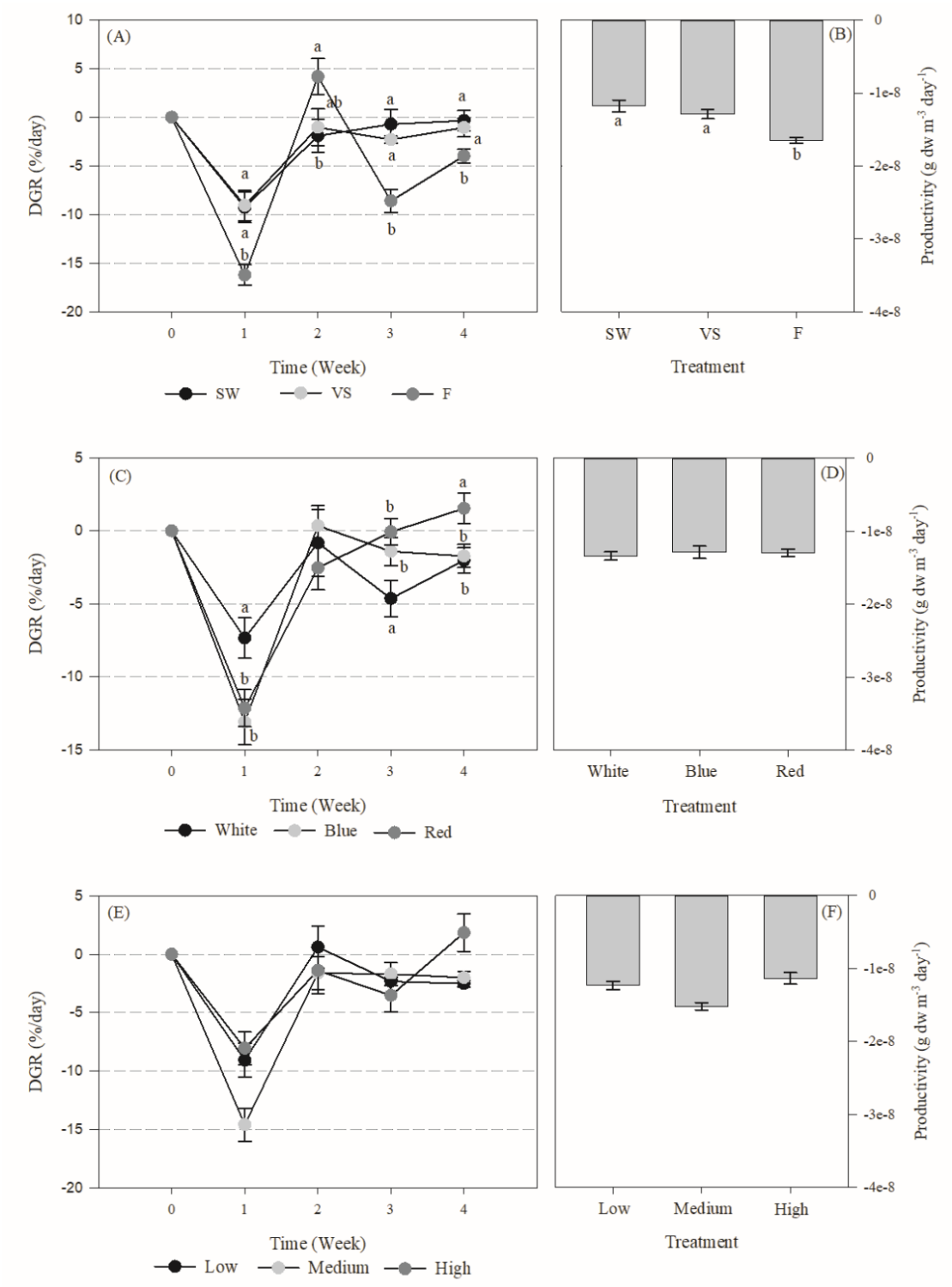

Figure 3.2. Daily Growth Rate (%/day) by Week of *Asparagopsis taxiformis* submitted to a three-way experiment, (A) Medium (SW = Sterilized Filtered Seawater, VS = Von Stosch, F = Fertilizer), (C) Light Quality (bulb light color emission: White, Blue, Red) and (E) Light Intensity (Low = 10 µmol photon m^{-2} s^{-1}, Medium = 20 µmol photon m^{-2} s^{-1}, and High = 40 µmol photon m^{-2} s^{-1}); Productivity (g dw m^{-3} day^{-1}) by Treatment of *Asparagopsis taxiformis* submitted to a three-way experiment, (B) Medium (SW = Sterilized Filtered Seawater, VS = Von Stosch, F = Fertilizer), (D) Light Quality (bulb light color emission: White, Blue, Red) and (F) Light Intensity (Low = 10 µmol photon m^{-2} s^{-1}, Medium = 20 µmol photon m^{-2} s^{-1}, and High = 40 µmol photon m^{-2} s^{-1}). Values are mean ± SE (n = 27). The absence of letters indicates no statistically significant differences. Different letters represent statistically significant differences among the treatments on the given day ($p < 0.05$).

3.1.3 Temperature

Among the temperatures tested, *A. taxiformis* only showed positive growth at 15ºC (overall mean: 0.653 ± 0.312 %/day; 0.00040 ± 0.00005 g dw m^{-3} day^{-1}). *Asparagopsis taxiformis* exposed to 20 ºC and 25ºC did not grow during the experimental period, with the latter (25 ºC) showing substantial structural degradation over time (Figure 3.3). Both DGR and Productivity showed significant differences between all treatments ($p < 0.05$) by the end of the experiment. Under no treatments did *A. taxiformis* showed any modifications of the reproductive characteristics.

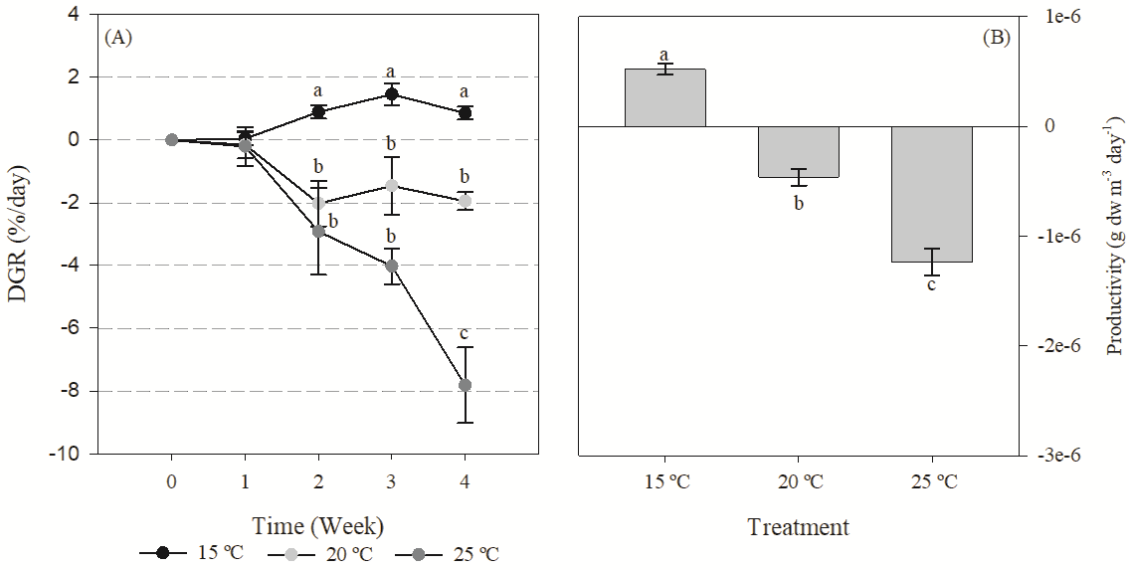

Figure 3.3. (A) Daily Growth Rate (%/day) by Week of *Asparagopsis taxiformis* submitted to three temperatures (15, 20 and 25 ºC). (B) Productivity (g dw m^{-3} day^{-1}) by Treatment of *Asparagopsis taxiformis* submitted to three temperatures (15, 20 and 25 ºC) at the end of the experimental period. Values are mean ± SE (n = 6). The absence of letters indicates no statistically significant differences. Different letters represent statistically significant differences among the treatments on the given day ($p < 0.05$).

3.1.4 Media

The growth rate of *A. taxiformis* was statistically similar ($p = 0.053$) both with the commercial fertilizer (at the two concentrations) and the commonly used Von Stosch Medium (Figure 3.4A). Despite the similar growth rates, over the entire experimental period, productivity of *Asparagopsis taxiformis* was significantly greater in 2F compared to F and VS, suggesting that it may be nutrient limited in the latter two treatments (Figure 3.4B). The treatments did not show any modifications to reproductive characteristics.

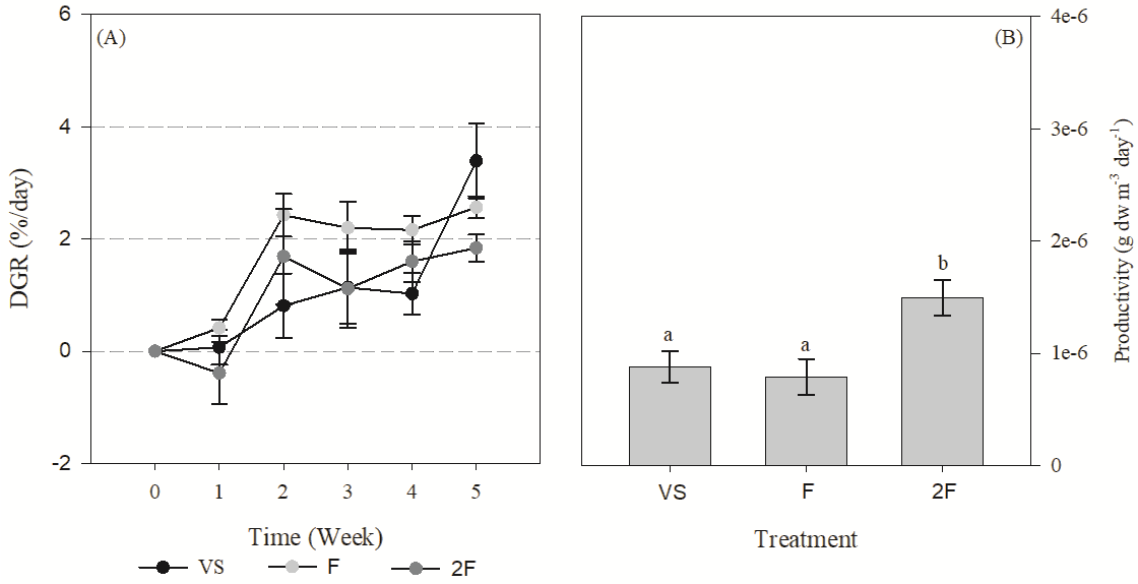

Figure 3.4. (A) Daily Growth Rate (%/day) by Week of *Asparagopsis taxiformis* submitted to three different culture media (Von Stosch (VS); Fertilizer (F); 2 times Fertilizer (2 F)). (B) Productivity (g dw m^{-3} day^{-1}) by Treatment of *Asparagopsis taxiformis* submitted to three treatments (Von Stosch (VS); Fertilizer (F); 2 times Fertilizer (2 F)). Values are mean ± SE (n = 6). The absence of letters indicates no statistically significant differences. Different letters represent statistically significant differences among the treatments on the given day ($p < 0.05$).

3.1.5 Photoperiod

There were no significant differences in the growth rate of *Asparagopsis taxiformis* grown under the three photoperiods ($p = 0.953$; Figure 3.5A). Overall, the productivity of *A. taxiformis* was negative and similarly so among the different

photoperiods (Figure 3.5B) Although the growth of *A. taxiformis* appears to show an increasing trend towards the last week of cultivation (third week), this experiment was abruptly terminated due to unforeseen circumstances (COVID-19 lockdown).

Despite the observed decreasing mass in *A. taxiformis* during the cultivation period across all treatments (Figure 3.5B), female reproductive structures could be observed in all treatments but with no increased development into carposporophyte.

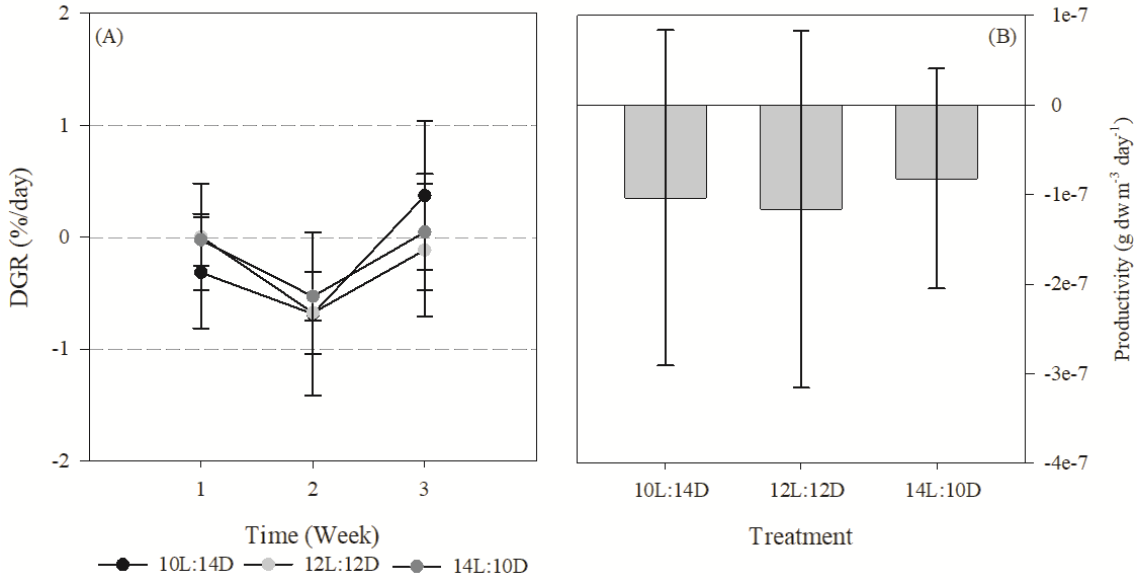

Figure 3.5. (A) Daily Growth Rate (%/day) by Week of *Asparagopsis taxiformis* cultivated under three photoperiods (Winter photoperiod - 10L:14D; Spring/Fall photoperiod - 12L:12D; Summer photoperiod – 14L:10D). (B) Productivity (g dw m^{-3} day^{-1}) by Treatment of *Asparagopsis taxiformis* cultivated under three photoperiods (Winter photoperiod - 10L:14D; Spring/Fall photoperiod - 12L:12D; Summer photoperiod – 14L:10D). Values are mean ± SE (n = 6). The absence of letters indicates no statistically significant differences.

3.1.6 Stock Density

The growth rate of *A. taxiformis* was significantly greater when cultivated at the lowest initial stock density of 1 g/L and similar when cultivated at the stock densities of 2 and 4 g/L (Figure 3.6A). When considering the productivity of *A. taxiformis* over the entire period of the experiment, there was no significant differences among stock densities (Figure 3.6B), although the productivity tended to be null at the greatest stock density of 4 g/L.

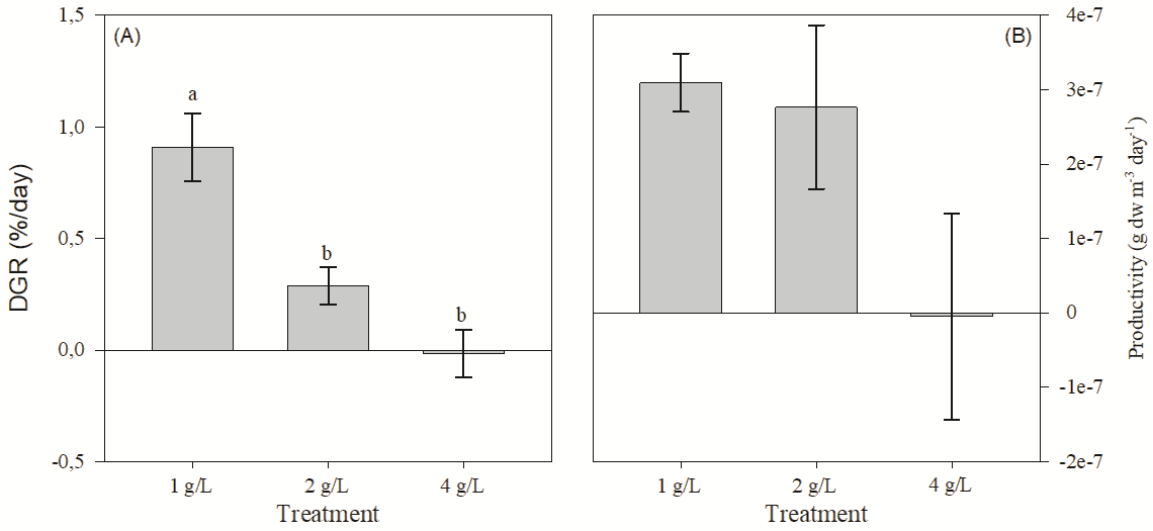

Figure 3.6. (A) Daily Growth Rate (%/day) by Treatment of *Asparagopsis taxiformis* cultivated under three stock densities (1 g/L; 2 g/L; 4 g/L). Values are mean ± SE (n = 30). (B) Productivity (g dw m^{-3} day^{-1}) by Treatment of *Asparagopsis taxiformis* submitted to three treatments (1 g/L; 2 g/L; 4 g/L). Values are mean ± SE (n = 6). The absence of letters indicates no statistically significant differences. Different letters represent statistically significant differences among the treatments on the given day (p < 0.05).

3.1.7 Light Intensity

Previous experiments were mostly done at 20 µmol photon m^{-2} s^{-1}, since preliminary studies showed that more light intensity tends to promote the individual loss of pigmentation and eventually death of *Asparagopsis taxiformis* (data not shown). This experiment showed that, generally, growth rate of *A. taxiformis* was greater when cultivated under 40 µmol photon m^{-2} s^{-1} but this result was only significantly so at the second week of cultivation (Figure 3.7A). This result, however, becomes apparent when considering the productivity, which was significantly greater at 40 µmol photon m^{-2} s^{-1} (Figure 3.7B). There were no modifications in reproductive characteristics over the experimental period.

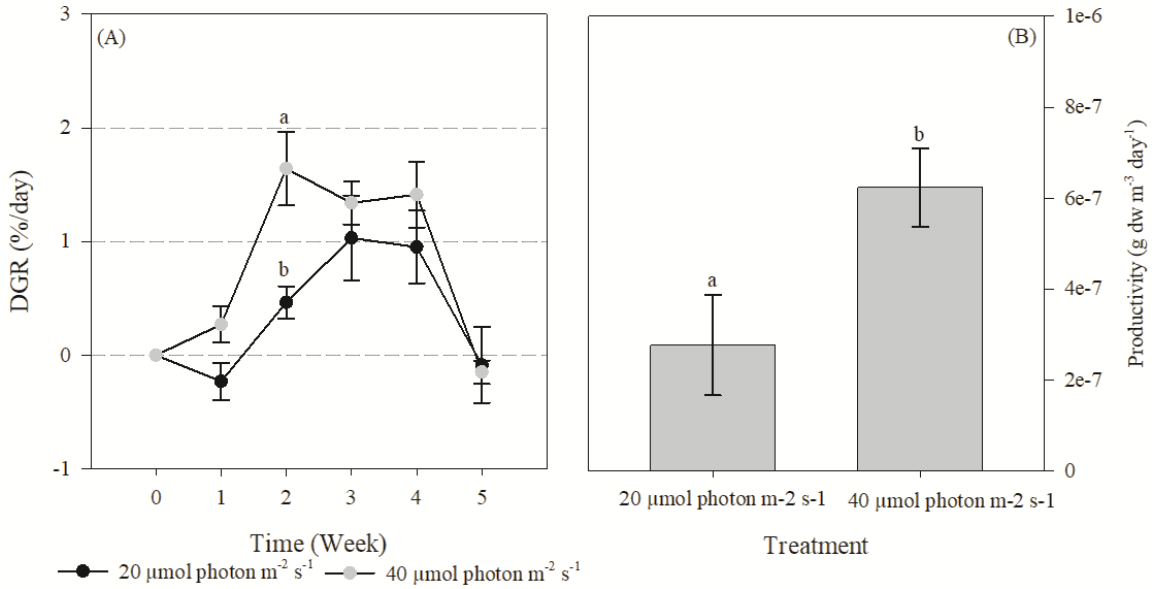

Figure 3.7. (A) Daily Growth Rate (%/day) by Week of *Asparagopsis taxiformis* submitted to two treatments (20 µmol photon m^{-2} s^{-1} and 40 µmol photon m^{-2} s^{-1}). (B) Productivity (g dw m^{-3} day^{-1}) by Treatment of *Asparagopsis taxiformis* submitted to two treatments (20 µmol photon m^{-2} s^{-1} and 40 µmol photon m^{-2} s^{-1}). Values are mean ± SE (n = 6). The absence of letters indicates no statistically significant differences. Different letters represent statistically significant differences among the treatments on the given day ($p < 0.05$).

3.2. Carposporophyte

Despite the presence of reproductive structures in all *A. taxiformis*, their development into carposporophytes was never observed during the cultivation period and regardless of treatment (Figure 3.8). In all cases, algae tend to die; in the nutrient enriched cultures, *A. taxiformis* became contaminated with epiphytes, whilst in the unenriched culture *A. taxiformis* lost pigmentation and bleached.

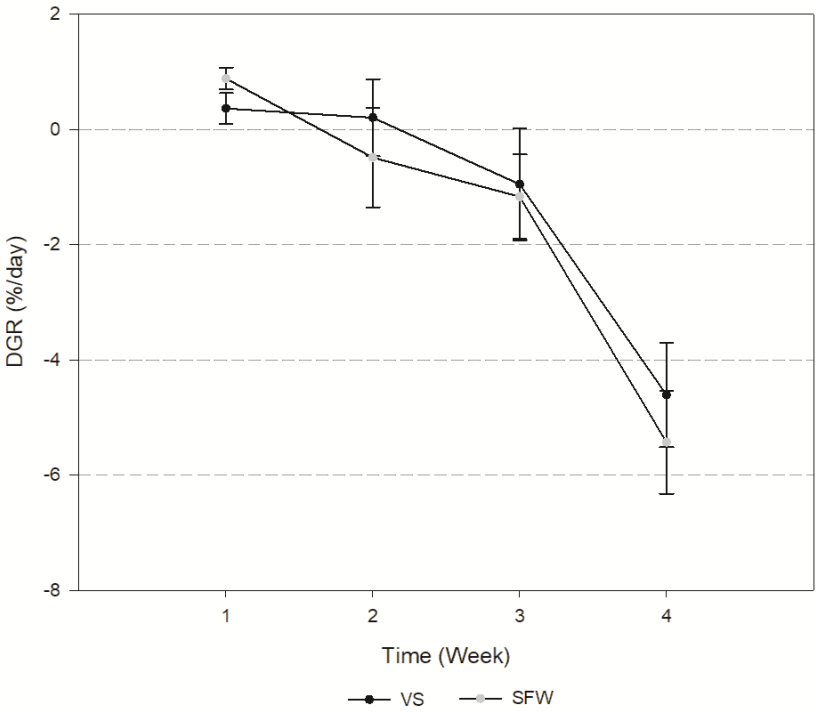

Figure 3.8. Daily Growth Rate (%/day) by Week of *Asparagopsis taxiformis* submitted to two treatments (SFW and Von Stosch). Values are mean ± SE (n = 6). The absence of letters indicates no statistically significant differences.

A number of other pilot and small studies were done to try to induce carposporophyte formation, but results were invariably unsuccessful.

4. DISCUSSION

The genus *Asparagopsis* has received much research attention in recent years (e.g. Mata *et al*., 2017; Alburquerque *et al*., 2019; Roque *et al*., 2019) with major breakthroughs in its cultivation having been made in the last decade but only on the *Falkenbergia* stage of its life cycle (Mata *et al*., 2006, 2007). The tetrasporophyte stage is known to be a more resistant-like state of the complex life cycle of the Florideophyceae (Agrawal, 2009). The *Falkenbergia* stage of the *Asparagopsis* spp. is mostly present in the fall-winter months in the Azores (Neto, 2000).

Despite the existence of some studies on the cultivation of the different stages in *Asparagopsis armata* (e.g. Mata *et al*., 2006), there is yet, to the best of my knowledge, no publications regarding the cultivation of the gametophyte of *Asparagopsis taxiformis* reporting a success in its cultivation. As such, this work presents itself as a breakthrough by showing that it is possible to maintain and grow the gametophyte of *A. taxiformis* under a strict set of cultivation conditions.

4.1. Gametophyte

4.1.1 Pretreatments

Cleaning pretreatments are a usual component of all seaweed cultivation starting point, since there is a need to clean macroalgae from debris and epizoa/epiphyte that may contaminate and ruin the cultivations (Andersen, 2005). This is a particularly important procedure when pure cultures are required.

The genus *Asparagopsis* is known to have many chemical and antifouling properties that naturally eliminate their possible "parasites" (Paul *et al*., 2006). My results showed that the common pretreatments used to clean macroalgae in literature (e.g. Shea & Chopin, 2007; Mata *et al*., 2017; Patarra *et al*., 2017) all had negative effects on the gametophyte of *A. taxiformis*. Since all treatments except the control had total mortality after 2 days, this may, perhaps, be associated with the use of Freshwater (across all pretreatments), taking in consideration the delicate nature of the gametophyte, which could have increased the sensibility to the remaining treatments.

Given these results, all macroalgae were never pretreated during the subsequent experiments of this work, except the cleaning of epizoa and the selection of healthy/low epiphyte presence individuals in SFW baths. These were enough to have experimental trials with no significant growth of epiphytes during many cultivation weeks.

4.1.2 Light Quality, Intensity and Medium

This was the first experiment of this thesis and was designed to cross test a number of variables that are known to be important in the cultivation and reproduction of marine macroalgae. For instance, some brown seaweeds are known to be affected in different ways by differences in light colour (Lüning & Dring, 1972). Likewise, light intensity can be a stimulus for the induction or inhibition of growth in algae (Goh *et al*., 2012). Unfortunately, the growth of *A. taxiformis* was negative across all the treatments. As demonstrated by the subsequent experiment (see point 4.1.3), growth of *A. taxiformis* is negatively affected by temperatures above 20 ºC. Since this experiment was done at 20 ºC without this *a priori* knowledge of the potential negative effect of this temperature, it is likely that the overall negative results were a consequence of the cultivation temperature. This experiment should be repeated if possible, under a different set of temperatures.

4.1.3 Temperature

Asparagopsis spp. is known to have greater biomass between winter and spring months (January to April) in the Azores, although *Asparagopsis taxiformis* is observed all year round (Neto *et al*., 2000; Neto, 2000, 2001). Results showed that cultivation of *A. taxiformis* was only positive (and with good visual aspect) when it was done at 15 ºC. There was no growth when cultivated at 20 ºC and 25 ºC with its mass eventually decreasing with time. These results seem to be in accordance to its ecology in the Azores where temperatures of the seawater never reach 25 ºC for more than a couple of days a year and being mostly around 15 ºC during the spring months (Amorim *et al.*, 2017).

The effect of lower temperatures and a wider range of temperatures in the growth of *A. taxiformis* should be tested to further define optimal cultivation temperature.

4.1.4 Media

Nutrient supplementation in seaweed cultivation is a common practice to achieve higher growth rate or for laboratory management of cultures. The most used media for red seaweeds, the Von Stosch Medium (Guiry & Cunningham, 1984), as many other artificial media, is time-consuming and volume-restricted in its makings. For that purpose, a commercial concentrated fertilizer, with similar components to Von Stosch, was tested for the cultivation of *Asparagopsis taxiformis*. Results showed that *Asparagopsis taxiformis* could be grown with both media, facilitating the process of media exchange for the other experiments. Interestingly, doubling the amount of the commercial fertilizer led to increased productivity, even compared to that observed in the Von Stosch suggesting that the latter may limit the growth of *A. taxiformis* in some way.

4.1.5 Photoperiod

Light is an important factor for autotrophic organisms such as seaweeds. Photoperiod is known to have implications in many aspects of seaweed life cycles, including a response in growth rate (Breeman *et al.*, 1988; Agrawal, 2009). The gametophyte of *Asparagopsis taxiformis* is normally present all year round, although the *Falkenbergia* state only appears during brief periods of the year (Neto, 2000). This pattern appears to suggest that a changing photoperiod over the year could have some effect on the development of the *Falkenbergia*. However, my results show that, at least for the duration of the experiment and under the cultivation settings used, changing the

photoperiod did not affect the growth or the reproductive behavior of the gametophyte. It is possible that a concomitant change in photoperiod and other abiotic factors (e.g. temperature) is required to trigger the onset of reproduction in *A. taxiformis*. The orthogonal test of the effects of photoperiod and temperature was planned but logistical constraints (COVID-19) made it impossible.

4.1.6 Stock Density

Stock Density is an important factor for seaweed cultivation. Stock density works in a way to reduce time of direct exposure to light while allowing space for growth of algae (Mata *et al*. 2006). In the case of *Asparagopsis taxiformis* gametophyte, the lowest stock density of 1 g/L showed the best result. It is possible that the delicate fronds of *A. taxiformis* are negatively affected by abrasion at greater densities. To further explore this, a system where the fronds are fixed to a substrate, and thus reducing the abrasion among fronds, could be tested.

4.1.7 Light Intensity

Light Intensity needs to be high enough to sustain the photosynthetic process and maximize growth but not as high as to cause stress, photoinhibition and damage to the photosynthetic apparatus (Goh *et al.*, 2012).

Results showed that the gametophyte of *Asparagopsis taxiformis* could be successfully cultivated at low light intensity (≤ 40 μmol photon m-2 s-1). Studies on the *Asparagopsis* spp. tetrasporophyte showed similar light intensity needs (Oza, 1989; Mata et al., 2007).

Trials should be made to assess the effect of a wider range of light intensities on the growth of gametophyte of *Asparagopsis taxiformis*.

4.2. Carposporophyte

Cultivation of the genus *Asparagopsis* has focused mostly on the tetrasporophyte stage of its life cycle (e.g. Mata *et al.* 2006), with a lack of knowledge on the conditions needed to stimulate the change from the gametophyte stage into the tetrasporophyte stage. The genus *Asparagopsis* has been showed to be monoecious (Zanolla *et al*, 2014, 2017), which was confirmed in our collected samples. Unfortunately, although it was possible to release carpospores from fertile carposporophytes to produce *Falkenbergia*, it was not possible to uncover the conditions under which the gametophyte undergoes

carposporogenesis. More trials regarding the photoperiod, temperature and nutrient supplementation should be done to understand the possible mechanisms of gametophyte induction.

Finally, under the laboratory conditions, growth rate of *Asparagopsis taxiformis* gametophyte was very low (close to 1% /day in most of the trials). This is very low when compared to growth rates observed in the cultivation of other species worldwide (e.g. *Ulva pertusa* – 12%/day (Le *et al.*, 2018)). Given these growth rates, at the moment, the indoor cultivation of the gametophyte of *A. taxiformis* seems likely unfeasible from a commercial point of view.

5. CONCLUSION

Although the life cycle of *Asparagopsis taxiformis* could not be closed in this work, the optimum abiotic conditions for the growth the gametophyte were tested.

Temperature and light intensity were shown to play a key role for the successful cultivation of the gametophyte. The Azorean form of *Asparagopsis taxiformis* only grew at temperatures lower than 20 ºC and under very low light intensities (< 40 µmol photon m^{-2} s^{-1}). Yet, even under the best growing condition, growth rates were close to 1% per day, which is a very low value taking in consideration the rest of the seaweed cultivation market.

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