Propagation of macroalga *Codium tomentosum* for aquaculture production

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Mestrado em Recursos Biológicos Aquáticos

Departamento de Biologia, 2022

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Agradecimentos

Em primeiro lugar quero agradecer a toda a gente que de alguma forma me ajudou, apoiou e encorajou. A todos os presentes na minha vida, obrigada.

À Doutora Isabel Sousa Pinto um profundo obrigado por me ter aceitado neste projeto e me ter dado a oportunidade de trabalhar na minha área de preferência, foi um trabalho extremamente gratificante.
Ao Doutor Gonçalo Marinho, a pessoa que me guiou durante todo este projeto, um agradecimento muito especial por toda a ajuda e disponibilidade, mas também pela liberdade e confiança.

À Xica, por toda a ajuda e ensinamentos que partilhaste comigo, pela motivação e confiança! Não sei se teria conseguido fazer tudo o que fiz sem ti. Do fundo do coração, obrigada!
À Stefany, pela companhia, sessões fotográficas e todas as histórias malucas trocadas.
A toda a gente do LBC, o melhor laboratório do CIIMAR, vocês são sem dúvida as melhoras pessoas que já conheci neste último ano. Obrigada por estarem sempre disponíveis a ajudar e pela vossa constante boa disposição, em especial à Adriana, à Libânia e ao Aires. É uma verdadeira honra pertencer a este laboratório! Gosto muito de vocês todos!
A todas as pessoas que conheci no CIIMAR e que de alguma maneira me ajudaram, muito obrigada, estou a olhar para ti Ricardo!

A todos os meus amigos, desde as de sempre, as minhas unicórnias, obrigada por fazerem parte da minha vida, pela inspiração de vos ver seguir os vossos sonhos e pelo orgulho que sei que têm em mim, aos amigos mais recentes que a universidade me deu, obrigada pelo apoio e por terem estado ao meu lado em todos os grandes momentos. Um obrigado muito especial à Joana e a Sara, por serem as minhas “the one” e se manterem firmes nesta amizade! À Lúcia e à Rita, as melhores surpresas do mestrado, por tudo o que passamos nestes dois anos, pela proximidade que criamos e por todos os momentos vividos, vocês sabem o que valem para mim!

Ao Diogo “PATO”, o meu braço direito (e esquerdo), o meu companheiro de todos os momentos, obrigada por me teres escolhido para melhor amiga (eu também te escolhi) e por me teres acompanhado desde o primeiro ano da faculdade até ao fim do mestrado. Sem dúvida alguma que esta aventura não teria sido a mesma sem ti (não teria tido nem metade da piada)! Eternamente agradecida patito… por tudo.

Às pessoas mais importantes da minha vida, os meus pais! Sem vocês não estaria aqui (literalmente). Obrigada por me assegurarem sempre do que valho, por me terem apoiado durante toda a minha vida, por todo o orgulho e confiança que têm em mim e por me tornarem na pessoa que sou hoje, adoro-vos muito! Beijos de paixão!

Finalmente, gostaria de dedicar esta tese à minha avó, a minha “mãe duas vezes” , que sempre se orgulhou tanto em mim, eu sei que se ainda cá estivesse seria a primeira a felicitar-me com mil beijos e a telefonar a toda a gente para se gabar!

This thesis is integrated in the research and innovation AquaVitae (AV) project funded by the European Union’s Horizon 2020 Research and Innovation Programme under Grant Agreement No 818173.

Resumo

O *Codium* é um género de macroalga verde (Chlorophyta) que é maioritariamente apanhada do mar, o que pode levar à sobrexploração dos seus stocks naturais. O *Codium* é usado nas indústrias alimentar, cosmética, farmacêutica e na biomedicina, visto que as espécies deste género, tal como o *Codium tomentosum*, são importantes fontes de polissacáridos sulfatados e outros compostos com múltiplas propriedades bioativas. Para além disto, o *Codium tomentosum* é considerada uma alga com grande potencial para a produção em aquacultura. No entanto, pouco é sabido sobre certos aspetos desta espécie, nomeadamente o seu estado reprodutivo, estratégias reprodutivas e condições de cultura que melhor induzem o seu desenvolvimento, tanto através de reprodução sexual como micropropagação.

Assim, este projeto foi concebido na tentativa de expor este conhecimento em falta relativamente ao ciclo de vida do *Codium tomentosum*, através da avaliação do seu estado reprodutivo no seu habitat natural (uma continuação de um ensaio de dois anos, fechando o ensaio) e os efeitos de diferentes métodos de cultivo tanto na reprodução sexual como na micropropagação da espécie. Com estas informações, novos protocolos de maternidade podem ser desenvolvidos, facilitando a transição da apanha de stocks naturais para a aquacultura.

Os resultados mostraram que uma população natural de *Codium tomentosum* da costa norte de Portugal é reprodutiva desde o fim do verão/início do outono até ao fim do inverno (Agosto a Março), confirmando a presença de um padrão sazonal. Relativamente às condições de crescimento apropriadas, o *Codium tomentosum* mostrou uma preferência pelas intensidades luminosas mais baixas, 20 µmol m^{-2} s^{-1} para germlings filamentosos (reprodução sexual) e 40 µmol m^{-2} s^{-1} para os filamentos de utrículos (micropropagação). Estes últimos também demonstraram um melhor crescimento quando expostos a luz vermelha (em comparação com luz azul e verde), sem movimento de água, e mostraram uma preferência por meio PES com concentração total, ao invés de PES com menores concentrações e água do mar autoclavada. Otimizámos a libertação de gametas recorrendo a choque hídrico seguido de exposição a ultrassom, que provou ser um método mais eficiente, visto que o número de gametas femininos libertados após um

terceiro ultrassom foi superior ao número libertado após 2 e 1 ultrassom a seguir ao choque hídrico.

Por fim, criamos com sucesso, uma maternidade em escala piloto e provamos que, a partir de micropropagação em linhas de cultura, os filamentos de utrículos de *C. tomentosum* aderiram ao fio, cresceram nele e passaram por morfogénese (formação de talo esponjoso).

Mais estudos sobre este último tópico devem ser feitos, visto que parece haver um grande potencial para a micropropagação de *C. tomentosum* em substratos sementados, abrindo o caminho para a criação de protocolos de maternidade otimizados para a produção em aquacultura desta espécie.

Palavras-chave: *Codium*; aquacultura de algas; micropropagação; ciclo de vida; condições de cultivo.

Abstract

Codium is a genus of green macroalgae (Chlorophyta) that is mainly foraged from the sea, which can lead to the overexploitation of the wild stocks. *Codium* is used in the food, biomedicine, cosmetic and pharmaceutic industries as *Codium* species such as *Codium tomentosum*, are important sources of sulfated polysaccharides and other compounds with multiple bioactive properties. In addition, *Codium tomentosum* is also thought to have great potential to be produced in aquaculture. However, not much is known about certain aspects of this species, mainly its reproduction status, reproduction strategies and culture conditions that best suit its development either by sexual reproduction or micropropagation.

Therefore, this project aims to fill the gaps of knowledge regarding the life cycle of *Codium tomentosum*, by assessing its reproductive status in its natural habitat (a continuation of last year, closing a two-year trial) and the effects of different cultivation conditions in both sexual reproduction and micropropagation of the species. With this information, new hatchery protocols can be developed, helping the transitioning to aquaculture from wild stock harvesting.

The results showed a wild natural population of *Codium tomentosum* from the northern Portuguese coast to be reproductive from late summer/early autumn to the end of winter (August to March), confirming the presence of a seasonal pattern. Regarding the suitable growing conditions, *Codium tomentosum* showed a preference for lower light intensities, 20 µmol m^{-2} s^{-1} for filamentous germlings (sexual reproduction) and 40 µmol m^{-2} s^{-1} for filamentous thallus from utricles (micropropagation). The latter also demonstrated a better development under red light (when compared to green and blue light), no water movement and showed preference for full strength PES nutrient medium as opposed to PES with less strength and autoclaved seawater. We optimized the release of gametes resorting to hydric shock followed by ultrasound, which proved to be a more efficient way, as the number of female gametes released after a third ultrasound was greater than after two and one following the hydric shock. Finally, we successfully created a pilot-scale hatchery and proved that, through micropropagation in culture lines, the filamentous thallus from

utricles of *C. tomentosum* adhered to string, grew in it and experienced morphogenesis (formation of spongy thalli).

Further studies on the last topic should be made as there´s seems to be a real potential for the micropropagation of *C. tomentosum* in seeded substrates, paving the way for the creation of optimized hatchery protocols for aquaculture production of this species.

Key-words: *Codium*; seaweed aquaculture; micropropagation; life cycle; culture conditions.

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FCUP – Faculty of sciences of the University of Porto
UP – University of Porto
ASW - Autoclaved seawater
FAO - Food and Agriculture Organization
DW- dry weight
IMTA - integrated multi-trophic aquaculture
g - Grams
PES - Provasoli´s Enriched Seawater
WM- water movement
Nm- no water movement
Mm- medium water movement
Hm- high water movement
GeO2 - Germanium Dioxide
L:D - Light: Day
LD - Long-day photoperiod
SD - Short-day photoperiod
SEM - Standard Error of the Mean
sp. - Species
Subsp. - Subspecies
t - Time
UV - Ultraviolet
US- ultrasound
BOGA – aquatic organisms bioterium

1. Introduction

1.1. Literature revision

1.1.1. Seaweed Aquaculture

Over the past few years, seaweeds have gained a wide popularity as they continue to show to have such wide and diverse applications, from raw material to fertilizers and biostimulants, animal as well as human food, materials, fabric, pharma, biotechnology, and, on top of that, providing several key ecosystem services.

Algae are major contributors to the global primary production while also having a critical role in the uptake of dissolved nutrients from their surrounding environments, coastal defense from hazardous waves and in carbon sequestration (Chapman, 1995; Christie et al., 2013; Dayton, 1985; Cai et al., 2021; Teagle et al., 2017). Macroalgae (also known as seaweeds) are important habitat-structuring species in coastal ecosystems as well (Bertocci et al., 2015; Reisewitz at al., 2006) and, on top of this, are being used in an increasing number of commercial applications. Seaweeds do not present intrinsic toxins, making them safe to consume. Humans have been eating seaweeds for centuries, with about 700 known edible seaweed species (Pereira, 2016). Their consumption is seen as a tradition in many coastal communities. In eastern Asia, seaweeds are consumed in a wide variety of ways, from soups to salads, sushi wraps, snacks, etc.

Despite being already well-established in Asia, mainly in the food industry, seaweed biomass is increasingly being integrated in western diets either for direct human consumption or used in a wide range of food applications (Philis et al., 2018). Interest in including various algae species in the European gastronomy has been growing in recent years due to their nutritional and therapeutic properties as well as their potential as a new source of sustainable and natural food (Mouritsen et al., 2019; Rioux et al., 2017; Wells et al., 2016). Seaweeds are seen as a healthy food due to their richness in dietary fibers, micronutrients, and bioactive compounds, favored for low-carbohydrate and plant-based diets. Seaweed consumption has many health benefits such as reducing obesity, the risk of diabetes type ll and improving gut health (Cherry et al., 2019; Gómez-Zorita et al., 2020; Pirian et al., 2017; Wan-Loy and Siew-Moi, 2016; Zubia et al., 2007). Besides consumption, their extracts (i.e., iodine, fucoxanthin, among others) are also used for

health benefits as food supplements (Cherry et al.,2019). Algae biomass is also being used in feed for aquaculture (Matsubara et al., 2000; Wan et al., 2019) and as a feed supplement for cattle to improve weight gain whilst reducing enteric methane emissions (Kinley et al., 2020; Machado et al., 2014; Roque et al., 2019). Additional uses include fertilizers and plant biostimulant applications along with a source of high quality and high-value bioactive products for cosmetics and nutraceuticals (Chatterjee et al., 2017; Milledge et al., 2015; Thomas et al., 2013).

Aquaculture production of seaweeds is at an early stage of development in Europe. According to FAO, Europe´s seaweed production is clearly dominated by wild collection whereas the seaweed aquaculture production contributes to less than 4% of the total European seaweed production, as seen in figure 1.

Country/area	Total (farmed and wild) production (tonnes)	Share of world total (%)	Aquaculture share in total production (%)
World	**35 762 504**	**100.00**	**96.97**
Asia	**34 826 750**	**97.38**	**99.10**
China	20 296 592	56.75	99.14
Indonesia	9 962 900	27.86	99.55
Korea, Republic of	1 821 475	5.09	99.52
Philippines	1 500 326	4.20	99.98
Korea, Dem People's Rep	603 000	1.69	100.00
Japan	412 300	1.15	83.80
Malaysia	188 110	0.53	100.00
Americas	**487 241**	**1.36**	**4.69**
Chile	426 605	1.19	5.08
Peru	36 348	0.10	0.00
Canada	12 655	0.04	0.00
Mexico	7 336	0.02	0.14
United States of America	3 394	0.01	7.75
Europe	**287 033**	**0.80**	**3.88**
Norway	163 197	0.46	0.07
France	51 476	0.14	0.34
Ireland	29 542	0.08	0.14
Russian Federation	19 544	0.05	54.10
Iceland	17 533	0.05	0.00
Africa	**144 909**	**0.41**	**81.29**
United Republic of Tanzania	106 069	0.30	100.00
Morocco	17 591	0.05	1.55
South Africa	11 155	0.03	19.32
Madagascar	9 665	0.03	91.72
Oceania	**16 572**	**0.05**	**85.32**
Solomon Islands	5 600	0.02	100.00
Papua New Guinea	4 300	0.01	100.00
Kiribati	3 650	0.01	100.00
Australia	1 923	0.01	0.00

Figure 1. Global seaweed production (2019), Data source: FAO 2021. FAO Global Fishery and Aquaculture Production Statistics (FishStatJ; March 2021; www.fao.org/fishery/statistics/software/fishstatj/en).

With the number of benefits and advantages reported for seaweeds, an increasing popularity is obvious, but with this popularity also comes a growing demand for traceable, high quality and predictable yields of algae biomass form the processing industry. However, this growing demand can lead to the overexploitation of wild seaweed resources.

Therefore, transitioning to aquaculture from wild stock seaweed harvesting, in order to satisfy all these needs and meet this growing demand from the processing industry, avoiding seaweed overexploitation, is more than necessary. Still, the sustainable development of seaweed cultivation must deal with various challenges that require

combined forces of policymakers, stakeholders and experts in order to address and overcome them (Cai et al., 2021).

Furthermore, interest in environmentally friendly farming practices such as integrated multi-trophic aquaculture (IMTA) where macroalgae play a key role has been increasing, which is being promoted at both European and national level (Alexander et al., 2016; Buck et al., 2018; Kleitou et al., 2018). Moreover, recent studies have shown the potential of seaweed aquaculture as a contributor to solve societal challenges such as climate change (Duarte et al., 2017; Pirian et al., 2017; Roque et al., 2019) and eutrophication of coastal waters (Duarte and Krause-Jensen, 2018). Also, aquaculture contribution to global food security is expected to continue increasing, given the limitations of agricultural systems to further increase their food production. Additionally, similarly to agriculture of land-based crops seaweed aquaculture facilitates the selection and production of biomass with improved and desired traits (Reddy et al., 2008).

Another important contribution from aquaculture is job creation. As seaweed aquacultures are usually labor intensive, they employ many workers. In addition, as a relatively newer industry in the western countries, there is a big potential for the creation of many future companies and, therefore more job opportunities. According to FAO, in 2019, the world seaweed production generated 14.7 billion dollars first sale value, a big part of which were used for wages. Also, the further activities post-harvest generates more income and employment.

As it was already referred, only a few species of macroalgae are responsible for the vast majority of aquaculture production. Consequently, there is a need to diverse the species that are being produced, especially when so many of them present valuable characteristics. For this to happen, we need to improve our knowledge about the reproduction strategies and culture conditions that best suit the development of seaweed species under controlled conditions, ultimately leading to the expansion of the seaweed aquaculture industry. With higher knowledge comes the chance to improve the economic prospects of seaweed cultivation.

1.1.2. *Codium* genus

The genus *Codium* (Codiaceae, Bryopsidales, Chlorophyta), as of now, includes around 150 species of marine green macroalgae widely distributed, with the exception of the polar regions, across the world's seas, being predominantly observed in temperate and subtropical areas (Choi et al., 2013; Costa et al., 2015; Oliveira-Carvalho et al., 2012; Verbruggen et al., 2007). Seaweeds of this genus can have a wide variation of forms and can occur in various habitats, and, as the majority of those belonging to the suborder Bryopsidineae, they exhibit no calcification.

Codium is found in marine habitats varying from the calmest lagoons to the rockiest coasts exposed to full wave-forces, from deep reefs to intertidal habitats, from tropical to arctic waters and from eutrophic estuaries to nutrient-depleted coral reefs (Verbruggen et al., 2007).

In the Northern Portuguese coast, *Codium* is generally represented by two species: *C. tomentosum* and *C. fragile*, which can only be set apart under the microscope (Trowbridge, 2001). *C. tomentosum* is a native species to the Portuguese coast, while *C. fragile* has become an invasive species on the coasts of the Northern Atlantic Ocean, as it is originally native to the Pacific Ocean (Watanabe et al., 2009).

Codium sp. are perennial algae, mostly organized in branches (some species form mats) that form spongy, dark green, coenocytic thalli of intertwined filaments that can assume a variety of forms, quite erect, globular or prostrate. In the first, the species grow upright forming cylindrical dichotomously branched axes and are fixed to the substrate by a small disc, the holdfast. In the latter two, the thalli grow over hard surfaces covering the substrate and forming a dark green velvety mat (González and Santelices, 2004; Pereira, 2009; Trowbridge, 2001). *Codium* species have two morphologically different thalli. The filamentous thalli, which is composed of a central medulla of narrow split threads and the spongy thalli that has a peripheral cortex of club-shaped utricles which are the swollen apices of the split threads (Chapman and Chapman, 1973; Guiry, 2014).

In morphological terms, all species of *Codium* feature a spongy and dark green color and an algal body (thallus).

1.1.2.1. Uses and aplications

Edible seaweeds come from the green, brown, and red groups of algae. These marine plants are rich in protein, fibers and iodine and have been known for their contemporary food preparations such as salads, soups, pickles, jams, etc. In fact, Celikler et al., (2009) reported that for *Codium* the protein content can be up to 18,8g/100g DW (dry weight). When compared to the main animal sources of protein, meat (~30g/100g DW), fish (~20-25g/100g DW), shellfish (~15-18g/100g DW), eggs (~14g/100g DW) and dairy (~3-27g/100g DW), this value is not too far off. When compared to the main plant sources of protein, pulses (~7g/100g DW), beans (~5-8g/100g DW), grains (~3-12g/100g DW) and nuts (~14-21g/100g DW), *Codium´*s protein content is higher than most of these (Finglas et al., 2015).
The Chinese and Japanese are among the prolific seaweed-eating people and are accredited with recognizing its food value.
Codium, known in the Philippines as 'pok-poklo' (Ilokano), is one of the most common edible green algae among the South-east Asians. These, along with other brown and red seaweeds, are simply prepared as a salad with just vinegar and salt. These edible seaweeds provide the coastal inhabitants in Asia with a high iodine intake which is believed to be the reason for the low incidence of goiter cases among them. (P.A. Cordero, 2003).

Codium is a genus of siphonous green macroalgae (Chlorophyta) that is mainly foraged from the wild. *Codium* species are known to be an important source of sulfated polysaccharides (Wells et al., 2016) and other compounds with multiple bioactive properties, such as antioxidant, anticoagulant, antiviral, antiangiogenic, antitumoral and hypoglycemic activities, which can be involved in the protection against various diseases (Celikler et al., 2009; Ganesan et al., 2011; Matsubara et al., 2000; Otha et al., 2009; Rey et al., 2020; Watanabe et al., 2009). On top of that, there is a growing demand for natural sources of antioxidants as the potential toxic effects of synthetic antioxidants concerns the consumers (Zubia et al., 2007). So, a diet composed of a wide range of natural phytochemical sources, such as seaweeds like *Codium* is idyllic to obtain optimal health benefits (Celikler et al., 2009). The observed properties are of major interest in the cosmetic, pharmaceutic, biomedicine and food industries (as human food or additives),

which makes *Codium* a very attractive seaweed with great potential as its already being used in these industries (Kang et al., 2008).
In Portugal, *C. tomentosum* is already being farmed at a small scale and sold as food by the Portuguese company "ALGAplus", and its production in aquaculture is thought to have great potential for expansion.

1.1.2.2. Identification of *Codium tomentosum*

Codium tomentosum Stackhouse or "Chorão do mar", as its commonly known in Portugal, is a small marine green alga (in literature is said to be up to 30 cm long, however up to 50 cm long individuals have been registered along this project) with a dichotomously branched and cylindrical frond (figure 2). The frond is solid but spongy with a felt-like touch and has many colorless hairs, seen when the plant is immersed in water. The holdfast is disc-like and made from numerous fine threads (The Marine Life Information Network).

The thallus of *C. tomentosum* is composed of coenocytic cells, large multinucleated cells that result from multiple nuclear divisions without cytokinesis, the transverse walls are rarely formed, hence it is said that the talus is siphoned. The highly entangled/interweaved siphons are expanded to the surface to form utricles (Graham and Wilcox, 2000). Utricles, which are cortical swellings at the ends of filaments, are usually cell wall projections or cellular thickenings usually colorless and rounded, which can be used as taxonomic characters (González and Santelices, 2004).

C. tomentosum is usually found on exposed rocks and/or deep rock pools on the lower seashore (Guiry and Guiry, 2014). This species is native to the Northeast Atlantic Ocean but has already been found around the coasts of Africa and in various other parts of the world. It is the species of the *Codium* genus most frequent in the Iberian Peninsula (APROMAR, 2014; Pereira, 2009). However, another species, *C. fragile*, native to the Pacific Ocean, has become an invasive species to the Northern coast of the Atlantic Ocean (Watanabe et al., 2009). Both species look similar, making it difficult to tell them apart, however possible. To differentiate *C. tomentosum* and *C. fragile*, the tips of the utricles must be observed under the microscope and note the presence or absence of the

mucron (figure 3), a small, transparent pointed tip present on the tips of the utricles from *C. fragile* and absent in *C. tomentosum*.

Figure 2. *Codium tomentosum* individuals (full thallus). Photo taken by Teresa Pacheco.

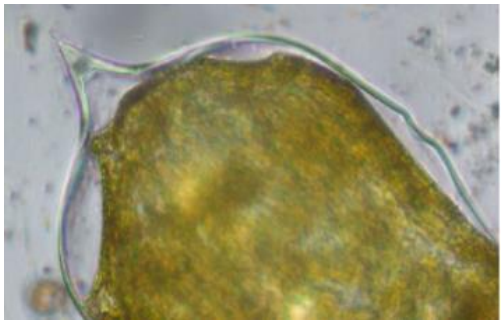


Figure 3. Detail of the mucron in *Codium fragile*. Photo taken by Gonçalo Marinho.

1.1.2.3. Life cycle

Codium tomentosum is not a well described seaweed in terms of life cycle and reproduction. Based on Arasaki et al. (1956), Borden & Stein (1969) and Miravalles et al. (2012) work, the life cycle of the *Codium* genus is diplont with a gametic meiosis as seen in figure 4, which leads to assume *Codium tomentosum* has the same life cycle.

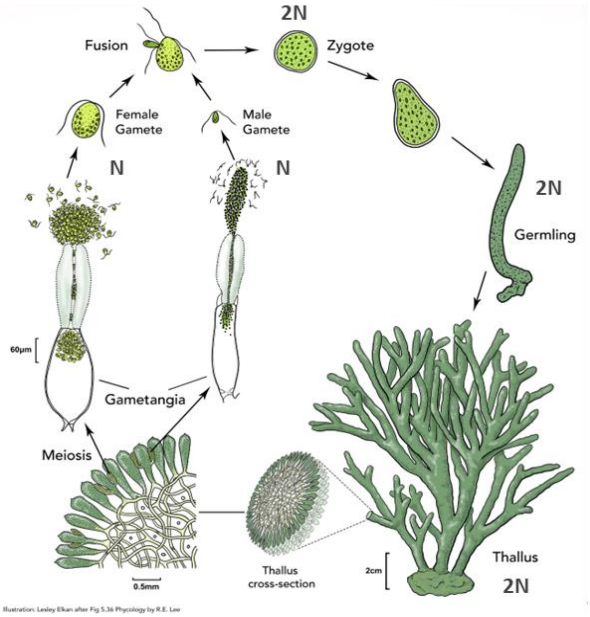


Figure 4. Life cycle of *Codium* sp. **Data source:** Lesley Elkan after fig.5.36 Phycology by R.E. Lee; https://www.rbgsyd.nsw.gov.au/learn/living-learning/primary-school-resources/project-codium-saving-seaweed/identify-and-define

The sexual reproduction is performed through the fusion of haploid biflagellated gametes that result in zygotes (Lobban and Harrison, 1997; Nanba et al., 2005). This zygote germinates into a germling (figure 5), a siphonated diploid filament (Lobban and Harrison, 1997).

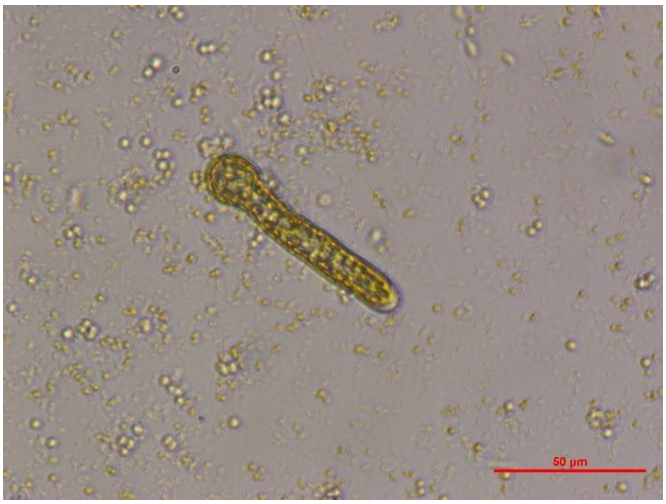


Figure 5. Germling of *Codium tomentosum* (7 days after gamete release). Photo taken by Teresa Pacheco.

The gametangia arise from the utricles almost like extensions, they can be dark or light in color, depending on the type of anisogametes they contain (figure 6) (Graham andWilcox, 2000). Male gametes are small and contain one or two chloroplasts, thus the male gametangia is lighter in color, while female gametes are large and have many chloroplasts (figure 7), their gametangia being darker (Encyclopedia Britannica*)*. The gametangia are separated from the rest of the stalk by transverse walls (septa), this allows the stalk to persist after release of the gametes.

However, the unfertilized gametes can germinate in vegetative stalks, which can be seen as a form of asexual reproduction (Graham and Wilcox, 2000).

The peak of reproduction and peak of growth of *Codium* occur at the same time, specifically during late summer/early autumn, when warm temperatures favor rapid growth (Hanisak, 1979; Kang et al., 2008).

Figure 6. *C. tomentosum* gametangia. Top (darker)- female; Bottom - male. Photo taken by Teresa Pacheco.

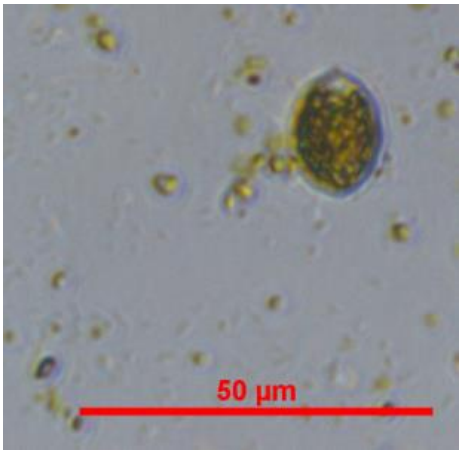


Figure 7. *C. tomentosum* gametes. Female gamete (big) surrounded by male gametes. Photo taken by Teresa Pacheco.

1.1.3. Culture conditions

Seaweeds interact with the marine organisms surrounding them and all are influenced by the physico-chemical environment, mainly light, temperature, salinity, water motion, and nutrient availability as well as all these factors combined. All seaweeds have their own pattern of growth, morphology and reproduction, (Hurd et al., 2014).
So, to get a good seaweed growth, composition and reproduction, these conditions (temperature, nutrients, light, etc.) must be taken into account, as well as the interaction between all (Lobban and Harrison, 1973; Lüning, 1990). Thus, understanding the combined effects of these parameters is essential for the optimized production of any seaweed (Abreu et al., 2011; Pereira, 2004).

There is limited knowledge regarding the ideal conditions for the early stages of development of *Codium* species, however vegetative growth conditions for *C. fragile* and *C. tomentosum* have already been investigated (Hanisak, 1979; Marques et al., 2020; Nanba et al., 2005). Though, there is much more data regarding *C. fragile* than *C. tomentosum*, which leads many authors to rely on the described conditions for *Codium fragile*.

Yang et al. (1997) have shown that optimal growth for *C. tomentosum* from isolated utricles to dissociated filamentous forms, occurred at 20°C, with a growth rate of 0,22-0,40 g after 15 days and a salinity of 30,6‰.
According to Hanisak, differentiated (spongy) thalli of *C. fragile* ssp. *tomentosoides* grow over a broad range of experimental conditions. However for the spongy thalli to achieve a growth/reproduction peak, the temperature must be between 18 and 24°C, the salinity between values of 24-30‰ and the photoperiod of 16H:8H (light:dark). Reinforcing this, Marques et al. also confirmed that the relative growth rate of adult *C. tomentosum* is significantly higher when cultivated under long-day (16 h light:8 h dark) photoperiod than under short-day (8 h light:16 h dark) conditions.

Concerning irradiance, Park and Sohn (1992) reported that the critical amount for the formation of spongy thalli was 60 µmol m^{-2} s^{-1}, for *C. fragile*. When it came to unialgal cultures from isolated utricles of *C. tomentosum* species, Yang et al. stated optimum growth to be at 44 µmol m^{-2} s^{-1}. In addition, in a more recent study by Marques et al. (2020) the results showed better growth between 120 - 230 µmol m^{-2} s^{-1} for domesticated thalli. In that same study, light spectra (white, blue, and red light) were investigated and *C. tomentosum* showed better growth in red light source, followed by white.

Many studies have demonstrated the importance of water movement in the formation of the adult thallus of *Codium* (Lüning, 1990; Scheibling and melady, 2008). A study performed by Nanba et al. (2005) with *C. fragile*, showed that growth of the spongy thalli occurred under high irradiance and high-water velocity (10 cm s^{-1}) combined, in contrast, the formation of filamentous thalli required calm water (0-3cm s^{-1}) and was inhibited when under the influence of high irradiance, suggesting that the different thalli types in *C. tomentosum* life cycle have different light and water movement requirements. Another study performed by Scheibling and Melady (2008) revealed that the attachment of vegetative propagules of *C. fragile* was higher when exposed to water flow conditions than under static water. The water flow enhanced the production of medullary filaments from the isolated utricles and their attachment to the substratum. For the same species with isolated utricles, water movement was necessary to stimulate growth and regenerate adult thallus (González et al., 2014).

Additionally, previous results from our research team, regarding the suitable conditions of the initial phase of sexual reproductive growth for *C. tomentosum*, demonstrate that development of germlings is promoted by long-day (16 h light:8 h dark) photoperiod when compared to short-day (8 h light:16 h dark); exposure to 20 µmol m^{-2} s^{-1} light intensity for white, green or red light spectra; exposure to water movement and when cultured in Provasoli's enrichment solution (PES). Although PES did promote a higher growth rate, it also promoted a greater development of contamination, when compared to germlings grown in PES/10 and autoclaved seawater (asw). In contaminated suspensions of gametes, the use of PES/10 is a better choice (Sá M. F. A. C., 2021).
Nevertheless, the conditions required to induce the morphogenesis of germlings to spongy thallus are yet to be identified.

These results suggest that the optimal conditions for the development of germlings are different from the ones who induce differentiation to spongy thalli and the growth of adult *Codium*.

1.1.3.1. Micropropagation

Micropropagation is an asexual method of reproduction. This method of propagation uses extremely small pieces of plant tissue and grows them under laboratory conditions to produce new organisms, in this case, isolated utricles were used. It is based on cell totipotency, which is the ability of a single vegetative cell to divide and differentiate, producing a new organism. This method facilitates the creation of biobanks, as it grants the production of a lot of individuals within a short period of time. These individuals can then be used as seedlings stored for future cultivation, which diminishes the need of gathering them from the wild (Reddy et al., 2007).
For successful seaweed tissue culture, it is of fundamental importance to know the physiology of seaweed and the factors (endogenous and exogenous) that control seaweed development.
Micropropagation is a key factor for the culture of seaweed tissue, it also has the potential to be used as a tool for mass production of selected high-yield or strains with interesting commercial potential.

1.2. Objectives

In this project, a population of *Codium tomentosum* was studied to determine the seasonal variation of its reproductive status and the best conditions for sexual reproduction as well as its micropropagation potential.

This study aims:
1) seasonal assessment of the reproductive status of the species in its natural habitat, in a selected area of the Portuguese coast (second year of a two-year study);
2) identify optimized culture conditions (including methods of optimization of gamete release, conditions for better growth and induction of morphogenesis) for the establishment of hatchery protocols based on sexual and asexual reproduction methods.

The effect of factors such as the time of sampling, as well as the growing conditions, such as the light regime (spectrum and intensity), hydrodynamics/water movement, etc. were evaluated for their impact on the reproductive status (time of sampling), gamete release, filament development and subsequent differentiation of spongy stems (*Codium* adult/macroscopic); as well as on the development of adult *Codium* from the differentiation of mechanically isolated utricles (micropropagation).

2. Technical description

All the experiences performed in this work were carried out in the Coastal Biodiversity Laboratory of the Interdisciplinary Center for Marine and Environmental Research (CIIMAR) in Matosinhos. The biological material was collected monthly from a shore in the north of Portugal, Aguçadoura (Praia da Paimó).

2.1. Material and methodology

Generally, the methodology applied followed, or was adapted from, internal protocols developed by our research team, unless stated otherwise.

2.1.1. Sampling

Monthly samples were made in Praia da Paimó (Aguçadoura), only in good conditions such as low tide (0,2-0,7m) and low ondulation (<1m). The sampling consists in the gathering of 20 specimens of *Codium tomentosum* (entire thallus). The selection was mainly random, however, as an overall reproductive study, small size individuals (<10cm) were avoided. The specimens were immediately stored in airtight bags with seawater and transported to the laboratory in a thermal cooler. Once in the laboratory, the samples were washed with autoclaved seawater to remove sediments and epiphytic organisms, afterwards they were kept in 10 L aerated flasks with autoclaved seawater in the climatic chamber, at 16 ºC, 12h:12h (L:D), and 60 µmol m^{-2} s^{-1} of light intensity, until further use.

2.1.2. Seasonal Variation of the reproductive status

First part – Presence of gametangia:

The first part began with the arrangement of each seaweed in a tray in order to number the seaweeds, measure them (the entire thalli is measured from the fixation disc to the tips) and captured by photography. Afterwards, three small fragments (2-3cm) from the first bifurcation were cut from each individual. These fragments were then dried with laboratory paper and weighed. After this, the fragments were put in a beaker with 20ml of autoclaved seawater and grinded. When completely homogenised, 5 mL of the mixture (suspension of utricles and medullary filaments) were transferred to a graduated petri dish. This step was repeated until all fragments from each individual were grinded and transferred to a petri dish.

Each petri dish was then observed under inverted microscope, Nikon Eclipse TE200 (Tokyo, Japan) connected to the computer with Nikon's NIS - Microscope Imaging Software in order to count and register the number of gametangia present in 4 squares in 6 random locations of the graduated petri dish. In the case of high density, the samples were diluted. Photos of the gametangia (with scale) using Nikon's NIS - Microscope Imaging Software were also taken to measure their length afterwards. All measurements registered in all assays were made with the software ImageJ (Image Processing and Analysis in Java).

Besides these data, the sex of each specimen, based on the type of gametangia present, was also registered, as well as confirmation of the species by observing the absence of mucron.

After the observations, the cultures were kept in the climatic chamber, at 16 ºC, 12h:12h (L:D), and 20 µmol m^{-2} s^{-1} of light intensity, until used again.

Second part – Induction of gamete release:

Similar to the first part, three small fragments (2-3cm) from the first bifurcation were cut from each individual. These fragments were then dried with laboratory paper, weighed and transferred to a petri dish. They then stayed at room temperature (outside the climatic chamber) for two hours. After this, 5ml of autoclaved seawater was added to each petri dish (inducing a hydric shock), which then went to the climatic chamber, where they were

maintained at 16 ºC, 12h:12h (L:D), and 20 µmol m^{-2} s^{-1} of light intensity and stay there for 3 days.

After the three days, each petri dish was observed under inverted microscope in order to see if gamete release as occurred and if so, the number of female gametes present in 6 random locations of the dish was counted and registered. Photos of the gametes (with scale) were also taken to measure their length afterwards. The sex of the gametes present in each dish was also registered. The fragments of every petri dish where gamete release occurred were removed in the laminar flow chamber. All petri dishes returned to the climatic chamber, maintained at 16 ºC, 12h:12h (L:D), and 20 µmol m^{-2} s^{-1} of light intensity.

Seven days after the beginning of the protocol, the petri dishes where gametes were present were observed again under inverted microscope in order to see if germination occurred and if so, the number of germlings present in 6 random locations of the petri dish were counted and registered.

By the seventh day, the cultures were fed with PES/10 culture medium. This medium was renewed every 2 weeks.

The cultures were maintained at 16 ºC, 12h:12h (L:D), and 20 µmol m^{-2} s^{-1} of light intensity.

The samples were monitored each week to measure the length of the germlings in order to determinate their growth, evaluate possible differentiation of spongy thallus, and the development of biological contamination.

2.1.3. Optimization of gamete release

For the optimization of gamete release, two linked assays were conducted, the first to test how different drying times, followed by different number of ultrasounds affect the number of female gametes released, and the second to test the best waiting time before retrieving the gametes from the final suspension.

It begins with selecting five random individuals. The full seaweeds were dried with laboratory paper and left to dry at room temperature for the amount of time to be tested: 0h/2h30min/5h.

Roughly the same amount of seaweed was cut from each individual and weighed. All the pieces of seaweed were then put in a beaker with 200ml of autoclaved seawater. The beaker was put under ultrasound for 3 min. After this, the suspension was let to set for approximately 10 min. From this suspension of gametes, 5ml were transferred to three graduated petri dishes, in order to count the number of gametes in 6 random locations of each graduated petri dish using the inverted microscope. The rest of the suspension was discarded and another 200ml of autoclaved seawater was added to the beaker containing the pieces of seaweed. The beaker was put under ultrasound for another 3 min and 5ml were transferred to three graduated petri dishes, to count the number of gametes. This was repeated for one last ultrasound. The discard of the suspension between ultrasounds is of most importance because only then can the results from each ultrasound be considered independent and not just a value added on the value of the previous ultrasound. In the end, there were 3 samples/replicates for each ultrasound for each drying time condition, a total of 27 samples as seen in the table below. The objective was to determine which set of conditions released the higher number of gametes.

Table 1. Distribution of each trio of samples according to the conditions they were exposed.

Drying time	US 1	US 2	US 3
0h	◯ ◯ ◯	◯ ◯ ◯	◯ ◯ ◯
2h30min	◯ ◯ ◯	◯ ◯ ◯	◯ ◯ ◯
5h	◯ ◯ ◯	◯ ◯ ◯	◯ ◯ ◯

The final part begins after the best time and ultrasound condition (based on the previous part) was selected. The procedure from part 1 was repeated only once, for the time and ultrasound condition chosen. After the final ultrasound, 5ml of gamete suspension were transferred from the beaker to 3 petri dishes following a 0min/15min/30min/1h/2h/3h and 24h waiting time. The samples were observed in the inverted microscope in order to count the number of gametes in 6 random locations of each graduated petri dish and create a growth curve to determine the best waiting time before transfer of the suspension of gametes.

2.1.4. Effect of light intensity and water movement on germling growth

Five random individuals were selected. The entire thalli were dried with laboratory paper and left to dry for 2 hours. Roughly the same amount of seaweed was cut from each individual and weighed. All the pieces of seaweed were then put in a beaker with 200ml of autoclaved seawater. The beaker was put under ultrasound for 3 min. The suspension from this first ultrasound was discarded and another 200ml of ASW was added before the beaker went under ultrasound for another 3 min. After about 10 min, the suspension was filtrated with a microplankton net and 5ml of the suspension were transferred to eighteen graduated petri dishes. On the first day, six samples were put under 20 µmol/m² s light intensity, six samples were put under 40 µmol/m² s light intensity and six samples were put under 60 µmol/m² s light intensity. After seven days, three samples of each light intensity condition were put in the orbital shaker, thus being subjected to water movement, and still under the same light intensity they were previously exposed to. The samples were kept at 16ºC and at 12h:12h (L:D). The culture medium (PES/10) was renewed every 2 weeks.

The length of the germlings was measured weekly using the inverted microscope and the Image J software, to determinate their growth and evaluate the best set of conditions.

2.1.5. Micropropagation

2.1.5.1. Laboratory assays

These assays were carried out to assess the best culture conditions for the development of filamentous thallus from isolated utricles, and subsequent differentiation of spongy thallus (adult *Codium*).

Three assays were conducted in total, to test different conditions, as followed:

1. **Nutrient media: ASW; PES; PES/2; PES/10,** (Maintained at 16 ºC, 12h:12h (L:D), white light, 60 µmol m^{-2} s^{-1} intensity and no water movement as constant conditions).
2. **Light intensity and water movement: 20; 40; 60 µmol/m²/s with or without water movement**. (Maintained at 16 ºC, 12h:12h (L:D) and white light as constant conditions).
3. **Light spectra and intensity: Blue; green; red at 20; 40; 60 µmol/m²/s**. (Maintained at 16 ºC, 12h:12h (L:D) and no water movement as constant conditions).

These assays began with obtaining an utricle suspension. For this, each seaweed was arranged in a tray and five similar looking individuals were selected. Afterwards, about the same amount of seaweed was cut from the five similar looking individuals, a total of 30g. The fragments were then dried, put in a beaker with 200ml of autoclaved seawater and grinded. When completely homogenised, 5 mL of the mixture (suspension of utricles and medullary filaments) were transferred to graduated petri dishes:

- 12 petri dishes for the nutrient media assay (4 conditions x 3 replicates);
- 18 petri dishes for the light intensity and water movement assay ((3x2) conditions x 3 replicates);
- 27 petri dishes for the light spectra and intensity assay ((3x3) conditions x 3 replicates).

Every week, the different concentrated nutrient media were added to the cultures of the nutrient media assay. The cultures from the light intensity and water movement assay spent the first week without motion, after this week, half of each of the light intensity condition cultures were exposed to water movement. As for the light spectra and intensity assay, all cultures were exposed to the different conditions from the beginning. For all three assays, each week, the utricle filaments were observed on the Leica stereo microscope to evaluate their growth, detect any differentiation and contamination. They were also photographed in 6 random sites of the graduated petri dish using the Leica Application Suite X (LAS X) software, to measure afterwards and determinate their growth. PES/10 was added every 2 weeks to all cultures where Nutrient media weren't being tested.

2.1.5.2. Hatchery assay (pilot-scale)

This assay was carried out in a pilot-scale hatchery to evaluate the effect of light intensity and water movement on the development of filamentous thallus from isolated utricles seeded into substrates (culture lines) and the role these culture parameters may play at inducing the subsequent morphogenesis/differentiation of spongy thallus (adult *Codium*).

Firstly, the ropes where the seedlings attach (culture lines) were wrapped in collectors (rectangular structures, as seen in figure 8).

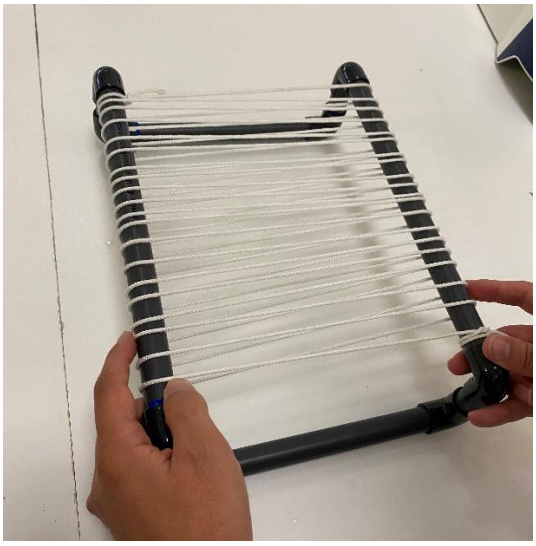


Figure 8. Collector with wrapped rope/culture line. Photo taken by Teresa Pacheco

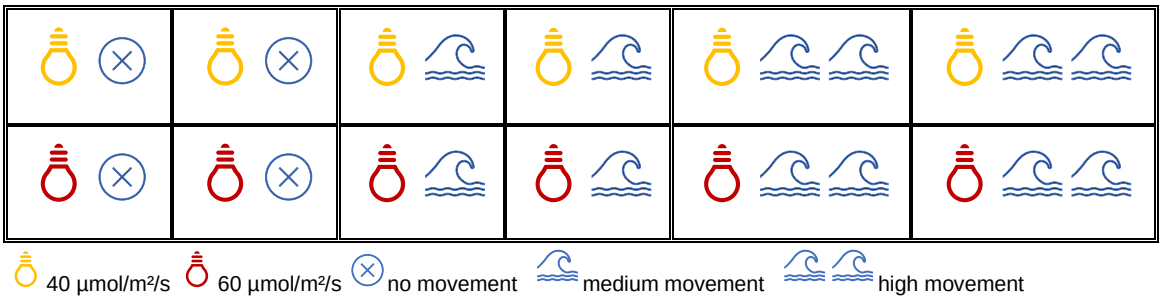
Focusing now on the seaweeds, the process began with the arrangement of all individuals (as clean and free of epiphytes as possible) in a tray. About 460 g of seaweed was collected for the trial. This amount of seaweed was put in a beaker, containing about 800ml of autoclaved seawater (asw) and 5% of betadine and stayed there for about 3min. They were then transferred to a beaker containing just asw, where they stayed for another 3 min and transferred again for another beaker containing just asw to remove any traces of betadine. Afterwards, the seaweed was grinded with about 2000ml of asw. When

completely homogenised, all twelve structures with rope were submerged in this suspension of utricles and medullary filaments (seeding). The structures with the seeded rope were left alone at room temperature for about 2 to 3h, helping the seeding material to fixate to the rope. Afterwards, each structure was placed in a tank. During the entirety of the trial, temperature was maintained at 15ºC, photoperiod was 12h:12h(L:D) with white light. Every 2 weeks, PES/10 was added to the system. Although PES tended to be preferred in the previous assay, those results weren't statistically significative when compared to PES/10, plus, this assay accounts for a lot of litres of water, thus, PES/10 is a more efficient choice.

During the first 3 weeks, all tanks were exposed to the same conditions, 40 µmol m^{-2} s^{-1} and no water motion. This amount of exposed time to similar conditions was intended to further help the fixation to the rope, as an immediate exposure to high water motion could have prevented this. Afterwards, each tank had a combination of the 2 different conditions, light intensity (40 µmol m^{-2} s^{-1} s or 60 µmol m^{-2} s^{-1}) and water movement (no, medium, and high motion), and a replicate (explained in table 2). The settings were chosen resorting to the results from the previous laboratory assays.

Each week the % of string coverage and the length of utricle filaments were measured, both using the software ImageJ.

Table 2. Disposition of the conditions tested in all 12 tanks



2.2. Statistical analysis

The software GRAPHPAD PRISM 8 was used for the analysis. The data was tested for normality using the Shapiro-Wilk normality test. Afterwards, the data was analysed by analysis of variance (ANOVA). A one-way ANOVA was applied to test the effect of season on the thalli length as the investigated dependent variable and to test the effect of waiting time on the number of gametes released. A two-way ANOVA was applied to test the effect of light intensity vs. spectrum, light intensity vs. water movement, drying time vs. number of ultrasounds on the growth of utricle filamentous. In case a significant difference between sample means or interaction of factors was revealed by ANOVA, a pairwise comparison among levels of factors was performed using the Tukey's test. Means were considered significantly different when $p < 0,05$.

3. Results

3.1. Seasonal studies (continuation of a two-year trial)

3.1.1. Reproductive status

The reproductive status of the *C. tomentosum* samples retrieved from Aguçadoura shore, during the timeline September 2021-July 2022 can be seen in table 1. Note that the samples were only considered reproductive (fertile), when it was observed the release of gametes, germination, and development of germlings. These data suggests that the specie *C. tomentosum* is fertile from August to March. May did not present gametangia. As for June and July, very little gametangia was found, and the ones found were considered immature gametangia, which explains why no germination occurred.

Table 3. Reproductive status of *C. tomentosum* sampled from the shore of Aguçadoura from 2021 to 2022. Sampling carried out during this master thesis project (data from the second half of the 2-year trial).

YEAR	MONTH	REPRODUCTIVE?
2021	September	Yes
	October	Yes
	November	Yes
	December	Yes
2022	January	Yes
	February	Yes
	March	Yes
	April	No
	May	No
	June	No
	July	No
	August	Yes

3.1.2. Seasonal variation of thalli length

The size of the thallus collected from Aguçadoura was measured every month from September 2021 to August 2022, as seen in Figure 9.

From September to November, the thalli average length of the samples collected increased, reaching its highest peak in length in the month of November (37,6 ± 1,2 cm), followed by December (35,4 ± 1,1 cm) and October (33,9 ± 1,1 cm), with no significant differences between all three, according to the one-way ANOVA conducted. After November the thalli length decreased gradually through the following months, with April being the month with the lowest thalli length registered (17,5 ± 1,1 cm), with no significative differences from the thallus from September, March, and May (21,4 ± 0,9; 21,3 ± 0,7 and 17,5 ± 1,1 cm respectively). Following April, the average lengths of the thallus increased consecutively with each month, until August.

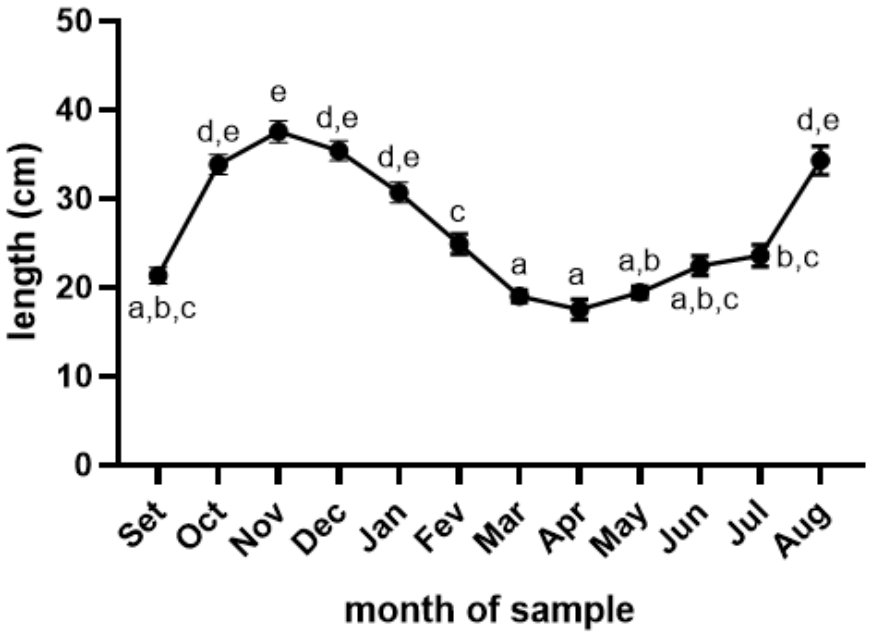


Figure 9. Average thalli length (cm) per month of sample (September 2021-August 2022). Values presented as means (n=20) and standard error (SEM). Different letters denote significant differences between lengths (p<0,05).

3.1.3. Seasonal variation of germling growth

The germlings obtained from the samples collected each month were measured, every week for a period of five weeks as seen in figure 10, in order to determinate their growth and, consequently, the months where the best growth was registered.

Overall, the germlings with the best average growth were the ones obtained from the samples/specimens collected in October and September, with the only significative difference between them on the 28th day ($p<0,05$). **On the first week**, November germlings were slightly bigger, however, none of the months presented significative differences. **On the second week**, the germlings from the first months, September, October and November had a higher growth, in order, November with the longer germlings, but still with no significative differences between all months. The germlings from December, January, March and August grew slightly. **On the third week**, the germlings from December stagnated, and the ones from March and August grew very little, these three months were the only months significantly different from November ($p<0,05$), who´s germlings kept growing as well as September´s and October´s, maintaining the same order from the previous week. **On the fourth week**, both germlings from November and December had a very small growth. From this week until the end of the trial, the germlings from the winter months (December, January, March) and August never showed significative differences between them, and the germlings from December were always significantly different from the germlings from the months that preceded him (September, October and November). The germlings from October topped all months with the biggest growth, followed by November´s and September´s. The germlings from October had a significative higher growth than December´s ($p<0,0001$), January´s, March´s, and August´s germlings ($p<0,05$), the latter three continued with their small growth. **On the fifth and final week**, the germlings from November and August had a very small growth. The ones from December, January, and March stagnated. October had the germlings with the biggest growth and September surpassed November with the second-best average germling length. The germlings from these three months had a significative higher growth than December ($p<0,0001$), March ($p<0,05$) and August ($p<0,05$). October was the only one of the three who had a significative higher growth than January ($p<0,05$).

The germlings from October were also significantly higher than the ones from November ($p<0,05$).

Note: In February 2022 it was not possible to obtain these data due to covid-19 and a malfunction in the culture camera, which prevented the continuous observation of the germling growth for the 5 weeks. The samples/specimens collected in April, May, June and July 2022 were infertile, thus gametes and subsequently germlings could not be obtained in these sampling months.

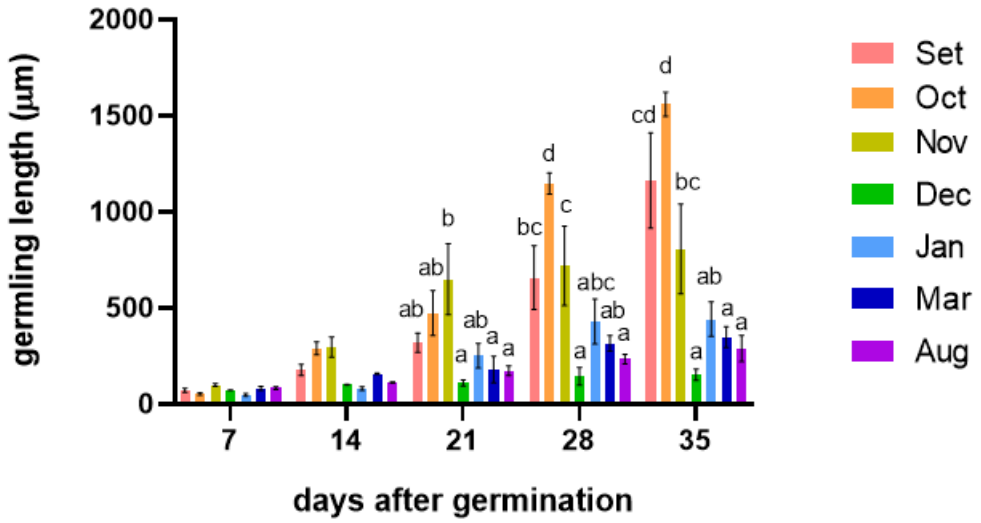


Figure 10. Monthly germling growth (µm). Values presented as means ($2<n<14$) and standard error (SEM). Different letters denote significant differences between lengths at each week ($p<0,05$).

3.2. Effect of light intensity and water movement on germling growth

For a period of 35 days (five weeks), the germlings from seaweed collected in November, were measured weekly, in all six different conditions, to assess their growth performance and determine the best culture conditions.

The two-way ANOVA showed no interaction and no significant differences between any of the conditions ($p\geq0,05$), not between water movements, nor between light intensities, nor between the sets of both movement and intensities. However, by the end of the trial, there was a growth tendency at 20 µmol m^{-2} s^{-1} and no water movement as seen in Figure

11, with the highest observed growth of 539,2 ± 164,9 µm in these set of conditions and the lowest observed growth of 225,2 ± 7,5 µm at 60 µmol m^{-2} s^{-1} with water movement.

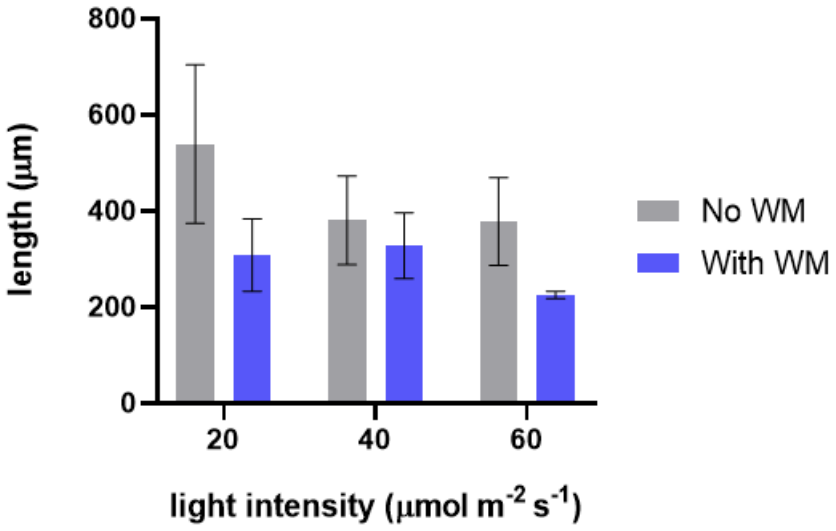


Figure 11. Effects of light intensity (µmol m^{-2} s^{-1}) and water motion (with or without) on the growth of germlings after 35 days of trial. (Two-way ANOVA, no interaction (p>0,05)). Values presented as means (n=3) and standard error (SEM). The absence of letters denotes no significant differences (p≥0,05). WM- water motion.

3.3. Optimization of gametes release

During this trial, the number of gametes released through each experimental condition was counted to identify the best performing treatment to induce gamete release. A combination of 2h30 drying time, followed by 3 ultrasounds, reached a maximum of 34390± 2000,4 gametes released per gram of biomass, which, following the two-way ANOVA results, outperformed all the other treatments (p<0,0001) as seen in Figure 12. Comparing only the drying times, the 2h30 period always showed significative differences for all ultrasounds (p<0,05), 2h30 1US was significantly higher (8848,0 ± 1166,2) than both 0h 1US and 5h 1US (3611,4 ± 253,9 and 372,5 ± 46,6), and the same happened for the 2US and 3US. As for the ultrasounds, both 2h30 and 5h released increasingly higher with each ultrasound and the third one released a significantly higher number of gametes (p<0,05), while the 0h drying time condition released a similar quantity of gametes for the first two ultrasound and almost nothing for the third and final ultrasound, significantly lower numbers (p<0,05).

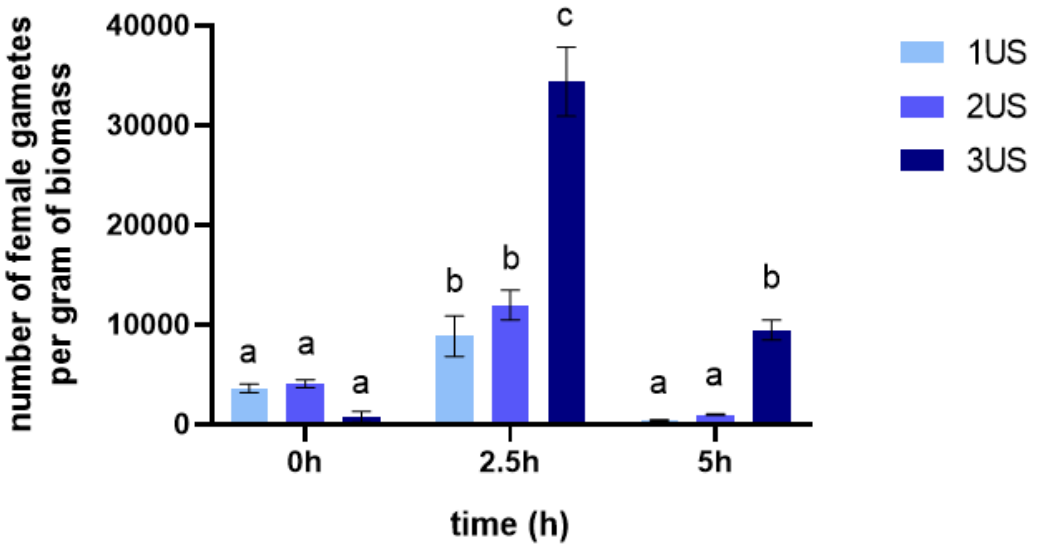


Figure 12. Effects of different drying times and number of ultrasounds on the release of female gametes. (Two-way ANOVA, number of ultrasounds ($p<0,0001$), drying time ($p<0,0001$), interaction ($p<0,0001$)) Values presented as means (n=3) and standard error (SEM). Different letters denote significant differences between different sets of conditions ($p<0,05$). US-ultrasound.

Following these results, the best waiting time was determined resorting to the count of gametes continuing to be released after the final ultrasound.

According to those results, the observed waiting times with the highest number of gametes are either 2h or 3h, as seen in Figure 13. The one-way ANOVA showed significative differences between waiting times, as expected. A Tukey´s multiple tests was conducted and showed significative differences for 0h, 0,25h and 0,5h when compared to each other and to all the other waiting times ($p<0,05$). As for the 1h, 2h, 3h and 24h, these showed no significative differences between them ($p\geq0,05$). The maximum average of gametes observed was 257989 ± 4032,7 per gram of biomass, after 3h waiting time.

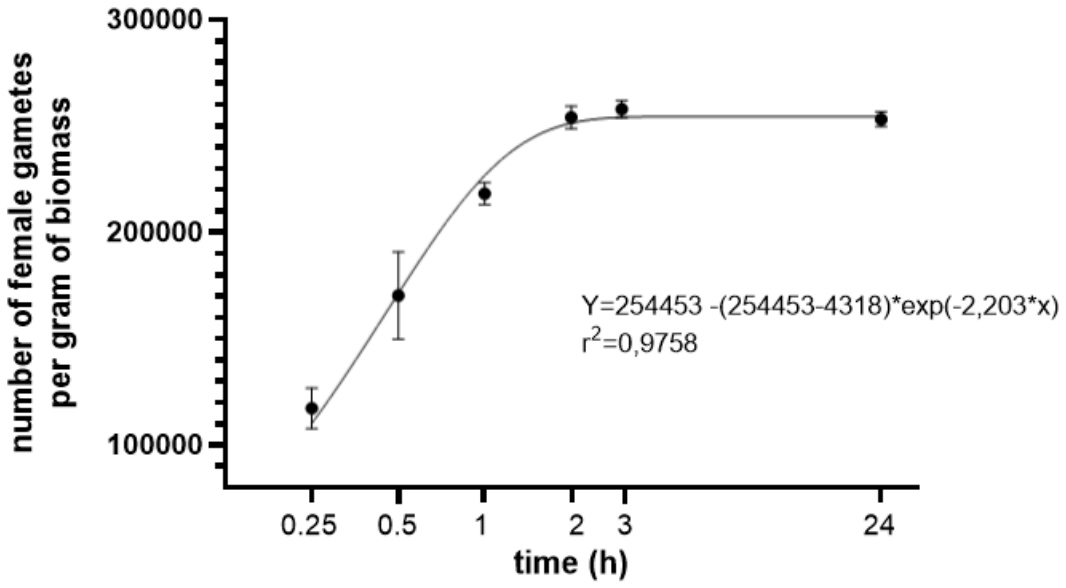


Figure 13. Growth curve (exponential plateau) of the number of female gametes per gram of biomass released after 6 different waiting times (0.25h;0.5h;1h;2h;3h;24h). Values presented as means (n=3) and standard error (SEM). X axis values presented in a logarithmic (log2) scale.

Besides the number of gametes, their activity/movement was also observed and registered. After 0.25h and 0.5h waiting times, the majority of the female gametes were moving around, showing their liveliness. After 1, 2 and 3 hours, the number of gametes showing movement was slightly lower, and after 24 hours wait, very little gametes were moving and, besides this, a significative number of them were starting to degrade.

3.4. Micropropagation

3.4.1. Laboratory assays

3.4.1.1. Nutrient media

During a period of 35 days (five weeks), the filamentous thallus that developed from the utricles exposed to different concentrations of nutrient media Provasoli´s enriched seawater were measured to determinate which concentration provided the best overall growth. The filamentous thallus seemed to grow better when cultured on full-strength PES compared with all other concentrations throughout the trial (Figure 14). The length of the filamentous thallus grown on PES was significantly higher compared to those from ASW in the last two weeks of the trial (3623,1 ± 457,7 µm and 1936,6 ± 88,4 µm, respectively), and was the highest among all culture media in the last week (5744,8 ± 625,2 µm, $p<0,05$).

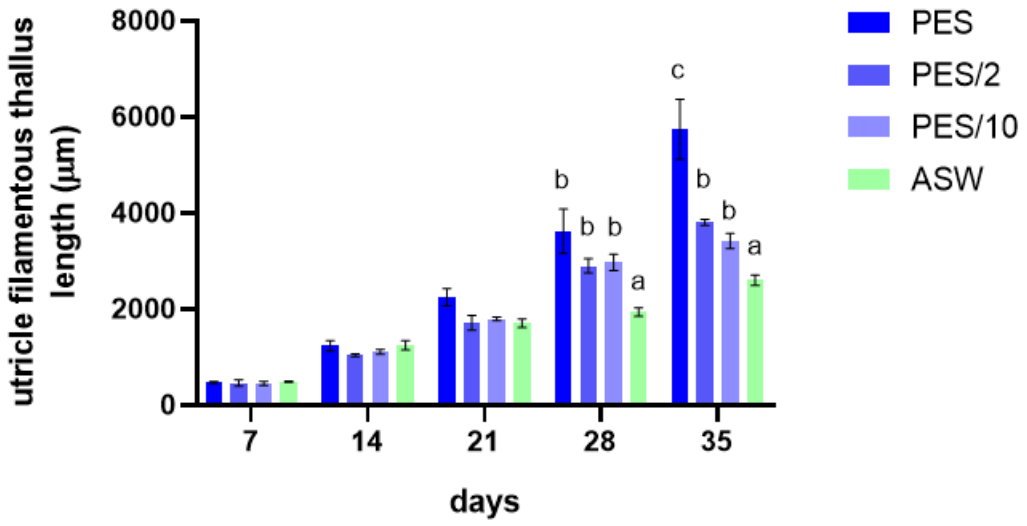


Figure 14. Effect of different concentrations of nutrient media (PES) on the growth of utricles. Values presented as means (n=3) and standard error (SEM). Different letters denote significant differences between nutrient media concentrations at each week ($p<0,05$). ASW- autoclaved seawater; PES- Provasoli enriched seawater; PES/2- half concentrated; PES/10- 1/10 concentrated.

3.4.1.2. Light intensities vs. water movement

During a period of 35 days (five weeks), the filamentous thallus from utricles exposed to each set of conditions (20/40/60 µmol m^{-2} s^{-1} with or without water movement) were measured to determinate which one provided the best overall growth. As showed in figure 15, the filamentous thallus from utricles showed a preference for light intensity 40 µmol m^{-2} s^{-1} with NO water movement by the end of the assay. This preference was significantly different from all the other sets of conditions ($p<0,05$), according to the Tukey´s multiple tests that was performed after the two-way ANOVA test showed interaction and significant differences between the factors light intensity and water motion in the growth of *C. tomentosum* in laboratory ($p<0,05$). Between types of movement, there was only significative differences between water movement and no water movement when exposed to intensity 40 µmol m^{-2} s^{-1} ($p<0,05$). Regarding the light intensities, there were no significative differences between 20 µmol m^{-2} s^{-1} and 60 µmol m^{-2} s^{-1} ($p>0,05$).

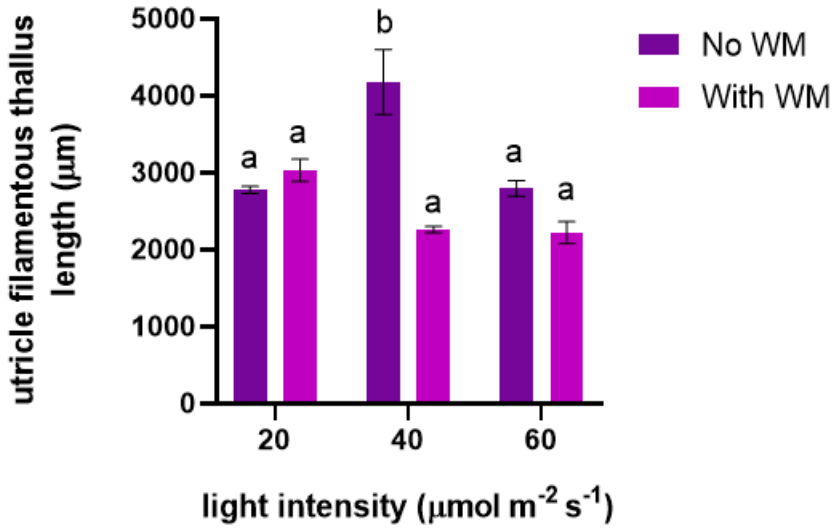


Figure 15. Effects of light intensity (µmol m^{-2} s^{-1}) and water motion (with or without) on the growth of filamentous thallus from utricles after 35 days of trial. (Two-way ANOVA, light intensity ($p=0,0123$), type of water movement ($p=0,0006$), interaction ($p=0,0005$)). Values presented as means (n=3) and standard error (SEM). Different letters denote significant differences between presence or absence of water motion ($p<0,05$). WM- water motion.

3.4.1.3. Light intensities vs. light spectrum

During a period of 35 days (five weeks), the filamentous thallus from utricles exposed to each set of conditions (20, 40, and 60 µmol m^{-2} s^{-1} in the blue, green, or red spectrum) were measured to determinate which one provided the best overall growth.

The two-way ANOVA performed to test the effect of light intensity and spectrum in the growth of *C. tomentosum* in the laboratory showed a significant effect of light intensity on the development/growth of filamentous thallus ($p<0,05$), however it was not significantly affected by spectrum. Additionally, there was no interaction of factors light intensities and spectrum ($p<0,05$). When solely comparing light intensities, the intensity 40 µmol m^{-2} s^{-1} showed significant differences with both 20 µmol m^{-2} s^{-1} and 60 µmol m^{-2} s^{-1} ($p<0,05$), whereas there were no significant differences between the intensities 20 µmol m^{-2} s^{-1} and 60 µmol m^{-2} s^{-1} ($p\geq0,05$).

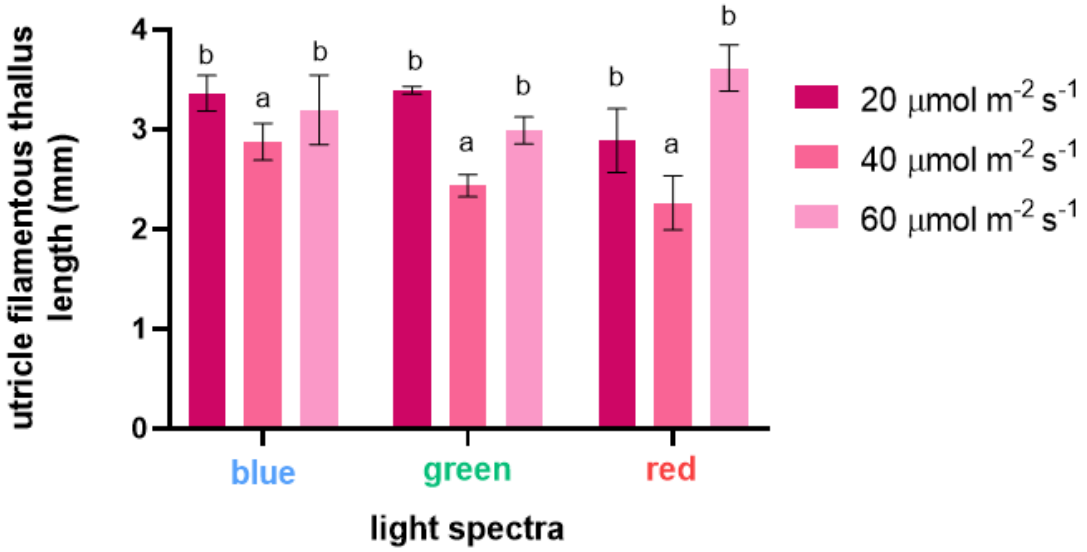


Figure 16. Effects of light intensity (µmol m^{-2} s^{-1}) and light spectrum (blue/green/red) on the growth of filamentous thallus from utricles after 35 days of trial. (Two-way ANOVA, light spectra ($p>0,05$), light intensity ($p=0,0011$), no interaction ($p>0,05$)). Values presented as means (n=3) and standard error (SEM). Different letters denote significant differences between light intensities ($p<0,05$).

3.4.2. Hatchery assay (pilot-scale)

This trial is considered a pre-trial as it was the first time following a new protocol, created by this team, and as some misfortunes occurred and aspects went wrong that need to be corrected to create a newer, well adjust protocol, to follow in the next trial. Still, as it did show some positive results, these will be shared in this paper.

Before the results, it is important to refer that, some temperature problems occurred in the CIIMAR facilities during this trial. To be more specific, the temperatures in the aquatic organisms bioterium (BOGA) rooms increased significantly, causing the water temperature to rise as well, which caused the loss of two replicates, and the occurrence of epiphytes.

Focusing now on the results retrieved from this trial. This is the first time, that we know of, that *Codium tomentosum* was cultivated in string as a form of micropropagation. The first observed result of this trial was the successful attachment of the filamentous thallus from utricles to the string, followed by their growth while attached to the string. These observations prove the success of this method for this specie.
For 49 days, the % of string coverage and the length of utricle filaments were measured each week, until they all reached their maximum (100% for coverage and more than 12mm for filament length, 12mm being the diameter of the stereo microscope lens) as seen in figures 17 and 18. Although the maximum was observed in all cultures by the 49th day, some conditions reached the maximum before the end of the trial. For the coverage percentage, the 40 µmol m^{-2} s^{-1} and no water motion condition, as well as both the 60 µmol m^{-2} s^{-1} medium and high-water motion reached 100% coverage by the 42nd day. For the utricle growth, the 40 µmol m^{-2} s^{-1} and no water motion condition reached 12mm by the 35th day and both the 60 µmol m^{-2} s^{-1} no and medium water motion reached 12 mm by the 42nd day. Until the 35th day, all tanks were exposed to the same conditions, however, by then, all presented, already, very different results, which compromised the results of the trial, as it made impossible to prove the existence of interaction between factors and the possibility of significative differences, invalidating completely a statistical analysis. Still, after the exposure to water motion and higher light intensity (after the 35th day), the growth occurred quite fast, as seen in the graph below, which leads us to believe, that a higher intensity and motion does indeed induce/fastens growth.

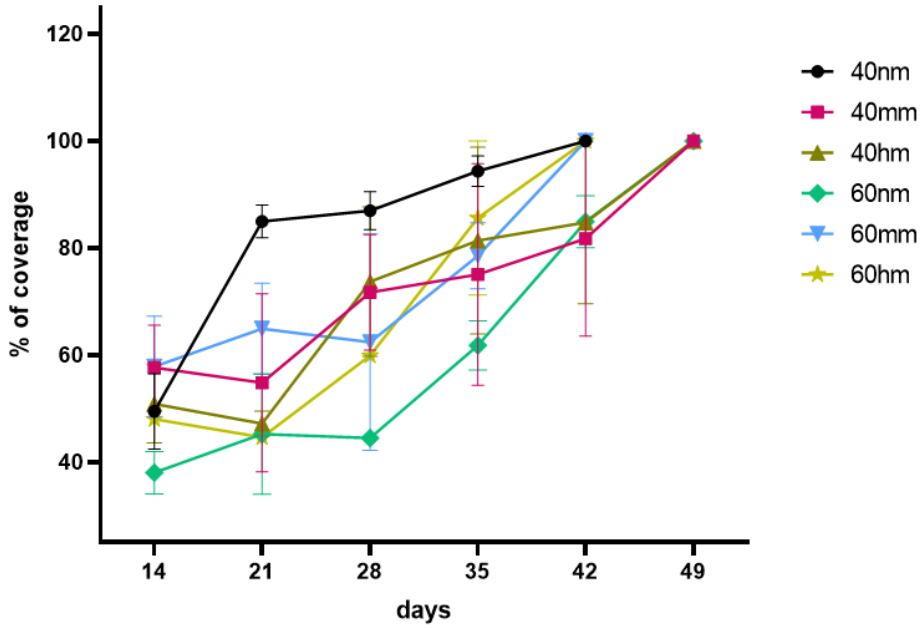


Figure 17. String coverage (%) through the duration of the trial (49 days) at different light intensities and types of water motion. Values presented as means (n=2) and standard error (SEM). Nm- no water motion; mm- medium water motion; hm- high water motion. Note: some conditions reached 100% coverage before the end of the trial (40nm,60mm,60hm).

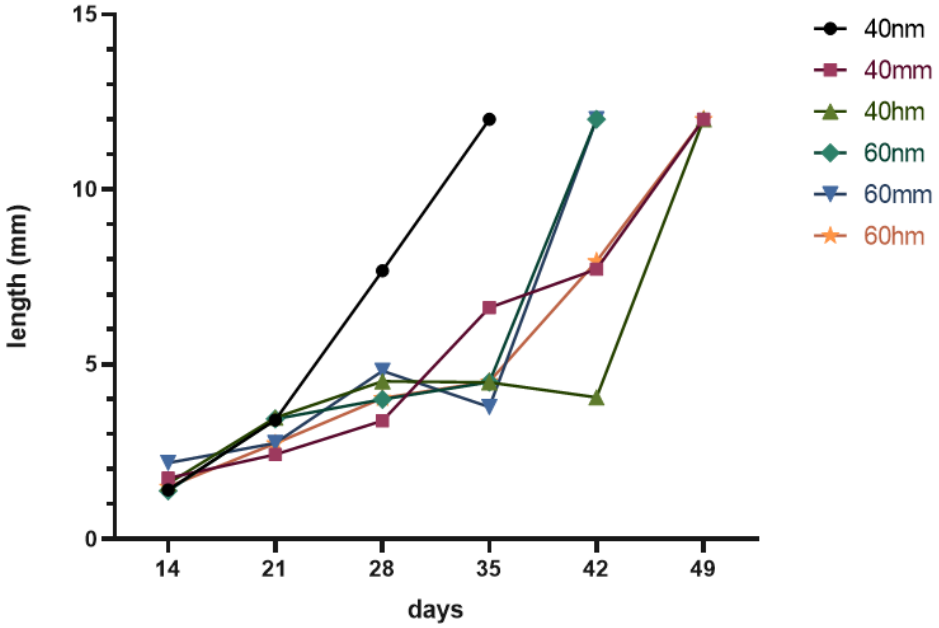


Figure 18. Utricle growth (mm) through the duration of the trial (49 days) at different light intensities and types of water motion. Values presented as means (n=2) and standard error (SEM). Nm- no water motion; mm- medium water motion; hm- high water motion. Note: maximum length possible for measure = 12mm, some conditions reached this value before the end of the trial(40nm,60nm,60mm).

Lastly, another exciting result observed during this trial. Small, spheric-like agglomerates of utricles were observed in the first weeks. A continuous observation of these agglomerates showed an evolution in organization and, after 4 weeks, what looks like spongy thalli was observed, leading us to believe that morphogenesis occurred sometime during the fourth week.

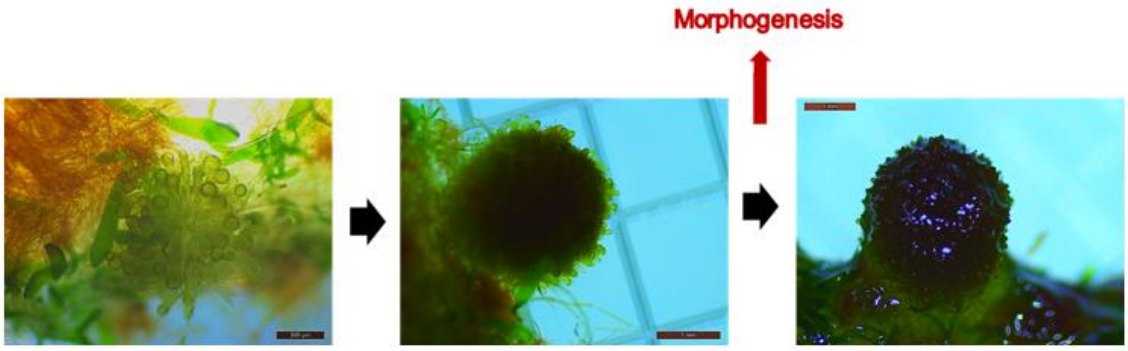


Figure 19. Sequence of utricle organization trough time. Agglomerates at 21(first photo) and 28 days (second photo), and "seemingly" spongy thalli at 35 days (third photo)

4. Discussion

SEASONAL STUDIES

Seasonal fertility

The natural population of *Codium tomentosum* retrieved from the Northern Portuguese coast studied in this paper showed to be reproductive from August to March. As of April, the specimens collected were no longer fertile, which coincides with the moment when the "older" *Codium* specimens on the shore are being replaced by the "younger", immature ones, thus (probably) explaining the infertility from April to July.

Unfortunately, there is a lack of studies about *C. tomentosum*, and therefore, about its reproductive habits. However, one study by Trowbridge et al. (2001) conducted in both *C. tomentosum* and *fragile* stated that *C. fragile* was mature in the summer months, while *C. tomentosum* was mature in the winter months, matching with the presented results in this trial. In all reproductive months for *C. tomentosum*, most individuals presented both male and female gametangia, sometimes in the same individual. Prince & Trowbridge (2004), in a study with *Codium* species, including *C. intricatum* and *C. latum* also observed both gametangia in the same specimen. This also happened for Borden & Stein (1969), with C. fragile, who also observed single utricles carrying both gametangia. The density of gametangia varied with the months, and May was the only month where no gametangia was detected. In April, the month when the older specimens of *Codium* on the shore start being replaced by the younger ones, the individuals collected varied greatly in size, while most of them were considered small (~12cm), and therefore young, larger individuals (~25cm), and older, still present in the shore were also collected. These larger individuals, corresponding to older specimens of *Codium*, presented gametangia and, the larger specimen collected that month even presented female gametes, however, none germinated. This was, most likely, due to its age, as older specimens, the quality of its gametangia and consequently gametes must decrease, lowering the chances of germination. As for June and July, very little gametangia was found, and the ones found were considered immature gametangia, which explains why no germination occurred. The low gametangia density could suggest that these specimens have started the gametogenesis process to eventually become mature adults.

By August, the gametangia density was high and they were mature, as germination occurred. Implying that the seaweeds reached adult maturity, capable of sexual reproduction.

As the second half of a two-year trial, it is of most importance to refer that these results go exactly accordingly with the data from the year before (first part of the trial), retrieved from our team (Sá M. F. A. C., 2021). This means that *Codium tomentosum* was fertile from January to March, infertile from April to July, and fertile again from August to December in 2020, 2021 and 2022 (until August at least). These findings provide robust evidence that *Codium tomentosum* follows, indeed, a seasonal reproductive/fertility pattern.

Thalli length

Interestingly, the infertile specimens collected between March and July also presented lower observed thalli length, corroborating our hypothesis that these are immature juvenile specimens.

Still focusing on the thalli length, when comparing this data with the results from 2020-2021 (Sá M. F. A. C., 2021), the average length of the specimens from the month of September coincides almost perfectly, the periods of time for the increases (sept-nov; apr-aug) and decreases (jan-apr) in length also coincide. In both datasets, April specimens had the lowest observed length. Regarding the month with the highest observed thalli length, while in the most recent data (2021-2022), the seaweeds from November had the highest thalli length, in the previous data (2020-2021), the ones from August prevail. The overall length is also higher in the 2021-2022 timeline when compared to 2020-2021.

Germling growth

An efficient germling growth has to do with optimal conditions and the overall quality of the seaweed itself. This leads to think that the different results from each month have to do with the quality of the seaweeds retrieved from the shore of Aguçadoura, as the laboratory conditions were the same throughout the two-year trial. There are many factors that could contribute to a poor or good quality of seaweed, temperature being one of the main. When comparing this year´s data with last year´s, there is a significative difference on the months that presented the specimens with the best growth, this year being September and October, and last year being December and January (Sá M. F. A. C.,

2021). What at first may look strange, quickly starts to make more sense after observing the registered monthly atmospheric temperatures from 2020 to 2022 in Portugal by IPMA. By lack of data from last year, it is only possible to compare the data from September, November, December, January, March and August. November had little difference in growth from 2020 to 2021. The other months, although, had considerable differences from the first year to the second. These months also had substantially different temperatures from the first year to the second. While in the 2020/2021 timeline, the winter months were considered cold, as they normally should, one year after they were considered hot, an abnormal temperature for this time of the year (most likely due to climate change). As for September, 2021 had lower temperatures than 2020 (~1ºC lower) and for August, 2022 had higher temperatures than 2021 (~1ºC higher), additionally, it is important to note that July, the previous month, experienced heat waves and was considered the hottest July in the last 92 years, with an abnormal average temperature of ~25ºC ,4ºC above the temperature from the year before, and a high temperature for *Codium*, as the temperature must be between 18 and 24°C for the spongy thalli to achieve a growth/reproduction peak, according to Hanisak (1979). These abnormal temperatures could have influenced the quality of the seaweeds, especially as an intertidal species that spends part of the day out of water, when the atmospheric temperatures can indeed have a great impact on their quality. *Codium tomentosum* reaches its adulthood/reproductive stage in late summer/early autumn and continues aging trough winter, until around march when the older individuals are replaced by the juveniles. We can assume that the older thalli must be used to the lower winter temperatures (~10ºC). While the younger/immature thalli must be used to the higher summer temperatures (~20ºC). When regarding the quality of the seaweeds, we could correlate this information and say that the quality of younger thalli is best with the normal, higher, summer temperatures and the quality of older thalli is best with the lower winter temperatures. Thus, during the winter of 2020/2021 the germling growth was best, as the temperatures were normal for that time of the year, improving its quality. Whereas in the winter of 2021/2022, the temperatures were exceptionally high, decreasing the quality of the parental seaweed/biomass/specimens, and consequently affecting the germling growth. This hypothesis could also explain why the best overall growth was observed in October as well as the better registered growth in September 2021, the first months after reaching adulthood, when the seaweeds are yet to be considered “old” and may prefer more neutral temperatures, such as 17 degrees Celsius,

the average temperature registered for October 2021, and a more close temperature to the ~20ºC registered September 2021 than the ~21ºC in September 2020,

Another possible reason for the low growth in both the winter months and August of 2021/2022 could be the greater presence of epiphytes due to the higher temperature, also affecting the seaweeds quality.

It is referred in the "Seaweed Ecology and Physiology" book (p.56) (Hurd et al., 2014) that some seaweeds have seasonal anticipators, environmental cues that can indicate the environmental features of the next months. Although this is not known for *Codium*, the fact that during 2020-2021 the rise in temperatures only occurred in the beginning of spring could have led to an expectation of the same happening in the next year, however the observed rise in temperature during 2021-2022 occurred in the winter. In short there was an anticipation in the temperature rise from one year to the next, that could have misled *Codium,* and possibly affecting its quality.

More studies on the hypothesis contemplated here should be done as there are none regarding *Codium tomentosum* itself and it would be most insightful. Lastly, the different results from the same species in what should have been similar conditions, prove the powerful impact climate change has on nature. In just the short span of a year, monthly temperatures have changed drastically, affecting this seaweed and its habits. Therefore, more studies on the impact of climate change should also be made, especially in order to best interpret the results of trials like this one, that are directly affected by these changes.

Effect of light intensity vs. water movement

Despite the lack of significant differences, there was an overall growth tendency for the light intensity 20 µmol m^{-2} s^{-1} and no water movement conditions when it came to germling growth. It is known that this species has two different types of thalli, the first and more juvenile is identified by the presence of a filamentous thalli, while the second stage, the adult *Codium* is identified by the presence of a spongy thalli. When cultivating this seaweed, it is to expect that the germlings must pass first for the filamentous stage, followed by a morphogenesis to spongy thalli. That being said, the first suitable conditions for the growth of germlings must be the suitable conditions for the formation of a filamentous thalli, the first stage. Nanba et al. (2005), did a similar trial with *C. fragile´*s isolated utricles, although this trial was not conducted with germlings, it was reported that the formation of filamentous thalli required calm water (0-3cm s^{-1}) and was inhibited when

under the influence of high irradiance, much like the results presented here, which leads to believe, that the suitable conditions for the filamentous thalli, must indeed be, low irradiance and low water movement.

GAMETE RELEASE OPTIMIZATION

According to the results, *Codium tomentosum* seems to have a response to hydric shock, as the highest number of gametes released occurred after 2h30 drying followed by submersion in autoclaved seawater. Supporting this, according to Moeller (1969) the release of spores occurred after drying the thalli of *C. fragile*. Moreover, Hanisak (1979) followed Moeller´s terminology and obtained zoospores from *C. fragile* with mature sporangia after drying the thalli. There is a possibility that this specie has some kind of survival behaviour. When exposed to critical conditions, this seaweed could form and release more gametes as a way to insure its legacy. Despite this, there seems to be a window of time for the induction of gamete release, as the 5h drying period seemed to be too much, since it released very few gametes. Supporting this, the no drying time condition, and consequently, no shock also led to a small release of gametes. *Codium*, also seems to respond to ultrasound, as the release of gametes was much better after 3 ultrasounds following both 2h30 and 5h drying time (the conditions that induced a hydric shock), compared to 1 or 2 ultrasounds. Ultrasound seems to stimulate and speed the release of gametes.

This trial suggests that both conditions, drying time and number of ultrasounds, are dependent of each other. Firstly, as it was already described, the ultrasound worked much better when performed on dried seaweed. Another fact that supports this dependence is the similar release pattern on the dried seaweeds, the number of gametes released were increasingly higher with each ultrasound and the third one released a significantly higher number. Where in the seaweeds that did not suffer a hydric shock, this pattern was not observed, instead, the first two ultrasound released a similar quantity of gametes and the final ultrasound released almost nothing. More studies contemplating both drying time and ultrasound favouring gamete release should be made.

The results for the waiting time after the last ultrasound showed that until the 2h wait, the number of gametes released kept on growing, showing that the seaweed was still releasing, yet, the number of released gametes at 1h wait was not significantly difference

from the following waiting times. From 2h to 3h, the number of observed gametes was barely different, the same goes for the 24h wait, as showed by the function applied, leading us to believe that after 2h, the seaweeds have released the maximum of gametes, since an hour later and even 22h later, the density of gametes presented in the suspension was roughly the same. Retrieving the gametes with no waiting time is not advised as the results showed that barely any gametes were released, and just 15 min later the numbers were significantly higher.

Besides the number of gametes, their activity was also considered. While the best number was observed after 2/3h, the activity of the gametes retrieved after that time, despite still being good, wasn't as good as the one observed by the ones retrieved after 15 and 30 min. In these two initial waiting times, the majority of the female gametes were quite active and moving around. Nevertheless, after the 24 hours wait, very little gametes were moving and, besides this, a significative number of them were starting to degrade. Because of this, waiting 24 hours is not advised, as this much waiting time seems to deteriorate gamete quality.

In general, these results show that resorting to hydric shock followed by ultrasound for gamete release, not only induces the release but it also releases a much higher number of gametes than not resorting to hydric shock or resorting solely to hydric shock as the number of female gametes released after a third ultrasound was greater than after two and one following the hydric shock. We can also conclude that, at least for this species, it needs time to start releasing gametes, and has a gradual release instead of a "one time event".

MICROPROPAGATION ASSAYS

Nutrients

The results showed Provasoli´s enriched seawater to be preferred over autoclaved seawater. In fact, natural seawater is not usually used for laboratory algal culture, as it has not enough nutrients to sustain proper seaweed growth, and as the renewal is not continuous as in the ocean. In laboratory conditions, the enrichment of seawater is usually required, as the lack of nutrients and trace metals in natural seawater challenges the growth of seaweed. For macroalgal cultures, enrichments based on the "Grund" medium of von Stosch (1963) and Provasoli's ES medium are most commonly used (Berges et al., 2001).

The Provasoli's ES medium is satisfactory for most marine macroalgae as it contains nitrate, phosphate, ironEDTA, trace metals, and vitamins (thiamine · HCl, biotin, and B12). Additionally, most of the articles on *Codium* propagation cited in this paper use PES as their preferred medium, such as Borden and Stein (1969), Hanisak (1979), Park and Sohn (1992), Nanba et al. (2000; 2005), securing the choice to use this medium.

During this trial it was evident that the filamentous thallus from utricles preferred the full concentrated PES, with a significative difference in the last week from all the other tested concentrations. This same trial was conducted by our team last year in germlings. The results were similar, the preferred concentration was also full strength, however, the fully concentrated medium also allowed the growth of contaminants, and for that, PES/10 was selected as the better choice, since it promoted a good seaweed growth with less contamination (Sá M. F. A. C., 2021). In the present study, however, there was no contamination observed, this could suggest that utricles are more resilient than germlings and can even outgrow epiphytes and other biological contamination. A possible reason for the lower presence of epiphytes in utricle assays could be the higher density of utricles, whereas in germling assays it´s much harder to achieve such high numbers, leaving more space for epiphytes to grow.

Light intensity vs water movement

The adult *Codium* is mainly constituted by utricles (Nanba et al., 2000), as it was referred earlier, it is known that this specie has two different types of thalli, the filamentous thalli and the spongy thalli. When cultivating this seaweed, either by sexual reproduction or asexual reproduction (micropropagation) it is to expect that both the germlings and isolated utricles must pass first for the filamentous stage, followed by a morphogenesis to spongy thalli. That being said, the first suitable conditions for the growth of the isolated utricles must be the suitable conditions for the formation of a filamentous thalli. According to Nanba et al. (2005), who did a similar trial with *C. fragile*, the formation of filamentous thalli required calm water (0-3cm s^{-1}) and was inhibited when under the influence of high irradiance. On the other hand, water motion and higher light intensity does promote the growth of spongy thalli (Marques et al., 2020; Nanba et al., 2000). This reported data, coincides with our results, as the cultures had a better growth without water motion. The preferred light intensity observed in the present trial (40 µmol m^{-2} s^{-1}) also coincides with

the one reported by Yang et al., which showed optimum growth to be at 44 µmol m^{-2} s^{-1} for unialgal cultures from isolated utricles of *C. tomentosum* species.

Light intensity vs. light spectrum

C. tomentosum showed no significative differences between light spectra within each light intensity, yet there were significant differences between light intensities, the filamentous thallus from the utricles exposed to 40 µmol m^{-2} s^{-1} experienced a significantly lower growth from the other intensities (20 µmol m^{-2} s^{-1} and 60 µmol m^{-2} s^{-1}). It is important to refer that, due to a technical problem, the cultures exposed to 40 µmol m^{-2} s^{-1} were exposed to temperature variations during 2 weeks of the trial, which could have influenced the results. Despite not having differences between 20 µmol m^{-2} s^{-1} and 60 µmol m^{-2} s^{-1} and all the spectrums, there was a slight tendency for the red spectrum in the light intensity 60 µmol m^{-2} s^{-1} in. Marques et al. did a light spectra trial on adult *C. tomentosum* under 120 µmol m^{-2} s^{-1} irradiance, and his results showed the relative growth rate to be higher under red light and lower in blue light. Huang et al. did a similar trial on *Ulva pertusa*, another green seaweed, and according to his results, *U. pertusa* seemed to maintain a relatively fast and stable growth under all the LED lights with constant growth rates. In the present study, *C. tomentosum* also maintained a stable growth under all lights.

Hatchery assay (pilot-scale)

As previously mentioned, this is the first time, that we know of, *Codium tomentosum* was cultivated in substrates (string) as a form of micropropagation, and the first time to report its success, as the filamentous thallus from utricles adhered to the string, grew in it and differentiated. Our results suggest that there is a real potential to micropropagate *C. tomentosum* in seeded substrates, which may form the basis for the establishment of new hatchery protocols for aquaculture production of this species.

It is also, the first time to report the presence of spongy thalli in multiple tanks from filamentous thalli (isolated utricles), leading us to believe that morphogenesis occurred sometime during the trial. This result is extremely exciting and important. Because of this, the trial must be repeated and focused on this as its objective, in order to know, with precision, the conditions that induced the morphogenesis and thereby optimize the protocol.

As it was already stated, this pre-trial suffered some complications due to temperature problems in the CIIMAR facilities during said trial, that most likely, influenced the results. To be more specific, for 2 weeks, the temperatures in the BOGA rooms were extremely high, and all trials that were being conducted in that space, such as this one, were exposed to these sudden rises in temperature. Because of this, immediately after the rises in temperature, a considerable amount of epiphytes grew in all tanks, competing with *Codium* for space, nutrients and light, struggling *Codium´*s growth. Due to the fact that the seeding protocol is still under development and not fully optimized, the density of suspension in each rope at seeding time was not homogeneous, the first ropes had a higher density, while the last ones had less. This difference affected not only the % of coverage from the very beginning of the trial, as it also affects the capability of competing with epiphytes, as the higher the density, the greater ability to compete.

Because it is a relatively long trial, restarting it or conducting a second one based on this pre-trial was not a possibility in time for this paper. However, it is of extreme importance to repeat this assay, in the right, constant conditions and, especially as, even with problems, some promising good results were achieved. That being said, the trial is scheduled to be repeated in the next few months.

5. Conclusion

We successfully assessed the seasonal reproductive status of a population of *C. tomentosum* in its natural habitat, for a duration of 2 years. With the data retrieve we can gladly report the presence of a seasonal pattern for this specie, which happens from late summer/early autumn to the end of winter.

We were also able to identify some optimal culture conditions for both sexual and asexual reproduction methods. Filamentous germlings (sexual reproduction) showed a preference for 20 µmol m^{-2} s^{-1} while the filamentous thallus that differentiate from utricles (micropropagation) preferred 40 µmol m^{-2} s^{-1} when it came to light intensity. The latter also demonstrated a better development under red light (when compared to green and blue light), no water movement and showed preference for PES nutrient medium-full strength. The release of gametes resorting to hydric shock followed by ultrasound proved to be a more efficient way to induce gamete release as the number of female gametes released after a third ultrasound was greater than after two and one following the hydric shock. We successfully created a pilot-scale hatchery and proved that the filamentous thallus from utricles of *C. tomentosum* adhered to string, grew in it and experienced morphogenesis (formation of spongy thalli). Our results suggest that there is a real potential for the micropropagation of *C. tomentosum* in seeded substrates, which may form the basis for the establishment of new hatchery protocols for aquaculture production of this species.

Further studies should be made on *Codium tomentosum,* as so little information is currently available for this specie. From its potential and characteristics of interest to the optimal growth and morphogenesis inducing conditions. As future perspectives, we hope to prove that, like the the filamentous thallus from utricles, germlings can adhere to string as well, and that both can survive, grow and differentiate not only in land, but, and perhaps most importantly, in the sea, as this environment is preferred for large scale aquacultures.

6. References

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