Bárbara Soares

Development and optimization of hatchery *Codium* sp. cultivation methods for offshore production

Faculdade de Ciências e Tecnologia

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Mestrado em aquacultura e pescas

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UNIVERSIDADE DO ALGARVE

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(Development and optimization of hatchery *Codium* sp. cultivation methods for offshore production)

Declaração de autoria

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

Bárbara Cristina Silva Soares

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Resumo:

A indústria europeia de macroalgas encontra-se atualmente numa fase de transição e crescimento, impulsionada pela crescente procura de biomassa sustentável para aplicações alimentares, farmacêuticas, cosméticas e biotecnológicas. Entre as macroalgas com maior potencial de cultivo em Portugal destaca-se o género *Codium*, nomeadamente as espécies *Codium tomentosum* e *Codium decorticatum*, devido ao seu elevado valor nutricional, capacidade de adaptação a diferentes condições ambientais e versatilidade de aplicação. Contudo, apesar do seu potencial, os métodos de cultivo offshore para estas espécies ainda se encontram numa fase inicial de desenvolvimento e otimização, especialmente em Portugal, contrastando com o que se observa em países asiáticos como a Coreia do Sul, onde o cultivo de *Codium fragile* já está estabelecido e industrializado.

O presente trabalho teve como principal objetivo o desenvolvimento e otimização de métodos de cultivo em maternidade para *Codium* sp. visando a produção offshore, focando-se em *Codium decorticatum*, uma espécie local com elevado interesse comercial. Para tal, foram exploradas e comparadas diferentes estratégias de propagação, nomeadamente métodos assexuados (propagação vegetativa por fragmentação) e sexuais (baseados em gâmetas), bem como analisados fatores críticos como métodos de libertação de gâmetas, tipos de substrato e densidades de biomassa.

A nível experimental, o estudo foi dividido em quatro experiências: (1) libertação de gâmetas (ultrassom e maceração), taxas de germinação e taxas específicas de crescimento; (2) propagação em cordas com fragmentos; (3) propagação em cordas com gâmetas; e (4) otimização da densidade de biomassa. Para cada uma destas experiências foram desenhados protocolos experimentais rigorosos, recorrendo a diferentes metodologias inovadoras, como a utilização de ultrassons para indução da libertação de gâmetas, e testando diferentes substratos (algodão, juta, PVA, nylon e sisal), bem como técnicas de fixação (imersão passiva e aplicação por spray).

Antes do início das experiências, a identificação molecular dos espécimes de *Codium* recolhidos foi confirmada por PCR, assegurando que todo o material experimental pertencia à espécie *Codium decorticatum*.

Foram testados dois métodos para indução da libertação de gâmetas: ultrassons e maceração. Os resultados demonstraram que o método de ultrassons foi significativamente mais eficiente, produzindo uma densidade superior de gâmetas em comparação com o método de maceração. Além disso, a utilização de ultrassons resultou numa redução notável da contaminação por outros organismos, como epífitas e microalgas, o que é crucial para o sucesso do cultivo em ambiente controlado. A análise das taxas de germinação revelou diferenças estatisticamente significativas entre os métodos de libertação e as concentrações de meio de cultura F2 utilizadas, sendo que concentrações mais elevadas favoreceram o desenvolvimento e crescimento das plântulas.

No que respeita à escolha do substrato, verificou-se que o algodão proporcionou os melhores resultados em termos de comprimento dos indivíduos cultivados, sugerindo que as suas propriedades hidrofílicas e capacidade de retenção de humidade criam um microambiente mais favorável ao crescimento do *Codium* em comparação com outros materiais como a juta ou o PVA. Esta observação é particularmente relevante para a escolha de materiais em larga escala, uma vez que o substrato pode influenciar não só o crescimento, mas também a morfologia e a facilidade de colheita da biomassa.

Relativamente às técnicas de fixação, foram comparadas duas técnicas: imersão passiva e aplicação por spray. A imersão passiva consistiu em submergir os fragmentos em solução durante 24 horas, seguida de um período de repouso sem água, enquanto o método spray envolveu a pulverização da solução sobre o substrato, com o período de repouso sem água. A propagação assexuada através da imersão passiva revelou-se a mais eficaz para a obtenção de morfologias naturais e robustas da alga, em oposição à aplicação por spray, que resultou em morfologias menos desenvolvidas. No entanto, importa sublinhar que a escolha da técnica de fixação e do substrato deve ser ponderada em função do objetivo final da produção, podendo ser ajustada para maximizar características específicas da biomassa, como o teor de compostos bioativos ou a facilidade de processamento.

Na terceira experiência, cordas foram inoculadas com gâmetas libertados por ultrassons, permitindo a fixação e desenvolvimento de zigotos diretamente no substrato. A fixação de zigotos foi bem-sucedida. A morfologia dos indivíduos desenvolvidos por via sexual foi semelhante à observada na propagação assexuada. Apesar de a propagação sexual ser mais demorada, permite a introdução de variabilidade genética, essencial para a resiliência e melhoramento futuro das culturas.

Foram testadas três densidades de biomassa: baixa, média e alta. Os resultados mostraram que densidades mais elevadas proporcionaram taxas específicas de crescimento superiores, sem comprometer a qualidade morfológica dos indivíduos. No entanto, densidades excessivamente altas podem aumentar o risco de competição por nutrientes e luz, pelo que é necessário um equilíbrio. Este resultado sugere que a otimização da densidade é uma estratégia viável para maximizar a produtividade em sistemas de cultivo offshore, desde que sejam asseguradas condições ambientais adequadas de luz, temperatura e nutrientes.

Do ponto de vista metodológico, o estudo destaca ainda a importância de práticas rigorosas de amostragem e monitorização, uma vez que eventuais erros ou limitações no desenho experimental podem influenciar a interpretação dos resultados, nomeadamente na avaliação do impacto de diferentes métodos de inoculação ou substratos. Assim, recomenda-se a implementação de protocolos de amostragem mais robustos e replicados em estudos futuros, de modo a validar e consolidar as conclusões obtidas.

Em síntese, este trabalho contribui de forma significativa para o avanço do conhecimento sobre o cultivo de *Codium* em maternidade e a sua transposição para sistemas offshore, fornecendo dados experimentais valiosos para a seleção de métodos de propagação, substratos e técnicas de fixação mais adequadas à realidade portuguesa.

Por fim, importa salientar que o desenvolvimento de métodos de cultivo eficientes para *Codium* não só responde às necessidades do setor da aquacultura, mas também contribui para a mitigação da pressão sobre as populações naturais, promovendo práticas sustentáveis e alinhadas com os objetivos de conservação e valorização da biodiversidade marinha. Os resultados deste estudo fornecem, assim, uma base sólida para futuras investigações e para a implementação de projetos-piloto de produção offshore de *Codium* em Portugal, com potencial de replicação noutras regiões e para outras espécies de macroalgas de elevado valor comercial.

Palavras-chave: Aquacultura, Macroalgas, Substrato, *Codium* sp.

Abstract:

The European seaweed industry is experiencing a shift towards seaweed aquaculture and one of the seaweed that is showing high potential for cultivation in Portugal, and presents diverse applications, is *Codium* spp.. Still, methods for its offshore cultivation are only now being developed and optimized. On the other hand, established *C. fragile* cultivation in South Korea involves the use of seed stock regenerated from isolated utricles and medullary filaments, that are seeded in twine in a nursery and then deployed in the sea using rope structures. *C. fragile* is also sometimes produced through the settlement of wild zygotes onto culture twine, however, the regeneration capacity method has shown higher biomass yields compared to zygote. This study aims to explore the reproductive mechanisms and attachment processes to twine of the local species *Codium decorticatum* for the optimization of offshore cultivation. The study explores key factors influencing *Codium* growth, including gamete release methods (ultrasound and blending), substrate types (cotton, jute, PVA, sisal), settling techniques (filaments passive immersion, filaments spray application, and zygote attachment), and seeding densities (low, medium, and high concentration). Results indicate that ultrasound gamete release enhances gamete density and reduces contamination, while substrate selection significantly affects growth metrics. Asexual propagation via passive settling yields more natural morphologies, while higher seeding densities improve specific growth rates.

Keywords: Aquaculture, Macroalgae, Substrate, *Codium* sp.

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1. Introduction

1.1 Seaweeds

Marine macroalgae, or seaweeds, are photosynthetic multicellular organisms widely distributed in saltwater (Bartsch *et al.*, 2008). They are taxonomically organized into three groups, Chlorophyta, Rhodophyta, and Ochrophyta-Phaeophyceae, distinguished mainly by the photosynthetic pigments they contain (Pereira and Van de Velde, 2011). Seaweeds are foundational organisms in coastal ecosystems, providing a range of critical ecological functions that support biodiversity and contribute to the health and stability of marine environments. As primary producers, seaweeds drive global primary production, essential for marine food webs and coastal biodiversity (Chapman, 1995; Dayton, 1985). By absorbing dissolved nutrients from surrounding waters, they improve water quality and reduce the risk of harmful algal blooms and eutrophication (Cai *et al.*, 2021; Teagle *et al.*, 2017). These nutrient-retention capabilities make seaweeds essential for maintaining balance in nutrient-rich coastal regions (Mejia *et al.*, 2012; Ware *et al.*, 2019).

Beyond nutrient management, seaweeds provide spatial refuge for numerous marine organisms, offering shelter against predators and harsh environmental conditions. By structuring habitats, they support diverse species populations and contribute to ecological complexity (Bertocci *et al.*, 2015; Reisewitz *et al.*, 2006). Seaweeds serve as a food source for both aquatic and terrestrial herbivores and contribute organic matter to support detritivore species (Pessarrodona *et al.*, 2019). They also play a role in coastal defense, with their presence mitigating wave energy and reducing shoreline erosion, which protects coastal ecosystems from hazardous waves (Chapman, 1995; Cai *et al.*, 2021).

The role of seaweeds in the marine carbon cycle has gained attention for its potential impact on climate regulation, particularly in ameliorating ocean acidification, a process driven by increased CO_2 absorption by seawater. By uptaking CO_2 during photosynthesis, seaweeds reduce the concentration of free hydrogen ions in the water, leading to a localized increase in pH. This buffering capacity is essential for protecting calcifying organisms, such as corals and shellfish, that are vulnerable to acidic conditions. Therefore, seaweeds also contribute to maintaining the health and stability of marine environments under changing climatic conditions (Pessarrodona *et al.*, 2024). While seaweeds sequester carbon through photosynthesis, their limited capacity for long-term carbon storage has historically excluded it from formal “blue carbon” classifications (Howard *et al*., 2017; Smale *et al*., 2018). However, recent studies suggest an indirect contribution to long-term carbon sequestration. As an organic carbon source, seaweed detritus may travel via allochthonous transport to "blue carbon" habitats, such as seagrass beds, mangrove soils, and deep-sea canyons, where it becomes buried and contributes to long-term storage (Krause-Jensen and Duarte, 2016; Hill *et al*., 2015; Chung *et al*., 2011). This source-to-sink mechanism underlines seaweed’s indirect but meaningful role in carbon sequestration and highlights its broader significance in global carbon cycling.

Globally, over 200 seaweed species find industrial and commercial applications, with approximately 32 species belonging to green algae, 125 to red algae, and 64 to brown algae. Among these, approximately 140 are directly utilized for human consumption. European gastronomy has seen a surge of interest in incorporating diverse algae species into diets, spurred by a growing demand for natural and eco-friendly food sources (Wells *et al*., 2016; Rioux *et al*., 2017; Mouritsen *et al*., 2019). The rest play crucial roles in various industries, including phycocolloid production, medicine, agriculture, and animal feed (Pereira and Van de Velde, 2011). Key species such as *Laminaria digitata*, *L. hyperborea*, *Ascophyllum nodosum*, and *Gelidium corneum* are widely utilized for extracting hydrocolloids like alginate and agar, which have applications in food, pharmaceuticals, and biotechnology (Kraan, 2012; Peteiro, 2018). Beyond food applications, algae biomass serves as aquaculture feed and has recently been explored as a cattle feed supplement to enhance weight gain while reducing methane emissions, showcasing its versatility in animal husbandry (Machado *et al*., 2014; Roque *et al*., 2019; Kinley *et al.,* 2020). Other applications include its use as fertilizers, plant biostimulants, and sources of bioactive compounds for cosmetics and nutraceuticals (Thomas & Kim, 2013; Milledge *et al*., 2015; Chatterjee *et al*., 2017).

Innovative uses of algae biomass are also emerging, including its role in bioremediation, biomonitoring, biofuel production, and bioplastics (Deng *et al*., 2007; Zeraatkar *et al*., 2016; Abdul Khalil *et al*., 2017). Early-stage research highlights the potential of algae in developing novel health products and sustainable materials, reinforcing its value as a cornerstone of the European bioeconomy (Bogie *et al*., 2019; Kwon *et al*., 2020). However, despite this growing interest, the current landscape of algae production and industry development within the EU remains underexplored, necessitating further research and innovation to fully harness its potential.

1.2 Aquaculture and Seaweed Cultivation

With the world's population rapidly growing, and the overexploitation of the wild fish stocks, aquaculture is considered a viable option to meet these growing demands sustainably (FAO 2020). In 2022, global aquaculture production reached 130.9 million tonnes in live weight. This value consists of 94.4 million tonnes of aquatic animals, and 36.5 million tonnes of aquatic algae (FAO, 2024).

According to the most recent global statistics compiled by the FAO, between 1990 and 2022, the production of aquatic algae increased by 4.1% (Figure 1). In this last year, farmed seaweeds accounted for 97.1 percent of the total volume of algae. The main seaweed production was the Japanese kelp *Saccharina japonica*, followed by Eucheuma (*Euchema* spp.), Gracilaria (*Gracilaria* spp.), and Wakame (*Undaria pinnatifida*) (FAO, 2024). According to Nayar and Bott (2014), the dominant cultivated green seaweeds worldwide include *Codium fragile* and others like *Enteromorpha clathrate*, *Monostroma nitidum*, and *Caulerpa spp*.

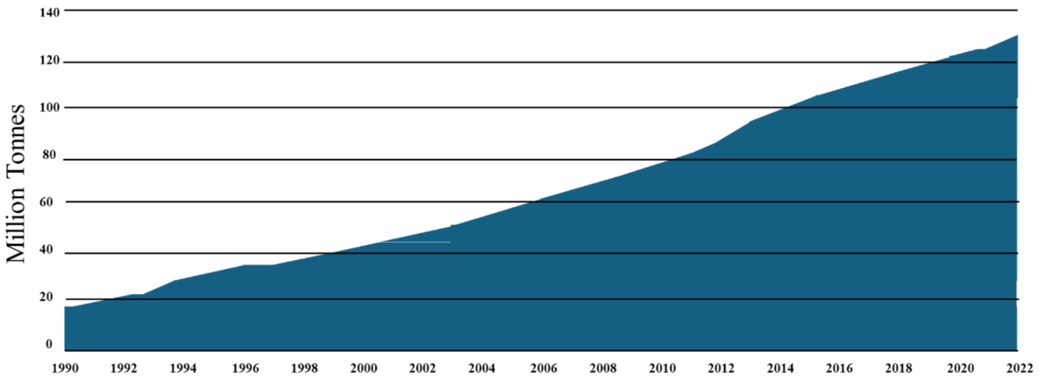

Figure 1 - Evolution of the world aquaculture production of aquatic algae from 1990 until 2022, adapted for FAO (2024).

Seaweeds are cultivated in diverse structures and environments, ranging from nets or ropes, whether floating, or fixed on stakes. Their cultivation is typically carried out using two main systems: land-based and at sea cultivation. Land-based cultivation involves growing seaweeds in controlled environments such as, bioreactors, tanks, ponds, or raceways, where water quality, nutrient levels, and growth conditions can be closely monitored. This system ensures consistent and high-quality biomass production, making it ideal for producing seaweeds for pharmaceuticals, nutraceuticals, and other high-value markets. However, these systems often require significant infrastructure investment and have high operational costs due to the need for water circulation and nutrient management. On the other hand, at sea cultivation utilizes natural marine environments, such as coastal or offshore areas, where seaweeds are grown on longlines, rafts, or grids. This approach is cost-effective and scalable, leveraging the natural nutrient flow and sunlight of the ocean. At sea systems are particularly suitable for large-scale production of species used in food, feed, and biofuel applications. However, at sea farming is exposed to environmental variables, including adverse weather conditions, and may pose challenges in monitoring and harvesting operations (Nayar & Bott, 2014).

Some seaweeds exhibit the ability to be cultivated through vegetative means, while others need careful control of their life cycles. In vegetative (asexual) cultivation, the propagation of new algae occurs through the fragmentation of older ones, directly fostering growth in a suitable environment. This method is exemplified by *Gracilaria spp*. and *Kappaphycus sp.* (Buschmann *et al*., 2017; Taelman *et al*., 2015). On the contrary, certain species, particularly kelps like *Undaria pinnatifida* or *Saccharina latissima,* require precise control of their life cycle, due to the alternation of generations (gametophytes and sporophytes). Successful cultivation of these species demands greater oversight (Loureiro *et al*., 2015; Taelman *et al*., 2015).

The primary seaweed-exporting nations around the world include Indonesia, Chile, and the Republic of Korea, being China, Japan, and the United States of America ranked as the foremost importers (FAO, 2020). While the predominant seaweed market is concentrated in Asia and the Pacific, there is an increased scientific and industrial interest in these organisms in Europe and North America (van den Burg *et al*., 2019; Lomartire & Gonçalves, 2022).

1.3 Seaweed aquaculture in Europe and Portugal

The European seaweed sector initially focused on harvesting wild marine seaweeds, is now shifting towards aquaculture, supported by the European Commission, which sees seaweed farming as an opportunity for sustainable job creation and economic growth (Van den Burg *et al*., 2019). Seaweeds are essential in the EU's Bioeconomy Strategy, which aims to advance bio-based sectors while promoting sustainable food systems and circular economy principles. Alongside this strategy, the Blue Growth Strategy emphasizes creating sustainable jobs in coastal economies through industries like aquaculture and biotechnology. Additionally, the European Green Deal sets goals for climate neutrality, biodiversity, and sustainable food systems, which align with the expanding role of algae cultivation (EC, 2018). Seaweed production can contribute to these priorities by supporting nutrient uptake, coastal protection, and even potential carbon sequestration, positioning it as an environmental asset as well as an economic one (Dayton, 1985; Steneck *et al*., 2002; Teagle *et al*., 2017).

Europe's seaweed industry has a rich history, with countries such as Spain, France, and Ireland traditionally harvesting wild seaweeds for food, animal feed, and fertilizer. Today, this production largely relies on manual or mechanical harvesting methods along the Atlantic coast, while aquaculture represents a smaller but growing fraction of European production, primarily led by Norway (Mac Monagail *et al*., 2017; Mac Monagail & Morrison, 2020). Although harvesting from wild stocks remains common, the shift to aquaculture brings new technological and ecological challenges but promises to enhance sustainability, especially through methods like Integrated Multi-Trophic Aquaculture (IMTA), where nutrient recycling from fish farms supports seaweed growth (Folke and Kautsky, 1989).

Despite the promise, Europe's seaweed sector faces significant challenges, including data fragmentation, regulatory and market gaps, and knowledge barriers that impact both production and market potential (Blue Bioeconomy Forum, 2019). Also, the high production costs and a lack of established markets have hindered its competitiveness against well-established Asian markets (NETALGAE, 2012).

Seaweeds in Europe primarily serve the food and nutraceutical markets, despite additional applications in cosmetics, bioremediation, biofuels, and animal feed, where their nutritional and environmental benefits continue to attract attention. With approximately 1700 seaweed species found along European coastlines, only a few are commercially utilized (Costello *et al*., 2006). *Saccharina latissima*, for instance, is popular for its high biomass yield, while *Laminaria hyperborea* supplies a quarter of the world's alginates (Muller *et al*., 2009; Frangoudes, 2011).

Portugal has emerged as a significant player in the macroalgae aquaculture sector, leveraging its extensive coastline, favorable climatic conditions, and a strong tradition of maritime innovation. The country is home to prominent companies and research institutions driving the industry forward. Alga+ is a company with success in land-based macroalgae production. Their focus is on producing high-quality macroalgae for diverse applications, including food, biofuels, cosmetics, pharmaceuticals, animal feed and supplementation, fertilizers, biostimulants, and innovative materials such as bioplastics. Alga+ produces *Ulva* sp., *Palmaria* *palmata, Gracilaria* sp., *Porphyra umbilicalis, Porphyra dioica, Fucus vesiculosus, Chondrus crispus,* and *Codium tomentosum*.

1.4 *Codium* spp.

Codium is a genus (Chlorophyta) belonging to the order Bryopsidales and the family Codiaceae, and represents a globally distributed green seaweed that encompasses various species and subspecies. It stands as one of the most prevalent genera, with approximately 150 described species (Goff *et al.* 1992; Costa *et al.*, 2015; Verbruggen *et al.*, 2007). *Codium* is found in seas worldwide, predominantly in temperate and subtropical zones (Oliveira-Carvalho *et al.*, 2012). Species within the *Codium* genus inhabit diverse marine environments, ranging from exposed rocky shores to tranquil lagoons, spanning intertidal to deep reef zones, and extending from eutrophic estuaries to nutrient-poor settings (González & Santelices, 2014; Verbruggen *et al.*, 2007).

In terms of morphology, all *Codium* species exhibit a spongy, dark green color and a thallus. The thallus can take on various forms, including erect, globular, or prostrate. Erect *Codium* species grow vertically, forming cylindrical dichotomously branched axes, and anchor to the substrate through a small disc called the holdfast (e.g. *C. fragile* and *C. tomentosum*) (González & Santelices, 2004; Verbruggen *et al.*, 2007). In prostrate or globular species, thalli spread over hard surfaces, covering the substratum and forming a dark green velvety mat (e.g., *C. adhaerens*) (Pereira, 2009).

Codium species consist of two morphologically distinct tissues (figure 2). The central region (medulla) is composed of long, colorless filaments (medullary filaments) arranged longitudinally with the thallus, densely intertwined. The peripheral region, the cortex, is formed of cylindrical or club-shaped siphonous swellings called utricles, tightly compressed together (Trowbridge *et al.*, 1998; Chapman and Chapman, 1973). The utricles are typically colorless, spine-like structures with rounded or pointed cell wall projections often used as taxonomic characters (González & Santelices, 2004).

Both the holdfast and the branches are composed of numerous utricles, each about 1 mm in size, connecting with the branching medullary filaments (Arasaki, 1955). *Codium* sp. exhibits apical growth, with the utricles being more concentrated in the apical tip region. Gametangia are developed on the utricles and are separated from them by complete septa (Borden and Stein, 1969).

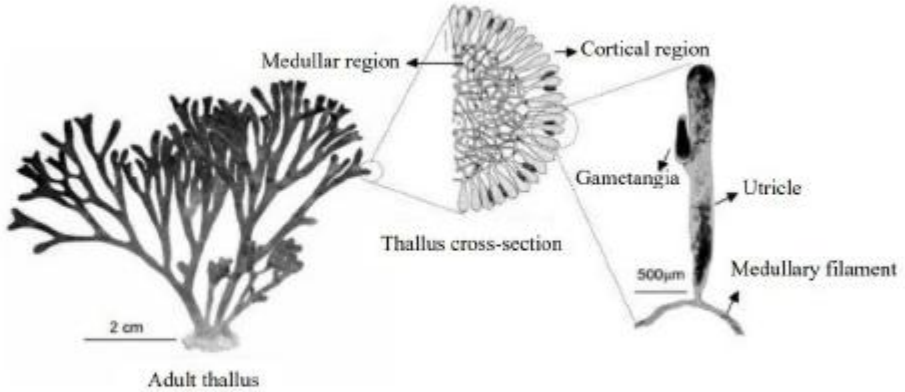

Figure 2 - *Codium* sp. morphology from Lee (1989).

According to the Naturdata platform, the *Codium* species found in Portugal include *C. tomentosum*, *C. decorticatum*, *C. bursa*, *C. adhaerens*, and the invasive *C. fragile*, in which *C. tomentosum, C decorticatum,* and *C. fragile* are the only erect species present.

1.4.1 *Codium tomentosum*

Codium tomentosum, commonly known in Portugal as Chorão, Chorão-do-mar, and Pingarelhos (Freitas *et al*., 2021), is indigenous to the northeast Atlantic Ocean. Its habitat ranges from the British Isles to the Azores and Cape Verde (Pereira, 2015), encompassing the coastal regions of Portugal. *C. tomentosum* holds the distinction of being the most prevalent species within the *Codium* genus in the Iberian Peninsula (Pereira, 2015). This species thrives in diverse environments, from sheltered to exposed locations, including tide pools, the lower horizon of the media littoral shelf, and the upper horizon of the infralittoral shelf (Pereira, 2009). Beyond its Atlantic range, this species has been identified along the coasts of Africa, specifically Morocco and Algeria, and in various other locations globally (Costa *et al*., 2015; Silva, 1955).

Typically abundant during the summer months in coastal areas moderately exposed to wave action, *C. tomentosum* can attain lengths of 30 to 50 cm in the autumn, often adorned with epiphytic organisms (APROMAR, 2014; Pereira, 2009). Widespread along the Portuguese coast, it thrives in both sheltered and exposed locations, appearing in the upper horizon of the infralittoral level, the lower horizon of the medium coastal level, and tide pools (Pereira, 2009). Morphologically characterized by a cylindrical or subcylindrical thallus with dichotomous branching and a spongy-elastic consistency, the plant displays numerous colorless hairs visible when submerged in water. Terminal segments are often elongated with rounded or slightly pointed apices (figure 3) (Pereira, 2009; Silva, 1955).

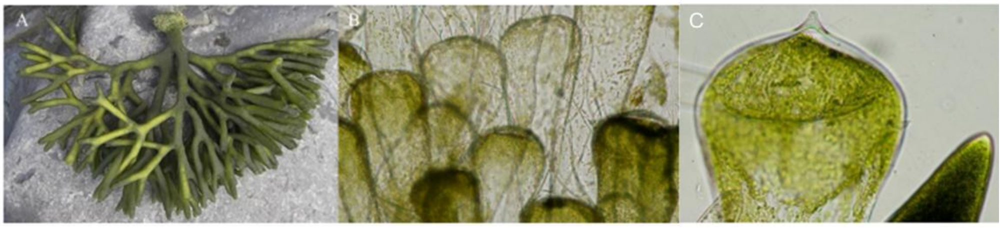

Figure 3 - Macro and microscopic view of C. tomentosum (A and B) and C. fragile (C). A- Cwmhiraeth (2011) B, C- Ignacio (2008).

Codium tomentosum may be mistaken for the invasive species *C. fragile*, which is native to the Pacific Ocean, ranging from the Sea of Japan to Alaska and Baja California, Mexico (Pereira, 2015). These two species bear a striking resemblance in appearance, with the main differentiation lying in morphological variations in the utricle, utilized as taxonomic attributes (García *et al.*, 2018; Trowbridge *et al.*, 2001). Notably, in the utricles of *C. fragile* species, there is an upper protrusion known as the mucron, which is absent in *C. tomentosum* species (figure 3 C) (Trowbridge, 2001). The apical mucron can be observed using a stereo microscope (Pereira, 2015).

1.4.2 *Codium decorticatum*

Codium decorticatum is commonly known in Portugal as Chorão or Macarrão verde (Oliveira, 1947). Its habitat ranges from the Azores and Cape Verde, encompassing the coastal regions of Portugal. *C. decorticatum* thrives in diverse environments, from hard substrates like rocks and shells, allowing it to thrive in shallow coastal environments with moderate wave action (Pereira, L., 2015). Beyond its Atlantic range, this species has been identified along the coasts of Africa, America, and the Middle East (Tsiamis *et al.*, 2014; Costa *et al.*, 2015; Silva, 1955).

Codium decorticatum is notable for its soft, spongy thallus structure (figure 4). The thallus can reach up to 1 m in length, and has a characteristic dichotomous branching pattern, with cylindrical branches that flatten at ramification nodes (Pereira, L., 2015; Oualid, Hwang *et al.*, 2020). The utricles, or specialized surface cells, are generally clavate and occasionally cylindrical, containing small chloroplasts. These utricles vary in size, with a diameter range of

108–343 μm and a length range of 585–900 μm (Hwang *et al*., 2020). As *C. tomentosum*, *C. decorticatum* utricles don't have mucron, a feature that adds to its unique velvety appearance, created by a dense arrangement of utricles covering its surface (Pereira, L., 2015).

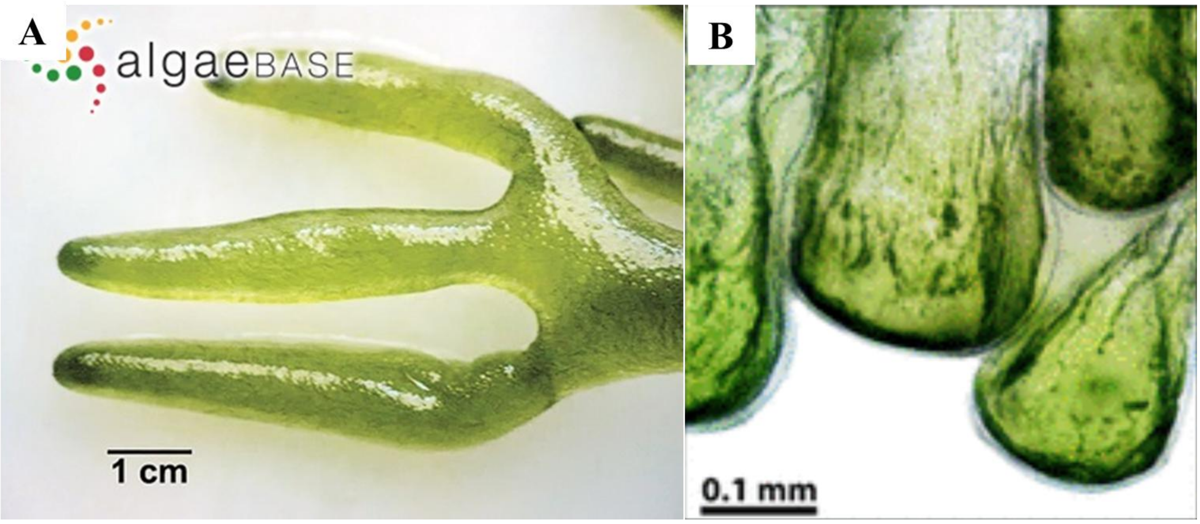

Figure 4 - Macro and microscopic view of *C. decorticatum.* A - AlgaeBase; B - Oualid *et al*., 2020.

1.4.3 Life cycle

Species belonging to the *Codium* genus undergo a diplontic life cycle featuring gametic meiosis and are commonly dioecious, meaning both male and female individuals coexist (Borden and Stein, 1969; Miravalles *et al*., 2012). Male and female gametangia are formed laterally from utricles, separated by transverse walls (septa) from the rest of the thalli (Chang *et al.,* 2003; Graham and Wilcox, 2000). These elongate-ovoid bodies, usually singular but sometimes multiple within an utricle, possess a two-layered wall. The inner layer is thick and expansible, while the outer layer is firm and thin (West, 1916). Both male and female gametes are biflagellate (Borden and Stein, 1969).

Female gametes are pyriform, approximately 20 μm long, with a dark green color due to discoid chloroplasts, and are released by the rupture of the gametangium apex caused by increased internal pressure from the swelling inner layer (Borden & Stein, 1969; Miravalles *et al*., 2012). In contrast, male gametes are much smaller (about 3 to 4 μm in length), obovate to elongate, exhibit a yellow-brown color, and are produced in larger numbers in similar gametangia (West, 1916; Prince & Trowbridge, 2004).

Both male and female gametes share the characteristic of having two flagella arising from a cap at the tip of the colorless extension of the hyaline apex (Prince & Trowbridge, 2004).

Discharge of gametes, accompanied by a slimy substance, has been reported for several *Codium* species, including *C. tomentosum* (Borden & Stein, 1969) and *C. decorticatum* (Miravalles, *et al*., 2002). Went (1889) discovered that, for *C. tomentosum*, both genders can coexist in the same specimen, while other studies have found individuals containing only one type of gamete, either female or male (Borden & Stein, 1969).

Sexual reproduction in the *Codium* genus involves the formation of zygotes resulting from the fusion of gametes (Nanba *et al*., 2002). The resultant zygote germinates into a germling, a siphonous filament that eventually develops into the diploid adult thallus (Borden & Stein, 1969; Chang *et al*., 2003). The peak density of gametangia for *C. tomentosum* occurs in winter, while in the invasive species *C. fragile*, it peaks in summer (Trowbridge, 2001, West, 1916).

In addition to sexual reproduction, reports indicate the capacity of thalli for asexual and vegetative reproduction (Nanba *et al*., 2002). *C. fragile* is known for asexual adult thallus formation, while other *Codium* species have been less studied (Chang *et al*., 2003). A study by Garcia *et al*. (2018) and Miravelles *et al*. (2002) suggested that the reproduction and "recruitment" of *C. tomentosum* and *C. decorticatum* in nature primarily involves vegetative regeneration from the perennial holdfast. In contrast, in *C. fragile*, reliance is on new settlers, parthenogenic gametes, buds, and thalli fragments (García *et al*., 2018). The significance of gametangia production and vegetative propagation remains unclear, necessitating further research (Prince & Trowbridge, 2004).

Parthenogenesis has been observed in at least three *Codium* species: *C. fragile, C. tomentosum, and C. vermilara* (Miravalles *et al*., 2012; Prince & Trowbridge, 2004). In cases where fertilization does not occur, the female and/or male gamete can develop into reproductive adult thalli, serving as a survival mechanism when the sexual phase cannot be completed. This reproductive strategy is maintained by natural selection (Brawley & Johnson, 1992). Studies on *C. fragile subsp. tomentosoides* in the Atlantic coast of North America and the Mediterranean Sea indicate the production of a single type of reproductive cell, resembling female gametes, referred to as "zoospores" by some authors. These reproductive cellsgerminate through parthenogenesis (Borden and Stein, 1969; Miravalles *et al*., 2012). Filamentous thalli development from isolated utricles with medullary filaments has also been described for *C. fragile* (Nanba *et al*., 2002; Yang *et al*., 1997), *C. bernabei* (González *et al*., 2014), and *C. tomentosum* (Pacheco *et al*., 2022) in cultivation. This type of vegetative reproduction involves a change in utricle morphology and the capacity to form elongated filaments, which can attach to the substratum and later branch into new utricles (González *et al*., 2014; Nanba *et al*., 2000). Results from a study by Nanba *et al*. (2002) suggest that utricles detached by strong waves and grazers can grow into fully developed adults through the development of filamentous thalli, contributing to the extensive and rapid spread of the species.

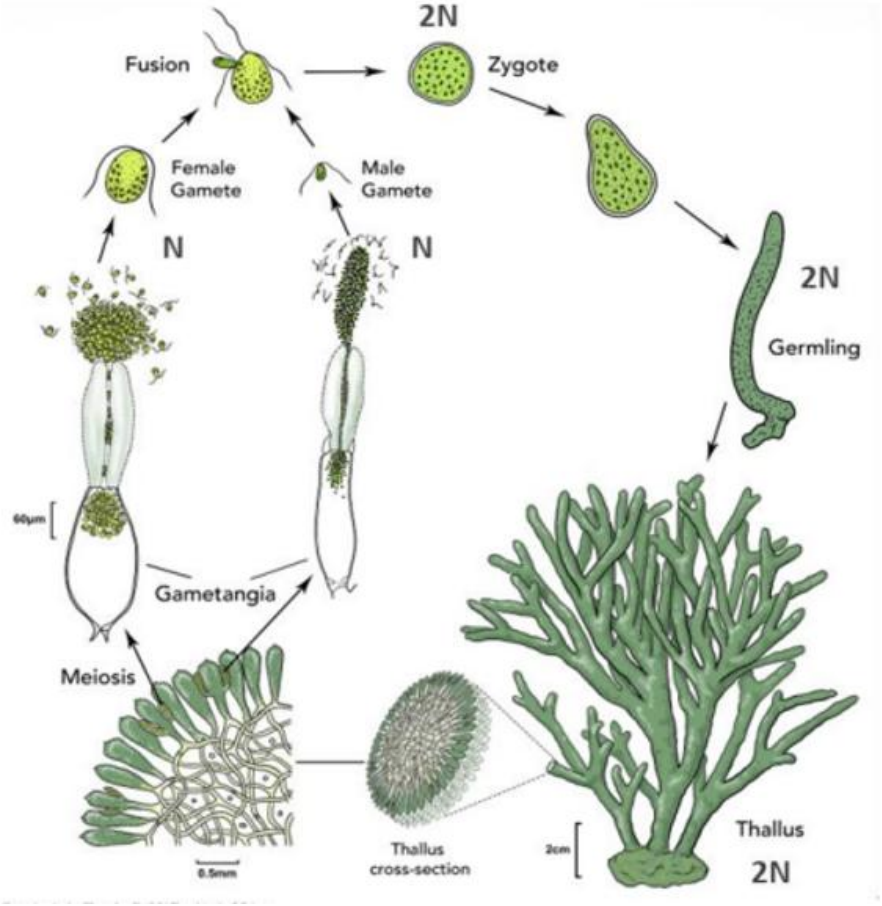

Figure 5 - Life cycle of *Codium* sp. (Lesley Elkan).

1.4.4 Applications

Codium tomentosum is cultivated in Portugal, Spain, and various other countries globally due to its recognition as an edible species (Mata, 2020; Freitas *et al*., 2021). It is consumed fresh and incorporated into salads in Malaysia and India, while in India, it is also dried for use in confectionary teas. In Japan, the species is dried and preserved in salt, consumed fresh with soy sauce and vinegar, or cooked in water in addition to soups (Freitas *et al*., 2021). *C. tomentosum* has emerged as a valuable source of neuroprotective and anti-inflammatory agents (Ballesteros *et al.,* 2022; Hafez *et al*., 2022). Lomartire *et al*. (2022) suggest that *C. decorticatum* has anti-inflammatory and antitumor agents. The cosmetic industry benefits from extracts of *C. tomentosum*, as they regulate skin water distribution, possess skin repair capabilities, and protect against dryness (Wang *et al.*, 2013). Notably, research by Christabell (2011) highlighted the antibacterial efficacy of *C. tomentosum*, particularly against Gram-negative bacteria. From the perspective of polar lipid content, *C. tomentosum* and *C. decorticatum* sourced from Integrated Multi-Trophic Aquaculture (IMTA) are considered a promising cash crop. It stands out as a potential element for both animal and human nutrition, offering various health advantages for human well-being (Costa, 2015; Lomartire *et al.*, 2022).

1.5 Cultivation methods and growth conditions of *Codium*

The cultivation of *Codium* species, though still developing, is gaining traction in Portugal and Europe due to its economic and ecological potential. In Portugal, Alga+ has pioneered the cultivation of *Codium tomentosum* using a free-floating land-based production system, which allows the seaweeds to grow in semi-controlled environments, maximizing biomass yield and quality (Oliveira *et al.*, 2023). In contrast, PortoMuinos, based in Spain, focuses on harvesting wild *Codium* sp., capitalizing on the natural abundance of these algae in coastal regions.

Outside Europe, notable advances in *Codium* at sea cultivation have been made in South Korea, particularly with *C. fragile*. In 2017 alone, South Korea reported a cultured production of 3,980 tons of fresh weight (Hwang & Park, 2020). The cultivation process typically starts with seed stock regenerated from isolated utricles and medullary filaments (Hwang *et al.*, 2008). This involves seeding the medullary filaments onto ropes in tanks, followed by a nursery phase. Once the filaments develop into erect thalli, the ropes are deployed at sea for further growth, culminating in the mature stage when the algae reach their maximum length.

The Korean method also employs an alternative approach involving the settlement of wild zygotes onto culture ropes. However, studies have shown that the regeneration capacity method achieves higher and more consistent biomass yields due to its resilience against environmental variability (Hwang *et al.*, 2008). Future research into optimizing reproduction in *Codium* local species could further enhance biomass production and support full life-cycle cultivation. Exploring innovative approaches, such as optimizing *Codium* local species settling into twine and utilizing offshore cultivation systems, could reduce costs and increase algal biomass production, fostering the broader adoption of *Codium* aquaculture in Europe.

1.5.1 Temperature

Temperature stands out as a pivotal abiotic factor affecting algae, operating in diverse and intricate ways. Numerous studies underscore the direct impact of climate changes on the physiological performance and survival of organisms, as well as their indirect influence on interspecies interactions such as predation and competition. The effect of temperature is particularly noteworthy on the rates of chemical reactions, consequently influencing metabolic pathways. Comprehensive studies aimed at unraveling the influence of temperature on seaweeds reveal that factors such as biomass production, reproduction, contamination levels (epiphytes), and algae growth exhibit an upward trend with increasing temperatures (Broitman *et al*., 2008; Werner *et al.,* 2015; Kim *et al*., 2007). The optimal temperature range for cultivating *Codium fragile* is between 18 and 24ºC (Hwang *et al*., 2005).

1.5.3 Irradiance and photoperiod

Photosynthesis and subsequent growth of seaweeds are reliant on the availability of light. The specific light requirements for each species depend on factors such as adaptation mechanisms, morphology, and distribution along intertidal zones and across the globe. In their natural habitats, the quality and intensity of light reaching seaweeds are influenced by factors like water turbidity, seasonality, and tides (Werner *et al*., 2004).

According to Hanisak, (1979), longer photoperiods increase *Codium* growth due to a rise in total daily irradiance. *C. tomentosum* was found to exhibit better growth under long-day photoperiods (16 h light:8 h dark) compared to short-day conditions (8 h light:16 h dark) (Marques *et al*., 2020; Sá *et al*., 2024).

Regarding irradiance, specific studies report critical amounts for thalli formation, such as 60 µmol m^{-2} s^{-1} for *C. fragile* (Park and Sohn, 1992) and 44 µmol m^{-2} s^{-1} for *C. tomentosum* in unialgal cultures (Yang *et al*., 1997). In the same study, different light spectra were investigated, revealing that *C. tomentosum* exhibited better growth under red light, intermediate in white light, and lower in blue light (Marques *et al*., 2020). Sá *et al.* (2024) show that *C. tomentosum* germlings exhibited faster growth when cultured under lower light intensity (20 µmol m^{-2} s^{-1}).

Codium anatomy is adapted for low-light environments, and exposure to strong light may lead to the development of protective hairs (West, 1916). A study by Nanba *et al.* (2002) with *C. fragile* demonstrated that spongy thalli growth occurred under the combination of high irradiance and high-water velocity, whereas the formation of filamentous thalli required calm water and was inhibited in the presence of high irradiance.

1.5.4 Water Movement

Consideration of the hydrodynamic environment is crucial in establishing seaweed aquaculture, as it significantly impacts both growth and productivity (Peteiro & Freire, 2011). Water movement plays a role in fertilization, propagule dispersal, and seaweed settlement (Hurd, 2000). In general, seaweeds tend to exhibit higher production levels in moderate water movement compared to low movement (Peteiro & Freire, 2011). This is because of the increased water velocity and turbulence, often associated with wave action, positively impact nutrient loading and photosynthetic rates. This enhancement in nutrient and carbon dioxide uptake, facilitated by a reduction in the boundary layer around the seaweed surface, leads to an increase in growth rates (Hurd, 2000).

For *Codium*, numerous studies underscore the importance of water movement in adult thallus formation (González *et al*., 2014; Nanba *et al*., 2002). Research by Scheibling and Melady (2008) demonstrated that the attachment of vegetative propagules of *C. fragile* was higher in the presence of water flow than in static water conditions. Water flow promoted the production of medullary filaments and their attachment to the substratum (Scheibling and Melady, 2008). In the case of *C. fragile* with isolated utricles, water movement was essential to stimulate growth and regenerate the adult thallus (González *et al*., 2014).

1.5.5 Settlement

The settlement of seaweeds to artificial substrata has been extensively studied, and such investigations are crucial for understanding the later establishment of organisms in artificial aquaculture environments. Various surfaces have been examined for different seaweed species (Hwang & Park, 2020; Watanuki & Yamamoto, 1990; Scheibling and Melady, 2008). The degree of roughness of these surfaces generally dictates the success of attachment, with irregular structures proving more favorable than smooth surfaces in facilitating the settlement of spores, gametes, and zygotes (Harlin & Lindbergh, 1977).

While no specific substratum preferences for the settlement of *Codium* spp. have been reported, observations by Ramus (1972) indicated that germlings grown in the laboratory displayed firmer attachment to irregular surfaces of a scratched microscope slide than to a smooth surface. In artificial structures, *C. fragile* colonizes boat hulls, wharves, buoys, and breakwaters (Scheibling & Melady, 2008). Kuralon twine (PVA) has already been shown to be a good substrate for *Codium* cultivation (Hwang, 2005; Mata, 2020).

1.6 Objectives

This study aims to elucidate key aspects of the reproductive mechanisms, settling, and development phases of local *Codium* sp.. Specific objectives are: 1) to identify male and female mature gametangia; 2) to investigate protocols for inducing gamete release and, 3) to develop methods (e.g. seeding method, substrate type, density) that optimize the settling process of *Codium* sp. (zygotes and filaments), establishing protocols for hatchery cultivation, and ultimately the aquaculture of this species in the open sea.

2. Material and Methods

This work took place at Estação Experimental de Moluscicultura de Tavira, Portuguese Institute of the Sea and Atmosphere (EEMT-IPMA), located in Parque Natural da Ria Formosa, Vale de Caranguejo, under the partnership agreement between IPMA and S2AQUAcoLAB.

2.1 Macroalgae collection and cleaning

Codium specimens were collected in South Portugal, in the Algarve. The selected collection site was the western region of Faro Island, on the Ria Formosa side (figure 6). The work was divided into four experiments. For experiment 1, *Codium* biomass was collected monthly from February 2024 until July 2024 in Faro Island. Wild biomass for experiments 2, 3, and 4 was collected between February 2024 and April 2024.

Figure 6 - Collection site in Faro Island indicated with red line.

To prevent damage to the algae and the surrounding environment, a careful removal process was employed, using a knife to detach the fronds from the substrate gently. Subsequently, the algae were placed in net bags and transported to the laboratory using buckets filled with seawater. The choice of collection days depended on weather conditions and tide parameters, considering factors such as low tide time and amplitude (between 0.2m and 0.4m).

The fronds with the least epiphytes were chosen and weighed for the experiments in the laboratory. The algae were inspected under the stereo microscope to ensure they were not invasive *C. fragile* (*C. fragile* can be distinguished by the presence of an apical mucron in *C.*

fragile's utricle, which is absent in this species of *Codium*), if it was, the specimen was discarded. Then the macroalgae biomass was cleaned using two steps to minimize contamination. First, it was cleaned manually using tweezers and seawater. Secondly, the biomass passed through a bath of 30 seconds of fresh water, then betadine 10%, and finally autoclaved seawater. The seawater used for cleaning had been previously filtered (5 μm filter) and UV-treated to eliminate sediment and attached organisms.

Three specimens were identified with the molecular tool Polymerase chain reaction (PCR), at S2AQUAcoLAB molecular laboratory. PCR is a molecular biology technique used to amplify specific DNA sequences. It consists of three main steps: denaturation (94–98°C, 20–30 seconds) to separate DNA strands, annealing (50–65°C, 20–40 seconds) to allow primers to bind to the template DNA, and extension (72°C) where DNA polymerase adds nucleotides to synthesize new DNA strands. These steps are repeated for 25–40 cycles, exponentially amplifying the target sequence. The results are typically analyzed via gel electrophoresis to confirm successful amplification (Erlich, 1987).

2.2 Twine preparation

Five different twines were used in this work, two were synthetic fibers (nylon and PVA (or Kuralon)) and three were natural fibers (sisal, jute, and cotton). To remove oils, manufacturing residues, and dirt that can affect the settlement and attachment response of the seaweeds (Kerrison et al., 2019), the ropes were submitted to a cleaning process, The procedure was adapted from the FAO (2004) report. The ropes were boiled, shaken, re-boiled, and dried at 50ºC.

2.3 Culture conditions

All experiments were conducted in climate chambers (FitoClima Bio 600, Aralab) set at 18°C with a photoperiod of 16L:8D (L= light, D= dark), and regular aeration. The nutrient media used was F2 (1ml/L, except when stated). To mitigate diatoms growth, 1ml/L of 1mg/l GeO_2 was added. The seawater was autoclaved at 121°C for 20 minutes and cooled to ambient temperature before use. The salinity was measured with a multiparametric probe (Thermo Scientific Orion KIT STAR A329) and adjusted with distilled water to 30. Seawater and nutrients were changed weekly, and the containers were cleaned.

2.4 Experiment 1 - Gamete release, germination rates, and specific growth rates

Experiment 1 was divided into two trials, both carried out at a light intensity of 20 μmol photons m^{-2} s^{-1}. The first trial (1.1) aimed to investigate the monthly presence of mature gametangia, gametes release, and germination of *Codium* sp. zygotes, in the South of Portugal, using Ria Formosa as a study area. The protocol used was based on Borden and Stein's study (1969) and Ramus (1972) for *C. fragile*. Every month, from February 2024 to July 2024, three individuals were collected. Each thallus was measured from the basal part to the tip and captured by photography. Four random fragments (2-3 cm) were ground using a mortar and pestle, adding 15ml of autoclaved seawater. After achieving complete homogenization, the resulting mixture (suspension of utricles and medullary filaments), was carefully transferred to petri dishes, with a microscope slide positioned in each dish. Two hours later, the sex was determined by examining them under a microscope (Leica DM750). Parameters such as gametes size, behavior, and the density of gametes were recorded by photo/video in each replicate weekly for 14 days.

The second trial (1.2) investigated the best gametes release method. Two methods were tested, the same used in experiment 1.1 (blended method) and ultrasound bath (based on Pacheco, 2022). The biomass was collected in February 2024. Six petri dishes with a microscope slide were prepared, three for the blended method and three for the ultrasound method. In the ultrasound method four fragments (per replicate), around 2-3cm, were transferred to a vial with 20ml of autoclaved seawater. The vials were subjected to an ultrasound bath for 3 minutes. This process was repeated three times, with a 10-minute interval between each ultrasound session. Approximately 10 minutes after the third ultrasound treatment, the contents were transferred to petri dishes. The blended method was the same as described for experiment 1.1. Two hours later, gamete gender was determined by examining the slide under the microscope. And three days later, the pieces of *Codium* were removed from the ultrasound method and the water of the blend method was filtered (20μm net). For each gamete release method, three F2 concentrations were tested, low, medium, and high. Parameters such as behavior and density of gametes, germination rates and germling growth were recorded by photo/video, in each replicate, weekly for 21 days.

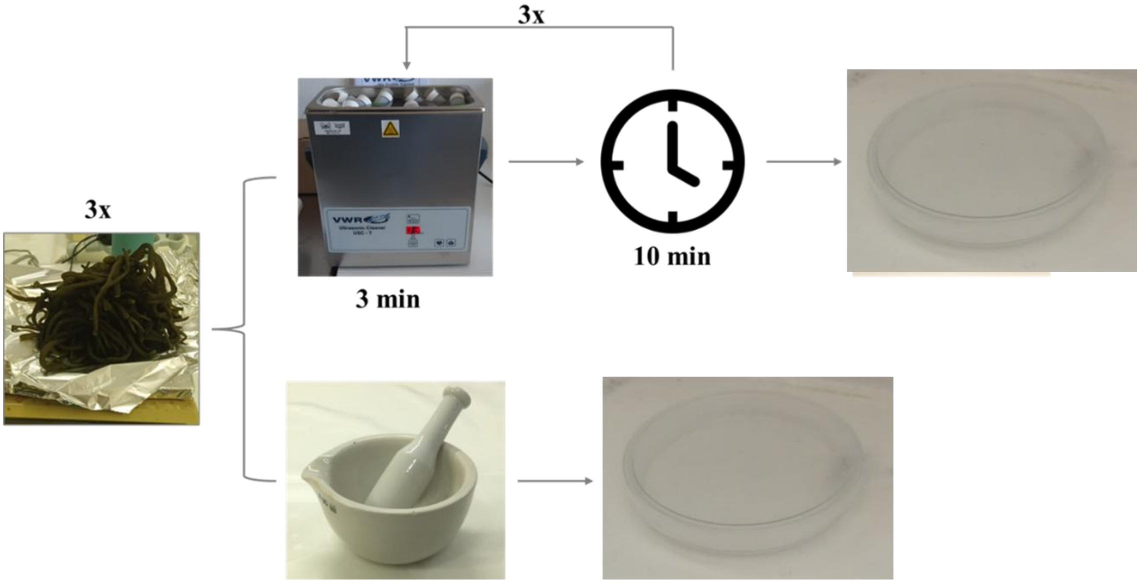

Figure 7 - Protocol scheme of experiment 1.2. Three individuals were collected. The first line shows the ultrasound bath in which the fragments were subjected to three rounds of ultrasound treatment for 3 minutes each, with 10-minute intervals. In the blended method, the fragments ground using a mortar and pestle until fully homogenized.

2.5 Experiment 2 - Twine seeding with fragments

Experiment 2 aimed to study the best twine substrate and best settling method for the cultivation of *Codium* sp., achieving the best option for offshore cultivation. The protocol was based on the work of Mata (2020), and Hwang (2005). *Codium* presents vegetative growth, so two different settling methods were tested, passive and spray. All the methods were tested, in triplicate, on nylon, sisal, jute, PVA, and cotton twine. This experiment was performed under a light intensity of 60 µmol photons m^{-2} s^{-1}.

The passive settling method involved creating a blended medium to produce a uniform solution concentrated with *Codium* sp. fragments under 7 mm. The substrate (twine) was immersed in the *Codium* solution for 24 hours in the climate chamber, with the conditions described above. Subsequently, the substrate rested in a container without water for 3 hours. It was then transferred to a larger container of 1L with 800ml of autoclaved seawater, nutrients, and GeO_2. The aeration was added two weeks later, by adding a glass tube to the bottom of the container, connected to a central air pump. The cultivation of the seeded substrate was kept for three months, with seawater changes and sampling carried out once a week.

The initial phase of the spray method was the same as in the passive method. The biomass was blended to form a uniform solution of *Codium* sp. with fragments under 7 mm. Using a spray bottle, the substrates were then sprayed with concentrated *Codium* solution. Following this, the seeded substrate rested for 3 hours without water. Ultimately, they were maintained in the same manner as described above for the passive method.

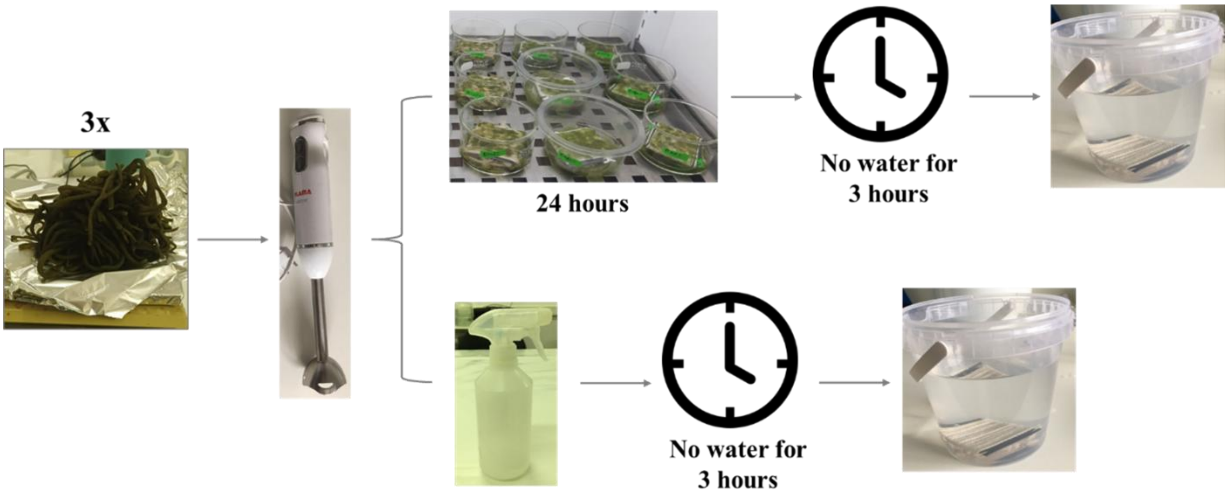

Figure 8 - Protocol scheme of experiment 2. In the passive settling method, a blended solution of *Codium* sp. fragments under 7 mm was prepared, and the substrate was immersed for 24 hours, then rested without water for 3 hours. In the spray method, the *Codium* solution was prepared similarly, but the substrate was sprayed with the solution and also left to rest without water for 3 hours.

Photos and videos along 5cm of twine were taken weekly to register the *Codium* length in each frame in the stereomicroscope. After the filament phase, the covered area with *Codium* sp. was measured.

2.6 Experiment 3 - Twine seeding with gametes

Experiment 3 aimed to study the best substrate for the cultivation of *Codium* sp. from gametes. The protocol is based on the work of Hwang (2005), and Pacheco (2022). This experiment was carried out at a light intensity of 20 µmol photons m^{-2} s^{-1}. The substrates used in this experiment were PVA, cotton, and jute. The gametes release method used was the ultrasound method, described above in section 2.4. Twine was immersed in autoclaved seawater and gamete solution (male and female) for 3 days, without aeration. Two weeks later, the aeration was added as above. The cultivation was maintained for six months, with sampling, water and media changed once per week.

Under a stereomicroscope (Leica S Apo), photos and videos were taken weekly, starting on week 4, to register the *Codium* length in each frame. After the filament phase, the covered area with *Codium sp.* was also measured.

2.7 Experiment 4 - Seeding density optimization

Experiment 4 aimed to identify the optimal seeding density of *Codium*. Based on preliminary results from previous trials, the best performing method chosen was the filaments blend passive seeding method, and the substrates used were PVA, jute, and cotton. Three different seeding concentrations were tested: low (half of the control biomass), medium (control), and high (twice the biomass of the control). The water was changed, and the medium was replaced once a week for 3 months. *Codium* development and growth were documented through photography and video using the stereomicroscope every week.

2.8 Data analysis

Photographs of the cultures were taken every week. The number of zygotes and filaments were counted, measured, and used to determine the germination rate (exp 1 and 3). The length of each individual was analyzed to calculate their specific growth rate (SGR) (exp 2, 3, and 4).

The germination rates were calculated using the following equation:

$$Germination\,rates = \frac{number\ of\ germlings\ (after\ 7\ days)}{number\ of\ female\ gametes\ (after\ 2\ hours)} * 100$$

The specific growth rate (SGR) utilized the most accurate formula among those available, as outlined by Yong (2013):

$$SGR(\%\alpha^{-1} = \left[\left(\frac{{L_t}^{\frac{1}{t}}}{L_0}\right) - 1\right] * 100$$

Where, L0 = initial length (µm); Lt = final length (µm); t = total days of culture.

The data were submitted to a One-way RM ANOVA if the data fit the assumptions for the parametric test (normality, equal variances (Brown–Forsythe)), or Friedmann's test if not. Except in experiment 1.2 when a t-test was used to analyze differences between the treatments. When significant differences were observed ($p<0.05$), pairwise comparisons among levels of factors were tested using Pearson correlation coefficient. Statistical analyses were conducted using Sigma Plot version 14.0 software, and figures were generated using Excel (Microsoft Office 365).

3. Results

3.1 Molecular ID using PCR

The PCR amplification of DNA extracted from all algal samples confirmed the analyzed specimens to be *Codium decorticatum*, hence in this work it is assumed all collected specimens belonged to the same species.

3.2 Experiment 1 - Gamete release, germination rates, and specific growth rates

The first trial (1.1) investigated the monthly presence of mature gametangia, gamete release, and germination of *Codium decorticatum* zygotes, from February to July 2024, by collecting, measuring, and analyzing thallus fragments microscopically to observe gametes' gender, size, behavior, and quantity.

Both female and male gametes were observed in the same individual. Both male and female gametes were confirmed to be biflagellate during the observations. Mature female gametes are easily distinguishable from their male counterparts by their dark green color, pear-shaped form, and larger size, measuring an average of 22.89± 4.85µm (n=45) in length (figure 9). In contrast, mature male gametes are significantly smaller, ranging an average of 3.91± 0.45µm (n=45) in diameter, and are characterized by their bright yellow color (figure 9).

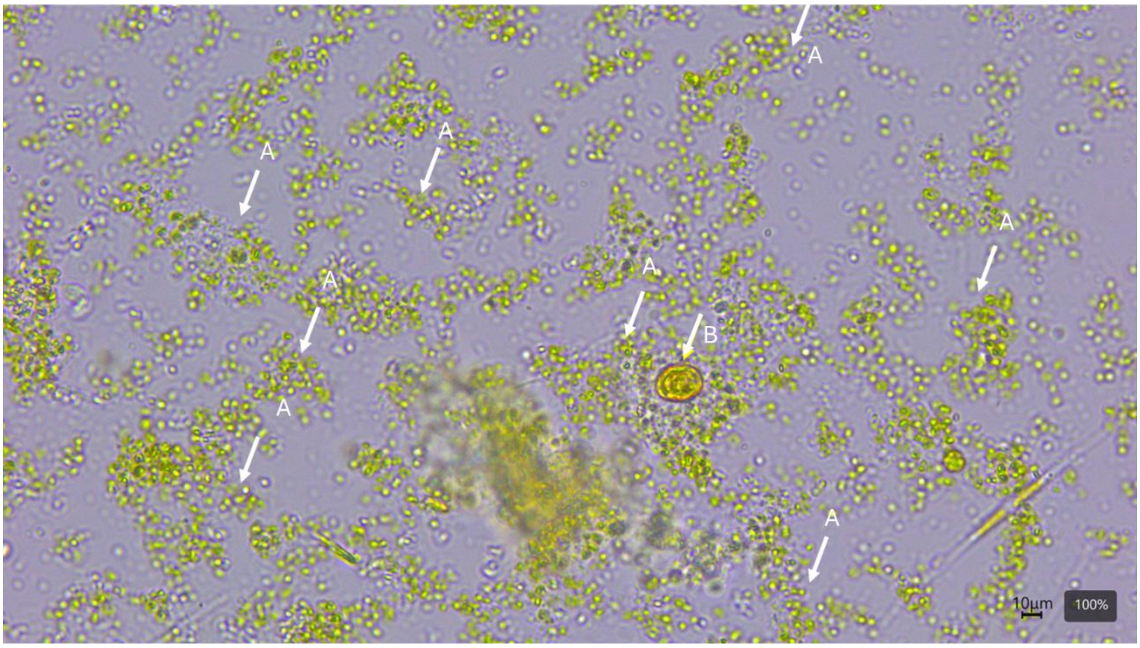

Figure 9 - Detail of *C. decorticatum* female and male gametes. A are male gametes, and B is a female gamete.

The gamete discharged occurred immediately, so the initial discharge moments were not observed. No discharge process of female gametangia was monitored in real time, but still numerous discharging male gametangia were registered (figure 10). The gametes exited the gametangium slowly, one at a time, in a stream of a slimy substance. Their tendency to remain together, shortly after discharge, suggests that they were also immersed in a slimy substance (figure 10). Initially, no movement was observed within the male gametes.

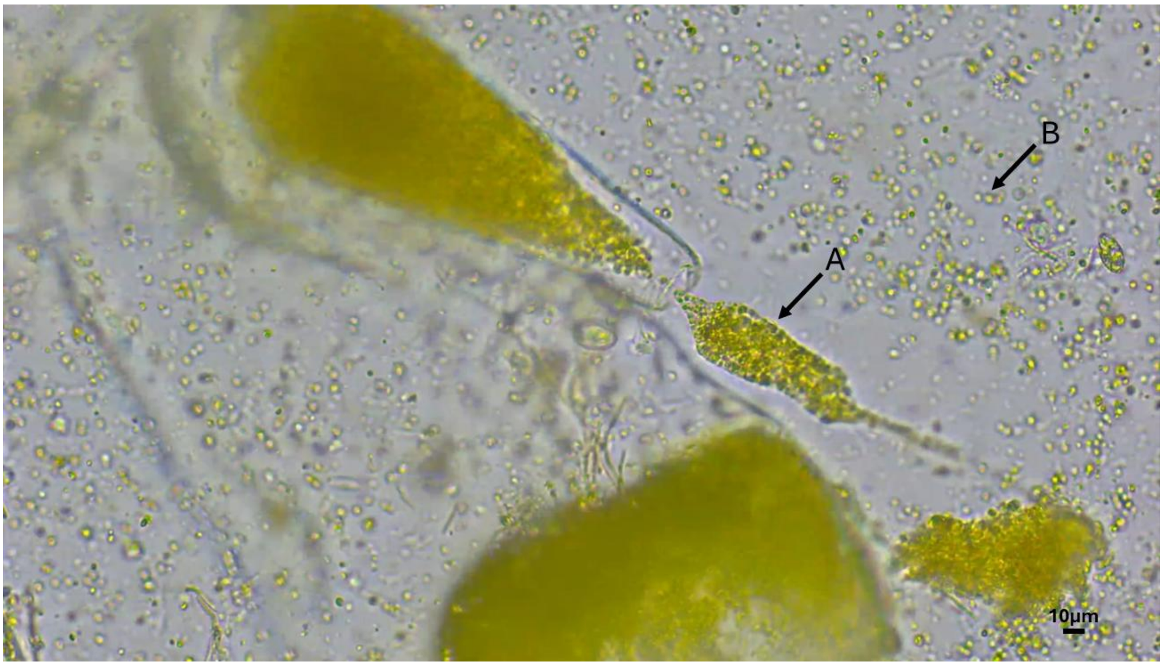

Figure 10 - Discharge process of *C. decorticatum* male gametes. A shows the slimy substance and male gametes; B shows male gametes after separation.

Over the six-month observation period (February to July), the quantity of gametes released each month (counted after 2h) exhibited significant variation (figure 11). Specifically, February, March, and April showed a reduction of female density (gametes/ml) ($6.61 \times 10^5 \pm 2.79 \times 10^5$; $3.50 \times 10^5 \pm 1.51 \times 10^5$; $6.31 \times 10^5 \pm 2.72 \times 10^5$, respectively) compared to the other months (p= 0.038, 0.034, 0.041, respectively), being the minimum female gametes per ml observed in March with $3.50 \times 10^5 \pm 1.51 \times 10^5$, and the maximum in May with $1.75 \times 10^7 \pm 4.78 \times 10^6$. While male gametes density (gametes/ml) were only lower in March and April ($3.78 \times 10^6 \pm 1.18 \times 10^6$; $1.26 \times 10^6 \pm 3.91 \times 10^5$, respectively), comparing to all other months (p= 0.037, 0.023, respectively), with the minimum male gametes per ml in April,$1.26 \times 10^6 \pm 3.91 \times 10^5$, and a maximum of $1.39 \times 10^7 \pm 1.71 \times 10^6$ in February.

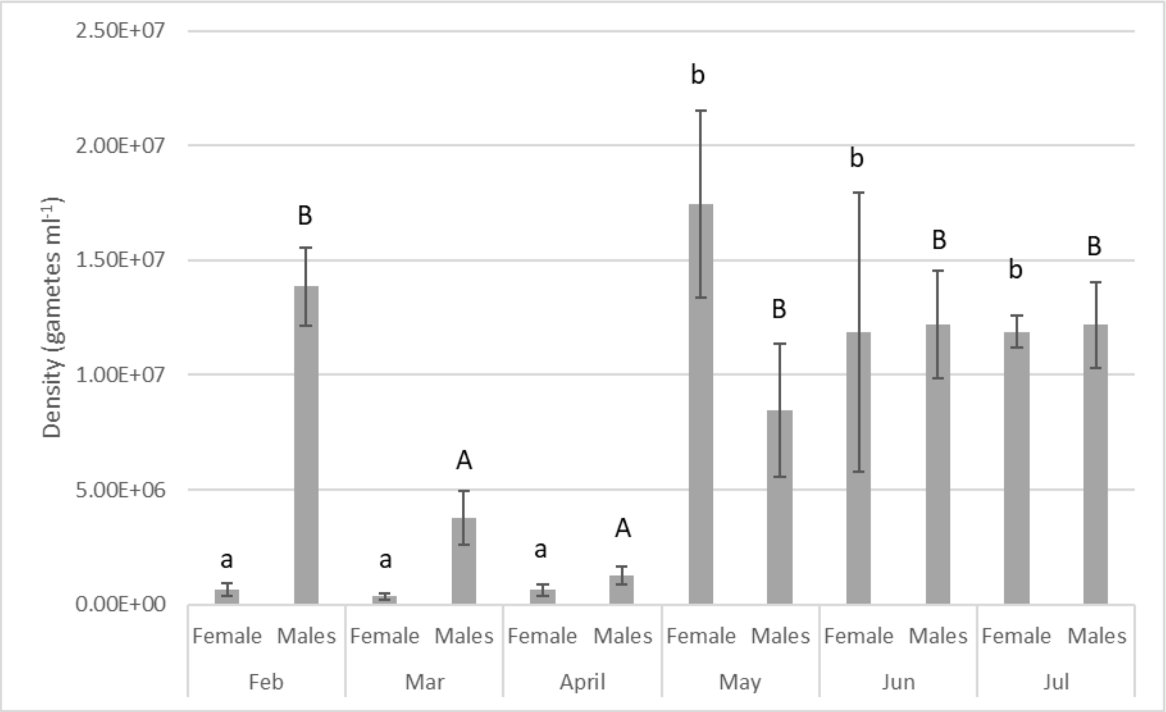

Figure 11 - Density of, female and male, *C. decorticatum* gametes (per ml) released each month from February to July (Mean ± SD). Different letters indicate significant differences between mean values of each month, within each sex, lowercase letters are for female gametes and uppercase letters are for male gametes ($p < 0.05$).

Observations were also made at 3, 7, and 14 days after the initiation of the culture. Female gametes and zygotes are distinguished by their behavior, female gametes are flagellated so they can move, while zygotes don't have flagella. The zygotes appeared round, with a prominent cell wall, measuring 20.56± 3.12µm (n=15) in diameter (figure 12, Day 3). Germination then occurred through the formation of one or two tubular outgrowths (figure 12, Day 7), which elongated and began to resemble the medullary filaments characteristic of the mature algae at day 14 (figure 12, day 14).

Germination rates were significantly higher in February (18% ± 3%) and March (29% ± 3%) compared to the other months (9% ± 3%, 12% ± 5%, 10% ± 7%, and 9% ± 7%) (p = 0.035 and 0.029, respectively), with the highest rate observed in March (figure 13).

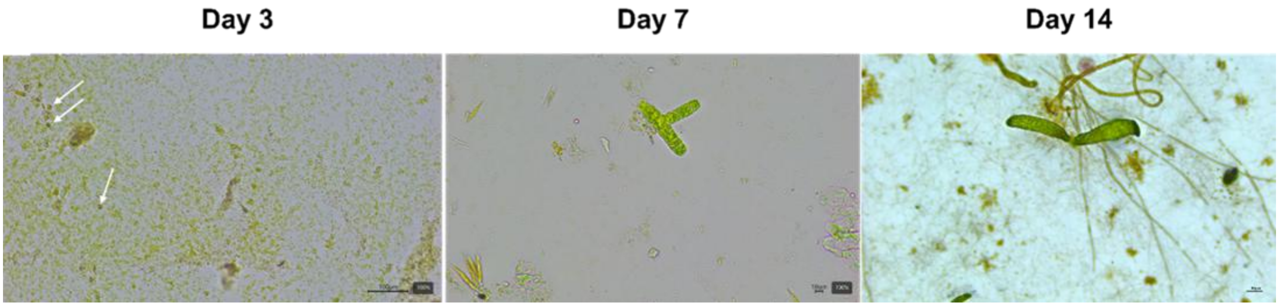

Figure 12 - Germination and development of *C. decorticatum*. over the 14 days observation period.

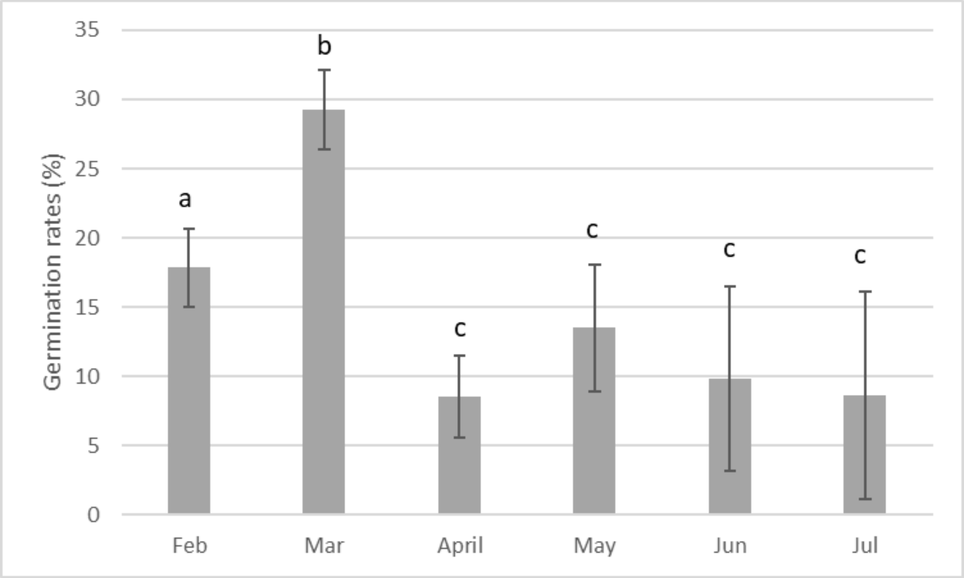

Figure 13 - Germination rates of *C. decorticatum* in each month from February to July (Mean ± SD). Different letters indicate significant differences between mean values of each month ($p < 0.05$).

The second trial (1.2) tested two gamete release methods, blended and ultrasound, on *Codium decorticatum*, comparing gametes' size, behavior, density, and germlings' growth over 21 days by examining them under the microscope. Three different nutrient concentrations were tested (Low, Medium, and High).

The comparison between germination rates in the blend and ultrasound methods (figure 14), 41.23%±18.78% and 33.99%±16.81%, respectively revealed no significant differences ($p=0.769$). Furthermore, the germination rates compared within the different nutrient concentrations (figure 14), revealed significant differences between low and high ($p=0.004$), and medium and high ($p=0.047$), with higher germination rates observed at the high nutrient concentration, compared to medium and low.

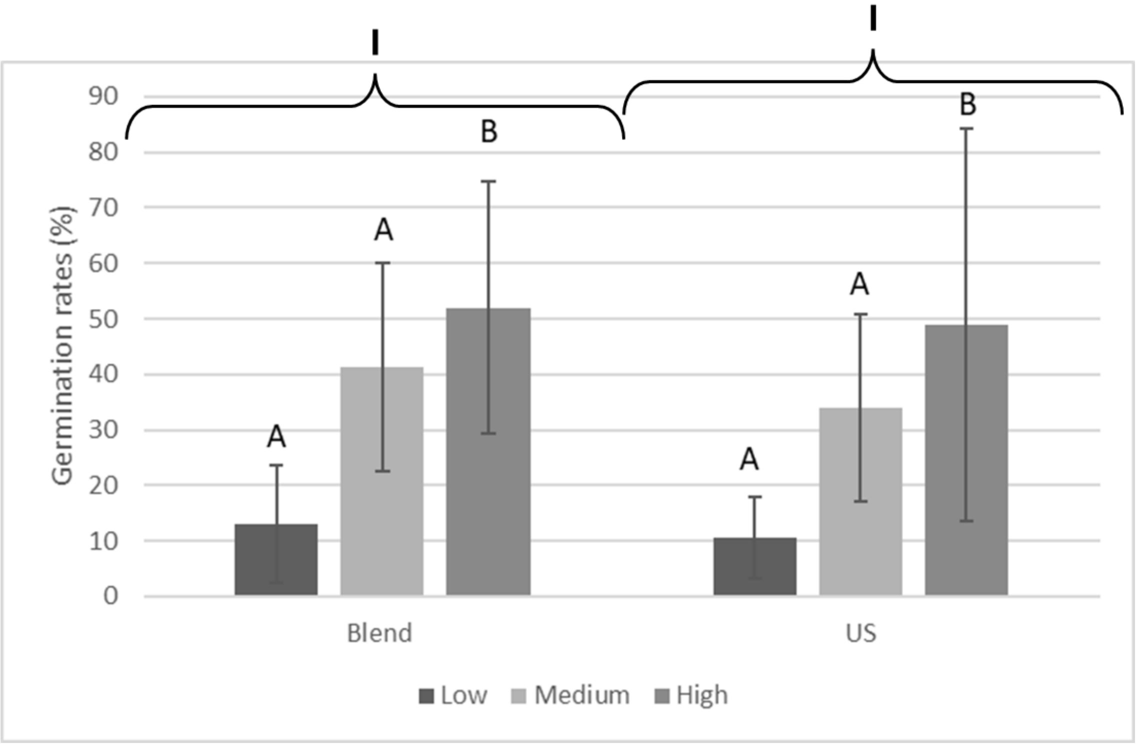

Figure 14 - Germination rates of *C. decorticatum* germlings, using blend and ultrasound gamete release methods, and cultivated at three F2 media concentrations, low, medium and high (Mean ± SD). Within each release method, different capital letters show significant differences between germination rates in the different F2 concentrations ($p<0.05$). Different roman numbers show significant differences between release methods ($p<0.05$).

Similarly, the specific growth rate ($p=0.67$) 54.04%±9.97 and 54.21%±14.14%, respectively in blend and ultrasound, did not show significant differences between the two methods (figure 15). Significant differences were observed between days, indicating a clear increase in growth over time (figure 15). The highest specific growth rate was recorded at the medium nutrient concentration in the blend treatment ($p = 0.030$), while in the ultrasound treatment, it was recorded on day 21 ($p = 0.046$) (figure 15).

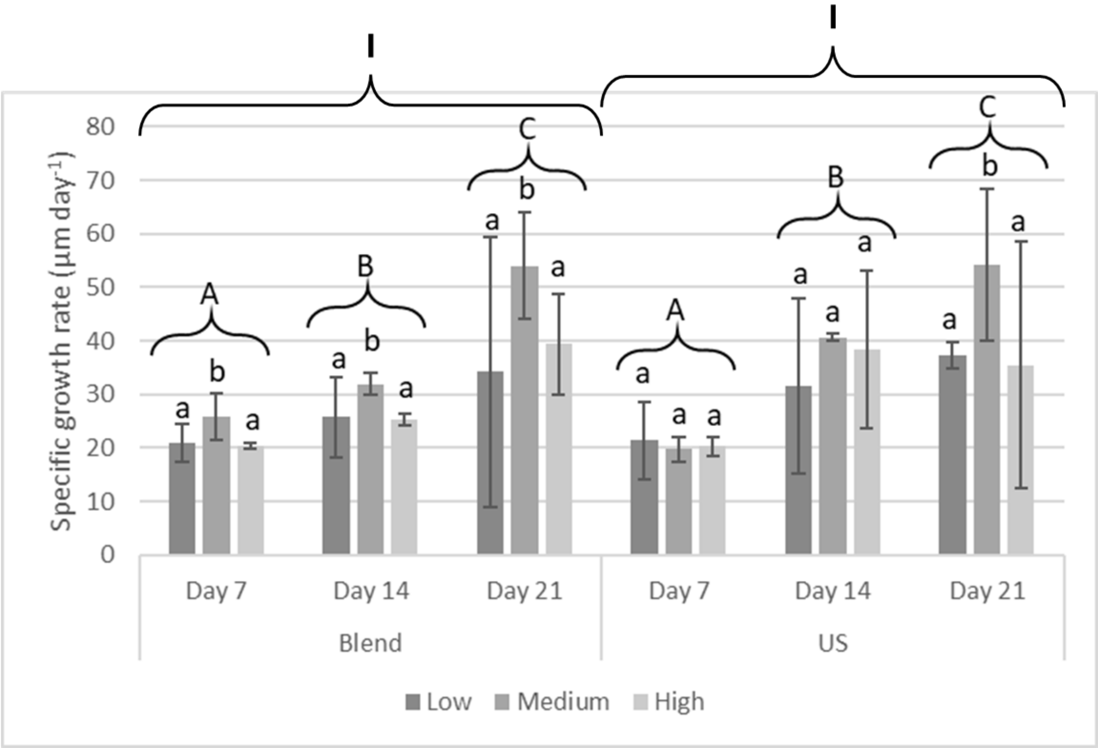

Figure 15 - Specific growth rate (SGR) of *C. decorticatum* germlings, using blend and ultrasound gamete release methods, and cultivated at three F2 media concentrations, low, medium and high (Mean ± SD). Within each release method, different capital letters show significant differences between SGR in the sampling days ($p<0.05$). Within each sampling day and release method, different lowercase letters show significant differences between SGR in the different F2 concentrations ($p<0.05$). Different roman numbers show significant differences between release methods ($p<0.05$).

During the water change, it was observed that the blend method showed a higher level of contamination compared to the ultrasound method. The contamination was not analyzed in detail, but the majority of the contamination was similar to *Ulva* sp..

3.3. Experiment 2 - Twine seeding with fragments

Experiment 2 aimed to determine the best twine substrate and settling method (passive or spray) testing nylon, sisal, jute, PVA, and cotton twine, with cultivation sustained for three months following initial immersion or spraying of *Codium* fragments.

The observed development of *Codium* through fragments can be divided into three distinct phases. Initially, the erect filaments grew independently of one another (figure 16A). By day 14, there were some differences between the twines. In the cotton, jute and PVA, the filaments began to come into contact, adhering to each other through a hooking mechanism (figure 16B). In nylon, this behavior was observed by day 21 (figure 17). As growth progressed, a tangled mass of intertwined and branching filaments formed and gradually enlarged (figure 16C). For cotton, jute and PVA, by day 21, differentiation of the tissue became evident, allowing a clear distinction between medullary and cortical cells (figure 17). During this phase, an organized movement of tissues was observed, with cortical tissue migrating toward the outer part of the knot (figure 16C). By day 35, a fully organized individual was formed (figure 16D). Both in nylon and PVA ropes, the last phase of development wasn't observed by day 91 (figure 17). Despite the good *Codium* development observed in sisal twine, after some time this twine disintegrated.

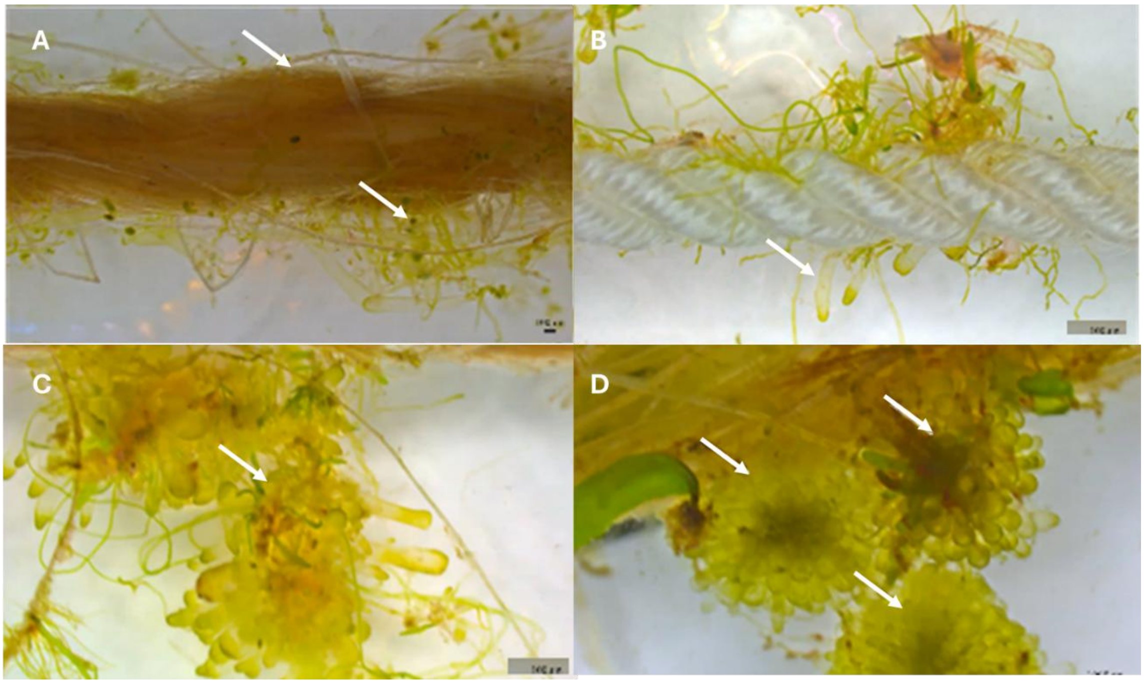

Figure 16 - Overall *C. decorticatum* development. (A) erect filaments growing independently of one another. (B) differentiation between medulatory and cortical cells. White arrow indicates a cortical cell. (C) filaments began to come into contact, adhering to each other. (D) an organized movement of tissues, with cortical tissue migrating toward the outer part of the knot.

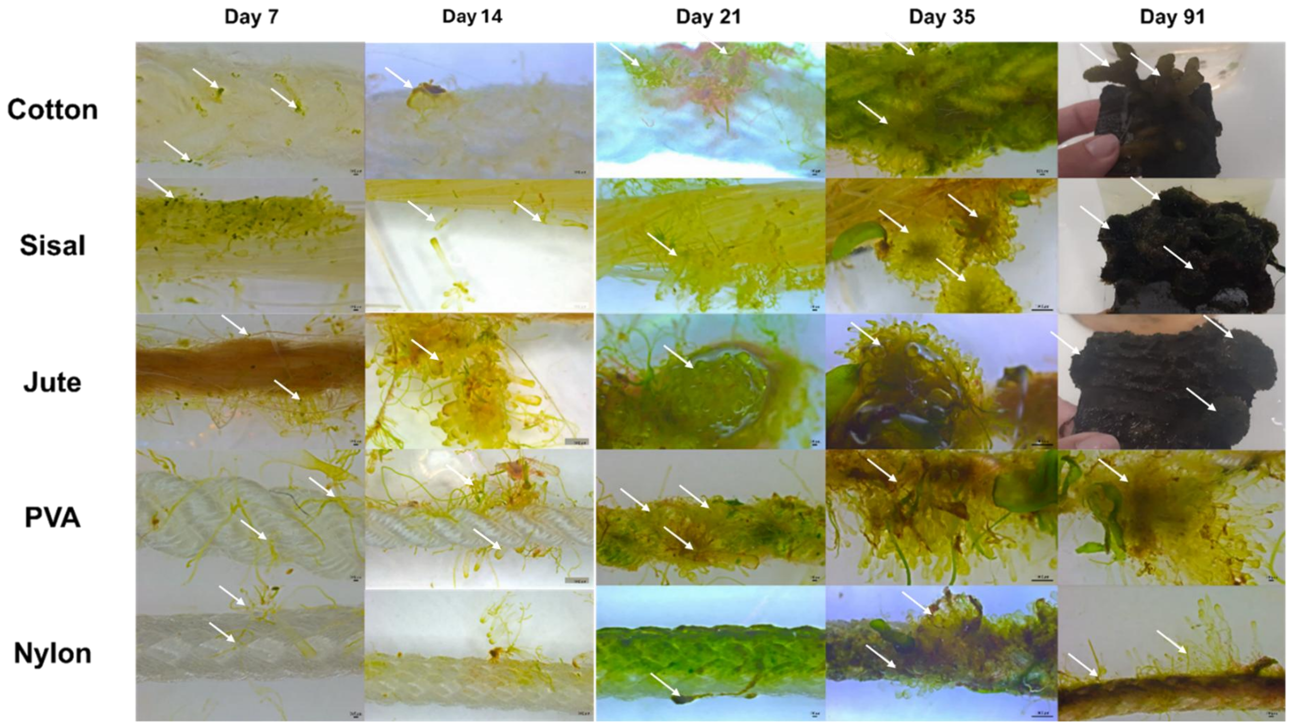

Figure 17 - Detail of *Codium* development, using the passive settling method with fragments, on days 7, 14, 21, 35, and 91 in the different twine. Arrows indicate *Codium*.

A comparison between the length of *Codium* within each settling method and twine revealed no significant differences ($p \geq 0.09$). Within each sampling day, there were significant differences, with length increasing with time (figure 18).

Also, no significant differences in specific growth rate between methods and the twine type were observed ($p \geq 0.35$) (figure 19). A higher specific growth rate was observed on days 21 and 35 for both methods (figure 19). In the passive method, the organisms exhibited characteristics more similar to those found in nature, including greater height and the presence of some branches (figure 20). In the spray method, the organisms tended to grow horizontally, displaying a distinct growth pattern (figure 20). Nonetheless, notable differences were observed in the final phase of development between the two methods in terms of organism morphology.

A comparison between seeding methods and twines, using the area, showed no significant differences ($p=0.297$ and 0.493, respectively). When compared within sampling days, there was a significant difference between day 21 and 91, for both seeding methods, ($p=0.007$), in which day 91 had a higher area (figure 21).

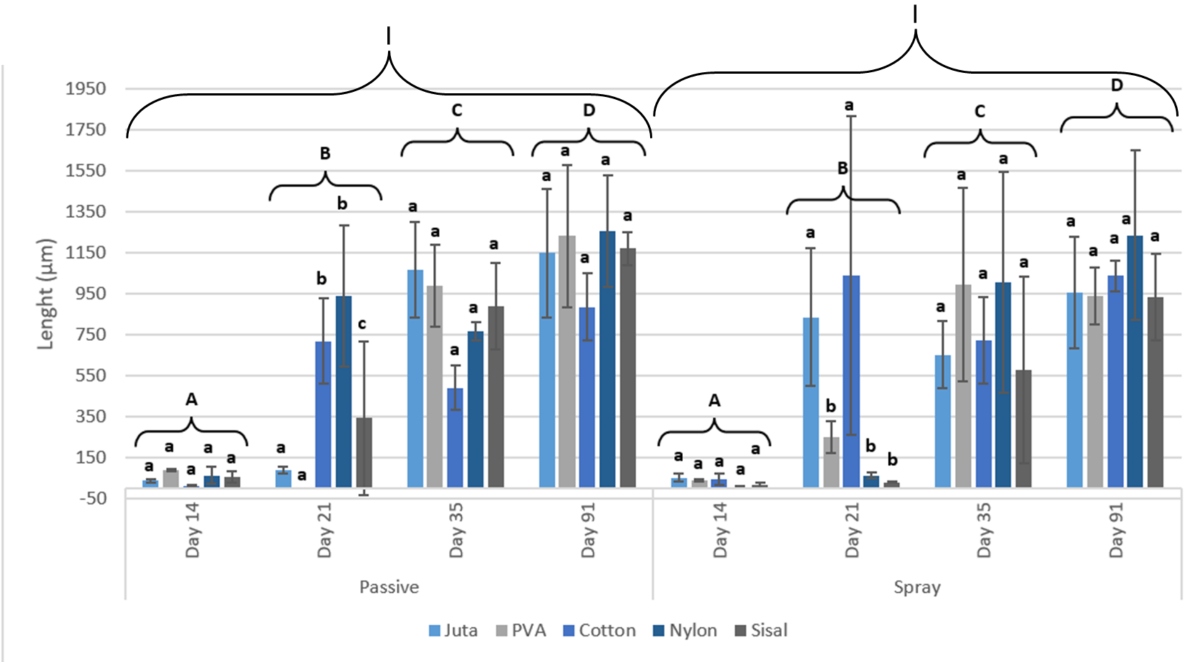

Figure 18 - Length of *C. decorticatum* fragments from the passive and spray methods (Mean ± SD). Within each seeding method, different capital letters show significant differences between lengths in the sampling days (p<0.05). Within each sampling day and seeding method, different lowercase letters show significant differences between twine lengths (p<0.05). Different roman numbers show significant differences between seeding methods (p<0.05).

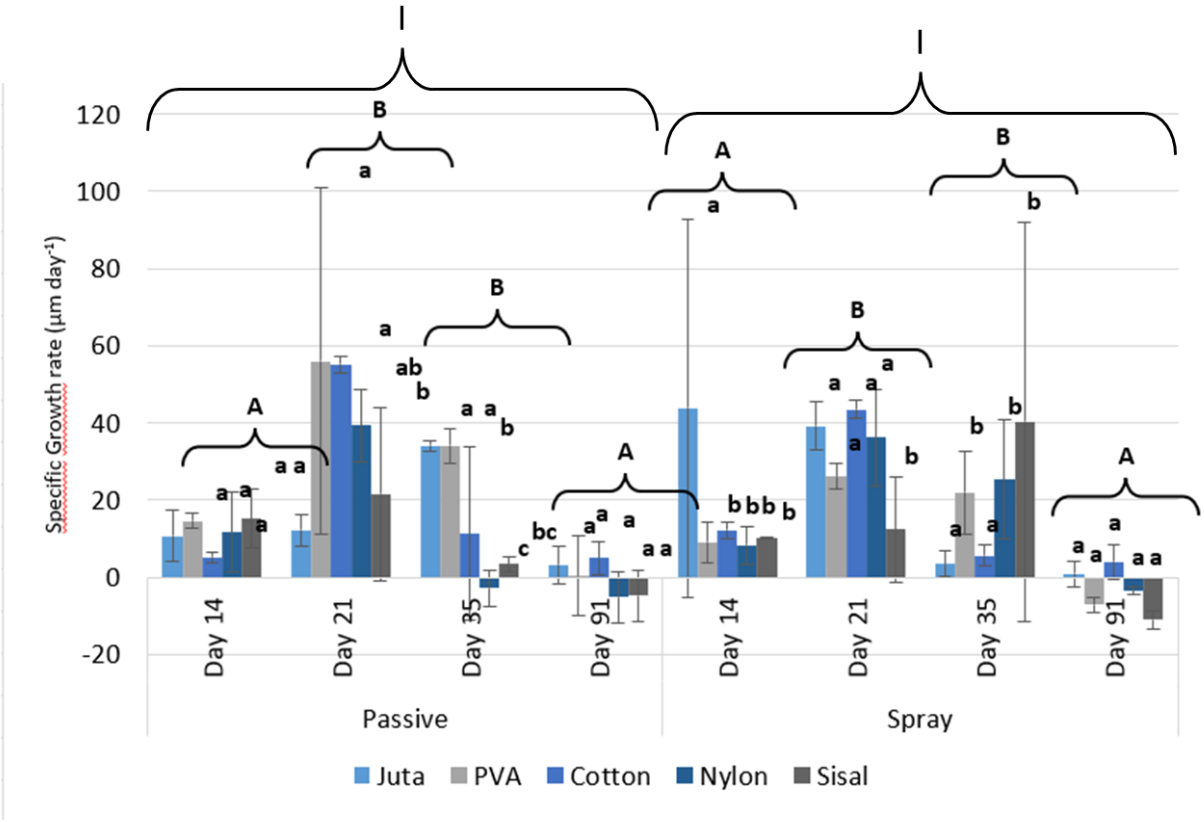

Figure 19 - Specific growth rates (SGR) of *C. decorticatum* fragments from the passive and spray methods (Mean ± SD). Within each seeding method, different capital letters show significant differences between SGR in the sampling days ($p<0.05$). Within each sampling day and seeding method, different lowercase letters show significant differences between SGR in the twines ($p<0.05$). Different roman numbers show significant differences between seeding methods ($p<0.05$).

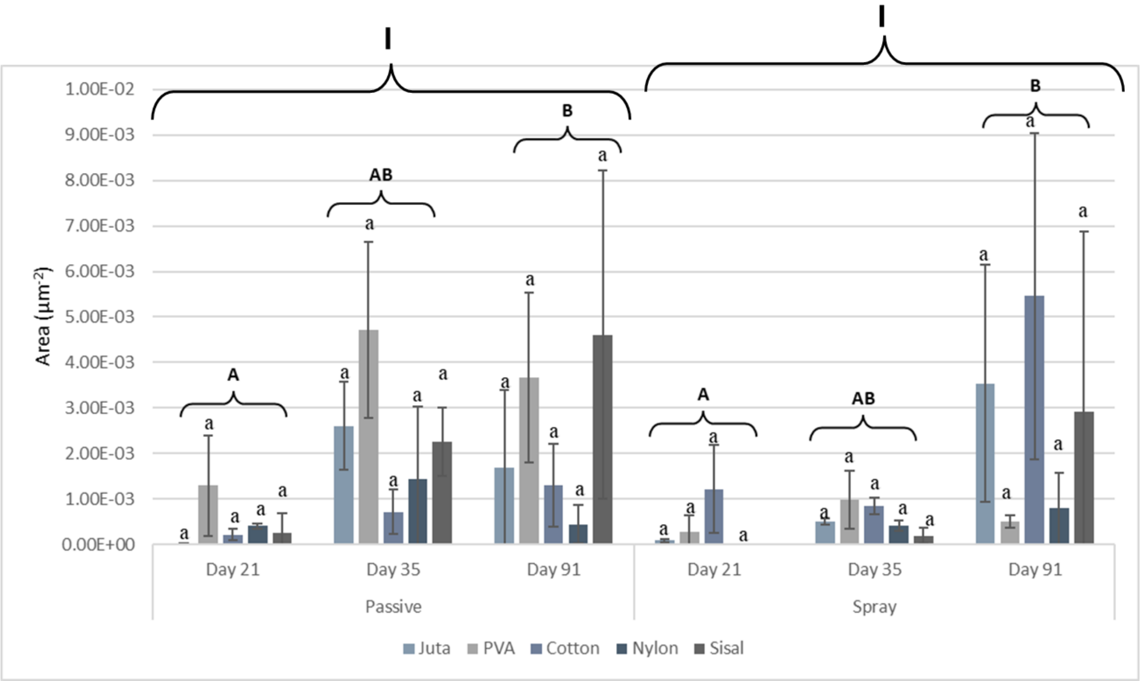

Figure 20 - Area of *C. decorticatum* fragments from the passive and spray methods (Mean ± SD). Within each seeding method, different capital letters show significant differences between areas in the sampling days ($p<0.05$). Within each sampling day and seeding method, different lowercase letters show significant differences between areas in the twines ($p<0.05$). Different roman numbers show significant differences between seeding methods ($p<0.05$).

Figure 21 - *C. decorticatum* after 91 days, culture form passive and spray method in seeding method with fragments, arrows indicate adult *Codium*.

3.4 Experiment 3 - Twine seeding with gametes

Experiment 3 aimed to identify the best substrate (PVA, cotton, or jute) for cultivating *Codium* sp. from gametes, using the ultrasound method for gamete release. Cultivation was maintained for six months and monitored weekly, after day 68, for length, and area.

Observations started at day 68. At that time, it was possible to observe filaments, with the third phase of development initiating at day 112 in all twines (figure 22). A comparison between the length of *Codium* in the different twines (figure 23) showed jute to have the longest individuals ($p<0.001$, mean=712.135µm). A comparison of length between weeks showed significant differences ($p<0.001$), indicating a clear increase in growth over time (figure 23).

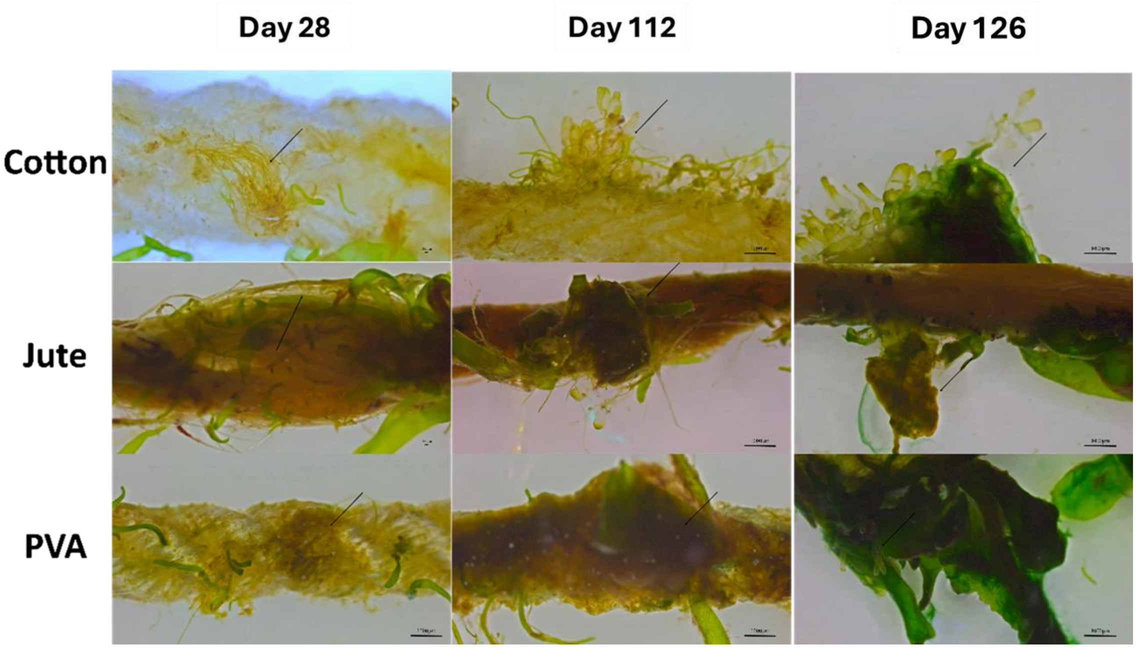

Figure 22 - Development of *Codium* from seeding method with gametes in the different twine, on days 28, 112, and 126. Arrows indicate *Codium*.

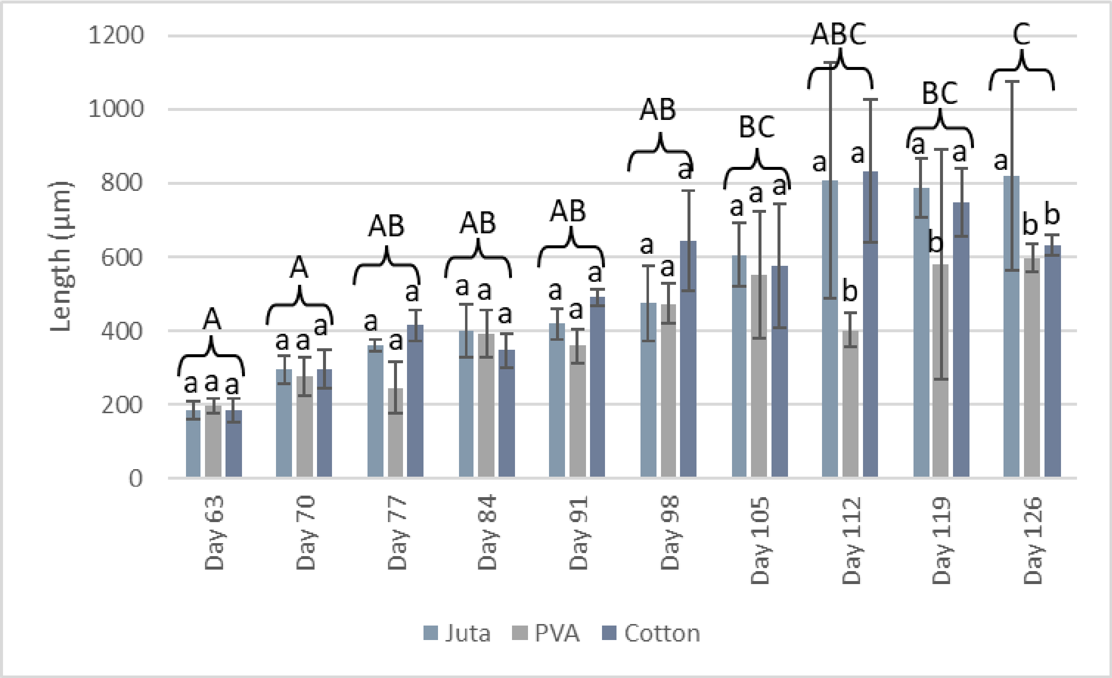

Figure 23 - Length of *Codium* sp. fragments from the seeding with gametes (Mean ± SD). Different capital letters show significant differences between lengths in the sampling days (p<0.05). Within each sampling day, different lowercase letters show significant differences between length in the twines (p<0.05).

Area comparison showed no significant differences between sampling days (p=0.315). When compared to twine, the *Codium* area in cotton was significantly higher than in the other twines (p=0.031) (fig. 24).

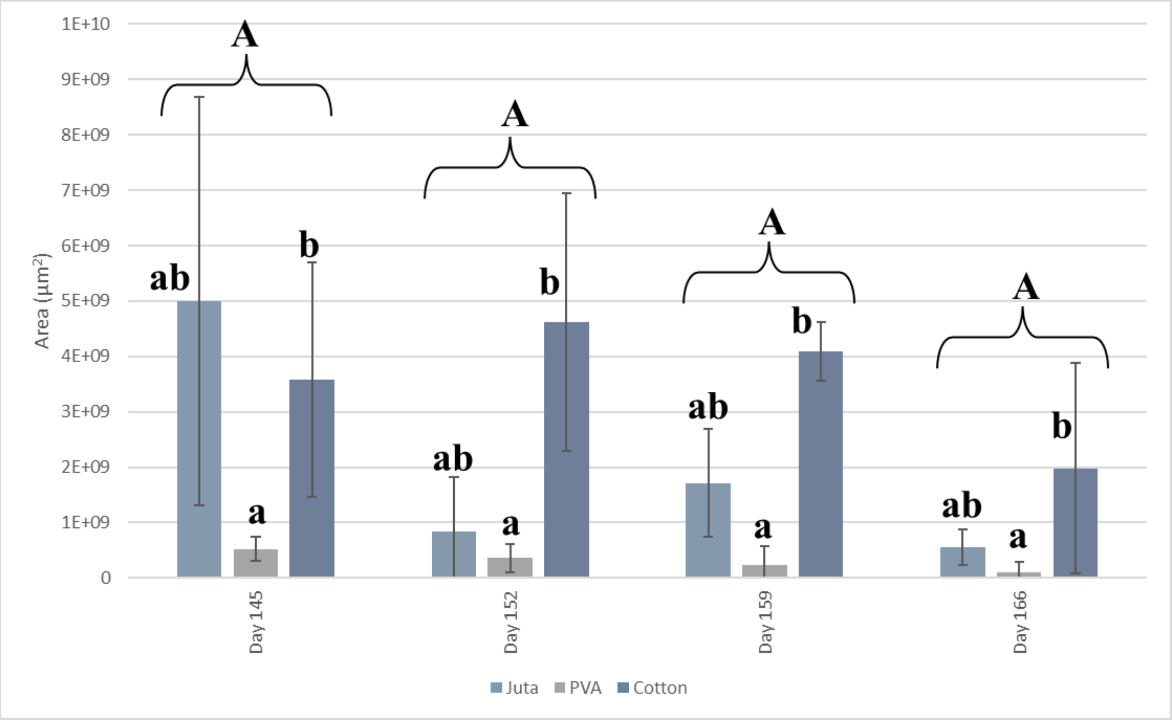

Figure 24 - Area of *Codium* sp. fragments from the seeding with gametes (Mean ± SD). Different capital letters show significant differences between areas in the sampling days (p<0.05). Within each sampling day, different lowercase letters show significant differences between areas in the twines (p<0.05).

3.5 Experiment 4 - Seeding density optimization

Experiment 4 aimed to determine the optimal seeding density of *Codium* by testing three biomass concentrations (low, medium, and high) on PVA, jute, and cotton twine using the passive seeding method, with weekly growth monitoring.

A comparison between the three twines revealed no significant differences in *Codium* length (1187.61µm±152.70µm and p=0.541 for cotton; 1387.49µm±218.18µm and 0.786 for jute; 1506.16µm±210.48µm and 0.566 for PVA) (figure 25) nor in specific growth rate (mean=5.27±1.67 and p=0.700 for cotton; 3.88±2.12 and 0.922 for jute; 5.88±2.64 and 0.726 for PVA) (figure 26). Comparing the three biomass concentrations revealed no significant difference between concentrations (figure 26).

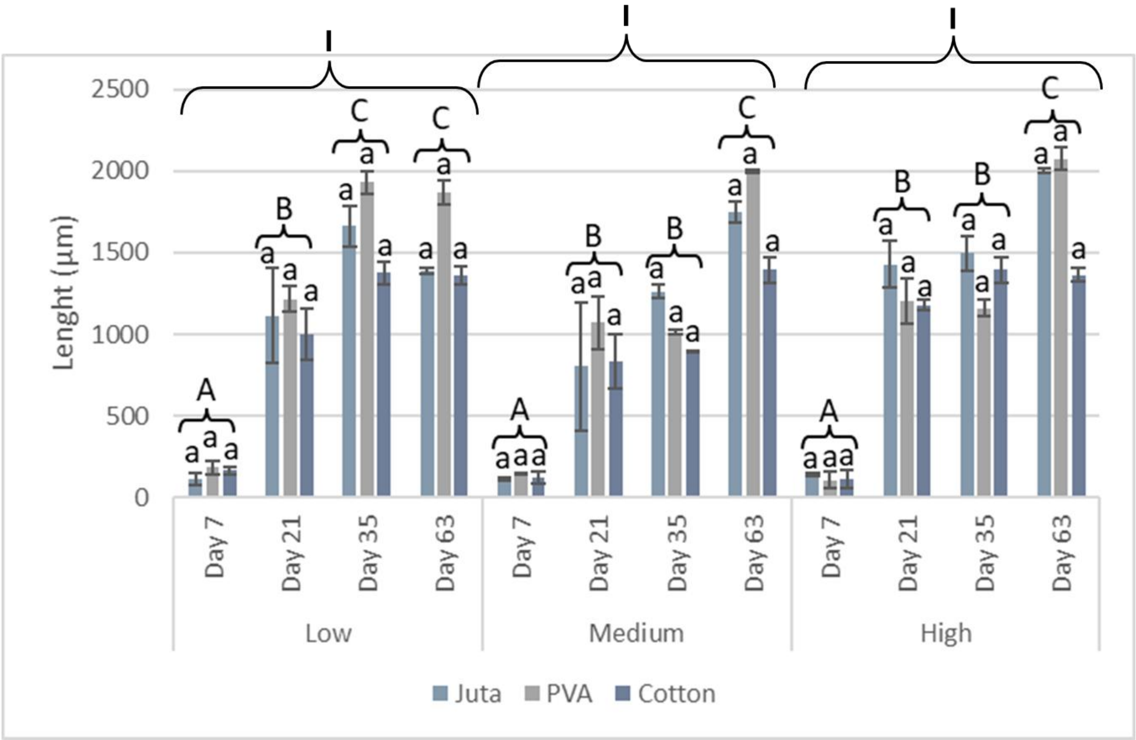

Figure 25 - Length of *C. decorticatum* fragments from the passive seeding method, using three *Codium* concentrations (Mean ± SD). Within each *Codium* concentrations, different capital letters show significant differences between lengths in the sampling days (p<0.05). Within each sampling day and *Codium* concentration, different lowercase letters show significant differences between lengths in the twines (p<0.05). Different roman numbers show significant differences between *Codium* concentrations (p<0.05).

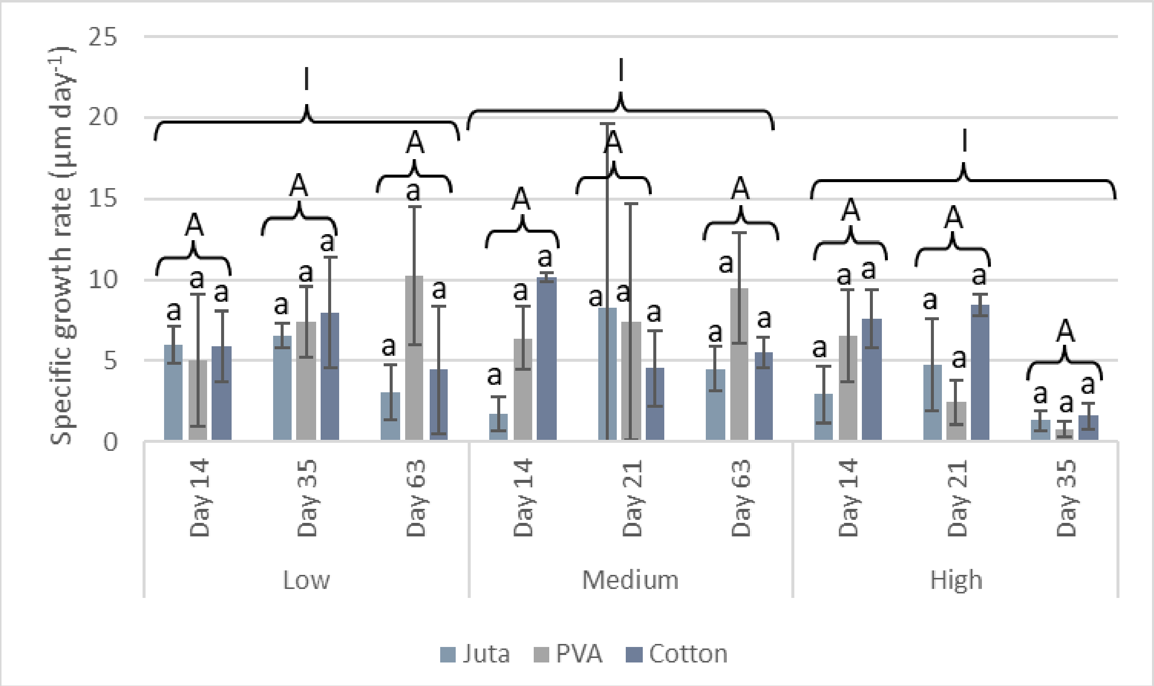

Figure 26 - Specific growth rate (SGR) of *Codium* fragments from the passive method in three different twines at three different *Codium* concentrations (Mean ± SD). Within each *Codium* concentration, different capital letters show significant differences between SGR in the sampling days ($p<0.05$). Within each sampling day and *Codium* concentration, different lowercase letters show significant differences between SGR in the twines ($p<0.05$). Different roman numbers show significant differences between *Codium* concentrations ($p<0.05$).

A comparison of the area showed no significant differences between biomass concentrations ($p=0.187$). When compared by sampling days, there is a significant difference between day 63 and days 21 and 35 for both treatments and twines ($p<0.001$), in which day 63 is higher (figure 27). Comparing the mean area for the three Codium concentrations within the twines, there is a significant difference between jute and cotton ($p=0.007$), with jute showing higher values (mean area=0.009 μm^2 at day 63) (figure 27).

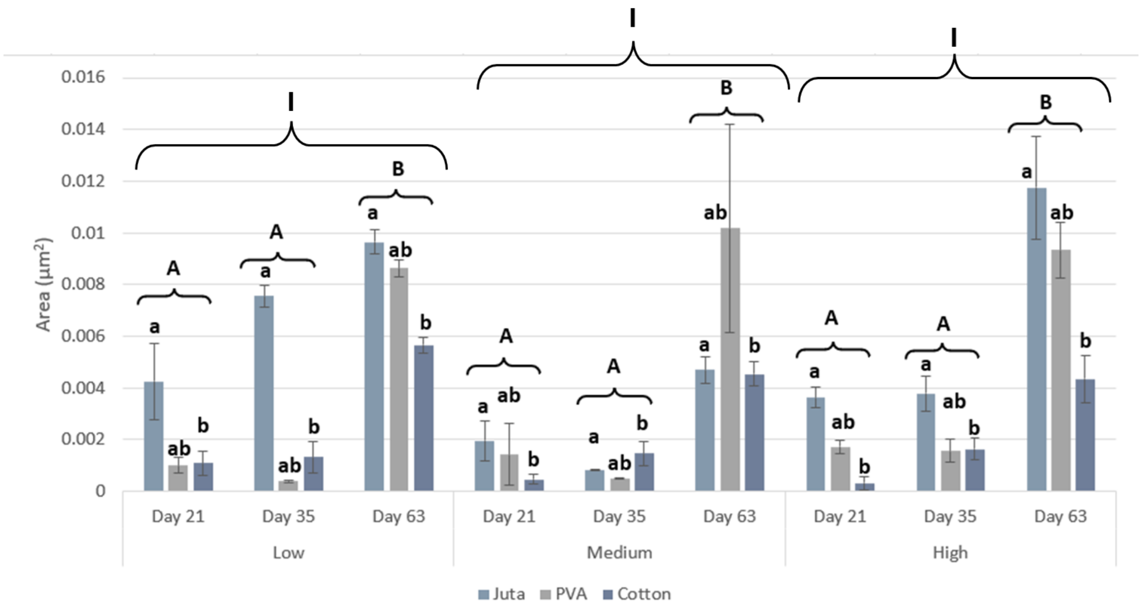

Figure 27 - Area of *Codium* fragments from the passive method in three different twines at three different *Codium* concentrations (Mean ± SD). Within each *Codium* concentration, different capital letters show significant differences between areas in the sampling days ($p<0.05$). Within each sampling day and *Codium* concentration, different lowercase letters show significant differences between areas in the twines ($p<0.05$). Different roman numbers show significant differences between *Codium* concentrations ($p<0.05$).

4. Discussion

The reproductive cycle of *C. decorticatum* exhibited significant seasonal variation, with gamete release fluctuating throughout the study period. A notable decline in female gamete density was observed between February and April, while male gamete release was lower between March and April. These findings suggest that these months are less favorable for gamete maturation, possibly due to suboptimal environmental conditions such as temperature and light availability. The observed reproductive trend aligns with the findings of Pacheco *et al.* (2022), who reported that *C. tomentosum* from the Northern Portuguese coast follows a distinct reproductive cycle, reaching peak maturity between August and March. The reduced reproductive activity observed for *C. decorticatum* between February and April on the South coast of Portugal reinforces the notion that seasonal shifts strongly influence *Codium* species. In contrast, May, June, and July exhibited significantly higher gamete release, indicating that these months likely provide optimal conditions for maturation and reproduction. Additionally, this study observed more frequent germination in February and March compared to later months, further supporting the idea that *C. decorticatum* experiences peak reproductive activity during cooler months. The overall decline in reproductive activity from April to July may reflect natural seasonal cycles, where fertility decreases as environmental conditions shift. The synchronization of increased gamete release and germination with specific environmental cues suggests an adaptive strategy to maximize reproductive success.

The gamete discharge process observed in this study highlights critical details of the reproductive biology of *C. decorticatum*. Male gametes were discharged with the presence of a slimy substance that facilitated their cohesive movement post-release. This phenomenon, also noted in the male gametes of other species such as *C. fragile* (Borden and Stein, 1969), may serve an adaptive function for gamete viability or aggregation to enhance fertilization efficiency. Notably, the slow, one-at-a-time discharge of male gametes from the gametangium and their initial immobility highlight a potential delay in activation. This behavior may be an adaptation to environmental triggers that optimize gamete motility and fertilization timing (Borden and Stein, 1969). The absence of observed real-time discharge from female gametangia underscores a gap in understanding the mechanics of female gamete release. However, the findings align with earlier descriptions by Borden and Stein (1969), who proposed that female gametes are expelled when the gametangium tip splits into two flaps. Further investigations with improved temporal resolution would be valuable to directly observe this process and confirm its occurrence in *C. decorticatum*. The presence of a slimy substance enveloping the gametes further supports its potential role in creating a favorable microenvironment for gamete survival and interaction.

The observed size differences between female and male gametes in *C. decorticatum* reflect distinct reproductive adaptations. Female gametes, measuring approximately 20 µm in length, are significantly larger than male gametes, which have a diameter of only 3 µm. This size disparity is consistent with findings from Borden and Stein (1969), who reported similar differences in gamete sizes for *C. fragile*. The larger size of female gametes likely reflects their role in reproduction, where they require more energy reserves for fertilization and early development. In contrast, the smaller male gametes may be adapted for efficient dispersal, allowing them to cover greater distances in the water column (Trowbridge *et al*., 2001). These size differences between female and male gametes may also influence their behavior during fertilization, with larger female gametes potentially acting as a more passive target for the smaller, motile male gametes (Prince *et al.,* 2004).

The comparison between the blend and ultrasound methods for *C. decorticatum* gamete release and cultivation revealed no significant differences in germination rates or specific growth rates. However, in the studies of Sá *et al.* (2024) and Pacheco *et al.* (2022), the ultrasound method was associated with a significantly higher density of gametes. These studies demonstrated significantly higher gamete yields when desiccation was combined with multiple ultrasound treatments. Despite the similarities in performance with the blend method, the higher levels of contamination observed in the blend method, predominantly resembling *Ulva* sp., suggest a practical limitation.

Nutrient concentration emerged as a more critical factor than the gamete release method for influencing germination and growth rates. Tailored nutrient regimes are vital for maximizing growth and ensuring the successful cultivation of *Codium* under different environmental and operational conditions.

The results of Experiment 2 provide valuable insights into the optimal twine substrates and settling methods for cultivating *Codium* fragments. The development of *Codium* from filaments was characterized by three distinct growth phases: initial independent filament growth, the establishment of interconnections and tissue differentiation, and the formation of fully organized individuals. This description aligns with the observations of González *et al.* for *Codium fragile* and *Codium Bernabei*. Natural fibers (sisal, jute, and cotton) and PVA twines supported a more rapid progression through these phases than nylon, highlighting their potential suitability as substrates for *Codium* cultivation. However, the eventual disintegration of sisal twine emphasizes the need for durable yet biodegradable materials to ensure sustainable cultivation. In experiment 3, the lack of significant differences in *Codium* length between twines (cotton, jute, and PVA) indicates that all tested twines can support comparable elongation rates under passive seeding conditions. Similarly, specific growth rates showed no significant variation between substrates, suggesting that substrate choice does not critically influence growth dynamics in this system.

This study didn't find significant differences in the length of *Codium* between settling methods and twine types within each sampling day, although significant differences were observed across sampling days. Similarly, no significant differences in specific growth rates were observed between twine types within each sampling date. These findings suggest that the choice of settling method and twine type may not be critical factors in determining the linear growth or specific growth rate of *Codium.* Instead, growth appears to be influenced more by cultivation conditions and cultivation time. This interpretation is consistent with previous studies, such as those by Trowbridge *et al*. (2001) and Hwang *et al*. (2008), which reported that seaweed growth rates are often more affected by factors like light, temperature, and other environmental variables than substrate characteristics. Additionally, when comparing the area occupied by *Codium*, no significant differences were found within seeding methods and twines. However, a significant difference in area was observed between day 21 and 91 for both methods, with day 91 showing a higher area. This indicates that while the choice of seeding method or twine may not significantly affect the area covered by *Codium*, time is a crucial factor in its expansion. It is important to note, however, that the lack of significant differences between methods and twine types could also be influenced by limitations or potential errors in the sampling methodology. Variability in sampling technique, sample size, or measurement accuracy may have affected the ability to detect subtle differences.

It was observed that morphological differences between the two methods: organisms grown using the passive method exhibited greater height and branching, resembling natural specimens, whereas those grown with the spray method displayed horizontal growth and distinct morphology. These results highlight how settling techniques can influence organism structure, which could be an important consideration depending on the intended application of the cultivated biomass.

The findings from experiment 3 show the influence of substrate type on the cultivation of *Codium decorticatum* from gametes, using the ultrasound method for gamete release. The observations revealed the early stages of filament development, with the third phase of development evident by day 112 across all substrates. This progression aligns with the typical growth patterns observed in *Codium* species, where initial independent filament growth transitions into a more organized structure (Bégin *et al.* 2003).

The significant differences in length among substrates, with cotton supporting the longest individuals, suggest that cotton provides a more conducive environment for elongation compared to PVA and jute. The preference for cotton may be attributed to its hydrophilic properties and ability to retain moisture, which is beneficial for seaweed cultivation (Fibre2Fashion, 2013).

The week-by-week analysis revealed significant differences in length over time, indicating steady growth progression throughout the cultivation period. This consistent elongation is characteristic of *Codium* species under favorable cultivation conditions (Sá *et al.* 2024). However, the lack of significant differences in area across sampling days suggests that the filament elongation occurred consistently.

The cultivation of *Codium* species presents diverse strategies for propagation, broadly categorized into asexual (vegetative) and sexual (gamete-based) seeding methods, each with distinct implications for growth dynamics and overall productivity. Asexual propagation, such as the passive and spray methods explored in Experiment 2, relies on the direct transfer of *Codium* fragments onto substrates. These methods bypass the gametogenesis and fertilization stages, allowing for potentially faster biomass accumulation in the short term. In contrast, sexual propagation, as utilized in Experiment 3 with ultrasound-released gametes, involves initiating cultures from spores, necessitating successful gamete fusion and subsequent development into mature thalli (Hoyle, 1978). Importantly, sexual propagation enables the introduction of genetic variation within cultivated populations, as offspring inherit a combination of genetic material from both parent gametes (Friedlander, 2018). This genetic diversity can be harnessed for selective breeding, allowing cultivators to choose for desirable traits such as enhanced growth rates, improved stress tolerance, or specific biochemical profiles (Valero *et al.,* 2017). The comparative analysis highlights the importance of considering both methods in *Codium* cultivation. Asexual methods offer simplicity and rapid establishment, sexual methods introduce diversity and potentially greater long-term resilience.

The results from Experiment 4 provide valuable insights into the effects of seeding density and substrate type on the growth of *Codium* using the passive seeding method. While no significant differences were observed in length or specific growth rates (SGR) across the three twines (cotton, jute, and PVA), variations in performance related to area measurements highlight important considerations for optimizing cultivation practices.

Biomass concentration significantly impacted lenght, with high-density seeding producing higher growth rates than low-density conditions. This aligns with findings in cell culture systems, where higher seeding densities enhance proliferation and resource efficiency through localized nutrient recycling and structural support (Hwang *et al.* 2008). For *Codium*, denser mats may create microenvironments that retain moisture or reduce shear stress, indirectly promoting growth.

Area measurements revealed distinctions between twines and sampling days. By day 63, jute demonstrated superior area values to cotton, suggesting its texture or chemical properties may enhance lateral expansion. This observation parallels studies on *Porphyra* cultivation, where substrate roughness and filamentous structure improved spore adhesion and radial growth (Ribeiro *et al.,* 2023). While hydrophilic substrates like cotton are often prioritized for vertical growth, jute's performance in area metrics highlights the importance of substrate texture in biomass spread, a critical factor for commercial yield optimization.

5. Conclusion

The comparison of gamete release induction methods revealed that ultrasound results in reduced contamination compared to the blend method. Nutrient concentration emerged as a more critical factor for influencing germination and growth rates, highlighting the need for tailored nutrient regimes to maximize *Codium* biomass production.

Our investigation into substrate types and settling methods highlighted the trade-offs between rapid establishment and long-term adaptability. Asexual propagation methods, such as passive and spray seeding, allowed for faster biomass accumulation. The selection of appropriate twine substrates further optimized growth outcomes.

These findings contribute valuable insights into the cultivation of *Codium decorticatum*. The optimal integration of gamete release methods, substrate selection, settling techniques, and seeding densities holds the key to unlocking the full potential of *Codium* for ecological restoration and commercial applications. Future research should focus on developing biodegradable twines, testing cotton and jute in offshore conditions, as well as exploring the synergistic effects of tailored nutrient regimes and environmental cues on *Codium* growth and development. It is essential to conduct an experiment to minimize contamination to safeguard the quality and safety of the cultivated biomass.

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