Biostimulant Activity of Microalgae and Cyanobacteria for Agricultural Purposes

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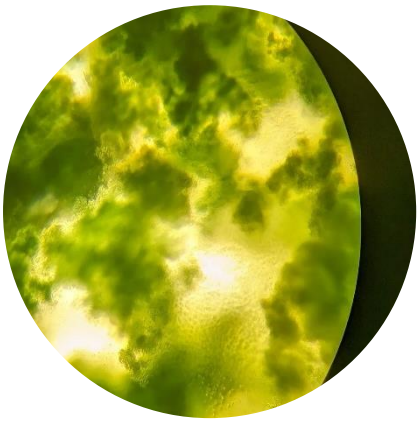

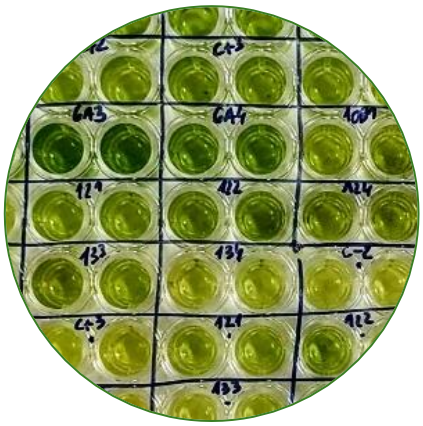

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Internship Report carried out as part of the Master in Applications in Biotechnology and Synthetic Biology

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“Study hard, my son,

Because while a doctor can do what a farmer does,

A farmer cannot be a doctor.”

You were right, Dad.

Acknowledgments

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Resumo

Atualmente, o sector agrícola enfrenta desafios significativos para satisfazer a procura crescente de alimentos a nível mundial e, ao mesmo tempo, enfrentar os impactos negativos das alterações climáticas, como a salinidade do solo. Os fertilizantes químicos têm sido utilizados para aumentar a fertilidade do solo e o rendimento das culturas; no entanto, esta prática tem tido efeitos adversos no ambiente e na saúde humana. Os bioestimulantes, um novo produto biotecnológico sustentável, foram desenvolvidos para aumentar o rendimento das culturas, melhorar a qualidade dos produtos alimentares e reforçar a tolerância aos fatores de stress abióticos. As microalgas e as cianobactérias são fontes importantes de compostos bioativos que podem ser utilizados para desenvolver bioestimulantes. O objetivo deste projeto foi desenvolver uma formulação bioestimulante eficaz utilizando extratos aquosos de microalgas e cianobactérias para uso agrícola. Extratos de 27 estirpes da coleção privada da empresa foram preparados por sonicação e avaliados quanto à sua atividade bioestimulante em ensaios de germinação de sementes. As estirpes mais promissoras foram selecionadas para testes em plantas de tomate e alface a uma concentração de 0,5 g/L (peso seco) para avaliar os seus efeitos promotores do crescimento das plantas. Além disso, foi avaliada a capacidade dos extratos para atenuar o efeito do stress salino nas sementes de tomate e nas plantas de tomate e alface. As formulações bioestimulantes foram desenvolvidas com 0,5 g/L de extrato aquoso de biomassa e testadas em ensaios de germinação de sementes e crescimento de plantas em condições padrão. Os resultados demonstraram que várias estirpes, particularmente S004, S009, S011 e S012, afetaram positivamente a germinação das sementes. Nas avaliações no solo, os extratos aquosos destas estirpes revelaram melhorias encorajadoras no crescimento das plantas de tomate e alface, bem como concentrações mais elevadas de pigmentos fotossintéticos. Sob stress salino, as plantas mostraram uma redução da germinação das sementes, das caraterísticas morfológicas e do conteúdo de pigmentos em comparação com os controlos. No entanto, as estirpes S011 e S012 inverteram parcialmente estes efeitos, sobretudo a concentrações de 3 g/L de NaCl. As formulações apresentaram efeitos fitotóxicos, indicando que é necessária uma maior otimização. Além disso, foram realizadas várias atividades empresariais durante o estágio, incluindo desenvolvimento de negócios, gestão de redes sociais, participação em eventos, participação em programas de financiamento e estudos sobre as necessidades dos clientes

Palavras-chave: Microalgas, Cianobactérias, Bioestimulantes, Agricultura, Stress Salino, Extração de Compostos Bioativos, Empreendedorismo

Abstract

Nowadays, the agricultural sector is facing significant challenges in meeting the rising global demand for food while simultaneously addressing the detrimental impacts of climate change, such as soil salinity. Chemical fertilizers have long been used to boost soil fertility and crop yields; however, this practice has led to adverse effects on the environment and human health. Biostimulants, a new sustainable biotechnological product, are being developed to boost crop yields, improve food product quality, and strengthen tolerance to abiotic stressors. Microalgae and cyanobacteria are important sources of bioactive compounds that can be used to develop biostimulants. The objective of this project was to develop an effective biostimulant formulation using aqueous extracts of microalgae and cyanobacteria for agricultural use. Extracts from 27 strains from the company's private collection were prepared through sonication and evaluated for their biostimulant activity in seed germination assays. The most promising strains were selected for testing in tomato and lettuce plants at a concentration of 0.5 g/L DW to assess their plant growth-promoting effects. Additionally, the ability of the extracts to mitigate salt stress in tomato seeds and tomato and lettuce plants was evaluated. Biostimulant formulations were developed with 0.5 g/L of biomass aqueous extract and tested in both seed germination and plant growth assays under standard conditions. Results demonstrated that several strains, particularly S004, S009, S011, and S012, positively affected seed germination. In soil evaluations, aqueous extracts of these strains exhibited encouraging growth improvements in tomato and lettuce plants, as well as higher concentrations of photosynthetic pigments. Under salt stress, plants showed reduced seed germination, morphological traits, and pigment content compared to controls. However, S011 and S012 strains partially reversed these effects, especially at 3 g/L NaCl concentrations. The formulations exhibited phytotoxic effects, indicating that further optimization is needed. Additionally, several entrepreneurial activities were undertaken during the internship, including business development, social media management, event attendance, funding program participation, and customer needs studies.

Keywords: Microalgae, Cyanobacteria, Biostimulants, Agriculture, Salt Stress, Bioactive Compounds Extraction, Entrepreneurship

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3. List of Abbreviations

Abs	Absorbance
ANOVA	Analysis of Variance
ASE	Accelerated Solvent Extractor
BBEH	Blue Biotechnology, Environment and Health
BSA	Bovine Serum Albumin
CAGR	Compound Annual Growth Rate
CEO	Chief Executive Officer
CIIMAR	Interdisciplinary Centre of Marine and Environmental Research
CNP	Cyanobacterial Natural Products
CSO	Chief Scientific Officer
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
DTT	Dithiothreitol
DW	Dry Weight
EDTA	Ethylenediaminetetraacetic Acid
EU	European Union
FBCC	Fykia Biotech Culture Collection
FW	Fresh Weight
gDNA	Genomic Deoxyribonucleic Acid
GI	Germination Index
GS	Glutamine Synthetase
hpa	13^2–hydroxypheophytin a
IAA	Indole-3-Acetic Acid
IPA	Indole-3-Propionic Acid
LEGE-CC	Blue Biotechnology and Ecotoxicology Culture Collection
NAD	Nicotinamide Adenine Dinucleotide
NPK	Nitrogen, Phosphorus, and Potassium

PBS	Phosphate-Buffered Saline Buffer
PCR	Polymerase Chain Reaction
PGPR	Plant Growth Promoting Rhizobacteria
PI	Principal Investigator
PPFD	Photosynthetic Photon Flux Density
RRP	Recovery and Resilience Plan
PVPP	Polyvinylpolypyrrolidone
ROS	Reactive Oxygen Species
rRNA	Ribosomal Ribonucleic Acid
SD	Standard Deviation
SDGs	Sustainable Development Goals
USA	United States of America
UN	United Nations
UV	Ultraviolet Radiation

Chapter 1

Screening of Microalgal and Cyanobacterial Strains for their Potential as Biostimulants for Agricultural Applications

1. Introduction

1.1. Global Agriculture Situation

1.1.1. Growing Population

The agricultural sector plays a pivotal role in global food security, serving as a primary source of food production. Approximately 40% of the world's territory is required to produce sufficient food to feed the current global population of 8 billion (Cardarelli et al., 2022). In recent times, the agro-industries have been facing relentless pressure to raise their food productivity, in part due to the rapid population growth. By 2050, the global population is projected to reach 9.7 billion (Anderson et al., 2020). Such a populational expansion will require an estimated increase of 60% in global crop production to attain a satisfactory level of food security (Chabili et al., 2024; Malhi et al., 2021; Mutale-Joan et al., 2022; Soumare et al., 2020). However, the available arable land is expected to increase only at a negligible rate of 0.10%, growing from 1,592 million hectares between 2005 and 2007 to 1,661 million hectares by 2050 (Parmar et al., 2023). Furthermore, the expansion of agricultural lands endangers biodiversity, particularly wildlife and plant species (Cristofano et al., 2021). Therefore, although it is crucial to increase the production of foodstuff, expanding cultivation areas is not a viable option (Santini et al., 2021).

1.1.2. Climate Change

The reality of climate change further compounds the complexity of this challenge. Precipitation anomalies, extreme temperatures, weather-related disasters such as droughts and floods, soil degradation, insect pest infestation, and plant diseases are all aggravated by climate change, which has a deleterious effect on agricultural production (Malhi et al., 2021; Mutale-Joan et al., 2022; Parmar et al., 2023). Agriculture is also a major contributor to global warming. The agricultural sector consumes significant amounts of resources, such as water and energy, and is responsible for 15% of the total global emissions, mainly in the form of methane and nitrous oxides (Cardarelli et al., 2022; Cristofano et al., 2021; Malhi et al., 2021).

The adverse cascading effects of climate change hinder plant growth and development, as well as crop quality, by causing abiotic and biotic stresses (Gedeon et al., 2022). As a result, crop yields, food production, and availability are inevitably reduced

(Chaudhry & Sidhu, 2022; Gedeon et al., 2022; Mutale-Joan et al., 2022). The agricultural industry is especially vulnerable to climate change due to its significant dependence on weather and temperature conditions, which results in considerable economic losses and social impacts, such as food shortages and famines, that threaten global food security (Corwin, 2020; Malhi et al., 2021; Mutale-Joan et al., 2022).

1.1.3. Salt Stress

The global agroecosystem is facing significant challenges due to extreme climate change, with soil health being particularly affected by erosion and salinization (Corwin, 2020). Soil salinity is a growing environmental concern, especially in arid, semi-arid and coastal regions (Krid et al., 2024; Sorrentino et al., 2021). It is considered one of the most significant abiotic stressors for plants, constraining their growth and development, and ultimately affecting crop yield (Chaudhry & Sidhu, 2022; Corwin, 2020; Sorrentino et al., 2021). Currently, approximately 20% of global cultivated lands and 33% of irrigated areas are affected by salinity in their soil, which translates to approximately 800 million hectares globally (Krid et al., 2024; Mutale-Joan et al., 2022). This number is expected to increase at a faster rate in the next decades due in part to climate change (Campobenedetto et al., 2021; Chanthini et al., 2022; Krid et al., 2024; Mutale-joan et al., 2021; Sorrentino et al., 2021).

Salt stress is characterized by the excessive presence of mineral salts in soil, comprising cations (Na^+, K^+, Mg^{2+}, and Ca^{2+}) and anions (HCO_3^-, Cl^-, NO_3^-, SO_4^{2-}, and CO_3^{2-}) (Chaudhry & Sidhu, 2022; Corwin, 2020). Soil salinization can result from either natural or anthropogenic causes. Natural causes such as the addition of salts from precipitation or the intrusion of saline groundwater and saltwater from the sea can be contributors for soil salinization. As for anthropogenic factors, it includes inappropriate irrigation practices, overuse of fertilizers and pesticides and the improper disposal of municipal and industry wastewaters (Corwin, 2020; Krid et al., 2024).

The vast majority of cultivated plants are glycophytes, exhibiting a diminished capacity to absorb water at NaCl concentrations of approximately 40 mM and above. Beyond 100-200 mM NaCl, these plants are unable to withstand soil salinities (Carillo et al., 2020; Chaudhry & Sidhu, 2022). Salinity stress induces both osmotic stress and ionic toxicity effects on plants, which hamper severely their germination, growth, photosynthesis activity and productivity (Chaudhry & Sidhu, 2022; Krid et al., 2024). The initial effect is a short-term one that occurs directly in the rooting zone. The

accumulations of such salts can limit water uptake from the soil by reducing the plant's osmotic potential (Chaudhry & Sidhu, 2022; Corwin, 2020; Sorrentino et al., 2021). A reduction in water uptake results in a decrease in stomatal aperture in plants, disrupting the normal transpiration rate (Carillo et al., 2020; Gedeon et al., 2022).

Moreover, high salinity levels also lead to long-term harmful effects due to ion-specific toxicity (Chaudhry & Sidhu, 2022; Corwin, 2020). In fact, elevated levels of salt concentrations in the soil can cause an excessive accumulation of Na^+ and Cl^- ions in the cytosol in disregard of other essential mineral salts such as K^+ and Ca^{2+}, thereby causing a nutritional imbalance (Gedeon et al., 2022; Krid et al., 2024; Mutale-joan et al., 2021). The absorption of an excessive quantity of Na^+ cations, for instance, causes an accumulation of these ions within the plant. This leads to membrane instability and permeability due to the substitution of Ca^{2+} ions and compromises important enzymatic reactions due to the displacement of K^+ ions, which jeopardizes protein synthesis and photosynthetic processes (Carillo et al., 2020).

Furthermore, an overproduction of reactive oxygen species (ROS) like hydroxyl radical (•OH), hydrogen peroxide (H_2O_2), superoxide radical (O_2•) and alkoxy radical (RO•) results from this ionic imbalance. This, in turn, causes oxidative damages to the cellular macromolecules such as proteins, lipids and nucleic acids (Krid et al., 2024; Mutale-joan et al., 2021; Parmar et al., 2023). Normal cellular processes such as photosynthesis and membrane structure are compromised due to chlorophyll breakdown and lipid peroxidation, respectively, both of which are induced by oxidative stress (Parmar et al., 2023). The final result of salt exposition is a reduction in plant growth and crop yields due to the alterations in plant metabolism by disrupting namely cellular respiration and biochemical pathways such as protein biosynthesis (Krid et al., 2024; Parmar et al., 2023). This can lead to chlorosis and even necrosis, ultimately culminating in the death of the plant (Chaudhry & Sidhu, 2022; Mutale-joan et al., 2021).

1.1.4. Chemical Fertilizers

Over the years, both farmers and farm companies have adopted agrochemicals such as synthetic fertilizers as a tool to improve the nutrient availability in the soil and thus enhance their crop yield (Braun & Colla, 2022; Mutale-Joan et al., 2022). Chemical fertilizers are synthesized industrially with a controlled quantity of nitrogen, phosphorus and potassium (NPK) (Bhardwaj, 2014). These inorganic fertilizers were only an interim solution as their overuse exacerbated long-term productivity issues by destroying the

natural and beneficial soil microflora and physicochemical properties, ironically leading to a counter-productive effect (Bhuyan et al., 2022; Chabili et al., 2024).

The soil plays a significant role in the plant, as it is the substrate from which it can absorb the needed nutrients. These nutrients need to be replenished for an arable land to be sustainable (Kholssi et al., 2022). Fertilizers degrade the soil through key soil nutrients leaching, resulting in a reduction in soil fertility, replacing them only with nitrogen, potassium and phosphorus (Bhuyan et al., 2022). However, only 17 and 20 percent of the applied nitrogen and phosphorus, respectively, are absorbed by the plants since when added to the soil, they complex with organic matter, compromising their assimilation by the plants (Prisa & Spagnuolo, 2023). Nutrient leaching, such as nitrates and phosphates, that compose the inorganic fertilizers, can contaminate surface waters and groundwaters and cause eutrophication, which leads to a loss of aquatic biodiversity (Parmar et al., 2023; Vassilev et al., 2015). Soil acidification is another consequence of the intensive application of chemical fertilizers in arable lands due to the loss of alkaline cations by ion exchange and ammonium nitrification by plants (Braun & Colla, 2022; Vassilev et al., 2015).

Nitrogenous chemical fertilizers are also responsible for the emission of greenhouse gases such as CO_2 that pollute the air to their industrial production from fossil feedstocks (Dagnaisser et al., 2022; Malhi et al., 2021; Suganya et al., 2016). Some studies correlated the consumption of food produced with chemical fertilizers and the use of water from contaminated water supplies to health hazards (Bhuyan et al., 2022; Mutale-Joan et al., 2022; Ochoa-Velasco et al., 2016). In addition to their toxic nature, fertilizers are becoming increasingly expensive due to the decline of fossil fuel reserves (Parmar et al., 2023). Therefore, inorganic fertilizers are unsustainable from a productive, food quality, health safety and environmental standpoint (Braun & Colla, 2022).

1.1.5. Organic Agriculture

The standards by which consumers evaluate products are undergoing a transformation. There has been a notable increase in the demand over the past few years for organically cultivated food, defined as food sourced from cultivation practices that do not rely on the application of artificial fertilizers or pesticides (Cao et al., 2023; Parmar et al., 2023). Organic farming makes use of strategies such as crop rotation and the incorporation of organic waste to enhance crop productivity while minimizing the

impact on soil health (Kumari et al., 2011). This general consumer interest emerges from the concern regarding the safety, nutritional value, and sustainability of food products (Coppens et al., 2015; Gundala & Singh, 2021; Iqbal et al., 2021; Parmar et al., 2023). Nevertheless, despite its eco-friendly nature, organic cultivation alone is not currently a sufficiently efficient to meet the substantial demand for food. Crops cultivated that way are more susceptible to abiotic stresses and receive fewer nutrients compared to those grown conventionally, which often results in yield losses of 20% (Santini et al., 2021).

1.1.6. Biofertilizers and Biostimulants

Biofertilizers and biostimulants are a new and promising biotechnological solution to ensure efficient and sustainable food production while keeping the environment in good condition. They are a cost-effective alternative to synthetic fertilizers and can be used to boost the low yields of organic farming (Ronga et al., 2019). Although the exact definition is still ambiguous, biofertilizers are generally considered as substances composed of beneficial living microorganisms, such as bacteria, fungi, microalgae and cyanobacteria, that promote plant growth and development and restore soil quality by colonizing the plant rhizosphere and its interior (Kapoore et al., 2021; Nosheen et al., 2021; Parmar et al., 2023). Such microorganisms generally constitute the microbiome of plant roots under natural conditions. These microbial inoculants can be applied either directly to seeds, as well as to plant leaves or soil (Nosheen et al., 2021). Based on the characteristics of the microorganisms, biofertilizers can act as a) N-fixing agents, b) potassium and phosphate mobilizing agents, and c) potassium and phosphate solubilizing agents, facilitating the uptake of these nutrients and other minerals by plants (Bhardwaj, 2014; Parmar et al., 2023; Prisa & Spagnuolo, 2023).

On the other hand, the scientific community tends to distinguish biofertilizers from biostimulants as the latter are not directly involved in nutrient delivery, nor do they improve soil fertility (Kapoore et al., 2021; Prisa & Spagnuolo, 2023). Plant biostimulants are defined under the European Regulation (EU) 2019/1009 as “a product stimulating plant nutrition processes independently of the product’s nutrient content with the sole aim of improving one or more of the following characteristics of the plant or the plant rhizosphere: a) nutrient use efficiency, b) tolerance to abiotic stress, c) quality traits, d) availability of confined nutrients in soil or rhizosphere.” (EU, 2019). Plant biostimulants exhibit effects that lie between those of plant protection products and fertilizers (Ronga et al., 2019).

Table 1. Main differences between biofertilizers and biostimulants according to the scientific bibliography.

	Biofertilizer	Biostimulant	Sources
Active Agent	Living microorganisms generally	Microalgal or cyanobacterial extracts generally	(Braun & Colla, 2022; Kapoore et al., 2021; La Bella et al., 2022; Parađiković et al., 2018; Ronga et al., 2019)
Main Functions	Improve soil quality and restore its fertility, which in turn enhance plant growth and development	Improve crop growth, development, yields, quality, protection against biotic and abiotic stresses, nutrient uptake and food shelf life directly affecting the plant physiology	(Braun & Colla, 2022; Prisa & Spagnuolo, 2023; Ronga et al., 2019)
Main Target	Soil and indirectly crops (plants, seeds, or root environment) and soil microbiome	Crop (plants, seeds or root environment) and soil microbiome	(Braun & Colla, 2022; Parađiković et al., 2018; Prisa & Spagnuolo, 2023)
Main Composition	Macronutrients, especially NPK elements	Biomolecules such as signaling molecules and metabolites	(Braun & Colla, 2022; Carillo et al., 2020)
Application Dose	Applied in larger doses	Applied in lower doses	(Kapoore et al., 2021; Prisa & Spagnuolo, 2023; Ronga et al., 2019)

In fact, the main function of biostimulants is to complement the macro- and micronutrient uptake of crops, and thus their yield and quality, as well as to strengthen their stress tolerance through the action of bioactive compounds that stimulate the production of plant growth regulators, phytohormones and stress-averting antioxidants (Chabili et al., 2024; Cristofano et al., 2021; Parađiković et al., 2018; Ronga et al., 2019; Rouphael & Colla, 2020; Santini et al., 2021). These compounds can be obtained from both organic and inorganic sources such as a) humic substances (humic acid, fulvic acids and humins) b) silicon, c) plant and animal-based protein hydrolysates (signal peptides and free amino acids), d) plant growth-promoting rhizobacteria (PGPR), e) arbuscular mycorrhizal fungi and f) macro- and microalgal extracts (Chabili et al., 2024; Kapoore et al., 2021; Prisa & Spagnuolo, 2023; Rouphael & Colla, 2020).

Biostimulant mechanisms of action can be directly correlated to physicochemical alterations in plants. Bioactive compounds from microorganisms, when absorbed by the plant through the leaves or the roots, can upregulate key genes involved in the primary and secondary metabolic pathways for the synthesis of beneficial molecules and the modulation of the release of phytohormones, which will enable an increased nutrient uptake, upgraded photosynthetic performance, improved antioxidant activities and

stimulation of chlorophyll and carotenoids synthesis (Chabili et al., 2024; Colla & Rouphael, 2020; Prisa & Spagnuolo, 2023; Righini et al., 2022). The increase of nutrient uptake leads to a stimulation of the nitrogen and carbon metabolism which in turn enhances the protein, carbohydrate, and photosynthetic pigments content in the leaves (Prisa & Spagnuolo, 2023).

The soil structure is also a target of biostimulants since they can increase the activity of some plant-beneficial microorganisms such as rhizobacteria and mycorrhizae due to the release of organic compounds such as extracellular polysaccharides and also through the rearrangement of the root system architecture that allows a more efficient nutrient and water uptake by the plants (Colla & Rouphael, 2020; Kapoore et al., 2021; Renuka et al., 2018).

Furthermore, biostimulants have a distinctive role in mitigating the effects of some abiotic stressors, including salt stress, through the activation of the plant's secondary metabolism, which includes plant defense molecules (Kapoore et al., 2021; Parmar et al., 2023). Plants have evolved defense mechanisms to cope with osmotic stress and oxidative damage caused by abiotic stressors, including *de novo* synthesis and regulation of phytohormones, production of heat shock proteins, buildup of osmolytes and an increase in the activity of enzymatic antioxidants (Parmar et al., 2023; Prisa & Spagnuolo, 2023). Biostimulants activate and improve the plant immune system in order to tackle the abiotic threats more promptly (Parmar et al., 2023).

1.2. Microalgae and Cyanobacteria

Microalgae and cyanobacteria are unicellular or multicellular photoautotrophic microorganisms that live in either free-living, colonial or filamentous form (Ronga et al., 2019). They are found in a wide range of terrestrial and aquatic ecosystems (marine and freshwater) and exhibit high diversity in terms of morphology, metabolism and physiology (Bello et al., 2021; Braun & Colla, 2022; Mutale-Joan et al., 2022; Righini et al., 2022). Although microalgae are eukaryotic microorganisms and cyanobacteria are prokaryotic organisms, both can use CO_2 and radiation energy to produce a wide variety of primary and secondary metabolites (Bello et al., 2021; Braun & Colla, 2022; Dagnaisser et al., 2022; Kapoore et al., 2021). The great diversity of secondary metabolites is the result of the evolution and adaptation of these microorganisms over the years to survive and

proliferate in extreme environments, such as saline, cold or hot habitats, acidic or alkaline soils or nutrient-deficient environments (Carillo et al., 2020; Renuka et al., 2018; Righini et al., 2022). Such metabolites are used for their own protection against the abiotic stresses to which they are exposed on a daily basis (Carillo et al., 2020).

These microrganisms are natural colonizers of the phytobiome and confer the plant growth promotion and biotic and abiotic stress alleviation effects through the release of beneficial biomolecules, including polysaccharides, phytohormone-like compounds, soluble amino acids, phenolic compounds, terpenoids and N-fixing enzymes (Chabili et al., 2024; Kholssi et al., 2022; Lee & Ryu, 2021; Parmar et al., 2023). Many research groups are currently researching new biostimulants derived from microalgae and cyanobacteria, as their metabolic flexibility, abundance of diverse bioactive compounds, and long-established beneficial relationship with plants make them promising candidates.

Microalgae and cyanobacteria naturally possess several compounds that serve the purpose of protection and survival in harsh environments (Kholssi et al., 2022). These high-value metabolites can be commercially exploited as biostimulant products. The biomass of microalgae and cyanobacteria is primarily composed of carbohydrates, proteins and lipids, with variations in composition observed across different strains (Bello et al., 2021). Nevertheless, a plethora of molecules such as polysaccharides, polyunsaturated fatty acids, amino acids, osmolytes, enzymes, pigments, vitamins, phytohormones and antioxidants have been extensively studied for potential applications in diverse industries, namely in the agro-industries (Bello et al., 2021; Chabili et al., 2024; Colla & Rouphael, 2020). However, the presence and activity of these biomolecules are contingent upon four main factors: the specific microorganism strain, the growth conditions, the harvesting time and the methodology employed in the extraction process (Chovancek et al., 2023; Colla & Rouphael, 2020).

1.3. Microalgae and Cyanobacteria Extracts Preparation Process

The initial phase of a study project on microalgae and cyanobacteria typically involves the isolation of the organisms from their natural environment and their subsequent maintenance in collections (Bello et al., 2021). A selection of the most

promising strains for biostimulant applications must be made. In addition to evaluating the biostimulant potential through seed germination and plant growth experiments, other factors such as the strain growth rate, productivity and cultivation scheme are also considered (Chabili et al., 2024). The strains are then harvested from the growth medium in accordance with their size and morphology through the implementation of techniques such as centrifugation, filtration, flocculation, flotation, and sedimentation (Parmar et al., 2023; Righini et al., 2022; Ronga et al., 2019). A dehydration process is essential to prevent the degradation of biomass and the consequent loss of quality. Freeze-drying, sun-drying or spray-drying are common techniques for drying biomass (Righini et al., 2022).

In order to gain full access to the vast array of bioactive compounds that these microorganisms possess, it is necessary to undertake a compound extraction from microalgae and cyanobacteria. This process comprise to disrupt the cell wall and release the bioactive substances into the aqueous medium (Bello et al., 2021; Braun & Colla, 2022). In fact, numerous bioactive compounds are present within the cell interior, occluded within the cell wall or associated with different structures, rendering them inaccessible (Righini et al., 2022; Ronga et al., 2019). These methods are classified into three main categories: mechanical (sonication, autoclaving, homogenization, liquid nitrogen and microwaving), chemical (sodium hydroxide (NaOH), hydrochloric (HCl), nitrous (HNO_2) and sulfuric acids and osmotic shock) and biological (enzymatic processes involving cellulases, proteases, β-glucosidases, β-glucanases, xylanases and pectinases) (Braun & Colla, 2022; Colla & Rouphael, 2020; Prisa & Spagnuolo, 2023; Righini et al., 2022).

The extraction method is selected depending on the type of biomass employed and the desired bioactive compounds, as it can exert a pronounced influence on the extraction yield, composition, and quality of the bioactive agents, and thus their biostimulatory activity (Bello et al., 2021; Braun & Colla, 2022; Carillo et al., 2020; Prisa & Spagnuolo, 2023). In accordance with the recommendations set by Lopes (2022) in her master thesis work, also carried out at Fykia Biotech, a sonication extraction method was selected for this project. A sonicator probe generates bubbles that grow and implode via acoustic cavitation in a liquid, resulting in pressure variations that can lead to cell lysis and the release of intracellular biomolecules into the medium (Bello et al., 2021; Carillo et al., 2020).

In terms of application, the liquid extracts can be applied as seed primers for pre-sowing treatment or as foliar sprays by direct nebulization on the foliage of the plants. Alternatively, they can be employed as liquid biostimulants, administered as soil drench treatments or through fertigation on the soil for root uptake (Cao et al., 2023; Chabili et al., 2024; Prisa & Spagnuolo, 2023). The bioactive mechanism underlying the soil drench treatment resides in the agglomeration of soil particles with organic particles such as exopolysaccharides, the recovery of the soil microbiome and the biological mineralization of inaccessible complex nutrients for use by the plant (Parmar et al., 2023).

In order to make an entrance in the market as a product, the extracts must be incorporated into a formulation as an end product, alongside other ingredients. The formulations are engineered to optimize the delivery, stability and efficacy of the bioactive compounds present in the extracts (Kour et al., 2020). A biostimulant formulation must have a prolonged shelf-life, maintain the bioactive metabolites in a stable state, be fabricated with low-cost raw materials that are readily available and in sufficient supply, and be entirely biodegradable, without causing pollution or toxicity to the environment (Kour et al., 2020).

1.4. Microalgae and Cyanobacteria as Biostimulants

For their application as biostimulants, microalgae and cyanobacteria can be used in a wide variety of forms such as alone or in consortium, as inoculates, dried biomass, or extracts and applied as foliar sprays, liquid formulations or seed primers (Braun & Colla, 2022; Parmar et al., 2023). Experiments with microalgae and cyanobacteria-based biostimulants have been carried out and tested under a variety of conditions, including the use of different crops (cereals, fruits, vegetables, medicinal plants, aromatic herbs), different cultivation environments (growth chambers, greenhouses, open fields and hydroponics) and with the influence of different abiotic stressors (salinity stress, heat stress and drought stress) (Bello et al., 2021). Alling et al. (2023) experimented the biostimulant potential of intact cells, lysed cells and the supernatant of Nordic strains of *Chlorella vulgaris* and *Scenedesmus obliquus*, two microalgae species, on tomato and barley seeds. Regardless of the state of the biomass, seeds treated with microalgae, especially *C. vulgaris*, exhibited a faster germination rate and a higher germination index compared to the control. In their study, Mutale-Joan et al. (2020) conducted a screening

of 18 microalgae and cyanobacteria crude liquid extracts to determine their biostimulant properties on tomato plants. A significant improvement in root and shoot length, as well as dry weight was discerned in treated plants, particularly in response to the cyanobacterial crude extracts of *Aphanothece* sp. and the microalgal crude extracts of *Chlorella ellipsoidea*. Additionally, the plants displayed enhanced nutrient uptake, with increases of 185.17%, 119.36% and 78.04% in comparison with the controls for nitrogen, phosphorus and potassium, respectively. The biostimulant potential of the cyanobacteria species *Arthrospira maxima* was assessed through foliar spray testing of different dilutions of sonicated biomass in basil plants (Marín-Marín et al., 2024). The results demonstrated an increase in growth parameters such as fresh and dry weight, stem length, leaf area, and leaf and node count. The chemical composition of the *A. maxima* was evaluated through a biomass characterization which revealed elevated levels of phytohormones, essential amino acids and macronutrients in the cyanobacteria, thereby substantiating its biostimulant potential. In recent years, plant growth enhancers and protectors derived from microalgae and cyanobacteria have been thoroughly explored and experimented on a diverse range of crops, vegetables and herbaceous plants, including tomatoes (Garcia-Gonzalez & Sommerfeld, 2016; Suchithra et al., 2022; Supraja et al., 2020), lettuces (Aloui et al., 2023; Chovancek et al., 2023; Mógor et al., 2017), sugar beet (Barone et al., 2017), beans (Lopes et al., 2023; Refaay et al., 2021), cucumber (Lv, 2020; Navarro-Lopez et al., 2020), pepper (Bello et al., 2022; Seğmen & Özdamar Ünlü, 2023), radish (Godlewska et al., 2019; Santos et al., 2024), onion (Cordeiro et al., 2022; de Oliveira Amatussi et al., 2023; Dineshkumar et al., 2018; Gemin et al., 2019), maize (Martini et al., 2021; Ördög et al., 2021; Silva et al., 2023), wheat (Viegas et al., 2021a, 2021b; Wang et al., 2024) and rice (Lamb et al., 2023; Sornchai et al., 2014), among others.

1.4.1. Bioactive compounds

Phytohormones

Some microalgal and cyanobacterial strains synthesize phytohormones or phytohormones-like molecules, which are low molecular weight signaling molecules that regulate and stimulate the growth and development of terrestrial plants (Braun & Colla, 2022; Kapoore et al., 2021; Lee & Ryu, 2021; Parmar et al., 2023). When produced at very low concentrations, phytohormones have been observed to stimulate root and shoot formation, tissue differentiation, and influence metabolic processes such as plant

respiration, photosynthesis, nucleic acid synthesis and nutrient uptake (Parmar et al., 2023; Ronga et al., 2019). Furthermore, these regulatory compounds can additionally provide the plant with an enhanced defense and tolerance against various biotic and abiotic stresses through the enhancement of endogenous hormone levels in plants (Parmar et al., 2023; Santini et al., 2021). Some microalgae and cyanobacteria are capable of producing and accumulating these chemical messengers intracellularly, while others can also secrete them into the surrounding environment (Kapoore et al., 2021; Renuka et al., 2018). Despite the presence and function of phytohormones in microalgae and cyanobacteria being currently uncertain, many scientific studies have demonstrated that these compounds play a regulatory role in these microorganisms that is analogous to that observed in higher plants. This suggests that they may serve as a viable biostimulant strategy (Kapoore et al., 2021). **Table 2.** lists the principal phytohormones identified in microalgae and cyanobacteria, along with their primary functions in plants.

Table 2. A summary of the major phytohormone families and examples of family members found in microalgae and cyanobacteria, as well as their biostimulatory effects in plants.

Phytohormones families	Examples of phytohormones	Biostimulant Action	Microalgae and cyanobacteria main sources	References
Abscisic acid	-	Plant development (induces stomatal closure and seed maturation), plant physiological processes (protein biosynthesis and storage in seeds for seed dormancy) and biotic and abiotic stress tolerance	Microalgae: *Chlamydomonas, Chlorella, Dunaliella, Haematococcus, Nannochloropsis* and *Scenedesmus*; Cyanobacteria: *Anabaena, Nostoc muscorum, Synechococcus* and *Trichormus variabilis.*	(Kapoore et al., 2021; Parmar et al., 2023; Ronga et al., 2019)
Auxins	Indole-3-acetic acid (IAA), 4-chloroindole-3-acetic acid (CIIAA), 2-phenylacetic acid (PAA) indole-3-butyric acid (IBA), indole-3-propionic acid (IPA), indol-3-acetamide (IAM) and 3-methylindole	Plant development (gametogenesis, embryogenesis, seedling growth, root formation and growth, and flower development), plant physiological processes (cell division and elongation, phloem differentiation, photo- and gravitropism, apical dominance and stress response) and stimulation of the growth of microorganisms from the soil microflora.	Microalgae: *Acutodesmus, Chlorella, Coenochloris,* and *Scenedesmus;* Cyanobacteria: *Anabaena, Chlorogloeopsis, Chroococcidiopsis, Nostoc, Oscillatoria, Phormidium* and *Synechocystis.*	(Braun & Colla, 2022; Kapoore et al., 2021; Lee & Ryu, 2021; Parmar et al., 2023; Ronga et al., 2019)
Brassinosteroids	Brassinolide, castasterone, homocastasterone and typhasterol	Plant development (division, elongation and differentiation of vascular system) and abiotic stress tolerance	Microalgae: *Chlorella* and *Klebsormidium.*	(Carillo et al., 2020; Kapoore et al., 2021; Ronga et al., 2019)
Cytokinins	Zeatin, kinetin, dihydrozetain, zeatin-o-glucoside, isopentenyladenosine (IPA), zeatin riboside, 6-benzylaminopurine and topolin conjugates	Plant development (root and shoot growth, cell division, seed germination, bud development photomorphogenic development, vascular differentiation, apical dominance, flower and fruit development leaf senescence), plant physiological processes (promotion of protein and chlorophyll synthesis, chloroplast biogenesis, nutrient	Microalgae: *Chlamydomonas, Chlorella, Desmococcus, Euglena, Mychonastes, Myrmecia, Nannochloropsis, Nautococcus, Protococcus, Scenedesmus, and Stigeoclonium*; Cyanobacteria: *Anabaena, Arthronema, Calothrix, Chlorogloeopsis Chroococcidiopsis, Phormidium, Oscillatoria,*	(Kapoore et al., 2021; Lee & Ryu, 2021; Parmar et al., 2023; Ronga et al., 2019)

		mobilization) and abiotic stress tolerance	*Rhodospirillum* and *Synechocystis.*	
Ethylene	1-aminocyclopropane-1-carboxylic acid (ACC) (ethylene precursor)	Plant development (cell division, cell elongation, opening of flowers, fruit ripening, senescence of vegetative tissues), plant physiological processes (photosynthesis regulation and chlorophyll biosynthesis) and abiotic and biotic stress tolerance	Microalgae: *Chlorella* and *Scenedesmus*; Cyanobacteria: *Arthrospira, Anabaena, Calothrix, Cylindrospermum, Nostoc, Scytonema* and *Synechococcus.*	(Kapoore et al., 2021; Parmar et al., 2023)
Gibberellins	GA1, GA3, GA4, GA5, GA6, GA7, GA8, GA9, GA12, GA12ald, GA13, GA15, GA19, GA20, GA24, GA29, GA34, GA44, GA51 and GA53	Plant development (seed germination, stem elongation, leaf expansion, floral organ development, initiation of flowering), physiological processes (protein biosynthesis, photosynthetic efficiency) and abiotic stress tolerance	Microalgae: *Chlamydomonas, Chlorella, Chlorococcum, Gyoerffyana, Myrmecia, Nannochloropsis, Nautococcus, Scenedesmus, Scotiellopsis* and *Stigeoclonium.* Cyanobacteria: *Arthrospira, Anabaenopsis, Cylindrospermum* and *Phormidium.*	(Braun & Colla, 2022; Kapoore et al., 2021; Parmar et al., 2023; Ronga et al., 2019)
Jasmonic acid	-	Plant development (seed germination, flowering, promotion of tuber formation and senescence), physiological processes (synthesis of proteinase inhibitors, upregulation of antioxidant genes, ion uptake and transport, glycolysis, photosynthetic rate, transpiration and plant defense regulation) and biotic and abiotic stress tolerance	Microalgae: *Chlorella, Dunaliella, Euglena, Gelidium* and *Scenedesmus*; Cyanobacteria: *Arthrospira.*	(Kapoore et al., 2021; Parmar et al., 2023)
Polyamines	Putrescine, spermine and spermidine	Plant development (plant growth and development), physiological processes (cell proliferation, differentiation and growth, lipid and sugar homeostasis, protein synthesis, modulation of enzymatic activities) and biotic and abiotic stress tolerance	Microalgae: *Chlorella, Cyanidium, and Euglena*; Cyanobacteria: *Arthrospira.*	(Braun & Colla, 2022; Carillo et al., 2020; Kapoore et al., 2021; Mógor et al., 2017; Parmar et al., 2023)
Salicylic acid	-	Plant development (seed germination and flowering), physiological processes (upregulation of antioxidant genes, ion uptake and transport, glycolysis, photosynthetic rate, transpiration and plant defense regulation) and biotic stress tolerance	Microalgae: *Scenedesmus*; Cyanobacteria: *Arthrospira.*	(Kapoore et al., 2021; Parmar et al., 2023)

Phytohormones can be applied to the plant by introducing microalgal or cyanobacterial living cells or extracts to the leaves or seeds (Braun & Colla, 2022). Mógor et al. (2017) investigated the potential for plant growth of polyamines obtained through the enzymatic hydrolysis of proteins from *Arthrospira platensis* in lettuce seedlings. The results demonstrated that foliar applications of the cyanobacteria hydrolyzed biomass elicited cytokinin-like effects in the plants, thereby promoting plant growth. Likewise, gibberellic acid extracted from the cyanobacterium *Scytonema hofmanni* was reported by Rodríguez et al. (2006) to possess beneficial properties in alleviating salt stress in rice cultures. The phytohormone was able to negate the inhibitory

effects of NaCl on the length and dry weight of the plant shoot, as well as the carotenoid and chlorophyll b content.

Polysaccharides

Microalgae and cyanobacteria are a rich source of polysaccharides, which can be found in their cell walls, or excreted as definite structures or mucilage (Santini et al., 2021). Polysaccharides are macromolecular polymers that possess a wide range of different bioactivities in plants (Lee & Ryu, 2021; Parmar et al., 2023). The main functions of these polysaccharides are to enhance root growth, promote photosynthesis through the synthesis of Rubisco enzymes, increase nutrient availability by chelating minerals and stimulate defense responses due to their role as plant immune elicitors for the induction of the plant resistance through key enzymes such as β-1,3-glucanases and chitinases (Braun & Colla, 2022; Kapoore et al., 2021; Lee & Ryu, 2021; Parmar et al., 2023). The predominant polysaccharides present in microalgae and cyanobacteria are heteropolymers of glucose, mannose, arabinose, xylose and galactose, along with homopolymers of galactose and β-(1,3)-glucan (Kapoore et al., 2021). Furthermore, exopolysaccharides produced by microalgae and cyanobacteria cells can also act as plant biostimulants due to their plant protection effect and plant growth promotion action (Braun & Colla, 2022). When present in the soil, their bioadhesive properties enable them to aggregate soil particles, and to bind toxic elements such as heavy metals and sodium ions, thereby alleviating plant stress induced by saline and polluted soils (Parmar et al., 2023; Santini et al., 2021). Crude polysaccharide extracts were prepared from three microalgae and cyanobacteria strains (*A. platensis*, *Dunaliella salina* and *Porphorydium* sp.). When irrigated on tomato plants, Rachidi et al. (2020) verified a significant improvement in morphological aspects of the plant, including shoot length and dry weight and node. These improvements were found to be 25.26%, 46.6% and 75% respectively. The biochemical properties of the plant leaves including protein, chlorophyll and carotenoid concentrations were enhanced in the plant leaves, as were the nitrate reductase and NAD-glutamate dehydrogenase enzymatic activities. Similarly, Guzmán-Murillo et al. (2013) recovered polysaccharides from *Dunaliella salina* and *Phaeodactylum tricornutum* extracts to evaluate their potential to alleviate salt stress alleviation potential on peppers during germination. The polysaccharide extracts positively influenced root growth and significantly reduced superoxide radicals and lipid peroxidation due to an increased antioxidant enzyme presence in the plants, efficiently mitigating the effects of saline stress.

Antioxidants

Vitamin C, vitamin E, chlorophylls, carotenoids, phenolic compounds and other secondary metabolites belong to the class of antioxidants that have been identified in microalgae and cyanobacteria (Kapoore et al., 2021). These crucial compounds act mainly as elicitors for the antioxidant and pathogenesis machinery in plants (Parmar et al., 2023; Renuka et al., 2018). Phenolic compounds represent a significant group of antioxidant metabolites capable of functioning as stress indicators and ROS scavengers during oxidative stress (Braun & Colla, 2022; Carillo et al., 2020; Kapoore et al., 2021; Parmar et al., 2023). In addition to their antioxidant properties, these biomolecules are known to possess antimicrobial, antiviral and radioprotective activities (Kapoore et al., 2021). Moreover, phenolic molecules can chelate ions, facilitating the absorption of nutrients by plants (Carillo et al., 2020; Parmar et al., 2023). Although antioxidants such as eckol and phloroglucinol retrieved from the macroalga *Ecklonia maxima* were correlated with physiological enhancements in cabbage (Rengasamy et al., 2016), few studies have been dedicated to the antioxidant compounds of microalgae and cyanobacteria have been explored at the moment (Kapoore et al., 2021; Parmar et al., 2023).

Proteins and Amino Acids

Microalgal and cyanobacterial extracts are also a source of proteins and amino acids that can be supplemented to the plants to conserve energy in some metabolic pathways such as the nitrogen and carbon metabolisms, while also facilitating increased nutrient uptake through the complexation and chelation of essential minerals, as well as the synthesis of important enzymes such as citrate synthase, isocitrate dehydrogenase and malate dehydrogenase (Braun & Colla, 2022; Kapoore et al., 2021; Parmar et al., 2023). The application of protein hydrolysates (polypeptides and oligopeptides) and amino acids might improve crop growth and development in two distinctive ways. They may directly increase the pigment, protein and phytohormone content of the crop and indirectly by promoting the growth of beneficial microorganisms in the soil and soil respiration (Kapoore et al., 2021). The biosynthesis of some phenolic compounds such as phenylamines and L-amino acids has been demonstrated to enhance abiotic stress resistance by mitigating the effects of injuries through a signaling role in the defense system (Braun & Colla, 2022; Ronga et al., 2019; Santini et al., 2021). The effects of lyophilized *A. platensis* suspension, which is known for its high levels of L-amino acids

content, were determined through its foliar application on red beets. The application of the lyophilized cyanobacterium suspension resulted in the promotion of plant growth as indicated by the increased levels of sugars, proteins, free amino acids and chlorophyll observed on the seedlings' leaves. In addition, the hypocotyl growth improvement was noted. The significantly higher yields observed may be attributed to the presence of free amino acids present in the cyanobacterium, as postulated by (Mógor, 2018).

1.4.2. Salt Stress Biostimulants

Biostimulants can be applied either as a priming solution to prevent the negative effects of a stress factor, during a stress event as a plant protector, or even after for the recovery of injuries (Carillo et al., 2020). Microalgal and cyanobacterial biostimulants boost the natural defense system of the affected plants through the delivery of protective biocompounds that promote antioxidant and osmoregulatory responses (Carillo et al., 2020). The results demonstrate that microalgae and cyanobacteria have biostimulant activities in plants, as evidenced by increased nutrient uptake, prevention of nutrient loss, boost in seed germination and seedling growth, accelerated flowering, increased crop yield, improved nutritional quality of the food and a greater tolerance to abiotic stress (Bello et al., 2021; Colla & Rouphael, 2020; Kapoore et al., 2021). El Arroussi et al. (2018) investigated the application of exopolysaccharides derived from the microalga *Dunaliella salina* to alleviate the adverse effects of salt stress on tomato plants. The treated plants exhibited enhanced morphological characteristics such as larger and heavier roots and shoots. Additionally, they demonstrated reduced sensitivity to salt stress, evidenced by decreased levels of Na^+, proline, phenolic compounds and antioxidant enzymes. Furthermore, the study also confirmed that the presence of microalgal exopolysaccharides activated various metabolic pathways such as jasmonic acid-dependent pathways associated with plant defense against abiotic stresses such as salt. The detrimental effects of salinity stress on lettuce were attenuated by the application of a terrestrial cyanobacterium, *Oculatella lusitanica*. This newly identified species of *Oculatella* did not promote a stronger plant growth compared to the controls. However, it did confer an increased tolerance to the plants through the activation of non-enzymatic antioxidant machinery (Brito et al., 2022). A similar outcome was observed in wheat plants irrigated with 10% or 20% salt water and subsequently treated with aqueous extracts of *Scenedesmus obliquus* and *Arthrospira platensis*. The promotion of antioxidant enzymes such as superoxide dismutase, catalase, total peroxidase and

ascorbate peroxidase, limited sodium ion concentrations and lipid peroxidation. Despite the irrigation with brackish water, the plants showed enhanced tolerance, as evidenced by a significant increase of the photosynthetic pigments (chlorophylls and carotenoids) and antioxidant compounds such as glutathione contents (Abd El Baky et al., 2014).

1.5. Advantages and Constraints of the Use of Microalgae and Cyanobacteria

The commercial use of microalgae and cyanobacteria as biostimulants is still in its infancy, as the majority of the scientific knowledge is recent and there are few microalgae and cyanobacteria-based companies in the market (Colla & Rouphael, 2020; Prisa & Spagnuolo, 2023). Of the approximately 800,000 estimated species of microalgae and cyanobacteria, only about 50,000 have been described and only a minute fraction has been commercially exploited to date (Bello et al., 2021; Prisa & Spagnuolo, 2023; Ronga et al., 2019). The two most studied microorganisms for biostimulant applications are the cyanobacteria *Arthrospira* spp. and the microalgae *Chlorella* spp. (Prisa & Spagnuolo, 2023).

Nonetheless, their utilization offers a multitude of benefits. Biostimulants derived from cyanobacteria and microalgae represent a green and sustainable alternative to synthetic fertilizers. Their ability to recycle nutrients, restore soil fertility and prevent soil pollution and degradation makes them an attractive option for agricultural applications (Righini et al., 2022). Moreover, microalgae and cyanobacteria capture carbon dioxide from the atmosphere through photosynthetic fixation, contributing in greenhouse levels reduction (Navarro-López et al., 2023). In addition to their useful metabolite content, the production of microalgae and cyanobacteria is generally straightforward and they often exhibit rapid growth and a short lifecycle which allows continuous harvesting (Bello et al., 2021; Dagnaisser et al., 2022; Navarro-López et al., 2023; Prisa & Spagnuolo, 2023). Furthermore, they do not require arable land for cultivation as they can be grown either in controlled culture systems facilities, including open systems such as circular and raceway ponds or closed systems such as photobioreactors, as in brackish or salt water (Navarro-López et al., 2023). This eliminates the potential competition with land for food production (Braun & Colla, 2022; Dagnaisser et al., 2022; Mutale-Joan et al., 2022; Prisa & Spagnuolo, 2023; Ronga et al., 2019).

In order to reduce production costs, studies are being conducted on the use of microalgae and cyanobacteria to support wastewater treatment. Wastewater is a cost-effective growth medium that is naturally rich in nutrients such as nitrogen and phosphorus, which these microorganisms can assimilate to increase their biomass while treating the polluted water by removing the excess of nutrients and toxic heavy metals (Braun & Colla, 2022; Chabili et al., 2024; Koller et al., 2014; Prisa & Spagnuolo, 2023; Ronga et al., 2019). Moreover, cyanobacteria and the majority of microalgae are autotrophic microorganisms with the capacity for photosynthesis, enabling them to capture and store CO_2 in the process. This natural process renders the production system carbon-negative, thereby partially eliminating the agricultural carbon footprint through carbon sequestration (Cao et al., 2023; Dagnaisser et al., 2022; Kholssi et al., 2022).

The development of effective microalgae and cyanobacteria-based biostimulants is hindered by several constraints that pose barriers to their marketability. The major bottleneck resides in the high cost of production (Braun & Colla, 2022). The large-scale production of microalgae and cyanobacteria is still undermined by the expensive cost of the culture medium, the use of significant volumes of water, and the high cost of energy, resulting in a total production cost of approximately 5-70 $\$.kg^{-1}$ (Braun & Colla, 2022; Cao et al., 2023; Chabili et al., 2024; Colla & Rouphael, 2020; Parmar et al., 2023). It is essential to consider critical factors such as high productivity, cost viability, low energy expenses, an increased ability to control environmental factors and sustainability when developing a microalgae and cyanobacteria production system (Bello et al., 2021).

Moreover, microalgal and cyanobacterial biostimulants remain largely underexploited due to the limited research conducted on a small fraction of species of microalgae and cyanobacteria. The biomolecular mechanisms underlying their bioproducts have yet to be fully elucidated (Bello et al., 2021; Colla & Rouphael, 2020). This is due to the elevated diversity of biocompounds and the intricate complexity of their modes of action (Chabili et al., 2024; Kapoore et al., 2021). It is difficult to pinpoint the specific bioactivity effect of a single molecule or class of molecules among the other myriad of compounds that compose the extract, as the biomolecules typically operate in a synergistic manner (Carillo et al., 2020; Righini et al., 2022).

There is still a lack of knowledge regarding crucial variables that may influence the efficacy of biostimulants, such as the phenological stage of the plant and

environmental conditions (Prisa & Spagnuolo, 2023). It is evident that the diverse range of plant species used for crop cultivation and the varying stages of their growth respond in different ways to the same biostimulant product making the attainment of a universal, standardized biostimulant a challenging objective (Bello et al., 2021; Colla & Rouphael, 2020; Prisa & Spagnuolo, 2023). Additionally, identifying the optimal timing, frequency and extract concentration for product development has proven to be a significant hurdle (Bello et al., 2021; Dagnaisser et al., 2022). To fully assess their potential as stress alleviators, further research is required to determine on a crop-by-crop base strategy the optimal timing of application of biostimulant on the affected crops (before, during or after the emergence of a stress factor) and the ideal dosage of microalgae and cyanobacteria extracts, i.e. the concentration that yields the most favorable outcomes (Bello et al., 2021; Carillo et al., 2020).

1.6. Objectives

The main objective set for the internship was to conduct applied research leading to the development of a novel biostimulant formulation derived from microalgae and cyanobacteria strains from the Fykia Biotech Culture Collection (FBCC). This formulation has the purpose to be launched as a final product for application in agriculture, which represents one of the three areas of interest for the Fykia Biotech company.

For that purpose, 27 strains were screened for their potential as biostimulants. The strains were cultivated and maintained under standard conditions, their biomass was harvested, and aqueous extracts were prepared through lyophilization and subsequent sonication. The microalgal and cyanobacterial aqueous extracts were evaluated in germination assays in Petri dishes. The four most promising candidates were then selected to assess their ability to promote plant growth, as well as their capacity to provide protection against salt stress. Subsequently, formulations of the selected aqueous extracts were prepared and tested for their capacity as plant growth promotors.

In addition to product development, the cultures were maintained. This involved sub-culturing and several attempts at isolating the contaminated cultures. The most promising microalgae and cyanobacteria strains were characterized through molecular biology approaches.

Secondly, the project encompassed business development for the emergent startup. Market research and competition assessment for biostimulants based on algae and cyanobacteria formed part of the research objectives. These activities included liaising with potential customers, participating in scouting activities, exhibiting the research output, representing the company externally as well as managing the company's social media presence.

2. Materials and Methods

The objective of this project is to develop a biostimulant product derived from cyanobacteria and eukaryotic microalgae (henceforth microalgae) dried biomass for agricultural applications. To this end, the company has established a private collection, the Fykia Biotech Culture Collection (FBCC), comprising microalgae and cyanobacteria obtained from environmental samples collected in the metropolitan areas of Porto and Vila Real, Portugal. A total of 31 microalgae and cyanobacteria strains were isolated from the different samples and are now a part of the collection.

2.1. Fykia Biotech Culture Collection (FBCC)

2.1.1. Culture Maintenance

The 31 isolates were cultivated in a Z8 liquid medium (Kotai, 1972) (see the protocol in **Appendix I**) in 40 mL culture flasks and maintained in a culture room with a temperature condition of 22 ± 2 °C and a light/dark cycle of 16h/8h. For the preparation of the stock Z8 culture medium, ultra-pure water and four stock solutions (Solution A, Solution B, Fe-EDTA, and Micronutrients solution) were autoclaved (Tuttarnauer, 3870ELV-D, Netherlands) separately at 121 °C for 20 minutes. After sterilization in a laminar flow chamber (Telstar, Bio II Advance, Spain), the medium was prepared with the assistance of a syringe coupled to a 0.22 µm sterile syringe filter, Solution A, Solution B, and a Fe-EDTA solution in a ratio of 10 mL/L, as well as a micronutrient solution in a ratio of 1 mL/L in one liter of distilled water. A volume of 40 mL of Z8 medium was poured into each culture flask.

To ensure the viability of the culture collection, each strain was cultivated in duplicate. Microalgae and cyanobacteria were sub-cultured every three and six months, respectively. Sub-culturing was conducted by first pouring 40 mL of Z8 medium into the cell culture flasks, followed by the transfer of culture biomass from a plastic Pasteur pipette into the respective flask.

2.1.2. Strain Isolation – Micromanipulation

Before harvesting the biomass for screening studies, all cultures from the collection were observed under a microscope (Leica, DMLB light, Germany) to verify

their purity. Contaminated cultures were re-isolated using a micromanipulation method. Two types of glass pipettes were prepared for micromanipulation. One pipette was constructed with an elongated and thin open tip for the aspiration of cells, while the other was made with an elongated, round, closed tip for the separation of cells. Two or three droplets of the contaminated culture were transferred to a glass Petri dish and isolated in the vicinity of a flame. Subsequently, each microorganism was then morphologically identified through the use of an inverted microscope (Motic, AE2000, China). The organism was then meticulously separated from the remainder of the culture through aspiration with the designated glass pipette coupled to a bulb and placed inside a 24-well tissue culture plate containing Z8 medium. The cells in filamentous strains were initially separated with the assistance of the glass pipette with the round, closed tip to facilitate the transfer. The 24-well plate was then positioned inside the culture room, where the isolated strains would grow optimally. Once sufficient biomass was observed, each isolated strain was transferred to a 40 mL culture flask.

2.1.3. Morphological Characterization

A digital camera (Leica Microsystems, Leica ICC50 HD, Germany) coupled to a microscope (Leica, DMLB light, Germany) and the software Leica Application Suite (Leica Microsystems, version 4.2.0, Germany) were used to microphotograph the strains of the collection to proceed to a basic morphological characterization. 400x and 1000x magnifications were used for that purpose.

2.2. Molecular Identification

Cells were collected from fresh cultures and washed with a fresh medium via centrifugation. Total genomic DNA (gDNA) was extracted using the PureLink Genomic DNA Kit (Invitrogen, Waltham, MA, USA), following the manufacturer's instructions for Gram-negative bacteria.

PCR reactions were conducted using a Veriti Thermal Cycler (Veriti 9902, Applied Biosystems, Thermo Fisher Scientific, Singapore). Each reaction had a final volume of 20 µL, comprising 5.9 µL of molecular biology-grade water, 4 µL of Green GoTaq Flexi Buffer, 2 µL of $MgCl_2$, 2 µL of each forward and reverse primer, 1.5 µL of deoxynucleotide triphosphates (dNTPs), 0.5 µL of bovine serum albumin (BSA), 0.1 µL of GoTaq Flexi DNA Polymerase (Promega, USA), and 2 µL of genomic DNA (Katana et al., 2001). The

16S rRNA gene sequence was amplified under the following conditions: an initial denaturation at 94 °C for 5 minutes, followed by 10 cycles of denaturation at 94 °C for 45 seconds, annealing at 57 °C for 45 seconds, and extension at 72 °C for 2 minutes. This was followed by an additional 25 cycles of denaturation at 92 °C for 45 seconds, annealing at 54 °C for 45 seconds, and extension at 72 °C for 2 minutes, with a final extension at 72 °C for 7 minutes.

The PCR products were separated on a 1% (w/v) agarose gel stained with SYBR Safe DNA Gel Stain (Invitrogen by Thermo Fisher Scientific, USA). DNA fragments of the expected size were excised and purified using the NZYGelpure kit (Nzytech, Portugal), according to the manufacturer's instructions. The purified fragments were then sent for sequencing with primers 359F and 781R (Nübel et al., 1997), 1494R and 27SF (Neilan et al., 1997), 23SR (Taton et al., 2003), and 1114F (Lane, 1991)

Sequencing was performed by Sanger dideoxy sequencing at GATC Biotech (Ebersberg, Germany). The resulting nucleotide sequences were manually inspected for quality and assembled using Geneious Prime 2023.2.1 software (Biomatters Ltd., Auckland, New Zealand).

2.3. Culture Scale-up

Depending on the required biomass for each assay the strains could be grown in flasks with 40 or 400 mL or in 6L flat-bottomed flasks with 4 L of Z8 medium. Once a stationary phase. was reached, the scale-up was performed in a maximum ratio v/v of 10 times, in sterile conditions, in a laminar flow chamber. The state of the cultures was always checked before scaling up.

2.4. Extracts Preparation

2.4.1. Biomass Harvesting

The process of biomass harvesting was dependent upon the quantity of biomass to be collected. In the case of the culture flasks, once sufficient biomass density had been reached, the filamentous strains were collected from the medium with the help of a drainer and a nylon net and subsequently transferred to a 50 mL sterile disposable

centrifuge tube with a spatula. In the case of coccoid strains, a Pasteur pipette was employed instead. The strains were centrifuged at 6000 g for 10 minutes at 4 °C (Heraeus, Megafuge 16R, Germany). The supernatant was discarded, and the biomass pellet was stored overnight in a freezer at – 20 °C, after which it was lyophilized in a freeze-dryer (Telstar, LyoQuest, Spain). The dried biomass was stored at room temperature and protected from light until it was required for further use.

For strains cultivated in 4 L volumes, the centrifugation was conducted in a centrifuge (Thermo Fisher Scientific, Sorvall BIOS 16, USA) with a 7186 g speed for 15 min at 4 °C. This process was repeated until the full culture was harvested, with the supernatant being discarded between each centrifugation. Subsequently, the biomass was transferred at the end to a 100 mL sample container for freeze-drying.

2.4.2. Cell Disruption

2.4.2.1. Sonication

The complete disruption of the cells was achieved through sonication. On an analytical scale (Sartorius, Germany) with a precision of 0.1 mg, 20 mg of the dried biomass was weighed in a 50 mL sterile disposable centrifuge tube. For a complete disruption of the cells, the sonication process was divided into two cycles. One milliliter of distilled water was added to the tube, the solution was vortexed and then sonicated in a sonicator (Bandelin Sonopuls, Germany) with an MS 72 probe for 10 min with an amplification of 80%, a pulse of 30 seconds on and 5 seconds off. The tubes were placed in an ice bath within a beaker to prevent overheating of the extracts and subsequent deterioration of the bioactive compounds. This process was repeated with the addition of one additional milliliter. At the end of the cell disruption process, the extracts were stored in the freezer at - 20 °C until further use.

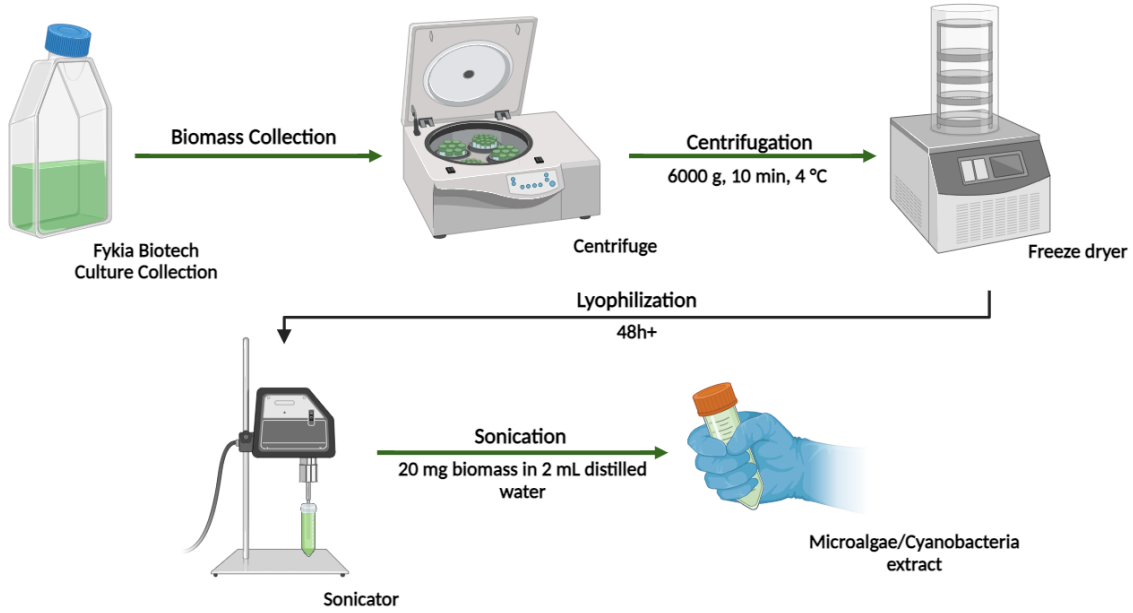


Figure 1. Microalgal and cyanobacterial extraction preparation methodology, from the harvesting, separation of the biomass from the supernatant, to the lyophilization and subsequent sonication and extract obtention.

2.4.2.2. Accelerated Solvent Extraction (ASE)

Instead of ultrasonic sound waves to disrupt cell membranes, the Accelerated Solvent Extractor (Thermo Fisher Scientific, Dionex ASE 350, USA) was used to extract intracellular components from the culture samples. Regarding the extraction process, 25 mg of the strain biomass was weighed on an analytical scale with a precision of 0.1 mg. In the fume hood, a cellulose membrane was positioned on the lower and upper cap of the cell belonging to the ASE equipment. A pestle was employed to ensure an effective seal of the membrane. A funnel was used to load two tablespoons of diatomaceous earth into the cell, which was then compacted. The weighted biomass and diatomaceous earth were loaded into the cell once more. The cells were then loaded into the equipment for extraction. The extraction process was accomplished at a temperature of 40 °C and a pressure of 1500 psi. The aqueous extract was collected in a tube and kept in the freezer (- 20 °C).

2.5. Formulations Preparation

The biostimulant formulation used (**Table 3**) was developed by Andreia Lopes as part of her Master's Thesis at Fykia Biotech (Lopes, 2022). In her study, Lopes (2022) followed the methodology of Michalak et al. (2016) in order to prepare a working formulation for algae.

Table 3. Composition of the biostimulant formulation containing aqueous extracts of microalgae and cyanobacteria.

Active Substance	(% mas.)	Function	Remarks
Biomass extract	12.00	Bioactive compound	-
Sorbitan monolaurate (Span 20)	5.00	Non-ionic emulsifier, surfactant	Sigma Aldrich, USA
Ethylenediaminetetraacetic acid (EDTA)	3.60	Stabilizer, preservative	Sigma Aldrich, USA
Citric Acid	0.50	Preservative, antimicrobial activity	Sigma Aldrich, USA
Demineralized Water	78.90	Solvent	-

The demineralized water and the biomass extract were combined. The weighed EDTA, citric acid, and sorbitan monolaurate were added to the solution. A total volume of 300 mL of the formulation solution was prepared for four extracts (S004, S009, S011, and S012) at a final concentration of 0.5 g/L. The solutions were kept in agitation for 30 minutes to create the emulsion. The prepared formulations were protected with aluminum foil to avoid light penetration and kept at room temperature.

2.6. Seed Germination Bioassays

2.6.1. Standard Conditions

The initial phase of the project entailed a comprehensive screening of the 31 microalgae and cyanobacteria strains sourced from the FBCC. Their assessment aimed to evaluate their biostimulant potential for seed germination in 90 mm Petri dishes. For that purpose, tomato (*Solanum lycopersicum* L. var. ACE VF 55, Luso Sementes, Portugal) and lettuce (*Lactuca sativa* L. var. Trocadeiro Bola de Manteiga, Luso Sementes, Portugal) seeds were purchased from Leroy Merlin, Portugal. The crop

species were selected on the basis of their rapid growth and their high agricultural value on a global scale (Alling et al., 2023). A new packet of seeds was opened at two-month intervals to guarantee the continued availability of healthy and viable seeds for the germination assays. Before starting the assay, in accordance with the methodology proposed by Alling et al. (2023), the seeds were weighed on an analytical scale in order to provide an initial estimation of their quantity. They were then sterilized by soaking them in 10 mL of 5% (v/v) of a commercial bleach solution for 10 min, after which they were thoroughly rinsed twice with distilled water (Alling et al., 2023; Garcia-Gonzalez & Sommerfeld, 2016). The pH of the distilled water used in the assay was adjusted with HCl and NaOH (Fluka Honeywell, ACS Reagent ≥ 97.0%, USA) to values between 5.5 and 6.0 for tomato seeds and 6.0 and 7.0 for lettuce seeds (Bhatia & Ashwath, 2005; Huang et al., 2024).

The Petri dishes containing Whatman™ Grade 1 filter paper (85 mm) were sterilized in the laminar flow chamber with UV for 20 minutes. Negative control plates were filled with 5 mL of distilled water. A commercial biostimulant (Siro, Portugal) derived from marine macroalgae – *Ascophyllum nodosum* – biomass was utilized as the positive control and the microalgae and cyanobacteria strains were tested in the form of aqueous extracts (obtained through sonication and ASE extraction) and formulations. The positive control was prepared by vortexing and gently pouring 5 mL of a diluted commercial biostimulant solution (3 µL/mL) onto the filter paper. The same volume of microalgal or cyanobacterial aqueous extracts or formulations was added at a concentration of 1 µg/mL. Ten seeds were placed per plate and distributed equally on the filter paper, which was then sealed with Parafilm®. Two replicates were prepared for each condition in each assay, and each condition was repeated in at least three different assays. The plates were incubated in an incubator (Eppendorf Innova S44i, Germany) at 25 °C in the dark for seven days.

2.6.1.1. Positive Control Concentration Optimization

The optimal commercial biostimulant concentration for the positive control was determined to be accurate in the seed germination assays. The effect of varying concentrations of the biostimulant on seed germination in tomato plants was investigated.

In accordance with the manufacturer's recommendations, 30 mL of the biostimulant product should be diluted in 5 L of water. To evaluate the best concentration

of biostimulant for seed germination assays, six different concentrations were evaluated: (i) 0 µL/mL (negative control), (ii) 2.0 µL/mL, (iii) 4.0 µL/mL, (iv) 6.0 µL/mL and (v) 8.0 µL/mL, and (vi) 10.0 µL/mL. Two replicates with 10 tomato seeds were prepared for each condition. The seeds were then placed in an incubator at 25 °C for 7 days in the absence of light.

Table 4. Main chemical composition and pH of the commercial biostimulant (SIRO®) provided by the manufacturer.

Composition	Chemical Characteristics	Value
***Ascophyllum Nodosum* Macroalga (100%)**	**N Total**	4%
	K_2O Total	5%
	Organic matter	70%
	Alginic Acid	22.9%
	pH	5.4

2.6.1.2. Microalgae and Cyanobacteria Extraction Optimization

Extraction optimization was performed by varying the sonication time and testing different biomass/solvent ratios. A seed germination experiment was carried out in tomato seeds to evaluate the efficacy of the various extraction methods in S011 strain.

Table 5. Different extraction biomass/solvent ratios and sonication times used for the cell lysis optimization procedure.

Treatment	Sample/Solvent Ratio	Sonication Time
T1	20 mg/2 mL	2 x 20 min
T2	20 mg/4 mL	2 x 20 min
T3	20 mg/10 mL	2 x 20 min
T4	20 mg/10 mL	2 x 10 min
T5	20 mg/20 mL	2 x 20 min
T6	20 mg/20 mL	2 x 10 min

2.6.2. Salt Stress Conditions

A concentrated stock solution of NaCl (25 mg/mL) was previously prepared according to the established protocol. From this stock solution, a series of dilutions were prepared to obtain the desired salt-concentrated solutions (0 mg/mL, 3 mg/mL, 6 mg/mL, 9 mg/mL and 12 mg/mL). For the standard assay, the positive control tubes were prepared with a concentration of 3 µL/mL of commercial biostimulant, and the extracts were with 1 µg/mL. The process used afterward was identical to that used for the standard condition.

2.6.3. PBS Buffer

The objective of preparing the PBS buffer was to stabilize the pH of the distilled water used for seed germination assays to a neutral value. To this end, 8.00 g of NaCl, 1.44 g of Na_2HPO_4, 0.24 g of KH_2PO_4, and 0.20 g of KCl were added to 800 mL of distilled water. To attain a pH of 7.2-7.4, HCl was employed. Finally, distilled water was incorporated until the total volume reached 1 L, after which the buffer underwent sterilization in an autoclave (121 °C for 20 minutes) and was stored at room temperature.

2.6.4. Germination Index

The germination of the seeds was observed on a daily basis, and the number of germinated seeds was recorded. The experiment was concluded after seven days. The roots of the seeds were photographed and subsequently measured using the ImageJ software (National Institutes of Health, Bethesda, MD, USA). In the case of tomato seeds, only radicles exceeding a length of 2 mm were deemed to have germinated (Alling et al., 2023; Garcia-Gonzalez & Sommerfeld, 2016).

The germination index (GI), initially described by Zucconi et al. (1981), is a reliable method for evaluating seed germination success. The number of germinated seeds after seven days (G) and the root elongation (L) registered for each treatment (T) were relatively compared with the negative and positive controls (C) using the following equation:

$$\mathrm{GI}\ (\%) = \frac{\mathrm{G_T} \times \mathrm{L_T}}{\mathrm{G_C} \times \mathrm{L_C}} \quad (1)$$

2.7. Plant Growth Bioassays

The objective of these assays was to test the potential of microalgal and cyanobacterial extracts as biostimulants for the growth of lettuce and tomato plants in soil conditions.

Table 6. Experimental conditions for the three plant growth assays.

Assay	Plants	Local of Growth	Temperature (°C)	Photoperiod	Light Intensity ($\mu mol.m^{-2}.s^{-1}$ PPFD)
1st Assay	Lettuce	Incubator	25.0/22.0	16h/8h	~30.0
2nd Assay	Lettuce and Tomato				
3rdAssay	Lettuce and Tomato	Greenhouse	25.4/16.9	~15h/9h	-

Three distinct parameters were evaluated under standard conditions: the optimal water volumes for lettuce growth, four different microalgae and cyanobacteria aqueous extracts and the application frequency, as well as their respective formulations, in tomato and lettuce plants.

2.7.1. 1st Assay: Optimal Water Volumes Assay

To ascertain the optimal quantity of water to be added to the lettuce pots, five different water application methodologies were tested. In the initial methodology, defined volumes of 5.0, 7.5 and 10.0 mL of water were selected. An alternative approach was to calculate the water lost by evapotranspiration. The pots were weighed on an analytical scale: initially after the seeds were sown and watered, and a second time 24 hours later. The weight difference would thus indicate the quantity of water required to be daily applied to each pot. Finally, with regard to the last method that was tested, the plants were irrigated manually as needed. These conditions were repeated twice, once with tap water and once with distilled water. In total, ten conditions were tested with two replicates for each.

Table 7. Experimental design (method of water application, treatments code (T), water volumes and the weekly frequency of application in the plants) of the first plant growth assay for the optimization of water volumes applications in lettuce plants.

Method Determined water volume	Treatment (Distilled/Tap Water)	Water Volume (mL)	Frequency (days/week)
Determined water volume	**T1/T2**	5.0	3
	T3/T4	7.5	2
	T5/T6	10.0	2
Weight difference	**T7/T8**	8.6	3
Watered as needed	**T9/T10**	-	-

The initial step involved the distribution of a non-sterilized universal substrate mixture of vegetal mulch and peat (ECO Grow, Siro, Portugal) (see composition in **Appendix II**) across the surface of the seedling trays (3×4 wells) with a volume of 10,1 cL. Three lettuce seeds were sown into each well and subsequently watered with 10 mL of water with a syringe. The seedling trays were then transferred to the incubator, which was set to the previously defined temperature and light intensity parameters (**Table 6**). The wells were randomly arranged in the incubator, with particular attention paid to the wells to receive equal radiation. Once germination had occurred outside the substrate, the lettuce seedlings were thinned to one plant per cell. The seedlings were covered with a transparent plastic lid. Additionally, a beaker containing water was placed in proximity to maintain humidity levels inside the incubator. After 28 days, the lettuce plants were harvested. The roots were cleaned to remove any particles of soil, and the fresh weight of the entire plant was measured using an analytical scale. The plant images were processed using ImageJ software and the following measurements were taken: the height and the fresh weight of the whole plant, the dimensions of the shoot and roots, and the area of the leaves.

2.7.2. 2nd Assay: Extract Application Frequency Assay

Three tomato and lettuce seeds each were sown in 24-cell seedling trays (Parkside, Lidl, Portugal) with 107 cm^3 volume. Three replicates were made for each condition for both the extracts (S011 and S012), as well as for the negative control (tap water) and the positive control (commercial biostimulant). The plants were watered three times a week (Monday-Wednesday-Friday) with a counted volume of 5.6 mL H_2O/pot for

tomato plants and 5.0 mL H_2O/pot for lettuce plants, determined by the evaporation method. The commercial biostimulant and the extracts were applied by soil drenching. Three different frequencies of extract application were tested: weekly, fortnightly and monthly, while the positive controls were treated every 2 weeks as suggested by the manufacturer. Seedlings were transplanted into 18 cL paper cups after 39 days (one seedling per cup). Plants were harvested after 75 days, the roots were washed and dried, and the height (cm), shoot and root size (cm), total fresh weight (g), number of leaves and nodes, and lettuce leaf area (cm^2) were recorded for all treatments. The leaves were then stored at - 80 °C and the rest of the plant was lyophilized and kept wrapped in aluminum foil.

Table 8. Experimental design of the different negative and positive control and aqueous extract treatment replicates and application timing employed for the second plant growth assay in tomato and lettuce plants.

Treatment		Description
T1	**C-**	Negative Control (Tap Water)
T2	**C+**	Positive Control (Commercial Biostimulant + Tap Water)
T3	**S011 7/7**	S011 Aqueous Extract applied every week + Tap Water
T4	**S011 14/14**	S011 Aqueous Extract applied every two weeks + Tap Water
T5	**S011 30/30**	S011 Aqueous Extract applied every month + Tap Water
T6	**S012 7/7**	S012 Aqueous Extract applied every week + Tap Water
T7	**S012 14/14**	S012 Aqueous Extract applied every two weeks + Tap Water
T8	**S012 30/30**	S012 Aqueous Extract applied every month + Tap Water

2.7.2.1. Biochemical Assays

Foliar Photosynthetic Pigments Content Determination

The concentrations of chlorophyll a, chlorophyll b, and β-carotene were determined following Tosin et al. (2021) methodology. Approximately 300 mg of plant leaf tissue was homogenized in a mortar in cold conditions with 6 mL of acetone (80% of the volume) and quartz sand. Subsequently, the supernatants were collected by centrifugation at 3500 g for 10 minutes in a centrifuge (Eppendorf 5415 R, Germany)

after which the volume was completed to 28 mL with acetone (80%). Absorbances were measured in a spectrophotometer (DeNovix DS-11+, USA) at 480 nm, 495 nm, 645 nm and 655 nm. Corrections were made (equations (1) and (2)) following Tosin et al. (2021) methodology to account for the influence of the chlorophylls and the remaining pigments on the absorbance of the extracts at 480 nm and 495 nm. Therefore, Abs480 and Abs495 represent the corrected absorbances of the original values Abs^{o}_{480} and Abs^{o}_{495}.

$$Abs480 = Abs^{o}_{480} - 0.566 \times Abs645 + 0.121 \times Abs655 \quad (1)$$

$$Abs495 = Abs^{o}_{495} - 0.112 \times Abs645 - 0.0036 \times Abs655 \quad (2)$$

The equations below, present in the study of Bulda et al. (2008), were used to calculate the concentrations (mg/L) of the different pigments.

$$[\text{Chlorophyll a}]\ (mg/L) = 19.00 \times Abs655 - 7.61 \times Abs645 \quad (3)$$

$$[\text{Chlorophyll b}]\ (mg/L) = 21.45 \times Abs645 - 5.92 \times Abs655 \quad (4)$$

$$[\text{ß-carotenes}]\ (mg/L) = 17.16 \times Abs495 - 3.96 \times Abs480 \quad (5)$$

The final concentrations of each pigment were expressed in mg/g of fresh weight.

Total Soluble Protein quantification and glutamine synthetase (GS) activity determination

Following Martins et al. (2021), the homogenate was prepared by grinding 500 mg of fresh leaf biomass in a mortar under cold conditions with the following reagents: approximately 1 mL of extraction buffer (25 mM Tris-HCl pH 6.4 (Fisher Bioreagents, USA), 10 mM $MgCl_2$ (BDH Chemicals Ltd, USA), 1 mM dithiothreitol (DTT) (Sigma Aldrich, USA), 10% glycerol (Fisher Bioreagents, USA) and 0.05% Triton X-100), 5-10% (w/v) polyvinylpolypyrrolidone (PVPP) and quartz sand. The homogenized solution was centrifuged at 15,000 g for 20 minutes at 4 °C, after which the resulting supernatant was collected in a 1.5 mL tube and kept on ice. To quantify the soluble proteins, 50 µL of a diluted supernatant (1:50) was added to 500 µL of Bradford reagent (Bradford, 1976). Two duplicates were prepared for each condition. Following a 15-minute incubation period, the absorbances were measured at 595 nm in the spectrophotometer.

The GS activity was determined through the transferase assay, as described by Cullimore and Sims (1981). In a 1.5 mL tube, 400 µL of the reaction mixture (Trizma-Base, L-glutamine, hydroxylamine, $MnCl_2.4H_2O$, ADP (pH 6.4)) was added to 50 µL of sodium arsenate (6.4% (w/v), pH 6.4) and 50 µL of the supernatant. The solutions were incubated at 30 °C for 20 minutes in an unstirred bath equipment. The GS reaction was put to an end by the addition of 500 µL of stop solution ($FeCl_3$, trichloroacetic acid (TCA), concentrated HCl). The absorbances were recorded at 500 nm and the enzymatic activity was expressed in nkat mg^{-1} protein.

2.7.3. 3rd Assay: Extracts and Formulations Assays, and Salt-Stress Induced Conditions

Tomato (*Solanum lycopersicum* L. var. Cherry Tomato) and lettuce (*Lactuca sativa* L. var. Butter Lettuce) seedlings were obtained from Horta do Bolhão, Porto, Portugal and transplanted into 26 cL paper cups containing substrate. A total of 10 conditions were tested, comprising a negative control, a positive control, three microalgal extracts, and one cyanobacterial extract, along with their respective formulations under standard conditions. In addition, 12 other conditions were tested under salt stress conditions, which included a negative control, a positive control, S011, and S012 at NaCl concentrations of 0, 3, and 6 g/L). Every condition was tested with four replicates each. The plants were randomly arranged in a greenhouse located in Matosinhos (41°10'48.4"N 8°41'41.3"W) for 40 days, from the 20th of June 2024 to the 5th of August 2024. The plants were irrigated daily with 15 mL of tap water for the lettuce plants and 20 mL for the tomato plants. The volumes were adjusted according to the weather and temperature conditions.

2.7.3.1. S004, S009, S011 and S012 extracts and formulations growth assay

Commercial biostimulant (6 mL/L), extract and formulation (0.5 g/L) treatments were applied by soil drench three times in total and fortnightly, commencing on the first day following transplantation.

Table 9. Experimental design of the different negative and positive control and aqueous extract treatment replicates employed for the third plant growth assay in tomato and lettuce plants.

Treatment		Description
T1	**C-**	Negative Control (Tap Water)
T2	**C+**	Positive Control (Commercial Biostimulant + Tap Water)
T3	**S004**	S004 Aqueous Extract + Tap Water
T4	**S009**	S009 Aqueous Extract + Tap Water
T5	**S011**	S011 Aqueous Extract + Tap Water
T6	**S012**	S012 Aqueous Extract + Tap Water
T7	**F04**	F04 Formulation + Tap Water
T8	**F09**	F09 Formulation + Tap Water
T9	**F11**	F11 Formulation + Tap Water
T10	**F12**	F12 Formulation + Tap Water

2.7.3.2. Salt-Stress Induced Growth Assays

The microalgal strains S011 and S012 were selected for testing to ascertain their potential protective effect on lettuce and tomato plants against saline stress. For each treatment (negative control, positive control, S011 extract, and S012 extract), plants were irrigated daily with three different NaCl concentrated solutions, with concentrations of 0 g/L, 3 g/L, and 6 g/L. The treated plants were administered three doses of a commercial biostimulant (6 mL/L) and S011 and S012 extracts (0.5 g/L) via soil drench on the first day following transplantation for 40 days.

Table 10. Experimental design of the different negative and positive control and aqueous extract treatment replicates and NaCl concentrations employed for the third plant growth assay in tomato and lettuce plants.

Treatment		Description
T1	**C- 0 g/L**	Negative Control (Tap Water)
T2	**C- 3 g/L**	Negative Control (NaCl solution (3 g/L NaCl in Tap Water))
T3	**C- 6 g/L**	Negative Control (NaCl solution (6 g/L NaCl in Tap Water))
T4	**C+ 0 g/L**	Positive Control (Tap Water + Commercial Biostimulant))
T5	**C+ 3 g/L**	Positive Control (NaCl solution (3 g/L NaCl in Tap Water + Commercial Biostimulant))
T6	**C+ 6 g/L**	Positive Control (NaCl solution (6 g/L NaCl in Tap Water + Commercial Biostimulant))
T7	**S011 0 g/L**	S011 Aqueous Extract + Tap Water
T8	**S011 3 g/L**	S011 Aqueous Extract + (NaCl solution (3 g/L NaCl in Tap Water)
T9	**S011 6 g/L**	S011 Aqueous Extract + (NaCl solution (6 g/L NaCl in Tap Water)
T10	**S012 0 g/L**	S012 Aqueous Extract + Tap Water
T11	**S012 3 g/L**	S012 Aqueous Extract + (NaCl solution (3 g/L NaCl in Tap Water)
T12	**S012 6 g/L**	S012 Aqueous Extract + (NaCl solution (6 g/L NaCl in Tap Water)

At the end of the 40-day period, the plants were harvested, and the following parameters were determined: plant height, fresh weight, leaf area (in the case of the lettuce), leaf number, and pigment content.

2.7.3.3. Biochemical Assays

Foliar Photosynthetic Pigments Content

The concentrations of chlorophyll a, chlorophyll b, and β-carotene were determined by applying established analytical techniques. Approximately 200 mg of plant leaf tissue was homogenized in a homogenizer (VWR, Precellys Evolution, USA) with 1.5 mL of acetone (80% of the volume) at 11,536 g in two cycles of 60 seconds with a 30-second pause between cycles. Subsequently, the supernatants were collected by centrifugation at 5,000 g for 10 minutes in a microcentrifuge (VWR, Micro Star 17R,

USA). In a 96-well tissue culture plate, duplicates of 60 µL of each supernatant were added to 120 µL of acetone (80%). Absorbances were measured at 480 nm, 495 nm, 645 nm and 655 nm with the appropriate corrections in a microplate reader (Marshall Scientific, BioTek Synergy HT, USA). The final concentrations of each pigment were expressed in mg/g of fresh weight.

2.8. Statistical Analysis

The results of the root lengths in standard and salt stress seed germination in tomato seeds, as well as the results of the leaf area and biochemical assays of tomato and lettuce plants were tested for normality by the Shapiro-Wilk test. Then One-way ANOVA followed by a Tukey's multiple comparisons test were used to detect significant differences ($p < 0.05$) for root lengths in standard seed germination assays.

Regarding the results of the root lengths obtained from seed germination assays with the standard lettuce, commercial biostimulant optimization, S011 extraction optimization, and formulation conditions, but also germination indices, the fresh weight, the height, root and shoot size, the number of leaves and nodes plant were analyzed by a non-parametric a Kruskal-Wallis test. The statistical analysis was carried out in GraphPad® Prism 8.0.2 (GraphPad Software Inc., USA).

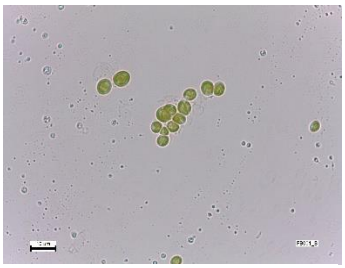
3. Results and Discussion

3.1. Fykia Biotech Culture Collection (FBCC)

At the beginning of the internship, the FBCC consisted of 31 isolates – 9 microalgae and 22 cyanobacteria (**Table 11**).

Table 11. Microalgal and cyanobacterial FBCC strains and their respective phylum, observable morphological characteristics, and culture medium in which they are maintained. Adapted from da Silva (2022).

Code Name	Phylum	Morphological Features	Culture Medium
S001	Chlorophyta	Filamentous	Liquid, Z8
S002	Chlorophyta	Short Filaments	Liquid, Z8
S003	Cyanobacteria	Coccoid	Liquid, Z8
S004	Cyanobacteria	Filamentous	Liquid, Z8
S005	Cyanobacteria	Filamentous	Liquid, Z8
S006	Chlorophyta	Coccoid	Liquid, Z8
S007	Chlorophyta	Filamentous	Liquid, Z8
S008	Cyanobacteria	Filamentous	Liquid, Z8
S009	Chlorophyta	Coccoid	Liquid, Z8
S010	Cyanobacteria	Filamentous	Liquid, Z8
S011	Chlorophyta	Filamentous	Liquid, Z8
S012	Chlorophyta	Filamentous	Liquid, Z8
S013	Chlorophyta	Filamentous	Liquid, Z8
S014	Chlorophyta	Filamentous	Liquid, Z8
S015	Cyanobacteria	Filamentous	Liquid, Z8
S016	Cyanobacteria	Coccoid	Liquid, Z8
S017	Cyanobacteria	Filamentous	Liquid, Z8
S018	Cyanobacteria	Filamentous	Liquid, Z8
S019	Cyanobacteria	Filamentous	Liquid, Z8
S020	Cyanobacteria	Filamentous	Liquid, Z8
S021	Cyanobacteria	Filamentous	Liquid, Z8
S022	Cyanobacteria	Coccoid	Liquid, Z8
S023	Cyanobacteria	Filamentous	Liquid, Z8
S024	Cyanobacteria	Filamentous	Liquid, Z8
S025	Cyanobacteria	Coccoid	Liquid, Z8
S026	Cyanobacteria	Coccoid	Liquid, Z8
S027	Cyanobacteria	Filamentous	Liquid, Z8
S028	Cyanobacteria	Filamentous	Liquid, Z8
S029	Cyanobacteria	Filamentous	Liquid, Z8
S030	Cyanobacteria	Filamentous	Liquid, Z8
S031	Cyanobacteria	Coccoid	Liquid, Z8



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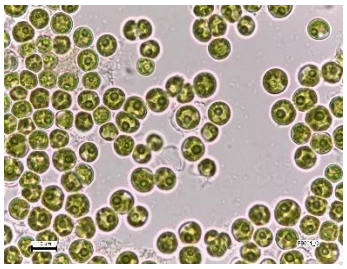

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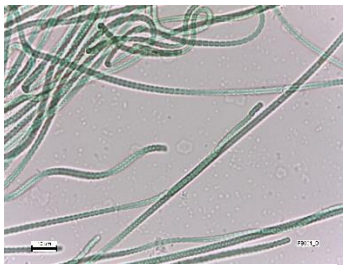

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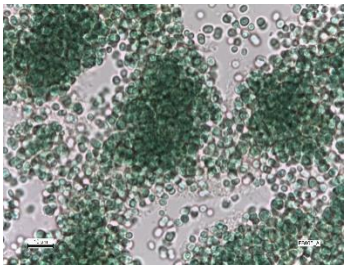

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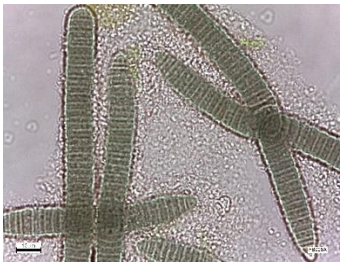

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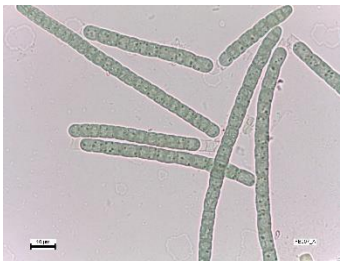

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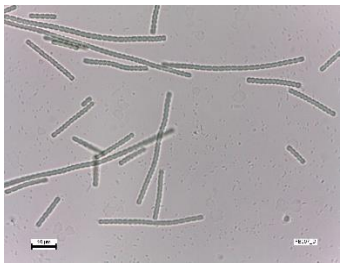

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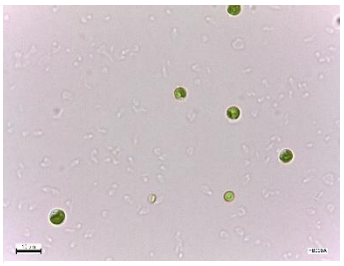

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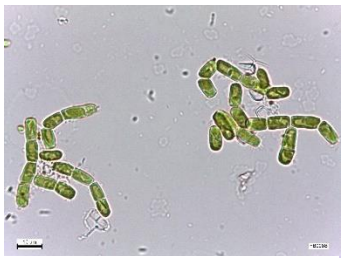

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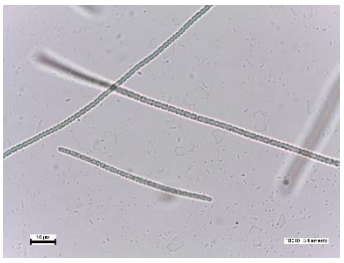

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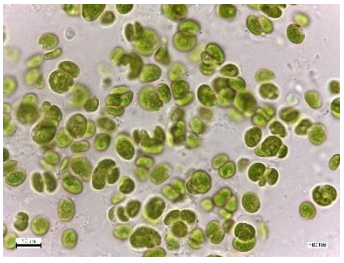

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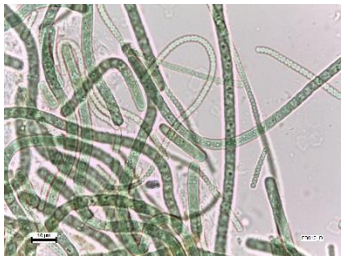

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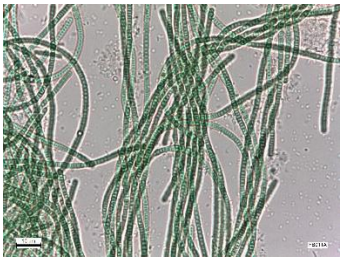

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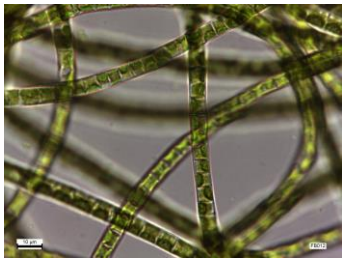

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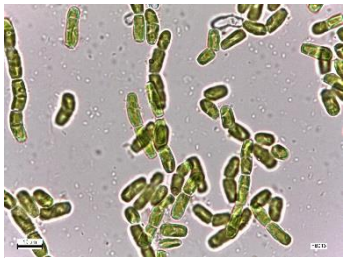

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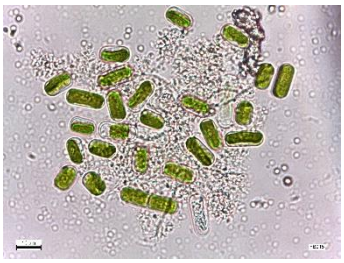

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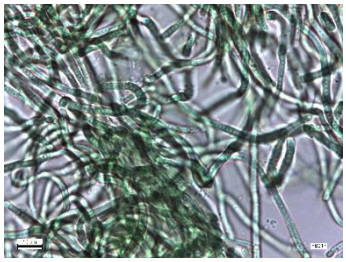

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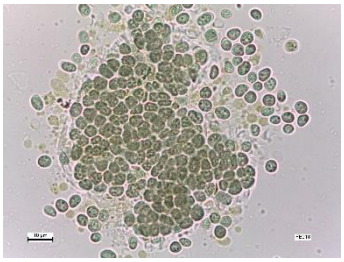

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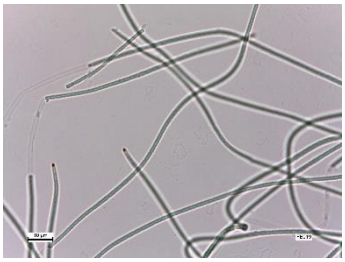

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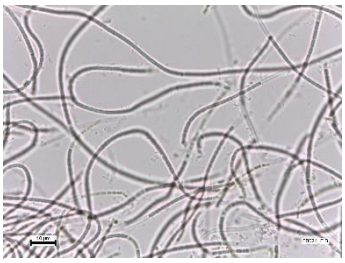

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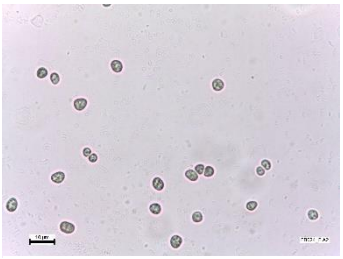

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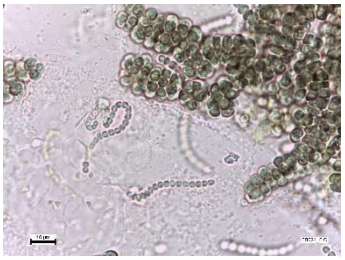

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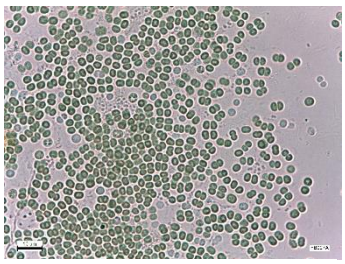
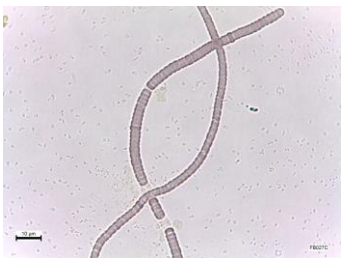


S022

S023



S024

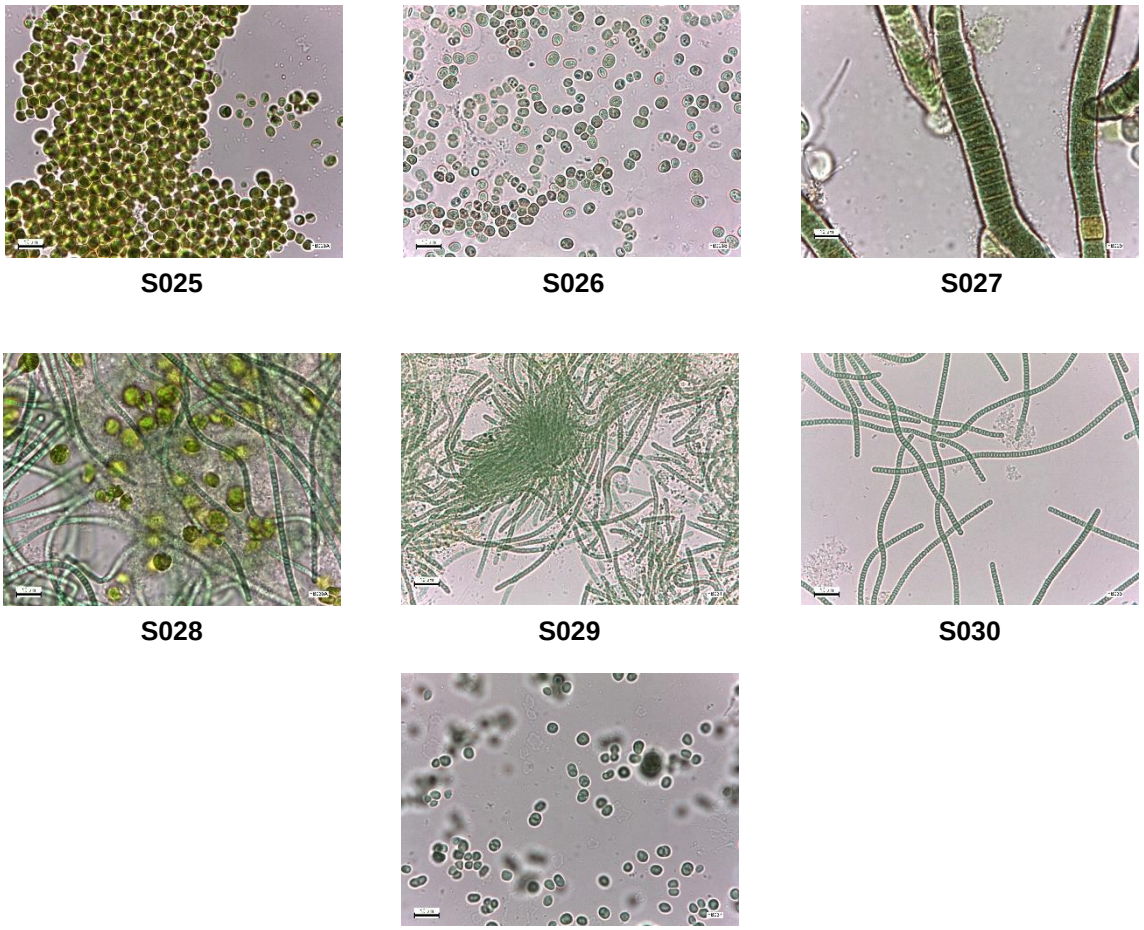


Figure 2. Microphotographs (1000x) of the 37 strains belonging to the FBCC.

3.1.1. Strain Isolation – Micromanipulation

Following observation under an optical microscope, five different culture strains – S001, S005, S008, S020, and S021 – were identified as contaminated with other microalgal or cyanobacterial strains after observation at the optical microscope. The original strains were isolated through a micromanipulation procedure (**Figure 3.**), and those at the origin of the contamination, seven in total, were maintained in Z8 medium and included in the FBCC as new members of the collection. At the end of the internship, the FBCC contained 37 strains. Subsequent molecular identification analyses will allow the elimination of duplicate strains within the collection.

Table 12. Microalgal and cyanobacterial strains isolated from a previously contaminated FBCC culture and observable morphological characteristics, and culture medium in which they are maintained.

New Strain Isolated	Original Strain	Morphological Features	Culture Medium
S001_A	S001	Coccoid	Liquid, Z8
S001_B	S001	Coccoid	Liquid, Z8
S001_C	S001	Filamentous	Liquid, Z8
S005_A	S005	Filamentous	Liquid, Z8
S005_B	S005	Filamentous	Liquid, Z8
S008_A	S008	Filamentous	Liquid, Z8
S008_B	S008	Filamentous	Liquid, Z8
S020_A	S020	Filamentous	Liquid, Z8
S020_B	S020	Filamentous	Liquid, Z8
S021_A	S021	Coccoid	Liquid, Z8
S021_B	S021	Filamentous	Liquid, Z8

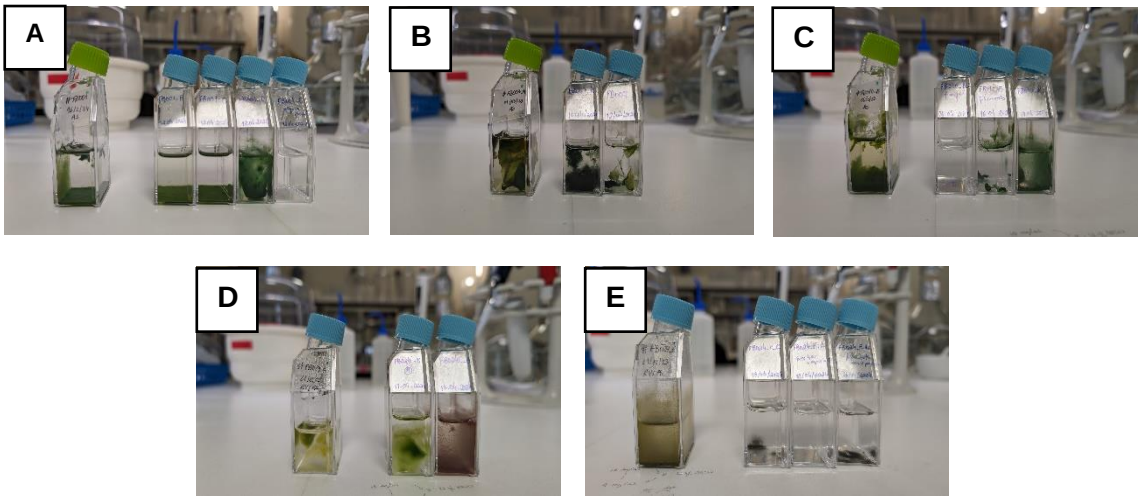


Figure 3. Culture flasks of the contaminated culture (on the left of each picture) and the resulting isolation (on the right of each picture). **A –** S001 (left), S001_A, S001_B, S001_C, unidentified fungus (right)**, B –** S005 (left), S005_A, S005_B (right)**, C –** S008 (left), unidentified fungus, S008_A, S008_B (right)**; D –** S020 (left), S020_A, S020_B (right)**, E –** S021 (left), S021_A, unidentified fungus, S021_B.

3.1.2. Molecular Identification

It was decided that only the chosen strains from the seed germination screening would undergo molecular identification. Of the four selected strains, three of them (S009, S011, and S012) had previously been identified by da Silva (2022) during her internship at Fykia Biotech. Consequently, only one strain, the cyanobacterium S004, was identified at the genus level. A comparison with the NCBI database revealed that the DNA

sequence did not correspond to any previously reported sequences. This suggests that the S004 strain may represent a novel cyanobacterial species. The molecular identification of the S004 strain is confidential and therefore it is not shared in this report.

3.2. Extracts Preparation

Of the 31 strains present in the collection, only 27 were harvested for aqueous extract preparation, as the remaining strains were found to be contaminated. Contaminated cultures impeded the direct correlation of an individual strain to potential biostimulant effects and thus these strains were discarded from the evaluation of their biostimulant potential.

3.2.1. Sonication Efficiency in Cell Disruption

All the isolated strains were subjected to sonication in order to recover the intracellular bioactive compounds present in the microalgae and cyanobacteria strains. In her internship at Fykia Biotech, Lopes (2022) evaluated three different extraction methods for their cell disruption efficiency: high-pressure extraction, enzyme-assisted extraction and sonication. The results demonstrated that sonication presented comparable extraction yields to high-pressure extraction methods and even higher yields than enzyme-assisted extraction.

The light microscopic observation (**Figure 4.**) revealed that sonication was an ineffective method for 12 strains (S002, S003, S006, S007, S012, S013, S014, S015, S016, S018, S022, and S029). It is noteworthy that five of the eight microalgal strains subjected to cell lysis through sonication retained their cellular structure intact. This is likely due to the fact that microalgae possess thicker cell walls than cyanobacteria, which makes it more challenging to access the bioactive compounds that are stored within the cell or bound to the cell membranes or the cell wall (Prisa & Spagnuolo, 2023; Stirk et al., 2020). This may suggest that sonication is not a suitable extraction method for microalgae, as it cannot guarantee the complete extraction of bioactive molecules (Prisa & Spagnuolo, 2023). Instead, sonication is more appropriate for microalgae and cyanobacteria species with low-resistance cell walls.

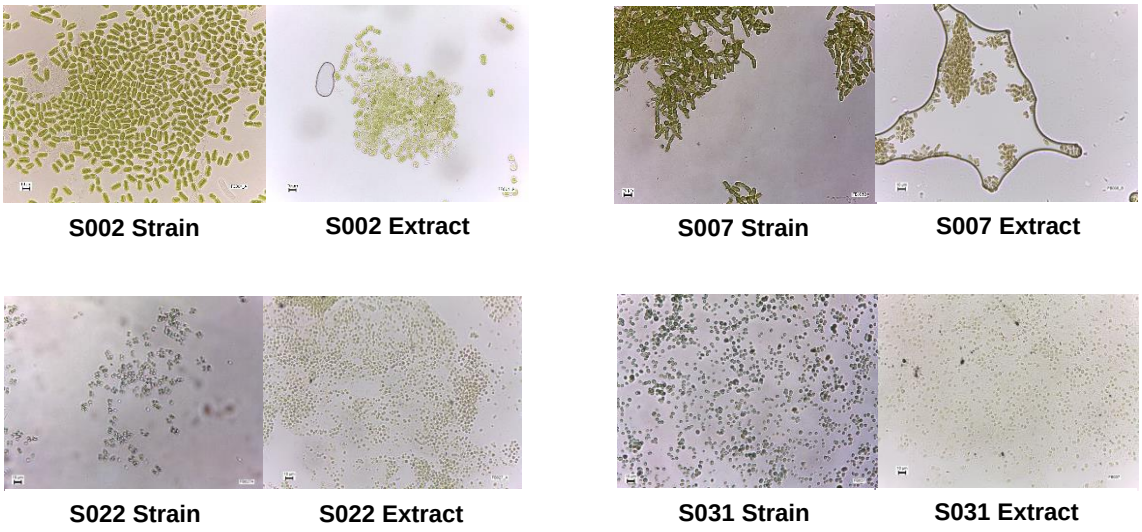


Figure 4. Comparison between microphotographs (400x) of strains prior to (on the left) and following (on the right) sonication.

3.3. Seed Germination Assays

The biostimulating properties of the microalgae and cyanobacteria strains from the collection were investigated at a concentration of 1 µg/mL. The extracts from these microorganisms were tested on tomato and lettuce seeds placed in Petri dishes as aqueous extracts obtained by sonication and ASE extraction, as well as formulations, for their seed germination-promoting activity. The performance of the extracts and formulations was determined based on their capacity to stimulate seed germination, quantified as the number of seeds that germinated after seven days in the incubator, and the final root lengths.

The Germination Index (GI) is a measure of the relationship between these two parameters and provides insight into the seed development success. A GI of 100% corresponds to seed germination and root length values obtained with distilled water (negative control) (Navarro-Lopez et al., 2020). A GI above 100% indicates plant growth stimulation, whereas values below 70-80% suggest phytotoxicity from the extract (Toribio et al., 2021).

3.3.1. Standard Assays

3.3.1.1. Positive Control Dose Optimization – Macroalgal Biostimulant Siro®

A macroalgal biostimulant was purchased to ensure the comparative efficacy of the microalgal and cyanobacterial treatments tested. Despite exhaustive efforts, no microalgae- or cyanobacteria-based biostimulant was found on the Portuguese market, and therefore a similar alternative was selected.

According to the manufacturer's recommendations, 30 mL of the commercial biostimulant should be diluted in 5 L of water. However, this dilution ratio applies to soil irrigation practices. In this *in vitro* context, the biostimulant is applied directly to the seeds, without any substrate forming a barrier between them. Therefore, a series of different concentrations of the commercial biostimulant were evaluated to determine the optimal dosage for use in the positive control.

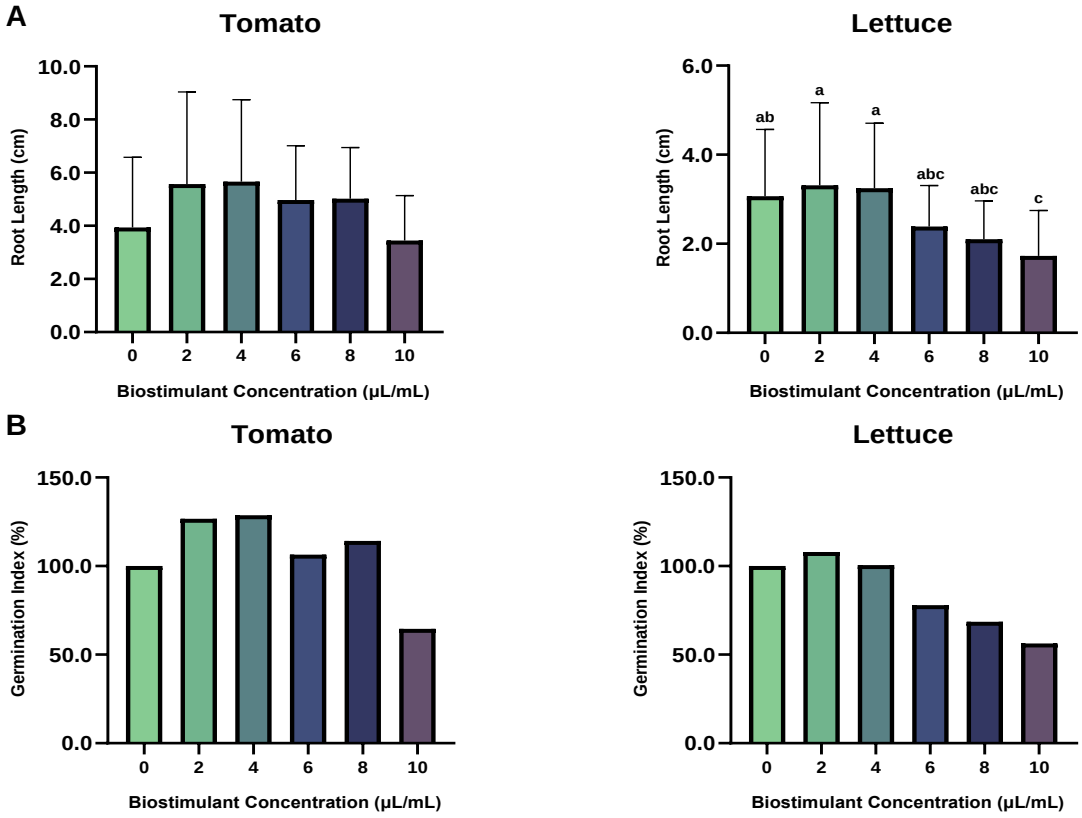


Figure 5. Influence of the treatment of the different concentrations of commercial biostimulant on the (**A**) root length and (**B**) germination index of the tomato and lettuce seeds. Results are expressed as means ± standard deviation of two replicates (n=2). Bars with different letters are significantly different ($p < 0.05$) in a Dunn's test. The absence of letters shows the lack of statistically significant differences.

The results of the root length and germination index analyses seem to indicate that lower concentrations of commercial biostimulant are generally more favorable for achieving higher germination rates of tomato seeds (**Figure 5.**). The treatment with 4 µL/mL of biostimulant, closely followed by 2 µL/mL concentration, resulted in longer roots (43.90% and 41.61%, respectively) than the control, thereby yielding a higher germination index in tomato seeds compared to the other treatments. The application of a biostimulant concentration of 10 µL/mL resulted, in contrast, in the lowest observed root elongation (12.44% lower than the control) and a germination index of 64.52%, which indicates a related phytotoxicity. This phenomenon is not particularly surprising, given that biostimulants should be applied in lower doses to guarantee a positive effect on plant development and to avoid phytotoxicity (Carillo et al., 2020; Prisa & Spagnuolo, 2023).

Lettuce seeds demonstrated a similar behavior, with the most effective treatments being 2 µL/mL and 4 µL/mL, which resulted in roots that were 8.00% and 5.90% longer than the control, respectively. The 10 µL/mL biostimulant treatment had significantly lower root lengths compared to the treatments with 2 and 4 µL/mL treatments ($p < 0.01$) and the negative control (0 µL/mL) ($p < 0.05$).

Although it could not be statistically proven that a dose of 2 and 4 µL/mL were the most effective for seed germination and root elongation in tomato and lettuce seeds compared to the negative control, the mean value of these concentrations (3 µL/mL) was utilized for the subsequent seed germination assays.

3.3.1.2. Sonication Extraction Optimization

Bioactive organic (phytohormones, polysaccharides, proteins) and inorganic (macro- and micronutrients) compounds are more readily available to the plant for absorption when the biomass is first lysed (Marín-Marín et al., 2024). Therefore, an optimization of the extraction method was undertaken with the objective of fully exploiting the bioactive molecules of the microalgae and cyanobacteria strains. For this purpose, different parameters were tested, including the sonication time and the sample/solvent ratio. The strain S011 was selected for the optimization experiment on the basis of its previous demonstration of biostimulant activity on tomato seeds.

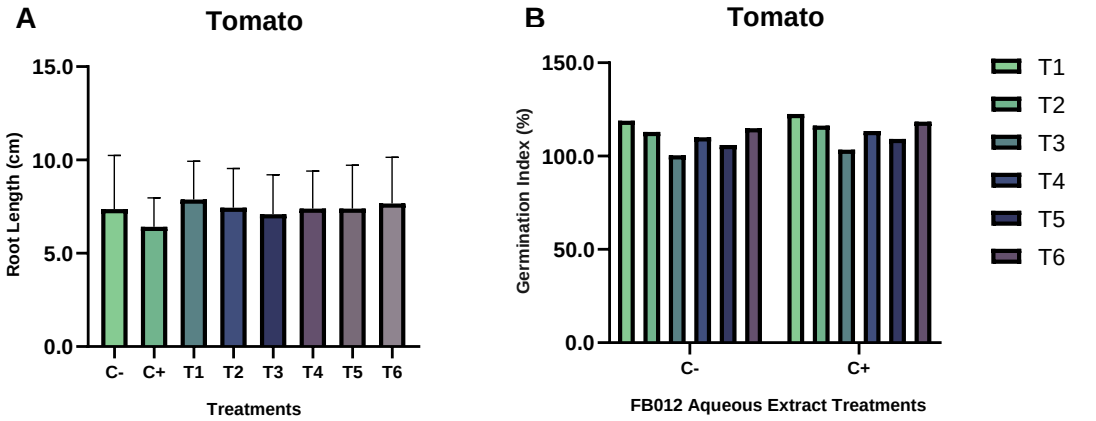


Figure 6. Mean root length (**A**) and germination index (GI) (**B**) of tomato seeds when treated with different aqueous extracts of S011 strain (T1-T6). GI values of each treatment are expressed relative to the values of the negative (C-) and the positive control (C+). Results are shown as means ± standard deviation of two replicates (n=2). (T1: 20 mg/2 mL, 2 x 20 min; T2: 20 mg/4 mL, 2 x 20 min; T3: 20 mg/10 mL, 2 x 20 min; T4: 20 mg/10 mL, 2 x 10 min; T5: 20 mg/20 mL, 2 x 20 min; T6: 20 mg/20 mL, 2 x 10 min.). The absence of letters shows the lack of statistically significant differences. The lack of standard deviation means that n=1.

Figure 6. shows the tomato root elongation and germination index for the six different treatments utilized in this assay. Upon examination of the results, it was observed that each extraction method (T1-T6) presented germination indices exceeding 100%, compared to both the negative and positive control. This indicates that all treatments had a beneficial effect on seed germination and root elongation in the tomato seeds. Extracts prepared with a biomass/solvent ratio of 10 mg/mL and a sonication duration of 20 minutes repeated twice (T1) showed a 6.97% higher values of root length (7.89 ± 1.58 cm) compared to the control and the highest germination indices (C-: 118.9% and C+: 122.5%). Therefore, the sonication method was deemed the optimal choice for the extraction of all strains. On the other hand, S011 biomass sonicated in two cycles of 20 minutes at a ratio of 2 mg/mL (T3) exhibited the lowest values of root elongation, a 3.80% reduction in comparison to the negative control, and also the lowest germination indices (C-: 100.4% and C+: 103.5%) relative to the other treatments.

The results of this assay may suggest, even though not statistically confirmed by a Dunn's test, that higher amounts of biomass in the same volume of solvent (in this case, distilled water) lead to an increased extraction of bioactive compounds from the biomass. Indeed, not only T1 but also T2 (5 mg/mL for 2 x 20 minutes of extraction time) gave the third-best results in terms of root length (7.49 ± 2.24 cm) and GI (C-: 112.9% and C+: 116.4%), just behind T6 (1 mg/mL extracted for 10 minutes repeated twice) with a RL of 7.63 ± 2.53 cm and a GI of 115.0 (C-) and 118.5 (C+).

Moreover, shorter sonication times appear to be more conducive to the efficient recovery of bioactive molecules. A comparison of T3 with T4 and T5 with T6, where the sole distinction is the duration of sonication (a total of 20 minutes versus 40 minutes), revealed that both treatments with shorter sonication times (T4 and T6) resulted in longer roots and higher GI than their respective counterparts. This could be attributed to the fact that the sonication process tends to heat the solution over time, causing the degradation of numerous molecular compounds. Surprisingly, the aqueous extract that demonstrated the most promising biostimulant effects (T1) was sonicated for a total of 40 minutes. It would be insightful to explore the potential of this extraction method by reducing the sonication time in future studies.

3.3.1.3. Aqueous Extractions

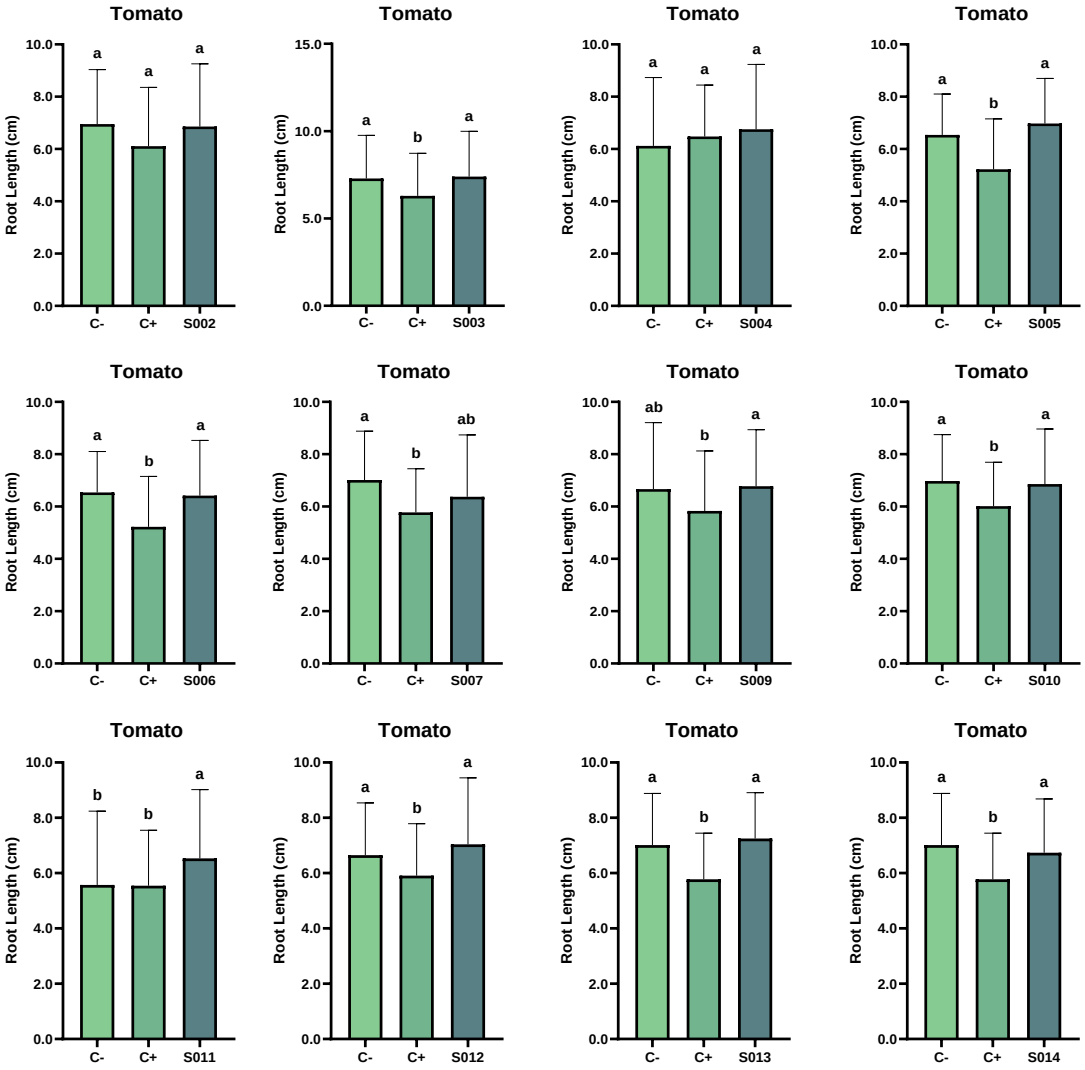
A full screening of the 27 isolates from the FBCC was performed to ascertain their influence on seed germination in tomato seeds. Some strains were also tested on lettuce seeds; however, due to time constraints, it was determined that the collection strains would only undergo a complete screening process on the seeds of a single plant. All the strains were subjected to the same extraction procedure and were applied to the seeds at the same concentration and volume, which is 1 µg/mL and 5 mL, respectively.

3.3.1.3.1. FBCC Strain Screening – Tomato Seed Germination Assays

The cultivation cycle starts with direct seed sowing for most vegetable crops. Homogeneous germination and vigorous seedling development play a pivotal role for crop establishment and performance due to a greater probability of capturing resources, tolerating biotic and abiotic stress and competing with weeds (Cardarelli et al., 2022; Krid et al., 2024). The process of germination is complex and involves the oxidation of lipids and carbohydrates within the seed, which results in the breakdown of storage proteins to obtain energy for plant development (Chaudhry & Sidhu, 2022).

The seed germination assays were able to indicate the influence of each microalgal and cyanobacterial extract on root elongation and seed germination. Of the 27 strains tested, only one – S011 – demonstrated statistically significant root growth-stimulating effects ($p < 0.05$) on tomato seeds in comparison to the negative and positive controls (**Figure 7.**). The mean root elongation of seeds treated with the S011 aqueous extract was 6.36 ± 2.38 cm, representing a 17.23% and 18.97% increase compared to the negative and the positive controls, respectively. Lopes (2022) investigated the presence of phytohormones such as IAA and IPA in S011 extracts obtained through

high-pressure extractions. The results indicated that the strain synthesized IAA, with a concentration of 1.24 µg/g dry weight. Auxins, including, IAA are phytohormones that are involved in cell division and growth in microalgae. In fact, a positive correlation has been identified between an elevated intracellular concentration of this hormone and a heightened growth rate in microalgal species (Kapoore et al., 2021). Similarly, auxins play a pivotal role in plant developmental processes such as root and shoot development (Casadesus et al., 2020; Kapoore et al., 2021; Refaay et al., 2021). The key role of IAA in root growth was corroborated by Li et al. (2023) in their study. The application of IAA to melon seedlings enhanced the expression of genes associated with root development in the treated plants. The presence of auxins such as IAA in S011 strains may be associated with the promotion of root growth.



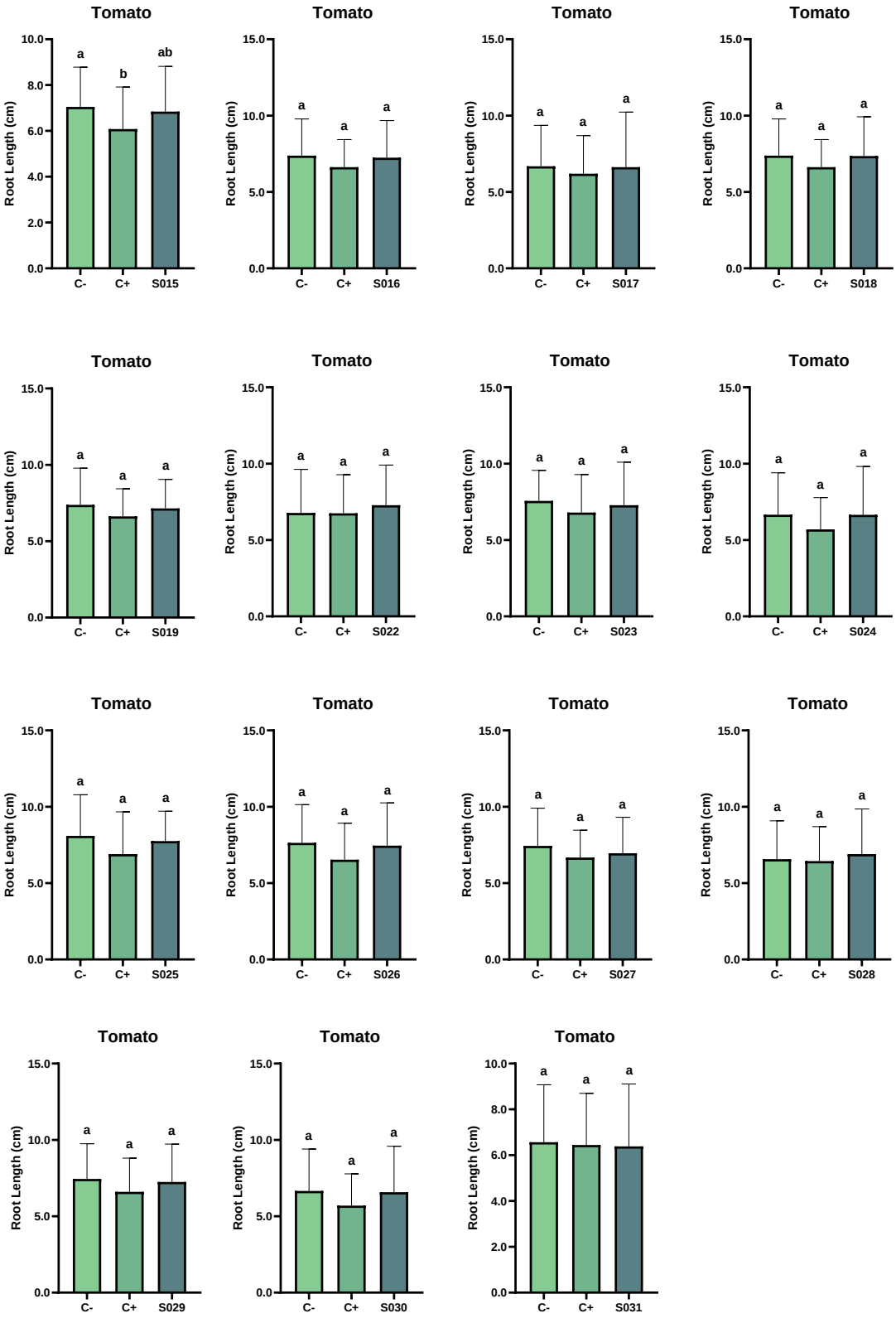


Figure 7. Mean root lengths of tomato seeds treated with aqueous extracts derived from 27 strains of microalgae and cyanobacteria. Results are shown as mean ± standard deviation (n=6). The same letter indicates no significance ($p \geq 0.05$) in a Tukey's multiple comparison test. (C-: Negative Control, C+: Positive Control).

Several extracts (S003, S005, S006, S009, S010, S012, S013, and S014) demonstrated a significant effect on the enhancement of root length in tomato seeds when compared to the positive control alone. However, this tells more about the inefficiency of the commercial biostimulant in promoting root growth than about the biostimulant power of the extracts. One potential explanation is that the commercial biostimulant is not formulated to act during the seed germination phase, but rather during the vegetative phase, as indicated in the product manufacturer's instructions. This also indicates that it is not sufficient to evaluate the biostimulant potential of a strain by assessing only the stimulation of seed germination, as this is highly specific to one phase of plant development. Therefore, a screening of the FBCC strains should also be carried out under soil conditions.

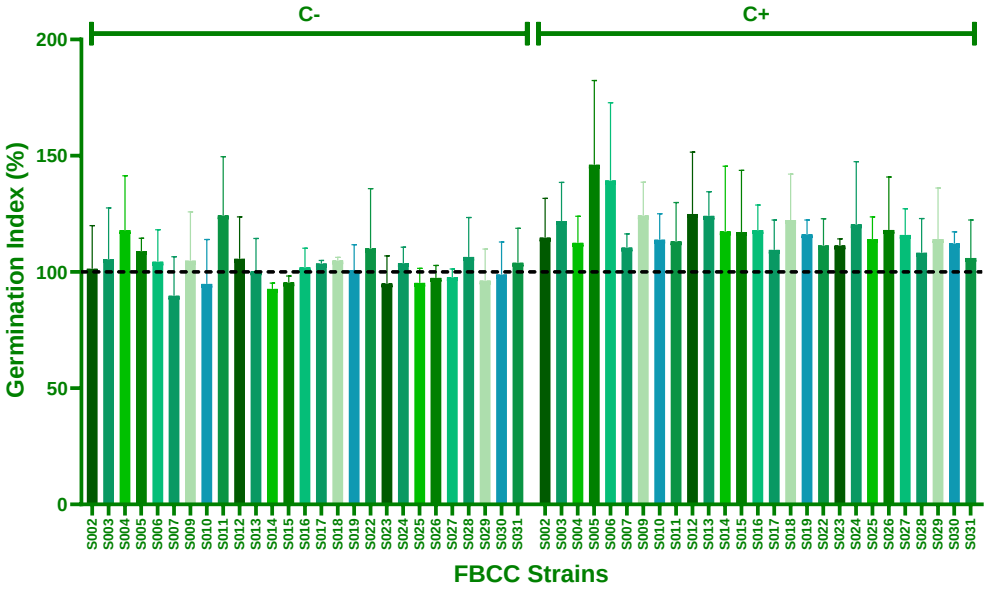


Figure 8. Germination Indices (GI) obtained from the aqueous extracts of 27 strains of the FBCC employed in seed germination assays on tomato seeds. The left part of the graph shows the GI of the negative control, while the right part shows the GI of the positive control. The GI is expressed as a percentage. The bars represent the GI mean ± standard deviation at n=3 and $p < 0.05$. The absence of letters shows the lack of statistically significant differences.

The objective of this screening was to select four strains for subsequent testing in plant growth and seed germination assays under standard and salt stress conditions, and as formulation products. The microalgal strain S011 was selected as the optimal candidate, exhibiting the greatest statistical difference in biostimulant activity compared to the control group. The remaining three strains, S012, S009, and S004 were selected for further study. The reason for studying the microalgal strain S012 was analogous to that of S011. Firstly, both strains had been previously investigated by da Silva (2022) and Lopes (2022), with both studies concluding that the strains possessed biostimulant

activity. Lopes (2022) also proceeded to conduct a chemical analysis of the composition of these extracts, with a focus on the presence of phytohormones and the NPK composition. Additionally, microalgae are typically regarded as a safer option for the general public and the scientific community due to the general absence of harmful toxins produced by these microorganisms, in contrast to cyanobacteria, which can produce cyanotoxins. Furthermore, European regulations about biostimulants are considerably more stringent for cyanobacteria-based biostimulants. Therefore, entering the market with a microalgae strain would be a more advantageous strategy. Finally, although the specific growth rates of these two strains were not quantified, they were observed to be among the fastest-growing strains in the collection, which is a key factor for industrial scalability. Considering the germination index results (**Figure 8.**), although no statistical difference was observed among the extract treatments, S004 and S009 strains were selected based on their positive early seed germination results in comparison to the other strains.

3.3.1.3.2. Lettuce Seed Germination Assays

Ten microalgae and cyanobacteria strains were tested for seed germination on lettuce seeds. The aqueous extracts of the S011 and S012 strains were the only treatments to demonstrate a significant positive impact on root growth in lettuce plants (**Figure 9.**). The mean root length of seeds treated with the S012 aqueous extract was 4.70 ± 1.80 cm, representing a significant ($p < 0.05$) increase of 56.59% compared to non-treated plants. In contrast, the S011 strain showed a significantly ($p < 0.01$) increase of root elongation (40,20%) compared to the positive control. Given that these two strains also promoted significantly higher root development in tomato seeds, it can be inferred that the biostimulant capacity of these two strains is transversal to more than one plant species. Consequently, there is a possibility that they could be used in the future as an active ingredient in a general biostimulant. However, further assays should be conducted to verify the replicability of the results.

The germination indices (**Figure 10.**) indicate that several strains, especially S002, S003, S011, S012, and S031, produced biostimulant effects in lettuce seeds, as evidenced by the increased root elongation and germination rates relative to the negative control. It should be noted, however, that only the seed germination assays involving S011 extracts were repeated in lettuce seeds, and thus there is no statistical evidence to suggest that the results would be replicated.

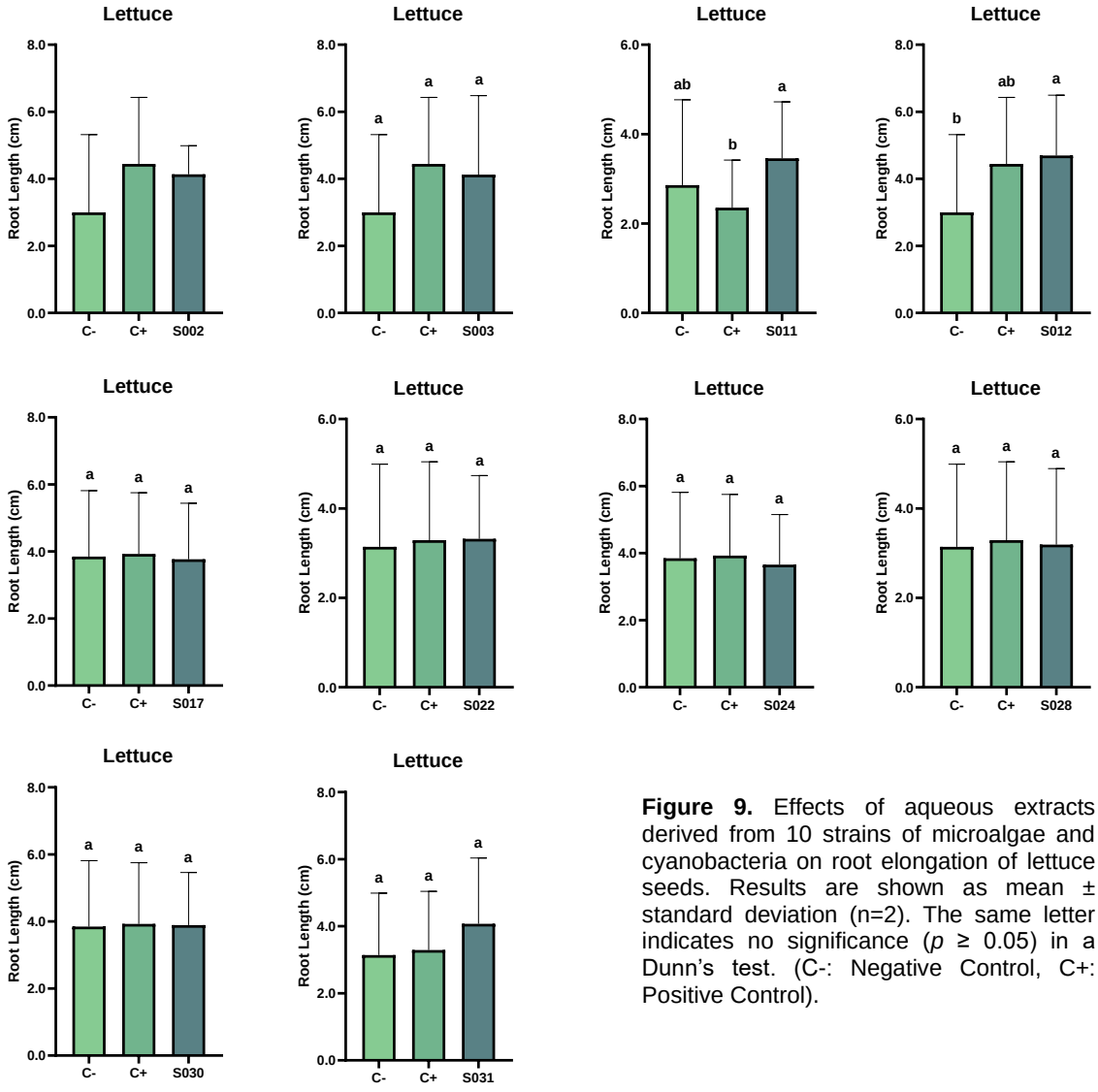


Figure 9. Effects of aqueous extracts derived from 10 strains of microalgae and cyanobacteria on root elongation of lettuce seeds. Results are shown as mean ± standard deviation (n=2). The same letter indicates no significance ($p \geq 0.05$) in a Dunn's test. (C-: Negative Control, C+: Positive Control).

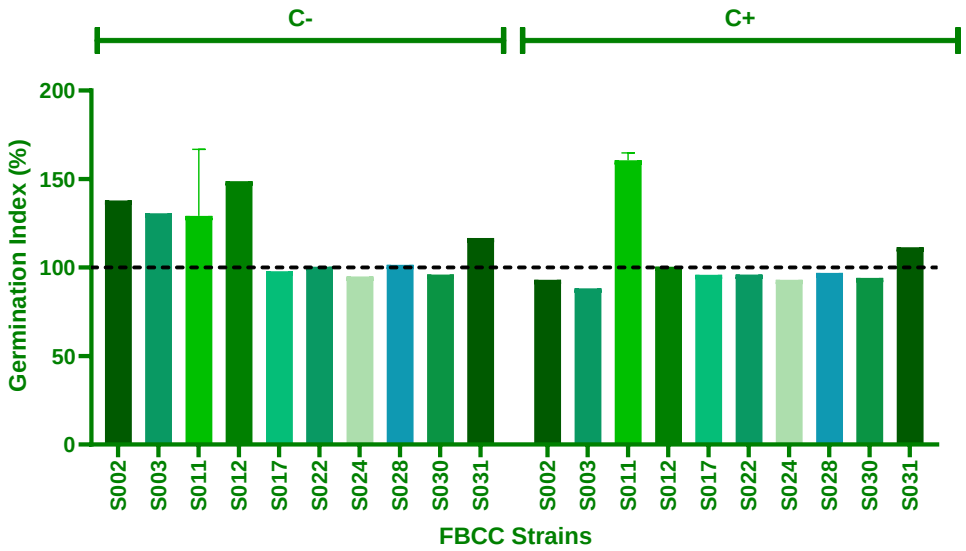


Figure 10. Germination Indices (GI) obtained from the aqueous extracts of 10 strains of the FBCC employed in seed germination assays on lettuce seeds. The left part of the graph shows the GI of the negative control, while the right part shows the GI of the positive control. The bars represent the GI mean ± standard deviation at n=2 and $p < 0.05$.

3.3.1.3.3. ASE Extracts

An alternative extraction method, aside from sonication, was experimented on the S011 strain. Only a single strain extract could be obtained through this process, after which the equipment ceased to function. The ASE extraction is a technique employed for the extraction of compounds from solid samples using solvents (in this case, distilled water) at elevated pressures. This alternative extraction method was tested on the grounds that the efficiency and speed of the extraction process were hypothesized to be superior to sonication. The aqueous extracts obtained through this extraction procedure were employed in seed germination assays in both tomato and lettuce seeds.

Upon examination of **Figure 11.**, it appears that although the treatment with S011 aqueous extracts obtained from ASE induced a higher germination index in tomato and lettuce (for the positive control only) seeds, suggesting a phytostimulating effect, the statistical analysis did not allow the assertion of a significant difference between the treated seeds and their respective controls. Given that the S011 strain had previously demonstrated stimulation of the germination and root development in seeds, it may be hypothesized that the observed inefficiency of the ASE is possibly due to a lack of optimization of the process. The concentration of the aqueous extract utilized for the seed germination treatments was identical to that employed for the extracts prepared

through sonication. It can be reasonably assumed that the two extraction methods have different cell lysis yields, which in turn results in the release of different quantities of bioactive compounds into each extract. It is also worth noting that plants are highly susceptible to fluctuations in phytohormone concentrations for instance. As previously discussed, a lower dose can promote plant growth and development, whereas a higher dose can inhibit several metabolic pathways. Consequently, the ASE method may offer a viable solution to compensate for the relatively low yields of cell disruption obtained by the sonication, especially for strains with thicker cell walls.

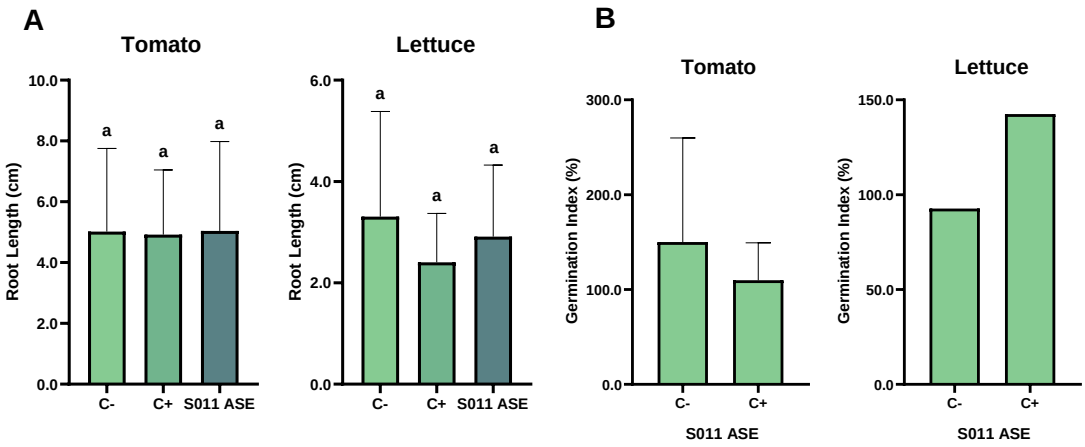


Figure 11. Mean root length (**A**) and germination index (**B**) of tomato and lettuce seeds treated with aqueous extracts of S011 strain obtained from ASE extraction. Results are shown as mean ± standard deviation (n=2 for lettuce, n=4 for tomato). The same letter indicates no significance ($p \geq 0.05$) in a Dunn's test. (C-: Negative Control, C+: Positive Control). The absence of letters shows the lack of statistically significant differences.

3.3.1.3.4. Formulations

Microalgal formulations of the strains S001, S011, S012, and a combination of the latter two were developed by Lopes (2022) for her Master's thesis. Following an investigation into the potential effects of these formulations on tomato seeds through a seed germination assay, the author concluded that the formulations did not negatively impact seed germination or root elongation. Indeed, it was observed that the S012 formulation, as well as the formulation of the combined strains of S011 and S012, stimulated significantly longer roots than those of the controls. Therefore, the formulations retained the same bioactivity as their respective crude aqueous extracts. This experiment was repeated for this project. Four products, F04, F09, F12 and F11, were formulated with the four selected strains, S004, S009, S011 and S012.

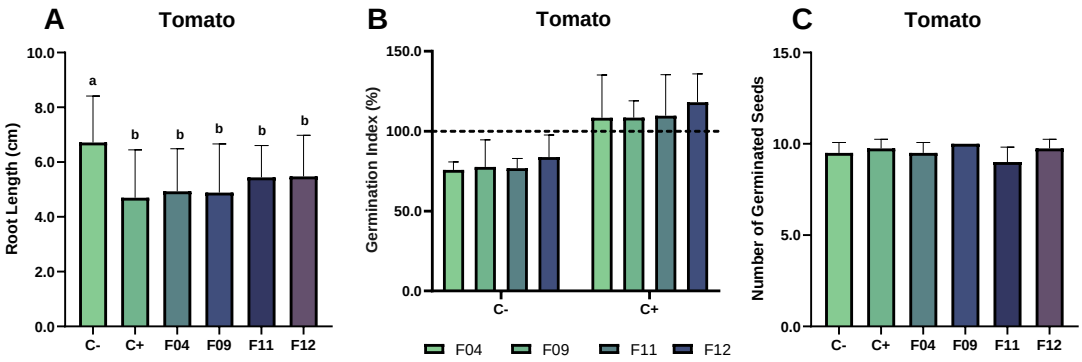


Figure 12. Root lengths (**A**), germination indices (**B**) and number of germinated seeds (**C**) after a 7-day incubation of tomato seeds treated with the formulations (F04, F09, F11 and F12) and the controls (C- and C+). Data are means ± standard deviation at n=2 and $p < 0.05$ and statistically tested for significance with Dunn's multiple comparisons test. The absence of letters shows the lack of statistically significant differences.

The results of these seed germination assays were not in accordance with those previously reported by Lopes (2022). After seven days of incubation, the seeds treated with the formulations showed significantly reduced root lengths (F04 and F09: $p < 0.0001$, F11: $p < 0.01$, F12: $p < 0.05$) compared to the non-treated seeds (C-), as it can be observed in **Figure 12.**. Low GI values (inferior to 80%) may indicate the formulations are in fact phytotoxic to the seeds (Toribio et al., 2021). Paradoxically, despite the apparent hindrance to root development caused by the extracts, the seed germination process was not adversely affected. There was no statistically significant difference in the number of germinated seeds across the various treatments. Consequently, it can be inferred that root germination and root development are not proportionally affected by the active compounds.

3.3.1.4. Salt Stress Assays

One of the most evident consequences of salt exposure in seeds is the reduction in root length, equally observed by Chanthini et al. (2022) and Hernández-Herrera et al. (2013). Non-stressed (0 g/L NaCl) seeds exhibited the greatest root lengths, and as the concentration of NaCl increased, the elongation of roots decreased. In terms of salinity tolerance, only the S012 aqueous extract demonstrated statistically significant ($p < 0.01$) root length changes (26.17%) in seeds grown at 3 g/L NaCl concentration compared to the positive control (**Figure 13.**). No discernible protective effect was recorded in the seeds after treatment with the four extracts. NaCl concentrations of 12 g/L were also evaluated, yet no seed germinated in these conditions across all treatments and therefore this salt stress condition was not considered in the graphs.

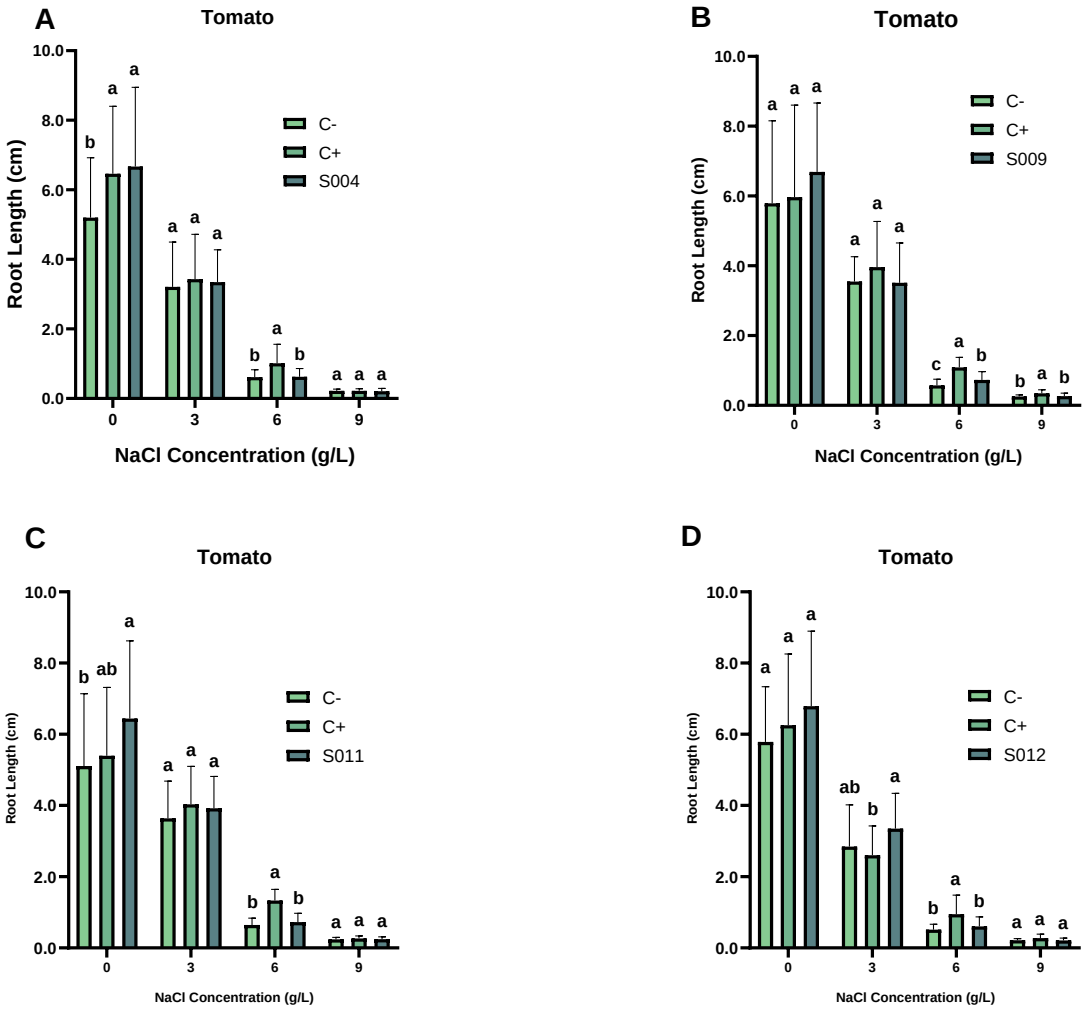


Figure 13. Mean root lengths in tomato seeds under different salt levels (0, 3, 6 and 9 g/L). The different letters indicate significant differences obtained by Tukey's multiple comparisons test between the controls (**C-** - Negative Control, **C+** - Positive Control) and the four aqueous extracts treatments (**A** – S004, **B** – S009, **C** – S011, **D** – S012) under the same NaCl concentrations. The bars represent the mean values ± standard deviations at n=4. Bars with the same letters show the lack of statistically significant differences.

Compared to the negative control, the majority of the extracts had a protective effect against salt stress at concentrations of 3 and 6 g/L of NaCl, although this was not statistically significant (**Figures 13.** and **14.**) Treatment with S012 strain demonstrated a higher capacity to mitigate stress in tomato seeds, conferring a germination index that was 20.7% superior to that observed with distilled water at 3 g/L NaCl concentration. Under 6 g/L NaCl conditions, the S009 treatment displayed a 31.29% more elevated GI in comparison to the negative control. However, at a concentration of 9 g/L, the extracts did not show any mitigation effect. In general, the commercial biostimulant demonstrated a better performance in terms of salt stress protection compared to the extracts.

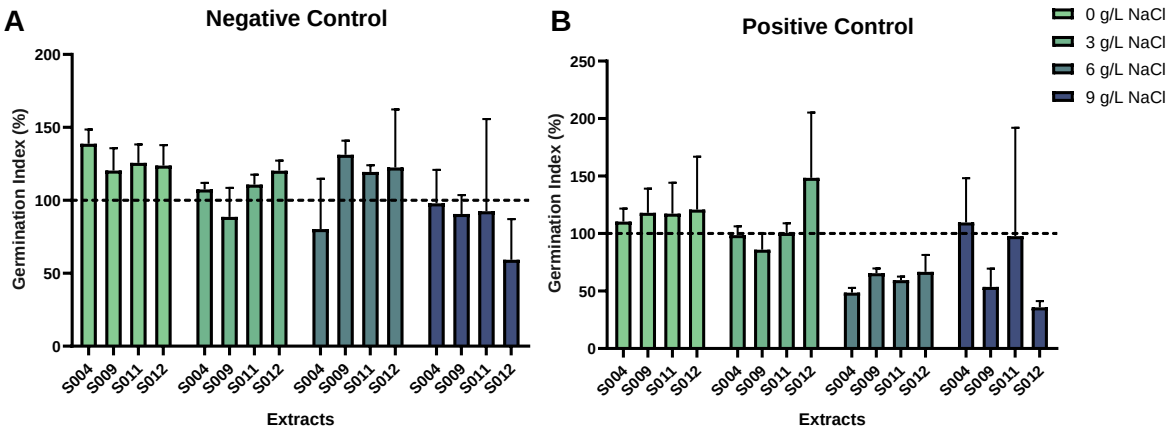


Figure 14. Mean Germination Indices relative to the negative control (**A**) and the positive control (**B**), obtained through a treatment of the tomato seeds with four aqueous extracts under four different NaCl-concentrated conditions. The mean ± standard deviation values at n=4 and $p < 0.05$ are represented as bars. The absence of letters shows the lack of statistically significant differences.

Although the results of the seed germination assays were statistically inconclusive, with no significant improvements found between the controls, this does not automatically rule out the possibility of the strains being effective in a salt stress context. Indeed, a number of factors, such as the extraction success of protective bioactive compounds, in addition to the inherent presence of bioactive compounds in the FBCC strains, may have affected the success of the extracts as biostimulants.

3.3.1.5. Standard Assays with PBS Buffer

During this internship, there was a period during which the germination rates of lettuce seeds were observed to be lower than expected. The pH of the distilled water used in the laboratory exhibited considerable inconsistency and a slightly acidic quality, with values spanning from 4.96 to 6.71. It was therefore initially hypothesized that the low pH of the distilled water employed in the germination assays was a principal factor contributing to the reduced germination rates, particularly in lettuce seeds. The use of a phosphate-buffered saline (PBS) buffer solution was considered a potential means of maintaining neutral pH levels.

After an experiment in which lettuce seeds were supplemented with PBS buffer instead of simple distilled water, it was concluded that this solution was not a viable option for this particular assay, as it hindered the root development of the lettuce seeds in the Petri dishes (**Figure 15.**). Moreover, it was determined that the presence of phosphate compounds in the buffer could potentially influence the outcomes, as they represent a crucial macronutrient for plant growth. The sustained low germination rates

observed in lettuce seeds served as a key factor in the decision to discontinue the full screening assays on these seeds while maintaining the use of tomato seeds exclusively.

Figure 15. Failed seed germination assay, where lettuce seeds were supplemented with PBS buffer.

3.4. Plant Growth Assays

3.4.1. 1st Assay: Water Volumes Optimization Assay

Before testing the biostimulant capacity of microalgae and cyanobacteria extracts under soil conditions, it was necessary to optimize several parameters such as the type and volume of water to be used, as well as its frequency of application. This was done to ensure the best conditions for plant growth inside the incubator.

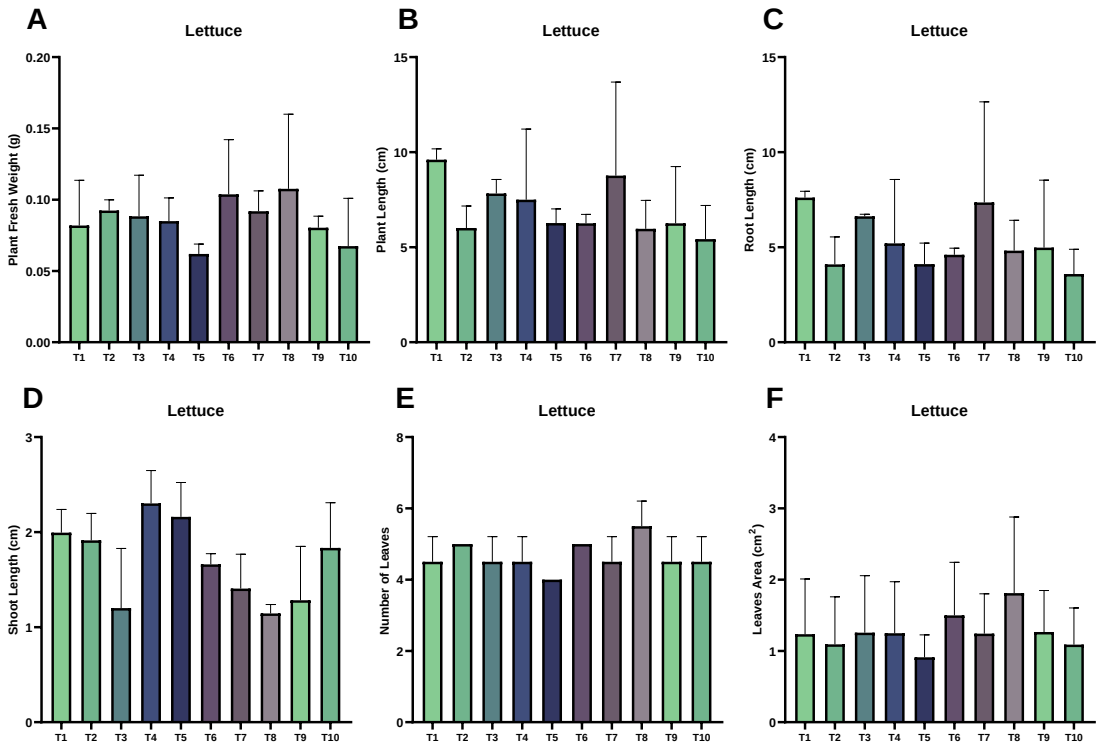


Figure 16. Mean values of plant fresh weight (**A**), plant (**B**), root (**C**) and shoot length (**D**), number of leaves (**E**) and foliar area (**F**) of lettuce seedlings watered with different water volumes and frequencies. Values presented are mean ± SD. T1 – 5.0 mL of distilled water three times a week; T2 - 5.0 mL of tap water three times a week; T3 – 7.5 mL of distilled water twice a week; T4 - 7.5 mL of tap water twice a week; T5 – 10.0 mL of distilled water twice a week; T6 – 10.0 mL of tap water twice a week; T7 – evaporation method: 8.6 mL of distilled water three times a week; T8 – evaporation method: 8.6 mL of tap water three times a week; T9 – water as needed with distilled water; T10 - water as needed with tap water. The absence of letters shows the lack of statistically significant differences.

No significant differences were observed among the different treatments with respect to the analyzed parameters. Nevertheless, as illustrated in **Figure 16.**, the lettuce seedlings that were irrigated with 8.6 mL of tap water (calculated after the evapotranspiration loss) three days per week (T8) had the highest fresh weight (0.108 ± 0.052 g) (**A**) and foliar area (1.812 ± 0.052 cm^2) values (**F**), as well as the highest number of leaves per plant (5.5 ± 0.5) (**E**). As illustrated in **Figure 16B** and **C**, the whole plant and roots exhibited greater development in seedlings treated three times per week with 5 mL of distilled water (T1). As for shoot length, the mean value of 2.302 ± 0.346 cm was observed for plants treated with 7.5 mL of tap water applied twice a week (T4), which was the highest among the different treatments (**Figure 16D**). Seedlings watered twice a week with 10 mL of distilled water showcased the least favorable outcomes in terms of fresh weight, number of leaves, and foliar area.

In light of the morphological findings, the T8 treatment was considered to be the most suitable for implementation in plant growth assays. The evapotranspiration method

is the most accurate of all the methods tested, providing a more precise calculation of the water requirements of the plant. It was observed that the soil moisture was retained for a longer period in the plants irrigated with tap water. This is evidenced by the morphological results of the lettuce seedlings, which demonstrated that treatments with tap water resulted in higher fresh weight values than their distilled water counterparts. Another reason to adopt tap water in plant growth assays is that it more closely resembles the conditions observed in farm fields thereby yielding results that better reflect agricultural conditions. Crops are irrigated with water that contains impurities such as minerals, salts, and other contaminants, which renders it less pure than distilled water.

3.4.2. 2nd Assay: Extracts Application Frequency

The frequency of application of a biostimulant is an important parameter that can influence the efficacy of the treatment. For that reason, S011 and S012 aqueous extracts were applied once, twice, and four times a month in lettuce and tomato seedlings to ascertain the optimal frequency of application. The assay followed the growth and development from seeds to full-grown plants for a period of two and a half months.

Morphological Parameters

The initial observation that can be drawn from **Figure 17.** and **18.** is that there are no discernible effects on plant growth effects following the treatment with S011 and S012 aqueous strains, regardless of the frequency of application. This is evidenced by the absence of statistical significance.

In tomato seedlings (**Figure 17.**), the S011 7/7 and S011 14/14 treatments demonstrated the best results in terms of fresh weight, number of leaves, shoot and plant length among all treatments. It can be observed that, in comparison to the controls, the aqueous extracts did not seem to promote plant growth effects in terms of fresh weight gain, exhibiting lower mean values. Nevertheless, treatments with S011 (7/7 and 14/14) demonstrated 22.50% and 23.75% higher mean values of the number of leaves respectively and 8.62% and 15.20% greater mean values of whole plant weight in comparison to the negative control (distilled water). When comparing the positive control to the other treatments, only the length parameters appeared to be slightly more enhanced following the application of S011 7/7 and S011 14/14, displaying a 3.11% and 9.37% increase, respectively. With regard to S012 aqueous extracts, tomato plants exhibited enhanced growth when treated with the extract twice a week (S012 7/7),

showing a 14.12% and 9.11% increase relative to the negative and positive controls, respectively.

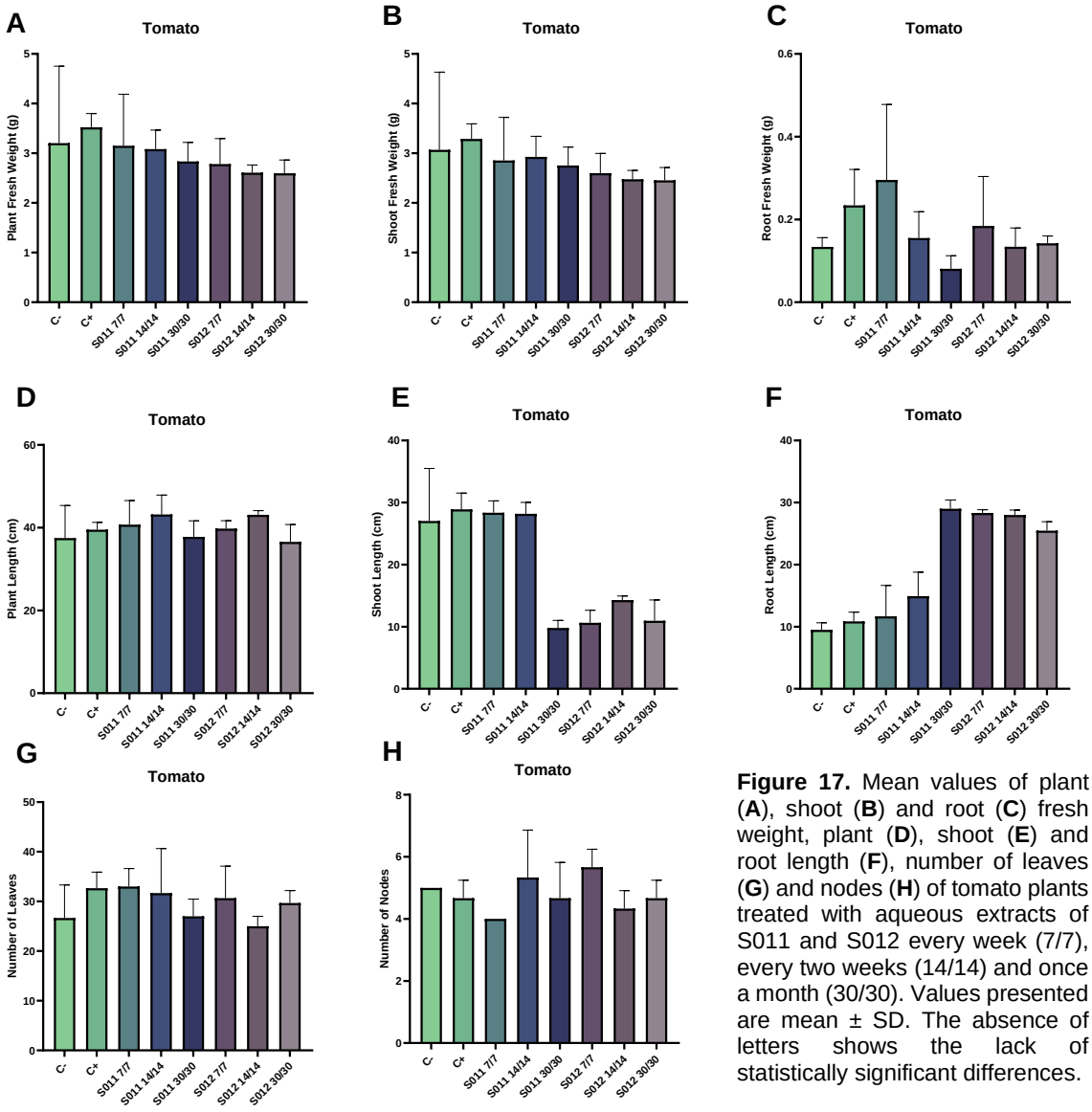


Figure 17. Mean values of plant (**A**), shoot (**B**) and root (**C**) fresh weight, plant (**D**), shoot (**E**) and root length (**F**), number of leaves (**G**) and nodes (**H**) of tomato plants treated with aqueous extracts of S011 and S012 every week (7/7), every two weeks (14/14) and once a month (30/30). Values presented are mean ± SD. The absence of letters shows the lack of statistically significant differences.

In the case of lettuce plants, the S012 strain seemed to be more efficient as a biostimulant than S011, as opposed to the results observed in tomato plants (**Figure 18.**). Indeed, the greatest increases in all the morphological parameters (plant growth, weight, number of leaves, and foliar area) were observed in lettuce plants treated with S012 aqueous extracts applied weekly (S012 7/7). However, these results must be interpreted with caution, as they are based on the single surviving replicate out of the three original replicates.

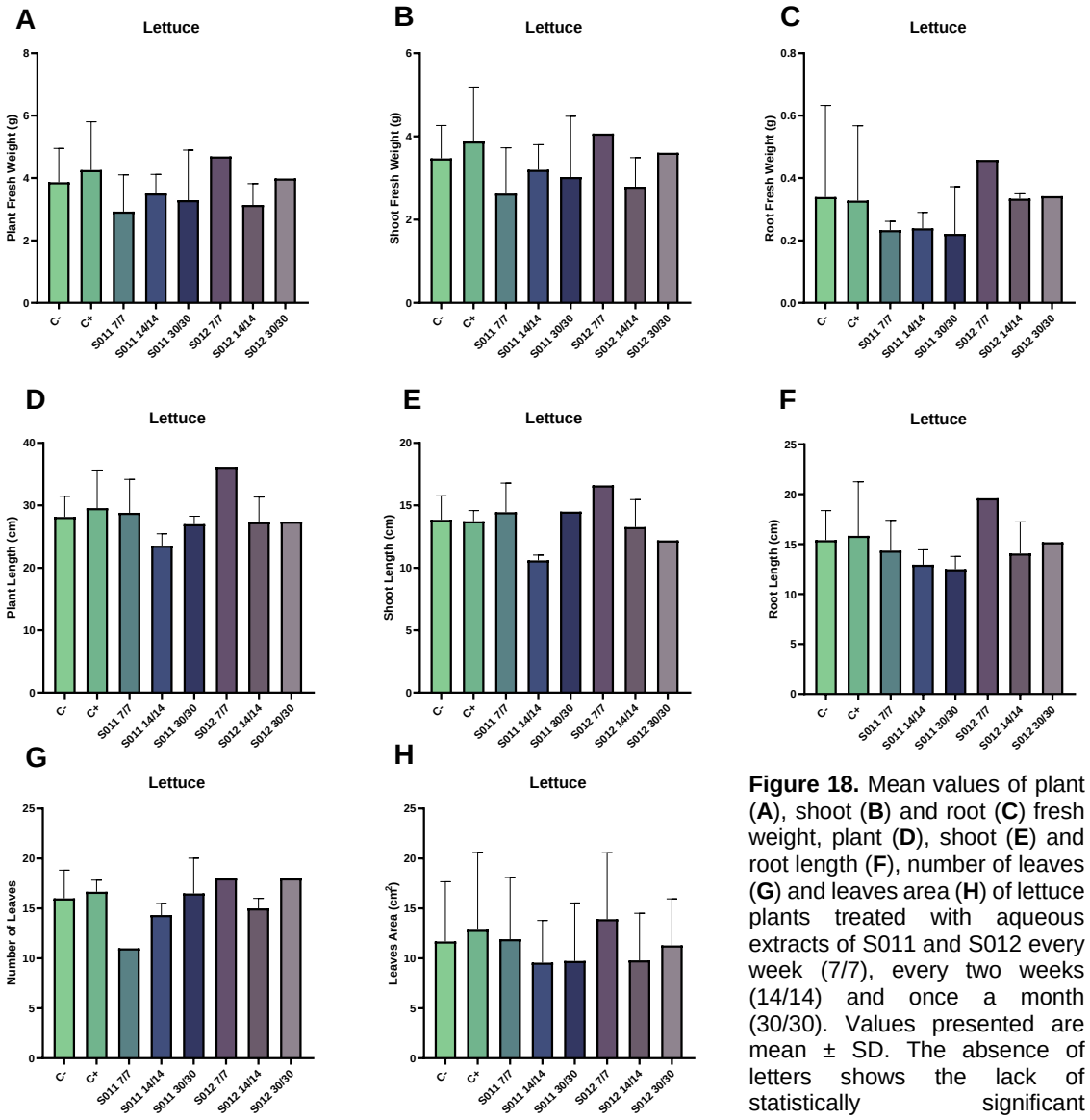


Figure 18. Mean values of plant (**A**), shoot (**B**) and root (**C**) fresh weight, plant (**D**), shoot (**E**) and root length (**F**), number of leaves (**G**) and leaves area (**H**) of lettuce plants treated with aqueous extracts of S011 and S012 every week (7/7), every two weeks (14/14) and once a month (30/30). Values presented are mean ± SD. The absence of letters shows the lack of statistically significant differences.

Photosynthetic Pigment Content in Leaves

Photosynthetic pigments such as chlorophylls a, chlorophylls b, and β-carotenes, are key molecules for plant growth. They are involved in the aerobic photosynthetic process in plants, which permits carbon assimilation and transformation into biomass. It is estimated that approximately 90% of plant biomass is derived from the assimilation of CO_2. Therefore, the presence of photosynthetic pigments in the plant leaves might indicate a more robust rate of photosynthesis and, consequently, a more pronounced plant growth (Kour et al., 2020).

The results of the pigment content analysis in tomato plants are depicted in **Figure 19.**. Although no significant differences were identified, the results indicate that all treatments contributed to an increase in the concentration of chlorophylls a and b and β-carotenes in the leaves of tomato plants. The concentrations of chlorophyll a and b were observed to be greater when fewer extract applications were administered (i.e., once per month). The S011 aqueous extracts applied once a month (S011 30/30) promoted the highest chlorophyll a and b concentrations in the plants, with increases of, respectively, 58.42% and 51.44%, compared to the negative control. With regard to β-carotene, the leaves of plants treated with S012 extracts applied every week (S012 7/7) displayed an increase of 73.10% in its content.

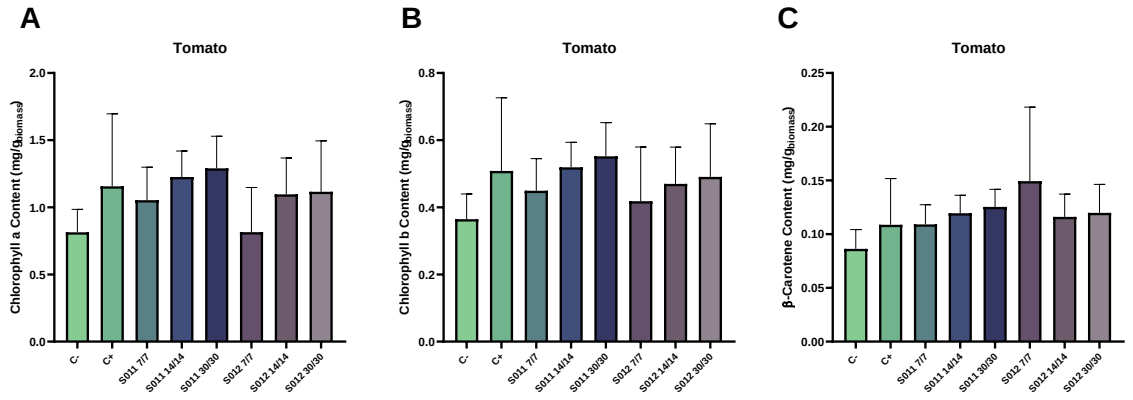


Figure 19. Mean values of the chlorophyll a (**A**), chlorophyll b (**B**) and β-carotene (**C**) content in the leaves of tomato plants treated with aqueous extracts of S011 and S012 every week (7/7), every two weeks (14/14) and once a month (30/30). Values presented are mean ± SD. The absence of letters shows the lack of statistically significant differences.

The application of the S011 aqueous extract applied at two-week intervals (S011 14/14) was the most effective treatment for increasing the levels of the chlorophylls a and b and β-carotenes in lettuce plants. The observed increases in these compounds were 9.63%, 7.93%, and 133.02%, respectively (**Figure 20.**).

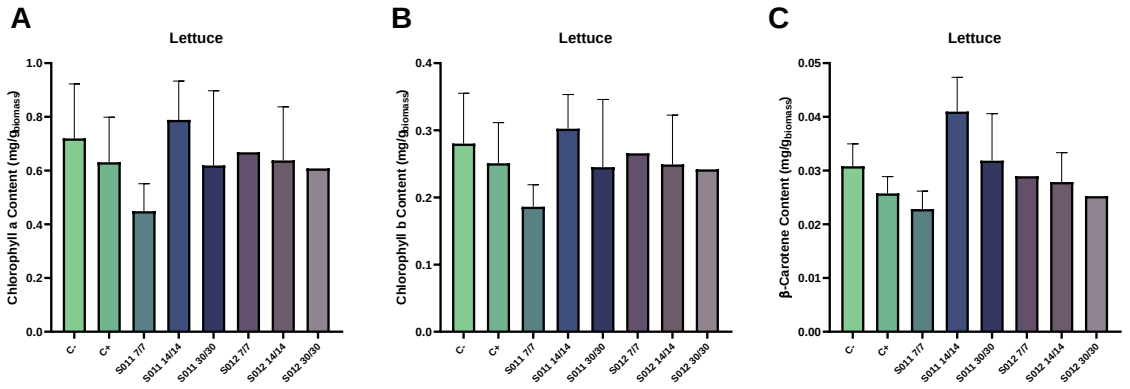


Figure 20. Mean values of the chlorophyll a (**A**), chlorophyll b (**B**) and β-carotene (**C**) content in the leaves of lettuce plants treated with aqueous extracts of S011 and S012 every week (7/7), every two weeks (14/14) and once a month (30/30). Values presented are mean ± SD. The absence of letters shows the lack of statistically significant differences.

Total Soluble Protein Content and GS Enzymatic Activity

Glutamine synthetase (GS) is a key enzyme in the nitrogen metabolic pathway, as it catalyzes the synthesis of glutamine from glutamate and ammonium ions in organisms such as plants. Plants are dependent on inorganic nitrogen uptake as an essential nutrient for growth and development (Yin et al., 2022). The product of the enzymatic reaction, glutamine, is used as a starting block for the biosynthesis of other amino acids, nucleic acids, tricarboxylic acid cycle intermediates, and chlorophylls, all of which are essential for plant development (Németh et al., 2018).

The aqueous extracts of the S012 strain have been observed to enhance the enzymatic activity of the GS in the leaves of tomato plants (**Figure 21.**). The effects were more pronounced when the extract was applied two or only once a month (S012 14/14 and S012 30/30), showing a 340.18% and 360.01% increase relative to the negative control. In terms of the presence of soluble proteins in the leaves, tomato plants were favored when treated with the S011 aqueous extracts applied once a month (S011 30/30), resulting in a 42.02% increase relative to the negative control.

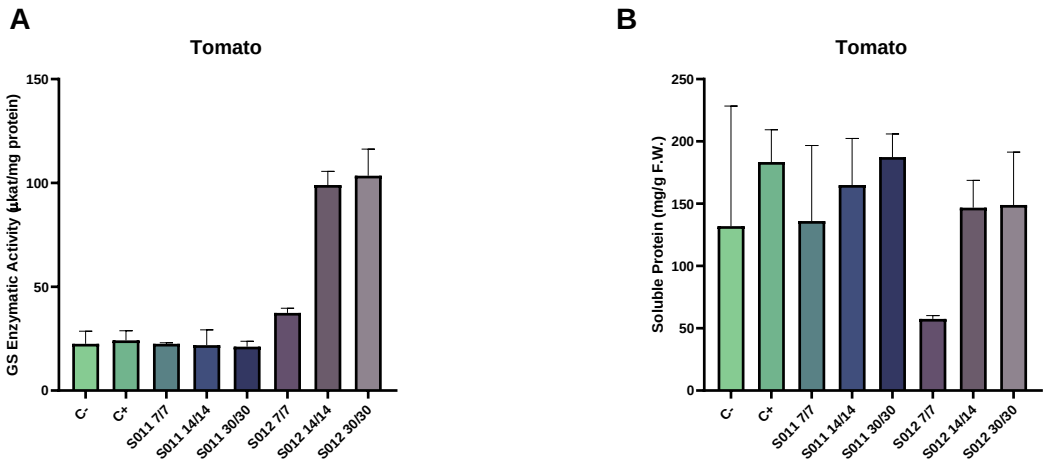


Figure 21. Mean values of the enzymatic activity of GS (**A**) and total soluble protein content (**B**) present in the leaves of tomato plants treated with aqueous extracts of S011 and S012 every week (7/7), every two weeks (14/14) and once a month (30/30). Values presented are mean ± SD. The absence of letters shows the lack of statistically significant differences.

In lettuce, the greatest GS activity was observed when the S011 aqueous extracts were applied once a week (S011 7/7), which showcased an increase in enzymatic activity of 54.84% in the leaves of treated plants, comparatively to non-treated lettuce (**Figure 22.**). The protein concentration in the leaves was higher in plants treated with S011 aqueous extract applied twice a month (S011 14/14), with 9.54% increase in protein content compared to the negative control. A similar positive effect of microalgae and cyanobacteria application on the GS activity was observed in previous studies (Brito et al., 2022; Puglisi et al., 2022).

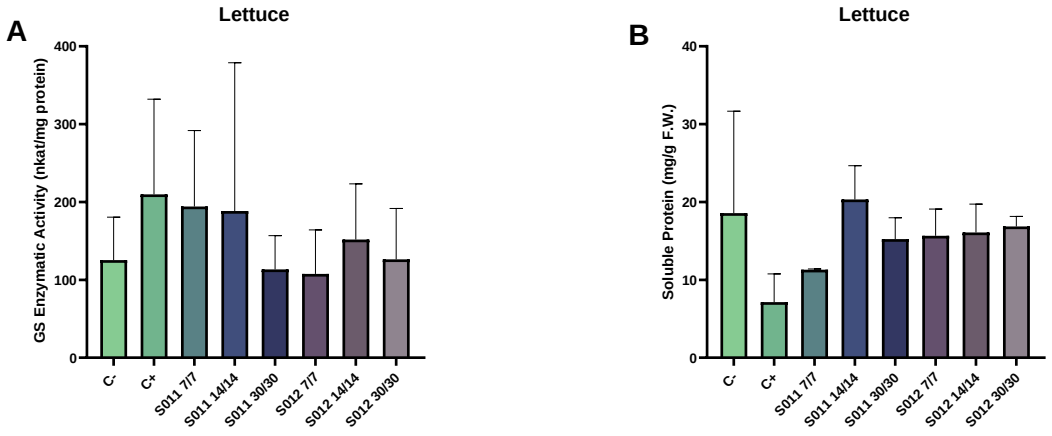


Figure 22. Mean values of the enzymatic activity of GS (**A**) and total soluble protein content (**B**) present in the leaves of lettuce plants treated with aqueous extracts of S011 and S012 every week (7/7), every two weeks (14/14) and once a month (30/30). Values presented are mean ± SD. The absence of letters shows the lack of statistically significant differences.

The results of the morphological and biochemical parameters do not allow for the determination of which treatment is more efficient for greater plant growth and development. Puglisi et al. (2020) reports that greater nitrogen absorption promotes the increase in total proteins and photosynthetic pigments, which in turn increases the fresh weights of the treated seedlings. These findings are not aligned with the conclusions of other studies, as no correlation can be established between the biochemical and the morphological results in tomato or lettuce plants (Puglisi et al., 2020; Puglisi et al., 2022; Roque et al., 2023). The influence of GS activity and the enhanced biosynthesis of total protein and photosynthetic pigment, as previously stated by Puglisi et al. (2020), could not be observed in either plant.

One potential explanation for the lack of statistically significant differences between the treatments and the controls may be due to the demise of several experimental replicates, which may have hindered the assay. The main reason for this is the inability of the incubator to provide the optimal environmental conditions for plant growth. On the one hand, the incubator was equipped with a constant air flux to maintain the selected temperature. The air flux affected the humidity of the soil, thereby subjecting the tomato and lettuce plants to drought stress. To address this issue, several measures were implemented. Initially, the plants were encapsulated inside a transparent plastic box (**Figure 23.**) to reduce their exposure to the air flux. Upon reaching a certain height, they were transferred to a closed glass aquarium. Additionally, a Becker filled with water was placed inside the incubator to maintain optimal humidity levels, since low relative air humidity can be the cause of water stress due to an increased rate of evaporation, and stomatal closing which impedes the absorption of further CO_2 and consequently the photosynthetic process (Ahmed et al., 2020).

Moreover, the incubator was unable to provide the requisite light intensity for optimal plant growth. Light is the principal source of energy required for photosynthesis and other physiological processes (Ahmed et al., 2020). The maximum intensity registered was approximately 30.0 $\mu mol.m^{-2}.s^{-1}$ PPFD, which is below the optimal light intensity range for the growth of tomatoes and lettuces, which is between 200-250 $\mu mol.m^{-2}.s^{-1}$ for lettuce and 300 $\mu mol.m^{-2}.s^{-1}$ for tomato plants (Ahmed et al., 2020; Schwarz et al., 2014). In other words, ten times more intense light was required for the plants to grow healthily. Consequently, the plants exhibited impeded development and growth hampered.

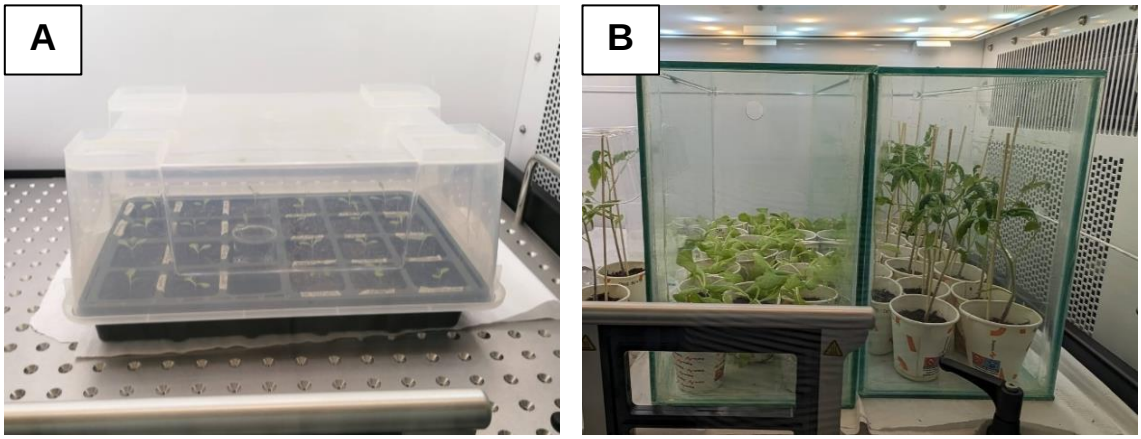


Figure 23. (**A**) Experimental setting of the seedling tray inside the incubator with lettuce seedlings growing in soil protected from air flux thanks to the plastic cap. (**B**) Lettuce and tomato plants placed inside two semi-closed aquariums in the incubator.

Due to the loss of several experimental duplicates, especially of lettuce plants, there is no compelling evidence demonstrating the stimulating effects of the strains. Even without clear results regarding the optimal application frequency to be applied in subsequent assays, it was decided to administer the biostimulant twice a month.

3.4.3. 3rd Assay: Standard Conditions and Salt Stress Conditions

3.4.3.1. Microalgal and Cyanobacterial Aqueous Extracts and Formulations Assay

The third assay occurred in a greenhouse between June and August 2024. The objective of this assay was to investigate the potential for plant growth enhancement resulting from the application of aqueous extracts of the four selected strains. Additionally, the assay served to assess the viability of the formulated biostimulant product *in planta*.

Morphological Parameters

The outcomes of tomato plant growth irrigated with the aqueous extracts are presented in **Figure 24.**. The treatments evidenced slight improvements in the morphological parameters, although these results were not statistically significant. The S009 and S011 aqueous extracts resulted in tomato plants with the highest whole plant, shoot and root fresh weight, with increases of 4.99%, 45.49%, 15.47% and 4.79%, 41.82% and 14.45% respectively for each of the strains in comparison to the non-treated plants. Furthermore, the aqueous extracts from the S009 and S011 strains also induced an improvement in the number of leaves per plant, with respective increases of 40,53%

and 36,21% compared to the negative control and 6.74% and 3.46% in comparison to the positive control. With regards to size, only the root length was found to be greater in plants treated with S011 aqueous extracts, exhibiting increases of 5.69% and 16.55% relative to the negative and positive controls respectively. Among the treatments, the strain S004 demonstrated the least pronounced morphological improvements in tomato plants.

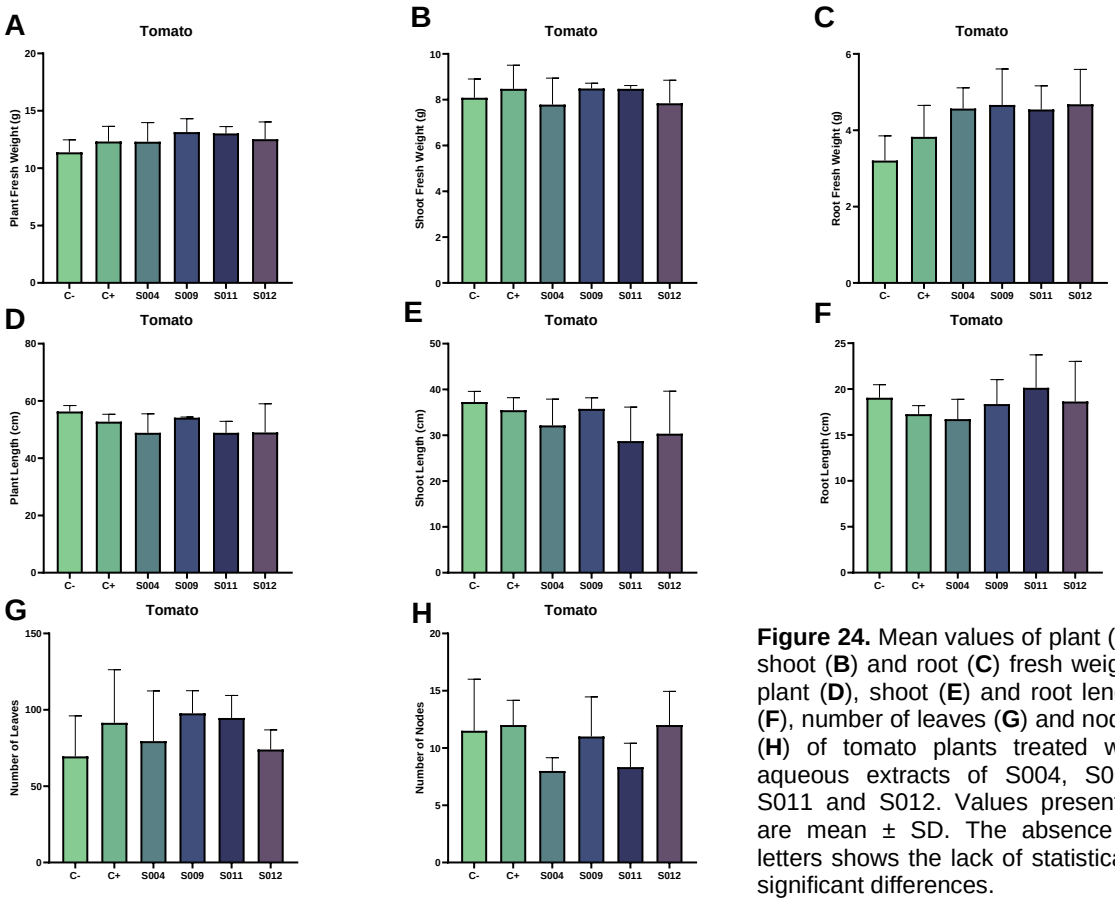


Figure 24. Mean values of plant (**A**), shoot (**B**) and root (**C**) fresh weight, plant (**D**), shoot (**E**) and root length (**F**), number of leaves (**G**) and nodes (**H**) of tomato plants treated with aqueous extracts of S004, S009, S011 and S012. Values presented are mean ± SD. The absence of letters shows the lack of statistically significant differences.

As for lettuce growth parameters (shown in **Figure 25.**), the plants that had not been treated demonstrated higher values of fresh weight in comparison to the treated plants, including the positive control. However, the S012 aqueous extracts demonstrated superior performance in promoting plant growth (29.62%) and especially root elongation, with significant improvements of 20.71%. In the case of the leaf count, the S009 and S012 aqueous extracts exhibited plants with 14 ± 1 and 13.5 ± 1.29 leaves, respectively, the negative control displayed 13 ± 1.41 leaves, while the positive control exhibited 10.75 ± 0.5 leaves. With the exception of the S011 extracts, all the other extracts enhanced the foliar area. The application of extracts of S004, S009 and S012 resulted in lettuce leaves that were 21.90%, 10.16% and 9.46% larger than those of the non-treated plants,

respectively. Notably, the aqueous extracts from S004 demonstrated a significant improvement in lettuce foliar area when compared to the plants treated with the commercial biostimulant, with an increase of 42.03%. The foliar area is a crucial parameter to be studied, particularly in lettuce plants, as leaves are the primary part of the plant that holds economic value.

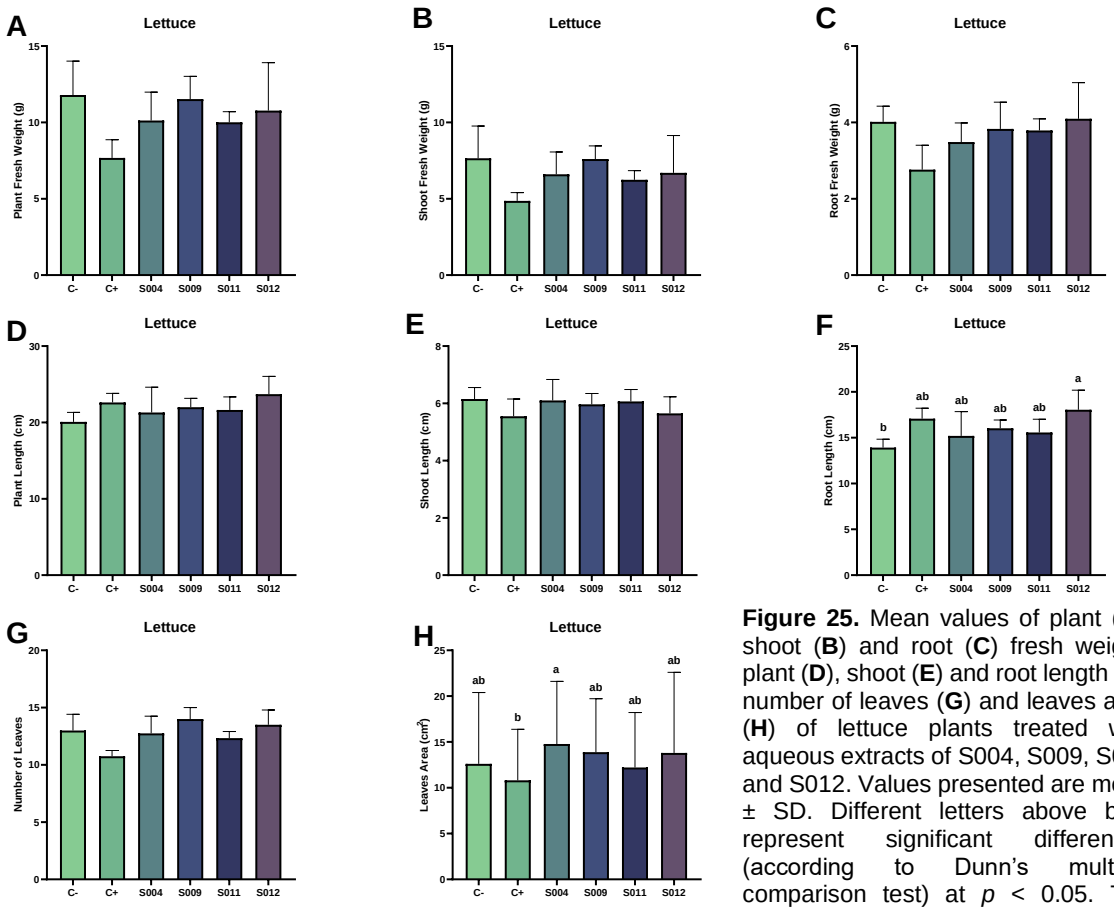


Figure 25. Mean values of plant (**A**), shoot (**B**) and root (**C**) fresh weight, plant (**D**), shoot (**E**) and root length (**F**), number of leaves (**G**) and leaves area (**H**) of lettuce plants treated with aqueous extracts of S004, S009, S011 and S012. Values presented are mean ± SD. Different letters above bars represent significant differences (according to Dunn's multiple comparison test) at $p < 0.05$. The absence of letters shows the lack of statistically significant differences.

Photosynthetic Pigment Content in Leaves

In this assay, all extracts exhibited the capacity to stimulate pigment synthesis in the foliar areas of the tomato plants (**Figure 26.**), corroborating other studies' results (Lopes et al., 2023; Mutale-Joan et al., 2020). The S004 aqueous extract demonstrated the greatest efficacy in enhancing chlorophyll a and b content. As for β-carotene, tomato

plants treated with S012 extracts were the most conducive to having higher amounts of this pigment in their leaves.

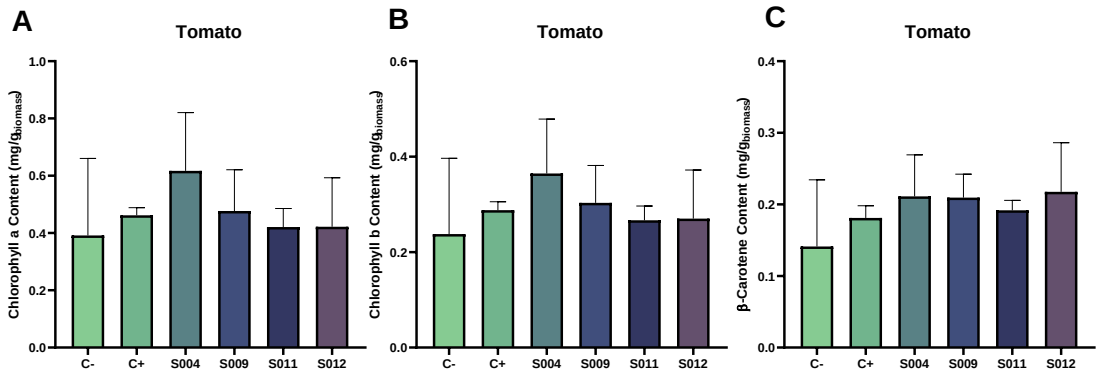


Figure 26. Mean values of the chlorophyll a (**A**), chlorophyll b (**B**) and β-carotene (**C**) content in the leaves of tomato plants treated with aqueous extracts of S004, S009, S011 and S012. Values presented are mean ± SD. The absence of letters shows the lack of statistically significant differences.

The aqueous extracts derived from S004 were observed to elicit the same outcomes in lettuce plants as in tomato plants, namely an increase in pigment concentration relative to the controls and the other treatments (**Figure 27.**). A significant difference ($p < 0.05$) was observed in the concentration of chlorophyll a when S004 extract was added to lettuce plants in comparison to the negative control. Furthermore, the concentrations of chlorophyll a and b, and β-carotene were present in concentrations 64.50%, 43.88%, and 8.58% higher, respectively, in the leaves of plants treated with this extract than in those that did not receive any treatment.

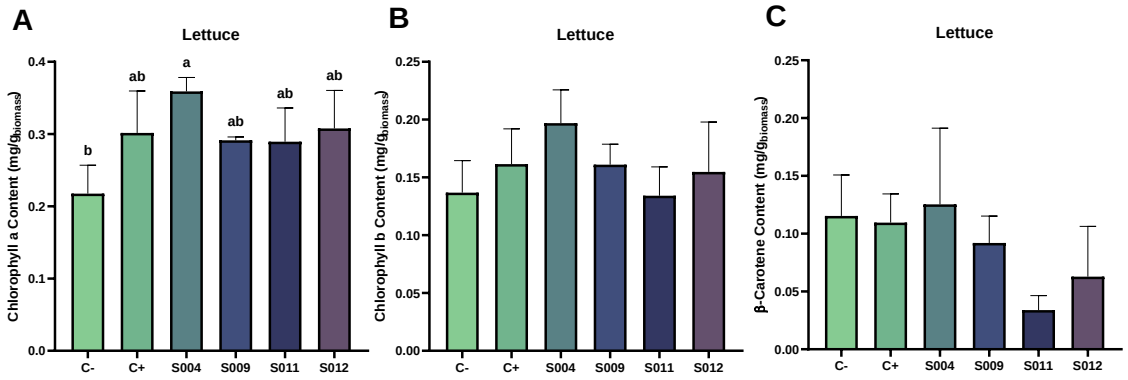


Figure 27. Mean values of the chlorophyll a (**A**), chlorophyll b (**B**) and β-carotene (**C**) content in the leaves of lettuce plants treated with aqueous extracts of S004, S009, S011 and S012. Values presented are mean ± SD. Different letters above bars represent significant differences (according to Dunn's multiple comparison test) at $p < 0.05$. The absence of letters shows the lack of statistically significant differences.

The assay permitted to confirm that each plant has its own biostimulant preference. In this case, while tomato plants exhibited accelerated growth and increased

size when treated with S011 and S009 aqueous extracts, lettuce plants demonstrated superior development with S004 and S012 extracts. It is also notable that the results of the first soil-based assay were sustained in this assay, with S011 demonstrating heightened efficacy in stimulating tomato growth and S012 exhibiting a more pronounced promotion of lettuce growth. The application of aqueous extracts derived from S004 seemed to enhance the chlorophyll a and b and β-carotene concentration in tomato (**Figure 26.**) and lettuce plants (**Figure 27.**). However, it is perplexing that the increase in photosynthetic pigments did not translate in enhanced morphological parameters of the plants treated with S004 extracts (**Figures 24.** and **25**). This may be attributed to the suboptimal conditions under which the plants were subjected during the experiment.

The application of several strains resulted in positive effects on the morphological and biochemical parameters of tomato and lettuce seeds, seedlings, and plants. Multiple studies have demonstrated the positive impact of extracts derived from microalgae and cyanobacteria extracts. For instance, Mutale-Joan et al. (2020) investigated the biostimulant potential of 18 microalgae and cyanobacteria strains as crude bio-extracts with three different application doses (0.1, 0.5, and 1 g/L) on tomato plants. The treated plants exhibited significantly enhanced morphological parameters such as root length, shoot length, dry weight, and photosynthetic pigments, including chlorophyll a, chlorophyll b, and carotenoid content, in comparison to the control. In a study conducted by Puglisi et al. (2022), lettuce seedlings were treated with a root drench of *Chlorella vulgaris* extract (1 mg C_{org}/L). The positive effects of the microalgae extracts were noticeable in the enhanced growth of lettuce seedlings and the increased dry weight, chlorophyll, carotenoid, and protein contents, and glutamine synthetase activity in the leaves.

3.4.3.2. Formulations

The phytotoxic effect of the formulations on plants was found to be the same as on seeds. None of the plants treated with the formulations survived until the end of the assay. This outcome might be due to a problem with dosage. The formulation was applied directly to the soil without a prior dilution. While the constituents of the formulation

are not generally toxic to plants, as noted by Lopes (2022), it is plausible that high concentrations of these constituents may negatively impact the plants.

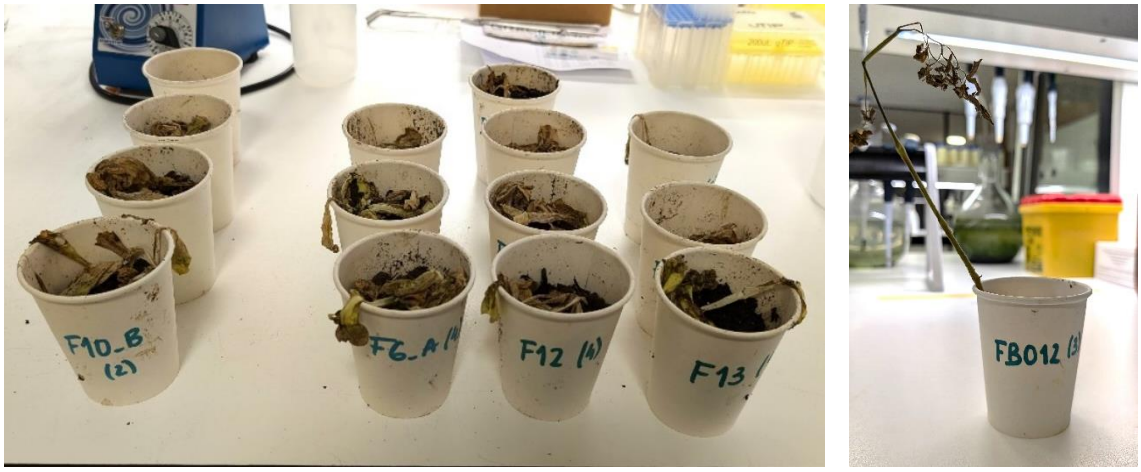


Figure 28. Lettuce plants (left) and tomato plants (right) after treatment with formulations.

3.4.3.3. Microalgal and Cyanobacterial Aqueous Extracts Effects under Salt Stress Conditions

Morphological Parameters

The aqueous extracts did not show significant differences in any of the morphological parameters evaluated, and thus the protective function against salt stress of the strains could not be statistically substantiated. Nevertheless, some differences were discerned in the treated tomato and lettuce plants.

As can be observed in **Figure 29.** and **30.**, the growth traits were seriously hindered by the increase in the concentration of NaCl, as observed in other studies (Brito et al., 2022; Chanthini et al., 2022). Compared to the control plants (0 g/L), the tomato plant fresh weight declined 8.16% and 24.14% at 3 g/L and 6 g/L NaCl exposure, respectively. However, the effect was even more prominent in lettuce plants, which demonstrated a 34.14% and 47.32% reduction. On the other hand, tomato plants seemed to be more sensitive to the effects of elevated salt concentrations, exhibiting reduced plant size and growth. In 3 g/L NaCl conditions, tomato plants exhibited a 27.58% reduction in size, while in 6 g/L NaCl, this reduction reached 66.19%. In contrast, lettuce plants demonstrated a less noticeable response, with a 2.37% and 8.18% reduction in size, respectively. The nefarious effects of salinity have been demonstrated

in many different plants (Aloui et al., 2023; Bello et al., 2022; Brito et al., 2022; Krid et al., 2024).

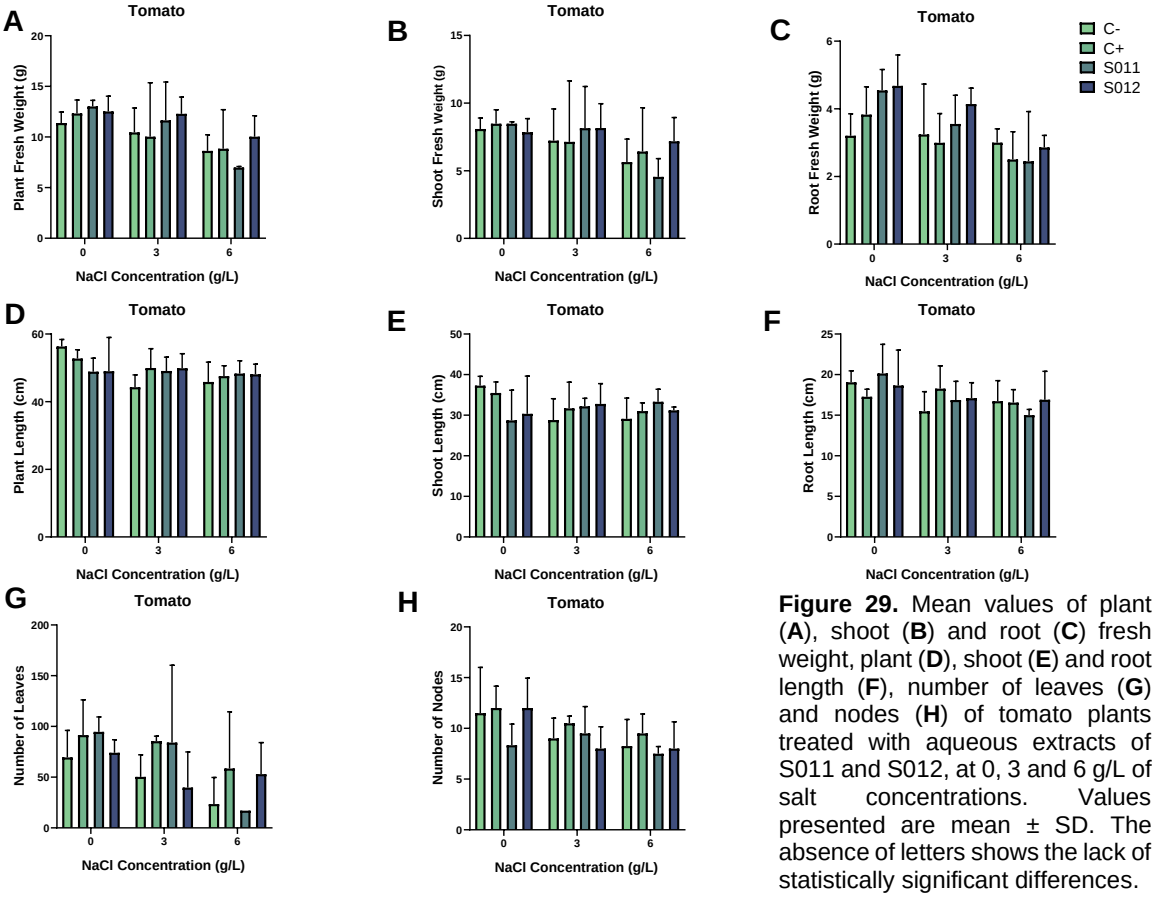


Figure 29. Mean values of plant (**A**), shoot (**B**) and root (**C**) fresh weight, plant (**D**), shoot (**E**) and root length (**F**), number of leaves (**G**) and nodes (**H**) of tomato plants treated with aqueous extracts of S011 and S012, at 0, 3 and 6 g/L of salt concentrations. Values presented are mean ± SD. The absence of letters shows the lack of statistically significant differences.

The aqueous extracts showed beneficial effects on both plants, enhancing their resilience against salinity stress which supports other studies (Chanthini et al., 2022; El Arroussi et al., 2018). The positive impact of the aqueous extracts of S011 and S012 were evident in both salt concentration conditions. The fresh weight of the tomato plant, shoot, and root increased with both treatments at 3 g/L. However, at the highest salt concentration, only the S012 aqueous extracts conferred protection to the plant, contributing to a 26.91% and 23.86% increase in weight relative to the negative and positive control counterparts, respectively. The same effects were portrayed in the size of the treated stressed plants. Applications of S012 at 3 g/L concentrations caused an increase in plant, root and shoot length of 12.65%, 10.50% and 13.80%, respectively, in comparison to the negative control at the same NaCl concentration. In the case of plants subjected to 6 g/L concentrations, both treatments demonstrated the stimulation of plant growth. Plants treated with S011 aqueous extracts exhibited an increase in height of

5.40%, while aqueous extracts of S012 strains attained a size of 4.96% larger than that of the negative control.

Regarding lettuce plants, the positive effects of biostimulant supplementation were also visible at both salt concentrations. In plants subjected to 3 g/L NaCl concentrations, only the S012 aqueous extracts showed the capacity to remediate the toxic effects of salt. Lettuce plants treated in this manner exhibited the greatest values for whole plant fresh weight, size, and number of leaves, with increases of 8.98%, 14.92% and 10.26%, respectively, in comparison to the negative control. In more severe saline conditions, the only morphological parameter that improved as a result of the extracts was the size of the lettuce plants, which demonstrated increases of 11.48% and 9.58% for the S011 and S012 extracts, respectively.

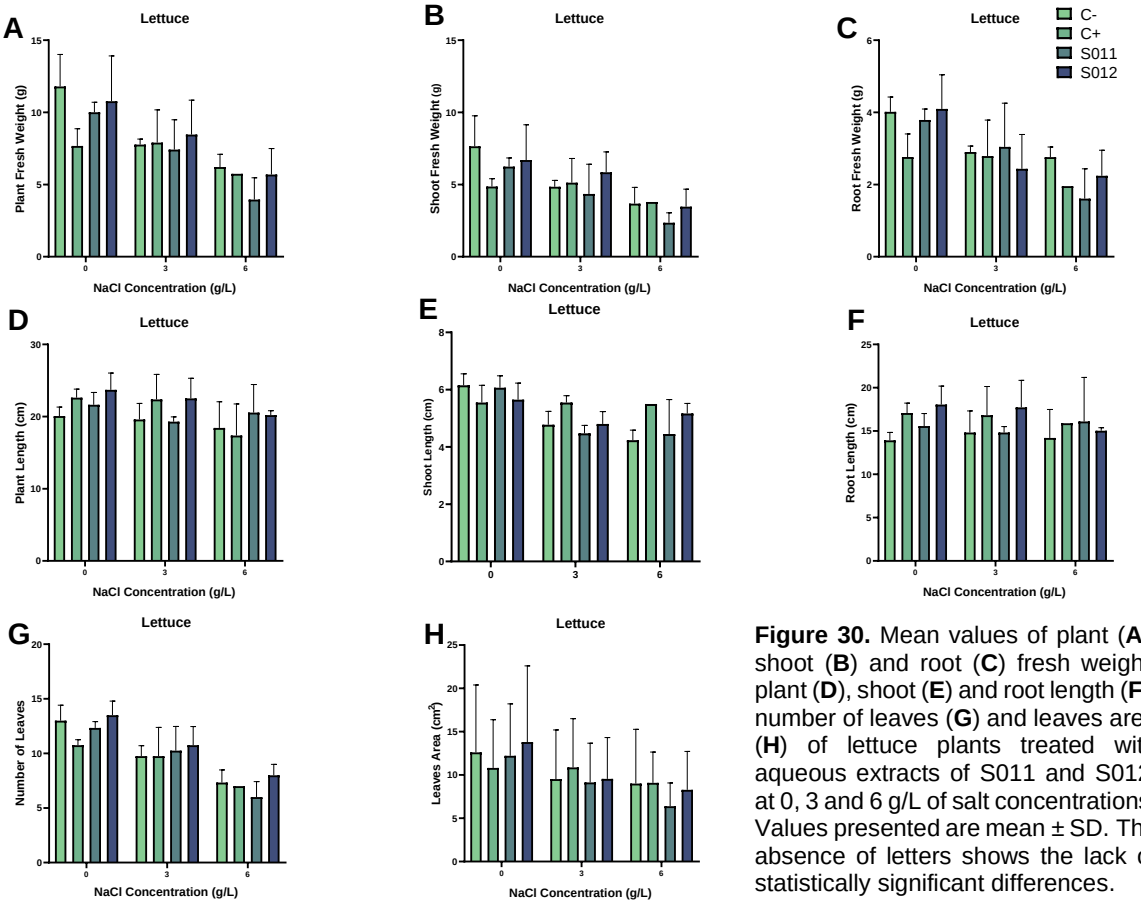


Figure 30. Mean values of plant (**A**), shoot (**B**) and root (**C**) fresh weight, plant (**D**), shoot (**E**) and root length (**F**), number of leaves (**G**) and leaves area (**H**) of lettuce plants treated with aqueous extracts of S011 and S012, at 0, 3 and 6 g/L of salt concentrations. Values presented are mean ± SD. The absence of letters shows the lack of statistically significant differences.

Photosynthetic Pigment Content in Leaves

No significant differences in the pigment content in the leaves of tomato and lettuce plants were attributed to the treatments or the stress caused by the elevated concentrations of NaCl in the soil. However, it can be noted in **Figure 31.**, that a reduction

of the chlorophylls a and b was a result of salt stress exposure in the tested NaCl concentrations. Indeed, the concentration of chlorophyll a was reduced by 62.01% and 19.62% in 3 and 6 g/L of NaCl, respectively, and that of chlorophyll b by 53.81% in 3 g/L in comparison to the control in tomato plants. A reduction in chlorophyll levels following exposure to salt has been previously observed in other studies (Aloui et al., 2023; El Arroussi et al., 2018; Krid et al., 2024). It can be concluded that a process of chlorophyll degradation occurred in non-treated plants (Aloui et al., 2023; Mutale-Joan et al., 2020), probably linked to an increase in the activity of chlorophyllases (Krid et al., 2024). The concentration of β-carotene did not differ significantly between the different salt concentrations, with the highest levels observed in plants subjected to 6 g/L of NaCl. In lettuce plants, no straightforward correlation could be identified between the NaCl concentration and the content of photosynthetic pigments (**Figure 32.**).

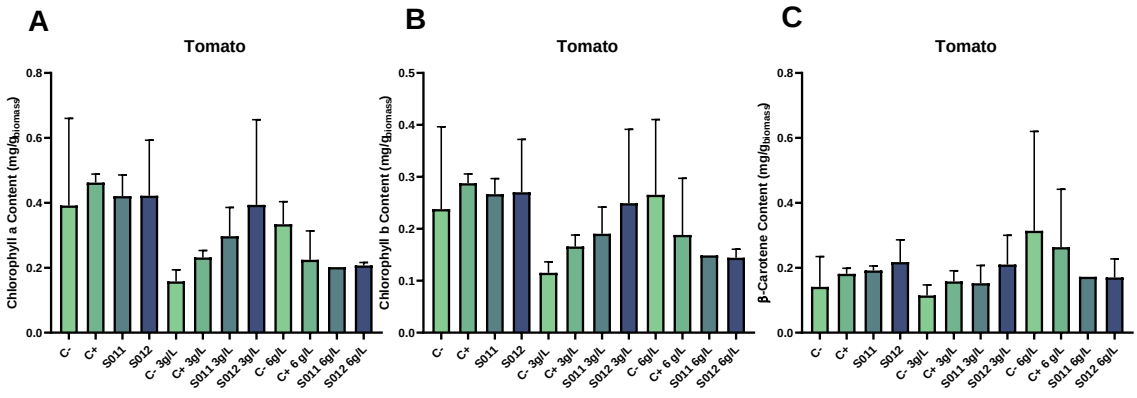


Figure 31. Mean values of the chlorophyll a (**A**), chlorophyll b (**B**) and β-carotene (**C**) content in the leaves of tomato plants treated with aqueous extracts of S011 and S012, at 0, 3 and 6 g/L of salt concentrations. Values presented are mean ± SD. The absence of letters shows the lack of statistically significant differences.

The extracts of S011 and S012 improved the photosynthetic pigment content in the leaves of tomato and lettuce plants at 3 g/L of NaCl, in comparison to both controls. In particular, S012 aqueous extracts exhibited the best performance in terms of pigment concentration improvement, showing increases of 149.32%, 115.96% and 83.05% for chlorophyll a, chlorophyll b and β-carotene, respectively, compared to the untreated tomato plants. A similar effect was observed in lettuce plants, with values 5.51%, 38.02% and 124.48% higher than in the negative control, for chlorophyll a, chlorophyll b and β-carotene, respectively. The increase in chlorophyll content may have boosted photosynthetic rate, while the production of β-carotene may have contributed to the scavenging of ROS and redox stability (Chanthini et al., 2022). Yet, the aqueous extracts appeared to lose their stimulation effect in both plants under severe salt stress conditions (6 g/L). In another study, the increase in concentrations of antioxidant and photosynthetic pigments such as chlorophylls in basil leaves after treatment with *Pseudomonas* sp., *Bacillus lentus,* and *Azospirillum brasilens* was correlated with the mitigation of abiotic stress such as water stress (Heidari & Golpayegani, 2012).

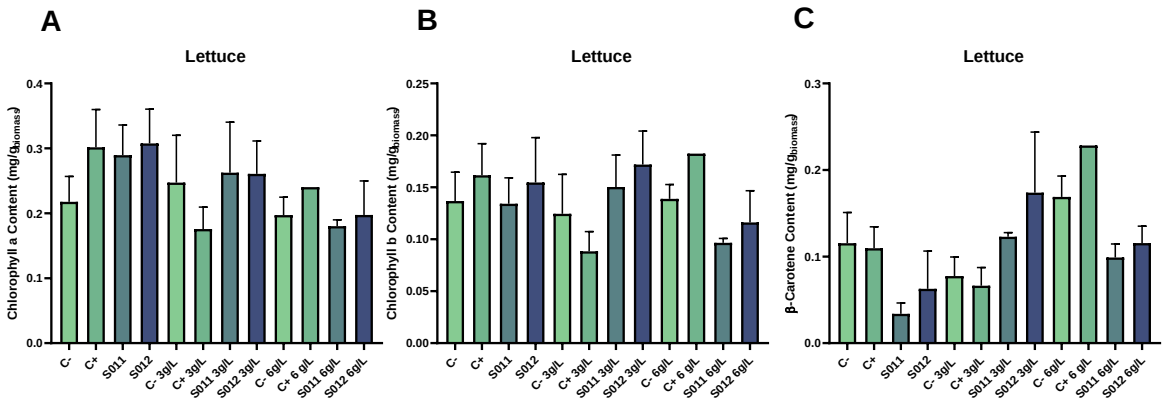


Figure 32. Mean values of the chlorophyll a (**A**), chlorophyll b (**B**) and β-carotene (**C**) content in the leaves of lettuce plants treated with aqueous extracts of S011 and S012, at 0, 3 and 6 g/L of salt concentrations. Values presented are mean ± SD. The absence of letters shows the lack of statistically significant differences.

Plants are susceptible to abiotic stressors such as salinity. Despite the development of an inherent defensive system, prolonged periods of stress can overcome it and result in harm to the plant. The presence of salt can have a detrimental impact on plant morphology, such as a reduction in leaf surface area, inhibiting indirectly photosynthesis, as well as water and mineral uptake (Aloui et al., 2023; Chanthini et al., 2022). A reduced photosynthesis may lead also to a decrease in plant biomass (Aloui et al., 2023). Consequently, the exogenous application of microalgae and cyanobacteria extracts represents a prevalent strategy for supporting plant growth and improving

productivity (Aloui et al., 2023; El Arroussi et al., 2018; Krid et al., 2024; Mutale-joan et al., 2021).

Numerous investigations addressed the capacity of microalgae and cyanobacteria to serve as protective agents against salt stress when introduced as extracts. *Dunaliella salina* exopolysaccharides were employed by El Arroussi et al. (2018) as a means of mitigating the effects of salt stress in tomato plants. The application of 3 and 6 g/L NaCl concentrations resulted in a reduction in plant growth and weight, as well as chlorophyll and protein, indicative of salt stress. However, plants treated with microalgal exopolysaccharides showed a significant improvement in shoot and root length, as well as fresh weight, accompanied by a restoration of chlorophyll and protein biosynthesis. In a related study, Brito et al. (2022) analyzed the effect of a recently identified terrestrial cyanobacterial species, *Oculatella lusitanica* LEGE 161147, on salt-stress tolerance in lettuce plants. The plants that were supplemented with perlite containing the isolated cyanobacterium exhibited a reduction in the deleterious effects of salt stress in comparison to the control plants. The treated plants demonstrated higher values for size, root, and shoot fresh weight, which may be attributed to the elicitation of the non-enzymatic antioxidant response system. The mitigation of the nefarious effects of salt stress may be attributed to the capacity of the extracts to confer an increase in antioxidant activity in plants (Mutale-Joan et al., 2022; Prisa & Spagnuolo, 2023).

Nevertheless, despite the observation of biostimulant capacity could be observed in the different stages of the plants, statistical analyses failed to corroborate this effect. There are reasons to believe that the low or even absent biostimulatory results are not only due to the absence of inherent bioactive compounds in the microalgae and cyanobacteria strains but also influenced by other external parameters that may have impacted the final results.

3.4.4. Sonication

As previously stated, the sonication extraction method is likely to be inefficient and therefore unsuitable for the recovery of bioactive molecules, especially in the case of microalgae, which typically possess thicker cell walls than prokaryotic cyanobacteria (Parmar et al., 2023). Microscopic observation of the extracts obtained from sonication revealed that 12 strains maintained their cellular integrity. A comparable finding was reported by Stirk et al. (2020) who tested three different cell disruption techniques such as lyophilization coupled with sonication or ball-milling and lyophilization without

additional cell disruption, for the extraction of bioactive compounds from *Chlorella* sp., *Chlorella vulgaris,* and *Scenedesmus acutus.* Microscopic examination indicated that only 10-20% of the cells had undergone rupture of their cell wall after sonication, a meager yield in comparison to the grinding treatment, which permitted the disruption of 70-80% of the cells. Indeed, the results demonstrated that sonication was the least effective method. In a similar experiment, Lee et al. (2010) found that among all the extraction methods analyzed, including autoclave, bead beating, osmotic shock, microwave, and sonication, the latter was the least efficient for extracting lipids from the green microalga *Botryococcus* sp. Therefore, the lower extraction yield of bioactive compounds from the interior of microalgal and cyanobacterial cells due to an incomplete cell lysis capacity of sonication may impede the seed germination and the plant growth-promoting potential of the extracts. Hence, it is essential to employ a tailored extraction method for each strain to fully access and evaluate its biostimulant capacity.

Although Lopes (2022) concluded that high-pressure extraction and sonication yielded similar extraction yields, she acknowledged that sonicated extracts exhibited inferior performance in terms of seed germination activity comparatively. Some sonicated extracts demonstrated significantly reduced germination of tomato and carrot seeds in comparison to the negative control after seven days of incubation, as well as lower germination indices. In contrast, the same extracts obtained from high-pressure extractions of the same strains resulted in positive effects on the seed germination assays. Similarly, Carneiro et al. (2021) demonstrated that sonicated extracts (0.5 and 2 g_{DW}/L) of *Chlorella vulgaris* and *Scenedesmus acutus*, two widely studied microalgae with documented biostimulating activities, did not improve in their experiment either seed germination or root development of mung beans in their experimental setup. Furthermore, sonication may also compromise the quality of the extracts. This is because the process not only results in the production of low yields of lysed cells but also causes heating of the solution, which in turn leads to the degradation of some heat-sensitive active constituents (Navarro-López et al., 2023; Stirk et al., 2020).

3.4.5. Microalgae and Cyanobacteria Extract Concentration and Quality

The extracts were prepared with a concentration of 1 mg/mL for all strains of microalgae and cyanobacteria. However, the dose of microalgal and cyanobacterial biostimulants constitutes a critical variable in determining the success of such products. It is, therefore, an object of ongoing investigation, with the objective of avoiding high

production costs and waste on the one hand, while, on the other, to avoid unexpected outcomes (Carillo et al., 2020; Kapoore et al., 2021). The success of a biostimulant is dependent on its concentration and the number of applications. There is a narrow range of concentrations and application frequencies that maximizes the efficiency of a biostimulant. An overdiluted biostimulant may fail to elicit any changes in plant growth, whereas the administration of high doses can potentially reduce, neutralize, or even inhibit it (Garcia-Gonzalez & Sommerfeld, 2016; Prisa & Spagnuolo, 2023). The inhibition of the plant performance may be attributed to an overdose of phytohormones or other bioactive compounds (Carillo et al., 2020; Supraja et al., 2020; Toribio et al., 2020). Generally, lower concentrations of biostimulants are more conducive to a favorable outcome.

In a recent study, Chovancek et al. (2023) assessed the biostimulant potential of aqueous crude extracts derived from six microalgal species on *Arabidopsis thaliana*. Four different extract concentrations (0.05, 0.1, 0.3, and 0.5 g/L) were applied via soil drenching on three separate occasions. The analysis of root growth revealed that only the 0.05 g/L concentration of the aqueous extract of *Chlorella sorokiniana* significantly increased root elongation and lateral root development, while higher concentrations showed the opposite effect. Similarly, Toribio et al. (2021) experimented the effect of sonicated extracts of microalgae and cyanobacteria on watercress seeds to evaluate their effect on the germination index. To this end, two different extract concentrations, 0.5 g/L and 2.0 g/L, were tested. The results demonstrated that the most concentrated extracts had a phytotoxic effect on the seeds, while extracts applied at 0.5 g/L showed stimulation of the germination.

On the other hand, it has been reported that multiple plant hormones, including auxins and cytokinins, are more soluble in alkaline solutions. Consequently, these hormones can be extracted more effectively from liquids with higher pH levels. As previously stated, the distilled water employed for the extractions was consistently slightly acidic, which may have influenced the yield of extraction of these crucial phytohormones and therefore impacted the final results (Kapoore et al., 2021).

While some FBCC extracts evidenced some biostimulant potential in the seed germination and plant growth assays, statistical analyses deemed the results inconclusive. The concentration and quality of the extract utilized in this project may have been a limiting factor in the success of the assays. It can be concluded that a plant

treatment with a specific extract concentration may elicit either a positive or negative response, depending on the microorganism employed, the extraction method employed. and the selected plant itself (Navarro-López et al., 2023). It is important to employ extraction methods that are optimized for the efficient recovery of the most important bioactive compounds such as phytohormones.

3.4.6. Plant Growth Experimental Design

The experimental design may have been flawed in several ways, which may have contributed to the less perceptible biostimulant and bioprotector effects observed on tomato and lettuce plants when cultivated in soil. Due to the limited space available inside the incubator and the greenhouses, the plants were overcrowded in a confined area. Schwarz et al. (2014) recommend that in long-term experiments, no more than 3.5 tomato plants should be grown per m^2, as they can overshade themselves and affect the light levels captured by each plant. Furthermore, the risk of transmission of infectious diseases is heightened. Indeed, this occurred during the second plant growth assay. Due to the warm and humid conditions in a confined space, approximately half of the tomato plants were infected seemingly by a fungus, which significantly affected their growth and survival.

Figure 33. Infected tomato plants in the second plant growth assay.

Plants tend to exhibit considerable variability among them, even when cultivated under identical conditions. Moreover, the absence of optimal conditions resulted in the demise of some plants. Therefore, it would have been preferable to conduct a greater number of replicates for each condition for all plant growth experiments in order to reduce

the variability of the population. This would allow for statistical proof of the stimulatory capacity of the extracts to be obtained.

Furthermore, it is plausible that the biostimulation was reduced due to a deficiency of nutrients in the soil. Nutrient uptake is one of the primary functions of a plant biostimulant. In other words, it optimizes the fertilizer consumption in plants (Mutale-Joan et al., 2020). Several authors added a nutrient solution jointly with the biostimulants they were testing (Casadesus et al., 2020; Chovancek et al., 2023; Puglisi et al., 2022; Rachidi et al., 2020). This approach could have facilitated the detection of potential variations in nutrient uptake between the treated plants and the control.

Finally, the third plant growth assay took place in two plastic greenhouses. The selected location proved to be suboptimal for plant growth. The plants were subjected to a multitude of stress factors, including drought and heat stress. The main function of greenhouses is to maintain a relatively constant temperature when external conditions are colder. However, in the event of elevated temperatures, the greenhouses tended to absorb the heat, thereby exposing the plants to temperatures that were too high for optimal growth. Air temperature can impact the leaf development and the absorption and transport of water through the roots of the plant (Ahmed et al., 2020). These unfavorable conditions were probably the principal cause of death of many tomato and lettuce plants. The plants endured an additional stressor: the location where the greenhouses were situated was particularly windy. During the assay, the greenhouses fell on two occasions, resulting in the plants being projected to the ground. While only two tomato plants died directly as a consequence of the falls, it is reasonable to assume that the plants were adversely affected by it. It is possible that these stressors may have contributed to the lack of consistent results at the end of the assay.

4. Conclusion

It is imperative that the farming industry implements immediate changes to its current fertilizing practices in order to ensure the sustainable provision of food for the forthcoming decades, while simultaneously respecting the environment. The phenomenon of climate change has led to an increase in the prevalence of salinity in arable soils, causing considerable economic losses for the agro-industrial sector. Consequently, it is vital to develop innovative technologies that can ensure crop protection against abiotic stressors and enable high yields of food products. Biostimulants represent an environmentally friendly alternative to chemical fertilizers, capable of enhancing crop physiology, nutrient uptake and stress tolerance, thereby facilitating the production of high-quality food. Microalgae and cyanobacteria are rich in bioactive compounds, such as phytohormones, antioxidants, polysaccharides, proteins and amino acids, which are valuable for plant growth and development. As a result, they are a promising source of bioactive ingredients for use in biostimulants.

At the beginning of the internship, Fykia Biotech had assembled a private collection of 31 strains of microalgae and cyanobacteria. The maintenance of the collection, which included sub-culturing, micromanipulation for the isolation of contaminated cultures and molecular identification of selected strains, were among the objectives of the internship. To evaluate the potential biostimulating effects of the isolates, they were subjected to sonication, whereby intracellular bioactive compounds were extracted. Subsequently, the aqueous extracts were screened in a seed germination assay, wherein the root elongation and seed germination of tomato and lettuce seeds were assessed. Based on the germination index obtained for each strain aqueous extract, the strains S004, S009, S011, and S012 strains were selected for further investigation to ascertain their biostimulant potential in plant growth assays. Tomato plants treated with S009 and S011 showed enhanced morphological characteristics compared to the control group. In contrast, the lettuce plants displayed the most notable differences when irrigated with S004, S009, and S012 extracts. The S004 extracts were observed to result in a greater improvement in pigment content in the leaves of both plants. Plants subjected to salt stress exhibited reduced plant growth, seed germination, and foliar pigment content in comparison to non-stressed plants. This effect was partially reversed by the application of aqueous extracts of S011 and S012, especially at NaCl concentrations of 3 g/L. Formulations of the four selected extracts

were also tested, but seed germination and plant growth assays demonstrated their phytotoxicity. Therefore, further optimization is necessary before these formulations can be launched to the market.

To conclude, there is experimental evidence, to which this study contributed, that indicates a relationship between the bioactive compounds extracted from the microalgae and cyanobacteria from the FBCC and positive effects on plant physiology. However, it is important to acknowledge that the effectiveness of a biostimulant product varies considerably depending on the microalgal and cyanobacterial strain selected, the extraction method employed, the plant species or cultivars treated, the dose and time of application (Carillo et al., 2020; Santini et al., 2021). Therefore, further studies to optimize the biostimulant preparation methods are required to release an efficient product in the market.

5. Future Perspectives

The purpose of this project was to develop an efficient biostimulant product for imminent market release. Nevertheless, the findings of this project indicate that further optimizations are necessary to maximize the efficiency of the product. The extraction process represents a fundamental step in the biostimulant industry. It is a crucial factor of differentiation between products, conferring a competitive advantage if it ensures high yields of extraction while maintaining the extract quality and low operational costs (Kapoore et al., 2021; Stirk et al., 2020). It would be beneficial to test a different extraction process, such as the high-pressure method, which Lopes (2022) has already tested and demonstrated to be more effective than sonication. Additionally, sonication is a process that is not easily scalable to industrial levels, in contrast to high-pressure extractions (Navarro-López et al., 2023).

Some authors have proposed that freeze-drying is sufficient to recover the biostimulant molecules present in the biomass (Marín-Marín et al., 2024; Stirk et al., 2020). During lyophilization, intracellular ice crystals form and sublimate due to the combination of low temperature and high pressure, resulting in the dehydration of the biomass. Although the cell wall remains intact, the freeze-drying process renders the cell membrane more porous, thereby allowing bioactive compounds to exit the cell and enter the surrounding media. Furthermore, it has been demonstrated that sonication can not only be redundant but also detrimental to the quality of the extract. If this hypothesis is validated, it would be advantageous for the company to reduce the number of required extraction steps, thereby minimizing the expenditure of time and resources.

The supernatant, in which the harvested biomass was cultivated, was consistently discarded following centrifugation. It is well established that microalgae and cyanobacteria secrete some beneficial compounds into their surrounding environment, including phytohormones and siderophores. The valuation of this currently discarded product could potentially enhance the profitability of the extraction process (Navarro-Lopez et al., 2020). Alling et al. (2023) found in their study that the supernatant of *Chlorella vulgaris* demonstrated equally a positive effect on tomato seed germination as the applied extracts.

Moreover, the use of consortia of microalgae and cyanobacteria may prove to be more efficient than the utilization of isolates of a single strain. In diverse ecosystems,

microalgae and cyanobacteria typically coexist and interact synergistically with other microorganisms, especially in microbiomes such as those found in the rhizosphere of plants (Chabili et al., 2024). A consortium can be defined as a group of microorganisms with different potential traits that grow together in a coordinated and complementary way (Toribio et al., 2022). It has been reported that consortia may improve metabolite production, biomass growth and nutrient cycling (Toribio et al., 2022). The use of microalgae and cyanobacteria-based consortia allows for the production of a greater variety of metabolites, thereby enhancing the performance of biostimulants (Chabili et al., 2024; Roque et al., 2023). The exudates of a consortium of microalgae and cyanobacteria have been demonstrated to increase plant growth and vigor, augment photosynthetic activity, and optimize nitrogen utilization in *Arabidopsis thaliana* and *Lolium multiflorum* (Roque et al., 2023).

Furthermore, foliar sprays may offer an appealing alternative to irrigation methods. The effectiveness of liquid biostimulants applied to soil is subject to the influence of a range of physical, chemical, and microbiological factors (Santini et al., 2021). Foliar spraying of microalgal or cyanobacterial extracts, in contrast, is a more direct method of application, as it is applied directly to the leaves of the plants. The absorption of bioactive compounds, such as phytohormones, by the stomata of plants results in more immediate physiological responses than those observed in root irrigation applications (Garcia-Gonzalez & Sommerfeld, 2016; Parmar et al., 2023; Renuka et al., 2018). Kholssi et al. (2022) suggest that foliar sprays are usually employed to enhance plant physiology and biochemistry, whereas soil drenching is the preferred method when the objective is to improve soil properties (Chabili et al., 2024). With regard to the study of salt stress mitigation effects, it would be worthwhile to explore the protective capacity of foliar sprays of aqueous extracts of the FBCC strains and compare them with the soil drench method. From an economic standpoint, foliar spraying may also be more sustainable, as lower doses of the product are required to achieve the same effects (Prisa & Spagnuolo, 2023).

As a final consideration, the influence that microalgae and cyanobacteria-based biostimulants have on the quality traits of the final marketable products should be studied in the future. In this project, the effects of the biostimulant products were tested and analyzed for seed germination, plant growth and abiotic stress tolerance. However, biostimulants are known to also improve food product quality and therefore assays should be conducted on the fruits and leafy vegetables. Commercial features such as

fruit size, color and firmness, as well as nutritional qualities of the fruits, including the presence of anthocyanins, carotenoids, organic acids, phenolic compounds, and titratable acidity, are important measures of the product quality (Santini et al., 2021).

Chapter 2

Business Development at Fykia Biotech

1. Introduction

1.1. Market Analysis

The global biostimulant market is currently in expansion: it is exhibiting a Compound Annual Growth Rate (CAGR) of 10.65% during the 2019 to 2027 forecast period and for that reason, it is considered as one of the fastest-growing agriculture-related sectors, expected to reach the $5.5 billion global market size by 2027 (Kapoore et al., 2021; Parmar et al., 2023; Santini et al., 2021). In contrast, the chemical fertilizer market, although 60 times wider, is expanding annually at a significantly lower rate of 1.3-1.8% (Santini et al., 2021). This trend is sustained by the current shift towards sustainable agricultural practices such as organic farming, endorsed by the general public that is aware of the actual environmental and food security issues, the farmers that demand more affordable fertilizing solutions and the government authorities (Kapoore et al., 2021). Nowadays, the biostimulant market is still predominantly dominated by macroalgae extracts and humic substances, which encompass approximately 70% of the global market (Kapoore et al., 2021). Microalgae and cyanobacteria-based biostimulants, in turn, represent only an almost insignificant share of the actual market, in part because of their lack of economic competitiveness in regard to the other biostimulants and commercial synthetic fertilizers, as well as insufficient scientific research around the topic (Cao et al., 2023; Dagnaisser et al., 2022; Ronga et al., 2019).

Biostimulants derived from microalgae and cyanobacteria are precious contributors to answer the needs and challenges raised by the United Nations (UN), resumed in 17 Sustainable Development Goals (SDGs) (UN, 2015). Their contribution can be applied directly to SDG-2 “Zero Hunger”, SDG-9 “Industry, Innovation and Infrastructure”, SDG-12 “Responsible Consumption and Production” and SDG-13 “Climate Action”. Furthermore, microalgal and cyanobacterial biotechnology such as this one is a fundamental step towards a circular bioeconomy since these microorganisms can recover nutrients from wastewater and CO_2 from the atmosphere, transform it into biomass from which it is possible to extract in return high-value bioproducts and bioactive compounds that can be applied in many industries (Cao et al., 2023; Dagnaisser et al., 2022).

Not only the industries but also the academia is committed to deepening our knowledge about the mechanisms surrounding the relationship between crops and biostimulants. In thirty years, there has been an increase of about 30 times more scientific papers tackling the biostimulant topic (Santini et al., 2021). By searching on the Scopus database the terms “Plant” AND “Biostimulants”, it can be noted that more than 2000 scientific articles were published in the last ten years.

The current EU Regulation 2019/1009 that took effect in 2022 facilitated the market entry of macroalgae and microalgae, as well as for cyanobacteria although limited, by removing marketing authorization for biofertilizers, therefore beneficiating the access to the agronomic sector (Righini et al., 2022).

1.2. The Company

Fykia Biotech Lda is a blue biotechnology startup based in Matosinhos, Porto, Portugal, that embraces the great biological diversity of exclusive microalgae and cyanobacteria from its own collection (FBCC) and their respective bioactive compounds to develop novel bio-based products. The company seeks to develop innovative solutions driven by cyanobacteria and microalgae to meet the evolving needs and demands of today's society. There are three different scientific areas that are currently being explored. The agricultural sector aims for the research and development of sustainable biofertilizers and biostimulants to be applied as liquid extracts in soils. Thanks to the discovery of a revolutionary cyanobacteria-derived natural compound, the nutraceutical area focuses its attention on the production of an anti-obesogenic nutraceutical to treat the obesity pandemic. Finally, the antioxidant, anti-inflammatory and anti-ageing molecules naturally synthesized by microalgae and cyanobacteria are of the main interest for the cosmetics and cosmeceuticals sector to apply them as natural ingredients for novel natural cosmetic products.

The company was founded in January 2021 as a spinoff of the Interdisciplinary Centre of Marine and Environmental Research (CIIMAR) by four scientific researchers: Pedro Leão, Principal Investigator (PI) of Cyanobacterial Natural Products (CNP) group, current chief executive officer (CEO) and Chief Scientific Officer (CSO) of the cosmetics and cosmeceuticals sector of the company; Vitor Vasconcelos, PI of Blue Biotechnology, Environment and Health (BBEH) group, Full Professor at the Faculty of Sciences of the University of Porto, current Director of CIIMAR and CSO of the agriculture sector; Ralph Urbatzka, PI of Biodiscovery for Health (B4H) group and CSO of the nutraceutical sector

and João Morais, former Research Technician and Curator of the Blue Biotechnology and Ecotoxicology Culture Collection (LEGE-CC).

2. Business Development

2.1. HiTech Acceleration Program

HiTech is a program provided by the HiSeedTech non-profit association that supports Portuguese research teams to transfer their scientific findings to the market world. This program enables the teams to acquire new skills in commercialization and knowledge to create a link between science and technology in order to create a product.

The nutraceutical team of Fykia Biotech, comprised of the founders Pedro Leão and Ralph Urbatzka as well as three interns, took part in one edition of this program in 2022 with a project entitled “hpa, a compound to fight obesity”. This year, the company did not participate in the program again, but the same strategy was followed to develop a product concept adapted to the biostimulant product, covering topics such as technology and application fields; opportunity space; business model and strategic assessment; product selection; value propositions; market assessment and validation; intellectual property; pricing point; sales plan; roadmap and financials.

2.1.1. Product Concept Development - MicroCrop®

The product concept was developed to offer a sustainable alternative to synthetic fertilizers. There is an ongoing demand for plant fertilizers to boost the agriculture sector and chemical fertilizers remain the main solution to this problem. However, they present hazardous effects on the environment and on human health. Therefore, the use of microalgae and cyanobacteria-based biostimulants, while presenting the same positive effects in plants, has minimal to no impact on human health and on the environment. Compared to other biofertilizers/biostimulants in the market, biostimulants derived from microalgae and cyanobacteria have the advantage of being carbon negatively produced, thus decreasing the carbon footprint.

The MicroCrop® product will be a liquid biostimulant derived from microalgae and/or cyanobacteria capable of improving crop physiology, nutrient uptake and salt stress resistance in order to enhance crop production yields and food quality through the use of a unique set of microalgal and cyanobacterial strains present in a private collection. It

is destined for farming companies or private farmers, adequate for organic farming and its biostimulating effects are expected to reach a large panoply of crops.

A brief analysis of the concurrent companies was performed in order to develop a product that is unique and commercially attractive (**Table 13**).

Table 13. Biostimulant and biofertilizer products currently present in the market, as well as the company that produces them, the country of origin, the company turnover and the claims of the product.

Product	Company	Country	Company Turnover	Capabilities Claimed
AllFertis®	AllMicroalgae®	Portugal	< $1 M	Natural biofertilizers and biostimulants derived from algae, in powder or liquid form, aimed at the agricultural and gardening sectors”
VitaSoil®	Symborg®	Spain	$3.8 M	A soil regenerator enriched with selected rhizospheric microorganisms designed to enhance microbial populations in soils and substrates, specifically for use in intensive agricultural systems
Ferticell®	Agroplasma®	Spain	$2.7 M	Fertilizer solution composed of living cells and cells extracts of 16 unicellular algae and bacteria”
Alganact®	Algaia®	France	$10.1M	Liquid biostimulant derived from seaweeds
Surety MA®	AlgaEnergy®	Spain	$6M	Microalgae-based protein hydrolysates for plant biofertilization through soil or foliar applications
PhycoTerra®	Heliae Development®	United States of America	$21.8M	Liquid microalgal biostimulant to activate the natural microbiome of the soil
Algafert®	Biorizon Biotech®	Spain	$3M	Biofertilizer based on enzymatic hydrolysate of microalgae that improves the quality of roots and tolerance of plants.

Figure 34. Packaging representation of the biostimulant product (MicroCrop®) of Fykia Biotech.

2.1.2. Customer Needs Assessment

When developing a new product, the main objective is to respond directly to the clients' necessities. Therefore, a contact was established with Portuguese farm companies to assess their fertilization habits and the challenges they face daily in crop production. A survey was prepared (see in **Appendix III**) and sent to 37 companies. Four answers were obtained.

2.2. Events

During the internship, the company attended different events to present and share their vision and products in development.

Blue Wink-E 2023 Conference

This event was promoted by B2E CoLAB, a Portuguese private non-profit organization, and the topic of this year's edition was “How to Finance Blue Bioeconomy”. The conference had the aim of exploring innovative solutions for the sustainable financing of projects in the bioeconomy of the sea. A pitch was presented by Ana Rita Vieira, a collaborator at the time of Fykia Biotech, a moment for networking between

companies, investors and research centers followed with a view to creating strategic partnerships and collaborations.

EIT Health – Hack a Pitch Porto 2023

In this networking event, Fykia Biotech was able to present a pitch about the nutraceutical product in development in front of investors to create potential partnerships and investment opportunities.

TOXICROP

The TOXICROP project was funded by the European Union through the H2020 program and had as main objective to monitor and improve water treatment processes to promote more sustainable and safer agriculture globally. Fykia Biotech was one of the collaborators of this project. There was an opportunity to share the startup vision and biostimulant product development process with other collaborating entities to explore new potential partnerships.

2.3. Funding Programs

With a view to obtain investment for the company, an application to “Vouchers for Startups – New Green and Digital Products”, a financial support cofinance by the Recovery and Resilience Plan (RRP) and by the European Union, was submitted.

2.4. Internal Meetings

The founders and I reunited once a month to discuss the advances in product development, applications to funding and networking programs, and business strategies, among others.

2.5. LinkedIn Management

LinkedIn is nowadays an essential platform for companies such as Fykia Biotech to showcase their scientific expertise to potential partners, investors, and clients in order to build a solid network. The Fykia Biotech LinkedIn page was released in April 2022. Since the start of this internship, in November 2023, the company launched four posts related to new member’s presentations, participation in events, and an open call for PhD students’ applications. In the meantime, Fykia Biotech’s LinkedIn page has been visited 2.607 times with 773 unique visitors. At the end of August 2024, the page had a total of 1.163 organic followers, this is, without applying any kind of sponsorship strategy.

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Appendix

Appendix I – Z8 Medium Culture

lege.
LABORATÓRIO DE ECOTOXICOLOGIA GENÓMICA E EVOLUÇÃO

Z8 Medium (Kotai, 1972)

PROTOCOL

- Sterilize all glass material and/or use sterile plastic material
- Use ultrapure water
- Prepare the solutions in volumetric flasks
- Autoclave separately the A,B, Fe-EDTA and micronutrients solutions
- Prepare medium in the flow chamber under aseptic conditions
- Solutions should be added at room temperature to prevent precipitation
- In the end stock solutions should be stored in the refrigerator

Add the solutions to water according to the following concentrations:

Solution A	10 ml/l
Solution B	10 ml/l
Fe-EDTA Solution	10 ml/l
Micronutrients Solution	1 ml/l

Composition of stock solutions:

Solution A

Reagent	Name	g/l	g/2L
$NaNO_3$	Sodium nitrate	46.7	93.4
$Ca(NO_3)_2.4H_2O$	Calcium nitrate tetrahydrate	5.9	11.8
$MgSO_4.7H_2O$	Magnesium sulfate heptahydrate	2.5	5.0

Solution B

Reagent	Name	g/l	g/2l
K_2HPO_4	Potassium phosphate dibasic	3.1	6.2
Na_2CO_3	Sodium carbonate	2.1	4.2

Fe-EDTA Solution

Solution	ml/l	ml/2L
$FeCl_3$*	10	20 ml
EDTA-Na**	9.5	19 ml

Micronutrients Solution

Solution	ml/l	ml/500ml
1 a 12	10	5 ml
13 e 14	100	50 ml

lege.
LABORATÓRIO DE ECOTOXICOLOGIA GENÓMICA E EVOLUÇÃO

Composition of basic solutions

* FeCl3 Solution

Reagent	Name	100ml	250ml
$FeCl_3$.6H2O	Ferric chloride	2.8 g	7 g
HCl (0.1 N)	Hydrochloric acid	100ml	250ml

** EDTA-Na Solution

Reagent	Name	100 ml	250 ml
EDTA	Ethylenediamine tetraacetic acid	3.9 g	9.75 g
NaOH (0.1 N)	Sodium hydroxide solution	100 ml	250 ml

Solutions 1-14

	Reagent	g/l	g/ 100ml
1	Na_2WO_4.2H2O Sodium tungstate	0.33	0.033
2	$(NH_4)_6Mo_7O_{24}$.2H2O Ammonium heptamolybdate	0.88	0.088
3	KBr Potassium bromide	1.2	0.12
4	KI Potassium iodide	0.83	0.083
5	$ZnSO_4$.7H2O Zinc sulfate	2.87	0.287
6	$Cd(NO_3)$.4H2O Cadmium nitrate	1.55	0.155
7	$Co(NO_3)_2$.6H2O Cobalt (II) nitrate	1.46	0.146

	Reagent	g/l	g/ 100ml
8	$CuSO_4$.5H2O Cupric sulfate	1.25	0.125
9	$NiSO_4(NH_4)_2SO_4$.6H2O Ammonium nickel sulfate	1.98	0.198
10	$Cr(NO_3)_3$.9H2O Chromium (III) nitrate	0.41	0.041
11	V_2O_5 Vanadium (V) oxide	0.089	0.0089
12	$AlK(SO_4)_2$·12 H2O Potassium aluminium sulfate	9.48	0.948
13	H_3BO_3 Boric acid	3.1	0.31
14	$MnSO_4$.4H2O Manganese sulfate	2.23	0.223

Appendix II – Chemical Composition and Physical Characteristics of the Soil Substrate used in the Plant Growth Assay

Soil Chemical Composition	Concentration
Nitrogen (N)	80-150 mg/L
Phosphorus (P_2O_5)	80-150 mg/L
Potassium (K_2O)	300-500 mg/L
Organic Matter	>70%

Soil Physical Characteristics	Value
pH in $CaCl_2$	5.5 - 6.5
Humidity	50 – 60%
Conductivity	0.6 -1.2 CE

Appendix III – Survey about the Portuguese Farm Companies on their Use Habits of Fertilizers and Potential Interest in Biofertilizers and Biostimulants derived from Microalgae

Pesquisa de Mercado - Potencial Interesse num Biofertilizante/Bioestimulante com base em Microalgas

Este questionário enquadra-se numa pesquisa de mercado que a **Fykia Biotech**, uma jovem start-up de biotecnologia originária de Matosinhos, realiza para estudar as necessidades e desafios atuais de empresas de produção agrícola e produtores agrícolas profissionais e o potencial interesse em investir num **biofertilizante/bioestimulante com base em microalgas**.

O tempo estimado de resposta ao questionário é de aproximadamente **5 minutos**. A sua participação é totalmente voluntária. A remoção do seu consentimento e paragem de preenchimento do questionário é respeitada, não havendo nenhuma contrapartida caso tal se verifique. A informação recolhida será tratada de forma confidencial, não será de nenhuma forma partilhada para terceiros e utilizada exclusivamente para fins de estudo de mercado.

Caso tenha alguma dúvida, pode entrar em contacto através do nosso e-mail: algae@fykia.pt

Agradecemos a sua disponibilidade e colaboração.

Declaração de consentimento informado:

Compreendi a explicação que me foi fornecida acerca da investigação com o título: "Pesquisa de Mercado - Potencial Interesse num Biofertilizante com base em Microalgas" conduzida pela equipa da Fykia Biotech, spinoff do CIIMAR.

Tomei conhecimento que os dados que fornecer durante este estudo serão totalmente confidenciais, exclusivos à Fykia Biotech.

Foi me dada a oportunidade de fazer perguntas através do e-mail da Fykia Biotech.

Tenho o direito de decidir livremente aceitar ou recusar a minha participação no estudo sem qualquer penalização, nem despesas pela participação neste estudo.

* Indica uma pergunta obrigatória

1. Email *

2. **Declaração de consentimento informado:** *

Compreendi a explicação que me foi fornecida acerca da investigação com o título: "Pesquisa de Mercado - Potencial Interesse num Biofertilizante com base em Microalgas" conduzida pela equipa da Fykia Biotech, spinoff do CIIMAR.

Tomei conhecimento que os dados que fornecer durante este estudo serão totalmente confidenciais, exclusivos à Fykia Biotech.

Foi me dada a oportunidade de fazer perguntas através do e-mail da Fykia Biotech.

Tenho o direito de decidir livremente aceitar ou recusar a minha participação no estudo sem qualquer penalização, nem despesas pela participação neste estudo.

Li e entendi o consentimento informado e aceito participar no estudo

Marcar apenas uma oval.

- ◯ Aceito participar no estudo.
- ◯ Não aceito participar no estudo.
  *Avançar para a secção 5 (**Fim do Questionário**)*

Identificação

3. Indique o nome da sua empresa *
   Se não aplicável, por favor preencha a resposta com "N/A"

4. Indique o seu email

5. Onde fica localizado o seu local de cultivo? *

Marcar tudo o que for aplicável.

- [ ] Norte de Portugal
- [ ] Centro de Portugal
- [ ] Sul de Portugal
- [ ] Madeira
- [ ] Açores
- [ ] Fora de Portugal

6. Qual é aproximadamente o tamanho do seu terreno de exploração (em hectares)? *

Marcar apenas uma oval.

- ◯ <5 ha
- ◯ 5-20 ha
- ◯ 20-100 ha
- ◯ 100-1000 ha
- ◯ >1000 ha
- ◯ Opção 6

7. Que tipo de vendas efetua? *

Marcar tudo o que for aplicável.

- [ ] Vendas locais
- [ ] Vendas nacionais
- [ ] Vendas internacionais

12. Usa fertilizantes nas suas culturas? *

Marcar apenas uma oval.

- ◯ Sim
- ◯ Não

13. Se utilizar fertilizante, de que tipo(s) usa?

Marcar tudo o que for aplicável.

- ☐ Fertilizantes químicos/sintéticos
- ☐ Biofertilizantes (à base de bactérias, fungos, algas, microalgas, ...)
- ☐ Fertilizantes orgânicos (subprodutos de origem animal ou vegetal como compostagem)
- ☐ Bioestimulantes
- ☐ Outra: _____

14. Se utilizar fertilizante, que tipo de formulações costuma usar?

Marcar tudo o que for aplicável.

- ☐ Fertilizantes líquidos
- ☐ Fertilizantes sólidos (grânulos ou pellets)
- ☐ Spray foliares
- ☐ Fertilizantes em pó
- ☐ Outra: _____

15. Se utilizar fertilizante, quantas vezes costuma aplicar durante o tempo de cultivo todo?

Marcar apenas uma oval.

- ◯ 1x
- ◯ 2x
- ◯ 3x
- ◯ 4x ou mais

16. Se utilizar fertilizante, a que momento(s) costuma aplicar?

Marcar tudo o que for aplicável.

- [ ] Aplicação nas sementes
- [ ] Aplicação diretamente após a sementeira/plantação
- [ ] Aplicação antes da transplantação
- [ ] Aplicação durante o cultivo
- [ ] Outra: _____

17. Qual é o seu grau de satisfação com o seu fertilizante atual?

Marcar apenas uma oval.

- ◯ Muito satisfeito
- ◯ Satisfeito
- ◯ Pouco satisfeito
- ◯ Muito pouco satisfeito

18. Se não estiver satisfeito, qual é a razão?

_____

19. Se só usa fertilizantes químicos/sintéticos, consideraria utilizar fertilizantes biológicos?

Marcar apenas uma oval.

- ◯ Sim
- ◯ Não
- ◯ Já utilizei no passado

20. Se só usa fertilizantes químicos/sintéticos, porque razão prefere estes aos biofertilizantes?

Marcar tudo o que for aplicável.

- [ ] Por serem mais económicos
- [ ] Por terem um aplicação mais fácil/eficaz
- [ ] Por serem mais eficazes
- [ ] Por estar satisfeito com o seu fertilizante atual
- [ ] Por manter um certo ceticismo face aos biofertilizantes
- [ ] Por não encontrar biofertilizantes à venda
- [ ] Por desconhecer a existência de biofertilizantes
- [ ] Outra: _____

21. Está atualmente ativamente à procura de tornar a sua produção agrícola mais sustentável? *

Marcar apenas uma oval.

- ◯ Sim
- ◯ Não
- ◯ Talvez

22. Teria interesse num novo produto biofertilizante com base em microalgas? *

Marcar apenas uma oval.

- ◯ Sim
- ◯ Não
- ◯ Talvez

23. Que valor estaria disposto a investir num novo biofertilizante líquido de origem natural que demonstre eficácia igual ou superior ao fertilizante que utiliza atualmente? *

Marcar apenas uma oval.

- ◯ 1-5 €/L
- ◯ 5-7 €/L
- ◯ 7-10 €/L
- ◯ 10-13 €/L
- ◯ 13-15 €/L
- ◯ 15-20 €/L
- ◯ >20 €/L

24. Quais são as qualidades/atributos que mais valoriza num (bio)fertilizante? *

Marcar tudo o que for aplicável.

- ☐ Qualidade-preço
- ☐ Teor de nutrientes
- ☐ Origem (biológica/orgânica ou sintética)
- ☐ Modo de aplicação (spray foliar, líquido, granular, ...)
- ☐ Compatibilidade ambiental
- ☐ Compatibilidade com sistemas de irrigação
- ☐ Outra: _____

25. Estaria disposto a participar em testes de campo com uma amostra do nosso produto? *

Marcar apenas uma oval.

- ◯ Sim
- ◯ Não
- ◯ Talvez

Bioestimulantes

26. Já ouviu falar de bioestimulantes? *

Marcar apenas uma oval.

- ◯ Sim
- ◯ Não *Avançar para a secção 5 (**Fim do Questionário**)*

27. Se sim, já utilizou?

Marcar apenas uma oval.

- ◯ Sim
- ◯ Não

28. Teria interesse em aplicar bioestimulantes nas suas culturas? *

Marcar apenas uma oval.

- ◯ Sim
- ◯ Não
- ◯ Não sei

Fim do Questionário

Muito obrigado pela participação!