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Study of the biopesticide and biostimulant activity of microalgae in horticultural species

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Declaração de autoria de trabalho

Study of the biopesticide and biostimulant activity of microalgae in horticultural species

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

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Catarina Viana

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Resumo

As algas microscópicas e as cianobactérias fazem parte de um diverso grupo de organismos unicelulares designados por microalgas. Este grupo é composto por organismos fotossintéticos, responsáveis por 60 % da produção da primária da Terra e podem ser encontrados em diferentes habitats, na sua maioria aquáticos. Devido à sua capacidade de captação de CO_2 atmosférico, à disponibilidade de água e a luz solar, produzem grandes quantidades de biomassa e com isso apresentam uma maior atividade fotossintética que as plantas. O seu crescimento heterotrófico, a sua natureza versátil e o seu rápido desenvolvimento, têm vindo a chamar à atenção e a ser utilizados em indústrias diversificadas, como a farmacêutica, cosmética, dietética, biocombustíveis, de entre outras. Para além destas aplicações , as microalgas estão a ser alvo de estudos tendo em conta a sua relação com o processo de mineralização, na circulação de matéria orgânica e inorgânica, a sua rica composição em macro- e micronutrientes e a sua capacidade de produção de compostos bioativos, como polissacarídeos, fitohormonas e até mesmo compostos fenólicos, o que os levou a serem vistos como possíveis candidatos a substitutos dos produtos químicos e sintéticos, nomeadamente dos pesticidas e fertilizantes.

A crescente preocupação com a necessidade de aumentar tanto a produtividade como a qualidade e combater as doenças que afetam a culturas, fez com que os produtores recorressem a produtos que lhes garantam um maior crescimento das suas culturas, nomeadamente os fertilizantes e os pesticidas. Como consequência, houve um uso abusivo de sustâncias que, em grande quantidade, são prejudiciais tanto para o ambiente como para a saúde humana. Essas consequências podem ser evitadas se se recorrer ao uso de fertilizantes orgânicos, sendo também referenciados como excelentes alternativas para a reciclagem de nutrientes e o aumento de carbono (C) no solo e mitigando as alterações climáticas. Para além destas vantagens, os compostos produzidos pelas microalgas também contribuem para um solo saudável e por acréscimo, uma microbiota benéfica.

O agravamento do efeito estufa na Terra e as alterações climáticas provocadas, levou a União Europeia a formular um acordo em 2019 (*European Green Deal*), com o principal objetivo de tornar a Europa um continente com um impacto ambiental neutro até 2050. Para tal, é fundamental reduzir a emissão de gases em 55% até 2030 em comparação com 1990. Paralelamente, a aplicação da Diretiva n.º 2009/128/CE, do Parlamento Europeu e do Conselho, de 21 de outubro, através da Lei n.º 26/2013 de 11 de abril, reduziu

significativamente a quantidade de substâncias ativas homologadas. Estas restrições abriram portas para a introdução de uma agricultura mais sustentável. Bioestimulantes e biopesticidas têm sido estudados como possíveis alternativas aos fertilizantes sintéticos e pesticidas, devido à sua já reportada eficácia. Estes compostos biológicos são seguros para o ambiente e ricos em nutrientes. A biomassa das microalgas contém inúmeros aminoácidos promotores do crescimento das plantas, bem como fitohormonas que aumentam a produção, estimulando o desenvolvimento das raízes e dos ramos. Para além disso, já foi reportado que o uso da biomassa destes microrganismos reduziu o desenvolvimento de diversos fungos fitopatogénicos.

Este trabalho visou o estudo do potencial biopesticida das microalgas *Scenedesmus obliquus* e *Chlorella vulgaris*, através da avaliação *in vitro* e *in vivo* da sua ação inibitória de fungos fitopatogénicos e do seu efeito no desenvolvimento de plantas hortícolas. Para avaliar o seu efeito inibidor do crescimento de fungos e oomicetas, foram preparadas suspensões aquosas com biomassa das duas microalgas em diferentes concentrações, que numa primeira fase foram testadas *in vitro* contra *Fusarium oxysporum*, *Botrytis cinerea*, *Alternaria alternata, Sclerotium rolfsii, Clarireedia* spp.*, Colletotrichum gloeosporioides* e *Phytophthora cinnamomi*. Estes agentes fitopatogénicos são os responsáveis pelo aparecimento de doenças nas plantas cultivadas, ameaçando a produção de alimentos e a segurança alimentar das populações. Numa segunda fase, foram conduzidos testes *in vivo*, onde se inocularam substratos hortícolas em vaso com micélios ativos de *Fusarium oxysporum* e *Sclerotium rolfsii* onde se plantou espinafre e alface, respetivamente. Para além destes fungos, outras plantas de espinafre e alface foram pulverizadas com uma suspensão de *Botrytis cinerea*. Este modo de aplicação das suspensões foi escolhido tendo em conta o tipo de fungo: no caso de *F. oxysporum* e *S. rolfsii* que são fungos do solo, o modo de aplicação das suspensões de microalgas foi por rega; no caso de *B. cinerea*, devido a ser um fungo da parte aérea da planta, o modo de aplicação selecionado foi a pulverização direta na parte aérea da planta. Estas aplicações decorreram semanalmente, durante o período de cultivo, de modo a avaliar o desenvolvimento da doença quando em contacto com as suspensões. Neste trabalho, avaliou-se também o potencial bioestimulante das suspensões de microalgas em ensaios *in vitro* e *in vivo*, verificando *in vitro*, o efeito na germinação e no desenvolvimento da radícula, e *in vivo* avaliando o desenvolvimento da planta, através de medições biométricas no final dos ensaios. Apesar

de já existirem diversos estudos sobre o efeito estimulante das microalgas, os estudos sobre o seu efeito inibitório continuam a ser escassos.

Quando em contacto com as suspensões, foi identificada a supressão do crescimento dos fungos *F. oxysporum, S. rolfsii, B. cinerea* e *C. gloeosporioides* e do oomiceto *P. cinnamomi,* indicando uma aplicação promissora das microalgas. Nos ensaios *in vivo,* foi comprovado que as microalgas afetam o desenvolvimento das doenças nas plantas, mostrando resultados promissores quanto à aplicação das microalgas tanto para a sua proteção como para o seu crescimento. Assim sendo, o uso de microalgas eucarióticas revelou-se promissor, possibilitando a redução do uso de fungicidas sintéticos, limitando o impacto ecológico da agricultura. A sua composição demonstrou ser uma mais-valia para o desenvolvimento das plantas quando aplicadas nos testes do potencial bioestimulantes. O seu conteúdo em fitohormonas e aminoácidos são uma fonte nutritiva para as plantas promovendo o seu crescimento, sendo de esperar que melhorem o desenvolvimento das plantas e enriqueçam o meio onde são aplicadas.

Palavras-Chave: Microalgas; Biopesticidas; Bioestimulantes; Agentes fitopatogénicos; Fitofármacos; Agricultura sustentável

Abstract

Microscopic algae and cyanobacteria are part of a diverse group composed of single-celled organisms called microalgae. This group is composed of photosynthetic organisms, responsible for 60% of the earth's primary production, and can be found in different habitats, mostly aquatic. Due to their atmospheric CO_2 capture capacity, water availability and sunlight, they are producers of large amounts of biomass and thus present higher photosynthetic activity than plants. Their rich composition in macro- and micronutrients and their ability to produce bioactive compounds such as polysaccharides, phytohormones and even phenolic compounds, led them to be seen as possible candidates for substitutes for chemicals and synthetics, namely pesticides and fertilizers.

The growing concern about the need to increase both productivity and good quality and combat diseases that will impact crops has caused producers to use compounds that would ensure accelerated growth of their crops, fertilizers and pesticides. Therefore, there has been an abusive use of substances that in large numbers are harmful to both the environment and human health.

This work aims to study the biopesticide potential of the microalgae *Scenedesmus obliquus* and *Chlorella vulgaris*, through *in vitro* and *in vivo* evaluation, the inhibiting action of phytopathogenic fungi and their effect on the development of vegetables. The inhibiting effects of these microalgae were tested on phytopathogenic fungi: *Fusarium oxysporum*, *Botrytis cinerea*, *Alternaria alternata*, *Sclerotium rolfsii*, *Clarireedia* spp., *Colletotrichum gloeosporioides* and oomycete: *Phytophthora cinnamomi*. These agents are responsible for numerous diseases in agriculture, causing several losses. This work describes the promising inhibiting effect of suspensions on the development of *Fusarium oxysporum*, *Botrytis cinerea*, *Sclerotium rolfsii*, *Colletotrichum gloeosporioides* and *Phytophthora cinnamomi.* The same effect was observed during the *in vivo* trials, when applied microalgae suspensions in plants inoculated with *F. oxysporum*, *S. rolfsii* and *B. cinerea*. And the biostimulant effect on agricultural plants was also shown in this study.

Keywords: Microalgae; Biopesticides; Biostimulants; Phytopathogenic agents; Phytopharmaceuticals; Sustainable agriculture

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Chapter I: State of the Art

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Abbreviations

APX- Ascorbate Peroxidase

AUDPC- *area under disease progress curve*

CFU- colony forming units

Cv- *Chlorella vulgaris*

DAI- Days After Inoculation

DAS- Days After Sowing

DMSO- Dimethyl sulfoxide

DW- Dry Weight

EC- Electrical conductivity

GA- Gallic Acid

GAE- Gallic Acid Equivalents

GI- Germination Index

IP- Inhibition Percentage

PCA- *Plate Count Agar*

PDA- *Potato Dextrose Agar*

PDO- Peroxidase Enzyme

RRG- Relative Radicle Growth

RSG- Relative Seed Germination

SG- Seed Germination

So- *Scenedesmus obliquus*

SPSS- Statistical Package for Social Sciences

STD- Standard Deviation

Chapter I: State of the Art

1.) Microalgae general description

Microalgae are microscopic algae belonging to phytoplankton. They are part of a complex group of microorganisms of which 15.7 x 10^4 species have been recognized so far, but the number of species that are produced industrially is much lower (Menaa et al., 2021). Microalgae are photosynthetic microorganisms that convert sunlight, water, and carbon dioxide into large amounts of biomass. The main microalgae groups are *Cyanophyceae* sp. (blue-green algae), *Bacillariophyceae* sp. (diatoms), *Chlorophyceae* sp. (green algae), and *Chrysophyceae* sp. (gold-brown algae). These microorganisms are recognized for having the ability to capture atmospheric CO_2 and they are applied in wastewater treatment. They can be found in various habitat, such as salt water, fresh water and soils (Alassali & Cybulska, 2015; Khan et al., 2018; Priyadarshani & Rath, 2012) because microalgae can live in a range of temperatures, pH, and salinity. This ability makes it possible to survive in extreme ecosystems and still they can live alone or in symbiosis with other organisms (Khan et al., 2018; Pushkareva et al., 2016). According to their use of the carbon source, microalgae can be classified as autotrophic, heterotrophic or mixotrophic.

The terminology "seaweed" does not describe a taxonomic group, but a set of producers of O_2 and photosynthetic organisms with chlorophyll, a pigment which do not present a common ancestor. Contributing to 50 % of the Earth's photosynthetic production, algae can range from microscopic organisms to macroscopic aggregates (Barsanti & Gualtieri, 2010).

Microalgae are vital to the Earth's ecosystems, producing about 50 % of atmospheric oxygen together with cyanobacteria. Through photosynthesis atmospheric carbon is captured and fixed using water and light as sources of energy and electrons (Lehmuskero et al., 2018; Raja et al., 2014), producing lipids, proteins, carbohydrates and nucleic acids. According to Song et al. (2022), microalgae can accumulate from 50 % to 60 % of lipids. Compared to terrestrial plants, microalgae can produce a greater amount of biomass, which requires good access to nutrients, CO_2, and water, most of them living in aquatic ecosystems (Gouveia, 2011; Raja et al., 2014).

2.) Agriculture

The world's population has been increasing significantly over the years, with an estimated 9 billion inhabitants by 2050 (Koop & van Leeuwen, 2017; UNDP, 2012). This increase has proved to be a challenge in terms of providing food security for all people. To ensure food security for the population and consequently a decent quality of life, it is necessary to increase both livestock and agricultural production. It is estimated that food security will only be obtained with 60 % increase in food production or that measures are taken against food waste (Alexandratos & Bruinsma, 2012). To increase production, natural resources will come under increased pressure, namely agricultural land, water, forests, and even climate. Given this situation, it was necessary to create a balance, thus the need to adapt to a sustainable agriculture. As the name implies, sustainable agriculture is a type of agriculture that aims to respect the environment while it is economically viable (Brodt et al., 2011).

2.1. Problems of maintaining conventional agriculture

In many cases, to increase production many farmers increase the use of synthetic fertilizers. Despite their fundamental role in the success of agricultural systems, these fertilizers have also proved to be a problem for the environment (Bouwmeester et al., 1985; Morari et al., 2011)

Fertilizers can be classified into synthetic fertilizers or natural fertilizers. Synthetic fertilizers, also known as chemical fertilizers, contain mostly nitrogen (N), phosphate (P), and potassium (K), essential nutrients for crop growth (Morari et al., 2011). Nitrogen is an important nutrient for plants and one of the main contributors to crop productivity. The use of nitrogen fertilizers has become a common practice since they played an important part in the success of the Green Revolution (GR), being essential for the quality, quantity and sustainability of crop yields (Liao et al., 2020). The concept Green Revolution was introduced by Norman Borlaug, when man-made changes in agriculture, such as the introduction of dwarf genes that would lead to an increase in production through irrigation and fertilizers, along with disease resistance, made possible to increase crop production (Evans & Lawson, 2020). However, the excessive use of mineral fertilizers has drastically altered the nutrient balance, leading to environmental changes such as air pollution; eutrophication of water; acidification and soil degradation; dissolution of the natural

nitrogen and phosphate cycle; among others (Aryal et al., 2021; Bouwman et al., 2005; Guo et al., 2010; Ju et al., 2009; Lu & Tian, 2013, 2017; Sutton et al., 2011; Tian et al., 2012; Vitousek et al., 2009), in addition to concerns with human health. It is estimated that about 30 % to 50 % of the nutrients of these chemical fertilizers are either leached into the underground water or volatilized into the air (Wang Y. et al., 2018). It is estimated that around 60 % of nitrogen pollution is due to agricultural production through the application of fertilizers. In addition, studies indicate that the application of nitrogen fertilizers in soils is the main pathway for the loss of nitrogen as ammonia (NH_3), which in turn, when in the environment, leads to the origin of nitrogen oxide (Beusen et al., 2008; Kang, 2022; Kang et al., 2022). N_2O, a gas that has a more potent effect than CO_2 on the greenhouse gas increase, and methane are the two gases that are estimated to be the most emitted into the environment representing about 50 % and 40 % of total emissions, respectively.

As mentioned above, nitrogen and potassium are plant nutrients used in agriculture, and consequently these elements enter the surface water and aquifers, increasing the concentration of nitrates and phosphates in water. Eutrophication will make impossible to use the affected water for most activities, due to the accelerated development of algae blooms, which death will reduce the oxygen in the aquatic environment leading to the death of living beings present in the aquatic ecosystem. In addition, some species of algae produce toxins, that can affect various systems of the body of living beings, such as neurotoxins that will affect the nervous system, and can lead to death causing a severe problem, not only for the environment but also for the economy and human health (Fleming et al., 2011; Ruivo, 2017).

Another factor that is influenced by the excessive use of chemical fertilizers are the microorganisms present in the soil. The microbial community has a key role in maintaining soil health and plant productivity, being the main responsible for processes such as the decomposition of organic matter or nutrient recycling (Liao et al., 2018; Wu et al., 2020). Microorganisms in soil promote plants' health by establishing a mutualist association of symbiotic character, providing plants with some degree of resistance against diseases (Yuan et al., 2017). According to Sun et al. (2015), the decrease in soil pH is proven to have a direct relationship with the reduction of bacterial diversity, causing a change in community. A disturbance in the microbial community will enable the growth of other microorganisms, harmful or not to the plants. Without the resistance induced by

the microbial community, many plants end up dying. However, Geisseler & Scow (2014), reported that for a brief period, the application of mineral fertilizers causes an increase of 15.1 % in microbial biomass compared to conditions without any type of fertilization.

Unlike chemical fertilizers, organic fertilizers are mostly originated from animal manure, plant residues, products from composting, etc. (Morari et al., 2011). When using these fertilizers, plant response is not direct and there is no rapid increase in productivity therefore their use was not preferred. However, the application of organic fertilizers will release various nutrients, continuously, in the soil, during its mineralization. Organic fertilizers may change the soil structure, pH, and electrical conductivity, among other soil characteristics, benefiting soil microbiota and improving water retention and fertility (Bell et al., 2015; Wu et al.,2020; Kang et al., 2022).

Increased productivity is not the only concern regarding population growth. The increase in crop productivity leads to the increased occurrence of pests and diseases, such as microorganisms, insects, rodents or weeds. According to Oerke & Dehne (2004), annually diseases in plants lead to a loss of 15 % of crops, with direct economic losses. So, to maintain crop quality and yield, farmers apply pesticides to control these diseases. The use of synthetic pesticides is another cause of soil and aquifer contamination (De Albuquerque et al., 2018). The negative effects on natural resources and human health have led to an increased demand for environmentally friendly biopesticides. They are called biopesticides because they are originated naturally or are obtained from biological sources, such as living organisms or their metabolites (Senthil-Nathan, 2015). From their composition, they can be called microbial, biochemical, and botanical biopesticides (Thirumurthy & Mol, 2020). Biopesticides act in several ways in disease control, and this can be obtained by inhibiting their metabolic activities, using nutrients, or even activating the plant's immune system (Mnif & Ghribi, 2015; Senthil-Nathan, 2015). The different modes of action on their targets will prevent pests and diseases from gaining resistance to biopesticides. Pesticides of biological origin are mostly target-specific and are supposed not to cause negative effects on water, air, environment or human health, and are still biodegradable. Several microalgae cyanobacteria have been considered promising biopesticides due to their rich composition in bioactive compounds, which have antifungal and insecticide properties (Renuka et al., 2018).

3.) Phytopathogenic fungi

Plant pathogens are usually microorganisms, mostly bacteria, fungi or virus. They attack plants to obtain nutrients, invading their tissues, growing and developing in them. Inside plant tissues, they cause diseases by destabilizing the cell structure by releasing enzymes, toxins, growth promoters, and other active substances (Agrios, 2009). It is estimated that there are 1.5 million fungi species, 70,000 are known species of which 10,000 are known to be disease-causing agents (Agrios, 2009).

It is estimated that about 70 % of diseases in plants are caused by fungi. According to Henson et al. (1999), Phyla Ascomycota and Basidiomycota contain most of the phytopathogenic species. Diseases caused by fungi and pathogenic oomycetes continue to cause large crop losses around the world. Phytopathogenic agents have developed different ways of life and different ways of interacting with their hosts, ranging from pathogens that can synthesize and secrete metabolites, killing their hosts and feeding on them (necrotrophic), while others feed on the nutrients of their hosts and do not produce toxins (biotrophic) and finally, the hemibiotrophic that are in between the two previous types (Doehlemann et al., 2008; Horbach et al., 2011)(Figure 1.1). Biotrophics are pathogens that are dependent on the host, obtaining their nutrients through specialized infectious structures called haustoria, which transfer nutrients from the host to the fungus. The haustoria is a specialized hypha that when entering the interior of the plant will expand (Szabo & Bushnell, 2001). For hemibiotrophic, in the initial stage of adhesion cellular and stabilization inside the host, it is alive, and no substances are produced that will damage their cellular appearance, so they are classified as biotrophic in the first phase, in which they form a less specialized structure than haustoria, which enters inside the cells. Once established, these pathogens change to a necrotrophic phase and begin the process of infection, which is divided into germination, proliferation, and sporulation (Perfect & Green, 2001).

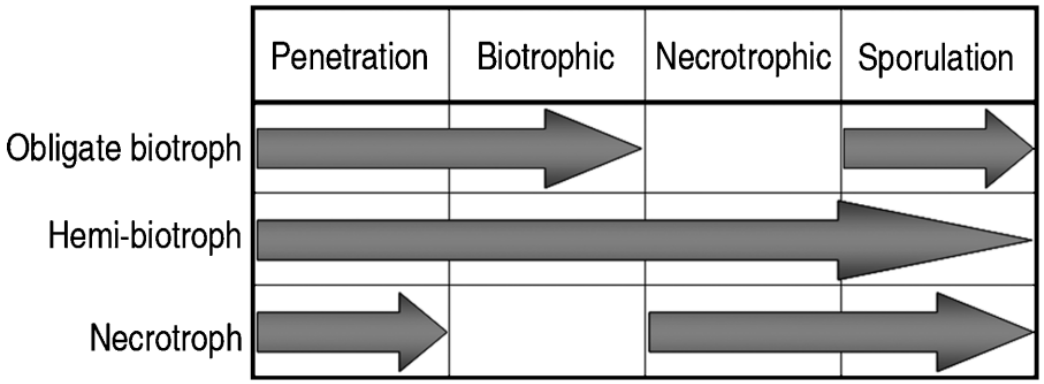


Figure 1.1: Infectious phases of the different types of plant pathogens (Divon & Fluhr, 2006).

In the case of necrotrophic, there is no formation of an haustorium. Appressoria formed by necrotrophic species are common, and hyphae that penetrate the host (infection hyphae) are uniform. The mechanism of infection usually used is that of direct penetration of the host surface, and may produce reactive oxygen species (ROS), activators of host immune responses (DAMPs) (Kleemann et al., 2012) or virulent effectors that will suppress as immune responses of the host, leading to significant tissue damage and consequently to the host death (de Cal et al., 2022; Sharma, 2021). In the first step, it is necessary to add the pathogen to the surface of the host, followed by the application of pressure and accompanied by enzymes for the degradation of the cuticle and cell wall (CWDE's). Adhering is granted by the appressoria, an organ of the hypha responsible for pressure in the host wall (Horbach et al., 2011) (Figure 1.2).

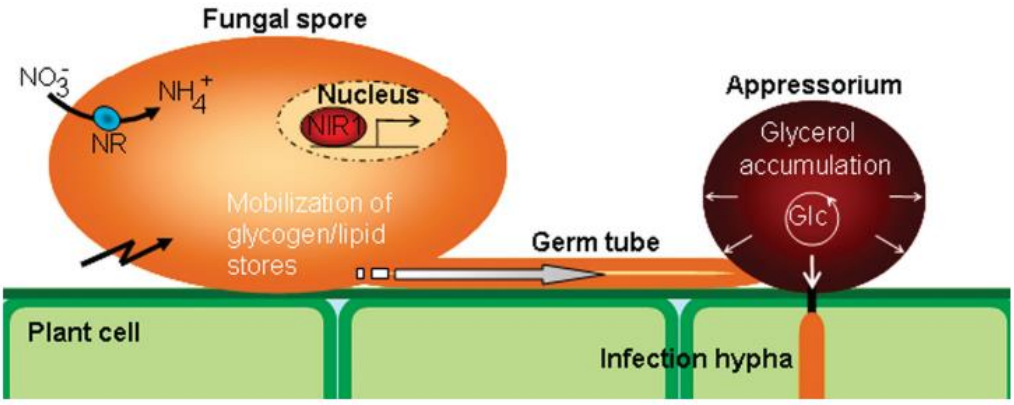


Figure 1.2: Stabilization of the fungus spore and penetration of the plant wall (Divon & Fluhr, 2006).

Species such as *Botrytis* spp., *Alternaria* spp., *Fusarium* spp., *Sclerotinia* spp., *Phytophthora* spp., and *Sclerotium* spp. are known to be necrotrophic species, while *Colletotrichum* is a hemibiotrophic species.

Botrytis cinerea

Botrytis cinerea is a phytopathogenic fungus responsible for the losses in around 200 crops due to its broad host range, that appears in second place on the list of diseases with the most scientific/economic importance (Dean et al., 2012). Also known as "gray mold", *Botrytis cinerea* is an airborne plant pathogen with a necrotrophic lifestyle, belongs to the family *Sclerotiniaceae*, and is responsible for several symptoms (Williamson et al., 2007). In addition, it has several mechanisms to invade and cause severe damage to the host plant. This fungus affects many important crops, namely grapes, strawberries, tomatoes, and cucumbers (Hou et al., 2020).

Alternaria alternata

Alternaria species are saprophytes that infest important crops such as cereals, vegetables, tobacco and fruits, like tomatoes or apples. Usually, their targets are leaves and fruits, leading to their necrosis. It is a leaf-borne pathogen and will reduce the photosynthetic potential of plants, leading to the slow destruction of tissues (Thomma, 2003). The

reduction in photosynthesis II is due to toxins produced by this specie (Wang R. et al., 2020).

Fusarium oxysporum

Fusarium oxysporum is known to be a soilborne fungus, present in all soil types of the world. It is a saprophyte fungus and survives for long periods of time in organic matter (Fravel et al., 2003). Its mechanism of infection is by penetrating the root of the plants and invading the vascular system, thereby preventing water from reaching the above parts of the plant, leading to a progressive yellowing and wilting of the plant (Edel-Hermann & Lecomte, 2019). According to (Dean et al., 2012), it is in fifth place as one of the most important diseases, harmful to cereals and non-cereals crops, such as tomatoes, cotton or bananas.

Clarireedia spp.

It is a destructive fungus, which causes economic losses related to grasses, particularly on golf courses, causing the disease called "Dollar spot," being very resistant to pesticides. It causes brownish stains on the grass fields and produces ascospores and conidia. His family is still being debated, however, according to genetic evidence, it seems to belong to the family *Rutstroemiaceae* (Salgado-Salazar et al., 2018).

Phytophthora cinnamomi

The genus *Phytophthora* includes species of oomycetes that are very aggressive pathogens, known to cause serious damage in agricultural crops and forest trees. *Phytophthora cinnamomi* is a specie adapted to the climate of the Iberian Peninsula and attacks mainly (Neves et al., 2014). This oomycete attacks the roots of the cork oak leading to the death of the plant. This pathogenic grows saprophytically in the soil and persists in the infected plant in the form of chlamydospore and manages to survive up to 6 years in moistened soil. In addition, recent studies have found that the zoospores of this pathogenic are mobile, reacting to chemical and electrical (Hardham, 2005).

Sclerotium rolfsii

Sclerotium rolfsii, like the other fungus already mentioned, has a wide range of hosts, comprising more than 500 species, causing "Southern blight disease". This fungus attacks the root of the plants and leading it to rot. Its main symptoms are the appearance of grayish lesions and water-soaked lesions at the base of the stem, near the soil line, and the damping-off of the plant. Sometimes it is possible to identify mycelium growth in soil and sclerotia (Paparu et al., 2020). It can affect crops such as lettuce, peanut, tomatoes and potatoes.

Colletotrichum gloeosporioides

Colletotrichum gloeosporioides is a hemibiotrophic fungus with a short period of time in the biotrophic stage, in the eighth place of importance (Dean et al., 2012),. This fungus causes anthracnose and causes great economic losses in crops, especially fruit, vegetables and ornamentals, like oranges and papaya (Silva-Jara et al., 2020). It is an asexual genus and belongs to the *Coelomycetes*.

4.) Microalgae as Biostimulants and Biopesticides

Microalgae have been widely studied by several industries, due to their characteristics. According to Chiaiese et al. (2018) and García et al. (2017), the versatile nature of microalgae and their rapid adaptation to both industrial and household waste, showed the capacity that these organisms must be good bioremediation agents in combating pollutants that are present in wastewater or in the soil (Martinez-Porchas et al., 2014). Su & Jacobsen (2021) showed in their study that the use of *Chlorella vulgaris* and *Scenedesmus obliquus* residual food water resulted in the removal of more than 54 % of the concentration of nitrogen and phosphorus.

Microalgae produce a variety of bioproducts, such as polysaccharides, lipids, pigments, proteins, vitamins, bioactive compounds and antioxidants (Khan et al., 2018). Various products from microalgae are available for use in agriculture and improve crop yields. Therefore, it is known that microalgae can act in different ways:

a) Soil improvement

Its application increases the availability of nutrients in the soil, making the soil more fertile. With this, soil microbial activity is stimulated, favoring seed germination, plant growth and productivity (Renuka et al., 2018). Microalgae participate in the biogeochemical cycle of nutrients, ensuring their availability in the rhizosphere. The use of microalgae leads to improvements in the soils where they are applied, allows an improvement in water retention and prevents nutrient loss (Table 1.1). The biomass of microalgae can be an important source of organic matter in the soil, providing the appropriate concentration of compounds for plant development (Gonçalves, 2021). In addition, the availability of nutrients in the substrate will lead to the growth of the community of microorganisms present and an increase in their activity (Prasanna et al., 2013). Plant growth-promoting rhizobacteria play an important role due to their excretion of hormones that promote not only plant growth but also the suppression of pathogens (Vessey, 2003). Micronutrients are essential elements in plant metabolism and their availability in the soil directly affects the quality of the plant and its products, along with its physiology (Prasanna et al., 2013; Rana et al., 2012).

Table 1.1: Crop improvements after microalgae application

Microalgae	Improvement	Target Crop	Reference
Calothrix ghosei, *Hapalosiphon intricatus* and *Nostoc* sp.	Organic carbon content in the soil; -Increase grain yield	Wheat	(Karthikeyan et al., 2007)
Cyanobacterial consortia including the species: *Anabaena doliolum*, *Cylindrospermum sphaerica* and *Nostoc calcicole*	Water retention capacity Increase in grain yields Nutritional properties	Wheat and millet	(Nisha et al., 2007)
Chlorella, *Scenedesmus*, *Chlorococcum*, *Chroococcus*, *Phormidium*, *Anabaena*, *Fischerella* and *Spirogyra*	Product Quality	Wheat	(Renuka et al., 2015)
Microalgal-bacterial flocs and *Nannochloropsis oculata*	Fruit quality (increase in sugar and carotenoids contents)	Tomato	(Coppens et al., 2016)
Scenedesmus quadricauda	-Nutritional properties	Lettuce	(Puglisi et al., 2020)

Chlorella vulgaris	-Nutritional properties	Lettuce	(Bella et al., 2021)

b) Phytohormones

It is also known that several green algae are able to produce and excrete plant growth-promoting hormones (Ronga et al., 2019), such as cytokinins and auxins. Recent studies have proven that its use in open field plantations or greenhouses led to an improvement in germination, stem growth and root biomass in lettuce, tomato, cucumber and wheat, among other crops (Bella et al., 2021; Bumandalai & Tserennadmid, 2019; Supraja et al., 2020; Puglisi et al., 2020; Renuka et al., 2015). Plant growth was associated with the stimulation of carotenoids and biosynthesis of chlorophyll pigments, which may have improved photosynthetic activity (Wuang et al., 2016).

c) Activation of plant defense mechanisms

Exposure of plants to extreme environments is another factor that negatively affects them (Bulgari et al., 2019; Górka et al., 2018), such as its decrease in its metabolic activity; the degradation of cell membranes; protein and enzymatic synthesis; gas exchange and photosynthesis among others (Bulgari et al., 2019; Górka et al., 2018; Kaya et al., 2007). The application of algae-derived products showed that increase tolerance against abiotic and biotic stress occurs. It was found that when appling *Dunaliella saline* to tomato under salt stress, it resulted in a higher stem and root mass and a higher chlorophyll a and b content, and reduced damage caused by excess salt in the plant (El Arroussi et al., 2018). This response was associated with seaweed exopolysaccharides. Cyanobacteria will activate the defense mechanisms of plants, thus increasing the activity of plant defense enzymes such as catalase, peroxidase, and chitinase, among others (Renuka et al., 2018). Li et al. (2014), described that when *Chlorella vulgaris* was applied to beans, it led to an increase in the frequency of stomata closure and efficient use of water, reducing perspiration and increasing drought tolerance.

5.) Microalgae as Biopesticides

Plant susceptibility to diseases increases with deficiencies in micronutrients, becoming especially susceptible to phytopathogenic fungi. The increase in nutrients in the soil

brings a resistance of plants to diseases in the roots, caused by competition for nutrients available by beneficial microorganisms and pathogens (Rana et al., 2012).

Microalgae produce a range of bioactive compounds with antifungal properties, such as phenolic and alleloquimic compounds. Its accumulation capacity of compounds makes it possible to use them as a biopesticide (Renuka et al., 2018). Bioactive compounds can act in a variety of ways, such as cytoplasmic membrane disruption or inhibition of protein synthesis (Costa et al., 2019).

Protection against phytopathogenic organisms is usually activated through polysaccharides that recognize the signaling molecules in the wall of pathogens and induces plant defenses, which consists of inducing the synthesis of secondary metabolites with antioxidant, antifungal, and antimicrobial properties (phenolic compounds, terpenoids, etc.) (Lee & Ryu, 2021; Thirumurthy & Mol, 2020). Therefore, having a rich composition in polysaccharides, when applied to plants, will lead to the activation of plant defense mechanisms. Despite the activation of defense mechanisms, microalgae also present phenolic compounds that are responsible for the degradation of the cell wall of pathogens.

Some studies (Table 1.2) proved the activity of microalgae against phytopathogens and insects responsible for crop destruction.

Table 1.2: Activity of microalgae

Cyanobacteria			
Algae	**Pathogen/insect type**	**Pathogen/insect name**	**References**
Anabaena variabilis RPAN59 and *A. oscillarioides* RPAN69	Fungal Pathogen	*Pythium debaryanum; Fusarium oxysporum* f. sp. *lycopersici; Fusarium moniliforme* and *Rhizoctonia solani*	(Chaudhary et al., 2012)
Anabaena sp.	Fungal Pathogen	*Candida albicans* and *Aspergillus Flavius*	(Shishido et al., 2015)
Aphanocapsa albida; Anabaena oryzae; Nostoc muscorum and *Calothrix marchica*	Pathogenic nematode	*Meloidogyne incognita*	(Hamouda, 2013)

Calothrix brevissima	Fungal Pathogen	*Alternaria alternata; Botrytis cinerea; Colletotrichum gloeosporioides; Fusarium oxysporum; Phytophthora capsici* and *Pythium ultimum*	(Kim, 2006)
Calothrix elenkenii	Fungal Pathogen	*Pythium aphanidermatum*	(Manjunath et al., 2009)
Microcoleus vaginatus	Pathogenic nematode	*Meloidogyne arenaria*	(Khan et al., 2005)
Nostoc commune FA-103	Fungal Pathogen	*F. oxysporum* f. sp. *Lycopersici*	(Kim & Kim, 2008)
Nostoc commune FA-103 and *Oscillatoria tenuis* FK-109	Pathogen	*Phytophthora capsica*	(Kim, 2006)
Nostoc muscorum and *Oscillatoria* sp.	Fungal Pathogen	*Alternaria porri*	(Abdel-Hafez et al., 2015)
N. muscorum; Spirulina platensis and *Oscillatoria* sp.	Fungal Pathogen	*Cercospora beticola*	(Hussien et al., 2009)
Nostoc strain. ATCC 53789	Fungal Pathogen	*Sclerotinia sclerotiorum*	(Biondi et al., 2004)
Oscillatoria chlorina	Pathogenic nematode	*Meloidogyne arenaria*	(Khan et al., 2007)
Green/Red Microalgae			
Caulerpa racemosa and *Gracilaria edulis*	Fungal Pathogen	*Fusarium oxysporum* f. sp. *Udum*	(Ambika & Sujatha, 2014)
Chlorella vulgaris	Pathogenic nematode	*Xiphinema indexin*	(Bileva, 2013)
C. vulgaris and *Tetradesmus obliquus*	Fungal Pathogen	*Fusarium oxysporum*	(Ferreira et al., 2021)
C. vulgaris and *Chlorella minutíssima*	Fungal Pathogen	*Fusarium oxysporum* and *Aspergillus niger*	(Vehapi et al., 2018)
Chlorella vulgaris; C. protothecoides; C. minutissima	Fungal Pathogen	*Aspergillus niger; Alternata alternata* and *Penicillium expansum*	(Vehapi et al., 2020)

Chlorella fusca	Fungal Pathogen	*Colletotrichum orbiculare*	(Jeung Kim et al., 2018; Lee et al., 2016)
C. fusca	Fungal Pathogen	*Botrytis squamosa*	(Kim et al., 2018)
C. fusca	Bacterial Pathogen	*Pseudomonas syringae* pv. Tomato	(Lee et al., 2020)

I.I: Objectives of this Thesis

The main goal of this study was to determine the biopesticide and biostimulant activity of two microalgae: *Scenedesmus obliquus* and *Chlorella vulgaris*. In order to achieve the above-mentioned goal, the specific aims were: (1) to evaluate the control of plant diseases through aqueous microalgae solutions under laboratory conditions; (2) Assess the control of plant diseases in lettuce and spinach crops *in vivo*; (3) Evaluate the biostimulant potential of aqueous suspensions of microalgae under laboratory conditions and (4) Evaluate the biostimulant potential of aqueous suspensions in lettuce crops *in vivo*.

Chapter II: Materials and Methods

In order to evaluate the biopesticide and biostimulant effects of the microalgae *Scenedesmus obliquus* (L201990256) and *Chlorella vulgaris* (L20190079), *in vivo* tests were conducted in a first phase of the work to determine the inhibitory effect on the growth of phytopathogens and the stimulation of seed germination. After determining the efficacy of the microalgae, the study proceeded to *in vivo* assays to prove the results obtained in the *in vitro* phase.

2.1. Isolation of microorganisms

2.1.1. Microbiological characterization of microalgae suspensions

The microbiological analysis of the microalgae suspensions consisted on the quantification of fungi, bacteria, and actinomycetes in these suspensions. For the two microalgae, suspensions were prepared with sterile distilled water at the concentrations of 1.5 g. L^{-1} and 2.0 g. L^{-1}. *Potato Dextrose Agar* (PDA) medium was used to quantify fungi, *Plate Count Agar* (PCA) for bacteria, and 1/2 PCA for actinomycetes, according to Coelho et al. (2013). Quantifications were performed in triplicate, in a total of 36 *Petri* dishes. Sterile distilled water was also inoculated as a negative control. From each suspension, 250 µL was added to each *Petri* dish, with inoculation by spreading on the surface of the medium. The entire process was conducted under aseptic conditions. *Petri* dishes were incubated in an incubation chamber (Binder KB 115-/E2, Germany) at 25 ± 2 ºC for 24 hours. Except PDA plates, that remained for 48 hours, colony-making units (CFU.g^{-1} microalgae) were determined.

2.1.2. Isolations of pathogenic organisms

Seven isolates of plant pathogenic species were used to test the microalgae suspensions: six pathogenic fungi (*Fusarium oxysporum*, *Botrytis cinerea*, *Alternaria alternata*, *Sclerotium rolfsii, Clarireedia* spp., *Colletotrichum gloeosporioides*) and a pathogenic oomycete (*Phytophthora cinnamomi*), obtained from the collection of the Soil Laboratory of the Faculty of Science and Technology, University of Algarve. All the microorganisms mentioned grew in PDA medium at 25 ± 2 ºC, for seven days, from which the discs with mycelium and inoculum were obtained for both *in vitro* and *in vivo* assays.

2.2. Chemical characterization of microalgae

2.2.1. Total Polyphenols Determination

In order to determine the total amount of polyphenols present in the microalgae, it was necessary to make an extraction from the biomasses. In the first phase, about 150 mg of each microalga was weighed into an Eppendorf, to which 0.7 grams of glass beads were added to facilitate the extraction. Then, 1 mL of methanol was added as an extraction solution. In total, six Eppendorf were prepared for each microalga, totaling 12 Eppendorf. These Eppendorf were placed in a bead miller (Retsch MM400, Germany) with a frequency of 30HZ for 5 minutes, for extraction. After 5 min, the Eppendorf were centrifuged at 10,000 rpm (Scansci Hermle Z167M, Portugal) for another 5 min. The supernatant was collected to a previously weighed glass tube and the pellet was resuspended by the extraction solution (methanol) and the process was repeated until the supernatant became transparent.

After collecting the entire supernatant, it was filtered with a nylon filter of 0.2 μm and dried using a nitrogen flow (stuart SBHCONC/1). The dry extract was weighed, and the yield was calculated:

$$yield\ (\%) = \frac{Dry\ extract\ (g)}{initial\ dryed\ biomass\ (g)}\ x\ 100 \qquad (1)$$

Then, the volume of DMSO required to resuspend the extracts was calculated for a final concentration of 20 mg. mL^{-1}.

$$Volume_{resuspension} = \frac{Dry\ extract\ (g)}{10}\ x\ 1000 \qquad (2)$$

Finally, this suspension was stored at -20 ºC prior to analysis.

For the quantification of polyphenols, the method of Velioglu et al. (1998) was used, which is based on the reaction of the reagent Folin-Ciocalteu (F-C reagent). To apply this method, 96 wells plate were used, to which was added, in a first step, 5 μL of the extracts under study, followed by 100 μL of the F-C reagent. After resting at room temperature for 10 min., 100 μL of sodium carbonate was added, and the plate was placed in the dark for 90 min so that the reaction occurs. After 90 min., the absorbance was read at 725 nm (BioTek Synergy 4, United States).

Similarly, a 96 wells plate containing gallic acid (GA) was prepared to obtain the calibration curve, since gallic acid's total content of phenols is expressed in mg. Therefore, GA solutions with different concentrations between 0.002 and 2.0 mg. mL^{-1} were added to the plate.

2.2.2. Amino acids Determination

The amount of free amino acids present in each sample was estimated from lyophilized microalgae biomass, according to the OPA method (Nielsen et al. 2001). L-serine was used as a standard and a calibration curve established between the different concentrations of the standard and its absorbance at 340 nm.

2.2.3. Total Protein (%)

The protein content was estimated by measuring total N in an Elemental Analyzer Vario EL III (Vario EL, Germany). The % of total protein was then calculated by multiplying the total N by the conversion factor of 4.97 as determined by Templeton and Laurens (2015).

2.3. Biopesticide Potential

2.3.1. *In vitro* assays

To verify the biopesticide potential by antagonist activity of microalgae *in vitro*, the diffusion method was adapted from Ambika & Sujatha (2014) and Machado et al. (2019).

2.3.1.1. Culture Media

PDA was used as a culture medium to perform this experiment, prepared according to the manufacturer's recommendation, to which 0.02 g. L^{-1} of chloramphenicol was added (Laboratories ESFAR- Pharmaceutical Specialties, S.A.). *Petri* dishes were duly marked in such a way that a volume of 12.5 mL of PDA medium was placed in each one, with the purpose that all pathogens had the same conditions during the test. The entire procedure was performed inside a flow chamber (FASTER, BH-EN 2005).

2.3.1.2. Preparation of microalgae suspensions and the controls

Microalgae were added to 15 mL *Falcons* containing 9 mL of sterile distilled water, to obtain the concentrations for the tests. Eight concentrations were evaluated for each microalga. The whole procedure was done under aseptic conditions. In each Petri dish

was added 450 µL of each concentration. With the help of an L-glass spreader, the homogeneous microalgae suspensions were spread. Tripled replicates were prepared for each of the treatments performed, as well as for sterile distilled water (negative control) and positive controls (biopesticide and commercial pesticide suitable for each phytopathogenic agent), totaling 57 plaques per studied disease. As a biopesticide control, T34 BIOCONTROL® (Biocontrol Technologies S.L.) was used, a biological fungicide based on *Trichoderma asperellum*, prepared in a solution using sterile distilled water to which 0.01 g. L^{-1} was added. Commercial pesticides differed according to the phytopathogenic agent under study, as shown in Table 2.1. These pathogens were selected because of their wide presence in agriculture.

Table 2.1: Phytopathogens and their pesticides

Phytopathogen	**Commercial pesticide**	**Active Substance**	**Concentration (g. L^{-1})**
Phytophthora cinnamomi	Aliette®	Fosetyl-aluminum	2.5
Botrytis cinerea	Rovral®	Ipodrione	1.5
Clarireedia spp.			
Sclerotium rolfsii			
Fusarium oxysporum			1.0
Alternaria alternata;	Mancozan®	Mancozeb	2.0
Colletotrichum gloeosporioides			

2.3.1.3. Inoculation

After 24 hours, 6 mm in diameter PDA discs were inoculated with the mycelium of pathogenic fungi, and placed in the center of *Petri dishes*, previously marked (Figure 2.1).

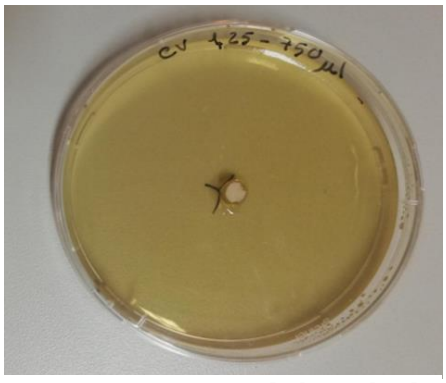


Figure 2.1: *Petri dish* with the suspension of *C. vulgaris* concentration of 1.25 g. L^{-1}, inoculated with an active mycelium disc of *Fusarium oxysporum.*

After inoculation, the plates were stored, in the incubation chamber, at 25 ± 2 ºC for three days, being removed every 24 hours to mark and measure the radius of the growth zones of each pathogen, to determine the percentage of inhibition, according to the formula:

$$IP\ (\%) = \frac{(Rc - R1)}{Rc} x100 \qquad (3)$$

where:

Rc= radius of the growth zone(cm), of the pathogen that grew on a plate containing only water;

R1= radius of the growth zone (cm), of the pathogen in contact with microalgae suspensions.

2.3.2.*In vivo* assays

In this assay, two species of microalgae, *C. vulgaris* (Cv) and *S. obliquus* (So) were used at different concentrations on lettuce (*Lactuca sativa* L. 'Romana Orelha de Mula', Semillas battle Sá, Spain) and spinach (*Spinacia oleracea* L. 'Lizard') plants. The experiment took place in a plastic greenhouse (nº 4) of FCT (37°02′35.45″N, 7°58′20.64″W), at the Gambelas Campus of UAlg, from April to June, under natural photoperiod conditions. In order to increase and stabilize humidity and air temperature, the pots were kept under a tunnel inside the greenhouse. Plants were irrigated by fine micro sprinklers, to maintain high humidity and moisture in the substrates. The mean air temperature was ≥ 20 °C and the average humidity was above 57 %.

To evaluate the suppressive capacity of the microalgae, the experimental design consisted of four complete blocks, randomized, with a total of 12 treatments per microalgae: 3 phytopathogenic agents; 3 concentrations, 2 positive controls (synthetic fungicide and a commercial biopesticide), and one negative control (distilled water). All these treatments were also applied in thermic-treated substrates (white peat, Hansa Torf, Floragard, Germany) at 60 ºC in the dry chamber (MMM Medcenter Venticell, Germany), for 7 days.

These treatments included the application of suspensions of the two microalgae, in three different concentrations that varied with the chosen disease, according to the *in vitro* results (Table 2.2). For the assay of *S. rolfsii* and *F. oxysporum*, pots of 100 mL were filled with white peat (4 pots per modality) and 5 seeds were sown per pot. to avoid the irregularity in seed germination, for the *B. cinerea* assay the plants from both species above mentioned were transplanted, by placing two plants per 100 mL pot (4 pots per treatment).

Table 2.2: Design of treatments for the biopesticide trials

Microalgae	**Diseases**	**Concentrations (g. L^{-1})**			**Application of microalgae**	**Plants**
Chlorella vulgaris	*Botrytis cinerea*	0.25	0.5	0.75	Foliar spray	Lettuce/Spinach
	Fusarium oxysporum	1.0	1.25	1.5	Substrate irrigation	Spinach
	Sclerotium rolfsii	1.0	1.25	1.5	Substrate irrigation	Lettuce
Scenedesmus obliquus	*Botrytis cinerea*	0.01	0.1	0.25	Foliar spray	Lettuce/Spinach
	Sclerotium rolfsii	0.5	0.75	1.0	Substrate irrigation	Lettuce
	Fusarium oxysporum	0.5	0.75	1.0	Substrate irrigation	Spinach
Positive Controls						
T34®	*Botrytis cinerea*	0.01			Foliar Spray	Lettuce/Spinach
	Sclerotium rolfsii				Substrate irrigation	
	Fusarium oxysporum					
Rovral®	*Botrytis cinerea*	1.5			Foliar Spray	Lettuce/Spinach
	Sclerotium rolfsii				Substrate irrigation	Lettuce

	Fusarium oxysporum	1.0	Substrate irrigation	Spinach

The pathogenic agents were inoculated one week before sowing, to allow the pathogen to grow in the substrate. The pathogens were inoculated with discs (6 mm in diameter) of the mycelium of the selected pathogens and cultivated in a PDA medium for 7 days. After 7 days, the applications of the different concentrations of microalgae started, applying about 20 mL of the solution in the corresponding pots.

For *Botrytis cinerea*, microalgae applications started 2 days after plant inoculation, through weekly sprays on the aerial part of the plant. The plants were observed daily to determine the initial symptoms of the disease and, since then, weekly determine the incidence of the disease [(number of plants affected by the disease/total number of plants) x 100] and its severity (according to the scale defined by Baayen & van der Plas (1992). The area under the disease progress curve (AUDPC- *area under disease progress curve*) was calculated per pot, based on the severity of the disease measured between the onset of symptoms and the end of the assay, divided by the number of days, according to the formula:

$$\sum_{i=1}^{n} = \left(\frac{y_i + y_{i+1}}{2}\right)(t_{i+1} - t_i), \quad (4)$$

where:

y= severity of the disease;

t= time (days).

2.4. Biostimulant Potential of microalgae

2.4.1. *In vitro* trial

An adapted phytotoxicity test technique was used to evaluate the biostimulant potential of the two algae *in vitro*, based on seed germination (Zucconi et al., 1981). Garden watercress seeds (*Lepidium sativum* L.) were used to perform this method, with the suspensions of the microalgae under test (*Chlorella vulgaris* and *Scenedesmus obliquus*).

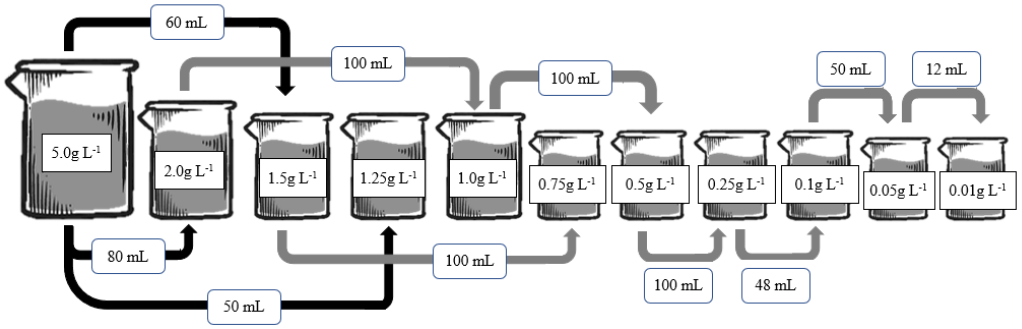
Different concentrations of each microalga were prepared, to calculate their effect on seed germination.

This procedure was divided into three major steps: preparation of the suspensions; germinating seeds on the extracts; and measuring and calculating the different indicators based on seed germination.

2.4.1.1. Preparation of suspensions

To obtain suspensions with different concentrations, 2 g of each microalga were weighed and mixed in 400 mL of sterile distilled water, to obtain the mother solution (5.0 g. L^{-1}). Then, for each microalga, the mother solution was successively diluted, to obtain the different concentrations of 0.01 g. L^{-1}; 0.05 g. L^{-1}; 0.1 g. L^{-1}; 0.25 g. L^{-1}; 0.5 g. L^{-1}; 0.75 g. L^{-1}; 1.0 g. L^{-1}; 1.25 g. L^{-1}; 1.5 g. L^{-1} and 2.0 g. L^{-1} (Figure 2.2).

Figure 2.2: Preparation of the different suspensions.



The test took place in square *Petri* dishes (11x11 cm), with 2 Whatman Nº1 filter papers on the bottom, which were moistened with 10 mL of each of the prepared suspensions.

Five replicates were performed for each microalga and each concentration studied, totaling 50 *Petri* dishes. In total, the test consisted of 65 *Petri* dishes, for algae suspensions and controls, which consisted of sterile distilled water (negative control), and two positive controls, namely, Algaman B; a biostimulant based on seaweed extract *Ecklonia maxima* and boron (Hubel®); and gibberellic acid (Sigma Aldrich, United States). To prepare the positive controls was used 2.0 g. L^{-1} of Algaman B according to the manufacturer indications; in the case of the gibberellic acid, it was used at approximately 0.0009 g. L^{-1}.

2.4.1.2. Incubation of the seeds

In this trial performed on square *Petri* dishes, 10 seeds were laid on filter paper next to each other, about 1.5 cm from the top of the plate, exerting a small pressure to ensure contact of the seed with the paper soaked with solution to be tested (Said-Pullicino et al., 2007). A total of 650 seeds were used for the 65 *Petri* dishes.

Unlike outdoor tests, these plates were positioned inside the growing chamber (Aralab 3505, Portugal) forming an angle of 70º to 80º degrees with the horizontal. The seeds were incubated in the dark, at a temperature of 25 ºC ± 2 for 72 hours. This position should facilitate the geotropic growth of the radicle, to the bottom of the plates.

2.4.1.3. Germination index of seeds

After 72 hours, *Petri* dishes were removed for the quantification of the number of germinated seeds and calculation of the germination index for each microalgae concentrations.

The radicle in each seed was measured, using a digital caliper (Toolland 3472B, United States), The average size length was determined, in mm. With these values, it was possible to calculate the indicators by Eqs (5-8), including: seed germination (SG), the relative seed germination (RSG), the relative radicle growth (RRG), and the seed germination index.

$$SG = \frac{Ga}{Number\ of\ total\ seeds}\ x\ 100\ (\%) \quad (5)$$

$$RSG = \frac{Ga}{Gb} x\ 100(\%) \quad (6)$$

$$RRG = \frac{La}{Lb}\ x\ 100\ (\%) \quad (7)$$

The germination index is then calculated using the following equation:

$$IG = \left(média \left(\frac{Ga\ x\ La}{Gb\ x\ Lb} \right) \right) x100\ (\%) \quad (8)$$

Where:

Ga= number of seeds germinated in the plates with samples;

La= length, in mm, of the radicle of the samples;

Gb= number of seeds germinated in the negative control (water);

Lb= length, in mm, of its radicle.

2.4.2. *In vivo* trials

As in the *in vivo* biopesticide assay, the treatments assessed included the application of suspensions of the two microalgae, a negative control (without biostimulant), a positive control Algaman B (Hubel, Portugal) and additionally SEAnergy, a commercial biostimulant produced by Kimitec Agro, that combines a microalgae *Spirullina platensis* and a macroalgae, *Ascophyllum nodosum* for the lettuce trial.

Each microalga was evaluated on lettuce plants. This application took place weekly during the entire test. It was applied 40 mL of the different concentrations on lettuces. Lettuce were seeded in 200 mL pots, with white peat as substrate, and were watered by sprinklers controlled by a clock timer. In order to avoid nutritional deficiencies throughout the experiment, each pot was watered once a week with 40 mL of full-strength Hoagland solution with (in mM): 5 $Ca(NO_3)_2$, 5 KNO_3, 1 KH_2PO_4, 2 $MgSO_4$, and (in mM): 46 H_3BO_3, 0.8 $ZnSO_4$, 0.4 $CuSO_4$, 9 $MnCl_2$, and 0.02 MoO_3. Iron was supplied at 10 µM as Fe(III)-EDDHA (Basafer® from Compo, with 6% of Fe; 5.0% of Fe chelated by ortho-ortho EDDHA).

In this study, two species of microalgae, *C. vulgaris* (Cv) and *S. obliquus* (So) were used at different concentrations for the cultures under study, positive control, and negative control (Table 2.3).

Table 2.3: Concentrations under study

Plant	**Microalgae**	**Concentrations (**g. L^{-1}**)**				**Positive Control**	**Negative Control**
Lettuce	*C. vulgaris*	0.1	0.5	2.0	5.0	Algaman 2.0 g. L^{-1}	Water
	S. obliquus					SEAnergy 2.0 mL.m^2	

The trial was installed with four replicates. For lettuce, 4 pots containing one seed each, were used per replicate; for spinach, 4 pots each containing 5 seeds were used per replicate.

2.4.3. Biometric measurements in plants

During the assay, the SPAD index was recorded weekly, allowing to estimate the chlorophyll content of lettuce plants, with a portable chlorophyll meter SPAD (Minolta SPAD-502, United States). This method allows the determination of the pigment content of the leaves, in a non-destructive way (Rubio-Covarrubias et al., 2009). This equipment calculates an index from the radiation transmitted in the infrared zone and the red of the luminous spectrum. The SPAD meter relates the radiation values obtained by the device with chlorophyll values (Jesus & Marenco, 2008). Chlorophyll measurement allows indirectly to estimate the amount of nitrogen in the plant, being possible to monitor the nutritional state of a plant (Afonso et al., 2016; Uddling et al., 2007; Yuan et al., 2016). At the end of both trials, the plants were cut about 2 cm above the neck, and the height of the plants and fresh weight were determined. The roots were carefully removed from the substrate and washed, and their length and fresh weight were registered. After drying at 103 ºC, the dry weights of the canopy and root were recorded, and their dry matter content was determined.

2.4.4. Leaf area

At the end of the trial and after the fresh weight of the lettuce leaves was recorded, the leaf area was measured. The leaf area was measured using the computer program WinDIAS 3 (Delta-T Devices, UK) connected to a video camera. With this program, through a real image, previously calibrated, it was able to process the image and determine the desired area.

2.5. Physical, chemical, and microbial analyses of the substrate

Before and after each assay, samples of each type of substrate were collected. These samples consisted of the collection of the substrate in the pots. For the study, the substrates of 3 pots per treatment were randomly collected. Samples were analyzed in triplicate.

2.5.1. pH determination

The pH was determined on a 1:2 water suspension, according to Martinez (1992). For each treatment, 50 g of substrate were weighed. The samples were placed in 250 mL *Erlenmeyer's,* adding 100 mL of distilled water.

The suspensions were placed in continuous agitation of 175 rpm for 20 minutes. After this time, the suspensions were left to rest for 1 hour, at room temperature (about 20ºC).

After that time, the pH was measured with a potentiometer (Crison Micro pH 2001, Spain) on each suspension, always taking care to respect the time of stabilization of each of the instruments and to clean the electrode between measurements, reducing the risk of errors.

2.5.2. Determination of electrical conductivity

The suspensions prepared for pH were used for the measurement of electrical conductivity (EC). EC was measured on the filtered suspensions. After filtering, more clear solutions were obtained, and no traces of substrates were spotted. On the filtered suspensions, using a conductimeter (Crison Conductimeter 522, Spain), the EC was measured at room temperature. All necessary care measures were taken to reduce errors, such as when measuring pH.

2.5.3. Microbiological analysis of substrates

The microbiological analysis consisted of the quantification of the different microorganisms present in the substrate, as for the microalgae suspensions. Fungi, bacteria, and actinomycetes were quantified in the samples collected from the different treatments.

A 50 mL *Falcon* was filled with 40 mL of distilled water and taken to the autoclave to sterilize. To each *Falcon* was added about 4 grams of the fresh substrate, repeating the process three times for each of the treatments. Then, each of the *Falcons* was stirred in the *vortex* so that the mixture was as homogeneous as possible, followed by serial dilutions preparations. In the end, two dilutions were obtained, which were used in quantification.

For this analysis, the same media used in the microbial quantification of microalgae were used: PDA, PCA, and $^1/_2$ PCA.

From the suspension obtained, 500 µL were placed in each of the different *Petri* dishes and with the help of a previously sterilized L-glass spreader, the suspension was spread over the surface of the media on the plates. The procedure was repeated for both dilutions

of each treatment. The entire process was conducted under aseptic conditions. In the end, 30 *Petri* dishes were obtained for each type of medium, totaling 90 *Petri* dishes.

After being prepared, the plates were incubated in the incubation chamber at 24º C ± 2 °C for 24 hours, except for the PDA plates, which required 48 hours for the colonies to be visible. After that time, colony forming units (CFU) were calculated, subsequently converting the value according to their dilution, calculating the substrate $CFU.g^{-1}$. This determined the number of microorganisms per gram of substrate.

2.6. Statistical analysis

After the *in vitro* and in *vivo* tests, the results were subjected to statistical analysis. Data were analyzed with the statistical software SPSS® (Statistical Package for Social Sciences, version 27, SPSS Inc., United States), and a Variance Analysis and Duncan Multiple Range Test (DMRT) at $p < 0.05$ for mean comparison test were performed.

Chapter III: Results and Discussion

Recently, several studies have emerged showing that cyanobacteria, macro-, and microalgae may have a pesticide activity on fungi and a stimulating activity on plants (Renuka et al., 2018, Lee & Ryu, 2021). In this work, *in vitro* and *in vivo* trials were performed to evaluate these effects using selected microalgae.

3.1. Microalgae analysis

3.1.1. Microbiological characteristics of microalgae

The analysis referred on point 2.1.1 were performed on the biomasses of *S. obliquus* (L201990256) and *C. vulgaris* (L20190079). Both microalgae did not show a significant number of micro-organisms populations that could impact the *in vitro* assays (Table 3.1). Furthermore, the biomasses did not differ significantly.

Table 3.1: Populations of microorganisms in microalgae

g. L^{-1}	CFU.g^{-1} microalgae		
	Fungi	Actinomycetes	Bacteria
Cv0.14	1.028 x 10^3a	0a	0a
Cv0.18	1.817 x 10^3a	0a	6.48a
So0.14	1.86 x 10^3a	0a	0a
So0.18	9.642 x 10^3a	5a	6.67a

Cv0.14, *Chlorella vulgaris* at a concentration of 0.14 g. L^{-1}; Cv0.18, *Chlorella vulgaris* at a concentration of 0.18 g. L^{-1}; So0.14, *Scenedesmus obliquus* at a concentration of 0.14 g. L^{-1}; So0.18, *Scenedesmus obliquus* at a concentration of 0.18 g. L^{-1}. In each column, values followed by the same letter do not show significant differences for $p<0.05$.

3.1.2. Chemical characterization of microalgae

To better understand the composition of the microalgae, the analyses referenced in point 2.1.2 were performed and the results obtained are found in table 3.2.

Table 3.2: Chemical composition of each microalga

	Total Polyphenolic (mg GAE/g DW)	Amino acids (mg/g DW)	Total Protein (%)
Chlorella vulgaris	73.01 ± 2.6	32.9 ± 0.7	41.9 ± 1.4
Scenedesmus obliquus	53.67 ± 6.4	16.9 ± 1.0	39.9 ± 0.8

Table 3.2 shows the phenolic and amino acid content of *Chlorella vulgaris* and *Scenedesmus obliquus*. It is verified that the phenolic content in *C. vulgaris* is 73.01 ± 2.6 mg GAE/g DW, being higher than the content of *S. obliquus*, which presents 53.76 ± 6.4 mg GAE/g DW.

As for its amino acid content, the same was observed. The biomass composition of *C. vulgaris* is richer in amino acids than that of *S. obliquus*, with 32.9 ± 0.7 and 16.9 ± 1.0 mg/g DW, respectively. In addition to that, the total content in protein (%) was higher in *C. vulgaris* with a 41.9 ± 1.4 % and 39.9 9 ± 0.8 % in *S. obliquus.*

These results may help explain some of the results obtained in the remaining trials.

3.2. Biopesticide Potential

3.2.1. *In vitro* trials

In the i*n vitro* trials, the biopesticide activity of microalgae was evaluated using the contact method. Different percentages of inhibition were obtained, resulting from the effect of each microalga suspension on the studied phytopathogenic fungi.

Fusarium oxysporum

The inhibition of *F. oxysporum* growth in all treatments was observed with both microalgae, *C. vulgaris* and *S. obliquus* (Figure 3.1). However, this growth inhibition was lower than the positive controls. At the lowest concentrations, the fungi had its growth reduced by approximately 12.4 ± 6.74 % and 27 ± 8.5 %, respectively on *C. vulgaris* and

S. obliquus. This inhibition was higher as the concentration of the microalgae increased up to the average concentrations and then decreased (Figure 3.1).

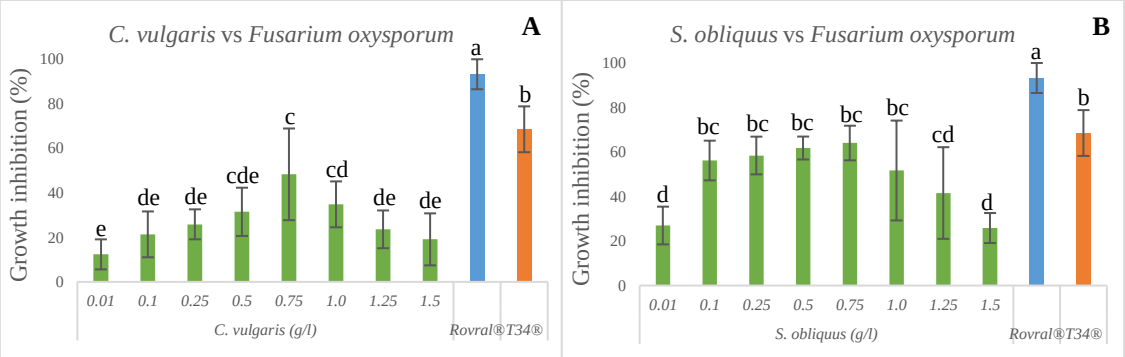


Figure 3.1: *Fusarium oxysporum* exposed to microalgae biomass on the 3rd day of growth. **(A)** *In vitro* results of growth inhibition (%) caused by *C. vulgaris*. **(B)** *In vitro* results of growth inhibition (%) caused by *S. obliquus*. At concentrations 0.01, 0.1, 0.25, 0.5, 0.75, 1.0, 1.25 and 1.5 g. L^{-1}; the Biocontrol Agent T34®; a synthetic pesticide Rovral®. For each treatment value, means (n=3 ± STD) with different letters were significantly different at $p<0.05$, using Duncan's multiple range test. Each line on top of the bars represents the standard deviation.

Figure 3.1A shows that treatment Cv 0.75 g. L^{-1}, *C. vulgaris* promoted a reduction in the growth of *F. oxysporum*, by about 48.3 ± 20.6 % compared to water. With *S. obliquus*, no statistically significant differences were identified between the biocontrol treatment and most of the microalgae suspensions (Figure 3.1B).

Although *C. vulgaris* had an effect on *F. oxysporum*, the highest inhibition was observed with *S. obliquus*, and it should be noted that most of the treatments with this microalga did not differ from the positive control T34® ($p>0.05$), except for treatments So 0.01 g. L^{-1}, So 1.25 g. L^{-1} and So 1.5 g. L^{-1}.

Botrytis cinerea

The results with these microalgae were significantly lower than those obtained with the positive controls (3.2A and 3.2B). However, also, in this case, the microalgae presented a small inhibition effect on the growth of the fungus.

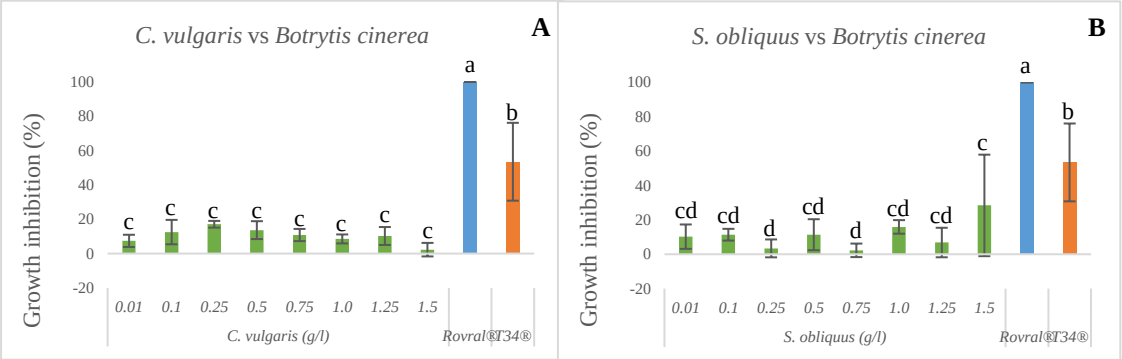


Figure 3.2: *Botrytis cinerea* exposed to microalgae biomass on the 3rd day of growth of its mycelium. **(A)** *In vitro* results of growth inhibition (%) caused by *C. vulgaris.* **(B)** *In vitro* results of growth inhibition (%) caused by *S. obliquus.* At concentrations 0.01, 0.1, 0.25, 0.5, 0.75, 1.0, 1.25 and 1.5 g. L^{-1}; the Biocontrol Agent T34®; a synthetic pesticide Rovral®. For each treatment value, means (n=3 ± STD) with different letters were significantly different at $p<0.05$, using Duncan's multiple range test. Each line on top of the bars represents the standard deviation.

With *C. vulgaris*, the inhibition rate varied between 2.28 % and 17 %. The highest inhibition effect was observed in Cv 0.5 g. L^{-1} with 17.04 ± 1.97 % and, the lowest effect was observed in 1.5 g. L^{-1} (2.27 ± 3.93 %), which means that at this concentration the growth of the phytopathogen mycelium was similar to the growth of in the plates of the negative control (water), which was used for the calculation of the growth inhibition rate (Figure 3.2A).

For *B. cinerea*, the inhibition growth rates varied between 2.27 ± 3.93 % and 28.4 ± 29.5 %, where the lowest inhibition was obtained in So 0.75 g. L^{-1} (Figure 3.2B). In the case of *S. obliquus*, according to Duncan's test, the concentration of So 1.5 g. L^{-1} promoted an inhibition growth significantly higher ($p<0.05$) than the concentrations of 0. 25 g. L^{-1} and 0.75 g. L^{-1}.

Phytophthora cinnamomi

Duncan's test for mycelium growth on the 3rd day after inoculation indicates no significant statistical differences between the positive control T34® and microalgae suspensions ($p<0.05$), showing that the results obtained with the aqueous suspensions of microalgae were comparable with those of the commercial biocontrol agent. As it could be expected, no significant differences were observed between the fungicide (Aliette®) and the other treatments.

In the trials with *C. vulgaris*, the growth of *Phytophthora cinnamomi* mycelium was inhibited by about 40.1 ± 0.00 % by T34®, whereas, with *Chlorella vulgaris*, the highest significant inhibition was obtained at the lowest concentration (0.01 g. L^{-1}), with the same suppression percentage (40.1 ± 11.9 %). The lowest inhibition rate was found at 0.1 g. L^{-1} with a 21.5 ± 1.7 % reduction in the growth of *P. cinnamomi.* (Figure 3.3A). Most of the *C. vulgaris* suspensions showed a similar effect on *P. cinnamomi* as the commercial positive control (T34®).

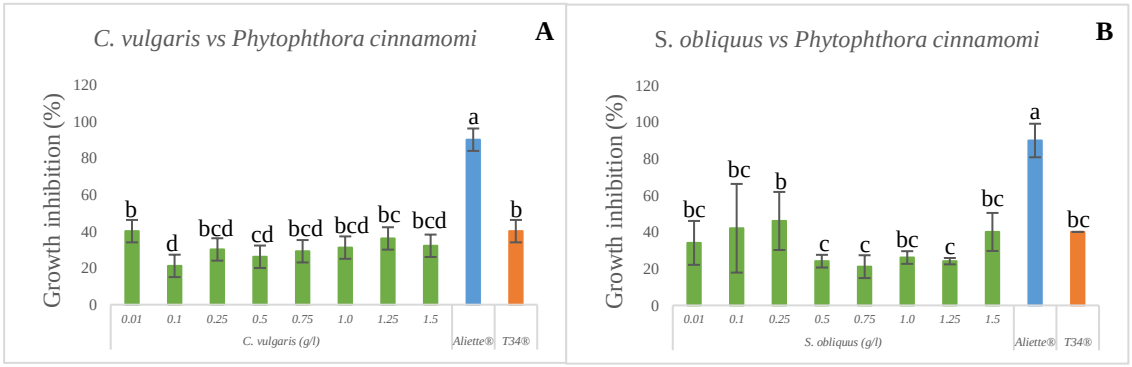


Figure 3.3: *Phytophthora cinnamomi* exposed to microalgae biomasses on the 3rd day of growth of its mycelium. **(A)** *In vitro* results of growth inhibition (%) caused by *C. vulgaris.* **(B)** *In vitro* results of growth inhibition (%) caused by *S. obliquus*. At concentrations 0.01, 0.1, 0.25, 0.5, 0.75, 1.0, 1.25 and 1.5 g. L^{-1}; the Biocontrol Agent T34®; a synthetic pesticide Aliette®. For each treatment value, means (n=3 ± STD) with different letters were significantly different at $p<0.05$, using Duncan's multiple range test. Each line on top of the bars represents the standard deviation.

In figure 3.3A, it can be observed that the microalgae concentrations caused some reduction in the growth of *P. cinnamomi* mycelium. In Figure 3.3B, when *S. obliquus* was applied, the concentration with a higher inhibition value was 0.25 g. L^{-1} (46.1 ± 15.8 %), showing a higher inhibition rate than the commercial positive control T34®, although with significant differences ($p>0.05$). With *S. obliquus*, all concentrations were comparable with the T34 biocontrol®. The lowest percentage obtained was 21.2 ± 6.23 %, verified in So 0.75 g. L^{-1}.

Alternaria alternata

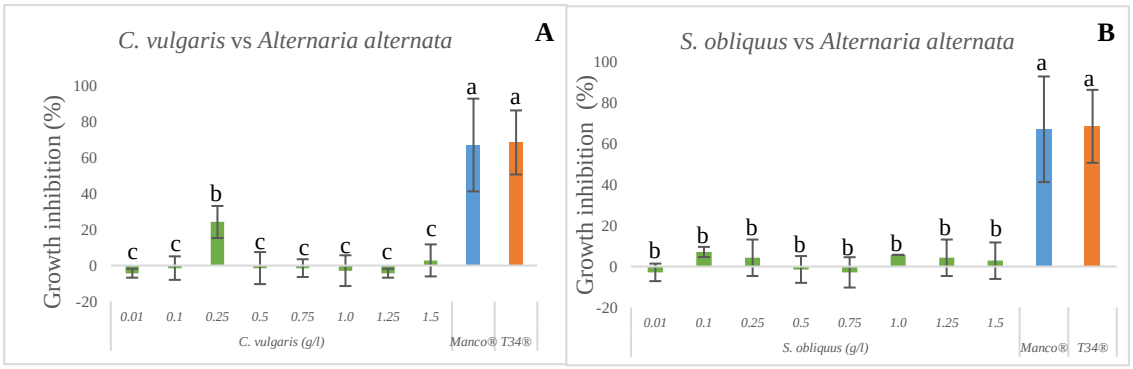


Figure 3.4: *Alternaria alternata* exposed to microalgae biomasses on the 3rd day of growth of its mycelium. **(A)** *In vitro* results of growth inhibition (%) caused by *C. vulgaris.* **(B)** *In vitro* results of growth inhibition (%) caused by *S. obliquus*. At concentrations 0.01, 0.1, 0.25, 0.5, 0.75, 1.0, 1.25 and 1.5 g. L^{-1}; the Biocontrol Agent T34®; a synthetic pesticide Rovral®. For each treatment value, means (n=3 ± STD) with different letters were significantly different at $p<0.05$, using Duncan's multiple range test. Each line on top of the bars represents the standard deviation.

Contrary to what happened in the previous trials, when tested against *Alternaria alternata*, *C. vulgaris* showed a stimulating effect on the pathogen growth of approximately 4.3 ± 2.47 % compared with the negative control (water) (Figure 3.4A). However, at 0.25 g. L^{-1}, with *C. vulgaris* was observed an inhibition rate of 24.3 ± 8.9 % in the pathogen growth.

For *S. obliquus*, a similar response occurred: an increased growth of the fungus mycelium in the concentrations of So 0.01 g. L^{-1}, So 0.5 g. L^{-1} and So 0.75 g. L^{-1}, a growth of approximately 3 ± 4.3 %, 1.4 ± 6.5 %, and 3 ± 7.4 %, respectively. However, the other suspensions showed a small inhibition rate, being the highest at 7.1 ± 2.47 % in So 0.1 g. L^{-1} (Figure 3.4B).

Colletotrichum gloeosporioides

Against *C. gloeosporioides*, it was found that most of the suspensions of *C. vulgaris* (0.01, 0.25, 1.25 g. L^{-1}) and one with *S. obliquus* (0.1 g. L^{-1}) did not differ significantly from the syntetic fungicide (Mancozan®), reaching inhibition rates between 13.3 % and 16 %. Treatments Cv 0.75 and Cv 1.0 g. L^{-1} showed a tendency to stimulate the fungus (Figure 3.5A).

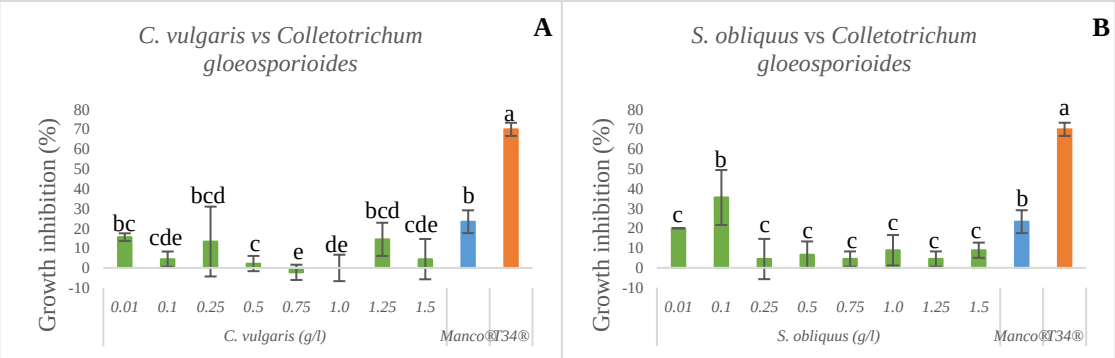


Figure 3.5: *Colletotrichum gloeosporioides* exposed to microalgae biomasses on the 3rd day of growth of its mycelium. **(A)** *In vitro* results of growth inhibition (%) caused by *C. vulgaris*. **(B)** *In vitro* results of growth inhibition (%) caused by *S. obliquus*. At concentrations 0.01, 0.1, 0.25, 0.5, 0.75, 1.0, 1.25 and 1.5 g. L^{-1}; the Biocontrol Agent T34®; a synthetic pesticide Rovral®. For each treatment value, means (n=3 ± STD) with different letters were significantly different at $p<0.05$, using Duncan's multiple range test. Each line on top of the bars represents the standard deviation.

S. obliquus and *C. vulgaris* showed some suppressive effect in most of the concentrations used in the assay. The concentration So 0.1 g. L^{-1}, according to Duncan's test, obtained an inhibition rate comparable to the one obtained with the fungicide approved for *C. gloeosporioides*, with a suppression of the growth of mycelium of the pathogen of 35 ± 13.9 %. Although significantly lower than the concentration of 0.1 g. L^{-1} and both positive controls, the other treatments with *S. obliquus* showed inhibition rates ranging from 4.4 % and 20 %, obtained in So 0.25, So 0.75, So 1.25 g. L^{-1} and So 0.01 g. L^{-1}, respectively (Figure 3.5B).

Sclerotium rolfsii

At the 3rd day of growth of the mycelium *of S. rolfsii*, no differences were observed with any of the microalgae suspensions ($p>0.05$) (Figure 3.6)

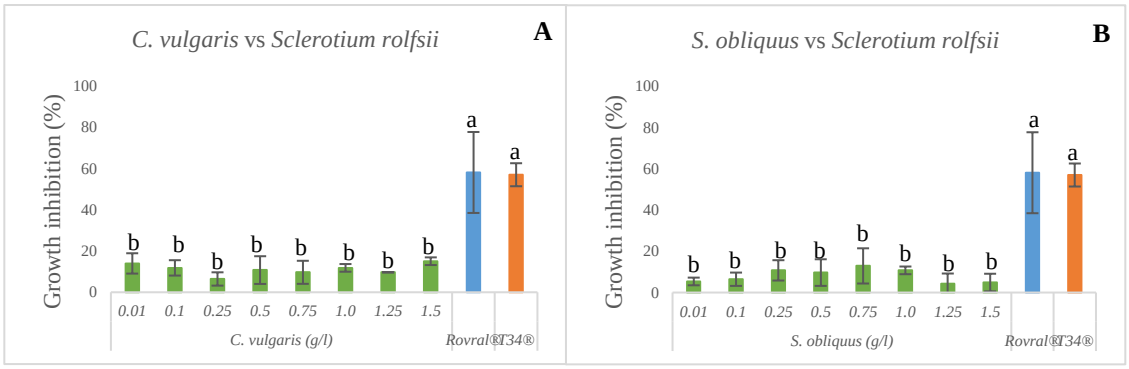


Figure 3.6: *Sclerotium rolfsii* exposed to microalgae biomass on the 3rd day of growth of its mycelium. **(A)** *In vitro* results of growth inhibition (%) caused by *C. vulgaris*. **(B)** *In vitro* results of growth inhibition (%) caused by *S. obliquus*. At concentrations 0.01, 0.1, 0.25, 0.5, 0.75, 1.0, 1.25 and 1.5 g. L^{-1}; the Biocontrol Agent T34®; a synthetic pesticide Rovral®. For each treatment value, means (n=3± STD) with different letters were significantly different at $p<0.05$, using Duncan's multiple range test. Each line on top of the bars represents the standard deviation.

Growth inhibition rates with the microalgae suspensions were significantly lower when compared to positive controls, Rovral® and T34®, which presented values of 58.1 ± 19.6 % and 57 ± 5.6 %, respectively ($p<0.05$).

With *C. vulgaris*, the highest percentage of inhibition was achieved with 1.5 g. L^{-1}, with an inhibition percentage of 15 ± 1.86 %, while the lowest was observed with 0.25 g. L^{-1}, reaching approximately 7 % (Figure 3.6A).

Figure 3.6B shows that (as in *C. vulgaris)*, all suspensions of *S. obliquus* were able to inhibit the growth of *S. rolfsii*, obtaining an inhibition of 13 ± 8.5 % at 0.75 g. L^{-1}, with no significantly different variation between microalga concentrations. However, this inhibition was much lower than the one obtained with the controls.

***Clarireedia* spp.**

According to Duncan's test, for *Clarireedia* spp. both microalgae showed no inhibition as shown in Figure 3.7.

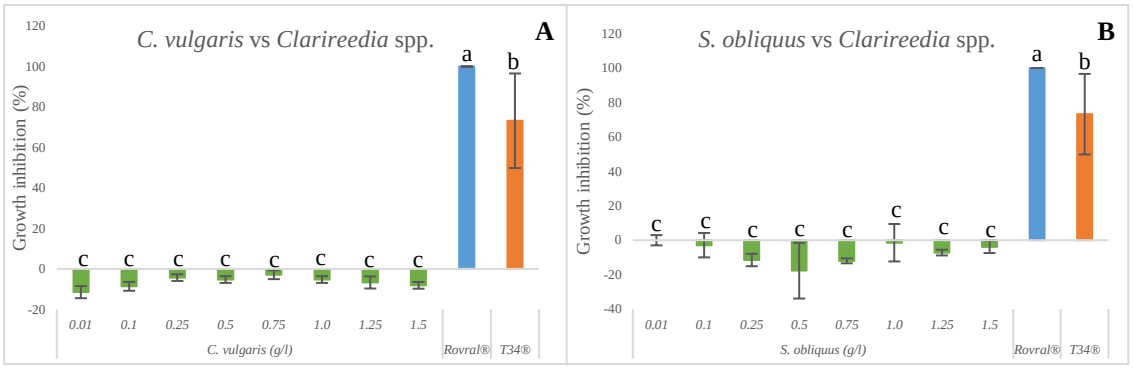


Figure 3.7: *Clarireedia spp*. exposed to microalgae biomass on the 3rd day of growth of its mycelium. **(A)** *In vitro* results of growth inhibition (%) caused by *C. vulgaris*. **(B)** *In vitro* results of growth inhibition (%) caused by *S. obliquus*. At concentrations 0.01, 0.1, 0.25, 0.5, 0.75, 1.0, 1.25 and 1.5 g. L^{-1}; a Biocontrol Agent T34®; a synthetic pesticide Rovral®. For each treatment value, means (n= 3 ± STD) with different letters were significantly different at $p<0.05$, using Duncan's multiple range test. Each line on top of the bars represents the standard deviation.

Against *Clarireedia* spp, microalgae suspensions had the opposite effect to what was intended, in all the concentrations assessed. *C. vulgaris* (Figure 3.7A) stimulated the growth of the fungus by up to approximately 12 ± 2.98 % in relation to the growth in the plates that contained only water. The highest rates of stimulation of *Clarireedia* spp. growth were observed with *S. obliquus* (Figure 3.7B), that at 0.75 g. L^{-1} increased the size of mycelium by 18 ± 16.1 %.

In Figure 3.7B, it is possible to see that in some of the concentrations the values obtained were close to zero, which might indicate that there may be an inhibiting effect if the concentrations of *S. obliquus* are increased or even if even lower concentrations are tested.

Effect of microalgae

The microalgae under study showed some suppressive effects on fungi, *F. oxysporum*, *B. cinerea*, *P. cinnamomi*, *C. gloeosporioides*, and *S. rolfsii*.

S. obliquus had an antifungal effect against all targeted phytopathogenic fungi, except against *Clarireedia* spp.. The results of its antagonistic effect were more significant against *F. oxysporum* and *P. cinnamomi*. *In vitro* inhibition of the growth of *F. oxysporum* was observed by 64 ± 8.8 % and *P. cinnamomi* by 46.1 ± 15.8 %, when in contact with *S. obliquus*.

The activity of *C. vulgaris* against *F. oxysporum* was previously reported using the same and different media (Ferreira et al., 2021; Vehapi et al., 2018), with higher concentrations, but for *S. obliquus* no studies were found. Schmid et al. (2022) described the antifungal effect of *S. obliquus* and *C. vulgaris* against *S. rolfsii* and *B. cinerea,* causing an inhibition rate similar to those obtained in this assay. Vehapi et al. (2020) reported the effects *of C. vulgaris* on disease-causing fungi in apples, such as *Aspergillus niger*, *Alternaria alternata,* and *Penicillium expansum, being* its antifungal activity due to its rich composition in terpenes, alkaloids, and polypeptides. According to Costa et al. (2019), pesticide activity is due to its production capacity of bioactive compounds such as phenolic compounds, polyphenols, and tocopherols, among others. It is known that pesticides can act in several ways, such as the dissolution of the cytoplasmic membrane, inhibition of enzymes, or even the activation of the plant's immune system when they are applied (Lee & Ryu, 2021). Farid et al. (2019) described the effect of crude polysaccharides from *C. vulgaris*, observing that when applied to tomato plants, they will increase the expression of PR genes and the coding of genes of antioxidant enzymes such as β-1,3-glucanase, APX, and PDO. Additionally, these polysaccharides from microalgae may also increase plant growth and tolerance for abiotic stress, thus acting as biostimulants.

Looking at point 3.2 where the phenolic content of microalgae is present, it can be seen that this may be one of those responsible for the inhibiting effect they have against fungi.

3.2.2. *In vivo* trials

Scenedesmus obliquus vs Fusarium oxysporum

For the *in vivo* trials, the results obtained in the white peat without heat treatment were compared with those obtained in thermic-treated white peat (Fig. 3.8, A and B respectively), substrate placed at 60 ºC in the dry chamber for 7 days.

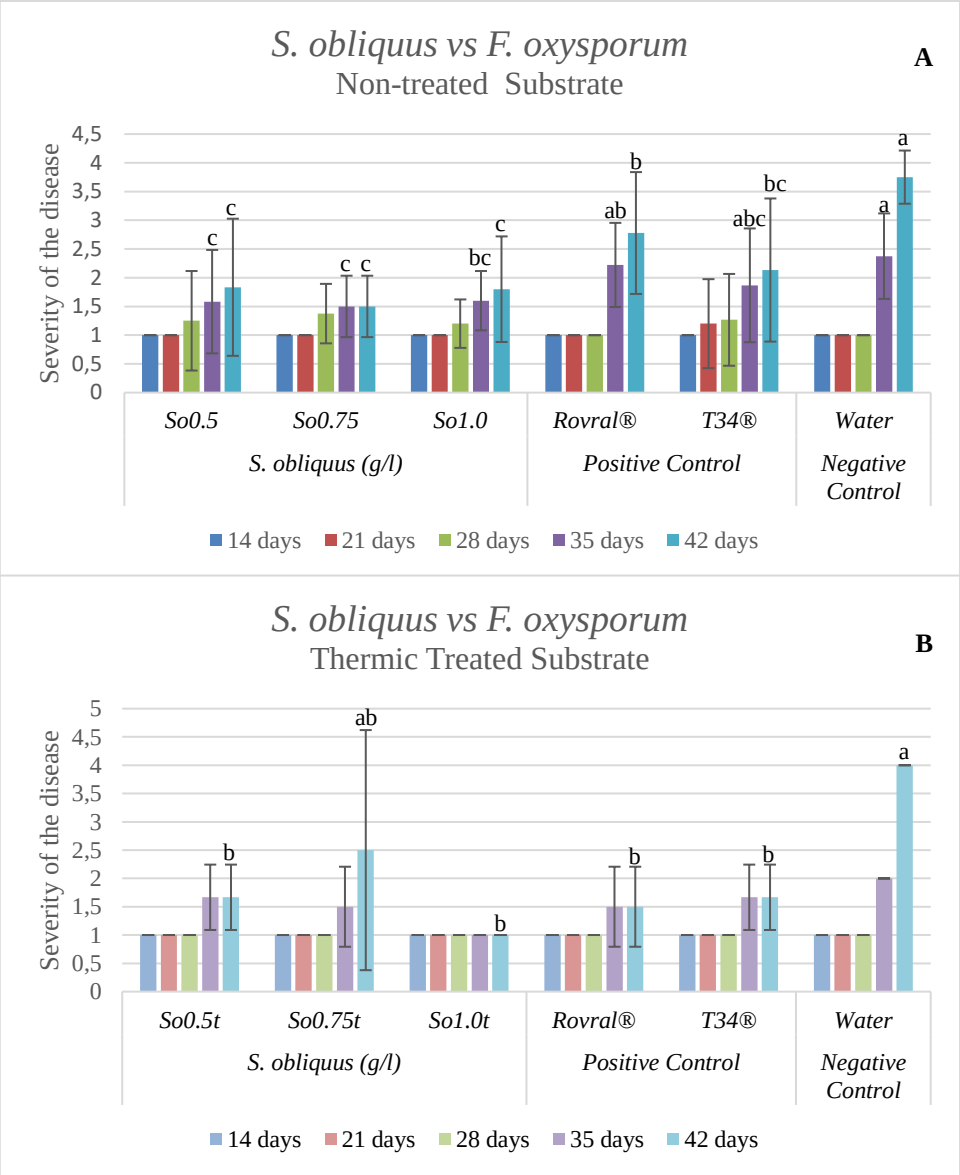


Figure 3.8: *In vivo* results of the severity of the disease caused by *F. oxysporum*. **(A)** In non-treated substrate when applied with: *S. obliquus* at 0.5, 0.75 and 1.0 g. L^{-1}. **(B)** In thermic treated substrate when applied with: *S. obliquus* at 0.5, 0.75 and 1.0 g. L^{-1}. For each treatment value are means (n=20 ± STD) for each day, the values followed by the same letter do not present significant differences, for $p<0.05$, using Duncan's multiple range test. Each line on top of the bars represents the standard deviation.

According to the results (figure 3.8A) in non-heat-treated peat, the symptoms caused by *F. oxysporum* began to be evident 28 days after sowing (DAS) in all treatments, except for water and fungicide (Rovral®). On thermic-treated peat (Figure 3.8B) the symptoms were observed only at 35 DAS, in all treatments, except for So 1.0t.

Analyzing the severity of the disease, on the non-heat-treated substrate it was observed that t *F. oxysporum* developed more rapidly in water, T34® and Rovral® which received

no suspensions of microalgae (*Scenedesmus obliquus*) (Figure 3.8A). On the thermic-treated substrate, this rapid disease development was only observed in the negative control (water) (Figure 3.8B).

However, in both substrates' treatments, at the end of the assay (42 DAS,) there was a reduction in severity compared with the water treatment when the different concentrations of the microalgae were used (Figure 3.8). It was also observed that with the microalgae the severity was always lower than level 2, except for treatment So 0.75 g. L^{-1} in the thermic-treated substrate, with a severity of 2.5. However, although the disease severity was high, the incidence of the disease with microalgae suspensions was lower than in the controls, reaching 50 % or less (Table 3.3).

It was expected an increased growth of *F. oxysporum* in the thermic treated substrate. However, this was not observed, indicating that *S. obliquus* suspensions had a suppressive effect on the pathogen, even without the influence of other microorganisms present in the peat. The highest suppressive capacity was observed in the pots where the algae treatments were applied, as well as the incidence of the disease (Tables 3.3 and 3.4).

Table 3.3 shows the variation in the incidence of the disease during the trial (DAS). As expected, the water treatment presented an incidence of 100 %, followed by fungicide (Rovral®) which at 35 DAS presented an incidence of 94 %, significantly higher ($p<0.05$) than those achieved in the other treatments. The lowest incidences were identified in the treatments with microalgae, which in turn did not differ from each other and between the positive control T34®.

Table 3.3: Incidence of *Fusarium oxysporum*, in the different treatments, throughout the trial on non-treated peat.

	Incidence of the disease on the non-heat-treated substrates (%)					
	So ($g.L^{-1}$)			Positive control		Negative control
DAS	0.5	0.75	1.0	Rovral®	T34®	Water
14	0a	0a	0a	0a	0a	0a
21	0a	0a	0a	0a	7a	0a
28	8a	12a	20a	0a	13a	13a
35	42c	50c	50c	94ab	60bc	100a
42	42c	50c	50c	94ab	60bc	100a

DAS, days after sowing; So, *Scenedesmus obliquus*; T34, biocontrol agent; Rovral commercial fungicide. In each row, values followed by the same letter do not show significant differences for $p<0.05$.

The thermic-treated peat showed higher disease incidence values (Table 3.4). Again, the negative control showed the higher incidence, according to the results obtained in severity, where the plants died at the end of the assay (Figure 3.8B).

Table 3.4: Incidence of *Fusarium oxysporum*, in the different treatments, throughout the trial in thermic treated peat.

	Incidence of the disease on heat-treated substrates (%)					
	So (g. L^{-1})			Positive Control		Negative Control
DAS	0.5t	0.75t	1.0t	Rovral®	T34®	Water
14	0a	0a	0a	0a	0a	0a
21	0a	0a	0a	0a	7a	0a
28	0a	0a	0a	0a	0a	0a
35	67ab	50ab	0b	50ab	67ab	100a
42	67ab	50ab	0b	50ab	67ab	100a

DAS, days after sowing; So, *Scenedesmus obliquus*; T34, biocontrol agent; Rovral commercial fungicide. In each row, values followed by the same letter do not show significant differences for $p<0.05$.

The best result was obtained with the suspension on *S. obliquus* at 1.0 g. L^{-1}, with no disease incidence, which is very interesting regarding its agricultural application.

After 42 DAS, the AUDPC in the water treatment was 0.016 ± 0.0024 and 0.009 ± 0.01 respectively in the non-treated and treated peat, which is significantly higher than in the other treatments (Figure 3.9). Treatments So 0.5 and So 0.75, were significantly lower AUDPC than negative and positive control (fungicide) (Figure 3.9A). The smallest AUDPC was observed at the concentration of 0.5 g. L^{-1} (0.005 ± 0.0038), significantly lower than all other treatments.

In figure 3.9B, it is shown that the treatments with *S. obliquus* showed less AUDPC when compared to the water treatment, however, due to the irregular germination of spinach seeds, Duncan's test did not show significant differences were determined. In addition, in So 1.0 g. L^{-1} the lowest AUDPC occurred.

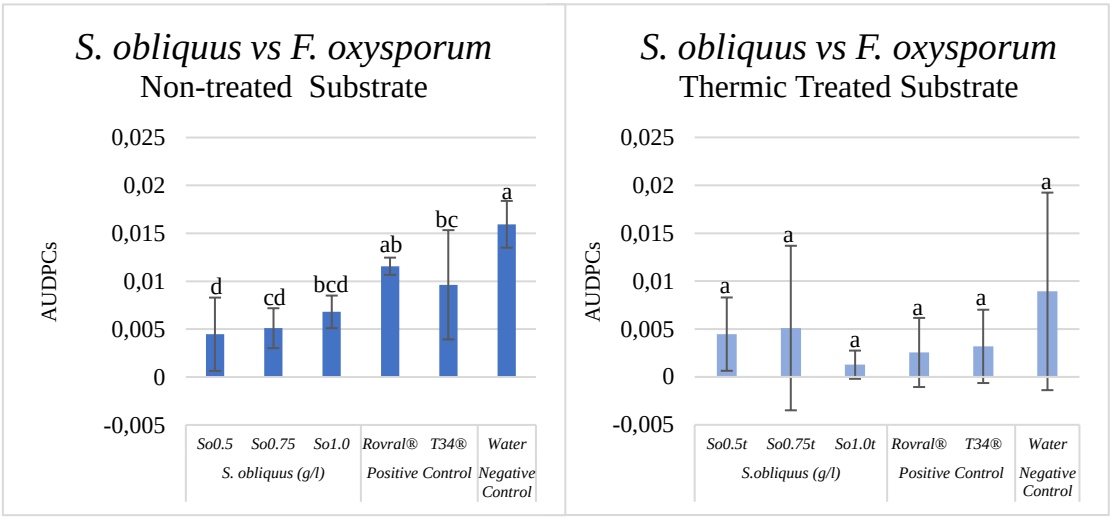
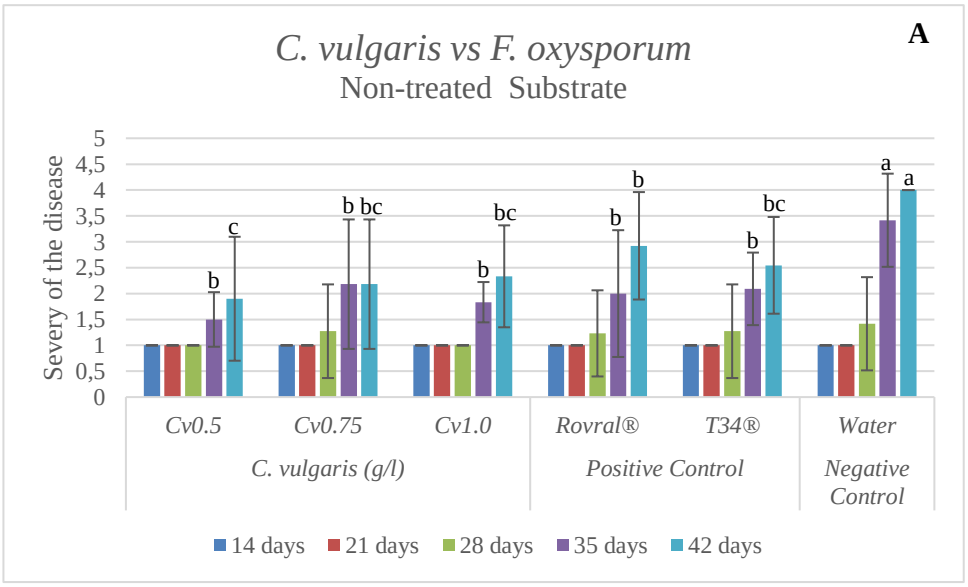


Figure 3 9: *In vivo* AUDPC results of the disease caused by *F. oxysporum*. **(A)** In non-treated substrate when applied with: *S. obliquus* at 0.5, 0.75 and 1.0 g. L^{-1}. **(B)** In thermic treated substrate when applied with: *S. obliquus* at 0.5, 0.75 and 1.0 g. L^{-1}. For each treatment value are means (n=20 ± STD) for each day, the values followed by the same letter do not present significant differences, for $p<0.05$, using Duncan's multiple range test. Each line on top of the bars represents the standard deviation.

Chlorella vulgaris vs Fusarium oxysporum

Figure 3.10A and 3.10B show the results obtained from the severity of *F. oxysporum* in spinach in contact with microalgae *C. vulgaris*.



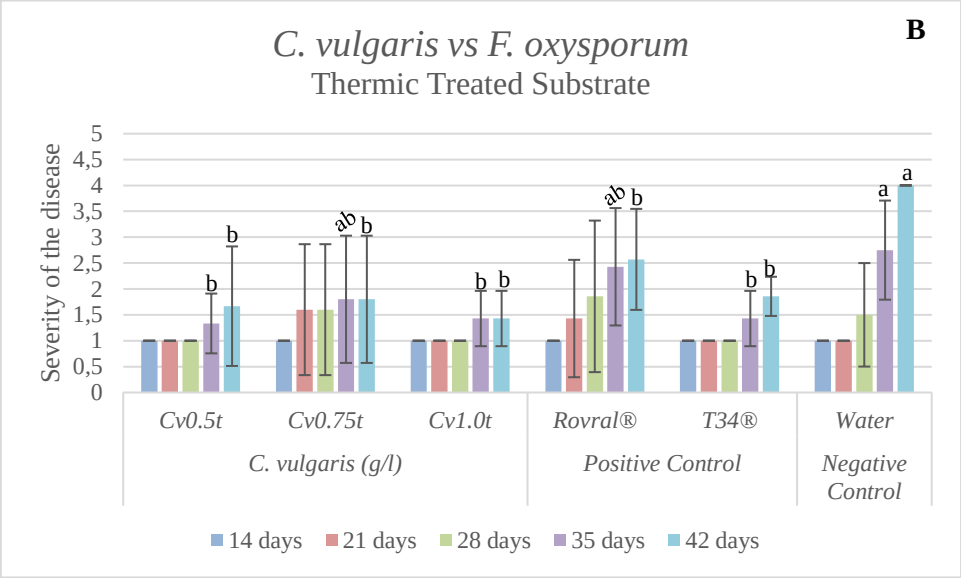


Figure 3.10: *In vivo* results of disease severity caused by *F. oxysporum*. **(A)** In non-treated substrate when applied with: *C. vulgaris* at 0.5, 0.75 and 1.0 g. L^{-1}. **(B)** In thermic treated substrate when applied with: *C. vulgaris* at 0.5, 0.75 and 1.0 g. L^{-1}. For each treatment value are means (n=20 ± STD) for each day, the values followed by the same letter do not present significant differences for $p<0.05$, using Duncan's multiple range test. Each line on top of the bars represents the standard deviation.

When observing the results in Figure 3.10A, the non-treated substrate, showed significant differences between the different treatments, namely between the negative control (water) and the others. There were no significant differences between the three concentrations of *C. vulgaris* at 0.5, 0.75, and 1.0 g. L^{-1} ($p>0.05$). However, statistical significant differences were observed between the commercial fungicide Rovral® and the treatment Cv 0.5 g. L^{-1}. In the treatments using *C. vulgaris*, the severity at 42 DAS, was approximately 2, showing that the plants presented symptoms, but they survived the disease, while the plants of negative control died. It is well known that, as occurred with T34 biocontrol®, the results obtained by the application of *C. vulgaris* significantly reduced the severity of the disease.

Figure 3.10B shows the results in the thermic-treated substrates. A higher disease severity of the disease was observed again in water (negative control), being significantly higher than in the other treatments (by Duncan's test at $p<0.05$), especially when compared with the results obtained in the application of *C. vulgaris* suspensions. After 42 DAS, there were no significant differences between concentrations of 0.5, 0.75, and 1.0 g. L^{-1} and positive controls (Rovral® and T34®) ($p>0.05$).

The results obtained in both trials suggest that the application of *C. vulgaris* led to a significant reduction in the severity of the disease caused by *F. oxysporum*, even in the thermic-treated substrate. These results are supported by the results presented in the following tables.

Table 3.5: Incidence of *Fusarium oxysporum*, in the different treatments, throughout the trial on non-treated peat.

	Incidence of the disease on the non-treated substrates (%)					
	Cv (g. L^{-1})			Positive Control		Negative Control
DAS	0.5	0.75	1.0	Rovral®	T34®	Water
14	0a	0a	0a	0a	0a	0a
21	0a	0a	0a	0a	0a	0a
28	0b	9ab	0b	8ab	9ab	27a
35	50b	64ab	83ab	54b	100a	100a
42	50c	64bc	83ab	100a	100a	100a

DAS, days after sowing; Cv, *Chlorella vulgaris*; T34, biocontrol agent; Rovral commercial fungicide. In each row, values followed by the same letter do not show significant differences for $p<0.05$.

Tables 3.5 and 3.6 show the percentages of disease incidence for each treatment, on the non-treated and treated substrate, respectively.

Results in figure 3.10 suggest that there was a lower disease incidence when microalgae were applied, leading to greater control of the disease. Results shown in Table 3.5, show that the first symptoms of the disease were visible from 28 DAS, appearing first in the positive and negative controls, together with the concentration of 0.75 g. L^{-1}, but without significant differences ($p>0.05$), between these treatments.

At 35 DAS, the incidence was significantly higher ($p<0.05$) in the T34® and in water, where the incidence was 100 %. The incidence of the disease increased with the concentration of *C. vulgaris*. However, despite the high incidence of the disease, when compared with the severity graph (figure 3.10A), it is verified that this higher disease incidence did not lead to an increase in the disease symptoms. At the end of the trial, 42 DAS, the lowest concentrations of microalgae, 0.5 and 0.75 g. L^{-1} had the lowest incidence percentage, 50 % and 64 %, respectively. They were significantly lower than that observed in the controls ($p<0.05$).

The results regarding disease incidence of the thermic-treated substrate are depicted in Table 3.6.

Table 3.6: Incidence of *Fusarium oxysporum*, in the different treatments, throughout the trial in thermic treated peat

	Incidence of the disease on heat-treated substrates (%)					
	Cv (g. L^{-1})			Positive Control		Negative Control
DAS	0.5t	0.75t	1.0t	Rovral®	T34®	Water
14	0a	0a	0a	0a	0a	0a
21	0a	20a	0a	14a	0a	0a
28	0a	20a	0a	29a	0a	0a
35	33b	40b	43b	86ab	43b	100a
42	33b	40b	43b	100a	86ab	100a

DAS, days after sowing; Cv, *Chlorella vulgaris*; T34, biocontrol agent; Rovral commercial fungicide. In each row, values followed by the same letter do not show significant differences for $p<0.05$.

The main difference observed on this substrate was the higher suppressive capacity of the treatments against *Fusarium oxysporum*. This plant pathogen could not develop as much as in the non-treated substrate, a different behavior from what was expected, suggesting once again that *C. vulgaris* suspensions had a higher suppressive capacity on its growth, without the influence of other factors, namely the presence of other microorganism's population. In these substrates, the onset of symptoms began 21 DAS. As shown in Table 3.6, there is also a greater inhibition capacity of the pathogen with the Cv 0.5 g. L^{-1} treatment, which was significantly different from the commercial fungicide Rovral ® and the negative control (water).

Figure 3.11 shows the progression of the variable AUDPC of *F. oxysporum* in these substrates, non-treated and thermic treated, respectively.

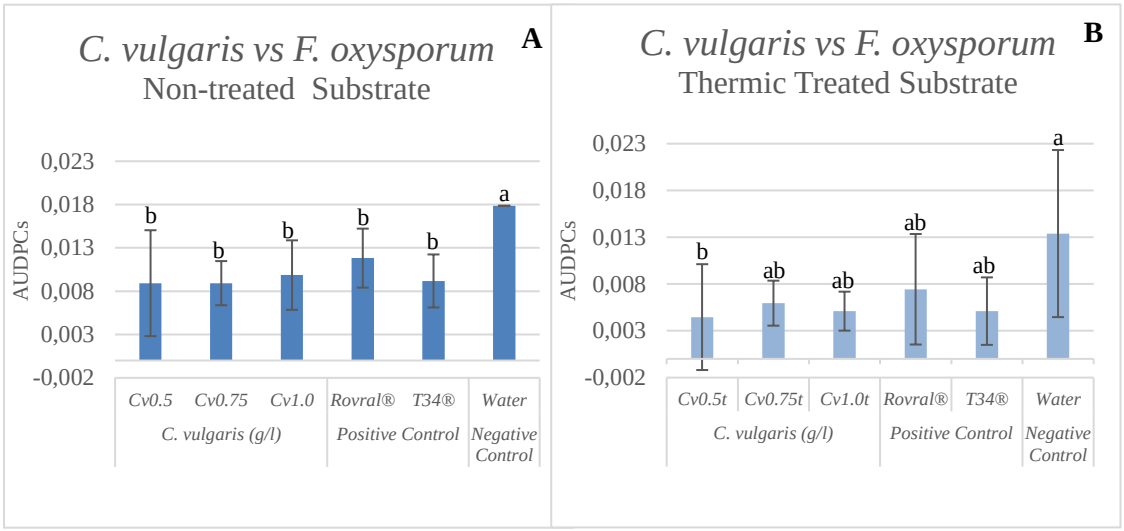


Figure 3.11: *In vivo* AUDPC results of the disease caused by *F. oxysporum*. **(A)** In non-treated substrate when applied with: *C. vulgaris* at 0.5, 0.75 and 1.0 g. L^{-1}. **(B)** In thermic treated substrate when applied with: *C. vulgaris* at 0.5, 0.75 and 1.0 g. L^{-1}. For each treatment value are means (n=20 ± STD) for each day, the values followed by the same letter do not present significant differences for $p<0.05$, using Duncan's multiple range test. Each line on top of the bars represents the standard deviation.

The thermic-treated substrate showed a lower progression of the disease. Both substrates presented significant differences. The disease had a significantly higher progression in water in the non-treated substrate (Figure 3.11A). In the thermic-treated substrate, the differences in AUDPC were obtained only with Cv 0.5t (Figure 3.11B). In the non-treated substrate, the water treatment obtained an AUDPC of 0.018 ± 0.00, while the Cv 0.5 g. L^{-1} reached 0.009 ± 0.006, a reduction of about 50 %. For the thermic-treated substrate (Figure 3.11B), when comparing the same treatments (water and Cv 0.5 g. L^{-1}), with values of 0.013 ± 0.009 and 0.005 ± 0.006 respectively, this difference of 0.008, corresponds to a reduction of about 61 % in the development of *F. oxysporum*.

It must be noted that even without significant differences when compared to the T34® and the fungicide Rovral®, all *C. vulgaris* solutions showed a reduction in disease progression in both substrates. This means that when these concentrations of *C. vulgaris* are applied, the disease was not so aggressive, and did not grow as quickly as in negative control (water).

Scenedesmus obliquus vs Sclerotium rolfsii

To evaluate the effect of microalgae on the growth of the fungus *Sclerotium rolfsii*, this pathogen was inoculated in the substrate (white peat), thermic-treated and not heat treated. Lettuce seedlings were planted n days after substrate inoculation with the pathogen. Figure 3.12 shows the severity indices determined during the trial period, for the non-treated substrate and the thermic-treated substrate (Figure 3.12A and 3.12B).

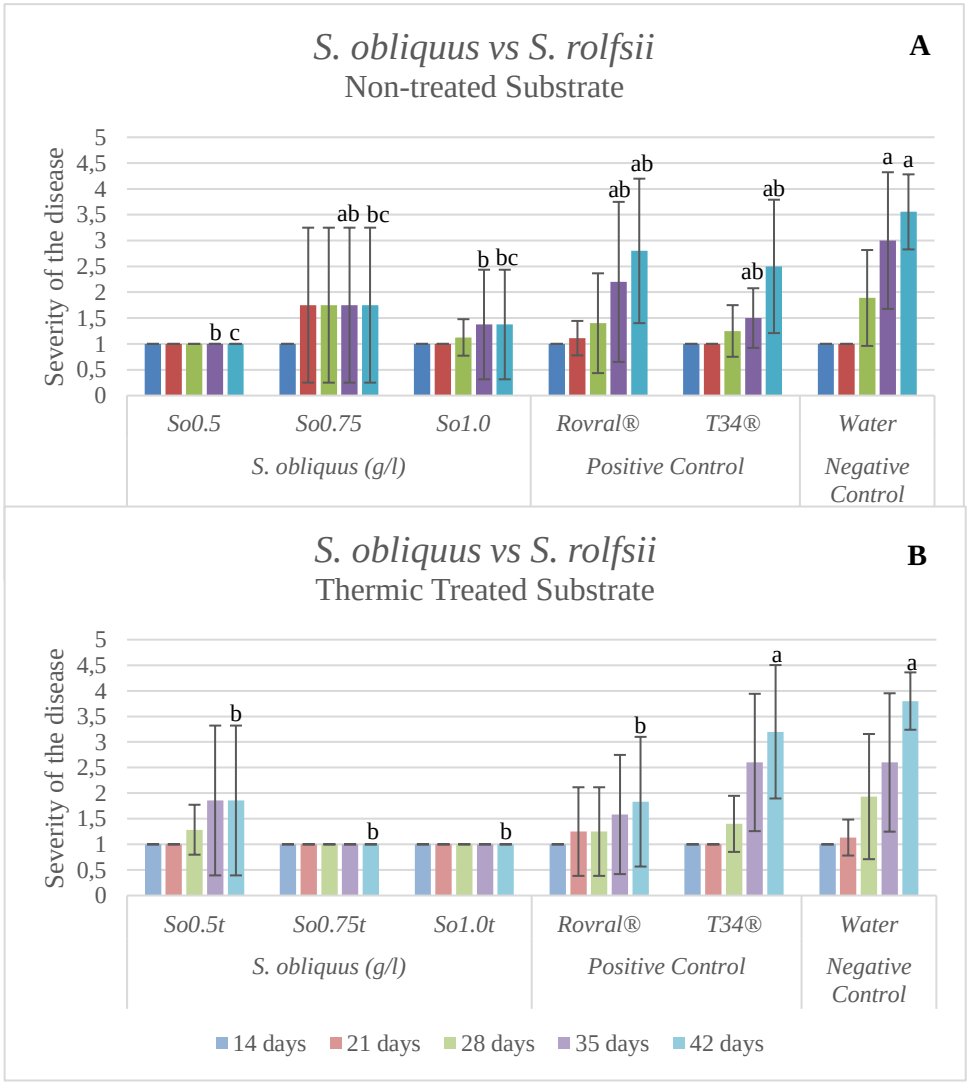


Figure 3.12: *In vivo* results of the severity of the disease caused by *S. rolfsii*. **(A)** In non-treated substrate when applied with: *S. obliquus* at 0.5, 0.75 and 1.0 g. L^{-1}. **(B)** In thermic treated substrate when applied with: *S. obliquus* at 0.5, 0.75 and 1.0 g. L^{-1}. For each treatment value are means (n=20 ± STD) for each day, the values followed by the same letter do not present significant differences for $p<0.05$, using Duncan's multiple range test. Each line on top of the bars represents the standard deviation.

According to Figure 3.12A, the application of microalgae *S. obliquus* led to a decrease in the severity of the disease caused by *S. rolfsii* on lettuces. At 42 DAS, the severity of the disease was significantly higher in the positive controls Rovral® and T34®, and the negative control water. However, the positive controls results were not significantly different from concentrations of 0.75 and 1.0 g. L^{-1} of *S. obliquus* ($p>0.05$). Therefore, the lowest severity index, statistically significant ($p<0.05$), was calculated in So 0.5 g. L^{-1} presenting a level of 1 (1=healthy).

In the thermic-treated substrate (Figure 3.12B), occurred a significantly higher disease severity in the T34® and the negative control. Microalgae-based treatments did not differ statistically between themselves or the commercial fungicide Rovral®. It must be noted that in the thermic-treated substrate, at concentrations of 0.75 and 1.0 g. L^{-1} no disease symptoms were observed. These results are confirmed by the incidence of the disease shown in Table 3.8, which shows that *S. rolfsii* was unable to grow when the microalgae suspensions were used, indicating once again an antifungal activity by *S. obliquus.*

Tables 3.7 and 3.8 show the percentages of disease incidence of *S. rolfsii* during the assay on the non-treated and treated substrate when the different treatments were applied.

Table 3.7: Incidence of *Sclerotium rolfsii*, in the different treatments, throughout the trial on non-treated peat.

	Incidence of the disease on the non-treated substrates (%)					
	So (g. L^{-1})			Positive Control		Negative Control
DAS	0.5	0.75	1.0	Rovral®	T34®	Water
14	0a	0a	0a	0a	0a	0a
21	0a	25a	0a	11a	0a	0a
28	0b	25ab	12.5b	20ab	25ab	67a
35	0b	25ab	12.5b	40ab	50ab	78a
42	0c	25bc	12.5c	70ab	75a	100a

DAS, days after sowing; So, *Scenedesmus obliquus*; T34, biocontrol agent; Rovral commercial fungicide. In each row, values followed by the same letter do not show significant differences for $p<0.05$.

Table 3.8: Incidence of *Sclerotium rolfsii*, in the different treatments, throughout the trial in thermic treated peat-

	Incidence of the disease on heat-treated substrates (%)					
	So (g. L^{-1})			Positive Control		Negative Control
DAS	0.5t	0.75t	1.0t	Rovral®	T34®	Water
14	0a	0a	0a	0a	0a	0a

21	0a	0a	0a	8.3a	0a	13a
28	29ab	0b	0b	8.3b	40a	47a
35	29b	0c	0c	25b	80a	67ab
42	29b	0b	0b	33b	80a	100a

DAS, days after sowing; So, *Scenedesmus obliquus*; T34, biocontrol agent; Rovral commercial fungicide. In each row, values followed by the same letter do not show significant differences for $p<0.05$.

The results in tables 3.7 and 3.8 confirm the ones expressed in figure 3.12. It is found that when microalgae suspensions are applied, the incidence of the disease was significantly lower than in controls (Table 3.7). It is also observed that throughout the trial, the incidence of the disease in the treatments with *S. obliquus* was stabilized, meaning that the plants did not increase its disease severity level. The lowest disease incidence was obtained with the concentration of 0.5 g. L^{-1}, followed by the concentration of 1.0 g. L^{-1}. As expected, the water treatment had an incidence of 100 %, leading to the death of all plants as reported in severity, reaching the index of 4 (Figure 3.12A).

In the thermic-treated substrate, similar results to the ones on the non-treated substrate occurred. Once again, it was found that the application of microalgae suspensions led to a lower disease incidence in plants, and this reduction was significant when compared to controls. Table 3.8 shows this information, and it must be mentioned that treatments So 0.75 and So 1.0 g. L^{-1} did not show any symptoms, again coinciding with the information demonstrated in disease severity, where these same concentrations, in the thermic-treated substrate, indicated a significant reduction of the disease (Figure 3.12B).

The greatest progression of AUDPC caused by *S. rolfsii* was observed in the thermic-treated substrate, as expected, calculating a progression of 0.017 ± 0.003 (Figure 3.13B). However, both substrates showed significant differences between treatments.

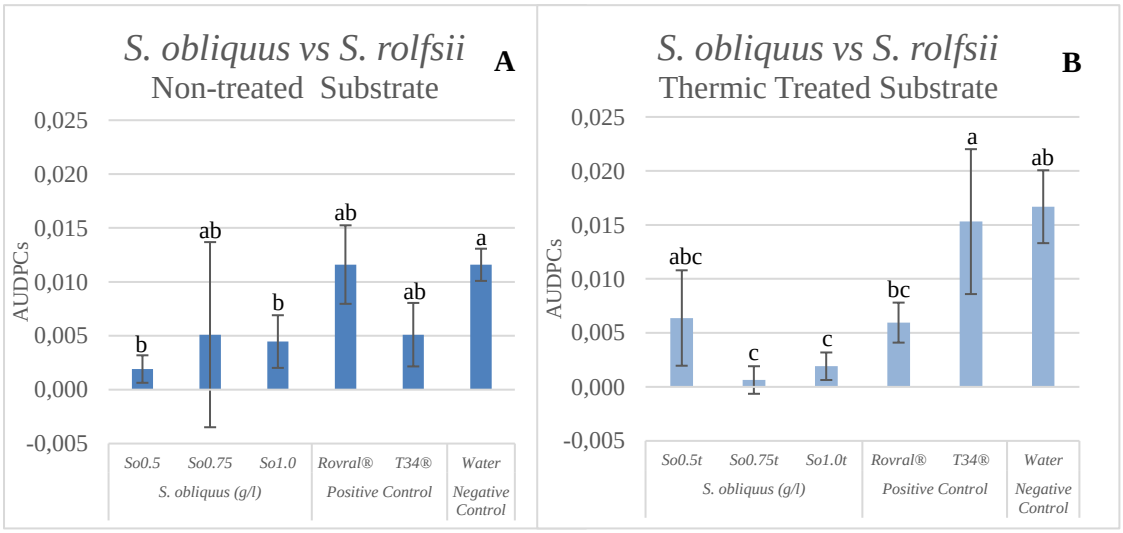


Figure 3.13: *In vivo* AUDPC results of the disease caused by *S. rolfsii*. **(A)** In non-treated substrate when applied with: *S. obliquus* at 0.5, 0.75 and 1.0 g. L^{-1}. **(B)** In thermic treated substrate when applied with: *S. obliquus* at 0.5, 0.75 and 1.0 g. L^{-1}. For each treatment value are means (n=20 ± STD) for each day, the values followed by the same letter do not present significant differences for $p<0.05$, using Duncan's multiple range test. Each line on top of the bars represents the standard deviation.

In Figure 3.13A, it is possible to see that regarding the AUDPC in the non-heat-treated substrate, no significant differences were observed between the positive controls (Rovral® and T34®) and the *S. obliquus* suspensions. Moreover, the *S. obliquus* concentrations of 0.5 and 1.0 g. L^{-1} showed a better result than the negative control. The lowest significant AUDPC was determined in So 0.5 g. L^{-1}, with a value of 0.002 ± 0.001, that represents a reduction of 84 % to the negative control (water).

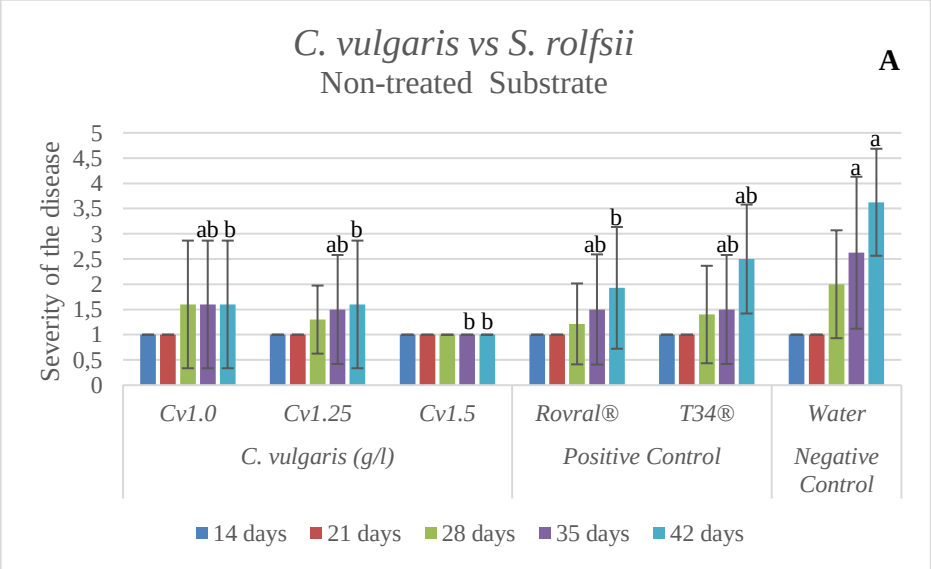
In the thermic-treated substrate (Figure 3.13B), treatments So 0.75 and So 1.0 g. L^{-1} showed a better result compared to one of the positive controls (T34®) and similar results to the other positive control, the fungicide. Moreover, all So suspensions showed a similar result to the fungicide. These results suggest a promising role of So suspensions on *S. rolfsii* control.

The lowest AUDPC occurred with the application of the *S. obliquus* at the concentration of 0.75 g. L^{-1}, with a value of 0.001 ± 0.0013, represents a reduction of 96.5 % when compared to the water treatment.

Chlorella vulgaris vs. Sclerotium rolfsii

The test of the treatments based on *C. vulgaris* was carried out in the same way as on the *S. obliquus* assays, except regarding the concentrations of the microalgae that were used, which were selected according to the results obtained in the *in vitro* assays with *C. vulgaris* and *S. rolfsii*.

Figure 3.14 shows the different severity indices evaluated in heat- and non-heat-treated thermic substrates (Figures 3.14A and 3.14B). In both trials, the symptoms were visible only after the 28 DAS, and occurred in most of the treatments.



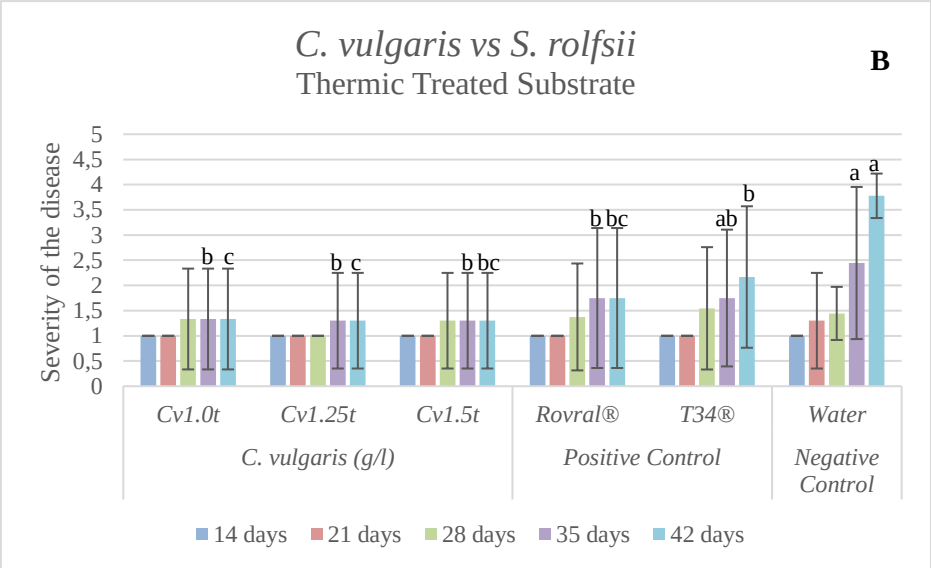


Figure 3.14: *In vivo* results of severity disease caused by *S. rolfsii*. **(A)** In non-treated substrate when applied with: *C. vulgaris* at 1.0, 1.25 and 1.5 g. L^{-1}. **(B)** In thermic treated substrate when applied with: *C. vulgaris* at 1.0, 1.25, 1.5 g. L^{-1}. For each treatment value are means (n=20 ± STD) for each day, the values followed by the same letter do not present significant differences for $p<0.05$, using Duncan's multiple range test. Each line on top of the bars represents the standard deviation.

In both substrates (heat- and not heat-treated) the results with Cv suspension were significantly better than the ones observed in the negative control at 42 DAS. Moreover, the AUDPC values with Cv suspension were even better than with both positive controls, however, they did not statistically differ.

These results show that Cv suspensions are promising options regarding *S. rolfsii* control. These results are also indicated by the observed disease incidence (table 3.9). According to the results in table 3.9, a significant reduction in the incidence of *S. rolfsii* is evident when applying *C. vulgaris*.

Table 3.9: Incidence of *S. rolfsii*, in the different treatments, throughout the trial on non-treated peat.

	Incidence of the disease on the non-treated substrates (%)					
	Cv (g. L^{-1})			Positive Control		Negative Control
DAS	1.0	1.25	1.5	Rovral®	T34®	Water
14	0a	0a	0a	0a	0a	0a
21	0a	0a	0a	0a	0a	0a
28	20ab	20ab	0b	7ab	20ab	63a

35	20bc	20bc	0c	21ab	78a	80a
42	20bc	20bc	0c	50ab	80a	88a

DAS, days after sowing; Cv, *Chlorella vulgaris*; T34, biocontrol agent; Rovral commercial fungicide. In each row, values followed by the same letter do not show significant differences for $p<0.05$.

Comparing the treatments water and T34®, a higher incidence was determined regarding the Cv suspensions and the fungicide treatments, with incidence percentages of 88 % and 80 %, respectively. However, a significant reduction occurs when the microalga is applied. This reduction is also observed in Figure 3.15A, with the disease progression variable values, in which it may be observed that all Cv suspensions treatments had similar results to the ones obtained with both positive controls. The concentration of 1.5 g. L^{-1}, in the non-heated substrate, promoted the lower development of the disease, reaching only 0.0026 ± 0.00, which represents a reduction by approximately 84 % of the disease's growth on the negative control.

Significant differences between treatments and the negative control occurred in the thermic-treated substrate (Figure 3.14B). At the end of the trial (42 DAS), the severity was significantly higher in water treatment (negative control) than the other treatments. In agreement with the non-treated substrate, the treatments with Cv showed the lowest severity, not different from the positive controls ($p>0.05$).

S. rolfsii growth was significantly reduced as expressed by the severity index and disease incidence (Table 3.10). The concentration of Cv that most affected the phytopathogenic were those of 1.25 and Cv 1.5 g. L^{-1}, with an incidence of 10 %, being comparable to the commercial pesticide Rovral®.

Table 3.10: Incidence of *S. rolfsii* throughout the trial in thermic treated substrate.

	Incidence of the disease on heat-treated substrates (%)					
	Cv (g. L^{-1})			Positive Control		Negative Control
DAS	1.0t	1.25t	1.5t	Rovral®	T34®	Water
14	0a	0a	0a	0a	0a	0a
21	0a	0a	0a	0a	0a	10a
28	11b	0b	10b	13b	18b	44a
35	11b	10b	10b	25ab	25ab	56a
42	11c	10c	10c	25bc	50b	100a

DAS, days after sowing; Cv, *Chlorella vulgaris*; T34, biocontrol agent; Rovral commercial fungicide. In each row, values followed by the same letter do not show significant differences for $p<0.05$.

The decrease in disease incidence in the thermic treated substrate was also expressed by the variable AUDPC (Figure 3.15B).

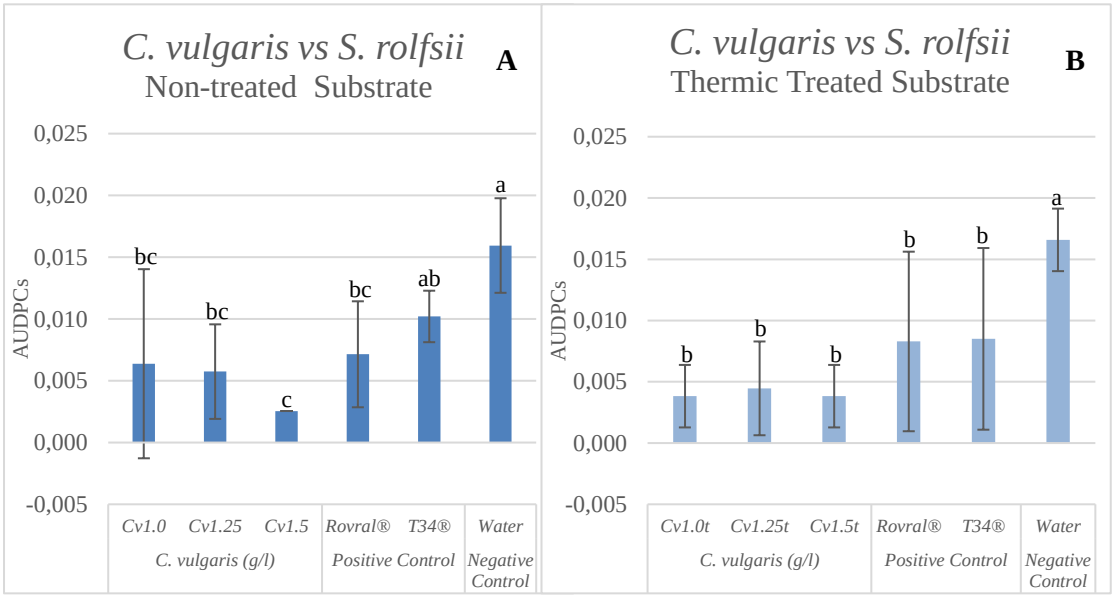


Figure 3.15: *In vivo* AUDPC results of the disease caused by *S. rolfsii*. **(A)** In non-treated substrate when applied with: *C. vulgaris* at 1.0, 1.25 and 1.5 g. L^{-1}. **(B)** In thermic treated substrate when applied with: *C. vulgaris* at 1.0, 1.25 and 1.5 g. L^{-1}. For each treatment value are means (n=20 ± DST) for each day, the values followed by the same letter do not present significant differences for $p<0.05$, using Duncan's multiple range test. Each line on top of the bars represents the standard deviation.

On the negative control (water) the progress of the disease reached the highest value, 0.017 ± 0.0025. Disease progression in Cv treatments ranged from 0.0038 and 0.0045, not significantly different from positive controls (T34® biocontrol, and Rovral commercial pesticide® ($p>0.05$).

Scenedesmus obliquus vs. Botrytis cinerea

To test the effect of microalgae *S. obliquus* and *C. vulgaris* on *Botrytis cinerea*. The phytopathogenic fungus was inoculated in spinach and lettuce plants grown in pots with substrate.

- Spinach trials

Figure 3.16A shows the severity over the days in the non-treated substrate, showing that there were significant differences.

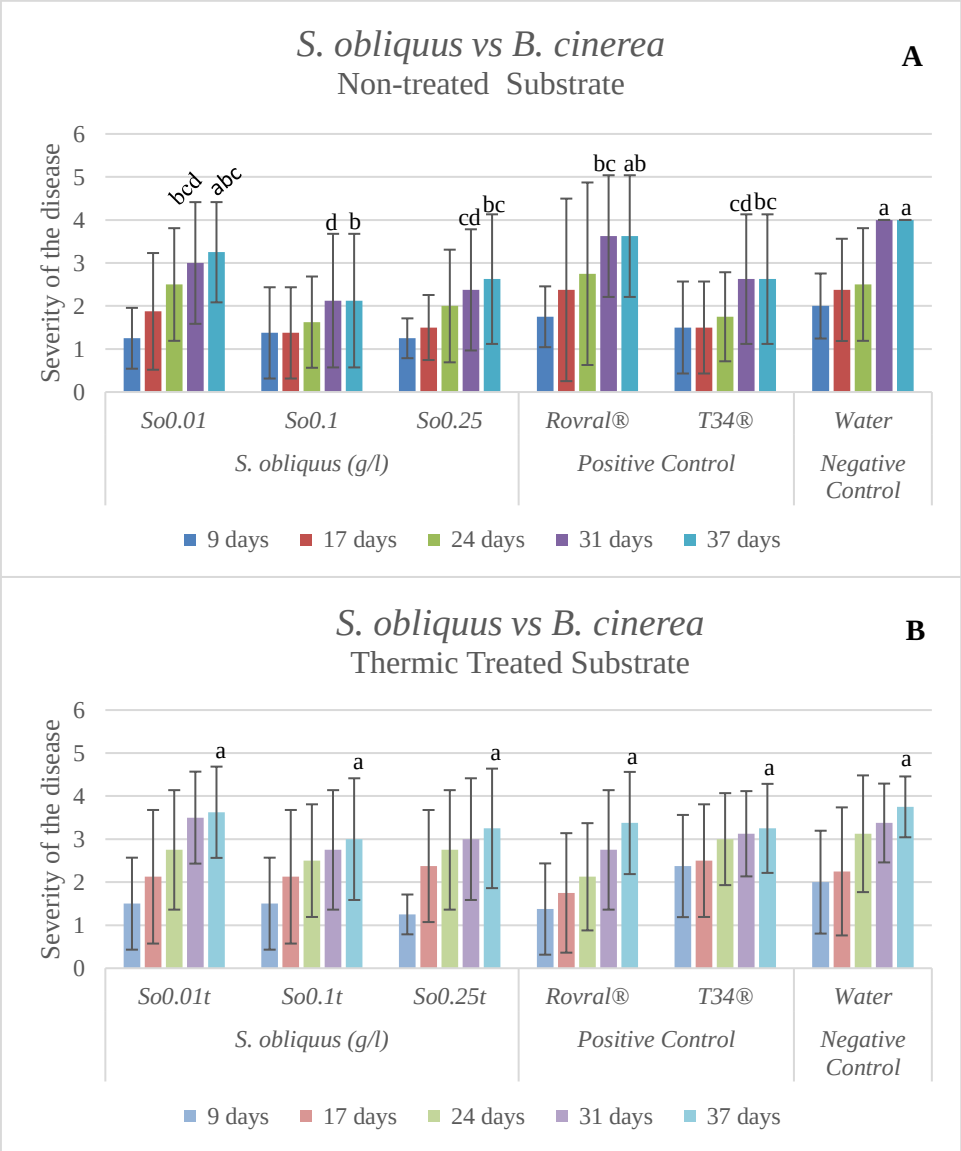


Figure 3.16: *In vivo* results of severity disease caused by *B. cinerea* in spinach plants. **(A)** In non-treated substrate when applied with: *S. obliquus* to 0.01, 0.1 and 0.25 g L^{-1}. **(B)** In thermic treated substrate when applied with: *S. obliquus* to 0.01, 0.1 and 0.25 g L^{-1}. For each treatment value are means (n=20 ± STD) for each day, the values followed by the same letter do not present significant differences for $p<0.05$, using Duncan's multiple range test. Each line on top of the bars represents the standard deviation.

Throughout the trial the disease severity index increased over time in the different treatments, however this increase decreased with time in some treatments, suggesting that the treatment led to a reduction in the effects of the pathogen on plants.

By 37 DAI (day after inoculation) a significant increase in severity was observed in water treatment, significantly higher to the positive biocontrol T34® and the So concentrations of 0.1 and 0.25 g. L^{-1}. The concentrations of 0.1 g. L^{-1} and 0.25 g. L^{-1} of microalgae *S. obliquus* promoted a significant disease severity reduction. This reduction was confirmed in Table 3.11, where it may be observed that, as expected, the incidence of the disease in plants was significantly lower in the concentration of 0.1 g. L^{-1}.

Table 3.11: Incidence of *B. cinerea*, during the spinach trial, on non-treated peat.

	Incidence of the disease on the non-treated substrates (%)					
	So (g. L^{-1})			Positive Control		Negative Control
DAI	0.01	0.1	0.25	Rovral®	T34®	Water
9	25bc	13c	25bc	63ab	25bc	75a
17	38ab	13b	50ab	63ab	25ab	75a
24	75a	38a	50a	88a	50a	75a
31	75ab	38b	63ab	100a	63ab	100a
37	88a	38b	63ab	100a	63ab	100a

DAI, days after inoculation; So, *Scenedesmus obliquus*; T34, biocontrol agent; Rovral commercial fungicide. In each row, values followed by the same letter do not show significant differences for $p<0.05$.

Although disease incidence in So 0.25 g. L^{-1} was not significantly different from the one on controls or the concentration of 0.01 g. L^{-1}, with an incidence percentage of 63 %, it did not lead to the death of plants, as indicated in Figure 3.16A.

Comparing the substrates, there was a higher severity in the thermic-treated substrate (Figure 3.16B). Figure 3.16B showed no statistical differences between treatments. The severity of the disease varied between levels 3 to 4 in the treatments, at 37 DAI.

According to table 3.11, when treated with *S. obliquus*, the incidence on So 0.1 g. L^{-1} was lower than in Rovral® and water controls, by the end of the trial. The lowest incidence was observed in So 0.1 g. L^{-1}, with an incidence of 38%.

On the contrary, on heat-treated substrates (table 3.12) no significant differences were calculated between treatments.

Table 3.12: Incidence of *B. cinerea*, in spinach plants during the trial, in thermic treated peat.

	Incidence of the disease on heat-treated substrates (%)					
	So (g. L^{-1})			Positive Control		Negative Control
DAI	0.01t	0.1t	0.25t	Rovral®	T34®	Water
9	25ab	25ab	25ab	13b	75a	50ab
17	38a	38a	63a	25a	75a	50a
24	75a	75a	75a	63a	100a	75a
31	88a	75a	75a	75a	100a	100a
37	88a	75a	75a	88a	100a	100a

DAI, days after inoculation; So, *Scenedesmus obliquus*; T34, biocontrol agent; Rovral commercial fungicide. In each row, values followed by the same letter do not show significant differences for $p<0.05$.

Regarding AUDPC, no significant differences were found between the treatments in both substrates ($p>0.05$) (Figure 3.17), meaning that none of the treatments was effective against the pathogen.

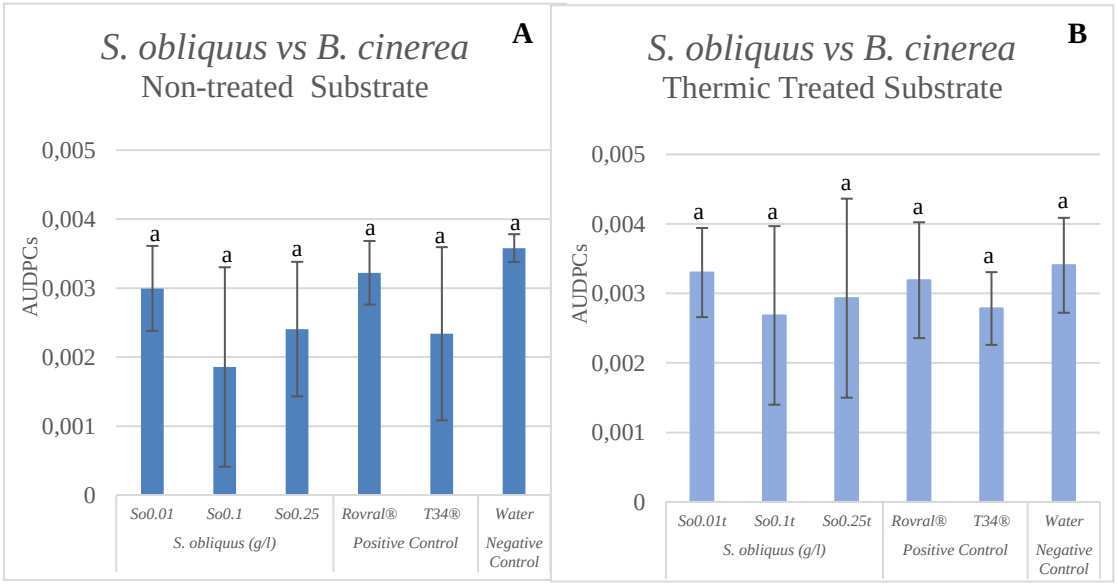
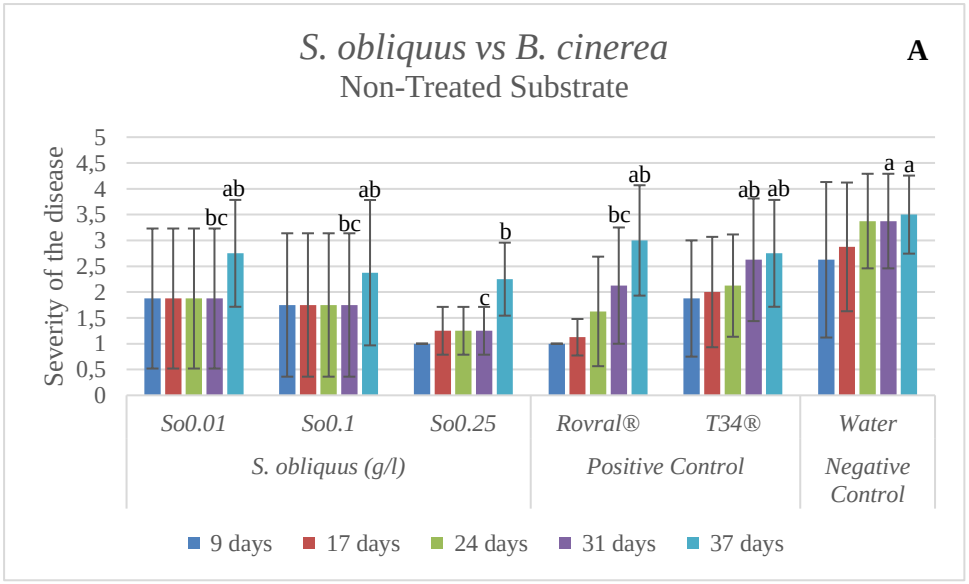


Figure 3.17: *In vivo* results of AUDPC of the disease caused by *B. cinerea* in spinach plants. **(A)** In non-treated substrate when applied with: *S. obliquus* 0.01, 0.1 and 0.25 g L^{-1}. **(B)** In thermic treated substrate when applied with: *S. obliquus* 0.01, 0.1 and 0.25 g L^{-1}. For each treatment value are means (n=20 ± STD) for each day, the values followed by the same letter do not present significant differences for $p<0.05$, using Duncan's multiple range test. Each line on top of the bars represents the standard deviation.

In both substrates, the disease had a similar progression in all treatments, i.e., there was no inhibition of the disease.

- Lettuce trials

When inoculated in lettuce plants, *B. cinerea* had different effects according to the treatment applied. Figure 3.18 shows the disease severity levels caused by *B. cinerea* in lettuce. The disease was more aggressive in the non-treated substrate (figure 3.18A), in which the water treatment reached the level of 3.5. However, in the thermic-treated substrate, a significantly higher severity was obtained at the concentration So 0.25 g. L^{-1}, below level 3.5.



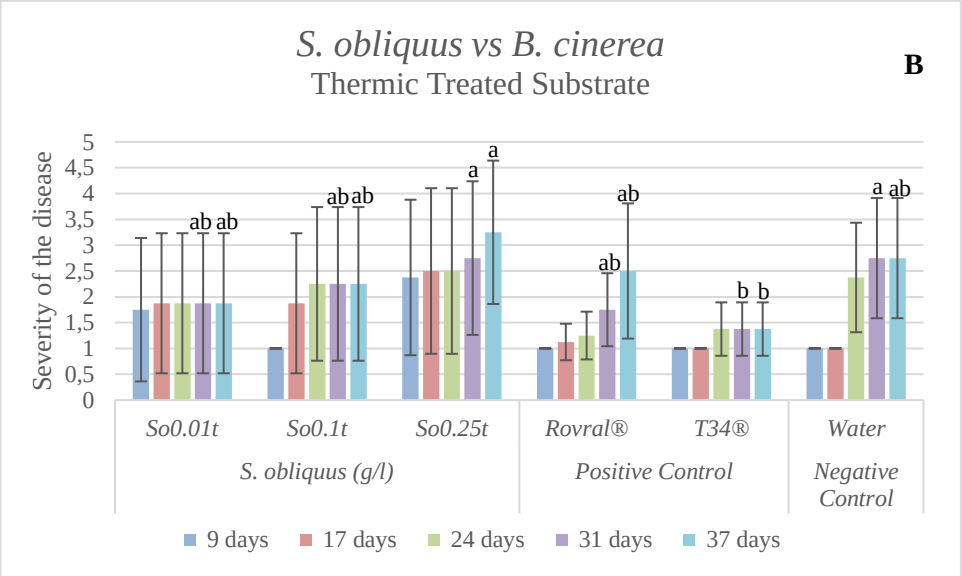


Figure 3.18: *In vivo* results of severity caused disease by *B. cinerea* in lettuce plants. (A) In non-treated substrate when applied with: *S. obliquus* to 0.01, 0.1 and 0.25 g. L^{-1}. (B) In thermic treated substrate when applied with: *S. obliquus* to 0.01, 0.1 and 0.25 g. L^{-1}. For each treatment value are means (n=20 ± STD) for each day, the values followed by the same letter do not present significant differences for $p<0.05$, using Duncan's multiple range test. Each line on top of the bars represents the standard deviation.

In the non-treated substrates, during the assay, several significant differences in disease severity were identified between treatments (Figure 3.18A). These differences were observed at the treatment So 0.25 g. L^{-1} at 31 DAI, where it differed from the T34® biocontrol and water, where a significantly higher severity was identified. At that time, results of So 0.01 and So 0.1 g. L^{-1} were also significantly different from T34® and water, together with the commercial pesticide Rovral®. At 37 DAI, the only significant difference occurred between the concentration So 0.25 g. L^{-1} and the biocontrol T34® and water.

According to figure 3.18B, the results in thermic-treated substrates contradict what was observed on the non-treated substrates. In the thermic-treated substrates, the most severe effects occurred in the So concentration where previously the least severity occurred: the suspension with 0.25 g. L^{-1}. Treatment T34® obtained the lowest severity by the end of the trial period, significantly lower than treatment So 0.25 g. L^{-1}. These results are shown in Table 3.14, where the percentage of incidence of the disease in each treatment is shown.

Table 3.13: Incidence of *B. cinerea*, on lettuce, in the different treatments, during the non-treated peat test.

	Incidence of the disease on the non-treated substrates (%)					
	So (g. L^{-1})			Positive Control		Negative Control
DAI	So 0.01	So 0.1	So 0.25	Rovral®	T34®	Water
9	38ab	25ab	0b	0b	50a	63a
17	38b	25b	25b	13b	63ab	88a
24	38bc	25c	25c	50bc	75ab	100a
31	38b	38b	25b	63ab	88a	100a
37	100a	63b	100a	100a	100a	100a

DAI, days after inoculation; So, *Scenedesmus obliquus*; T34, biocontrol agent; Rovral commercial fungicide. In each row, values followed by the same letter do not show significant differences for $p<0.05$.

Table 3.14: Incidence of *B. cinerea* on lettuce, in the different treatments, throughout the trial in thermic treated peat.

	Incidence of the disease on heat-treated substrates (%)					
	So (g. L^{-1})			Positive Control		Negative Control
DAI	0.01t	0.1t	0.25t	Rovral®	T34®	Water
9	25ab	0b	50a	0b	0b	0b
17	38ab	38ab	50a	13ab	0b	0b
24	38ab	38ab	50ab	25b	38ab	88a
31	38a	50a	63a	63a	38a	88a
37	38a	50a	75a	75a	38a	88a

DAI, days after inoculation; So, *Scenedesmus obliquus*; T34, biocontrol agent; Rovral commercial fungicide. In each row, values followed by the same letter do not show significant differences for $p<0.05$.

With the biocontrol T34 disease incidence was lower than with the concentration of 0.25 g. L^{-1}, however, results did not differ significantly.

Table 3.13 shows that the lowest incidence in the non-treated substrates occurred in So 0.1 g. L^{-1}, which is significantly lower than the other treatments where incidence reached 100 %. Throughout the trial, there was a faster increase in the disease's incidence in Rovral®, T34® and water treatments, showing that there is a possible delay in disease growth when microalgae-based treatments are applied.

On heat-treated substrates no differences between treatments were observed by the end of the trial (Table 3.14).

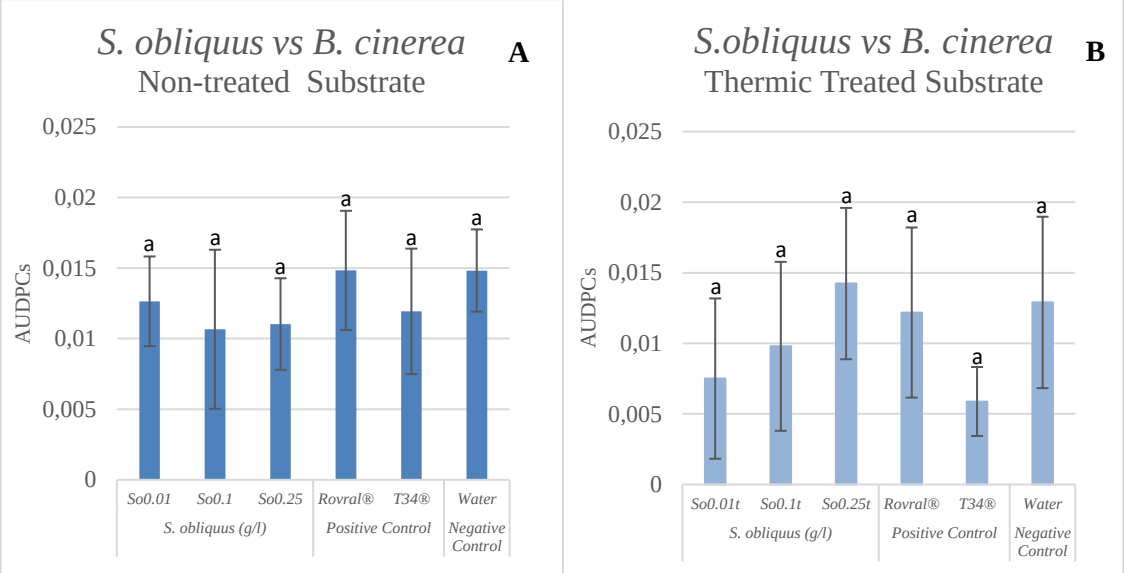


Figure 3.19: *In vivo* AUDPC results of the disease caused by *B. cinerea* in lettuce plants. **(A)** In non-treated substrate when applied with: *S. obliquus* 0.01, 0.1 and 0.25 g. L^{-1}. **(B)** In thermic treated substrate when applied with: *S. obliquus* 0.01, 0.1 and 0.25 g. L^{-1}. For each treatment value are means (n=20 ± STD) for each day, the values followed by the same letter do not present significant differences for $p<0.05$, using Duncan's multiple range test. Each line on top of the bars represents the standard deviation.

Again, the AUDPC of *B. cinerea* showed no difference between treatments This indicates that although *S. obliquus* shows to have a reduction in the severity of symptoms, it does not have a reducing on the affect leaf area by the disease.

Chlorella vulgaris vs. Botrytis cinerea

- Spinach trial

In the experiments with spinach, the effects of *C. vulgaris* in *B. cinerea* on disease severity indices on both substrates (non-treated and thermic treated), can be observed in figure 3.20.

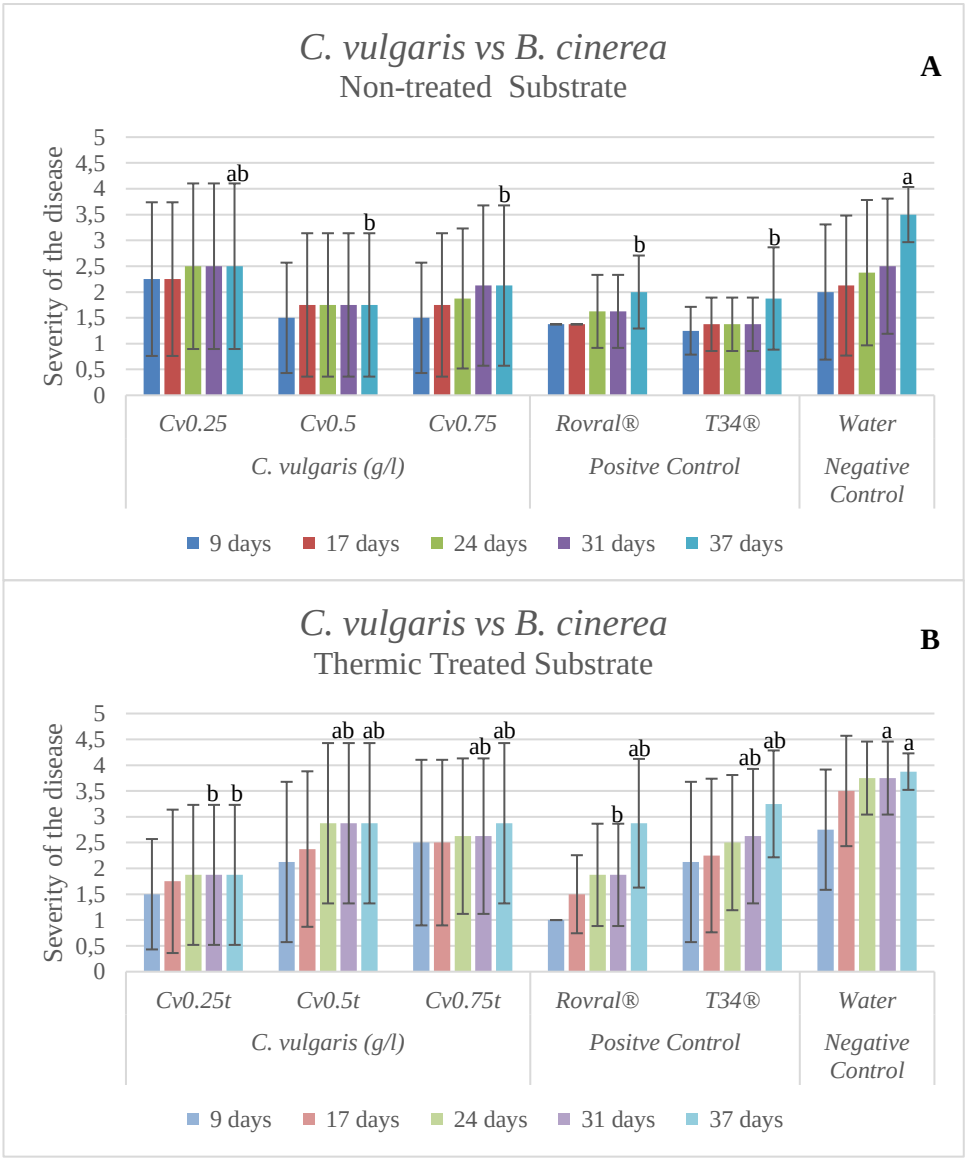


Figure 3.20: *In vivo* results of severity disease caused by *B. cinerea* in spinach plants. **(A)** In non-treated substrate when applied with: *C. vulgaris* at 0.25, 0.5 and 0.75 g. L^{-1}. **(B)** In thermic treated substrate when applied with: *C. vulgaris* at 0.25, 0.5 and 0.75 g. L^{-1}. For each treatment value are means (n=20 ± STD) for each day, the values followed by the same letter do not present significant differences for $p<0.05$, using Duncan's multiple range test. Each line on top of the bars represents the standard deviation.

As shown in Figure 3.20A, all treatments except the lowest Cv concentration (0.25 g. L^{-1}), had similar results, significantly lower than the negative control.

In the thermic-treated substrate, the concentration of 0.25 g. L^{-1} had a significant lower disease severity than the negative control water, however, it did not differ from the other treatments.

At 37 DAI, it was confirmed that applying suspensions of *C. vulgaris* to spinach, reduces the incidence of the disease (Table 3.15). At the end of the trial, the Cv concentrations of 0.5 and 0.75 g. L^{-1} were comparable with the positive controls (Rovral® and T34®) and significantly decreased the disease on plants, with reductions of 25 % and 38 % incidence (Table 3.15).

Table 3.15: Incidence of *B. cinerea*, in the different treatments, during the non-treated peat test in spinach plants.

	Incidence of the disease on the non-treated substrates (%)					
	Cv (g. L^{-1})			Positive Control		Negative Control
DAI	0.25	0.5	0.75	Rovral®	T34®	Water
9	50a	25a	25a	13a	50a	50a
17	50a	25a	25a	13a	63a	50a
24	50a	25a	38a	38a	63a	63a
31	50a	25a	38a	38a	63a	75a
37	50ab	25b	38b	38b	75ab	100a

DAI, days after inoculation; Cv, *Chlorella vulgaris*; T34, biocontrol agent; Rovral commercial fungicide. In each row, values followed by the same letter do not show significant differences for $p<0.05$.

This reduction is also visible in Figure 3.21A, where all treatments showed significantly lower values than the positive control, with a slower disease progression. The AUDPC values ranged from 0.007 to 0.010 on Cv suspensions. Although there were no significant differences between positive controls and the microalgae suspensions, in Cv 0.5 g. L^{-1} the progression of the disease was lower than the other treatments, reaching 0.007 AUDPC, a difference of 0.009 when compared with the greatest progression of the disease, in the water (0.016 ± 0.001), meaning a reduction of 56.4 %.

The incidence of when treated with Cv suspensions are represented in table 3.16. However, only Cv 0.5 g. L^{-1} presented a significantly lower disease incidence when compared to controls, with a value of 38 %. The remaining concentrations showed a higher disease incidence, from 63 % to 75 %, but not statistically significant.

Table 3.16: Incidence of *B. cinerea*, in spinach plants during the trial, in thermic treated peat.

	Incidence of the disease on heat-treated substrates (%)					
	Cv (g. L^{-1})			Positive Control		Negative Control
DAI	0.25t	0.5t	0.75t	Rovral®	T34®	Water
9	25c	38bc	50ab	0c	38bc	88a
17	25b	50ab	63ab	38ab	50ab	88a
24	38b	63ab	75ab	63ab	75ab	100a
31	38b	63ab	75ab	75ab	75ab	100a
37	38b	63ab	75ab	88a	100a	100a

DAI, days after inoculation; Cv, *Chlorella vulgaris*; T34, biocontrol agent; Rovral commercial fungicide. In each row, values followed by the same letter do not show significant differences for $p<0.05$.

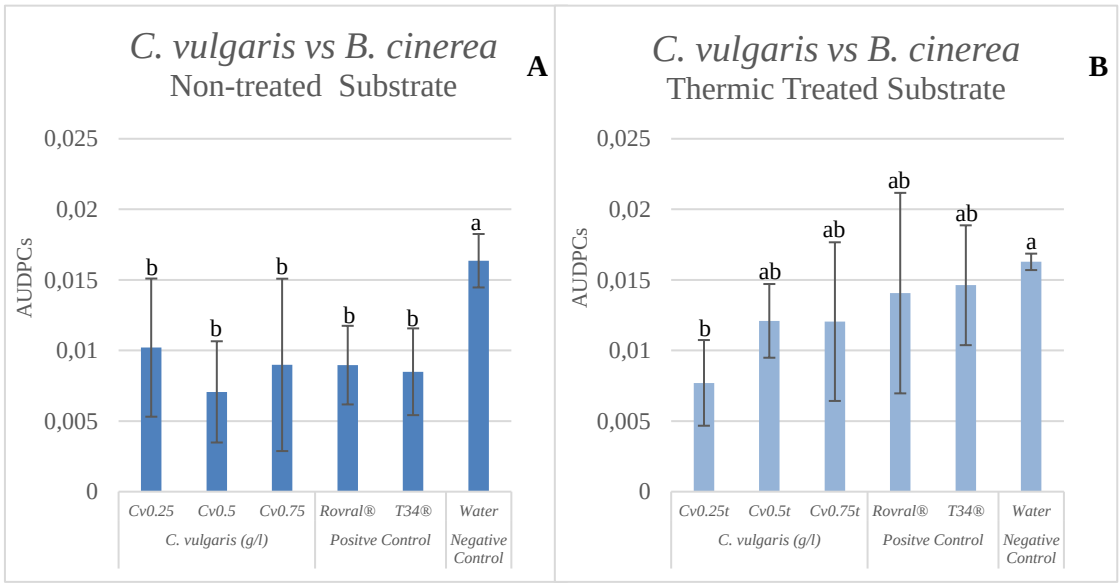


Figure 3.21*: In vivo* AUDPC results of *B. cinerea* disease in spinach plants. **(A)** In non-treated substrate when applied with: *C. vulgaris* at 0.25, 0.5 and 0.75 g. L^{-1}. **(B)** In thermic treated substrate when applied with: *C. vulgaris* at 0.25, 0.5 and 0.75 g. L^{-1}. For each treatment value are means (n=20 ± STD) for each day, the values followed by the same letter do not present significant differences for $p<0.05$, using Duncan's multiple range test. Each line on top of the bars represents the standard deviation.

Figure 3.21B confirms the results on non-treated substrates. The Cv concentration of 0.25 g. L^{-1} promoted the lower AUDPC (0.008 ± 0.003) significantly lower to the value observe with the water treatment (a 50 % reduction).

- Lettuce trials

As in spinach, lettuce also showed it's the symptoms of this disease (Figure 3.22).

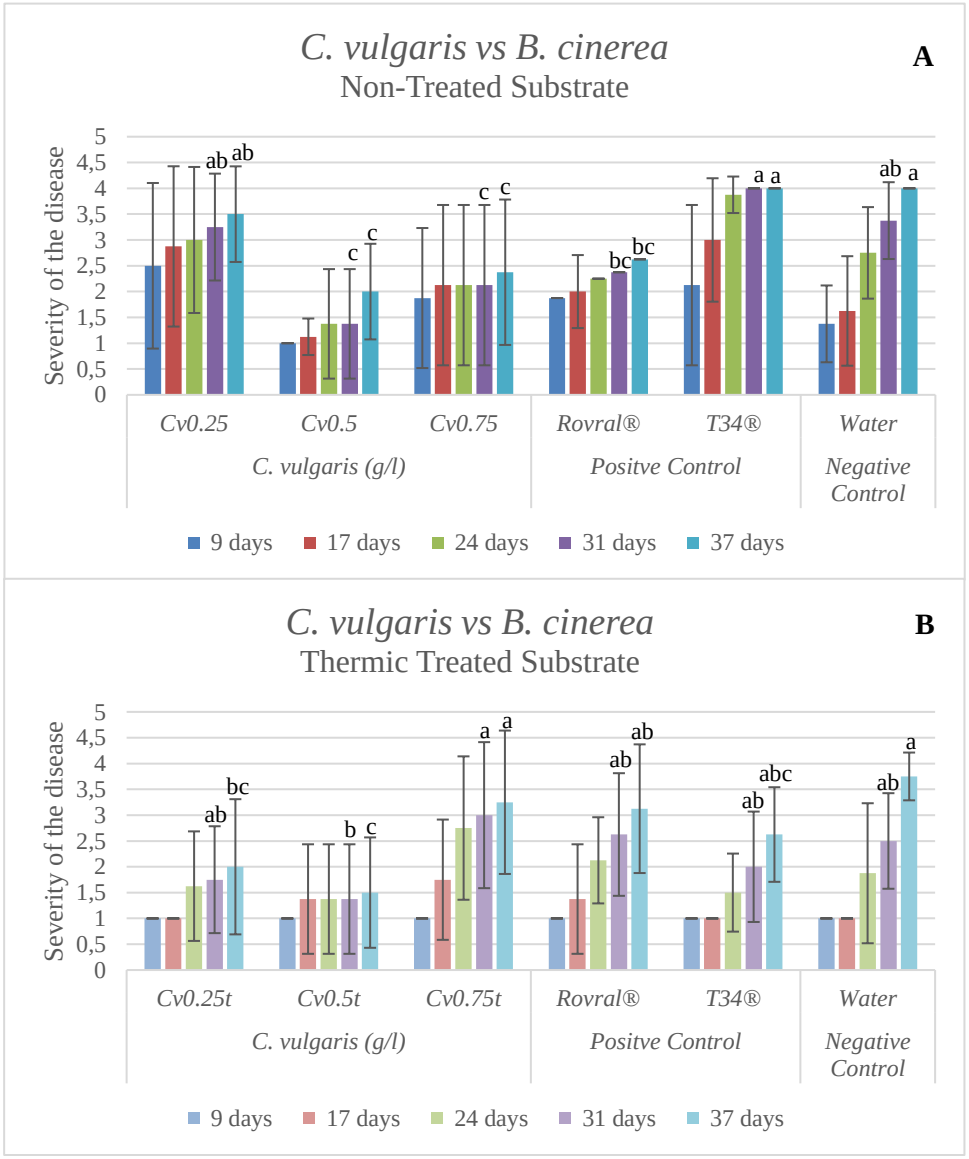


Figure 3 22: *In vivo* results of severity caused disease by *B. cinerea* in lettuce plants. **(A)** In non-treated substrate when applied with: *C. vulgaris* at 0.25, 0.5 and 0.75 g. L^{-1}. **(B)** In thermic treated substrate when applied with: *C. vulgaris* at 0.25, 0.5 and 0.75 g. L^{-1}. For each treatment value are means (n=20 ± STD) for each day, the values followed by the same letter do not present significant differences for $p<0.05$, using Duncan's multiple range test. Each line on top of the bars represents the standard deviation.

In both thermic-treated and –non heated substrates trials, significant differences between the different concentrations and the controls where observed. When applying Cv

concentrations of 0.5 and Cv 0.75 g. L^{-1}, the severity of the disease is significantly reduced. These results were also obtained in spinach, suggesting that this concentration of *C. vulgaris* influences *B. cinerea* expression.

A significant reduction in the effects of *B. cinerea* was observed (Figure 3.22). In the thermic-treated substrate, significantly less severe effects were observed in Cv 0.5 g. L^{-1} at 37 DAI (Figure 3.22B). Disease severity under this Cv concentration was significantly lower than in other treatments, apart from Cv 0.25 g. L^{-1}.

Table 3.17: Incidence of *B. cinerea*, in the different treatments, throughout the trial in non-treated peat in lettuce plants.

	Incidence of the disease on the non-treated substrates (%)					
	Cv (g. L^{-1})			Positive Control		Negative Control
DAI	0.25	0.5	0.75	Rovral®	T34®	Water
9	50ab	0b	38ab	63a	38ab	25ab
17	63ab	13b	38ab	63ab	88a	38ab
24	75ab	13c	38bc	75ab	100a	100a
31	100a	13b	38b	88ab	100a	100a
37	100a	75ab	63b	88ab	100a	100a

DAI, days after inoculation; Cv, *Chlorella vulgaris*; T34, biocontrol agent; Rovral commercial fungicide. In each row, values followed by the same letter do not show significant differences for $p<0.05$.

Table 3.18: Incidence of *B. cinerea*, in the different treatments, throughout the trial in thermic treated peat in lettuce plants.

	Incidence of the disease on heat-treated substrates (%)					
	Cv (g. L^{-1})			Positive Control		Negative Control
DAI	0.25t	0.5t	0.75t	Rovral®	T34®	Water
9	0a	0a	0a	0a	0a	0a
17	0b	13ab	38a	13ab	0b	0b
24	38ab	13b	75a	88a	38ab	38ab
31	50bc	13c	75ab	88ab	63ab	100a
37	50bc	25c	75ab	88ab	100a	100a

DAI, days after inoculation; Cv, *Chlorella vulgaris*; T34, biocontrol agent; Rovral commercial fungicide. In each row, values followed by the same letter do not show significant differences for $p<0.05$.

At the end of the trial (37 DAI), in both substrates, *C. vulgaris* led to a reduction in disease severity and development. In Cv 0.5 and Cv 0.75 g. L^{-1}, an incidence of 75 % and 63 %,

respectively were obtained (Table 3.17). A similar and significant reduction occurred in Cv 0.25 and Cv 0.5 g. L^{-1}, in disease incidence (Table 3.18).

Supporting these results, the variable AUDPC (Figure 3.23) shows that disease progression was significantly lower in Cv 0.5 g. L^{-1} treatment in both substrates, which means that this concentration has an antifungal effect against *B. cinerea*, even if there are no other organisms interacting, *C. vulgaris* concentrations did not show significant differences compared to Rovral® (Figure 3.23A). The lowest AUDPC was observed at Cv 0.5 g. L^{-1}, approximately 0.0095 ± 0.04. In the thermic-treated (Figure 3.23B), at the 0.5 g. L^{-1} concentration, the AUDPC reached 0.0064 ± 0.003 AUDPCs, which corresponds to a reduction of 50 % and 66.4 % on the AUDPC obtained in the treatment Cv 0.5 g. L^{-1} applied to the non-treated ant thermic-treated substrates, respectively.

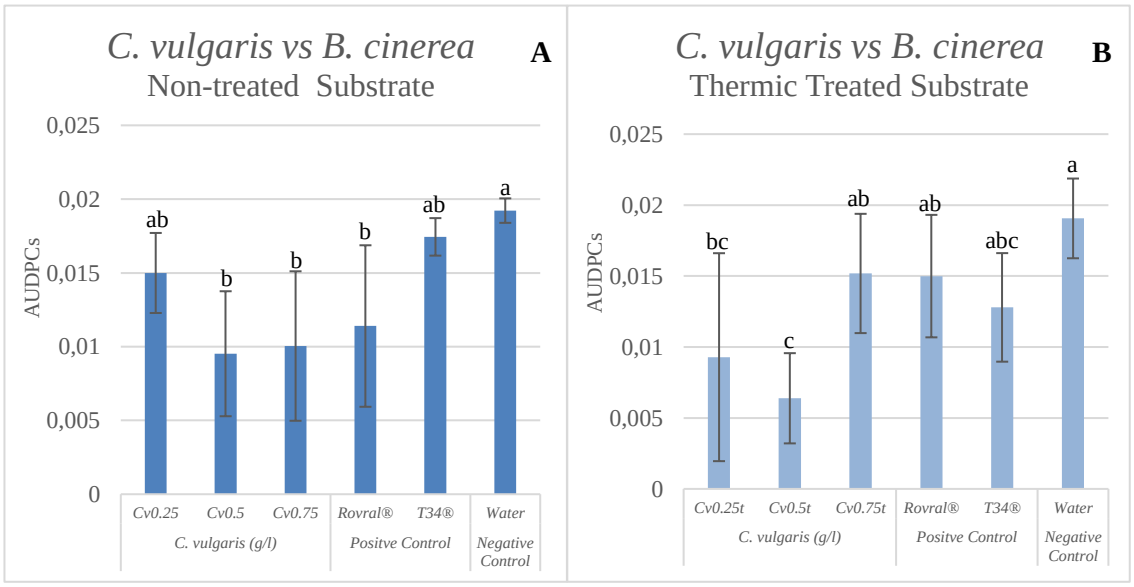


Figure 3.23: *In vivo* AUDPC results of the disease caused by *B. cinerea* in lettuce plants. **(A)** In non-treated substrate when applied with: *C. vulgaris* at 0.25, 0.5 and 0.75 g. L^{-1}. **(B)** In thermic treated substrate when applied with: *C. vulgaris* at 0.25, 0.5 and 0.75 g. L^{-1}. For each treatment value are means (n=20 ± STD) for each day, the values followed by the same letter do not present significant differences for $p<0.05$, using Duncan's multiple range test. Each line on top of the bars represents the standard deviation.

Microbiological characteristics

At the end of the *in vivo* assays of the biopesticide potential of *C. vulgaris* and *S. obliquus* against *Botrytis cinerea*, in the thermic-treated substrate, the larger number of microorganisms in the substrates was found in treatments with microalgae suspensions

(Table 3.19). The larger populations of bacteria and actinomycetes were counted in treatment Cv 0.75 g. L^{-1}, differing significantly from the treatments where *S. obliquus* was used and the controls. However, in Cv 0.75 g. L^{-1} population sizes did not differ significantly from the other concentrations of the same microalgae. In the thermic-treated substrate, it was in So 0.25 g. L^{-1} where a larger fungal population was counted, being significantly different from the positive biocontrol T34® and the negative control (Table 3.19).

In the non-treated-substrate, the treatment with the commercial fungicide Rovral® presented the largest number of colony-building units of actinomycetes and fungi, differing significantly differently from the other treatments, apart from Cv 0.25 g. L^{-1} treatment, where a high number of fungi were also observed. In the substrates that were treated with T34®, there was a decrease in microbial communities, indicating its antagonistic properties, suppressing the growth of other microorganisms, and may even lead to the disappearance of some communities (Table 3.19).

Table 3.19: Populations of microorganisms in the substrates of the different substrates.

	CFU.g^{-1} substrate					
	Thermic Treated Substrate			Non-Treated Substrate		
	Fungi	Actinomycetes	Bacteria	Fungi	Actinomycetes	Bacteria
Cv0.25	$4.26x10^7$ab	$7.50x10^7$ab	$4.26x10^7$b	$1.04x10^9$a	$1.39x10^9$b	$1.42x10^9$a
Cv0.5	$8.99x10^7$ab	$4.40\ x10^7$ab	$5.20x10^7$ab	$9.23x10^7$d	$1.45x10^7$b	$5.28x10^7$a
Cv0.75	$8.97x10^7$ab	$6.33x10^7$a	$9.49x10^7$b	$8.96x10^8$b	$1.24x10^9$b	$8.96x10^8$a
So0.01	$1.62x10^7$b	$4.25x10^6$b	$8.39x10^6$b	$3.50x10^7$d	$9.33x10^6$b	$8.87x10^7$a
So0.1	$6.30x10^6$b	$7.33x10^5$b	$3.21x10^6$b	$1.01x10^8$d	$1.86x10^8$b	$3.90x10^7$a
So0.25	$1.29x10^8$a	$2.23x10^6$b	$3.91x10^6$b	$3.31x10^7$d	$1.90x10^8$b	$1.31x10^9$a
Water	$1.35x10^6$b	$8.54x10^6$b	$2.71x10^6$b	$4.94x10^8$b	$3.74x10^8$b	$1.57x10^8$a
Rovral®	$5.15x10^7$ab	$6.20x10^6$b	$2.83x10^6$b	$2.13x10^9$a	$3.64x10^9$a	$3.75x10^8$a
T34®	$1.67x10^7$b	$1.55x10^7$b	$2.14x10^7$b	$3.10x10^8$cd	$2.28x10^8$b	$1.24x10^8$a
Peat	0b	0b	0b	$2.81x10^7$d	$2.60x10^7$b	$5.92x10^7$a

Cv0.25, *Chlorella vulgaris* at a concentration of 0.25 g. L^{-1}; Cv0.5, *Chlorella vulgaris* at a concentration of 0.5 g. L^{-1}; Cv0.75, *Chlorella vulgaris* at a concentration of 0.75 g. L^{-1};

So0.01, *Scenedesmus obliquus* at a concentration of 0.01 g. L^{-1}; So0.1, *Scenedesmus obliquus* at a concentration of 0.1 g. L^{-1}; So0.25, *Scenedesmus obliquus* at a concentration of 0.25 g. L^{-1}; T34, commercial fungicide. In each column, values followed by the same letter do not show significant differences for $p<0.05$.

Compared with peat, there was a clear increase in the number of microorganisms in the other treatments, although with no significant differences.

According to these results obtained on plants *in vivo*, the largest number of microorganisms were developed in the treatments using *Chlorella vulgaris* where the highest suppressive capacity was also observed, which indicates the possible microbiological nature of this suppressive capacity.

Antifungal activity of microalgae

The results obtained in the *in vivo* assays were according to the results obtained *in vitro*. In these trials, an antifungal effect of microalgae was found against *Fusarium oxysporum* and *Sclerotium rolfsii*. The only microalgae with an effect against *Botrytis cinerea* was *C. vulgaris*.

Although both microalgae species presented some suppressive capacity for the growth of *F. oxysporum* and *S. rolfsii*, *S. obliquus* exhibited a greater inhibiting effect on phytopathogenic fungi, based on the results of disease severity, disease incidence and AUDPCs, obtained in both non-treated and thermic-treated white peat thermic. This suggests that even if *C. vulgaris* has the highest concentration of phenolic compounds than *S. obliquus*, this composition is not in itself responsible for the inhibition that was verified.

Even if the plants were infected, presenting some degree of severity and disease incidence, the results show that the effects of the disease were significantly less severe when applying microalgae suspensions to the plants, showing that the algae under study have a biopesticide potential.

The most significant results of antifungal effects were obtained against *S. rolfsii*. An average severity of 1.37 and 1.25 was obtained with the application of aqueous solutions from *Scenedesmus obliquus* at 42 DAS, in the non-treated and thermic treated substrate;

followed by *Chlorella vulgaris* with an average severity of 1.4 and 1.31. For disease incidence, when *S. obliquus* was applied the mean value was 12.5 % and 9.5 %, while *in C. vulgaris* it was 13.3% and 10.4 %. However, these interactions show a possible dependence on the combination of microalgae and pathogen, suggesting a specificity degree, as in the case *of C. vulgaris* and *B. cinerea*.

C. vulgaris exhibited a potential biopesticide against all target fungi, including *B. cinerea* as opposed to *S. obliquus*, demonstrating that microalgae are not efficient against all fungi.

No studies were found reporting the effect of aqueous suspensions *of S. obliquus* on any of the phytopathogens studied in the *in vivo trials*.

The application of microalgae suspensions led to an increase in microbial populations, what can be explained by the supply of amino acids and proteins presented in the biomass of microalgae. This biomass of microalgae is composed by several micro- and macronutrients, which when applied to the substrates will increase the nutrition capacity of the substrates not only for the plant but also for the microorganisms present in it (Lee & Ryu, 2021).

Physic-chemical properties of substrates

At the end of the assay, the substrates from the different treatments presented variable pH values (Table 3.20), ranging from 4.61 to 7.43. Most of the thermic-treated substrates, presented an acidic pH, except for water treatment (7.43 ± 0.01). In the non-treated substrate, only the positive controls showed neutral pH values.

Regarding electrical conductivity (EC), it is possible that the substrates that received the sprays of microalgae reached higher EC than the water (negative control) in the heat-treated substrate, indicating that these substrates could be more nutrient-rich at the end of the assay, presenting mineral salts in greater quantity. However, these differences were not so evident in the nontreated substrate, probably because initially on this substrate there were already microorganisms, in which electrical conductivity differed only significantly between the treatment So 0.25 g. L^{-1} (0.511 ± 0.01 $dS.m^{-1}$) and the treatments with

Chlorella vulgaris (0.25, 0.5 and 0.75 g. L^{-1}) and the commercial fungicide Rovral® (0.332 ± 0.018 $dS.m^{-1}$).

Table 3.20: Physicochemical characteristics of the substrates obtained at the end of the assay

	Thermic Treated Substrate		Non-Treated Substrate	
Treatment	pH	EC ($dS.m^{-1}$)	pH	EC ($dS.m^{-1}$)
Cv0.25	4.95ef	0.480ab	5.61c	0.303b
Cv0.5	4.69f	0.425abc	5.10de	0.350b
Cv0.75	4.61f	0.449ab	4.69e	0.294b
So0.01	5.98d	0.534[a]	6.33b	0.401ab
So0.1	6.01d	0.439ab	5.20cd	0.386ab
So0.25	5.29e	0.328bc	4.96de	0.511a
Water	7.43a	0.277c	6.77a	0.403ab
Rovral®	6.91b	0.342bc	7.03a	0.332b
T34®	6.49c	0.330bc	7.08a	0.384ab

Cv0.25, *Chlorella vulgaris* at a concentration of 0.25 g. L^{-1}; Cv0.5, *Chlorella vulgaris* at a concentration of 0.5 g. L^{-1}; Cv0.75, *Chlorella vulgaris* at a concentration of 0.75 g. L^{-1}; So0.01, *Scenedesmus obliquus* at a concentration of 0.01 g. L^{-1}; So0.1, *Scenedesmus obliquus* at a concentration of 0.1 g. L^{-1}; So0.25, *Scenedesmus obliquus* at a concentration of 0.25 g. L^{-1}; T34, biocontrol agent; Rovral, commercial synthetic fungicide; EC, electrical conductivity. In each column, values followed by the same letter do not show significant differences for $p<0.05$.

3.3. Biostimulant Potential

3.3.1. *In vitro* trials

pH and EC of the microalgae suspensions

The pH measurements showed that the lowest concentrations presented a more acidic pH than the concentrations with more biomass of the microalgae. The more biomass present in the suspension, the more basic the pH.

In *Chlorella vulgaris* suspensions, from the concentration of 0.75 g. L^{-1}, the pH values are presented at neutral pH, the same is observed for suspensions of *Scenedesmus obliquus*, which reach neutral pH at So 0.25 g. L^{-1} concentration (Figure 3.24 and 3.25). According to Supraja et al. (2020), biostimulants have a better effect when at neutral pH.

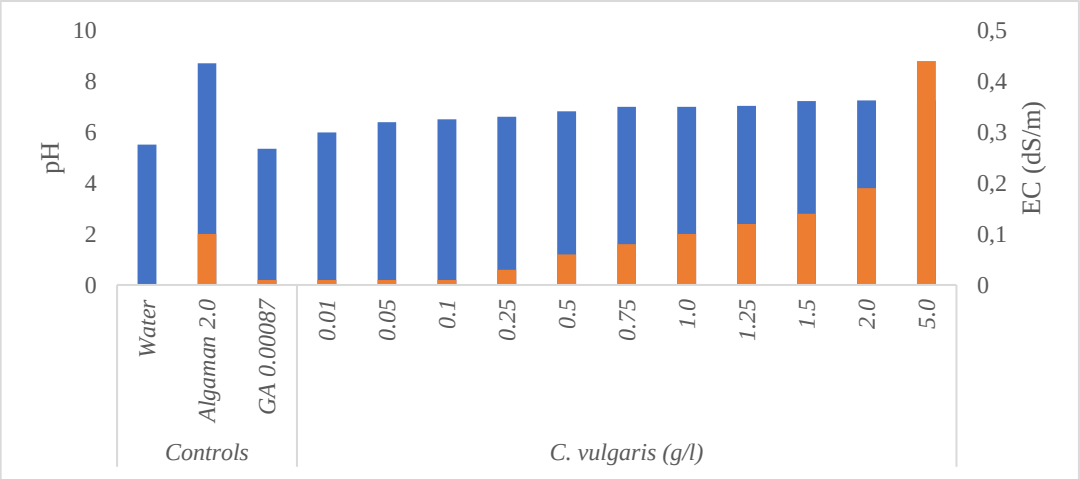


Figure 3.24: pH and electrical conductivity of *Chlorella vulgaris* suspensions and controls; GA0.00087, Gibberellic acid (0.00087 g. L^{-1}); Algaman B (2.0 g. L^{-1}); and Sterilized deionized Water. EC, electrical conductivity. The bars presented in blue correspond to the pH and the orange bars show the EC values.

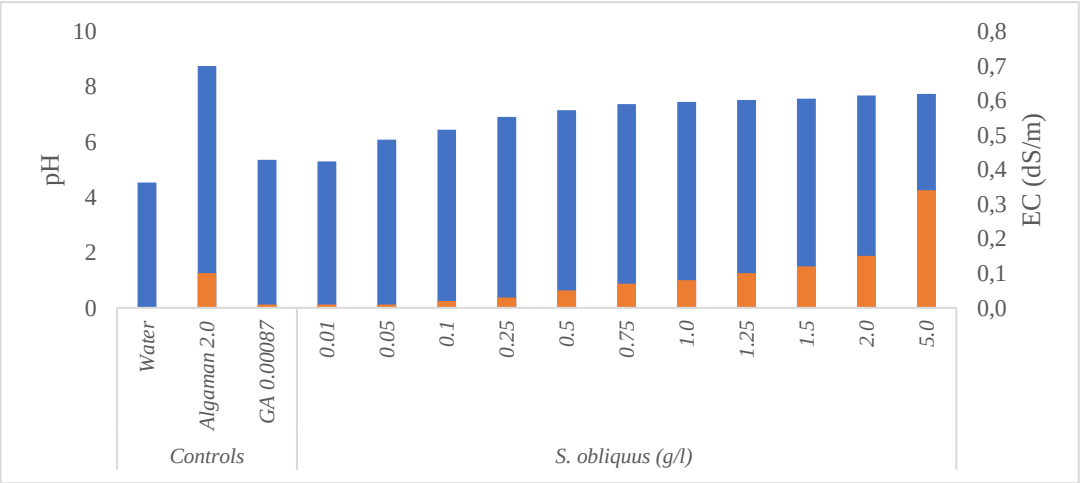


Figure 3 25: pH and electrical conductivity of *Scenedesmus obliquus* suspensions and controls; GA0.00087, Gibberellic acid (0.00087 g. L^{-1}); Algaman B (2.0 g. L^{-1}); and Sterilized deionized Water. EC, electrical conductivity. The bars presented in blue correspond to the pH and the orange bars show the EC values.

Germination index (GI)

For this method, the germination index of the negative control (sterilized deionized water) was considered 100 %, i.e., treatments with indexes higher than 100 % are the only ones considered to have a biostimulant activity on the seed development, as shown in Figure 3.26.

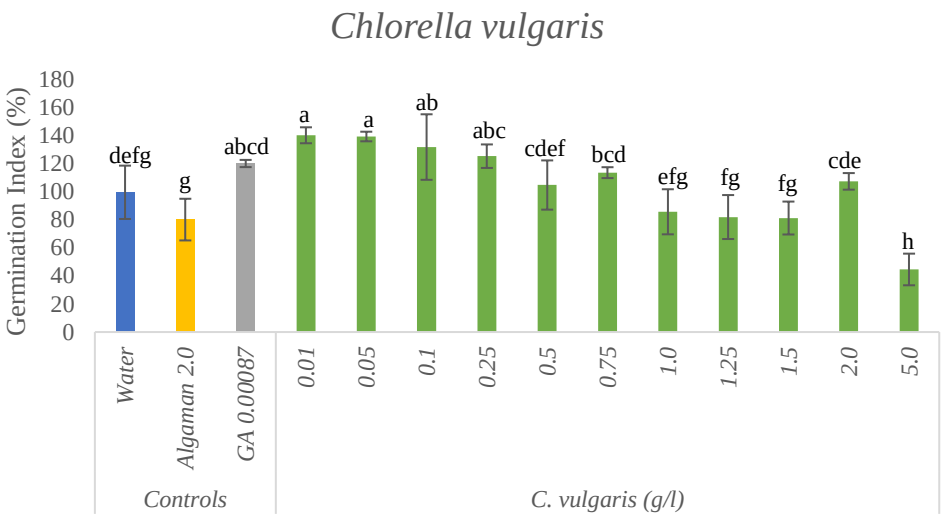


Figure 3.26: Germination index (%) of *Lepidium sativum* L. seeds, when in contact with *Chlorella vulgaris* suspensions. At concentrations 0.01, 0.05, 0.1, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 2.0 and 5.0 g. L^{-1}; Algaman B (2.0 g L^{-1}); Gibberellic acid, GA, (0.00087 g L^{-1}), and Sterilized deionized Water. For each treatment value, means (n=50 ± STD) with different letters were significantly different at $p<0.05$, using Duncan's multiple range test. Each line on top of the bars represents the standard deviation.

According to the results obtained in the germination test, it is observed that the microalga *C. vulgaris*, depending on the concentration, had a positive effect on the seeds of *Lepidium sativum* L. While for the microalgae *S. obliquus* this effect was not so notorious for most concentrations.

Figure 3.26 shows that *C. vulgaris* had a biostimulant effect from 0.01 g. L^{-1} to 0.75 g. L^{-1} concentration, where GI was greater than 100 %. Concentrations of 0.01 g. L^{-1} (140.1 ± 6.7 %) and 0.25 g. L^{-1} (125.25± 8.36%) differed significantly from negative control (Sterilized deionized Water). Noteworthy is that even at low concentrations of 0.01 and 0.05 g L^{-1} led to a significant increase in the GI of about 40 % when compared to the negative control. Concerning the positive controls gibberellic acid obtained a GI of about 40% whereas Algaman B obtained a GI of 80.1 ± 14.84 %. It should be noted that from the concentration of 1.0 g. L^{-1}, the germination index became less than 90 %, apart from the concentration of 2.0 g. L^{-1} which showed an increase of 7 ± 5.9 % of germination. According to, Luo et al. (2018) this value may be related to the phytotoxicity of the sample that is being analyzed, considering it as a germination inhibitor.

The increase in biomass of *S. obliquus* leads to a reduction in the germination index of watercress seeds (Figure 3.27).

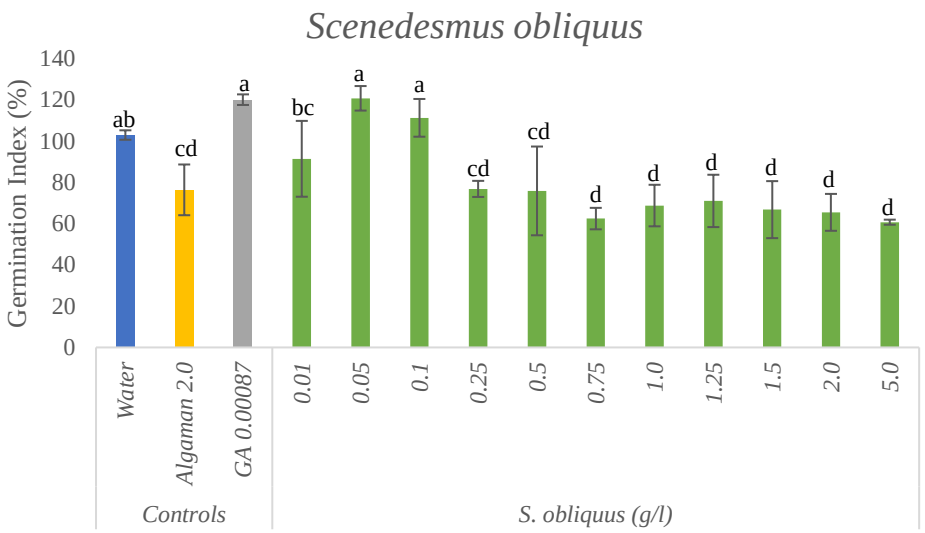


Figure 3.27: Germination index (%) *of Lepidium sativum* L. seeds, when in contact with *Scenedesmus obliquus* suspensions. At concentrations 0.01, 0.05, 0.1, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 2.0 and 5.0 g. L^{-1}; Algaman B (2.0 g L^{-1}); Gibberellic acid, GA, (0.00087 g L^{-1}), and Sterilized deionized Water. For each treatment value, means (n=50 ± STD) with different letters were significantly different at $p<0.05$, using Duncan's multiple range test. Each line on top of the bars represents the standard deviation.

In the plates where the lowest concentrations were applied, except for the concentration of 0.01 g. L^{-1} presented a GI of 91 ± 18.3 %, in 0.05 g. L^{-1} and 0.1 g. L^{-1}, there was an increase of 17 % (120.7 ± 5.94 %) and 8 % (111.24 ± 9.14 %), respectively, in comparison with water. Although there was an increase in relation to the negative control, these differences did not present statistical significance. In the case of *S. obliquus*, inhibition of germination started from the concentration of 0.25 g. L^{-1}, which only reached about 77 ± 3.89 %, a reduction of 26 %.

Microalgae and cyanobacteria are microorganisms capable of synthesizing bioactive compounds with a diversified biological value, such as phytohormones, fatty acids, and polysaccharides (Ferreira et al., 2021; S. M. Lee & Ryu, 2021; Renuka et al., 2018). According to Navarro-López et al. (2020), phytohormones are bioactive compounds that when in small concentrations will influence the response of the plant, however, when at high concentrations they can inhibit this response. Also in this study, the author encountered the same inhibition at high concentrations of *S. obliquus*. Considering that microalgae are described as having these components in their constitution, this inhibition observed when increasing biomass concentrations may be the result of the increase in phytohormone concentration. However, it is not yet really known how these mechanisms

work, so more studies are needed to be completely informed. It can only be assumed that *C. vulgaris* in its composition contains more compounds that favor plant germination.

Since gibberellic acid is a phytohormone responsible for regulating development processes, such as germination and elongation of the stem, it was used as a positive control, to determine whether the effect of microalgae would be similar. As can be seen in the previous figures, in both microalgae, the effect of germination did not differ significantly between the gibberellic acid and the same.

Root evaluation (RRG)

The result obtained after measuring the lengths of the root of the germinated seedlings for each of the microalgae (*C. vulgaris* and *S. obliquus*) are represented in figures 3.28 and 3.29. For both microalgae, the best results were obtained at lower concentrations, showing that with the increase of concentrations there is an inhibition in the growth of the root of the watercress. This inhibition was later confirmed by statistical analysis of the results, which evidenced the differences between the lowest and highest concentrations, which were significantly different from each other.

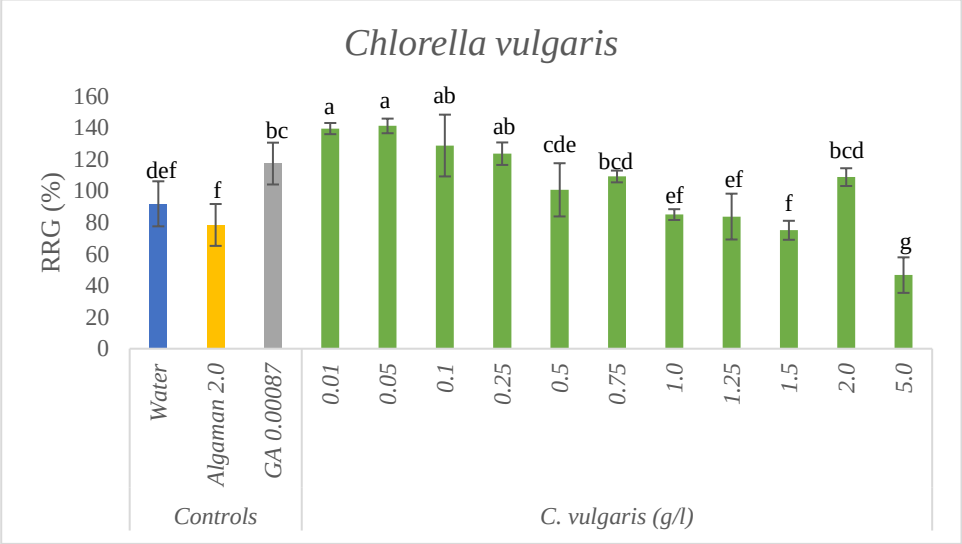


Figure 3.28: Relative length of radicles (RRG) of *Lepidium sativum* L. plants after exposure to *C. vulgaris*. At concentrations 0.01, 0.05, 0.1, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 2.0 and 5.0 g. L^{-1}; Algaman B (2.0 g L^{-1}); Gibberellic acid, GA, (0.00087 g L^{-1}), and Sterilized deionized Water. For each treatment value, means (n=50 ± STD) with different letters were significantly different at $p<0.05$, using Duncan's multiple range test. Each line on top of the bars represents the standard deviation.

When looking at figure 3.28, it is observed that the concentrations of 0.01 g. L^{-1}, 0.05 g. L^{-1}, 0.1 g. L^{-1}, and 0.25 g. L^{-1}, were the concentrations with a higher development of the radicle, with a significant increase of 49 % (140.96 ± 4.62 %), when compared to the length of 0.05 g. L^{-1} in relation to water (91.67 ± 14.25 %). The significantly lowest growth occurred at 5.0 g. L^{-1}, in which 46.57 ± 11.24 % was the maximum reached. In agreement with Duncan's test, it was verified that the radicles of concentrations of 0.1 and 0.25 g. L^{-1} obtained on average a growth of 32 %, not statistically diverging from the Gibberellic Acid (GA- positive control), which had an increase of 26 % (117.15 ± 13.24 %) when compared to water.

The lowest concentrations (0.05 g. L^{-1} and 0.1 g. L^{-1}) of *S. obliquus* solutions were the ones that obtained the greatest development, which was statistically different from the values obtained at higher concentrations (0.75 g. L^{-1} to 5.0 g. L^{-1}) (Figure 3.29). As in *C. vulgaris*, Gibberellic acid was comparable with the lowest concentrations, obtaining an average of 17 %, while in the seaweed there was an 18 % (118.29 ± 5.81 %) and 9 % (109.01 ± 8.95 %) increase in the growth of radicles in 0.05 g. L^{-1} and 0.1 g. L^{-1}.

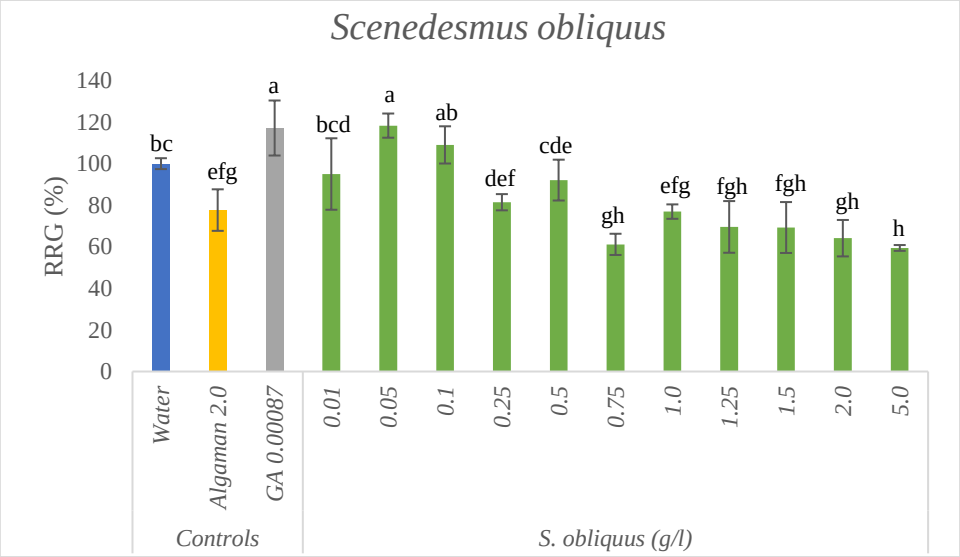


Figure 3.29: Relative length of radicles (RRG) of *Lepidium sativum* L. plants after exposure to *S. obliquus*. At concentrations 0.01, 0.05, 0.1, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 2.0 and 5.0 g. L^{-1}; Algaman B (2.0 g L^{-1}); Gibberellic acid, GA, (0.00087 g L^{-1}), and Sterilized deionized Water. For each treatment value, means (n=50 ± STD) with different letters were significantly different at $p<0.05$, using Duncan's multiple range test. Each line on top of the bars represents the standard deviation.

The assay in which *C. vulgaris* was applied originated longer radius than in the *S. obliquus* assay, with a difference of 23 % comparing the treatments where the longest lengths were reached (0.05 g. L^{-1}). In the present study, the concentrations where the best values were shown were 0.01 and 0.05 g. L^{-1}. Comparable results were described by Ferreira et al. (2021), where when in contact with *C. vulgaris*, the radicles of wheat and cucumber seeds increased their size by 100 % and 33.5 %. As analyzed in germination, there was also an inhibiting effect on root growth. This inhibition was mentioned in the study by Navarro-López et al., (2020), when the concentrations of *S. obliquus* increased there is a reduction in the root size of the plant under test, again indicating that the highest concentrations of microalgae contain an excessive amount of phytohormones or other bioactive compounds that exceed their optimal concentration, becoming toxic to crops, causing the opposite effect to the intended, that is, causing the reduction of their development rather than stimulating it (Luo et al., 2018).

3.3.2. *In vivo* trials

To evaluate the biostimulant potential of *Chlorella vulgaris* and *Scenedesmus obliquus*, a trial using lettuce plants was performed.

3.3.2.1 Biostimulant assay:

In the assay to evaluate the biostimulant effect of the microalgae applied on lettuce, it was tested in a wide range of concentrations: 0.1, 0.5, 2.0, and 5.0 g. L^{-1} (Figure 3.30). The results of the evaluated parameters are represented in Figure 3.31 - 3.36.

Figure 3.30: Biostimulant potential assays using four microalgae concentrations: 0.1, 0.5, 2.0, 5.0 g. L^{-1}; Algaman B (2.0 g. L^{-1}); SEAnergy (2.0mL. m^2) and water. **(A)** *S. obliquus* biostimulant trial. **(B)** *C. vulgaris* biostimulant trial.

The results at the end of the assay identified that *Chlorella vulgaris* microalgae had a greater biostimulant effect on plants compared to controls and with the other microalga under study (*S. obliquus*). Cv 5.0 g. L^{-1} was the treatment that presented the highest values in all parameters, apart from root length.

When the leaves were counted for each modality (Figure 3.31), as referenced above, Cv 5.0 g. L^{-1} was the concentration with the significantly higher average of leaves per plant (27 leaves), followed by another concentration of *C. vulgaris* with 22 leaves (Cv 2.0 g. L^{-1}). These concentrations of *C. vulgaris* only did not differ from the concentration So 5.0 g. L^{-1} which presented an average value of approximately 19 leaves. However, the remaining treatments were around the 15 leaves on average, not differing from each other. Evaluating in relation to the negative control (water), Cv 5.0 g. L^{-1} presented more 58.9 % leaves (Figure 3.31).

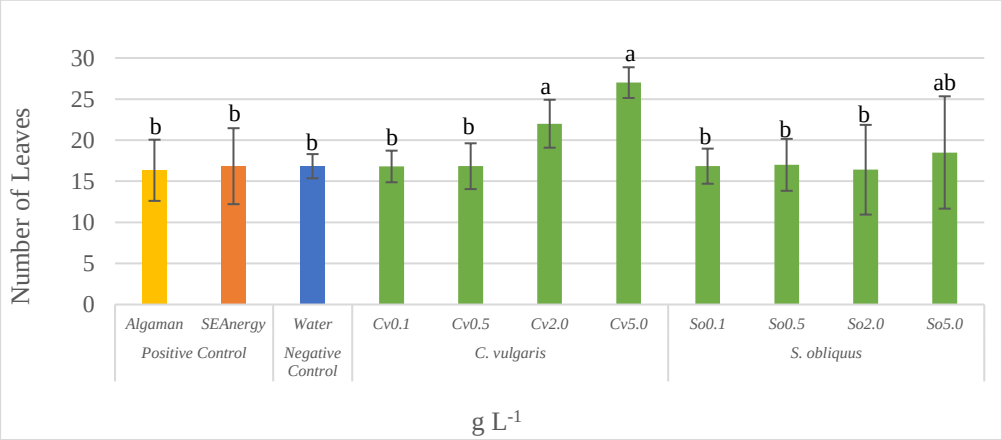


Figure 3.31: Number of leaves obtained in the biostimulant potential assays *in vivo*, when applying concentrations of 0.1, 0.5, 2.0 and 5.0 g. L^{-1} of microalgae *S. obliquus* and *C. vulgaris* in lettuce plants. For each treatment value are means (n=20 ± STD), the values followed by the same letter do not present significant differences for $p<0.05$, using Duncan's multiple range test. Each line on top of the bars represents the standard deviation.

This increase was confirmed by the average plant length (cm) reached by the plants (Figure 3.32A). *Chlorella vulgaris* treatments of 2.0 and 5.0 g. L^{-1} acted positively in the increase of the aerial part of the plant, with a size of 23.3 ± 2.33 cm and 23.5 ± 3.26 cm, respectively. However, in these trials, there was also the inhibiting effect that the highest concentration of biomass caused in the roots (Figure 3.32B). Navarro-López et al. (2020) also obtained an inhibiting effect of the radicle in germination tests when treated with

suspensions of *S. obliquus*, however, the same effect was observed when the *in vivo* trials were performed. Bumandalai & Tserennadmid (2019) observed that the 0.25 g. L^{-1} concentration of *C. vulgaris* led to a reduction in radicle growth in tomato seeds, but the same was not observed in cucumber seeds, leading to the belief that the optimum amount of microalgae biomass can be specified for each species.

With this, looking at figure 3.32B, it was verified that the concentrations of 2.0 and 5.0 g. L^{-1} of both microalgae (*S. obliquus* and *C. vulgaris*) obtained a root length of 15.2 ± 0.72 cm (Cv 2.0 g. L^{-1}), 14.1 ± 2.76 cm (Cv 5.0 g. L^{-1}), and 15.8 ± 2.8 cm (So 2.0 g. L^{-1}), 14.7 ± 5.0 cm (So 5.0 g. L^{-1}), presenting the lowest lengths of the treatments ($p<0.05$). The longest root length was observed in Cv 0.1 g. L^{-1} with 20.6 ± 2.34 cm, but this did not present statistical differences compared to the controls, presenting a value comparable to Algaman B with 20.4 ± 3.1 cm.

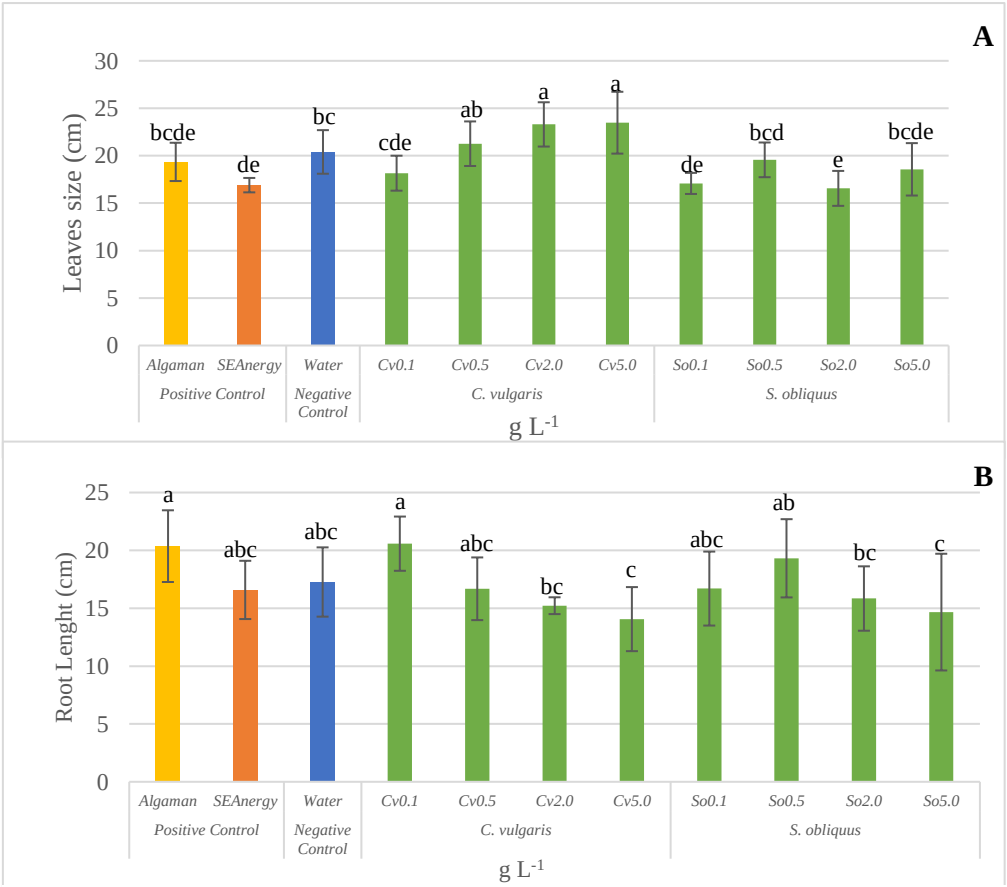


Figure 3.32: Size (cm) obtained in the trials of the biostimulant potential *in vivo*, when applying concentrations of 0.1, 0.5, 2.0 and 5.0 g. L^{-1} of microalgae *S. obliquus* and *C. vulgaris* in lettuce plants. **(A)** Average length of the aerial part (cm) of lettuce plants. **(B)** Length of the roots (cm) of lettuce plants. For each treatment value are means (n=20 ± STD), the values followed by the same letter do not present significant differences for $p<0.05$, using Duncan's multiple range test. Each line on top of the bars represents the standard deviation.

By the end of the assay, it was measured the leaf area (cm^2) that can be observed in figure 3.33. Once again, the concentration of Cv 5.0 g. L^{-1} (753 ± 121.5 cm^2) presented the largest leaf area ($p<0.05$), showing that the plants invested in development to obtain the highest sunlight uptake and energy production. The smallest leaf area was obtained in the positive control SEAnergy (332 ± 125.39 cm^2); however, it did not differ from the other treatments, except for Cv 2.0 and Cv 5.0 g. L^{-1}.

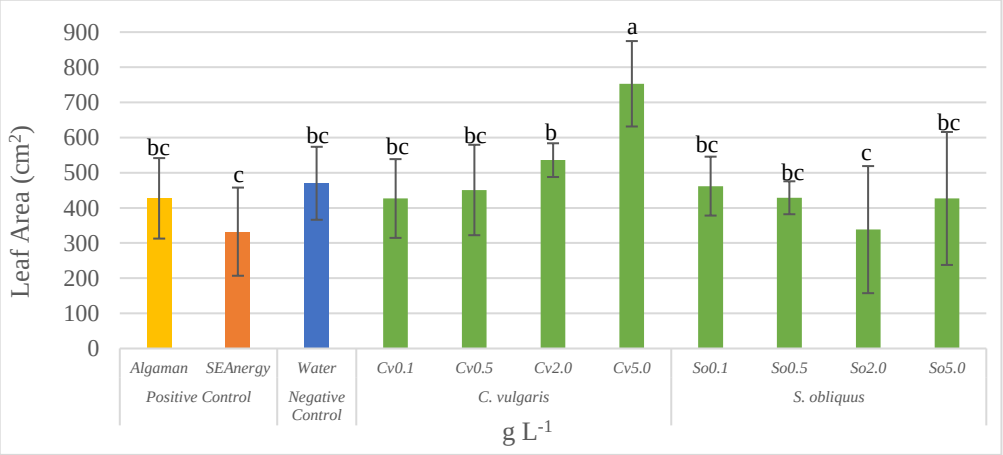


Figure 3.33: Average leaf area (cm^2) obtained in the *in vivo* biostimulant potential assays, when applying concentrations of 0.1, 0.5, 2.0 and 5.0 g. L^{-1} of microalgae *S. obliquus* and *C. vulgaris* in lettuce plants. For each treatment value are means (n=20 ± STD), the values followed by the same letter do not present significant differences for $p<0.05$, using Duncan's multiple range test. Each line on top of the bars represents the standard deviation.

SPAD readings occurred weekly throughout the trial, where fluctuations were observed between treatments. The SPAD value will estimate the chlorophyll content, thus identifying that the only statistical differences were obtained between So 2.0 and So 5.0 g. L^{-1} and the positive control Algaman B and SEAnergy (Figure 3.34). These values were already expected, since this treatment corresponds to the treatment that presented the largest leaf area, having a larger absorption area. Since the SPAD value is related to the nitrogen level in the leaves, the higher the SPAD value the higher the N content in them.

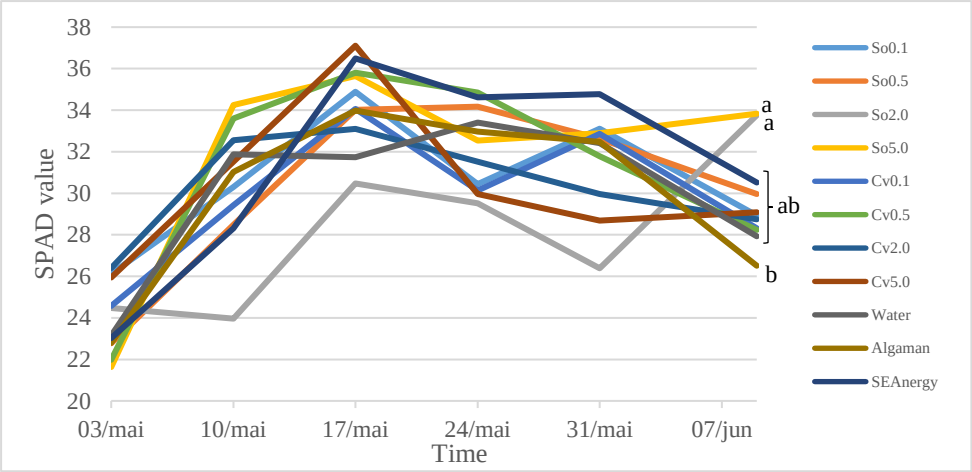
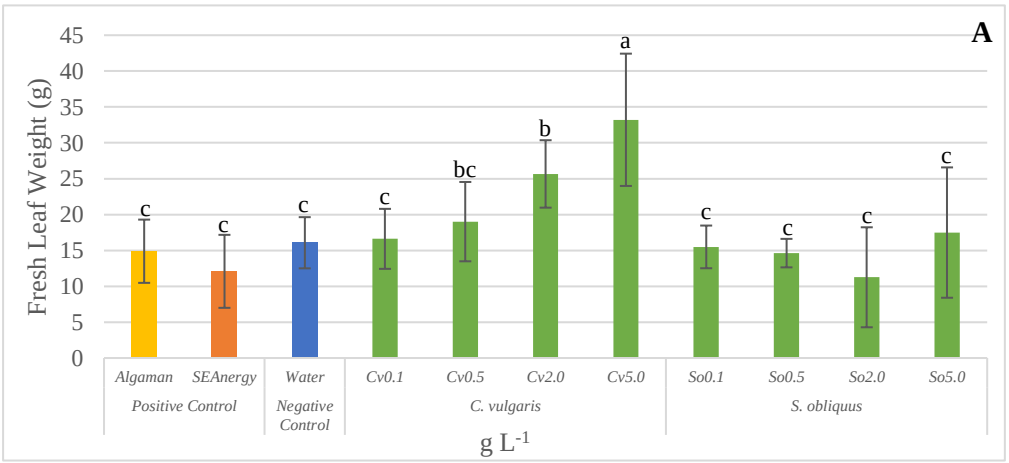


Figure 3.34: SPAD readings throughout the assay for the different treatments: concentrations 0.1, 0.5, 2.0, and 5.0 g. L^{-1} of microalgae *S. obliquus* and *C. vulgaris*; a negative control (water) and a positive control (Algaman B). For each treatment value are means (n=20 ± STD), the values followed by the same letter do not present significant differences for $p<0.05$, using Duncan's multiple range test. Each line on top of the bars represents the standard deviation.

Observing the fresh weight and dry weight of the aerial parts (figure 3.35A and 3.36A) and the root parts (Figure 3.35B and 3.36B), the Cv 5.0 g. L^{-1} concentration maintained the significantly higher weight value ($p<0.05$) in all these parameters, showing that although it did not have an expected root development, since microalgae have affected their development, it was able to capture and retain nutrients. For the shoot, the Cv 5.0 g. L^{-1} had a fresh weight of 33.21 ± 9.22 g and a dry weight of 4.9 ± 1.0 g.



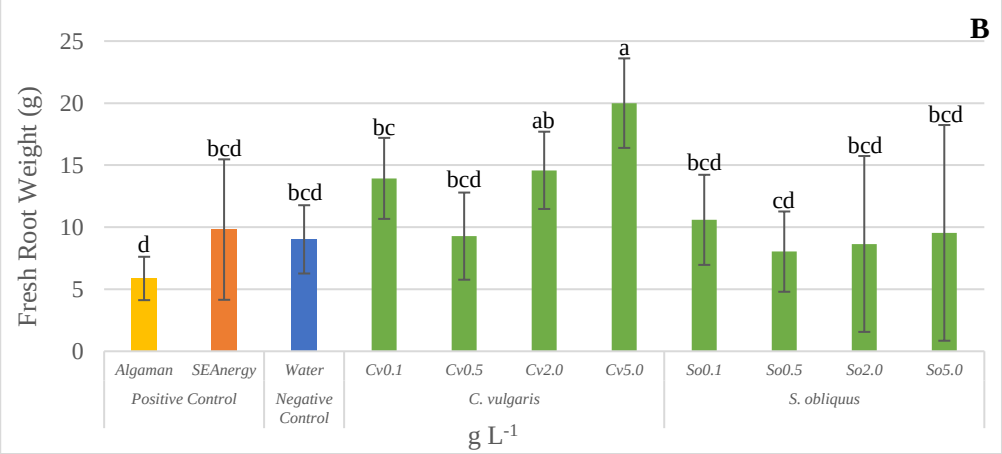


Figure 3.35: Fresh weight (g) resulting from the *in vivo* biostimulant potential assays, when applying concentrations of 0.1, 0.5, 2.0 and 5.0 g. L^{-1} of microalgae *S. obliquus* and *C. vulgaris* in lettuce plants. **(A)** Fresh weight (g) of the aerial part of lettuce plants. **(B)** Fresh weight (g) of the roots of lettuce plants. For each treatment value are means (n=20 ± STD), the values followed by the same letter do not present significant differences for $p<0.05$, using Duncan's multiple range test. Each line on top of the bars represents the standard deviation.

Also, in relation to the leaf dry weight (Figure 3.36A), the second highest weight was verified in Cv 2.0 g. L^{-1} with 4.4 ± 2.0 g. While the lowest fresh weight was observed in So 2.0 g. L^{-1} (11.3 ± 7.0g), but it did not differ from the other treatments, excluding Cv 2.0 and Cv 5.0 g. L^{-1}.

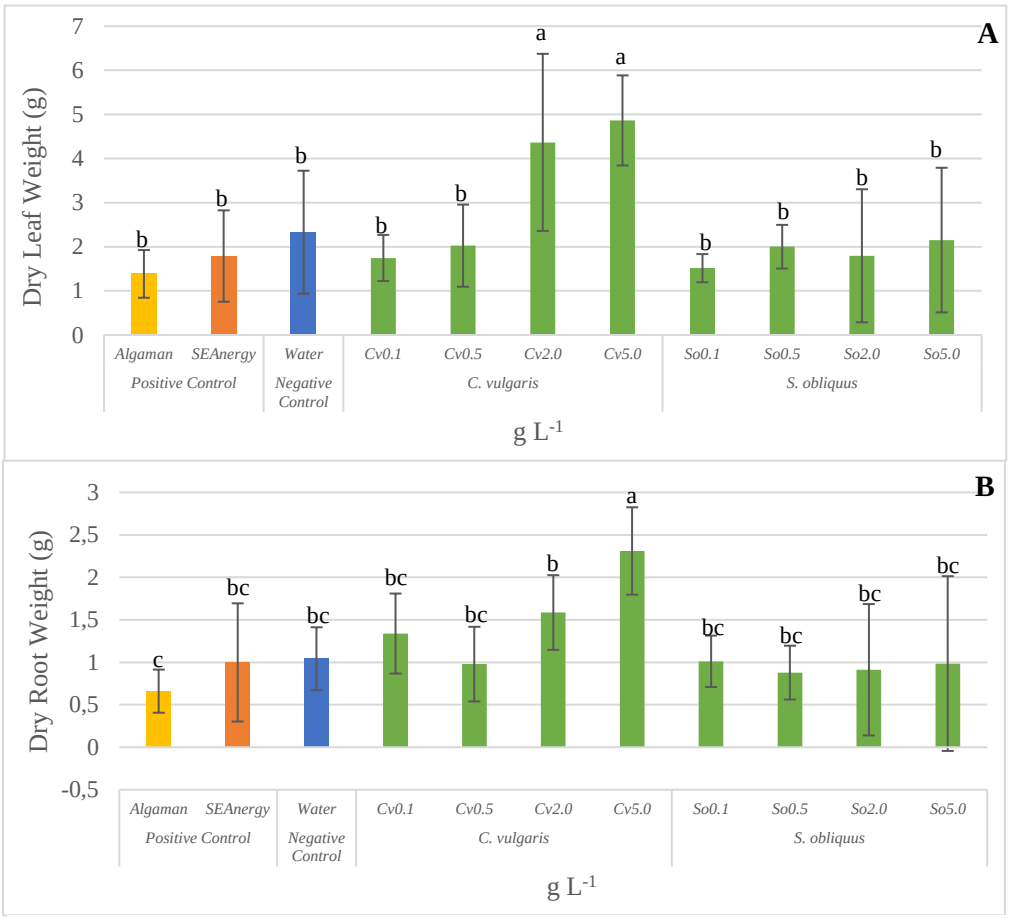


Figure 3.36: Dry weight (g) resulting from the *in vivo* biostimulant potential assays, when applying concentrations of 0.1, 0.5, 2.0 and 5.0 g. L^{-1} of microalgae *S. obliquus* and *C. vulgaris* in lettuce plants. **(A)** Dry Leaf weight (g) of lettuce plants. **(B)** Dry Root weight (g) of lettuce plants. For each treatment value are means (n=20 ± STD), the values followed by the same letter do not present significant differences for $p<0.05$, using Duncan's multiple range test. Each line on top of the bars represents the standard deviation.

Regarding the fresh and dry weight of the root part, the highest value was calculated in Cv 5.0 g. L^{-1} (20 ± 3.6 g and 2.31 ± 0.51 g). It is worth mentioning that Cv 5.0 g. L^{-1} is the treatment where the root least developed.

In addition to the analyses performed on the culture, additionally, physical, and microbiological analyses were performed to the substrate where they were located (Table 3.21 and 3.22).

Table 3.21: Populations of microorganisms in the substrates of the different substrates.

CFU g^{-1} / g. L^{-1}	PDA	PCA	$\frac{1}{2}$ PCA
Cv0.1	$2.21x10^7$c	$1.04x10^8$b	$6.95x10^7$b
Cv0.5	$6.89x10^7$c	$3.75x10^7$b	$3.28x10^7$b
Cv2.0	$6.99x10^8$b	$5.01x10^8$a	$1.01x10^9$a
Cv5.0	$9.14x10^7$c	$3.34x10^7$b	$1.37x10^8$b
So0.1	$1.83x10^8$c	$7.25x10^7$b	$8.98x10^7$b
So0.5	$9.44x10^7$c	$7.47x10^7$b	$2.69x10^7$b
So2.0	$4.42x10^7$c	$3.40x10^7$b	$5.25x10^7$b
So5.0	$2.95x10^7$c	$6.05x10^7$b	$6.47x10^7$b
Water	$1.36x10^8$c	$6.61x10^7$b	$2.26x10^8$b
Algaman B	$1.28x10^7$c	$4.26x10^7$b	$1.83x10^7$b
SEANERGY	$2.19x10^9$a	$1.80x10^8$b	$1.41x10^8$b
Peat	$2.8\ x10^7$c	$2.59x10^7$b	$5.92x10^7$b

For each column, the same letters have no significant differences, for $p<0.05$. PDA: medium to quantify fungi; PCA: medium to quantify bacteria; $^1/_2$ PCA: medium to quantify actinomycetes; Cv0.1, *Chlorella vulgaris* at a concentration of 0.1 g. L^{-1}; Cv0.5, *Chlorella vulgaris* at a concentration of 0.5 g. L^{-1}; Cv2.0, *Chlorella vulgaris* at a concentration of 2.0 g. L^{-1}; Cv5.0, *Chlorella vulgaris* at a concentration of 5.0 g. L^{-1}; So0.1, *Scenedesmus obliquus* at a concentration of 0.1 g. L^{-1}; So0.5, *Scenedesmus obliquus* at a concentration of 0.5 g. L^{-1}; So2.0, *Scenedesmus obliquus* at a concentration of 2.0 g. L^{-1}; So5.0, *Scenedesmus obliquus* at a concentration of 5.0 g. L^{-1}

As identified in the biopesticide potential assays, *Chlorella vulgaris* is the microalgae that present an increase in the microbial community of the soil where it is applied. Cv 2.0 g. L^{-1} obtained an increase in the number of bacterial microbial communities and actinomycetes (5.01×10^8 CFU g^{-1} and 1.01×10^9 CFU g^{-1}). In the number of fungi, the highest number of CFU was calculated in the positive control SEAnergy with 2.19×10^9 CFU g^{-1}, followed by Cv 2.0 g. L^{-1} with 6.99×10^8 CFU g^{-1}.

The initial substrate is represented by "Peat", and it was verified that the application of this concentration of *Chlorella vulgaris*, led to an increase in the communities, and observing the results of the parameters realized in the plants, it is possible that this increase/improvement of their communities have played an important role, not only facilitating in obtaining nutrients by plants as possibly helping in plant protection. According to the analyses performed on the amino acid and protein content (3.2), the biomass of *C. vulgaris*, it is verified that it presents a greater amount than *S. obliquus*,

bringing with it a higher rate of nutrients to the soil, explaining not only the greater development of the plant but also the increase of microorganisms.

Electrical conductivity (EC) was determined to evaluate the conditions of the different substrates. This parameter also indicates indirectly the salinity level and the soluble ions of the substrate. With this information is possible to describe if a substrate is going to have a phytotoxic or a phytoinhibitory effect on the plants (Ding et al., 2018; Zaha et al., 2013). The values collected from electrical conductivity showed that the final values were higher than the initial value, which indicates that the final substrates were more nutritious, presenting more mineral salts in larger quantities than in the initial substrate. With this, looking at Table 3.22, the substrate where the highest electrical conductivity was verified was in the substrate where 5.0 g. L^{-1} of *Chlorella vulgaris* (0.590 ± 0.2 $dS.m^{-1}$) was applied.

Table 3.22: Physicochemical characteristics of the substrates obtained at the end of the assay

g L^{-1}	**pH**	**EC** ($dS.m^{-1}$)
Cv0.1	5.97bc	0.372cd
Cv0.5	6.36abc	0.527ab
Cv2.0	6.38ab	0.419bc
Cv5.0	6.69a	0.590a
So0.1	5.99bc	0.333cd
So0.5	4.49e	0.320cd
So2.0	5.91c	0.319cd
So5.0	6.34abc	0.421bc
Water	6.42ab	0.259d
Algaman B	6.54a	0.413bc
SEANERGY	5.28d	0.274cd
Peat	5.31d	0.141e

For each column, the same letters have no significant differences, for $p<0.05$. Cv0.1, *Chlorella vulgaris* at a concentration of 0.1 g. L^{-1}; Cv0.5, *Chlorella vulgaris* at a concentration of 0.5 g. L^{-1}; Cv2.0, *Chlorella vulgaris* at a concentration of 2.0 g. L^{-1}; Cv5.0, *Chlorella vulgaris* at a concentration of 5.0 g. L^{-1}; So0.1, *Scenedesmus obliquus* at a concentration of 0.1 g. L^{-1}; So0.5, *Scenedesmus obliquus* at a concentration of 0.5 g. L^{-1}; So2.0, *Scenedesmus obliquus* at a concentration of 2.0 g. L^{-1}; So5.0, *Scenedesmus obliquus* at a concentration of 5.0 g. L^{-1}; ; EC, Electrical conductivity.

Almeida (2006) referred that, the maximum salinity for a production drop would be 1.3 $dS.m^{-1}$. Looking at the results of the different substrates, it is verified that they do not exceed this maximum level, maintaining the productivity of this crop.

According to Duncan's statistical test, the pH of the substrates varied in a range of 4.49 and 6.69. The initial pH of the white peat, after treatment with calcium carbonate ($CaCo_3$), was 5.31 ± 0.05. After being in contact with the suspensions for about six weeks, there were both differences in pH and EC.

Observing table 3.22, all substrates were around the pH of 6, excluding the positive control SEAnergy and the concentration So 0.5 g. L^{-1}. pH is an important factor in assessing soil quality and influencing nutrient availability. According to the statistical test and the results obtained in the previous parameters, lettuce had a better development at pH=6. Almeida (2006) reported that for lettuce, the maximum pH range of soil to take advantage of the species would be between 6.5 and 7.2. Considering the pH values for each substrate, it is observed that the substrates treated with *C. vulgaris* concentrations were the substrates closest to the optimum pH for lettuce. The substrates, where the highest concentrations of *C. vulgaris* were applied, were where the edible part of the plant was obtained. This could explain why does *C. vulgaris* obtain the better results on the lettuce crops comparing to the other treatments.

Ding et al. (2018) found that the increase or decrease of the EC is going to have a influence on the fresh weight (FW) and dry weight (DW) of the plant, as well as affect the values of the leaf relative chlorophyll.

According to Renuka et al. (2018), green algae represent a great source of organic matter due to their photosynthetic capacity, since they are involved in the capture of atmospheric CO_2 and convert it into biomass. When added to the substrate they will insert this CO_2 into it, increasing its microflora and fauna.

In addition to the renewal of CO_2 in the soil where they are applied, microalgae have been widely studied due to the rich composition of their biomass. They represent a source of several bioactive compounds, such as proteins, hormone-like substances, and polysaccharides, which are known for their antioxidant properties, promoters of plant growth, such as auxins, amino acids, and proteins. According to Ronga et al. (2019), the biomass of microalgae presents several micro- and macronutrients, such as phosphor (K), nitrogen (N) and potassium (K), and can be considered a slow-release organic fertilizer.

The additions of these compounds in the soil will lead to their improvement, the protection of crops against biotic and abiotic factors and the direct stimulation of plant growth (Gonçalves, 2021; la Bella et al., 2022).

Chapter IV: Conclusion

Regarding the objectives of this study, the main conclusions are:

Biopesticide potential

In vitro

The biopesticide potential of aqueous solutions of the two microalgae was evaluated by testing *in vitro*, eight concentrations (0.01; 0.1; 0.25; 0.5; 0.75; 1.0; 1.25 and 1.5 g L^{-1}) to evaluate the possible inhibiting effect of both microalgae on the phytophatogenic fungi *Fusarium oxysporum*, *Botrytis cinerea*, *Colletotrichum gloeosporioides*, and *Sclerotium rolfsii* and the oomycete *Phytophthora cinnamomi*.

C. vulgaris suspension showed a suppressive effect on *Fusarium oxysporum* at the concentration of 0.75 g L^{-1}, and against *Sclerotium rolfsii* at 1.5 g L^{-1}.

S. obliquus presented the highest suppressive effect *against Botrytis cinerea*, *Phytophthora cinnamomi* and *Colletotrichum gloeosporioides* at the concentrations of 1.5 g L^{-1}, 0.25 g L^{-1} and 0.1 g L^{-1}, respectively. For both microalgae, the results with the above-mentioned pathogens varied according to the concentration of the suspension and the phytopathogenic organism, showing that there is some specificity.

For the remaining fungi, *Alternaria alternata* and *Clarireedia* spp., the results suggested that microalgae promoted their *in vitro* growth. *A. alternata* and *Clarireedia* spp. grew when in contact with the concentration 0.01 g L^{-1} of *C. vulgaris*. For *S. obliquus*, there was a promotion of growth with *A. alternata* with *S. obliquus* at 0.01 g L^{-1} and 0.75 g L^{-1}. However, further studies are needed to better understand the relationship between microalgae and the phytopathogens and to confirm their effectiveness in controlling fungi and oomycetes growth.

In vivo

Both microalgae, *C. vulgaris* and *S. obliquus,* led to a reduction in disease severity, incidence, and AUDPC of *F. oxysporum*, *S. rolfsii* and *B. cinerea* when applied to letuce and spinach affected *by S. rolfsii* and *F. oxysporum*.

The better control for *B. cinerea* was obtained whit C. *vulgaris*, on both plant species. On spinach inoculated with *F. oxysporum*, *S. obliquus* showed a significant reduction in disease progression of up to 68% (So 0. 5 g L^{-1}). With *C. vulgaris* disease progression was reduced by 50% (Cv 0.5 g L^{-1}) when comparing the results on AUDPC on water (negative control). Even when the disease had a high incidence, it did not lead to an increase in disease severity or the death of plants. In lettuce, disease incidence reduction was also evident, reaching 84% (with So 0.5 g L^{-1} and Cv 1.5 g L^{-1}), and reducing AUDPC with both microalgae. On lettuce, there was a clear reduction of the effects of the disease, especially because lettuces treated with microalgae, especially *in C. vulgaris* showed an increased growth.

Spinach and lettuce plants inoculated with *B. cinerea*, showed a better response when *C. vulgaris* was applied in relation to *S. obliquus*. When treated with *S. obliquus*, no significant reduction of the disease was found. However, when *C. vulgaris* was applied, disease reductions were significant, of 75% (Cv 0.5 g. L^{-1}) for spinach and 50% (Cv 0.5 g. L^{-1}) for lettuce.

The global results suggest a potential for the use of microalgae as a biopesticide, an ecological alternative to the use of chemical pesticides. Although several studies had already described the antifungal activity of microalgae after extracting their bioactive compounds, this study shows that the direct use of biomass in aqueous suspensions can reduce diseases caused by some important phytopathogens. Moreover, microalgae biomass has several chemical components that may promote plant growth such as phytohormones and polysaccharides, leading to stimulation of crop growth, which was also proven in previous trials, as a secondary effect of microalgae suspensions use.

Biostimulant potential

In vitro

The evaluation of biostimulant potential *in vitro* showed a significant increase in the germination index when using both *C. vulgaris* and *S. obliquus.* In addition to germination, when in contact with the microalgae suspensions, the radicle also increases it length comparing to the water. It was also possible to identify an inhibition of root growth when increasing the concentration of microalgae suspensions above 0.5 g. L^{-1} to *C. vulgaris*, and 0.1 g. L^{-1} to *S. obliquus*. This inhibition confirms results by other authors.

The application of *C. vulgaris*, increased the germination index and radicle length, especially at low concentrations, such as 0.01 g. L^{-1}, indicating a possible biostimulant effect of this microalga.

In vivo

This inhibition was also proven in the *in vivo* assays, especially at the concentration 5.0 g. L^{-1}, which had inhibited root growth in the *in vitro* trials. The trials showed a stimulating effect of algae on lettuce when watered with their aqueous suspensions.

In short, there were positive effects from the use of microalgae suspensions both for the control of phytopathogens and for the growth of lettuce. The results from the *in vitro* assays were confirmed by the *in vivo* results. It was shown that as the concentration of suspensions increases the more an inhibiting effect of the root part of the plants is evidenced. This might be explained after complementary analyses of the biomasses of the microalgae. However, the inhibitory effect of the concentration of the suspensions on seed germination is not observed in the aerial part of plants, which is a benefit for the producers.

The use of microalgae suspensions as biostimulants will have a beneficial effect on e the environment by allowing the reduction of synthetic pesticides and fertilizers. The concentrations of the suspensions to be applied will depend on the crop, due to the possible inhibition when using high concentrations. For lettuce, recommended

concentrations would be the highest ones tested, 2.0 and 5.0 g. L^{-1}, in order to or obtain larger leaves, however, for other crops more studies need to be done.

Moreover, microalgae can be "programmed" to produce certain compounds through the composition of their culture medium, which is an aspect to be studied in the future since it would be possible to grow microalgae with an optimized composition that could more effectively control plant diseases and increase plant growth.

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