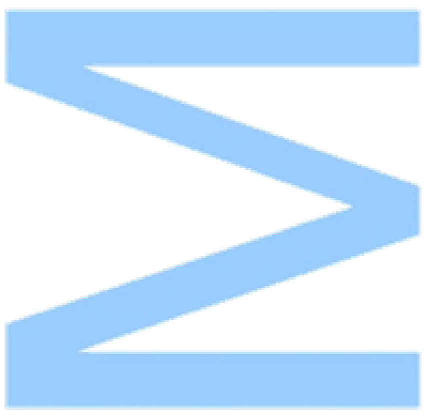

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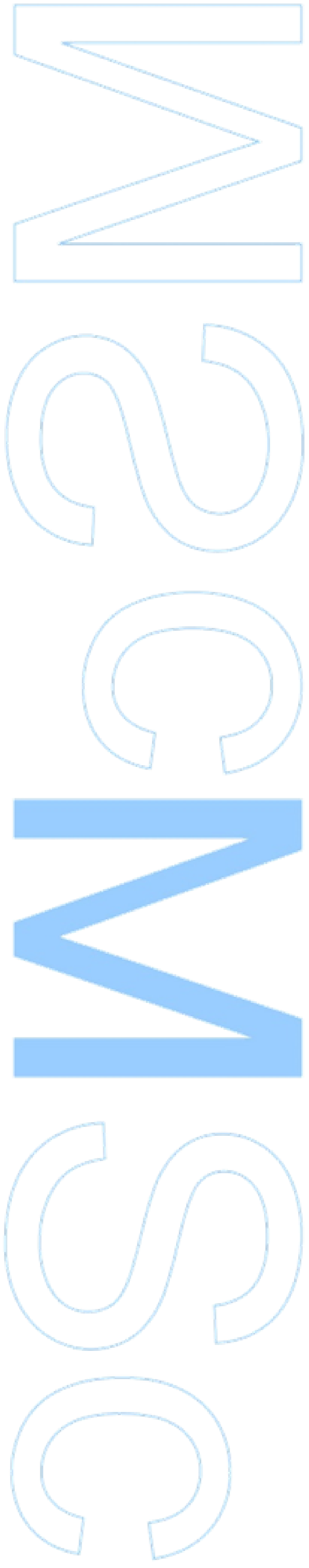
Biofertilizer and herbicide effects of microalgae and cyanobacteria from Portuguese soil

Telma Catarina da Rocha Nunes



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Telma Catarina da Rocha Nunes

Dissertação de Mestrado apresentada
à Faculdade de Ciências da Universidade do Porto em
Biologia funcional e biotecnologia de plantas
2022

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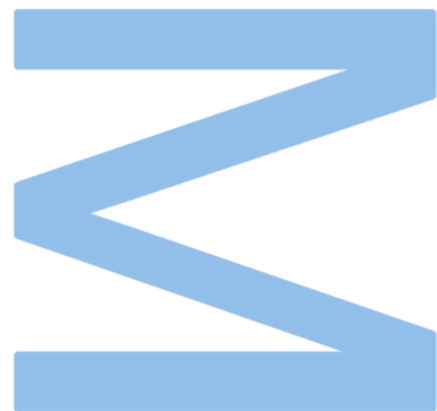
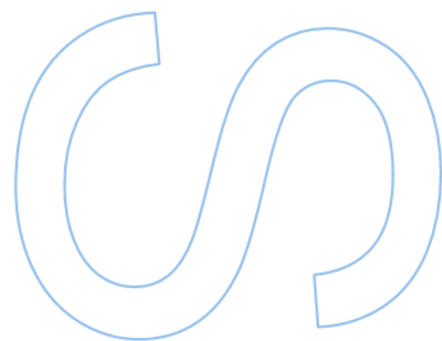

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Mestrado em biologia functional e biotecnologia das plantas
Departmento de biologia
2022

Supervisor
Paula Melo,
Professora Auxiliar, Faculdade de Ciências da Universidade do Porto

Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

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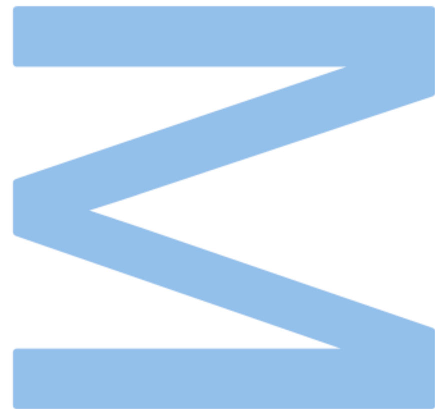
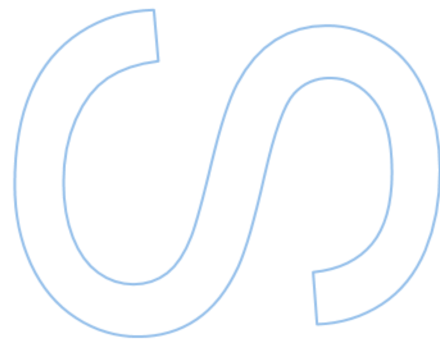

Dedicated to my parents who always do everything for me. Thank you!

Sworn Statement

I, [Telma Catarina da Rocha Nunes], enrolled in the Master Degree [Biologia functional e biotecnologia de Plantas] at the Faculty of Sciences of the University of Porto hereby declare, in accordance with the provisions of paragraph a) of Article 14 of the Code of Ethical Conduct of the University of Porto, that the content of this dissertation/ internship report/ project [Biofertilizer and herbicide effects of microalgae and cyanobacteria from Portuguese soil] reflects perspectives, research work and my own interpretations at the time of its submission.

By submitting this dissertation/ internship report/ project [Biofertilizer and herbicide effects of microalgae and cyanobacteria from Portuguese soil], I also declare that it contains the results of my own research work and contributions that have not been previously submitted to this or any other institution.

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[30/09/2022]

Acknowledgements - Agradecimentos

Há coisas na vida que são impossíveis de conquistar sozinhas e esta dissertação foi uma delas, apesar de gostar de poder retribuir mais, neste momento o que posso é mostrar o reconhecimento por tudo o que fizeram e dizer obrigada.

Em primeiro gostaria de agradecer à professora Paula Melo, pela oportunidade que me deu e por todo o apoio ao longo do projeto, mesmo com uma agenda cheia esteve sempre lá e por isso muito obrigada!

Gostaria também de agradecer à professora Ana Campilho por ter disponibilizado as sementes repórter de Arabidopsis, sem esse contributo teria sido impossível realizar alguns dos testes. Obrigada!

Não posso também deixar de agradecer à professora Olga Laje e aos seus alunos, especialmente o José, por me ajudarem com o seu conhecimento e material. Obrigada, sem essa ajuda seria impossível.

Agradeço também à professora Paula Tamagini e ao I3S por disponibilizarem as microalgas e cianobactérias necessárias para a realização deste trabalho. Obrigada!

Obrigada também ao meu parceiro de laboratório, Emanuel, pela paciência e experiência partilhada, sem ti teria sido muito mais difícil. Obrigada!

Devo também agradecer aos meus pais pelos sacrifícios feitos para me colocarem a estudar. Muito obrigada ao meu pai pela força dada para eu continuar e à minha mãe, foi contra a tua vontade que vim estudar a apesar de teres tentado muito mudar as minhas ideias assim que vim sempre me apoiaste enquanto aqui estive e por isso muito obrigada!

Finalmente, mas não menos importante agradeço aos meus amigos que estiveram ao longo do caminho, obrigada Rodrigo pela paciência, pelos abraços e por esperares as horas vagas na agenda cheia, obrigada Kayla pelo apoio e por seres minha gêmea, obrigada Sofia por seres a mais atinada das três e nos colocares nos eixos, obrigada Joana sem as tuas boleias e o conhecimento passado sobre transportes públicos eu nem sequer teria conseguido chegado à faculdade. Obrigada a vários outros que não disse o nome, vocês são lembrados!

Por último gostaria de agradecer o apoio financeiro dado a este projeto por parte da GreenUporto e pela bolsa de iniciação à investigação do projeto Verão com ciência 2022 – GreenUP. Este trabalho também foi promovido pela Fundação para a Ciência e

Tecnologia (FCT) através dos projetos UIDB/05748/2020, UIDP/05748/2020 e PCIF/RPG/0077/2017.

Resumo

Para alimentar a população humana em constante crescimento é cada vez mais importante encontrar novas estratégias agrícolas mais sustentáveis e amigas do ambiente. Para tal, é importante reduzir a grande quantidade de químicos usados na agricultura, como fertilizantes e herbicidas, por produtos mais sustentáveis, que podem ser encontrados em microalgas e cianobactérias, já que estes microrganismos têm potencial biofertilizante e bioherbicida. Apesar de esse potencial ser conhecido, pouco ainda se sabe ainda sobre o modo como as cianobactérias e as microalgas interagem com as plantas e como afetam o seu metabolismo. Assim, este projeto propõe-se a estudar os efeitos bioquímicos e moleculares de cianobactérias e microalgas nas plantas tentando decifrar o modo de ação destes microrganismos.

Numa primeira parte foi testado o efeito bioherbicida de uma microalga do género *Klebsormidium*, referida como isolado 1.5. Em experiências em placas de petri, os exsudados do *Klebsormidium* sp. (1.5) foram testados em diferentes espécies vegetais, tanto mono como dicotiledóneas, e em diferentes concentrações, através da análise dos parâmetros biométricos, verificando-se uma redução no crescimento de todas as dicotiledóneas testadas, enquanto as monocotiledóneas se mantiveram maioritariamente inalteradas. Depois de selecionadas as espécies vegetais mais tolerantes e as mais sensíveis, as plantas foram crescidas em vasos na presença da microalga, aplicada por aspersão, e usadas para avaliar diversos parâmetros bioquímicos e moleculares relacionados com a condição de stress e produtividade. Tal como nas experiências em placa, as mocotiledóneas não foram significativamente afetadas pela presença do *Klebsormidium* sp. (1.5), enquanto nas dicotiledóneas o seu tamanho foi reduzido e nas espécies mais sensíveis as plantas apresentaram um aspeto clorótico, que como foi possível ver através de testes bioquímicos se devia a danos celulares que as plantas sofreram por não conseguirem responder de forma eficaz ao stress causado pela microalga. Enquanto as plantas mais sensíveis só ativaram o sistema de defesa antioxidante não enzimático, não sendo este suficiente para enfrentar as consequências causadas pela microalga, as plantas mais resistentes, ativaram o sistema de defesa antioxidante enzimático.

Numa segunda parte foi testado o efeito biofertilizante de uma cianobactéria do género *Trichocoleus* em *Lactuca sativa*. Para tal as plantas foram crescidas em vaso, e pulverizadas com os exsudados da cianobactéria, e foram usadas para avaliar diversos parâmetros bioquímicos e moleculares, relacionados com a produtividade. A presença

Trichocoleus sp. levou a um ligeiro incremento no crescimento das plantas e ao aumento da atividade de alguns parâmetros relacionados com a produtividade, mas que não se traduziu num aumento de produção de açucares nem amido. Provavelmente a forma de tratamento terá de ser reavaliada, passando a usar-se em vez do meio de cultura a própria cultura sonicada, por exemplo.

Finalmente foi avaliado o efeito nas plantas de um consórcio de microalgas e cianobactérias isolado de solos queimados portugueses, e previamente testados individualmente, no âmbito do projeto GreenRehab. As plantas foram crescidas em sistema de hidroponia na presença do consórcio e usadas para avaliar diversos parâmetros bioquímicos e moleculares. A presença do consórcio levou a efeitos positivos na maioria dos parâmetros biométricos, sobretudo nas monocotiledóneas. Esta presença não levou a danos moleculares e sobretudo nas dicotiledóneas levou a um aumento dos níveis de produtividade. Estes resultados permitiram verificar que o consórcio escolhido está adequado para se testar a sua utilização com vista à recuperação de solos queimados.

Os resultados destes estudos irão contribuir para a clarificação dos efeitos bioquímicos e moleculares de microalgas e cianobactérias nas plantas e abrir novos caminhos para o desenvolvimento de alternativas sustentáveis aos produtos químicos usados atualmente na agricultura e na recuperação de solos danificados.

Palavras-chave: Cianobactérias, Microalgas, Biofertilizantes, Bioherbicidas, Agricultura, Crescimento vegetal

Abstract

To feed the ever-growing human population it is increasingly important to find new, more sustainable and environmentally friendly agricultural strategies. To do this, it is important to reduce the large amount of chemicals used in agriculture, such as fertilizers and herbicides, with more sustainable products, which can be found in microalgae and cyanobacteria, as these microorganisms have biofertilizer and bioherbicidal potential. Although this potential is known, little is still known about how cyanobacteria and microalgae interact with plants and how they affect their metabolism. Thus, this project proposes to study the biochemical and molecular effects of cyanobacteria and microalgae on plants, trying to decipher the mode of action of these microorganisms.

In the first part the bioherbicidal effect of a microalga of the genus *Klebsormidium*, referred to as isolate 1.5, was tested. In petri dish experiments, the exudates of *Klebsormidium* sp. (1.5) were tested on different plant species, both mono- and dicotyledonous, and at different concentrations, through the analysis of biometric parameters, verifying a reduction in the growth of all dicotyledonous plants tested, while monocotyledonous plants remained unchanged. After selecting the most tolerant and the most sensitive plant species, the plants were grown in pots in the presence of the microalgae, applied in the form of a spray, and used to evaluate several biochemical and molecular parameters related to stress and productivity. As in the petri dish experiments, monocotyledonous plants were not significantly affected by the presence of *Klebsormidium* sp. (1.5), while in the dicotyledonous plants their size was reduced and in the most sensitive species the plants showed a chlorotic appearance, which as it was possible to see through biochemical tests was due to cellular damage that the plants suffered by not being able to respond effectively to the stress caused by the microalga While the most sensitive plants only activate the non-enzymatic antioxidant defense. system, that does not seem to be sufficient to cope with the consequences caused by the microalga, the most resistant activated the enzymatic antioxidant system.

In the second part, the biofertilizer effect of a cyanobacterium of the genus *Trichocoleus* on *Lactuca sativa* was tested. For this purpose, plants were grown in a pot in the presence of the cyanobacterium and were used to evaluate several biochemical and molecular parameters, related to productivity. The presence of *Trichocoleus* sp. led to a slight increase in plant growth and an increase in some yield-related parameters but did not translate into an increase in sugar or starch production. Probably the treatment will have to be reassessed, using instead of the culture medium the sonicated culture.

Finally, the effect on plants of a consortium of microalgae and cyanobacteria isolated from Portuguese burnt soils, and previously tested individually, was evaluated under the scope of the GreenRehab project. The plants were grown in a hydroponics system in the presence of the consortium and used to evaluate several biochemical and molecular parameters. The presence of the consortium led to positive effects on the plants, especially on monocotyledonous, as most of the biometric parameters analyzed were increased. This presence did not lead to molecular damage and in dicotyledonous plants it led to an increase productivity. These results confirmed that the chosen consortium is adequate to be test in the recovery of burnt soils.

The results of these studies will contribute to the clarification of the biochemical and molecular effects of microalgae and cyanobacteria on plants and open new avenues for the development of sustainable alternatives to chemicals currently used in agriculture and in the recovery of damaged soils.

Keywords: Cyanobacteria, Microalgae, Biofertilizers, Bioherbicides, Agriculture, Plant-growth

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List of Abbreviations

ADP	ADENOSINE DIPHOSPHATE
APX	ASCORBATE PEROXIDASE
BSC	BIOLOGICAL SOIL CRUST
CAT	CATALASE
CTL	CONTROL
DTT	DL-DITHIOTHREITOL
F.W.	FRESH WEIGHT
FAD	FLAVIN ADENINE DINUCLEOTIDE
FCUP	FACULTY OF SCIENCES OF THE UNIVERSITY OF PORTO
GOGAT	GLUTAMATE SYNTHASE
GS	GLUTAMINE SYNTHETASE
GUS	B-GLUCURONIDASE
LP	LIPID PEROXIDATION
MDA	MALONDIALDEHYDE
NADH	NICOTINAMIDE ADENINE DINUCLEOTIDE (NAD) (REDUCED FORM)
NBT	NITROBLUE TETRAZOLIUM
PMSF	PHENYLMETHYLSULFONYL FLUORIDE
SD	STANDARD DEVIATION
SOD	SUPEROXIDE DISMUTASE
TBA	THIOBARBITURIC ACID
TCA	TRICHLOROACETIC ACID
UP	UNIVERSITY OF PORTO

1.Introduction

The soil is composed of several layers, one of which is called biological soil crust (BSC). This layer is located near the surface and is an environment with complex interactions between rock surface, water, soil and living organisms (Karsten et al., 2016). It is possible to find BSC in various ecosystems: tropical and temperate deserts, polar regions and arid areas where it can become the dominant feature, since it plays an important ecological role by protecting and stabilizing the soil against wind and water erosive forces (Abinandan et al., 2019), besides influencing local hydrologic patterns such as soil texture, absorptivity, cracking, roughness and aggregation (Belnap, 2006). The BSC also has a role in retaining soil moisture by influencing patterns of infiltration (Belnap, 2006) and improving vegetation by helping the germination of seeds and inhibiting weeds (Lababpour, 2016). After a disturbance, the BSC constitutes the pioneer communities that form the basis for a new ecosystem development (Abinandan et al., 2019), for example after a volcanic eruption microalgae together with cyanobacteria were the first organisms to initiate the formation of the BSC since they only need small amounts of moisture to initiate their living. They stay in the top layer preventing moisture evaporation and allowing crusts dominated by lichens and mosses to appear (Abinandan et al., 2019). Numerous species constitute this layer: lichen, mosses, fungi, liverworts, diatoms, bacteria, eukaryotic microalgae and prokaryotic cyanobacteria (Lababpour, 2016).

Cyanobacteria or blue-green algae are a diverse group of procaryotic microorganisms capable of making oxygenic photosynthesis, and can be found in many habitats: terrestrial, marine, freshwater and extreme habitats such as arctic or antarctic, some can even create symbiotic relationships with algae, fungi, animals, protists and plants (Singh, 2014). They can survive with a minimum requirement of carbon dioxide, light and water, in addition, they can improve the soil nutrient status and they can produce bioactive compounds that promote growth and protect the crops (Singh et al., 2016; Santini et al., 2021), such as polysaccharides, carbohydrates, vitamins, hormones, proteins and amino-acids (Singh, 2014; Win et al., 2018; Poveda, 2021; Santini et al., 2021), and other growth-promoting compounds that stimulate the growth of the soil microbial populations and crop development (Singh and Trehan, 1973).

Many studies confirmed the plant growth promoting effects of cyanobacteria since they could increase root and shoot growth (Singh et al., 2016), they are also able to, for example, promote the transport of nutrients from the soil to plants, plant germination, growth and development due to the cyanobacteria's capability of producing the bioactive

molecules. Furthermore, cyanobacteria can complex xenobiotics and metals which limits their mobility and transport in plants and they can protect plants from pathogenic insects and abiotic stresses, such as salinity and drought (Singh et al., 2016; Poveda, 2021; Santini et al., 2021). They also produce enzymes that confer resistance to pollutants or that biodegrade them (Abinandan et al., 2019).

Some cyanobacteria like *Nostoc*, *Plectonema*, *Anabaena, Aulosira, Syctonema* and *Tolypothrix* can fix atmospheric nitrogen (Bhardwaj et al., 2014). When in symbiosis with plants they can provide nitrogen, fixed in specialized cells called heterocysts, to their host (Poveda, 2021). These cyanobacteria also help increase the N content in the soil, since they are capable of releasing the N content in many forms, like free amino-acids and ammonia, either by secretion or by mineralization of the cyanobacterial biomass after death (Singh et al., 2016; Alvarez et al., 2021). In agricultural ecosystems, the best example of this increase in N availability happens mainly in rice fields (Singh et al., 2016). But cyanobacteria are also capable of increasing the phosphorus bioavailability to the plants and increasing the soil organic carbon content (Bhardwaj et al., 2014; Singh et al., 2016)

However, cyanobacteria are not the only organisms that can benefit the soil and plants, eucaryotic microalgae can also be used. Algae exist in various habitats such as the ocean's depths, hot and cold deserts, rock crevices and as mentioned soil crusts (Renuka et al., 2018). Microalgae are microscopic algae that can be found in nearly all aquatic, sub-aerial and terrestrial surfaces, they are photosynthetic microscopic organisms whose size can vary from 1 to 900 μm, they can be unicellular or filamentous and grow in aquatic (marine and fresh) and terrestrial environments including soil (Ronga et al., 2019; Alvarez et al., 2021).

Microalgae can also produce many bioactive compounds, such as lipids, carotenoids, proteins, polysaccharides and vitamins and like cyanobacteria, they are capable of producing structurally complex extracellular polysaccharides (EPS) (Rachidi et al., 2020). They are capable of reducing heavy metal translocation to plant tissues and also produce a variety stress tolerance metabolites, amino-acids, polyamines, free volatile fatty acids and phytohormones, such as cytokinin, abscisic acid, auxins and gibberellins (Plaza et al.; Ronga et al., 2019; Alvarez et al., 2021; González-Pérez et al., 2022), that are small molecules that act as chemical messengers in plants to regulate cellular activity and influence a variety of metabolic processes like nutrient uptake, photosynthesis, nucleic acid synthesis and respiration (Ronga et al., 2019).

Studies also showed that microalgae can enhance soil fertility by increasing microbial activity, organic matter, C, N and P contents and promote water retention. In addition, they are capable of promoting plant growth, by enhancing macronutrient and micronutrient mobilization and uptake (Ronga et al., 2019; Alvarez et al., 2021; González-Pérez et al., 2022).

Due to the positive effects caused by microalgae and cyanobacteria, their inoculation on the soil has been proposed in a process called cyanobacterization/algalisation (Alvarez et al., 2021), since they improve the aeration, the water holding capacity and agglomeration of particles, improving soil properties and fertility (Win et al., 2018; Poveda, 2021). The use of cyanobacteria on highly disturbed soils has been proposed as a means of increasing microbial populations and accelerating crust formation on desert and perturbed soils since they form a cohesive layer on the soil surface that provides soil stabilization and habitat for other organisms (Acea et al., 2003; Rossi et al., 2017; Abinandan et al., 2019; Chamizo et al., 2020; Alvarez et al., 2021; Kapoore et al., 2021). This process can be used as a promising tool in the post-fire rehabilitation process since have shown a potential to be used as a rehabilitation agent accelerating burnt soil recovery (Chamizo et al., 2020). This inoculation leads to positive effects due to the capability of cyanobacteria to introduce a significant amount of vitamins, amino acids, phytohormones, nitrogen, carbon and exopolysaccharides (EPS) into the soil (Rossi et al., 2017). EPS help improve soil moisture due to their hygroscopic nature and therefore help to bind together soil particles (Singh et al., 2016). This type of inoculation in very disturbed soils has been proposed as a means of increasing microbial populations and accelerating crust formation on desert and perturbed soils since they form a cohesive layer on the soil surface that provides soil stabilization and habitat for other organisms (Acea et al., 2003; Rossi et al., 2017; Abinandan et al., 2019; Chamizo et al., 2020; Alvarez et al., 2021; Kapoore et al., 2021).

The use of cyanobacteria and microalgae together as a consortium has also been reported, this way the microorganisms can work synergistically to improve soil fertility and plant growth, in general, the green algae are most effective in enhancing plant growth and quality of fruits while the cyanobacteria are mostly used for soil aggregation, N fertilization and biocontrol (Renuka et al., 2018).

As seen, microalgae and cyanobacteria have a variety of positive effects on the plants and the soil. This allows them to be used as a possible substitute for the fertilizers and pesticides currently used to help feed the growing human population that are chemically based which can translate into toxicity and accumulation in plants and soil, therefore,

becoming a threat to humans and the environment (Gonçalves, 2021). Cyanobacteria and microalgae can be used as biofertilizers that are environmentally friendly resources since they contain living microorganisms (Kapoore et al., 2021; Santini et al., 2021; González-Pérez et al., 2022) capable of stimulating plant growth, restoring soil fertility and improving soil biological and chemical properties (Bhardwaj et al., 2014; Renuka et al., 2018; Ronga et al., 2019). Biostimulants are natural products, that can be bioactive compounds produced by microorganisms, capable of improving crops' physiological processes when applied to plants or the soil. These are extracts obtained from organic raw materials that contain bioactive compounds, such as minerals and vitamins that can increase crop yield and quality, biotic and abiotic stress tolerance and plant nutritional efficiency (Hawrylak-Nowak et al., 2019; Ronga et al., 2019; Nephali et al., 2020). But contrary to fertilizers biostimulants do not provide nutrients to the plants directly, they act on plant metabolism hence having neglectable nutrient concentrations (Bulgari et al., 2015).

In addition to being used as biofertilizers and biostimulants, due to their beneficial effects on plants and soil and in agricultural biocontrol, cyanobacteria and microalgae have many other practical purposes such as serve for bio-remediation and has a bio-source of materials, animal and human food, medicines, cosmetics and energy production (Chaïb et al., 2021; Hachicha et al., 2022; López-Hernández et al., 2022; Saeed et al., 2022).

Although very useful in various areas and to stimulate plant growth, some microalgae and cyanobacteria may have harmful effects on certain plants, such as weeds, which allows the use of microalgae as potential bioherbicides for weed control (Costa et al., 2019). Since the presence of weeds, some of which are no longer controlled efficiently by synthetic products (Ulrich et al., 2021), reduces crop productivity causing considerable crop losses every year, these microorganisms have potential uses in sustainable agriculture (Stefanski et al., 2020). The use of microalgae as bioherbicides is an unexplored field, although it is known that microalgae can synthesize metabolites with herbicidal potential, such as photosynthesis inhibitors and antimitotics, they are not well studied (Chaïb et al., 2021).

Although, very important for an agricultural sustainable growth a study made by Abinandan et al concluded that from 1908 to 2018 microalgae and cyanobacteria received less attention when compared to fungi and rhizobia. This comparison also applies between cyanobacteria and microalgae with the last receiving less attention (Abinandan et al., 2019; Alvarez et al., 2021). Therefore, there are, so far, not many

studies about the use of cyanobacteria and microalgae as bioherbicides, biostimulants and biofertilizers. Furthermore, their effects on plants and the environment and their mode of action are still unknown. So, this study has the goal to contribute knowledge to this field still unexplored.

1.1. Objectives and work division

This study was divided into three different parts, the first had as organism in study a microalga from the genus *Klebsormidium,* collected from a Portuguese soil, that in previous studies has shown a selective bioherbicide potential since it inhibited the growth of a dicotyledonous species (*Arabidopsis thaliana*) and did not affect a monocotyledonous species (*Lollium multiflorum*). The goal was to study the biochemical effect of this microalga in both tolerant and susceptible plant species and to elucidate the microorganism´s mode of action. For that petri dish and pot assays were performed and some biochemical parameters were assessed to evaluate the physiological state of the plants in study.

The second part concerned a cyanobacterium from the *Thichocoleus* genus, also collected from Portuguese soil, that appeared to have a biofertilizer effect on plants in prior studies. This project aimed to study the effect of this cyanobacterium on plants and to clarify its biochemical and molecular mechanisms of action. This will be achieved by the realization of growth pot assays and the assessment of some biochemical parameters.

The third part studied the effects of a consortium of microalgae and cyanobacteria, collected from Portuguese burned soils in plants, previously individually selected by its beneficial effects on plants and soil. The consortium was tested in both mono and dicotyledonous plants in hydroponic cultures to evaluate its biochemical effects before being applied for the rehabilitation of burned soils in the scope of the GreenRehab Project (Green rehabilitation system for burned soils based on the inoculation of native cyanobacteria and microalgae).

2. Material and methods

2.1. Microorganism growth conditions

The microorganisms, cyanobacteria and microalgae, used in this study were collected as part of the Greenrehab project. These were maintained under continuous agitation at 100 rpm, in specific conditions of light (16h light and 8h darkness, intensity 40 µmol s^{-1}) and temperature (22ºC) with BG_{11} solution as the growth medium (table S1) (Rippka et al., 1979). Every two months the microalgae and cyanobacteria were transferred to a sterile Erlenmeyer with a new growth medium.

When necessary, the concentration of chlorophyll in the culture was assessed using the method provided by Meeks & Castenholz, (1978). Initially, 1 mL of microorganism culture was centrifuged at 12500 rpm for 10 min using the Gusto mini centrifuge (Heathrow Scientific, Illinois, USA). After, the supernatant was discarded, and 1 mL of water was added to the pellet and centrifugated again at 12500 rpm for 5 min. Then, the supernatant was discarded, and the pellet was resuspended in 1 mL of methanol 90% and left to incubate in the dark, at room temperature, for 1h with agitation of 100 rpm. At end of that time, the mix was centrifuged at 12500 rpm for 5 min. Later the supernatant absorbance was measured at 663 nm in the spectrophotometer Genesys 10S UV-Vis (ThermoFisher Scientific, Massachusetts, USA), using methanol 90% as blank. The results were expressed in µg/Ml^{-1}.

2.2. Plant growth conditions

All the plants used in the following studies were grown in a growth chamber under controlled conditions of light (16h of light and 8h of dark, intensity 100 µmol $m^{-2}s^{-1}$) and temperature (20-22ºC), using Hoagland solution (table S2) (Taiz and Zeiger, 2010) as nutritive medium.

2.2.1. Petri dish experiments

Seeds of various plant species, *Lactuca sativa* L. cv Marvel of Four Seasons (Vilmorin, France), *Nicotiana tabacum*, *Arabidopsis thaliana* of the ecotype Columbia [(Columbia 0 (Col-0)] of Nottingham Arabidopsis Stock Center (NASC) (Nottingham, United Kingdom), *Zea mays, Medicago truncatula, Lollium multiflorum* cv. Diamond T. of OreGro Seeds Inc. (Oregon, USA) and *Hordeum vulgare,* after being disinfected with a solution of bleach 20% v/v and washed with sterilized water, were placed in squared petri dishes containing agarized Hoagland solution. The dishes were enriched with 2 mL of BG_{11} solution in the control situation or with 2 mL of the medium in which the microalgae

and cyanobacteria were being grown (at least for a month), after a 10 min centrifugation at 12000 rpm using the Gusto mini centrifuge (Heathrow Scientific, Illinois, USA). Afterward, the plants were grown under controlled conditions (2.2) and after 2-3 weeks the plants were collected and several biometric parameters were assessed: the number of leaves, shoot fresh weight, shoot length, root length, and root fresh weight.

Using the same principle, some petri dish assays were performed using seeds from *Arabidopsis thaliana* lines DR5::GUS. These were sown in petri dish and the plant growth medium was enriched with 2 mL of the growth medium of *Klebsormidium* sp. 1.5 or 2.1 b or 2 mL of 2,4-D in the concentration of 1 mM; 0.1 mM; 0.01 mM or 0.001 mM. 2 mL of BG_{11} was used in the control dishes. After growing for 15 days in controlled conditions the plants were collected and the GUS stain was performed

The same petri dish experiments were performed to test the extracts obtained from *Klebsormidium* sp. (1.5), using *Arabidopsis thaliana* and 2 mL of the acetonic extracts. In the control dishes, the plant growth medium was enriched with 2 mL of DMSO 20%. After 15 days the plants were collected and several biometric parameters were assessed: the number of leaves, shoot fresh weight, shoot length, root length, and root fresh weight.

2.2.2. Pot trials

Seeds of *A. thaliana*, *L. multiflorum*, *N. tabacum*, and *L. sativa* were sown in pots with perlite and vermiculite (1:1) (both products from SIRO, Leal & Soares, S.A., Mira, Portugal) as substrate. These were watered with Hoagland medium and after a time, dependent on the plant species, the shoots were sprayed with the exudates from 1-month-old microorganism cultures containing 0.367 mg of chlorophyll, obtained by cells sonication at intensity 10 for 10 seconds, 10 times, (between each time the extracts were left to cool on ice before being sonicated again) with the Model XL-200 (Misonix incorporated, New York). In the end, the extracts were centrifugated at 3214g (5804R centrifuge, Eppendorf, Hamburg, Germany) before being applied. The application was initiated in *A. thaliana* 10 days, in *L. sativa* and *L. multiflorum* 15 days after and in *N. tabacum* 5 days after sown. The sonicates were applied two times a week during the first two weeks and then once a week until the end of the assay. The plants were grown in the growth chamber and were collected after 1-2 months depending on the plant species and some biometric parameters were assessed: the number of leaves, shoot fresh weight, shoot length, root length and root fresh weight, and in *L. multiflorum* the number of tillers was also assessed. Afterward, the plants were stored at -80ºC and used for biochemical quantifications.

The same method was applied for *Trichocoleus* sp., but there was no sonication and the only thing sprayed was the cyanobacteria growth medium after 1 month of growth.

2.2.3. Hydropony assays

Seeds of *L. multiflorum* and *A. thaliana* were sown in perlite and vermiculite and grown for 2 weeks until they reach an appropriate size to be transferred to the hydroponics system. On the test system, the Hoagland solution was enriched with the consortium, constituted by *Trichocoleus* sp., *Oscillatoria* sp., *Nodosilinea* sp., *Klebsormidium* sp. (2.1 b), *Parakomarekiella* sp. and *Nostoc* sp., isolated at I3S, when the chlorophyll content was of 0.6 mg. After growing in direct sunlight and outside temperature for 1 month, the plants were collected and the biometric parameters assessed: number of leaves, shoot fresh weight, shoot length, root length, and root fresh weight, and in *L. multiflorum* the number of tillers was also assessed. Afterward, the plants were stored at -80ºC and used for biochemical quantifications.

2.3. (β-glucuronidase) GUS staining

Three plants from each petri dish (control, *Klebsormidium* sp. (1.5); *Klebsormidium* sp. (2.1 b); 2,4-D 1 mM; 2,4-D 0.1 mM; 2,4-D 0.01 mM and 2,4-D 0.001 mM) were collected and incubated overnight at -20ºC in 0.2 µL microtubes with 90% acetone. After, the samples were washed 2 times with sodium phosphate buffer (0.2 M NaH_2PO_4; 0.2 M $NaHPO_4$) for 10 min. Next, while protecting the samples from the light the buffer was replaced by X-Gluc solution (50% phosphate/NaPi buffer; 1% Triton X-100; 0.8% potassium ferrocyanide; 0.8% potassium ferricyanide; 46.4% diH20 and 100% (w/v) X-Gluc) and the samples were left overnight at 37ºC. Afterward, the X-Gluc was removed and the samples were washed with 90% ethanol and 70% ethanol for 10 min each. Finally, the ethanol was discarded, the samples were submerged in chloral hydrate, left at 4ºC overnight and the results were observed in the ZeissTMAxioImager AZ microscope with differential interference contrast (DIC) optics and photographed with a ZeissTMAxiocam MRc3 camera through the Zen Imaging acquisition software (SP1, Zen 2011).

2.4. Compounds extraction

Three % of AmberLite XAD-16N resin was added to 1-month cultures of *Klebsormidium* sp. 1.5 and 2.1 b. The cultures were then left to grow in a growth chamber, under controlled conditions with BG_{11} as growth medium for 15 days in the presence of the resin. At the end of that time, the resin was extracted and left to incubate with acetone (proportion of 200 mL acetone per 20 g of resin) for one and a half hours.

Next, the acetone was collected, dried in a rotary steamer and left at -80ºC overnight. After, the water remaining was dried in a freeze dryer. Once the samples were dried, acetone in the same proportion used previously was added and dried in a rotary steamer until only 5 mL of acetone remained, these were left to dry in air at room temperature. Once the samples were completely dry, the extracts obtained were weighed and 6 mL of DMSO 20% were added.

2.5. Plant Biochemical Assays

2.5.1. Lipid peroxidation

The assessment of lipid peroxidation (LP) was made through the quantification of the malondialdehyde (MDA) levels, using the procedure developed by Heath and Packer, (1968), adapted for microplates spectrophotometry. 0.4 g of plant material were homogenized in trichloroacetic acid (TCA) 0.1%, with the help of quartz sand, and centrifuge for 15 min at 12000x *g* (5804R centrifuge, Eppendorf, Hamburg, Germany). Later, 50 µL of supernatants were mixed with 200 µL of thiobarbituric acid (TBA) 0.5% and left to incubate for 30 min at 95 º C. By the end of that time, the mixtures were cooled in ice for 10 min and then centrifuged for 5 min at 10000 *g.* Next, the mix was transferred to a microplate and the absorbance was measured at 532 nm and 600nm (Multiskan GO, Thermo Scientific), using 50 µL of TCA 0.1% mixed with 200 µL of TBA 0.5% as white. For LP quantification, the 600 nm values were subtracted from the 532 nm values to eliminate unspecific turbidity. MDA concentration was calculated considering $\varepsilon=155\ mM^{-1}\ cm^{-1}$, and the results were expressed in nmol. g^{-1} f. w.

2.5.2. H_2O_2 quantification

The H_2O_2 quantification was performed by following the procedure described by Alexieva et al. (2001), adapted for microplate spectrophotometry. 50 µL of the supernatants obtained in the previous point were added to 50 µL of phosphate buffer 100 mM (pH 7.0) and 200 µL of KI 1 M, and the mixture was left to incubate for 1h in the dark at room temperature. Once the time was finished the absorbance was measured at 390 nm (Multiskan GO, Thermo Scientific), using 50 µL of TCA 0.1%, mixed with 50 µL of phosphate buffer 100 mM (pH 7.0) and 200 µL of KI 1 M as white. The H_2O_2 content was expressed in $nmol^{-1}$. g^{-1} f. w. and was calculated using the molar extinction coefficient of 0.28 μM^{-1}. cm^{-1}.

2.5.3. Proline quantification

The proline quantification was performed by following the procedure described by Bates et al. (1973), adapted for microplate spectrophotometry. Initially, the extracts were obtained by homogenizing 50 mg of plant material in 640 µL of sulfosalicylic acid 3% with the help of quartz sand. Then, the extracts were centrifuged at 10000x *g* for 10 min (5804R centrifuge, Eppendorf, Hamburg, Germany) and 80 µL of the supernatants were mixed with 80 µL of glacial acetic acid and 80 µL of acid ninhydrin. After, 1h at 96 º C and being cooled in ice, 400 µL of toluene were added to the mix. Next, the mix was vortexed for 15-20 seconds and the two phases were allowed to separate. 250 µL of the top part with a reddish pink color were collected to a microplate and the absorbance was measured at 250 nm using the toluene as blank (Multiskan GO, Thermo Scientific). The proline content was expressed in µg/mg^{-1} f. w, using a standard curve obtained by different solutions of known concentrations of proline.

2.5.4. Reduced glutathione quantification

The reduced glutathione quantification was performed by following the procedure described by Rahman et al. (2006), adapted for microplate spectrophotometry. Reduced glutathione was quantified by mixing 300 µL of work solution (7 mL of EDTA 1mM in phosphate buffer 100 mM, pH 7.0 and 200 µL of DTNB), 80 µL of water and 20 µL of the supernatant used for proline quantification in a microplate. Then, the mix was left in the dark for 1h at room temperature. By the end of that time, the absorbance was measured at 412 nm (Multiskan GO, Thermo Scientific), using 300 µL of work solution mixed with 80 µL of water and 20 µL of sulfosalicylic acid 3% as white. The reduced glutathione content was expressed in µmol/g f. w using a standard curve obtained by different solutions of known concentrations of GSH.

2.5.5. Protein quantification

Soluble protein contents were quantified in the extracts obtained with the different enzymatic extraction buffers (catalase, ascorbate peroxidase, superoxide dismutase, nitrate reductase and glutamine synthetase). For this, 250 µL of Bradford solution were mixed with 8 µL of the supernatants and left to react at room temperature for 15 min. After, the absorbance was measured at 595 nm (Multiskan GO, Thermo Scientific), using 258 µL of Bradford as blank. The values for protein content were expressed in µg/µL^{-1} using a standard curve obtained by different solutions of known concentrations of bovine serum albumin protein (BSA).

2.5.6. Catalase (CAT) quantification assay

The catalase quantification was performed by following the procedure described by Aebi (1984), adapted for microplate spectrophotometry. First, the extracts were obtained by homogenizing, in ice, 0.2 g of plant material in 1 mL of extraction buffer (Phosphate buffer 100 mM, pH 7,3; EDTA 1 mM; glycerol 8%; PMSF 1mM; L-ascorbic acid 5 mM and PVPP 2% placed directly in the mortar). The extracts were then centrifuged for 20 min, at 16000x *g*, at 4 º C (5804R centrifuge, Eppendorf, Hamburg, Germany). After 50 µL of the supernatants were mixed with 200 µL of phosphate buffer, later H_2O_2 0.3% was added and the absorbance was read at 240 nm, every 15 seconds 21 times (Multiskan GO, Thermo Scientific). Later the catalase content was calculated considering ε=39.4 mM^{-1} cm^{-1} and was expressed in µmoles/min^{-1}/ug-1 of protein.

2.5.7. Ascorbate Peroxidase (APX) quantification assay

The APX quantification was performed by following the procedure described by Murshed et al. (2008), adapted for microplate spectrophotometry. APX was quantified by mixing 20 µL of the supernatants obtained in the previous point with 170 µL of buffer (20 mL of 100 mM phosphate buffer and 48 µL of Ascorbic acid 260 mM), later H_2O_2 0,3% was added and the absorbance was read at 290 nm, every 15 seconds 21 times (Multiskan GO, Thermo Scientific). Later the APX content was calculated considering ε=0.49 mM^{-1} cm^{-1} and the result was expressed in µmoles/min^{-1}/ug^{-1} of protein.

2.5.8. Superoxide dismutase (SOD) quantification assay

The SOD quantification was performed by following the procedure described by Donahue et al. (1997) and adapted for microplate spectrophotometry. This process has its base in the reaction of inhibition of the photochemical reduction of nitroblue tetrazolium (NBT). Initially, these extracts were obtained by homogenizing, in ice, 0,4 g of plant material in 2 mL of extraction buffer (same as used for catalase quantification). Then, the extracts were centrifuged for 20 min, at 16000x g, at 4 º C and supernatants were obtained (5804R centrifuge, Eppendorf, Hamburg, Germany). Later, 20 µL of supernatants were mixed with 280 µL solution A (phosphate buffer 50 mM, pH 7.8; EDTA 0.1 mM; methionine 13 mM and NBT 75 μM^{-1}), 7 µL of phosphate buffer and 3 µL of riboflavin solution. After, the mixes were left to react at room temperature in two conditions, light and obscurity. By the end of that time, the absorbance was measured

at 560 nm (Multiskan GO, Thermo Scientific), and the results were expressed as U SOD/mg^{-1} of protein.

2.5.9. Glutamine synthetase (GS) quantification assay

The GS quantification was performed by following the procedure described by Cullimore and Sims, (1980), adapted for microplate spectrophotometry. Initially, extracts were obtained by homogenizing, in ice, 60 mg of plant material in 500 µL of extraction buffer (10 mM Tris-HCL pH 7.5; 5 mM Na-glutamate; 10 mM MgSO4; 1 mM DTT; 10% glycerol; 0.05 % Triton X-100 and PVPP placed directly in the mortar). Then, the extracts were centrifuged at 20000x g, for 15 min, at 4ºC and supernatants were obtained (5804R centrifuge, Eppendorf, Hamburg, Germany). Later a mix of 150 µL activity solution (100 mM Tris-base, 100 mM L-glutamine, 60 mM hydroxylamine, 1 mM $MnCl_2.4H_2O$, 0.5 mM ADP), 10 µL of sodium arsenate and 20 µL of supernatant, were left to incubate at 30ºC for 30 min, at the end of that time the reaction was stopped with 180 µL of STOP solution (3.3 % FeCl2; 8% TCA; 2M HCL). Later the absorbance was read at 500 nm (Multiskan GO, Thermo Scientific), using 150 µL activity solution mixed with 10 µL of sodium arsenate, 20 µL of activity solution 180 µL of STOP solution as white. The GS content was expressed as U GS/mg^{-1} of protein.

2.5.10. Nitrate reductase quantification assay

The nitrate reductase quantification was performed by following the procedure described by Kaiser and Brendle-Behnisch (1991), adapted for microplate spectrophotometry. First, in ice, 0.2 g of plant material were homogenized with 1 mL of extraction buffer (HEPES-KOH Buffer 50 mM, pH 7.8; PMSF 1 mM and $MgCl_2$ 10 mM. The obtained extracts were then centrifuged, at 15000x *g* for 25 min, at 4 º C (5804R centrifuge, Eppendorf, Hamburg, Germany). After, 50 µL of supernatant are mixed with 300 µL of reaction solution (HEPES-KOH buffer 20 mM pH 7.8; NADH 500 µM, FAD 10 µM and KNO_3 2 mM). Later the absorbance was read at 340 nm, at 20-second intervals for 1 min and 40 seconds (Multiskan GO, Thermo Scientific). Later the quantity of nitrate reductase is calculated considering ε=6,22 mM^{-1} cm^{-1} and the values were expressed in nmoles/min^{-1}/ng^{-1} of protein.

2.5.11. Chlorophyll quantification

Ten mg of plant material were homogenized in 2 mL of acetone 80%, and the obtained extracts were then centrifuged for 10 min at 5000 rpm (5804R centrifuge, Eppendorf, Hamburg, Germany). After centrifugation, the supernatants were collected, and the volume was adjusted to 2 mL. Later, the absorbance was read at 645 and 663 nm

(Multiskan GO, Thermo Scientific), using acetone 80% was used as white. The concentrations of chlorophyll were calculated using the equations provided by Lichtenthaler and Buschmann, (2001) and the total chlorophyll content was expressed in mg chlorophyll a+b/mg^{-1} f. w.

2.5.12. Soluble sugar content quantification

The sugar quantification was performed by following the procedure described by Irigoyen et al., (1992) adapted for microplate spectrophotometry. First, 40 mg of plant material were homogenized in 5 mL of ethanol 80%, the obtained extracts were then left at 80ºC for 1 h. After that time the extract was vortexed and centrifuged at 5000x g, for 10 min at 4ºC (Universal 32 R, Hettich). After, the pellets and the supernatants were collected in different containers. Later, 30 µL of these supernatants were mixed with 750 µL of anthrone. The mixes were then left for 10 min at 100ºC. Next, they were cooled in ice for 10-15 min, shaken, centrifuged at 10000x g for 5-10 min (Eppendorf AG 22331) and the absorbance was read at 625 nm (Multiskan GO, Thermo Scientific). The soluble sugar content was expressed in mg/mg^{-1} f. w.

2.5.13. Starch content quantification

The starch quantification was performed by following the procedure described by Osaki et al., (2012), adapted for microplate spectrophotometry. The pellets collected in the previous point were resuspended in 3 mL of perchloric acid 30%, vortexed, left at 60ºC for 1 h, vortexed again, centrifuged at 10000x g, for 10 min at 4º and the supernatants were collected (Universal 32 R, Hettich). Later, 30 µL of these supernatants were mixed with 750 µL of anthrone. The mixes were then left for 10 min at 100ºC. Next, they were cooled in ice for 10-15 min, shaken, centrifuged at 10000x g for 5-10 min (Eppendorf AG 22331) and the absorbance was read at 625 nm (Multiskan GO, Thermo Scientific). The starch content was expressed in mg/mg^{-1} f. w.

2.6. Statistical Analysis

For the petri dish experiments, three independent assays were performed using three petri dishes for each sample and 15 seeds per dish in *Arabidopsis thaliana* and *Nicotiana tabacum,* 7 seeds for *Zea mays* and *Hordeum vulgare,* 20 seeds for *Lollium multiflorum* and 30 seeds for *Lactuca sativa*. The same principle was used for pot experiments where three independent assays were performed with at least three replicas each using 15 seeds per pot for *Lollium multiflorum*, *Lactuca sativa* and *Arabidopsis thaliana* and 4 seeds per pot for *Nicotiana tabacum* due to the plant size.

For biochemical quantification, each experiment was performed using a pool of plants from each independent experiment, with at least three independent technical replicates.

The results were expressed as mean ± standard deviation (SD) and Unpaired t-tests with Welch´s correction assuming two-tailed p values were performed.

All tests were performed with a 95% confidence level where $p<0,05$ and the statistical analysis was completed entirely with the GraphPad Prism 6 software (version 6.1.1).

3. Results and discussion

3.1. *Klebsormidium* sp. (1.5), a potential selective bioherbicide

In this work, different species of microalgae and cyanobacteria were studied to seek their potential use in the improvement of agricultural sustainable practices. In this first chapter, the species in study was a microalga belonging to the genus *Klebsormidium*, known as isolate 1.5, for which previous studies foresaw a selective bioherbicide potential (Rocha et al., 2021).

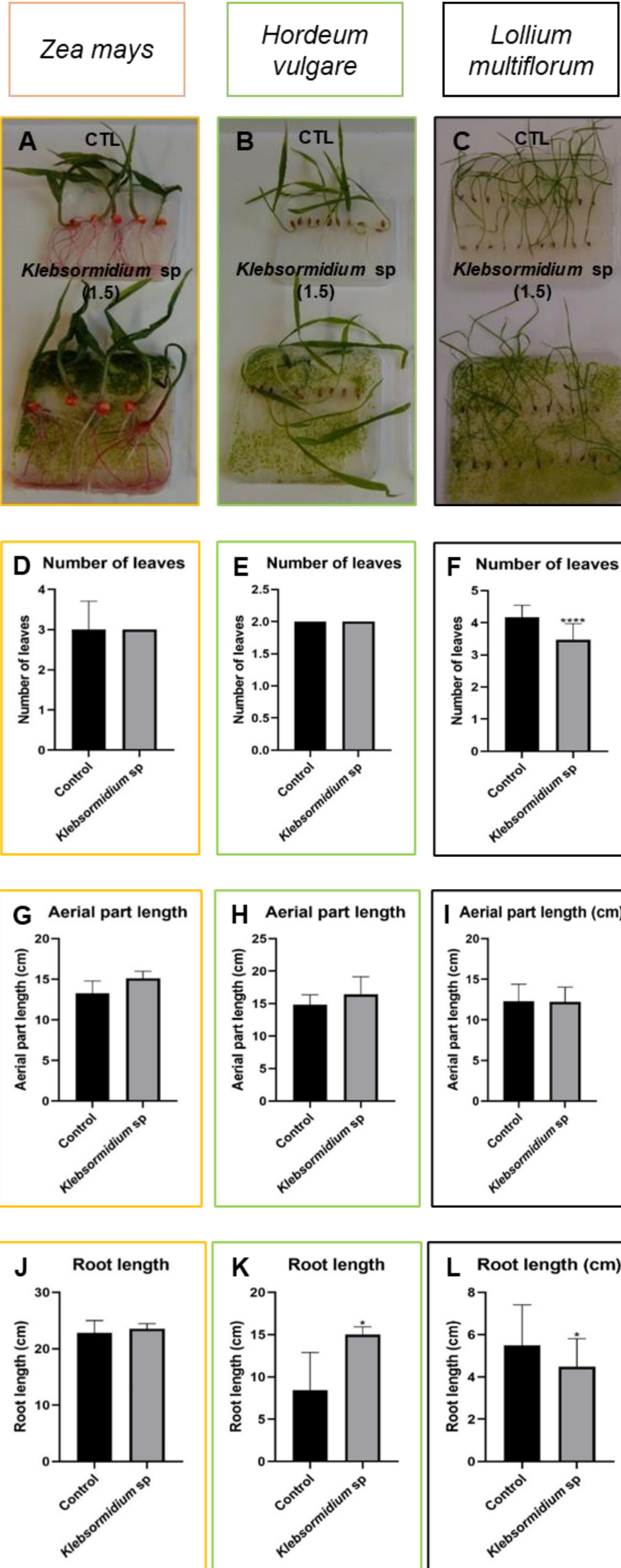
3.1.1. Petri dish assays

The goal of this work was to test the effects and the mode of action of the *Klebsormidium* sp. (1.5), in plant growth and a first approach was to perform small-scale petri dish assays using different plant species, monocotyledonous and dicotyledonous, to access their different susceptibility to the microalgae. For that, the plants were grown in petri dish with a plant nutritive medium, in the presence of *Klebsormidium*'s (1.5) growth medium, after centrifugation (exudates), for two weeks, and some plant biometric parameters were assessed.

A preliminary petri dish experiment was performed to evaluate the amount of *Klebsormidium* sp. (1.5) growth medium needed to observe the desired effects in plants. For that, we used *Arabidopsis thaliana*, since it was already known that this species was affected by the microalga presence. The *A. thaliana* plants were grown in the presence of three different amounts of *Klebsormidium* sp. (1.5) growth medium: 2mL, half of that quantity (1mL of the growth medium after centrifugation + 1 mL of BG_{11}) and 1/10 of that quantity (100 µL of the growth medium after centrifugation + 1900 µL of BG_{11}). After three weeks the plants were observed and the plant growth assessed. The normal and half concentrations led to the *A. thaliana* plants' death.

Despite showing effects at half concentration, for future petri dish assays, it was used 2 mL of medium to reduce the risk of other species not showing signs of effect at lower concentrations due to *A. thaliana* just being extremely sensitive compared to others.

The petri dish experiments were then performed with different plants species, the monocotyledonous *Zea mays*, *Hordeum vulgare* and *Lollium multiflorum* and the dicotyledonous *Lactuca sativa*, *Nicotiana tabacum* and *Arabidopsis thaliana*. The biometric parameters (number of leaves, aerial part and root fresh weight and aerial part and root length) were assessed after two weeks of growth.


Zea mays
Hordeum vulgare
Lollium multiflorum
A
B
C
CTL
Klebsormidium sp (1.5)
D Number of leaves
E Number of leaves
F Number of leaves
Number of leaves
G Aerial part length
H Aerial part length
I Aerial part length (cm)
Aerial part length (cm)
J Root length
K Root length
L Root length (cm)
Root length (cm)
Control
Klebsormidium sp
****
*

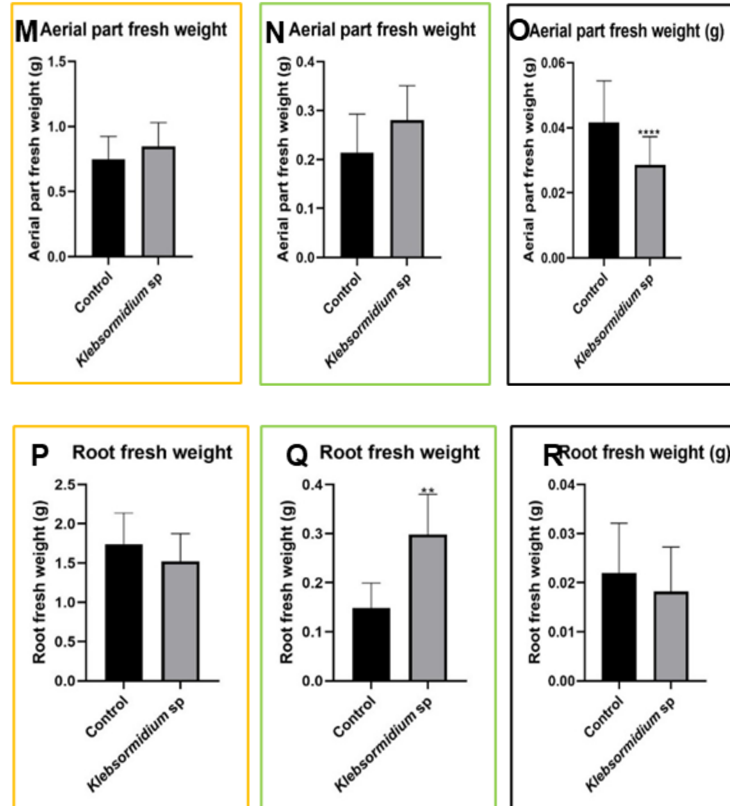
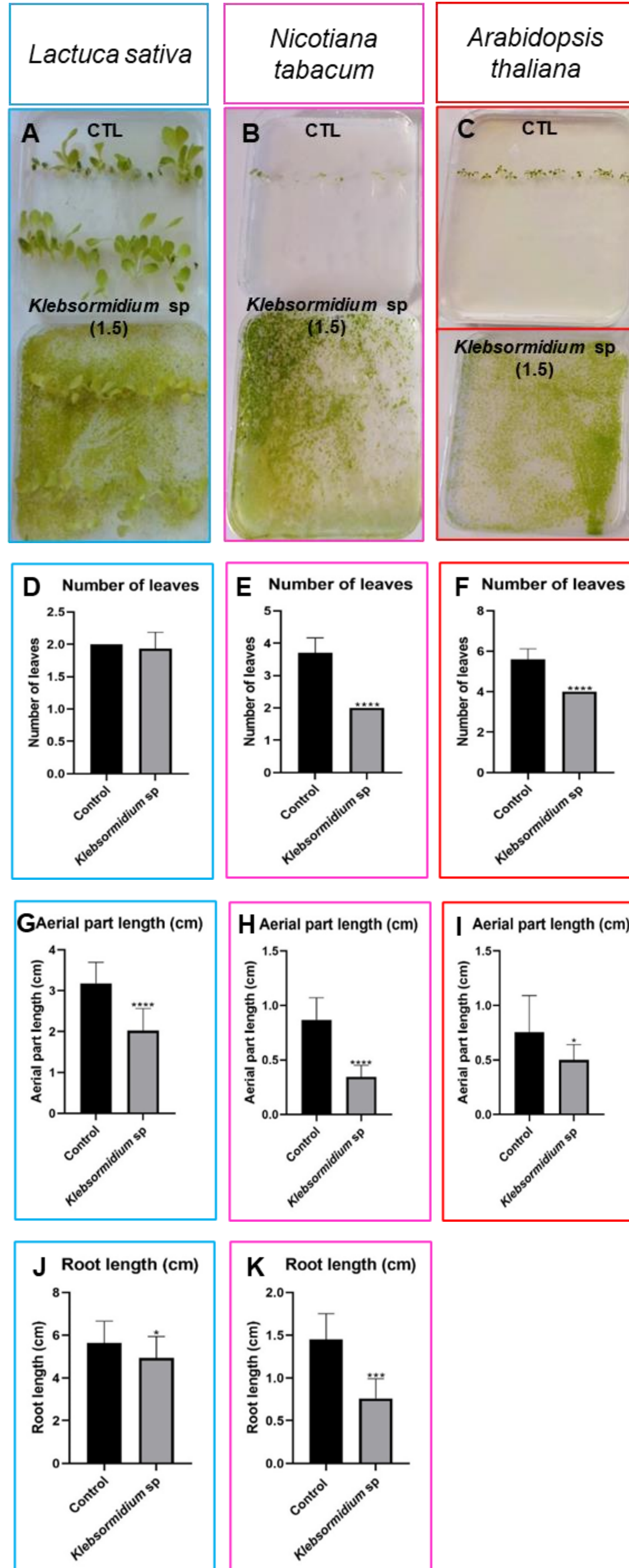


Figure 1 Effects of *Klebsormidium* sp. (1.5) on monocotyledonous plant growth, evaluated in petri dish experiments. Growth of *Zea mays (A), Hordeum vulgaris (B), and Lollium multiflorum (C),* in the absence (CTL) or presence of *Klebsormidium* sp. (1.5). Each color represents a different plant species: orange corresponds to *Zea mays*, green to *Hordeum vulgaris,* and black to *Lollium multiflorum.* Assessment of different growth parameters in the different plant species: number of leaves (D, E, F), aerial part length (G, H, I), root length (J, K, L), aerial part fresh weight (M, N, O), and root fresh weight (P, Q, R). Results are expressed as mean +/- SD. Unpaired Student's t-tests were performed with Welch's correction, for comparison with the control. Statistically relevant values are indicated * $P<0.05$; ** $P<0.01$; **** $P<0.0001$.

The results collected for the monocotyledonous can be seen in **figure 1**. Looking at the results gathered for maize (*Zea mays*) it is possible to observe that there are no visible changes in the visual aspect of the plants, as there are no significant differences in any of the biometric parameters assessed. The same was observed for the monocot barley (*Hordeum vulgare*), there were no visible changes in the visual aspect of the plants, and no negative effects were observed in the biometric parameters. Furthermore, *Klebsormidium* sp. (1.5) leads to a significant increase in barley root length and weight.

As for *L. multiflorum* the results were a bit different, in **figure 1** is possible to see that although the visual aspect remains the same, some biometric parameters were slightly reduced, such as the fresh weight of the aerial part and the length of the root.


Lactuca sativa
Nicotiana tabacum
Arabidopsis thaliana
A CTL
Klebsormidium sp (1.5)
B CTL
Klebsormidium sp (1.5)
C CTL
Klebsormidium sp (1.5)
D Number of leaves
E Number of leaves
F Number of leaves
Number of leaves
Control
Klebsormidium sp
****
G Aerial part length (cm)
H Aerial part length (cm)
I Aerial part length (cm)
Aerial part length (cm)
*
J Root length (cm)
K Root length (cm)
Root length (cm)
***

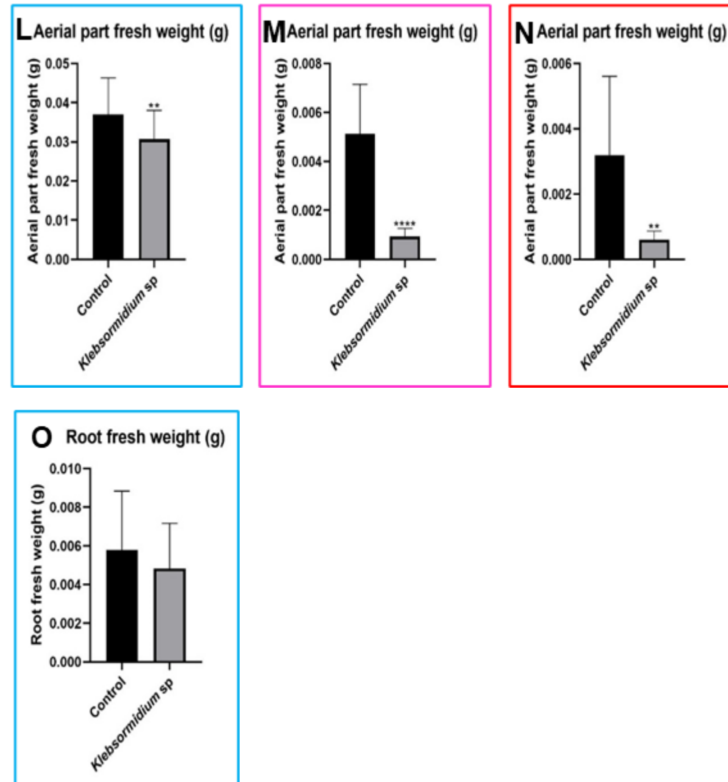


Figure 2 Effect of *Klebsormidium* sp. (1.5) on dicotyledonous plant growth, evaluated by petri dish experiments. Growth of *Lactuca sativa (A), Nicotiana tabacum (B), and Arabidopsis thaliana (C),* in the absence (CTL) or presence of *Klebsormidium* sp. (1.5). Each color represents a different plant species: blue corresponds to *Lactuca sativa*, pink to *Nicotiana tabacum,* and red to *Arabidopsis thaliana.* Assessment of different growth parameters in the different plant species: number of leaves (D, E, F), aerial part length (G, H, I), root length (J, K), aerial part fresh weight (L, M, N), and root fresh weight (O). Results are expressed as mean +/- SD. Unpaired Student's t-tests were performed with Welch's correction, for comparison with the control. Statistically relevant values are indicated * $P<0.05$; ** $P<0.01$; *** $P<0.001$; **** $P<0.0001$.

The results presented in **figure 2**, concern the effect of *Klebsormidium* sp. (1.5) in the growth of dicotyledonous plants. For lettuce (*L. sativa*), the results were obtained after 2 weeks and we can see that the plants grown in the presence of the microalgae became visibly chlorotic and the size was significantly reduced, especially the aerial part weight and aerial part and root length.

For *Nicotiana tabacum* results were collected after 3 weeks and their size remained extremely small when grown in the presence of the microalgae *Klebsormidium* sp. (1.5). Due to the small size, it was impossible to collect data concerning the root fresh weight. However, it is still possible to see that all the other parameters were significantly reduced, and the plants became visibly chlorotic.

The results collected for the dicot *A. thaliana*, show a significant decrease in the plant size. Like, *N. tabacum*, some parameters were impossible to evaluate due to the reduced size of the plants in the study, namely the root fresh weight and length. Like the other

dicotyledonous, the plants became visibly chlorotic and some died before the end of the essay, (roughly 10 days after sown).

Lastly, is possible to see that despite the centrifugation, the *Klebsormidium* sp. (1.5) still grew in the plates where it was applied.

Table 1 Percentage of variation of several growth parameters of the plants of different species treated with the microalgae, compared to the control plants. Statistically relevant values are expressed relative to control and expressed as * P<0.05; ** P<0.01; *** P<0.001; **** P<0.0001.

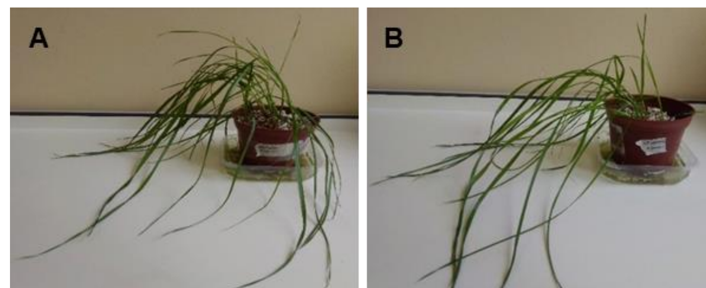
Plant species	Number of leaves (%)	Aerial part weight (%)	Aerial part length (%)	Root length (%)	Root fresh weight (%)
Hordeum vulgare	0.00	31.14	10.70	77.87*	101.90**
Zea mays	0.00	13.37	13.86	3.33	-12.53
Lollium multiflorum	-3.35 ****	-31.24****	-0.57	-18.52*	-17.15
Lactuca sativa	-16.80	-20.97**	-36.32****	-12.70 *	-16.42
Nicotiana tabacum	-45.95 ****	-81.60 ****	-60.06****	-47.59**	No value
Arabidopsis thaliana	-28.57 ****	-81.20 **	-33.73*	No value	No value

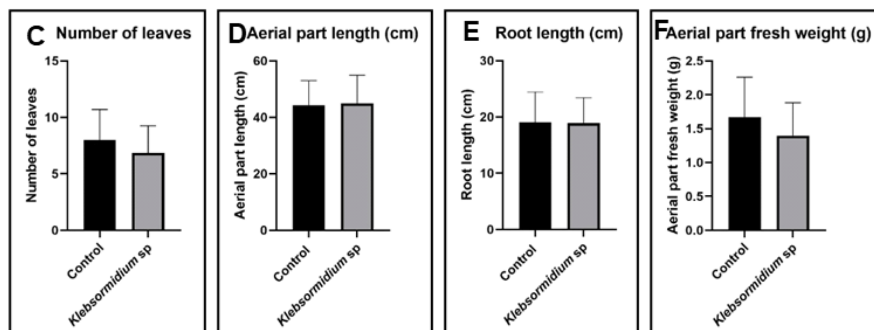
The overall results were compiled in a table (**table 1**) that shows the variation, in percentage compared to the control of several parameters in all the species tested. In general, it is possible to see a difference between monocotyledonous and dicotyledonous plants, since the dicotyledonous exhibit a reduction in size while most monocotyledonous show an increase in some of the parameters, especially in the root length and weight in barley (*H. vulgare*). The exception was *L. multiflorum* that showed a slight decrease in most of the parameters studied, like number of leaves, aerial part weight and root length, although the visual aspect was not affected. Within the dicotyledonous, lettuce was the species with the lowest decrease. *Arabidopsis* and *Nicotiana* showed a most drastic decrease in terms of growth percentage. The reduction was so drastic that it was impossible to collect values for root length and weight due to the plants' small size.

3.1.2. Pot assays

From the results collected for petri dish experiments, some plant species were chosen to be used for large-scale experiments - pot assays. For these assays, *L. multiflorum* was used as a representative of the monocotyledonous species, a tolerant species, even though being the only monocots showing some negative effects of the *Klebsormidium* sp. (1.5) application. *L. sativa* was chosen in these assays due to the economic interest and as a representative of a mildly susceptible species since it was the less affected dicotyledonous. *A. thaliana* was the species chosen as a susceptible species since in petri dish assays the plants died in the presence of *Klebsormidium* sp. (1.5) exudates and may represent an example of a weed from an agronomic perspective. However, during the pot experiments complications appeared with the growth of this species, so as a substitute in case of need *N. tabacum* was used as it proved to be also quite sensitive to the presence of the *Klebsormidium* sp. (1.5) exudates.

The plants were sown in pots with a mix of vermiculite and perlite and watered with a nutritive solution and after reaching an adequate size, a *Klebsormidium* sp. (1.5) extract, obtained by cell sonication followed by centrifugation, was sprayed. Once they reached 1-2 months old, the plants were collected and then some biometric and biochemical parameters were assessed.





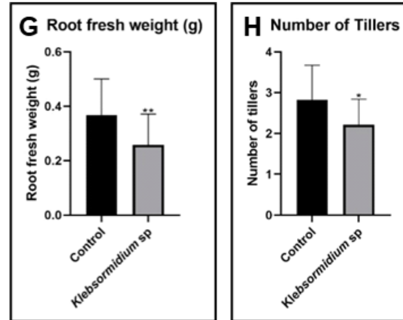
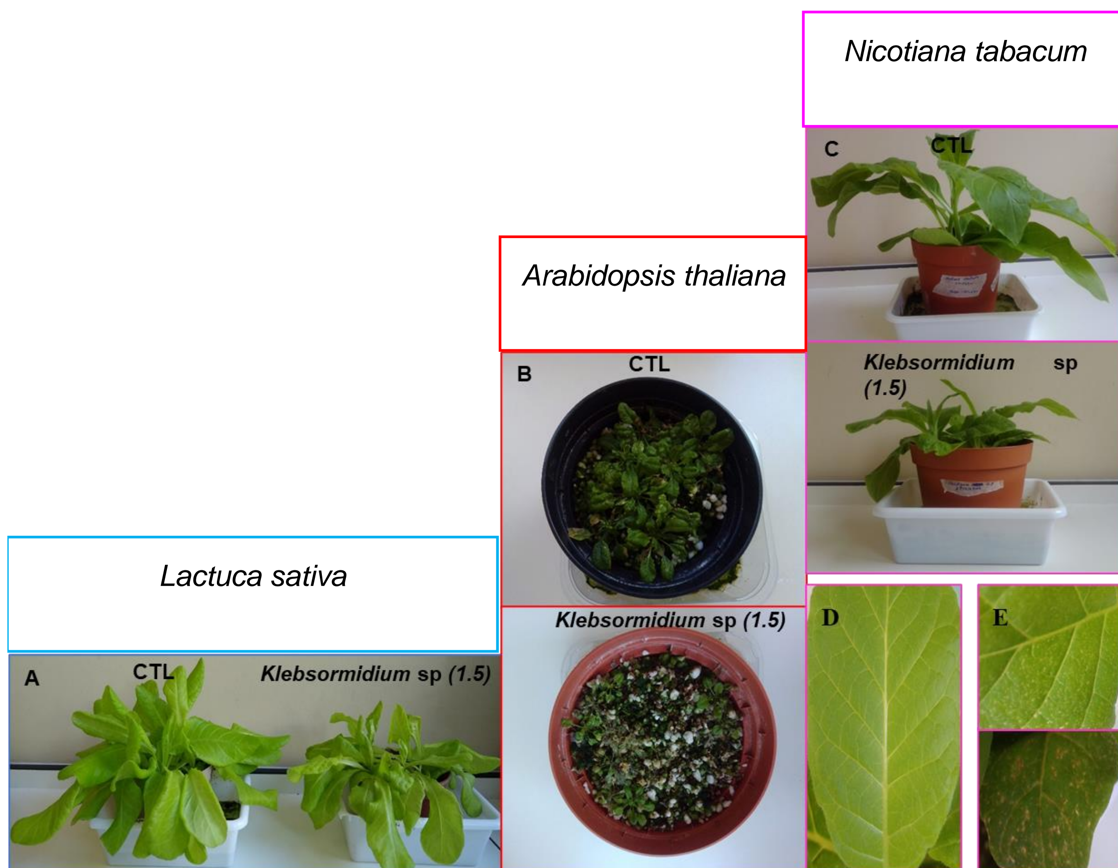
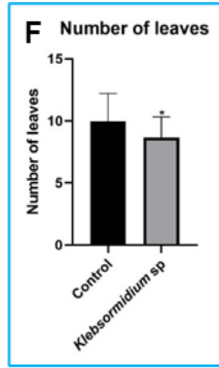
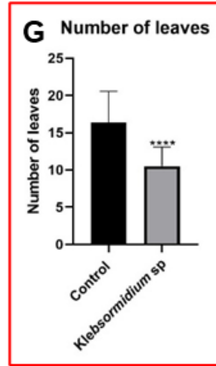


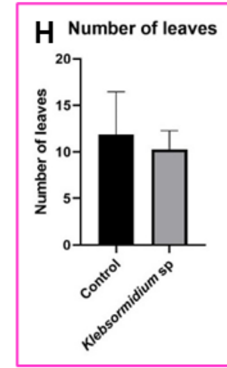
Figure 3 Effects of the application of *Klebsormidium* sp. (1.5) on plant growth evaluated in pot assays. Growth of *Lollium multiflorum* in the absence (A) or after application of *Klebsormidium* sp. (1.5) exudates (B). Assessment of different growth parameters: number of leaves (C), aerial part length (D), root length (E), aerial part fresh weight (F), root fresh weight (G), and number of tillers (H). Results are expressed as mean +/- SD. Unpaired Student's t-tests were performed with Welch's correction, for comparison with the control. Statistically relevant values are indicated * $P<0.05$; ** $P<0.01$.

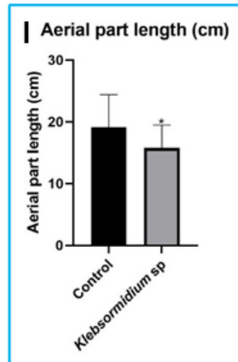
In **figure 3** it is possible to see the results collect for *L. multiflorum* after one month of *growth*, and there are no visual differences in the plants sprayed with the *Klebsormidium* sp. exudates. However, the root fresh weight and the number of tillers were slightly reduced in the presence of *Klebsormidium* sp.

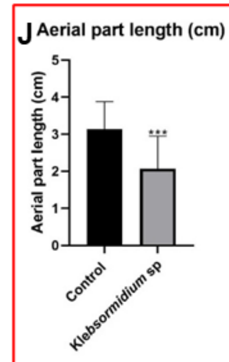


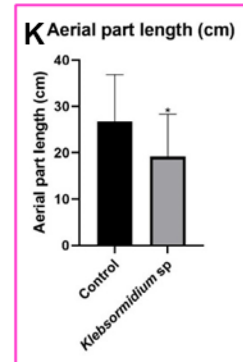

F
Number of leaves
Number of leaves
15
10
5
0
*
Control
Klebsormidium sp

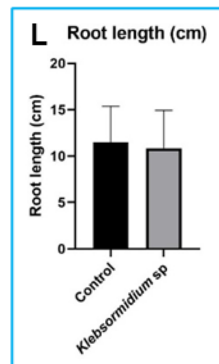

G
Number of leaves
Number of leaves
25
20
15
10
5
0
****
Control
Klebsormidium sp

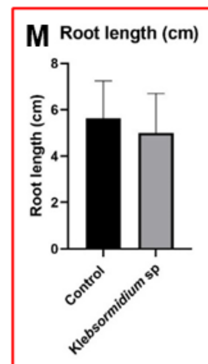

H
Number of leaves
Number of leaves
20
15
10
5
0
Control
Klebsormidium sp

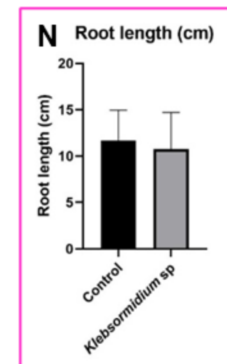

I
Aerial part length (cm)
Aerial part length (cm)
30
20
10
0
*
Control
Klebsormidium sp

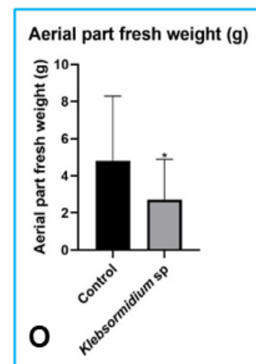

J
Aerial part length (cm)
Aerial part length (cm)
5
4
3
2
1
0
***
Control
Klebsormidium sp

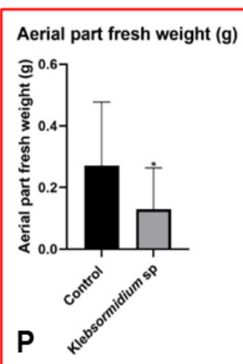

K
Aerial part length (cm)
Aerial part length (cm)
40
30
20
10
0
*
Control
Klebsormidium sp

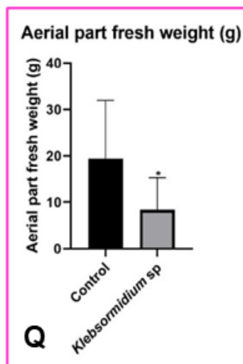

L
Root length (cm)
Root length (cm)
20
15
10
5
0
Control
Klebsormidium sp


M
Root length (cm)
Root length (cm)
8
6
4
2
0
Control
Klebsormidium sp


N
Root length (cm)
Root length (cm)
20
15
10
5
0
Control
Klebsormidium sp


Aerial part fresh weight (g)
Aerial part fresh weight (g)
10
8
6
4
2
0
*
Control
Klebsormidium sp
O


Aerial part fresh weight (g)
Aerial part fresh weight (g)
0.6
0.4
0.2
0.0
*
Control
Klebsormidium sp
P


Aerial part fresh weight (g)
Aerial part fresh weight (g)
40
30
20
10
0
*
Control
Klebsormidium sp
Q

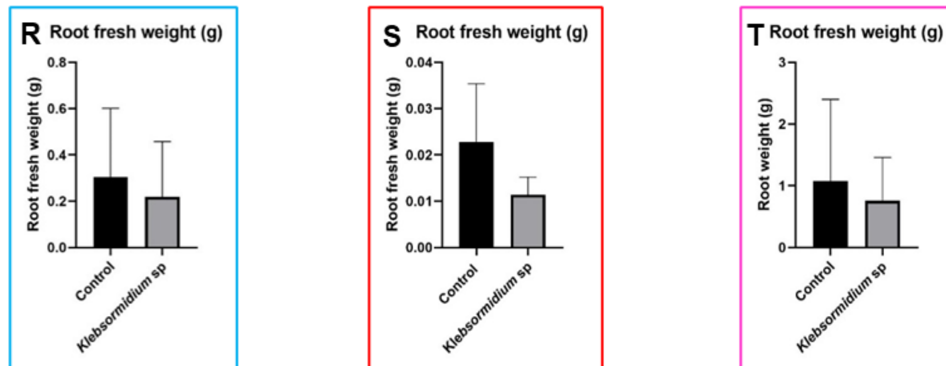


Figure 4 Effects of the application of *Klebsormidium* sp. (1.5) on dicotyledonous plant growth evaluated in pot assays. Growth of *Lactuca sativa (A), Nicotiana tabacum (B), and Arabidopsis thaliana (C)* in the absence (CTL) or after application of *Klebsormidium* sp. (1.5). Detail of a *N. tabacum* leaf not sprayed (D) and sprayed with *Klebsormidium* sp. (1.5) (E). Each color represents a different plant species: blue corresponds to *Lactuca sativa*, pink to *Nicotiana tabacum,* and red to *Arabidopsis thaliana.* Assessment of different growth parameters: number of leaves (F, G, H), aerial part length (I, J, K), root length (L, M, N), aerial part fresh weight (O, P, Q), root fresh weight (R, S, T). Results are expressed as mean +/- SD. Unpaired Student's t-tests were performed with Welch's correction, for comparison with the control. Statistically relevant values are indicated * $P<0.05$; ** $P<0.01$; **** $P<0.0001$.

The results collected for dicotyledonous species are shown in **figure 4**. In *L. sativa* it is possible to observe some differences between the control plants and the plants treated with *Klebsormidium* sp. (1.5). Visually it is possible to perceive a reduction in the size of the plants and quantification of the biometric parameters shows a statistically relevant reduction, namely, in the number of leaves and the weight and length of the aerial part.

When looking at the dicotyledonous *A. thaliana* it is visible, in addition to a reduction in size, necrosis of the leaves, showing an overall degradation of the plant status. All this ends up resulting in a statistically significant reduction in the number of leaves and the weight and length of the aerial part of the plants treated with *Klebsormidium* sp. (1.5).

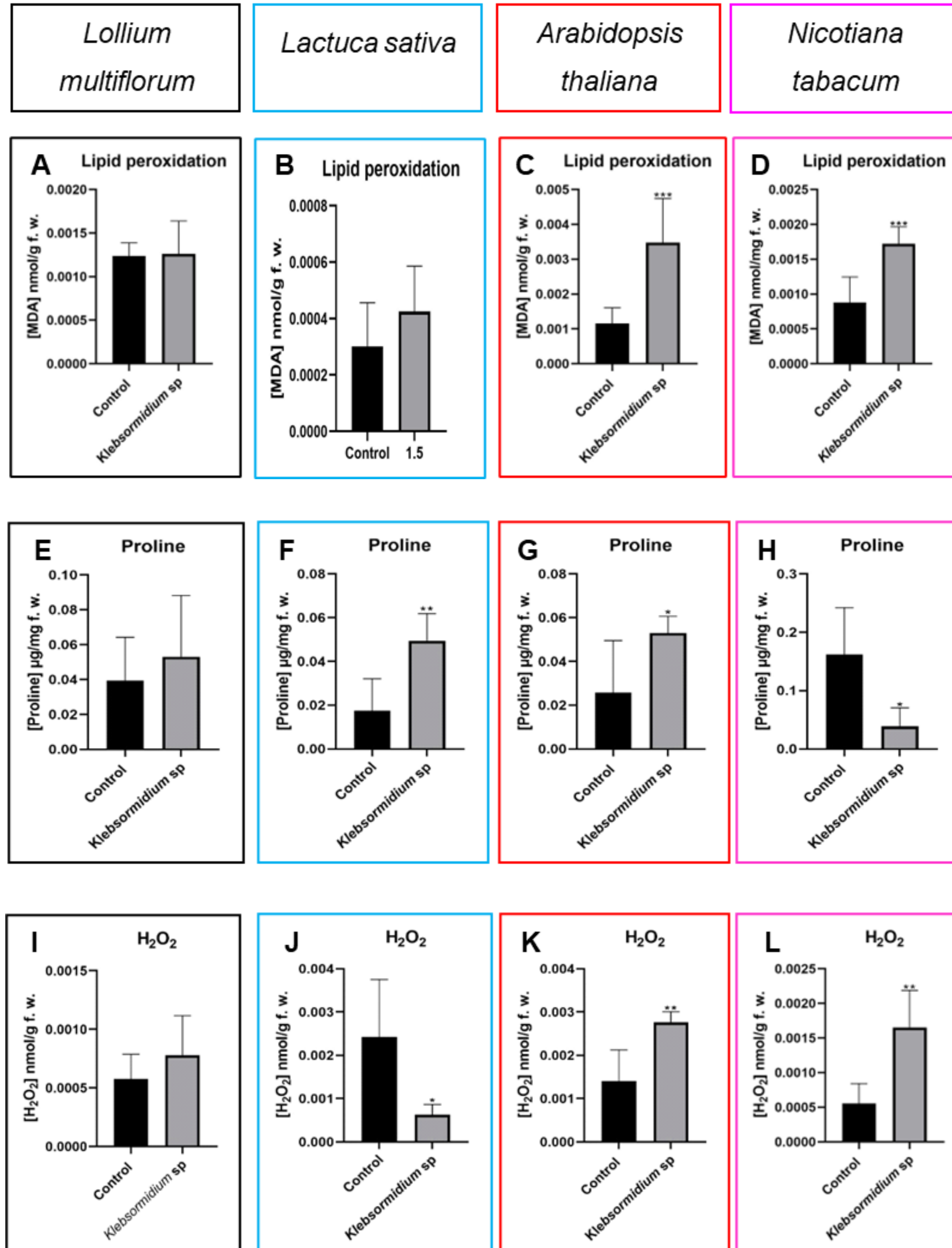
Also, in *N. tabacum*, it is possible to observe a significant reduction in plant growth. In addition to growth impairment, it is possible to observe that the leaves of plants treated with *Klebsormidium* sp. (1.5) showed marks of necrosis. Furthermore, a significant decrease in the length and weight of the aerial part of the plants was observed in the plants treated with the microalga.

3.1.3. Biochemical parameters assays

To further characterize the effect of the microalgae *Klebsormidium* sp. on the different plant species the plant material collected from the pot assays was used to quantify some biochemical parameters. Some enzymatic and non-enzymatic parameters associated with stress and productivity-related parameters were quantified since the plants treated with *Klebsormidium* sp. (1.5) showed a reduction in growth and symptoms of stress like chlorosis and necrosis.

3.1.3.1. Stress related parameters

To respond to stress caused by biotic and abiotic stress plants developed a variety of mechanisms, that can be enzymatic and non-enzymatic (Nadarajah, 2020). For this work, the enzymatic parameters evaluated were the activity of catalase (CAT), superoxide dismutase (SOD) and Ascorbate peroxidase (APX), while the non-enzymatic parameters assessed were lipid peroxidation, proline, H_2O_2 and reduced glutathione contents. The results obtained can be seen in **figure 5.**



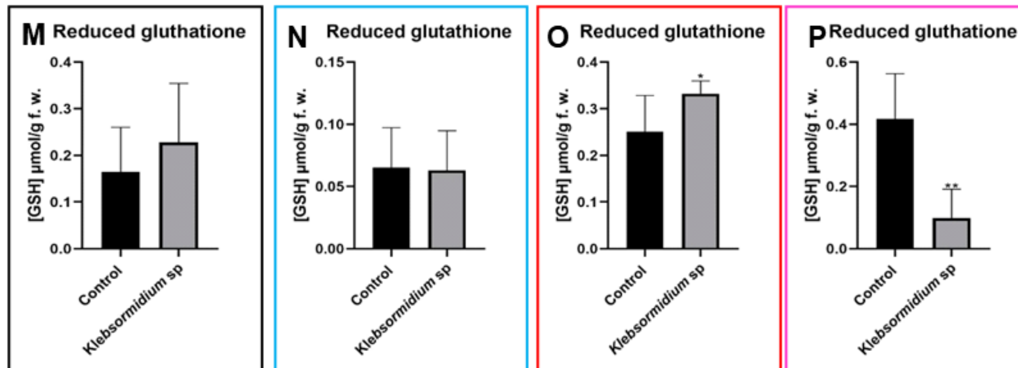


Figure 5 Quantification of stress-related parameters in *Lollium multiflorum, Lactuca sativa, Arabidopsis thaliana* and *Nicotiana tabacum* plants grown with the application of *Klebsormidium* sp. in the form of spray and without this application (Control). Each color represents a different plant species: black corresponds to *Lollium multiflorum*, blue to *Lactuca sativa*, red to *Arabidopsis thaliana* and pink to *Nicotiana tabacum.* Lipid peroxidation quantification (A, B, C, D), proline quantification (E, F, G, H), H2O2 quantification (I, J, K, L), and reduced glutathione quantification (M, N, O, P). Results are expressed as mean +/- SD. Unpaired Student's t-tests were performed with Welch's correction, for comparison with the control. Statistically relevant values are indicated * P<0.05; ** P<0.01; *** P<0.001; **** P<0.0001.

Concerning the non-enzymatic stress parameters presented in **figure 5**, lipid peroxidation a parameter that indicates cellular membrane damage (Pospíšil and Yamamoto, 2017) was quantified. It is possible to observe a significant increase in the most sensitive plants *A. thaliana* and *N. tabacum*, which indicates that the presence of *Klebsormidium* sp. led to damage in the cells of the susceptible plants.

Another parameter indicative of the stress status of the plants is the proline content (Verbruggen and Hermans, 2008). It is possible to observe that, contrary to the monocotyledonous *L. multiflorum* where there were no significant changes, in most dicotyledonous (*L. sativa* and *A. thaliana*) there was a significant increase in proline levels, except in the case of *N. tabacum* in which there was a significant reduction.

The H_2O_2 levels, also indicative of the activation of the defensive mechanisms of the plants (Černý et al., 2018) showed a significant increase in the most sensitive dicotyledonous (*A. thaliana* and *N. tabacum*) while in the less sensitive (*L. sativa*) there was a decrease, whereas in the resistant monocotyledonous there were no significant differences.

The last non-enzymatic parameter evaluated was the levels of reduced glutathione, a molecule that alleviates oxidative stress (May et al., 1998; Couto et al., 2016; Khan et al., 2020) and this only changed significantly in the most sensitive species, with an increase in *A. thaliana* and a decrease in *N. tabacum.*

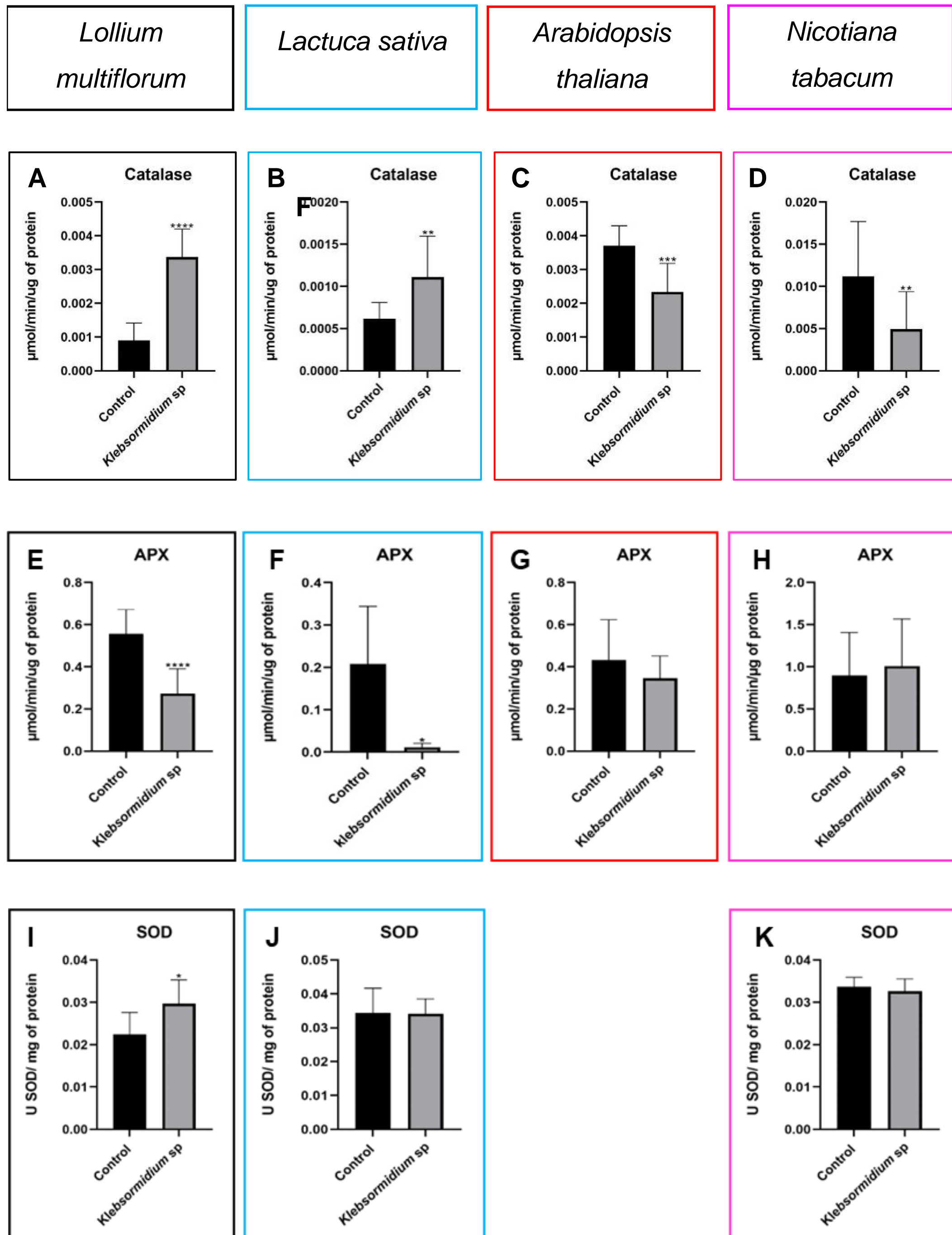


Figure 6 Quantification of some stress-related enzymes in *Lollium multiflorum, Lactuca sativa, Arabidopsis thaliana* and *Nicotiana tabacum* plants grown with the application of *Klebsormidium* sp. (1.5) in the form of spray and without this application (Control). Each color represents a different plant species: black corresponds to *Lollium multiflorum*, blue to *Lactuca sativa*, red to *Arabidopsis thaliana* and pink to *Nicotiana tabacum.* Catalase quantification (A, B, C, D), APX quantification (E, F, G, H), and SOD quantification (I, J, K). Results are expressed as mean +/- SD. Unpaired Student's t-tests were performed with Welch's correction for comparison with the control. Statistically relevant values are indicated * P<0.05; ** P<0.01; *** P<0.001; **** P<0.0001.

Also, the activity levels of some stress-related enzymes were studied and in **figure 6** it is possible to see some differences between the less sensitive and the more sensitive species. Concerning the activity of catalase, it is possible to observe that it increases

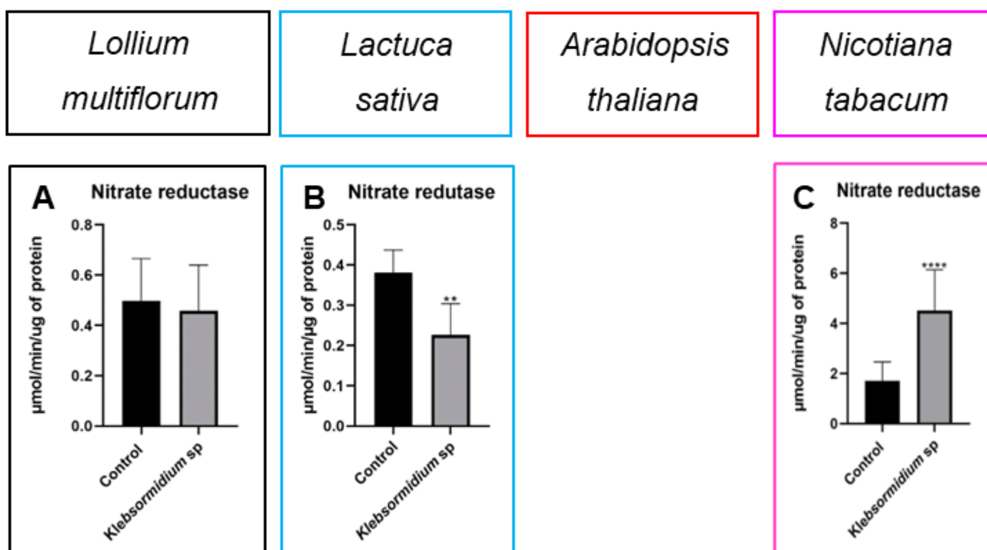
significantly in *L. multiflorum* and *L. sativa* and drops significantly in the two most sensitive dicotyledonous species, while significant changes in ascorbate peroxidase (APX) activity were only found in the least susceptible species, *L. multiflorum* and *L. sativa*, where there was a significant decrease.

The last parameter evaluated was the SOD enzyme activity, which was not possible to quantify in *A. thaliana*, as it was small and difficult to grow, so it was not possible to obtain enough plant mass to carry out the test. Even so, it is possible to see that only the monocotyledonous *L. multiflorum* had significant changes in SOD activity with a significant increase.

3.1.3.2. Productivity related parameters

However, not only aspects related to stress were quantified to allow a vision of the general physiological states of the plants, some parameters related to productivity were also assessed. Thus, the activity of certain enzymes related to productivity was evaluated (**figure 7**). Regarding the enzyme nitrate reductase, it is possible to see that such as SOD it was not possible to collect *A. thaliana* values but is still possible to observe that only the dicotyledonous studied had significant differences, namely a decrease in the more tolerant dicotyledonous species, *L. sativa* and an increase in the susceptible *N. tabacum*.

Another productivity enzyme studied was GS whose activity levels only changed significantly in the species *L. sativa* with a small decrease.



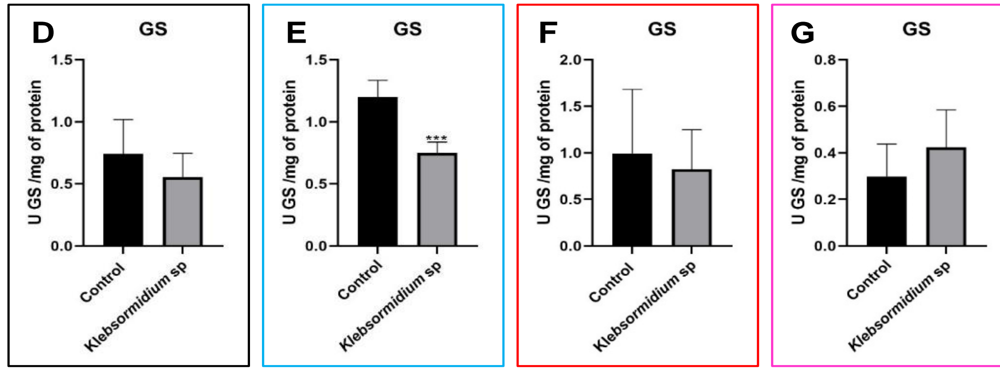


Figure 7 Quantification of some stress-related enzymes in *Lollium multiflorum, Lactuca sativa, Arabidopsis thaliana* and *Nicotiana tabacum* plants grown with the application of *Klebsormidium* sp. (1.5) in the form of spray and without this application (Control). Each color represents a different plant species: black corresponds to *Lollium multiflorum*, blue to *Lactuca sativa*, red to *Arabidopsis thaliana* and pink to *Nicotiana tabacum.* Catalase quantification (A, B, C, D), APX quantification (E, F, G, H), and SOD quantification (I, J, K). Results are expressed as mean +/- SD. Unpaired Student's t-tests were performed with Welch's correction for comparison with the control. Statistically relevant values are indicated ** P<0.01; *** P<0.001; **** P<0.0001.

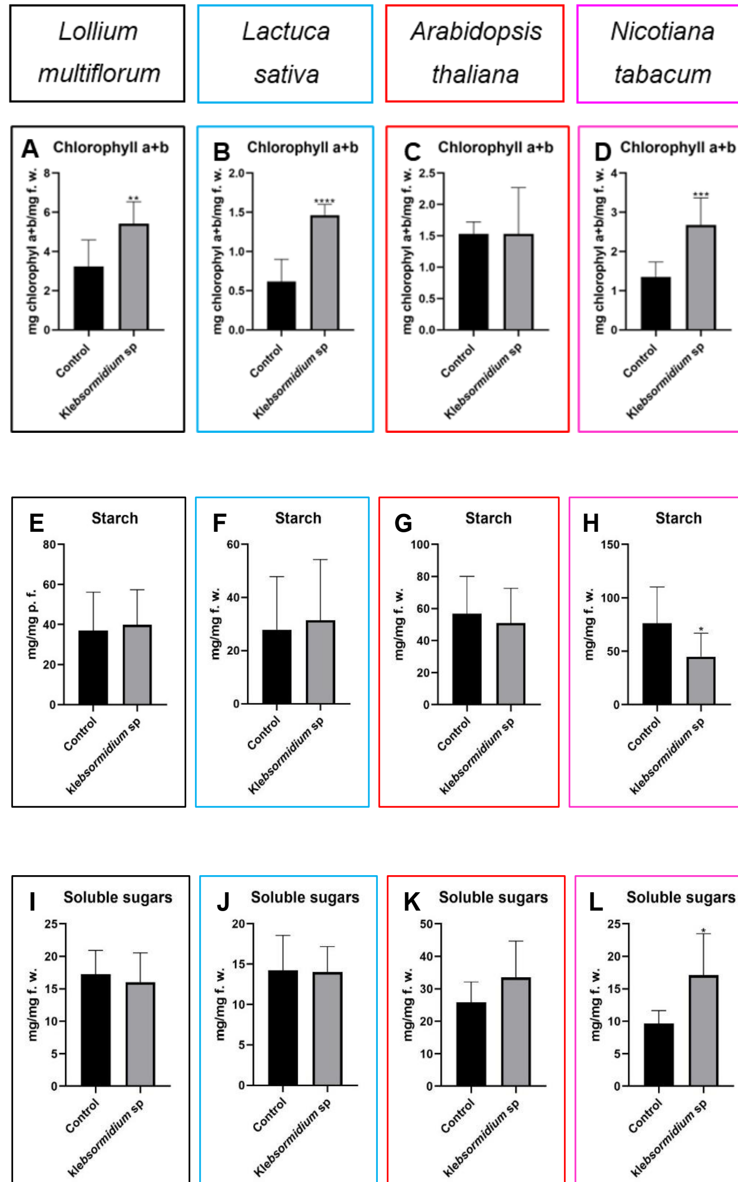


Figure 8 Quantification of some productivity-related parameters in *Lollium multiflorum, Lactuca sativa, Arabidopsis thaliana* and *Nicotiana tabacum* plants grown with the application of *Klebsormidium* sp. (1.5) in the form of spray and without this application (Control). Each color represents a different plant species: black corresponds to *Lollium multiflorum*, blue to *Lactuca sativa*, red to *Arabidopsis thaliana* and pink to *Nicotiana tabacum.* Chlorophyll a+b quantification (A, B, C, D), starch quantification (E, F, G, H), and soluble sugars quantification (I, J, K, L). Results are expressed as mean +/- SD. Unpaired Student's t-tests were performed with Welch's correction for comparison with the control. Statistically relevant values are indicated * P<0.05; ** P<0.01; *** P<0.001; **** P<0.0001.

Lastly, we studied the levels of some non-enzymatic productivity indicators (**figure 8**), chlorophyll, starch and sugar contents. Regarding chlorophyll a and b levels it is possible to observe a significant increase in all the studied species except *A. thaliana*. However, concerning the sugar/starch contents, the only significant change was detected in *N. tabacum* with a decrease in starch content and an increase in the level of soluble sugars.

3.2. Molecular effects of *Klebsormidium* sp. (1.5)

After observing the effects caused by *Klebsormidium* sp. (1,5) it was important to assess the cause for those effects. To accomplish that different experiments were performed. First, an acetonic extract of the compounds produced by the *Klebsormidium* sp. (1.5) was tested in A. *thaliana*, second, it was analysed if the effect caused by the microalga was similar to the effect caused by the 2,4-D, a chemical selective herbicide and finally the effect of the microalgae in the plants' auxin levels was assessed by using reporter plants expressing a reporter gene under the control of an auxin-responsive promotor. To compare the results in these experiments another microalga from the *Klebsormidium* genus, also collected in the scope of the GreenRehab project, named 2.1b, that does not show the bioherbicide effect was used.

3.2.1. Attempt to isolate the compound responsible for the *Klebsormidium* sp. (1.5) effect

First, petri dish assays comparing the effects of both *Klebsormidium* were performed, using *A. thaliana* to confirm that 2.1b and 1.5 have different effects in this plant species.

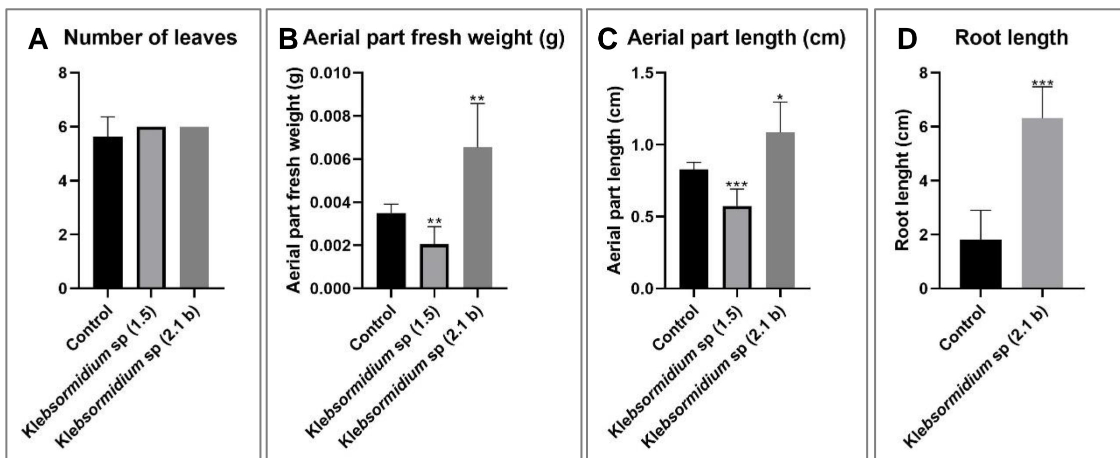


Figure 9 Effect of *Klebsormidium* sp. (1.5) and *Klebsormidium* sp. (2.1 b) on *Arabidopsis thaliana,* in the absence (control) or presence of the microalgae. Assessment of different growth parameters for the different microalgae species: number of leaves (A, D), aerial part length (B, E), aerial part fresh weight (C, F), and root length (G). Results are expressed as mean +/- SD. Unpaired Student's t-tests were performed with Welch's correction, for comparison with the control. Statistically relevant values are indicated * P<0.05; ** P<0.01; *** P<0.001.

As it is possible to see in **figure 9**, and as previously observed the *Klebsormidium* sp. 1.5 exudates lead to a reduction in the size of *A. thaliana* plants, and once again that reduction was so dramatic that prevented the collection of results concerning the root length.

On the other hand, the presence of *Klebsormidium* sp. 2.1b exudates, led to an increase in the growth of *A. thaliana*, increasing various parameters, namely the aerial part and root length and the aerial part fresh weight.

Having in mind that the two *Klebsormidium* have different effects in *A. thaliana* plants, we tried to extract the compounds produced by both species by acetonic extraction and use these extracts to confirm the algae effects. For that *A. thaliana* plants were grown in petri dish in the presence of the two extracts to see if the compound or compounds responsible for the negative effect were present in the extract and therefore could be further identified.

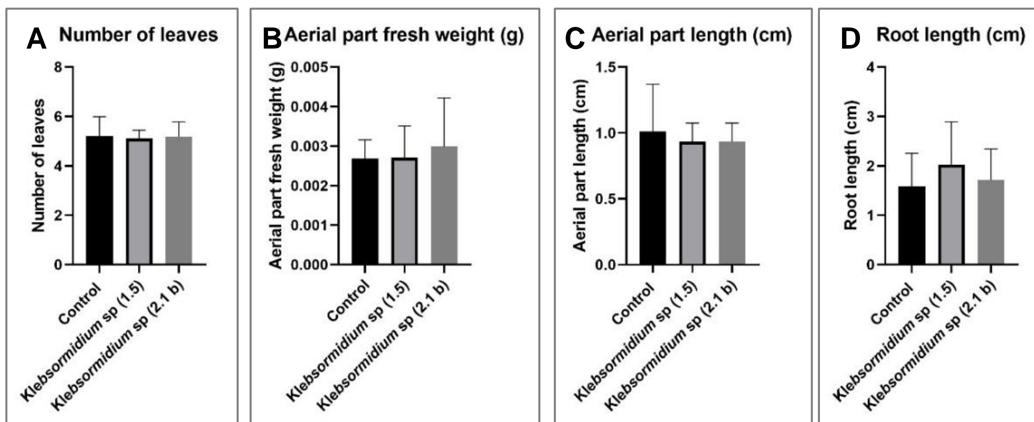


Figure 10 Effect of the compound extracted from *Klebsormidium* sp. (1.5) and *Klebsormidium* sp. (2.1 b) on *Arabidopsis thaliana,* in the absence (control) or presence of extracted compounds. Assessment of different growth parameters for the different microalgae species: number of leaves (A, E), aerial part length (B, F), aerial part fresh weight (C, G), and root length (D, H). Results are expressed as mean +/- SD. Unpaired Student's t-tests were performed with Welch's correction, for comparison with the control. No statistically relevant values are indicated.

In **figure 10**, it is possible to see that both extracts collected from *Klebsormidium* sp. 1.5 and 2.1b had no effect on plants since none of the parameters assessed had significant changes, meaning that we could not extract the compound(s) responsible for the *Klebsormidium* sp. (1.5) herbicide effect.

3.2.2. Assessing an auxin-like effect of Klebsormidium 1.5

To assess if the *Klebsormidium* sp. 1.5 could act in a similar way to the herbicide 2,4-D, a synthetic auxin, and also to detect changes in the auxin contents of *A. thaliana* treated with the microalgae, transgenic plants expressing the reporter gene GUS under the control of the auxin-responsive promoter DR5 (transgenic DR5 plants) were grown in petri dish in the presence of *Klebsormidium* sp. 1.5, *Klebsormidium* sp. 2.1b and in the presence of different concentrations of 2,4-D: 1 mM, 0.1 mM, 0.01 mM and 0.001 mM. The growth of the plants was observed and as can be seen in **figure 11** a marked decrease in growth was observed in plants treated with all 2,4D concentrations (except for the 0,001mM) and *Klebsormidium* sp. (1.5) that causes an effect similar to 0.01 mM concentration of 2,4D.

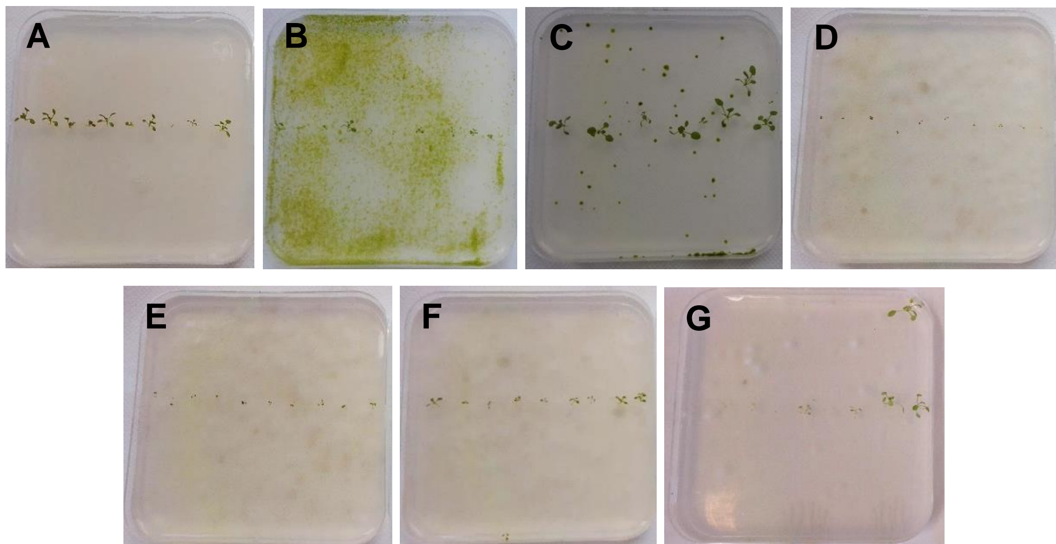
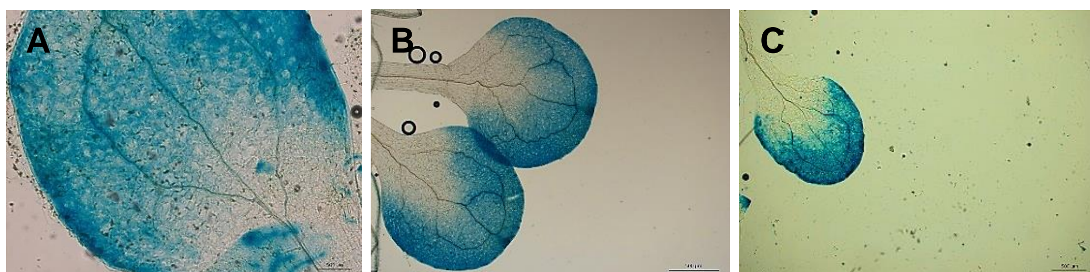


Figure 11 Growth of DR5 transgenic plants in the absence of compounds (control A), presence of *Klebsormidium* sp. (1.5) (B), *Klebsormidium* (2.1 b) (C) and presence of 2,4-D at 1 mM (D), 0,1 mM (E), 0,01 mM (F) and 0,001 mM (G).

Furthermore, the activity of the DR5 promotor was visualized by detecting the GUS stain (**figure 12**) in all these plants, trying to compare the levels of plant auxin with the herbicide effect.



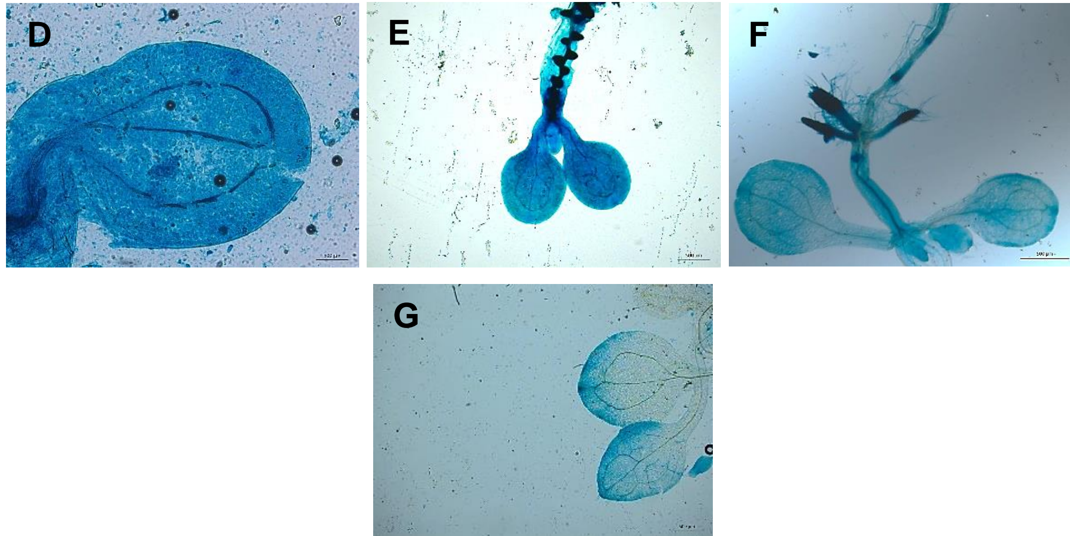


Figure 12 GUS staining indicative of auxin distribution in *Arabidopsis thaliana*. Control (A), plant grown in the presence of Klebsormidium sp. (2.1 b) (B), plant grown in the presence of Klebsormidium sp. (1.5) (C), plant grown in the presence of 2,4-D at 1 mM (D), 0,1 mM (E), 0,01 mM (F) and 0,001 mM (G).

As it was possible to observe in **figure 12**, in the 2,4-D treated plants there are differences in GUS activity, increasing the levels of GUS stain intensity according to the concentration of 2,4 D used, the higher the concentration of the synthetic auxin, the higher the plant auxin levels. However, both *Klebsormidium* treated plants had levels of auxin similar to the control, excluding an auxin-like effect of *Klebsormidium* 1.5 herbicide effect.

3.2.3. Discussion

Weeds are plants that severely limit agricultural production. They compete with crops for water, gases, nutrients, space, light, and other growth resources, and they can also serve as hosts for pests and diseases, which causes considerable yield losses. Therefore, weed control is an important agronomic practice in agriculture, so herbicide use has increased around the world (Hasan et al., 2021).

As it was seen in the first part of this work, the organism in study, a microalga from the genus *Klebsormidium*, known as isolate 1.5, has a severe effect on the growth of the tested dicotyledonous plants. As it was possible to observe, in small-scale petri dish experiments, the presence of the growth medium of a one-month *Klebsormidium* sp. (1.5) culture, which contains the microorganism exudates, led to a decrease in size of all the dicotyledonous plants and cause death in the most sensitive species *A. thaliana*, while the monocotyledonous *L. multiflorum*, *H. vulgare* and *Z. mays* remain almost unaffected, suggesting that *Klebsormidium* sp. (1.5) may have a selective bioherbicide effect on dicotyledonous plants. Their effect may be due to some compound exudated

by the algae, or by the algae *per se* as, despite the centrifugation, some microalgae grown in the petri dishes.

From these petri dish results, it was possible to select the plant species to be used in a larger scale pot assay, namely, *L. multiflorum* (a resistant species and representant of monocotyledonous crops), *L. sativa* (a species with intermedium sensitivity and a dicotyledonous crop), *A. thaliana* (a sensitive species and representant of weeds) and *N. tabacum* that acts a backup in case of need due to the difficulty in growing *A. thaliana*. First, we attempted to grow the plants with the *Klebsormidium* sp. (1.5) growing in the substrate, but no effects were observed, probably due to lixiviation, and for this reason, the collected data were not shown and another way of applying the microalga was attempted. The new way consisted of spraying the microalga culture, after sonication, on the leaves of the treated plants.

With this form of administration, the data from the Petri dish experiments were confirmed, since dicotyledonous growth was inhibited by the compounds produced by the microalgae with different degrees of sensibility depending on the specie, while the monocotyledonous, *L. multiflorum* was not affected. Once again, these results suggest that the monocotyledonous are resistant to *Klebsormidium* sp. (1.5) presence, while the dicotyledonous are sensitive to different degrees, being *A. thaliana*, a representative of weeds, the most sensitive. *N. tabacum* also demonstrates a significant sensibility while *L. sativa* was the most resistant of the three species. Once again, these results suggest a possible use of K*lebsormidium* sp. (1.5) as a selective bioherbicide and confirm the production by the microalgae of compounds with bioherbicide effect.

To elucidate the plant responses to the *Klebsormidium* sp. (1.5) action, several biochemical evaluations were performed in the different plant species, namely the evaluation of the stress-related defensive antioxidant system. Stress conditions lead to different types of responses in plants, this may include the activation of molecular antioxidant defence, but when the plant responses are not enough to cope with stress the cell may suffer damage, namely on the cell membrane that can be detected by the levels of lipid peroxidation that reflect the lipid oxidation status as well as the membrane integrity of plants after stress exposure (Alché, 2019).

The antioxidant system is composed of a variety of molecules of different natures, enzymatic and non-enzymatic, that act to prevent damage. The non-enzymatic antioxidant system includes proline, a molecule that has a role in cellular homeostasis and can be used as a signaling molecule. Its accumulation has been reported in response to various environmental stress (Szabados and Savouré, 2010), and it has

been proposed that proline accumulation is capable of reducing oxidative damage since it has demonstrated the capability to eliminate hydroxyl radicals and reduce H_2O_2 levels (Alvarez et al., 2022) along with the molecule reduced glutathione, which has many functions in plants namely being oxidized by ROS (reactive oxygen species) (Noctor et al., 2012). H_2O_2 is a ROS that may act as a signaling molecule but at higher concentrations provokes cell death according to literature (Petrov and Van Breusegem, 2012; Niu and Liao, 2016).

Still, cell damage can also be prevented by the action of the antioxidant enzymatic system. There is the enzyme SOD, which works as the first line of defence against ROS since it dismutes superoxide into H_2O_2 (Apel and Hirt, 2004), which can be scavenged by the enzymes catalase and APX (Mhamdi et al., 2010), and while APX has a higher affinity for H_2O_2 catalase has a faster turnover (Sofo et al., 2015).

Another factor affected by stress is productivity which can be quantified by enzymatic and non-enzymatic molecules productivity-related. For example, the NR is a very important enzyme involved in N obtainment since it is the first enzyme to act in a multistep process, reducing nitrate to nitrite which is then reduced to ammonia that is fixed into organic acids by the combined action of GS and GOGAT (glutamate synthase). Using ammonia as substrate GS can convert glutamate to glutamine which is used by GOGAT to synthesize two glutamate molecules that can be utilized to produce the amino acids aspartate and alanine that may generate enzymes related to photosynthesis. This is why the GS/GOGAT cycle is at the interface of the N and C metabolism (Lam et al., 1996; Chamizo-Ampudia et al., 2017; Ali, 2020). The proteins produced may be chlorophylls a and b which are the two most important types in terms of photosynthesis since chlorophyll a initiates the light reaction and chlorophyll b auxiliaries (Tezcan et al., 2019). The results of photosynthesis are sugars and the sugar content has proved to play a vital role in reducing the damage caused by stress (Siddiqui et al., 2020).

In *A. thaliana*, the most sensitive species, it was possible to see an increase in peroxidation levels, indicating that the *Klebsormidium* sp. (1.5) not only led to a reduction in size it also caused damage on a cellular level. That damage may be linked to the increase in H_2O_2 levels, which was not stopped by the increased in proline and reduced glutathione levels. Although *A. thaliana* activated the non-enzymatic antioxidant system it was not enough to cope with the effects caused by the microalga and the enzymatic antioxidant system action was not activated since there was a reduction in the catalase activity levels and the APX activity levels remained the same. Despite only being possible to quantify the activity levels of GS in *A. thaliana* it is possible to see that there were no

significant changes in the activity of the enzyme and the same can be reported in the content of non-enzymatic productivity-related molecules, chlorophyll a + b, soluble sugars and starch.

Concerning *Nicotiana tabacum*, also a sensitive species, it is possible to see that like *A. thaliana* the lipid peroxidation levels increased, indicating that there was cellular damage, once again that damage may have been caused by ROS like H_2O_2 since its levels were also increased. However, unlike *A. thaliana* which tried to contradict this rise with non-enzymatic antioxidant molecules in *N. tabacum* the levels of proline and reduced glutathione decreased alongside the defence molecule catalase. Even though the defence strategy adopted by *N. tabacum* seems worse than in *A. thaliana*, the levels of some productivity indicators were increased, namely, the nitrate reductase enzyme which may indicate bigger assimilation of N, that can eventually be used to create proteins like chlorophylls, whose levels also increased, leading to an increase in soluble sugar content, that like mentioned previously has proved to play a vital role in reducing the damage caused by stress.

Even though it is a dicotyledonous, *L. sativa* has a different response. Despite having a reduction in growth, these plants did not suffer cellular damage, since the lipid peroxidation levels are similar to the control. That may be explained by the fact that in *L. sativa* the levels of H_2O_2 decreased, which may be explained by the action of the non-enzymatic antioxidant system since the proline levels increased, and also by the action of the enzymatic antioxidant system since the catalase activity levels increased While CAT is activated by higher concentrations of H_2O_2, APX tends to respond to lower H_2O_2 concentrations, which may explain thee absence of changes in the activity of APX. Lastly, it is possible to see that even though the productivity-related enzymes (nitrate reductase and GS) decrease their activity the chlorophyll content increase which may indicate a higher photosynthesis level, that did not translate into higher sugar content.

Last, the monocotyledonous *L. multiflorum*, like *L. sativa* did not suffer cellular damage since the lipid peroxidation levels are similar to the control. However, unlike the previous species, *L. multiflorum* did not need the action of the non-enzymatic antioxidant system to keep the levels of H_2O_2 similar to the control. This fact seemed to be achieved by the action of the defense enzymes catalase and SOD, that work together since SOD works first by dismuting superoxide into H_2O_2, which can be later scavenged by catalase. This collective action of the antioxidant enzymes may help explain the resistance of this species.

To sum up, it is possible to conclude that *Klebsormidium* sp. (1.5) affects differently the physiology of monocotyledonous and dicotyledonous. The monocotyledonous plants are more resistant to the microalga effect possibly due to the activation of the enzymatic antioxidant system, namely increasing the activity of CAT and SOD, preventing the increase in oxidant molecules like H_2O_2 and cellular damages. The dicotyledonous plants are more sensitive to the microalga, being most of them incapable of activating the enzymatic defence system. Although activating the non-enzymatic system (increase in proline and reduced glutathione), most of these plants are not able to cope with the H_2O_2 increase and the consequent cellular damage, like *A. thaliana*. *N. tabacum* plants were not even capable of the activation of the non-enzymatic system. However, some more resistant dicotyledonous are able to activate both enzymatic and non-enzymatic defences, increasing CAT activity and proline contents, this way reducing H_2O_2 levels and the lipid peroxidation.

Thus, it is possible to conclude that *Klebsormidium* sp. (1.5) has the potential to be used as a selective bioherbicide since it kills and damages dicotyledonous but does not affect monocotyledonous, by acting differently in these plants

To discover the cause of those effects the extraction of the compounds produced by the microalga was attempted, however, this was not successful since the obtained extract did not cause changes in the growth of *A. thaliana* plants. Due to that, it is possible to conclude that the extraction process needs improvement and another solvent may be necessary.

It was also hypothesized that the *Klebsormidium* sp. (1.5) had a similar effect to 2,4-D, a selective bioherbicide effect, acting in the auxin levels, explaining the selective bioherbicide effect of the microalga. To test this hypothesis transgenic plants of *A. thaliana* carrying an auxin responsive promoter associated with a reporter gene were used, but no differences in the auxin levels induced by the *Klebsormidium* were detected. So, more tests are necessary to uncover the responsible for the *Klebsormidium* sp. (1.5) actions.

Nevertheless, the agronomic potential of this microalga should be highlighted, since the bioherbicide effect is notorious and after further study this microalga could be used as an environmentally friendly substitute for the herbicides used nowadays.

3.3. *Trichocoleus* sp. – a potential biofertilizer

In the second part of this work, the microorganism tested was a cyanobacterium from the genus *Trichocoleus* sp., collected from Portuguese soils in the scope of the GreenRehab Project, and for which previous studies suggested a potential biofertilizer effect in plants, although nothing was uncovered about its mode of action. To discover more about the effect of this cyanobacterium on plants, some pot trials were performed using *L. sativa* plants due to their economic relevance. The plants were grown in pots, with perlite and vermiculite, watered with a nutritive solution and regularly sprayed with *Trichocoleus* sp. growth medium. After growing for a month in pots, the plants were collected and some biometric and biochemical parameters were assessed.

3.3.1. Biometric parameters

The visual aspect and the evaluation of the number of leaves, aerial part and root length and weight of the plants control and treated with *Trichocoleus* sp. exudates are gathered in **figure 13**. Visually it is possible to see that the plants grown with the application of *Trichocoleus* sp. were bigger, which results in a significant increase in the number of leaves and the aerial part length. No significant changes were observed concerning the root development.

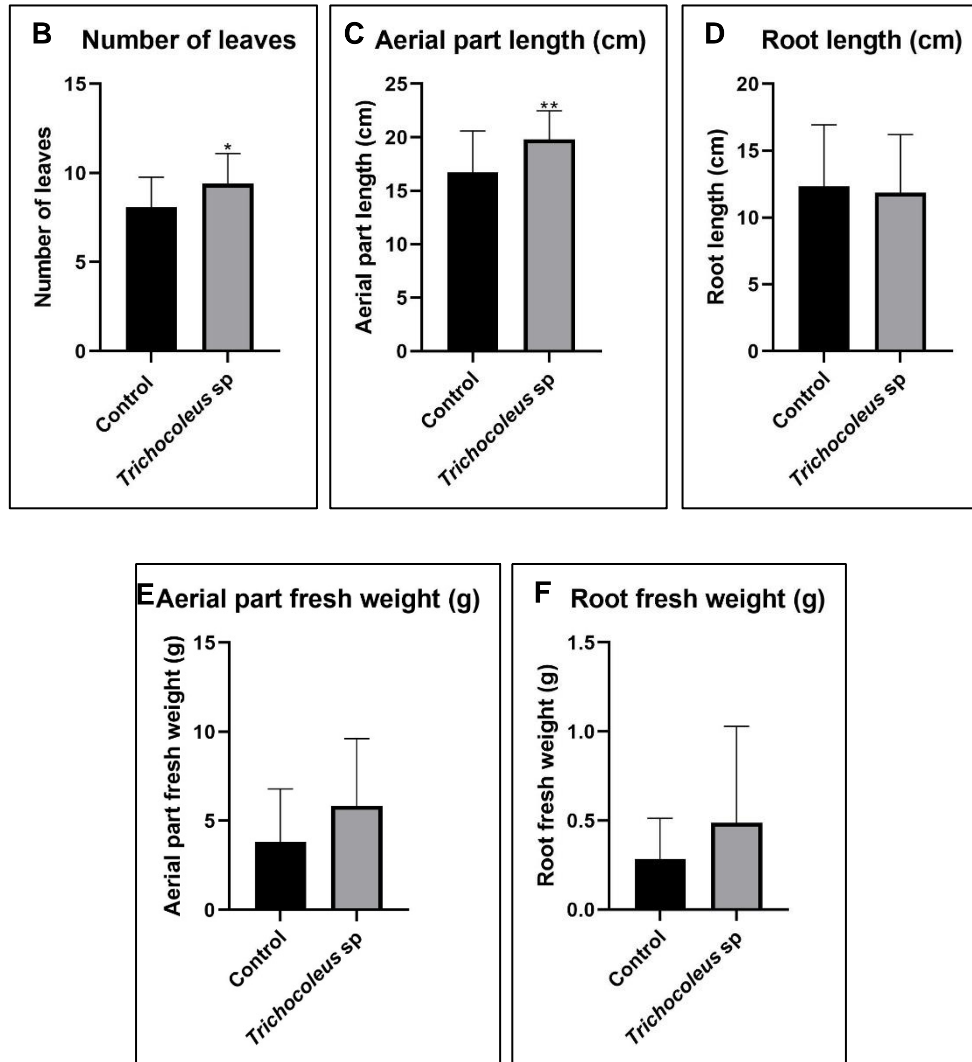


Figure 13 Effects of the application of *Trichocoleus* sp. on plant growth evaluated in pot assays. Growth of *Lactuca sativa* in the absence or after application of *Trichocoleus* sp. exudates (A). Assessment of different growth parameters: number of leaves (B), aerial part length (C), root length (D), aerial part fresh weight (E) and root fresh weight (F). Results are expressed as mean +/- SD. Unpaired Student's t-tests were performed with Welch's correction, for comparison with the control. Statistically relevant values are indicated * $P<0.05$; ** $P<0.01$.

3.3.2. Biochemical parameters – productivity indicators

The lettuce plants treated with the cyanobacteria were collected and used to evaluate some biochemical parameters related to productivity since these plants have shown an increase in size. Chlorophyll a + b, starch and soluble sugar contents, as well as the activity of enzymes enrolled in nitrogen metabolism, GS and NR, were assessed.

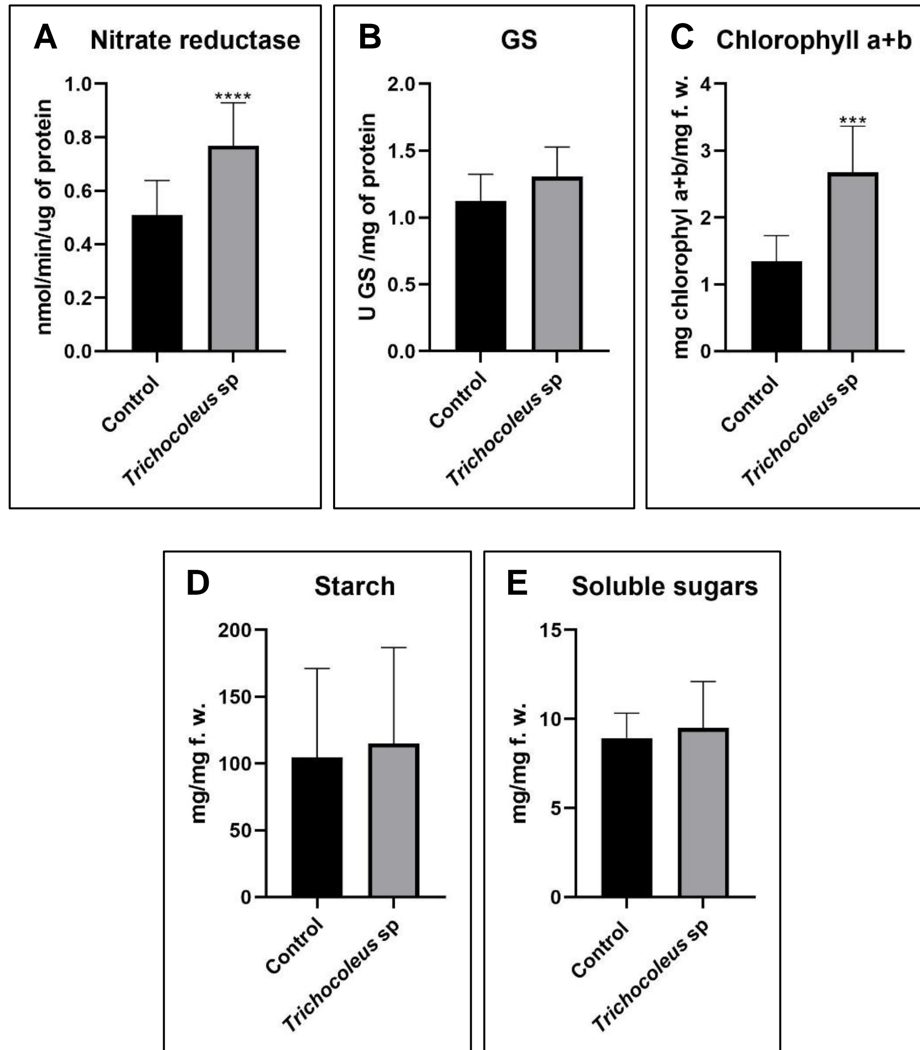


Figure 14 Quantification of some productivity-related parameters in *Lactuca sativa* plants grown with the application of *Trichocoleus* sp. in the form of spray and without (Control). Nitrate reductase activity (A), GS activity (B), Chlorophyll a+b quantification (C), Starch quantification (D), and Soluble sugars quantification (E). Results are expressed as mean +/- SD. Unpaired Student's t-tests were performed with Welch's correction, for comparison with the control. Statistically relevant values are indicated *** P<0.001; **** P<0.0001.

Of the evaluated indicators, only two showed significant changes, namely the activity of the nitrate reductase enzyme and the content of chlorophyll a and b that has increased in the plants treated with the cyanobacteria.

3.3.3. Discussion

The exponential growth of the human population has put an enormous pressure on agriculture to produce more food and consequently led to a greater use of agrochemicals that are not environmentally friendly (Costa et al., 2019; Ferreira et al., 2021) Nevertheless, using cyanobacteria as a biostimulant may help reduce chemical use. Many cyanobacteria have proved to have a positive effect on plant growth (Toribio et al., 2020; Santini et al., 2021) and are increasingly being used in agricultural soils due to

their potential contribution to plant growth since they release biologically active substances (elicitors molecules) that promote plant development and/or protect them against biotic and abiotic stresses (Bocchi and Malgioglio, 2010; Singh et al., 2017).

With this in mind, we decided to study the effects of a cyanobacterium from the genus *Trichocoleus* sp. that in previous petri dish assays had shown a positive effect on plant growth (Rocha, 2021).

To assess the effects provoked by *Trichocoleus* sp. some pot trials were performed and the growth medium of *Trichocoleus* sp. was sprayed on the leaves of *L. sativa*. After plant growth, some parameters were assessed and it was possible to see that the presence of the cyanobacterium exudates led to an increase in size, namely in the aerial part length and number of leaves. Although slight, this increase is very interesting considering that *L. sativa* is a species with big commercial interest. Effects like this were observed in other species of cyanobacteria, for example, Santos et al. verified an increase in leaf growth in lettuce plants grown with cyanobacteria biomass (Santos et al., 2022).

Therefore, and to see if this increase in size was related to an increase in productivity, some productivity-related parameters were assessed. By stimulating nitrogen uptake and nitrogen use efficiency, cyanobacteria have the potential to regulate and modify physiological processes in plants, thereby improving growth (Singh et al., 2016). Non-nitrogen-fixing cyanobacteria improving plant nutritional assimilation has been reported, but the molecular and physiological mechanisms involved, as well as how they enhanced plant growth, are still unknown (Omoarelojie et al., 2021). This fact can be seen in the plants treated with *Trichocoleus* sp. that not only had a slight increase in growth but also had a significant rise in the levels of NR activity which may be linked to the rise in chlorophyll a and b content. Similar results were observed by Grzesik et al for other cyanobacteria species in *Salix viminalis* L. plants (Grzesik et al., 2017). Also, Haroun and Hussein showed that filtrates of the cyanobacteria *Cylindrospermum muscicola* and *Anabaena oryzae* can increase the growth of the plant *Lupinus termis* (via increased chlorophyll-a and -b content, nitrogen and carbon content in leaves and photosynthetic activity) (Haroun and Hussein, 2003).

Still, despite the chlorophyll content increase in *L. sativa* plants after being treated with *Trichocoleus* sp., the levels of soluble sugars and starch remained the same which could indicate that the photosynthesis levels were not altered.

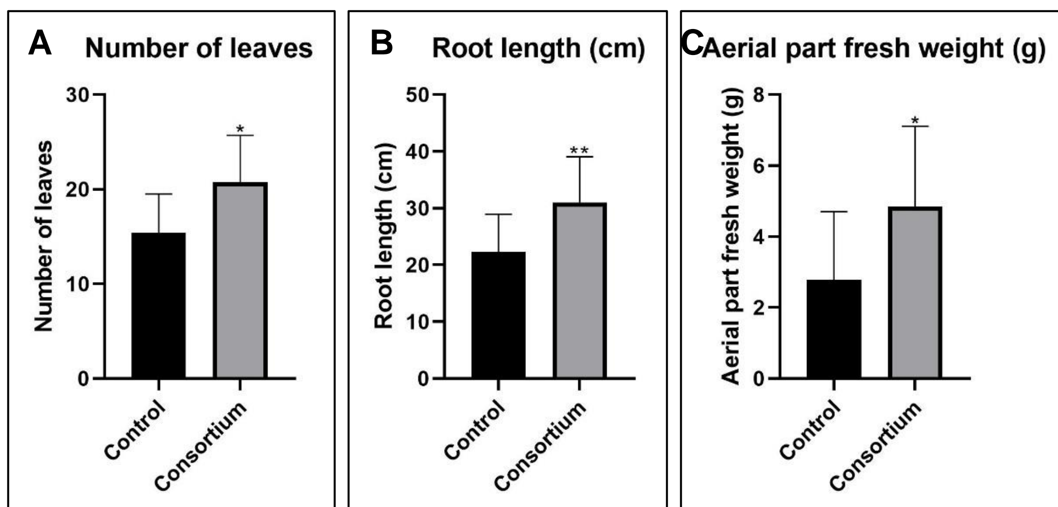
Overall, *Trichocoleus* sp. had a mild effect on *L. sativa* but it is important to notice that only the growth medium was applied and not the cyanobacterium, therefore the effect may be reduced. Still, the *Trichocoleus* sp. capability to increase its size is interesting because *L. sativa i*s a plant with a commercial interest which could mean a possible use of *Trichocoleus* sp. as a biostimulant in the future after some more study. It would also be a good idea to study the effects of the application the growth medium and the sonicated cells of *Trichocoleus* sp. to see if the observed effects would be stimulated.

3.4. Potential biofertilizer effect of a consortium of soil microalgae and cyanobacteria, an agronomic approach

In part 3 of this work, the effect of a consortium isolated from burned soils in the scope of the project GreenRehab was evaluated in plants. This consortium is composed of different species of microalgae and cyanobacteria that previously when tested individually, have shown a positive effect on plants or soil (Rocha et al., 2021).

The members of the consortium were *Trichocoleus* sp., *Oscillatoria* sp., *Nodosilinea* sp., *Parakomarekiella* sp., *Nostoc* sp. and *Klebsormidium* sp. (2.1 b).

To evaluate the effect of the 6 microorganisms together, a consortium was established and tested in two plant species, a monocotyledonous (*L. multiflorum*) and a dicotyledonous (A. thaliana). Plant hydroponic cultures were set up for a larger scale and an agronomic approach. Biometric and biochemical analyses were also performed to better understand the effects of the consortium on the two groups of plants.



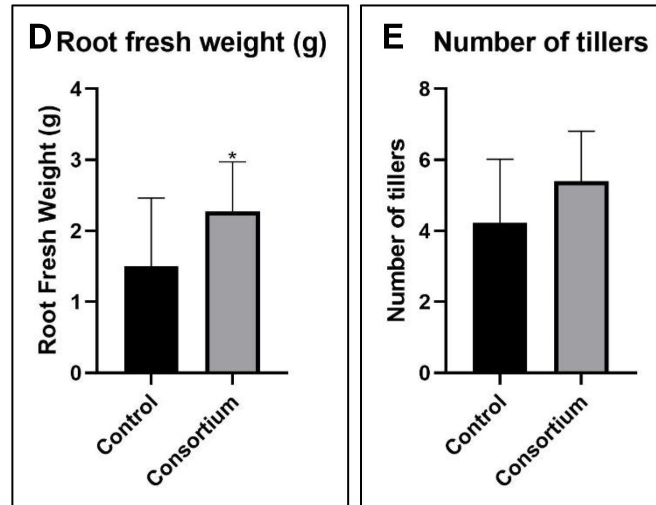
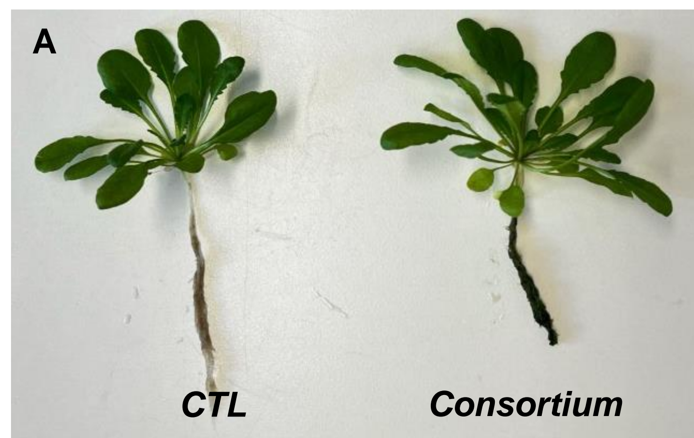


Figure 15 Effects of the consortium on *L. multiflorum* growth. Plants were grown in hydroponic culture with Hoagland medium supplemented with the microorganism consortium and the number of leaves (A), root length (B), aerial part (C) and root (D) fresh weight and number of tillers (E) were evaluated. These results are expressed as means +/- SD (n=10). Unpaired Student's t-tests were performed with Welch's correction, comparing treated plants to the control. Statistically relevant values are indicated * P<0.05; ** P<0.01.

Concerning the monocotyledonous *L. multiflorum*, after a month of growing in the hydroponic system, in the presence of the consortium the plants were collected and various biometric parameters were assessed, namely the: number of leaves, the aerial part and root fresh weight, the root length and the number of tillers. The collected results are in **figure 15** where it is possible to observe a significant increase in the majority of the parameters assessed, with the only exception being the number of tillers that had no significant increase.



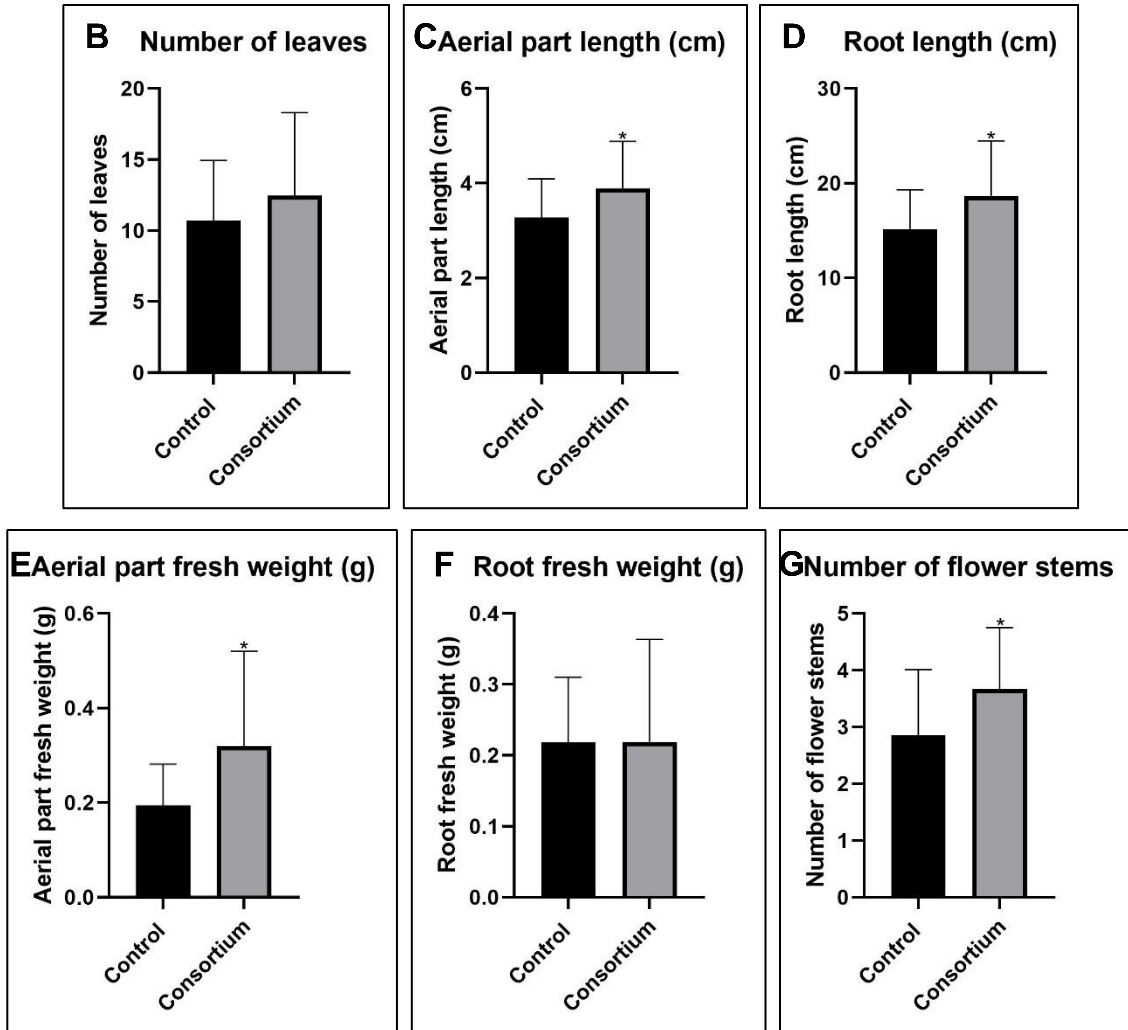
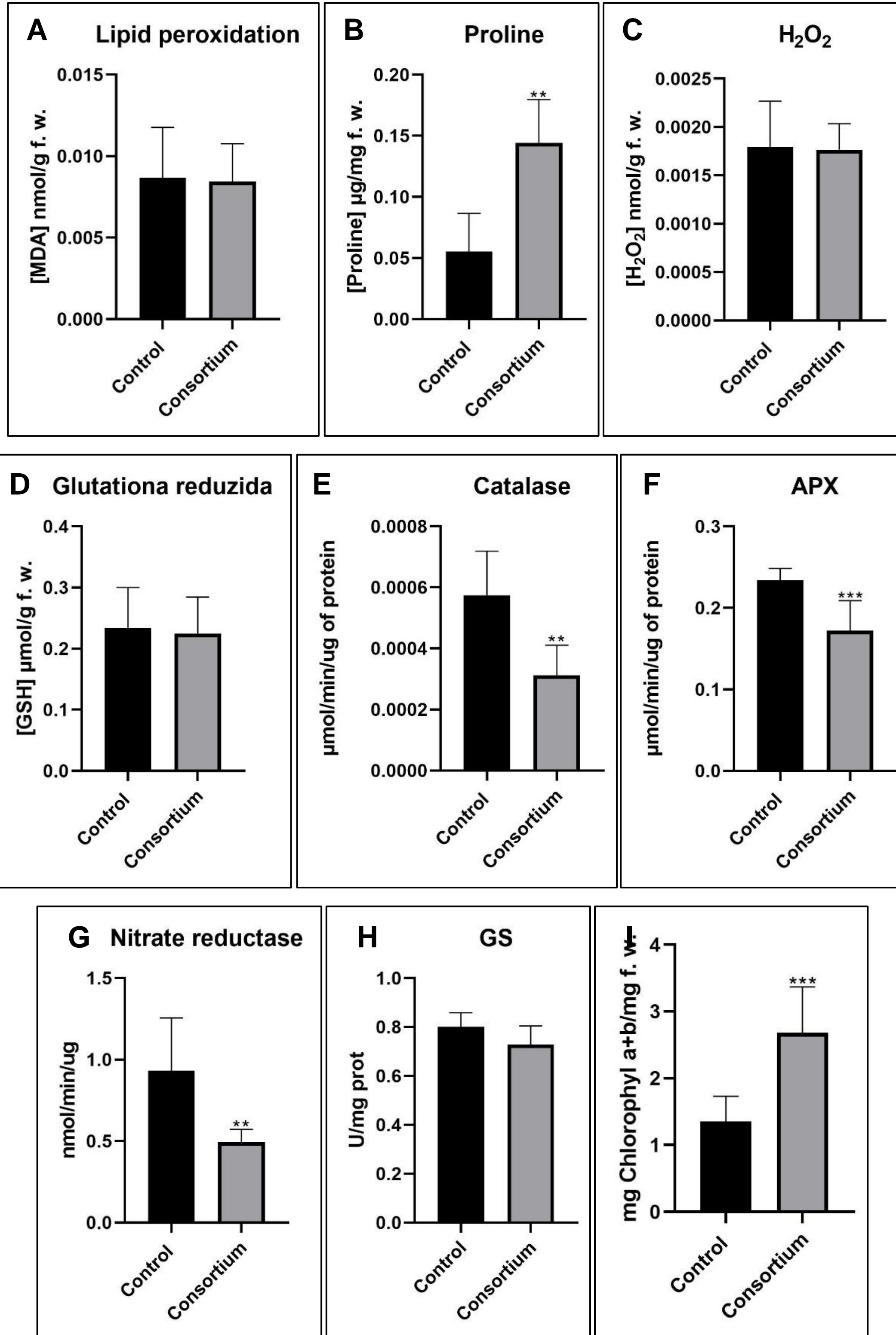


Figure 16 Effects of the consortium on *A. thaliana* growth. Plants were grown in hydroponic culture with Hoagland medium supplemented with the microorganism consortium and the number of leaves (A), aerial part (B), and root (C) length, aerial part (D) and root (E) fresh weight and number of flower stems (F) were evaluated. These results are expressed as means +/- SD (n=10). Unpaired Student's t-tests were performed with Welch's correction, comparing treated plants to the control. * indicates statistically significant differences ($p < 0.05$).

Concerning the *A. thaliana*, several biometric parameters were also evaluated, namely the number of leaves and flower stems, the aerial part and root fresh weight and length. The collected results can be seen in **figure 16** and after growing for a month in the hydroponic system, the root and aerial part length, the aerial part fresh weight and the number of flower stems showed significant increases.

3.4.1. Biochemical parameters

The collected plants were used to evaluate some biochemical parameters related to stress and productivity. The parameters assessed were enzymatic activities like catalase, APX, nitrate reductase and GS and non-enzymatic parameters like lipid peroxidation, proline, reduced glutathione, H_2O_2, chlorophyll a+b, starch and soluble sugar content, which results can be seen below.


A Lipid peroxidation
[MDA] nmol/g f. w.
0.015
0.010
0.005
0.000
Control
Consortium
B Proline
[Proline] µg/mg f. w.
0.20
0.15
0.10
0.05
0.00
**
Control
Consortium
C H_2O_2
[H_2O_2] nmol/g f. w.
0.0025
0.0020
0.0015
0.0010
0.0005
0.0000
Control
Consortium
D Glutationa reduzida
[GSH] µmol/g f. w.
0.4
0.3
0.2
0.1
0.0
Control
Consortium
E Catalase
µmol/min/ug of protein
0.0008
0.0006
0.0004
0.0002
0.0000
**
Control
Consortium
F APX
µmol/min/ug of protein
0.3
0.2
0.1
0.0
***
Control
Consortium
G Nitrate reductase
nmol/min/ug
1.5
1.0
0.5
0.0
**
Control
Consortium
H GS
U/mg prot
1.0
0.8
0.6
0.4
0.2
0.0
Control
Consortium
I
mg Chlorophyl a+b/mg f. w.
4
3
2
1
0
***
Control
Consortium

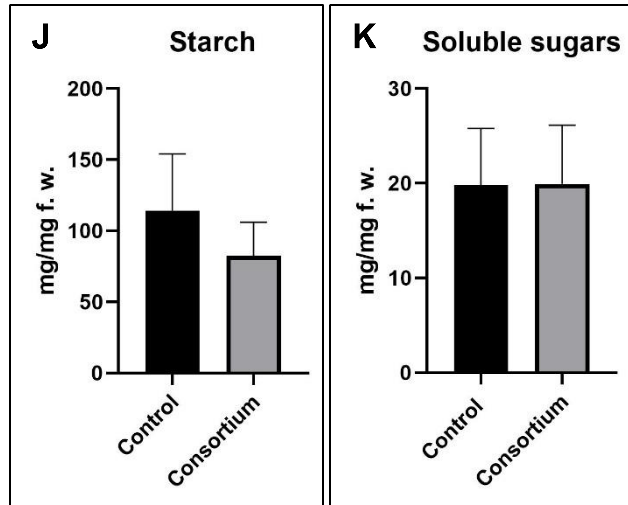
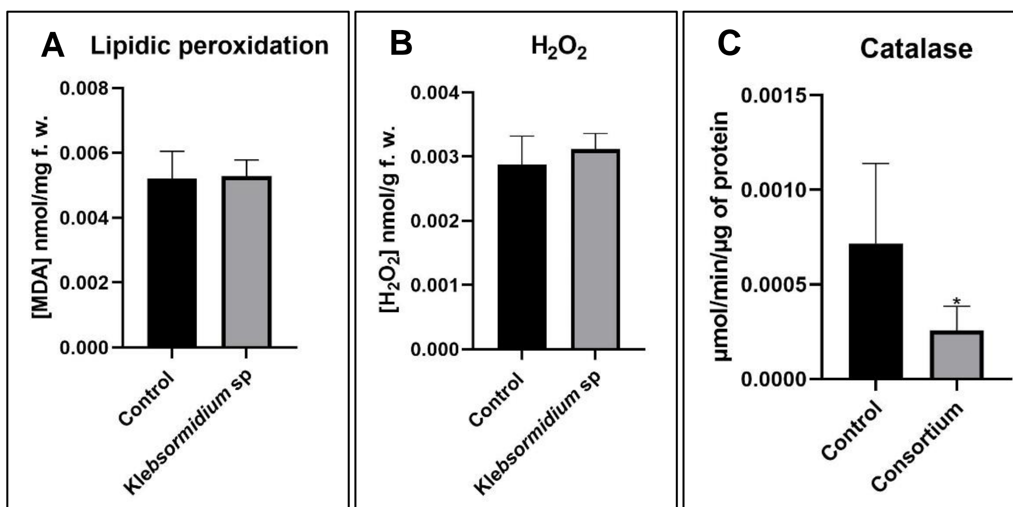


Figure 17 Quantification of some biochemical parameters in *Lollium multiflorum* plants grown in a hydroponic system in the absence (control) or the presence of the Consortium. Lipid peroxidation quantification (A), proline quantification (B), H_2O_2 quantification (C), reduced glutathione (D), catalase activity (E), APX activity (F), Nitrate reductase activity (G), GS quantification (H), Chlorophyll a+b quantification (I), Starch quantification (J), and Soluble sugars quantification (K). Results are expressed as mean +/- SD. Unpaired Student's t-tests were performed with Welch's correction, for comparison with the control. Statistically relevant values are indicated ** $P<0.01$; *** $P<0.001$.

Regarding the non-enzymatic stress-related parameters in *L. multiflorum*, it is possible to see in **figure 17** that only the proline levels were altered in a significant way with an increase in the concentration of the amino acid, while the levels of lipid peroxidation, H_2O_2 and reduced glutathione remained the same. However, when looking at the activity of some stress-related enzymes it is possible to see that the activity decreased significantly in both catalase and APX. On the other hand, in productivity-related enzymes only the nitrate reductase activity decreased significantly while GS activity remained the same. Lastly, in the non-enzymatic productivity-related parameters the chlorophyll a and b contents were significantly altered with an increase, while starch and soluble sugar content did not change.



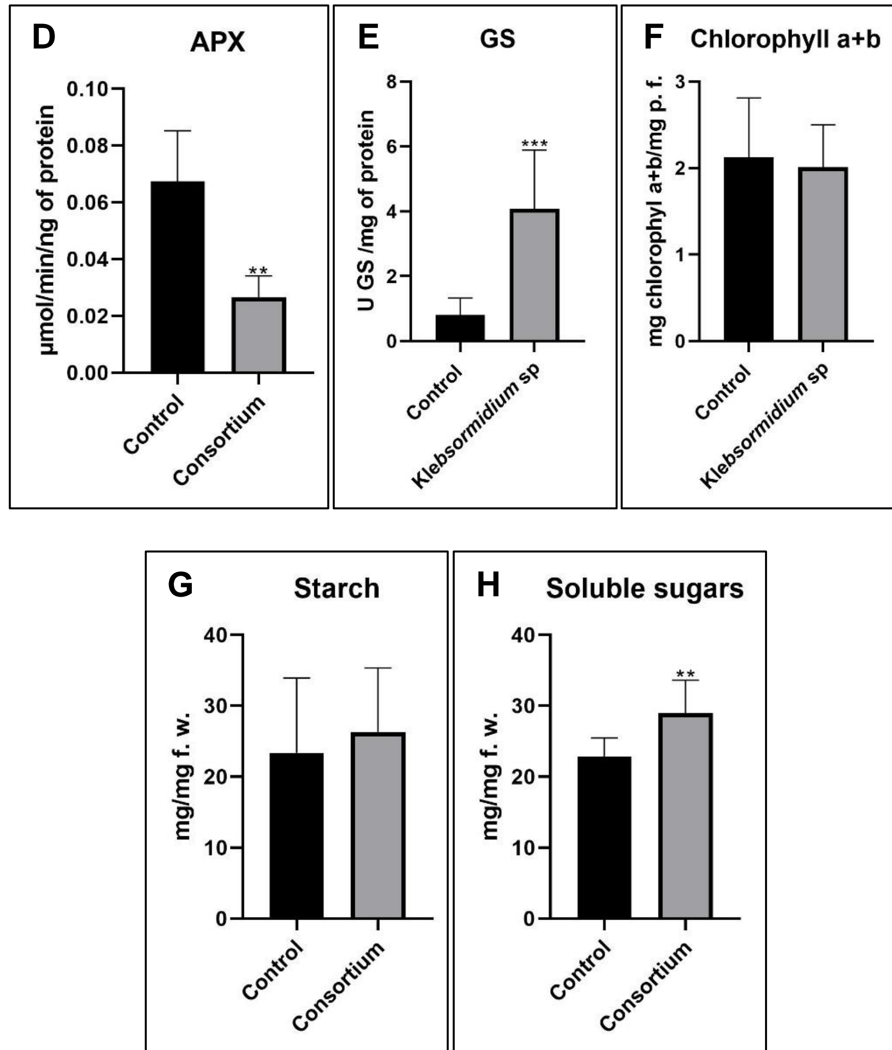


Figure 18 Quantification of some productivity-related parameters in *Arabidopsis thaliana* plants grown in a hydroponic system in the absence (control) or the presence of the Consortium. Lipid peroxidation quantification (A), H_2O_2 quantification (B), catalase quantification (C), APX quantification (D), GS quantification (E), Chlorophyll a+b quantification (F), Starch quantification (G), and Soluble sugars quantification (H). Results are expressed as mean +/- SD. Unpaired Student's t-tests were performed with Welch's correction, for comparison with the control. Statistically relevant values are indicated * P<0.05; ** P<0.01; *** P<0.001.

For *A. thaliana,* due to the small size of the plants and the high rate of death in the hydroponic cultivation climatization, it was only possible to evaluate certain parameters. While the non-enzymatic stress parameter, namely lipid peroxidation and H_2O_2 were unaltered the enzymatic stress parameters, activity levels of the enzymes catalase and APX, decreased significantly. Concerning productivity, it is possible to see that the content of chlorophyll a and b and starch remained equal and the GS activity and soluble sugar content increased.

3.4.2. Discussion

Wildfires have emerged as a serious concern for many populations, as their frequency and intensity have increased significantly in recent years due to climate change, (Pausas

and Keeley, 2021) and because it may take more than 150 years for a new and well-established species community to be developed under normal circumstances (Shugart, 2012). The cyanobacterization / algalization process seeks to accelerate the ecological succession process by shortening the time required for the establishment of a new species community in the affected region (Rossi et al., 2017).

Therefore, under the scope of the GreenRehab project, several species of cyanobacteria and microalgae were collected from a Portuguese burned soil and individually tested for their effect on the plants and in the soil (Rocha et al, 2021). The ultimate goal of the project was to create a consortium with the most suitable species to be used to recover burned soils. However, before applying it to the soil it was important to evaluate its effects as a consortium on plants. For that, plants of *L. multiflorum* and *A. thaliana,* representative of the monocotyledonous and dicotyledonous from the Portuguese flora, were grown in a hydroponic system, a large-scale and agronomically representative system, to further transfer the acquired knowledge to a sustainable agricultural utilization. In *L. multiflorum* the presence of the consortium lead to an improvement in growth, with a significant increase in the majority of the parameters, namely number of leaves, aerial part and root weight and root length, although there was no significative difference in the number of tillers. A positive effect also occurred in *A. thaliana* when in the presence of the consortium with an increase in the aerial part and root length, the aerial part fresh weight and the number of flower stems. Similar results of plant growth improvement were observed in rice plants when a consortium of two species of cyanobacteria and microalgae (*Anabaena variabilis*, *Chlorella vulgaris*, and *Azotobacter* sp.) were applied (Zayadan et al., 2014).

Afterward, some biochemical parameters were assessed to evaluate the physiological state of plants, to see if plants are under stress caused by the presence of the microorganisms and how their metabolism was affected in terms of productivity. For *L. multiflorum* is possible to see that the proline levels increased significantly while the levels of catalase and APX (antioxidative enzymes) decreased, despite this decrease the levels of lipid peroxidation remained the same, which suggests that the plant is not in a stressed state and the action of proline is enough to protect the plant from possible damages in response to the presence of the microorganisms. Some productivity-related parameters were also assessed, among those only nitrate reductase and chlorophyll a and b content changed with a decrease and an increase, respectively. Although, the levels of chlorophyll a and b increased the content of sugar did not increase, which

suggests that the consortium presence did not lead to an increase in the photosynthesis level.

As for *A. thaliana* and although the number of parameters assessed was inferior, due to lack of plant material, it is possible to see that the presence of the consortium did not lead to cellular damage since the level of lipidic peroxidation did not increase and the levels of H_2O_2 which can be harmful in high levels remained unchanged. As for the levels of catalase and APX activity, like in *L. multiflorum*, they decreased significantly, which may indicate that the plant is not under stress since the action of these defense enzymes is not necessary. In productivity-related parameters is possible to see an increase in the levels of GS activity and in the content of soluble sugars, which may indicate an increase in photosynthesis levels.

Despite the mild effect of the consortium in the monocotyledonous and dicotyledonous plants assessed, the results are still positive since the consortium did not negatively affect the plants, which allows its use for the intended goal of recovering burned soils under the scope of the GreenRehab project.

4. Conclusion

With a human population in exponential growth, it is important to find alternatives to the chemicals used in agriculture, namely herbicides and fertilizers. As seen throughout this work microalgae and cyanobacteria can be good alternatives to the chemicals currently used. Although there are many studies regarding the use of microalgae and cyanobacteria as biostimulants the same cannot be said for bioherbicides.

This last field is especially understudied, so studies like this are very important, as it was possible to see the *Klebsormidium* sp. (1.5) as shown the capability of being potentially used as selective bioherbicide since it affected the dicotyledonous, in various degrees and did not affect the monocotyledonous (a group where many plants with agronomic interest are inserted). Although the presence of *Klebsormidium* sp. (1.5) affected different parts of the metabolism, the difference in the level of activity of the antioxidant enzymes seemed to be what caused the plants to be more resistant or more susceptible, since the most sensitive plants lowered the catalase levels of activity, which led to an increase in H_2O_2 and eventual cellular damage.

With this study there is now more knowledge on the mode of action of *Klebsormidium* sp. (1.5), but what leads to this effect is still unknown, so it would be interesting to continue to study this species. Some hypotheses for the cause were theorized and tested but more studies are required.

Another part of this study aimed to study the effects of cyanobacteria from the genus *Trichocoleus* sp., and from the results collected it was possible to see that the effects of the cyanobacteria were mild and the productivity levels were not altered. Nonetheless, the presence of *Trichocoleus* sp. led to an increase in the size of *L. sativa* and since this plant has economical interest it is appealing to continue to acquire more knowledge on the effects of this cyanobacterium in other species of plants and to further investigate the mode of action, possibly with the application of the sonicated *Trichocoleus* sp. cells and its growth medium.

Lastly, in part 3 it was possible to see that the presence of a consortium of cyanobacteria and microalgae collected from a burned soil led to a slight improvement in plant development so, since the species present in the consortium did not affect negatively the plants it is possible to use them in studies to research their potential use as a way to recover burned soils, since that was the main goal of the GreenRehab project.

As seen, microalgae and cyanobacteria lead to different effects on plants, whether positive or negative it is possible to use these beings in various research fields and for many purposes. Therefore, it is important to continue to study these beings, especially in fields less explored, such as bioherbicides. Hopefully, this work helped to contribute with more knowledge to an unexplored field that deserves a deeper study.

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6. Attachments

Table S 1 Composition of the BG_{11} solution

Composts		Final Concentration (µM)
Sol. 1	$K_2HPO_4.3H_2O$	230
Sol. 2	$MgSO_4.7H_2O$	300
Sol. 3	$CaCl_2.2H_2O$	240
	Citric Acid.H_2O	31
Sol. 4	Ferric Ammonium Citrate	21
	$Na_2EDTA.2H_2O$	2.7
Sol. 5	Na_2CO_3	190
Sol. 6	H_3BO_3	46
	$MnCl_2.2H_2O$	9
	$ZnSO_4.7H_2O$	0.77
	$Na_2MoO_4.2H_2O$	1.6
	$CuSO_4$	0.3
	$Co(NO_3)_2.6H_2O$	0.17
NaNO3		17 600

Table S 2 Composition of the Hoagland solution

Composts		Final concentration (µM)
Macronutrients	KNO_3	16000
	$Ca(NO_3)_2.4H_2O$	6000
	$NH_4H_2PO_4$	4000
	$MgSO_4.7H_2O$	2000
Micronutrients	KCl	50
	H_3BO_3	25
	$MnSO_4.H_2O$	2
	$ZnSO_4.7H_2O$	2

	$CuSO_4.5H_2O$	0,5
	$Na_2MoO_4.2H_2O$	0,5
	FeEDTA	50