



MESTRADO EM
BIOTECNOLOGIA
PARA A
SUSTENTABILIDADE

Ana Sofia Rodrigues Dos Santos

Licenciatura em Biologia Marinha e Biotecnologia

**Deciphering putative biostimulant effects of a seaweed
extract, from *Gracilaria gracilis*, in tomato plants**

Dissertação para obtenção do Grau de Mestre em Biotecnologia para a Sustentabilidade

Oeiras, 31 de março de 2023

Orientador: Dra. Ana Paula Santos (Researcher, Plant Functional Laboratory, ITQB-NOVA)

Co-orientador: Dr. Juan Ignacio Vílchez (Head of iPlantMicro Laboratory, ITQB-NOVA)



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Abstract

Chemical fertilizers have become frequent in agriculture to meet the escalating demand of an ever-growing world population, projected to reach 9.7 billion by 2050. However, these conventional agricultural practices have led to severe problems in human's health, animals, plants, and the entire environment, including plant-related microbiota. In Portugal and many other seaside countries worldwide, seaweeds were traditionally used in agricultural fields, providing crops with organic matter, minerals, trace elements, plant-growth regulators, vitamins and other bioactive compounds. More recently, with the high need for sustainable alternatives in the agricultural sector, seaweed extracts have been explored as a potential alternative to ensure food quality and safety for present and future generations. However, the use of these extracts poses several concerns regarding characterization, mode of application, optimal plant stage to apply, effective concentration, effects on microbiota, and on environment over time. In this context, this project aimed to develop a pipeline to assess the effects of a red seaweed extract, *Gracilaria gracilis*, on tomato plants (*Solanum lycopersicum*). Two distinct approaches were conducted, one was focused on evaluating culturable microbiota in *G. gracilis* extract with subsequent studies on its impact on planta and the other one intended to evaluate the impacts of extract application on planta. In the second approach, different methods and dosages of extract were tested, aiming to determine impacts on plant phenotype and root/soil microbiota. GE2_ *Klebsiella oxytoca*, isolated from *G. gracilis* extract, was able to boost by 32% the shoot length of tomato plants. Furthermore, it was shown that *G. gracilis* extract positively affected tomato seed germination rate (%). Overall, *G. gracilis* extract at 0.5% applied in soil induced a significant improvement of shoot length of tomato (45% and 14%) in the early and late stages of development, respectively. *G. gracilis* extract was also able to induce changes in soil and root microbiota profile. Data obtained in this work may contribute to the better use of seaweed extracts in agriculture, although further studies must be carried out to have a broader understanding of seaweed effects on planta.

Keywords: *Gracilaria gracilis*; seaweed extracts; culturable microbiota; biostimulants; plant growth.

Resumo

Os fertilizantes químicos tornaram-se frequentes na agricultura para satisfazer a crescente procura de uma população mundial em constante crescimento, projetada para atingir 9,7 mil milhões em 2050. Contudo, estas práticas agrícolas convencionais levaram a graves problemas de saúde humana, animais, plantas, e todo o ambiente, incluindo a microbiota relacionada com plantas. Em Portugal e em muitos outros países costeiros do mundo, as algas marinhas eram tradicionalmente utilizadas nos campos agrícolas, fornecendo às culturas matéria orgânica, minerais, oligoelementos, reguladores do crescimento das plantas, vitaminas e outros compostos bioativos. Mais recentemente, com a grande necessidade de alternativas sustentáveis no sector agrícola, os extratos de algas marinhas foram explorados como uma alternativa potencial para assegurar a qualidade e segurança alimentar para as gerações presentes e futuras. No entanto, a utilização destes extratos suscita várias preocupações relativamente à caracterização, modo de aplicação, fase ideal da planta em que o extrato deve ser aplicado, concentração eficaz, efeitos sobre a microbiota, e sobre o ambiente ao longo do tempo. Neste contexto, este projeto visava desenvolver uma pipeline para avaliar os efeitos de um extrato da alga vermelha, *Gracilaria gracilis*, nas plantas de tomate (*Solanum lycopersicum*). Foram desenvolvidas duas abordagens distintas, uma focada na avaliação da microbiota cultivável em extrato de *G. gracilis* com estudos subsequentes sobre o seu impacto na planta e a outra destinada a avaliar os impactos da aplicação do extrato na planta. Na segunda abordagem, foram testados diferentes métodos e dosagens para aplicação do extrato, com o objetivo de determinar os impactos no fenótipo da planta e na microbiota do solo e raízes. GE2_ *Klebsiella oxytoca*, isolada do extrato de *G. gracilis*, foi capaz de aumentar em 32% o comprimento da parte aérea das plantas de tomate. Além disso, foi demonstrado que o extrato de *G. gracilis* afetou positivamente a taxa de germinação das sementes de tomate (%). No geral, o extrato de *G. gracilis* a 0,5% aplicado no solo induziu uma melhoria significativa do comprimento da parte aérea do tomate (45% e 14%) nas fases iniciais e tardias de desenvolvimento, respetivamente. O extrato de *G. gracilis* também foi capaz de induzir alterações no perfil da microbiota do solo e raízes. Os dados obtidos neste trabalho podem contribuir para uma melhor utilização de extratos de algas marinhas na agricultura, embora devam ser efetuados mais estudos para se ter uma compreensão mais ampla dos efeitos das algas marinhas na planta.

Palavras-chave: *Gracilaria gracilis*; extratos de algas marinhas; microbiota; bioestimulantes; crescimento da planta.

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Abbreviations

ACC - Aminocyclopropane-1-carboxylate

E – Evenness

GE – *Gracilaria gracilis* extract

H – Shannon Diversity Index

IAA - Indole-3-Acetic Acid

IMTA – Integrated Multi-Trophic Aquaculture

IRS - Induced Systemic Resistance

LB - Luria-Bertani broth

PGPB – Plant Growth-Promoting Bacteria

PGPR – Plant Growth-Promoting Rhizobacteria

Introduction

Plant biostimulants to improve plant performance

According to the United Nations, the world population is projected to reach 9.7 billion by 2050, which means that food production will need to increase by 70% to meet the needs of a growing population. To accomplish this challenge a combination of several approaches will be required, including increasing the agricultural productivity and efficiency on food production and distribution, reducing food waste, and promoting sustainable agricultural practices. Only with the implementation of innovative and sustainable solutions, it will be possible to ensure that everyone has access to safe, healthy, and sustainable food production (Godfray et al., 2010). In this regard, biostimulants have been widely explored as a sustainable solution to address the challenges faced by the agricultural sector (Yakhin et al., 2017).

Biostimulants are emerging as beneficial for a sustainable agriculture practice since they can contribute not only to reduce the use of fertilizers but also to increase crop production. The definition and concept of plant biostimulants is still a matter of debate, partly due to the wide range of inputs that can be described as biostimulants, expanding the ranges of substances and modes of action still very much unknown (du Jardin, 2012, 2015; Roupael & Colla, 2020). Plant biostimulants were initially defined as substances able to enhance plant growth and development through non-nutritional means. This definition has been expanded to include microorganisms and other products that may provide plant nutritional benefits. Currently, plant biostimulants have been described as any substance or microorganism applied to plants with the aim to enhance nutrition efficiency, abiotic stress tolerance and/or crop quality traits, regardless of its nutrients content (du Jardin, 2015). This definition was then reinforced by "considering that plant biostimulants can also include commercial products containing mixtures of such substances and/or microorganisms" (du Jardin, 2015).

Plant biostimulants can be made from a variety of natural sources, e.g. including: a) seaweed extracts as they contain a wide range of plant growth-promoting compounds, such as hormones, enzymes, and minerals; b) humic substances, a group of naturally compounds found in soil and compost, which can improve plant growth and health by increasing nutrient uptake and promoting root development; c) microorganisms (beneficial bacteria and fungi), since they can promote plant growth and health by increasing nutrient availability, producing growth-promoting compounds, and improving soil health; d) protein hydrolysates and other N-containing compounds, which can modulate N (nitrogen) uptake and assimilation, protect plants against heavy metals but also contribute to micronutrients mobility and acquisition, mitigate environmental stress, increase microbial biomass and activity, soil respiration and soil fertility; e) chitosan and other biopolymers, that can enhance plant growth and health increase plant immunity to disease, promote root growth, and improve the efficiency of nutrient uptake; and f) beneficial chemical elements (Al, Co, Na, Se and Si), that can promote plant growth, quality of plant products and tolerance to abiotic stress (du Jardin, 2015; Shahrajabian et al., 2021).

Plant biostimulants have been used in a variety of plant species, including trees and vine crops (citrus, pome fruits, stone fruits, and grapes), crops (e.g. barley, maize, rice, wheat, oilseed rape, and sugar beet), vegetables and legume crops (such as broccoli, carrots, peppers, tomato, potato, lettuce, and

others) and other horticultural crops (ornamentals, nursery, and turf) (du Jardin, 2015). Plant biostimulants can be applied in various forms, such as liquids by foliar or soil application and by seed soaking before planting (Shahrajabian et al., 2021). It is a fact that plant biostimulants are becoming increasingly popular in modern agriculture as a way to improve soil nutrition and structure through composting and plant biomass yield and quality while also reducing the use of synthetic inputs (Shahrajabian et al., 2021). However, it is worth noting that there are various challenges in what concerns the establishment of biostimulants in agriculture namely: a) the lack of regulatory framework regarding research about its composition; b) lack of consistent definitions and standards e.g in what concerns the chemical analysis of biostimulants; c) lack of standardized testing methods e.g. related to methods of application, best concentration to use or establishing the optimal plant developmental stage in which the biostimulant would be more effective; d) limited scientific understanding about the mechanisms of action of biostimulants, as well as the unknown potential risks and/or benefits over time; e) difficulty in assessing the biostimulants efficacy since that may not be necessarily related to a direct and immediate impact on plant growth; f) variable biostimulants effectiveness since the effect will be dependent on product specificity, plant species, specific plant growing conditions (soil and environmental factors) being difficult to predict their impact; and finally g) cost-effectiveness since some biostimulants can be expensive to produce and apply preventing their adoption by farmers (Povero et al., 2016; Yakhin et al., 2017). Overall, biostimulants show potential to be a more sustainable and environmentally friendly approach to agriculture and thus addressing these challenges is crucial to unlock their full potential.

Seaweed extracts as biostimulants to improve plant performance

Marine macroalgae, also known as seaweeds, are multicellular, macroscopic, and autotrophic plant-like organisms (Pereira et al., 2013). It is accepted to classify seaweeds into three large and independent groups: Chlorophyta (green algae), Rhodophyta (red algae), and Ochrophyta (brown algae) (Pereira et al., 2013). As mentioned in parenthesis, the three phyla have a commonly accepted correspondence with the color of the species, even though the trait color can easily mislead the observer. These three different colors reflect different chloroplast pigments (Cikoš et al., 2022). Pigments derived from macroalgae are highly valued in the industrial sector; nonetheless, macroalgae possess other valuable compounds. Among all these compounds, there are nutrients, vitamins, minerals, phenolic compounds, sterols and other bioactive agents. It is also worth noting that algae can produce aminoacids and proteins, saturated/unsaturated fatty acids, and several types of polysaccharides (Leandro et al., 2019).

Historically, seaweeds were traditionally used to improve nutrient-poor soils; for instance, along the French Atlantic coast, seaweeds were harvested after storms and scattered over dunes to dry, allowing preservation through the year. In Norway, around 1926 and 1937, some industries started to process *Ascophyllum nodosum* (brown seaweed) for soil conditioner and fertilizer (Delaney et al., 2016). In Portugal, since the XIV century, seaweeds have been collected to be used in soil fertilization (Gaspar

et al., 2019). Macroalgae were mainly harvested from natural environments being traditionally used two specific mixtures of seaweeds as soil fertilizers, such as the "moliço" including e.g. *Gracilaria verrucosa*, *Ulva* sp., *Enteromorpha* sp., *Lola lubrica*, and the marine angiosperms *Potamogeton pectinatus*, *Ruppia cirrhosa* and *Zostera noltii*, and the "sargaço" including e.g. *Laminaria hyperborea*, *L. ochroleuca*, *Saccorhiza polyschides*, *Fucus vesiculosus*, *Codium tomentosum*, *Palmaria palmata*, *Chondrus crispus* and *Gelidium sesquipedale* (R. Santos & Duarte, 1991).

Seaweeds have an important ecological role in mitigating environmental problems. Besides recycling nutrients, they are on the bottom of the marine food web acting also as shelter and nursery grounds for many animal species (Balina et al., 2016; Pereira & Yarish, 2008). The seaweed harvesting is subject to annual fluctuations, which is not suitable for optimal and predictable production (Favot et al., 2019) and thus there is a spreading interest in developing suitable and sustainable cultivation techniques. Currently, several techniques for the cultivation of seaweeds are standardized, routine and economical, making all the process easier (Pereira & Yarish, 2008). Towards the end of the twentieth century, an integrated aquaculture system started to appear with a sustainable mindset, named land-based integrated multitrophic aquaculture (IMTA) system (Pereira et al., 2013). An IMTA system (Fig. 1) is an alternative approach for sustainable aquaculture, which combines the cultivation of two or more different trophic levels (Araújo et al., 2021). It is based on the re-utilization of the "wastes" or by-products of animal (fed) aquaculture. These "wastes" work as a nutrient source for the growth and development of seaweeds or other extractive, filter, or detrital feeding organisms. According to the literature, the IMTA concept is extremely flexible. It can be performed offshore, near-shore or in-land, in marine and freshwater systems. Species of *Porphyra*, *Laminaria*, *Undaria* and *Gracilaria* are already cultivated in this sustainable concept (Chopin et al., 2008; Pereira & Yarish, 2008).

The development of protocols and procedures to liquefy seaweed biomass and transform it into seaweed extracts was the first step to promote the use of seaweeds in agriculture. Milton (1952) pioneered liquid seaweed extracts to concentrate plant growth-stimulating compounds, facilitating their usage (Milton, 1952). Since then, seaweed extracts have been increasingly gaining attention as a potential solution for the challenges faced in agriculture (Table 1). Nowadays, there are a lot of available, newly emerging seaweed extract products in the market with an accelerating expansion in production and competitive market shared with chemical fertilizers and pesticides. Several companies, including BioAtlantis Ltd, Acadian Seaplants Ltd., and many more, have developed commercial plant biostimulants based on the brown seaweed *Ascophyllum nodosum* (<https://www.bioatlantis.com/>; <https://acadianseaplants.com/>). Another company, namely Nutimax Fertilizantes, developed the commercial biostimulant Reabilit® Algas derived from the red and brown seaweeds *Kappaphycus alvarezii* and *Sargasum vulgare*. The extracts based on *A. nodosum* have been shown to improve plant growth, mitigate some abiotic and biotic stresses while also improving plant defenses by modulating e.g., metabolic and physiological processes (Shukla et al., 2019). In tomato, the foliar spray application of *A. nodosum* extract was able to induce larger root systems, higher concentrations of minerals in the shoots and higher fruit yield and quality (Ali et al., 2016). In another studies, the application of the Reabilit® Algas to pepper (*Capsicum annuum* L.) plants, at a low concentration (0.5%), was associated

to a significant increase in agronomic efficiency, stomatal conductance and higher fruit production (Melo et al., 2020). The same authors also reported an increased in nutrient uptake directly related to the effects of the applied biostimulant (Melo et al., 2020). *Padina gymnospora* and *Ulva lactuca* extracts at 0.2% were also able to increase shoot and roots lengths, and plant yield when applied separately in tomato plants (Hernández-Herrera et al., 2022; Mireya Hernández-Herrera et al., 2013). Besides, *Padina gymnospora* significantly improved early-flowering and reduced negative effects induced by salinity stress in tomato plants (Hernández-Herrera et al., 2022). A significant enhancement in nitrogen (N), phosphorus (P) and Potassium (K) uptake was also observed in maize plants (*Zea mays* L.) when treated with *Kappaphycus alvarezii* and *Gracilaria edulis* extracts at 10% (Basavaraja et al., 2018). Another study from Ali et al., (2021b), reported that foliar applications of *Sargassum vulgare* and *Acanthophora spicifera* extracts at 0.5% were able to not only enhanced growth and yield parameters in pepper plants, but also reduced disease severity by the pathogens *Xanthomonas campestris* pv. *vesicatoria* and *Alternaria solani* (Ali et al., 2021b). Moreover, several studies demonstrated that extracts derived from the seaweeds *Ulva lactuca*, *Padina gymnospora*, *Gracilaria gracilis*, *Gracilaria* sp., *Ulva intestinalis* and *Ascophyllum nodosum* had positive effects on the germination phase of tomato, kale (*Brassica oleracea* L.) and lettuce (*Lactuca sativa*) (Mendes et al., 2022; Mireya Hernández-Herrera et al., 2013; Turco et al., 2022).

Despite all the potential benefits of seaweed extracts previously mentioned, there is still much to be understood about their mechanisms of action and how to apply them in different agricultural contexts (Ali et al., 2021a). The quality, consistency and reproducibility of seaweed extracts can vary depending on the species, environmental factors, the extraction method, and the processing conditions, which can lead to variations in the composition and reliability of the extract. In addition, there is limited regulation of applying seaweed extracts in many countries, which make it difficult to ensure that they are safe for the environment and for present and future generations. It is important to consider that seaweed extracts can also interact with other inputs, such as fertilizers and pesticides, making it difficult to optimize them (Ali et al., 2021a; Battacharyya et al., 2015). Therefore, defining a well-established pipeline to apply seaweed extracts and monitoring their impact on both crops and environment over an extended period, will be crucial for sustainable agriculture moving forward.

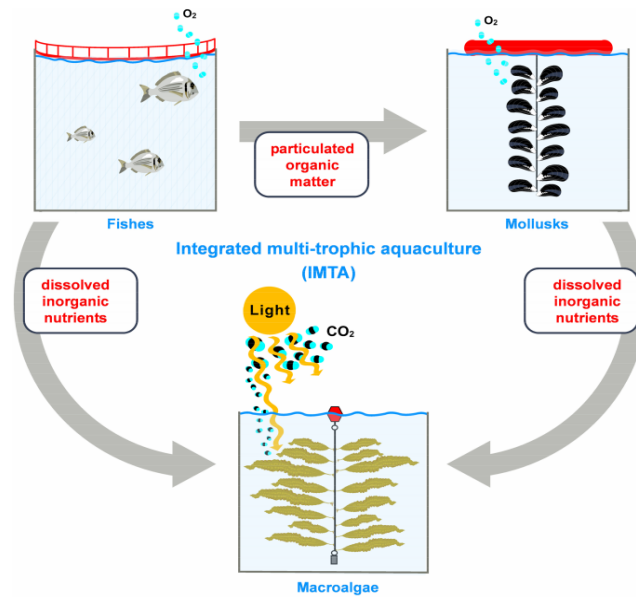


Figure 1. The concept of an integrated multitrophic aquaculture (IMTA) system. Source: Araújo et al., (2021)

Table 1. Effects of seaweed extracts on plant growth, development and protection against biotic and abiotic stresses. In the third column (Tested/optimal concentrations), concentrations in bold represent the study's optimal concentration.

Plant species	Seaweed extract	Tested & optimal concentrations of seaweed extracts	Experimental conditions/mode of application	Plant developmental stage	Plant Phenotype	References
Tomato (<i>Lycopersium esculentum</i>)	<i>Ascophyllum nodosum</i>	0.2%, 0.5%	Greenhouse/Foliar spray	Not mentioned	Larger root systems, higher concentrations of minerals in the shoots and higher fruit yield and quality attributes	(Ali et al., 2016)
Tomato (<i>Lycopersium esculentum</i>)	<i>Ascophyllum nodosum</i>	0.2%, 0.5%	Field experiment/ Foliar spray	Not mentioned	Higher plant height and plant fruit yield	(Ali et al., 2016)
Pepper (<i>Capsicum annuum</i>)	"Reabilit@ Algas": <i>Kappaphycus alvarezii</i> and <i>Sargassum vulgare</i>	0.5% , 1%, 1.5%, 2%	Greenhouse/Foliar spray	Before flowering and during fruit formation	Increase in agronomic efficiency e.g. stomatal conductance and fruit production	(Melo et al., 2020)
Maize (<i>Zea mays</i>)	<i>Kappaphycus alvarezii</i> / <i>Gracilaria edulis</i> (applied separately)	2.5, 5.0, 7.5, 10 , 15% (v/v)	Field experiment/ Foliar spray	Early vegetative growth, tassel initiation and cob formation stages	Enhanced N, P and K uptake (grain + stover) for both extracts	(Basavaraja et al., 2018)
Tomato (<i>Solanum lycopersicum</i>)	<i>Padina gymnospora</i>	0.2% w/v	Greenhouse/Soil drench	Vegetative and reproductive	Increased growth of shoots and roots, early flowering, increased yield, alleviation of salinity effects	(Hernández-Herrera et al., 2022)
Tomato (<i>Solanum lycopersicum</i>)	<i>Ulva lactuca</i> , <i>Caulerpa sertularioides</i> , <i>Padina gymnospora</i> , and <i>Sargassum liebmannii</i> (applied separately)	0.2 , 0.4, 1.0 %	Laboratory/Filter paper	Seed germination	<i>U. lactuca</i> and <i>P. gymnospora</i> : better response in germination rate, high germination index and germination energy, and increased seedling vigor, greater plumule and radicle length	(Mreya Hernández-Herrera et al., 2013)
Tomato (<i>Solanum lycopersicum</i>)	<i>Ulva lactuca</i> , <i>Caulerpa sertularioides</i> , <i>Padina gymnospora</i> , and <i>Sargassum liebmannii</i> (Applied separately)	0.2 , 0.4, 1.0 %	Greenhouse/ Foliar spray vs Soil drench	Early vegetative (15-day-old)	Soil drench was more effective than foliar spray application; <i>U. lactuca</i> and <i>P. gymnospora induced</i> increased weight, shoot and root length	(Mreya Hernández-Herrera et al., 2013)
Rice (<i>Oryza sativa</i> subsp. <i>Japonica</i>)	<i>Sargassum homeri</i>	5%	Field experiment/Root application	Tillering and Heading Stages	Increased rhizosphere bacterial community and diversity; Increased nutrients in the soil; enhanced rice quality and yield	(Chen et al., 2022)
Kale (<i>Brassica oleracea</i>)	<i>Ulva lactuca</i> , <i>Fucus ceranoides</i> , <i>Gracilaria gracilis</i> (applied separately)	Not mentioned	Laboratory/Filter paper with cotton bellow	Seed germination	<i>Gracilaria gracilis</i> extract enhanced kale seed germination and seedlings weight/length.	(Mendes et al., 2022)
Lettuce (<i>Lactuca sativa</i>)	<i>Gracilaria</i> sp., <i>Ulva intestinalis</i> , <i>Ascophyllum nodosum</i> , "Fitoalgas Green" (applied separately)	10 , 20%	Laboratory/Filter paper with cotton bellow	Seed germination	Increased seed germination rate	(Turco et al., 2022)
Tomato (<i>Solanum lycopersicum</i>) and Sweet pepper (<i>Capsicum annuum</i>)	<i>Sargassum vulgare</i> and <i>Acanthophora spicifera</i> (applied separately)	0.5%	Greenhouse/Foliar spray	Not mentioned	Alleviated disease severity caused by pathogens, <i>Xanthomonas campestris</i> and <i>Alternaria solani</i> . Enhanced plant growth and yield	(Ali et al., 2021)
Tomato (<i>Solanum lycopersicum</i>) and Sweet pepper (<i>Capsicum annuum</i> L.)	<i>Stella Maris</i> ®: <i>Ascophyllum nodosum</i>	Not mentioned	Greenhouse/ On the tray	Not mentioned	Increased plant productivity, increased biodiversity and structure of the microbial community (fungal and bacterial at rhizosphere)	(Renaut et al., 2019)

Seaweed extracts as modulators of soil and root microbiota

Studies have demonstrated that applying seaweed extracts into the soil can enhance the development and health of various crops, as previously described. Additionally, these extracts have been demonstrated to impact the soil and root microbiota, leading to changes in abundance and diversity (Alam et al., 2013; Chen et al., 2022; Renaut et al., 2019). The organic matter and minerals present in seaweed extracts can serve as a source of nutrients for soil microorganisms, increasing their populations. This can lead to soil health and fertility improvements, which can, in turn, benefit the growth and health of crops (Renaut et al., 2019). As reported by Margulis & Fester, (1991), it may be appropriate to redefine plants and microbes as holobionts, which represent a combination of several species that function as a unified ecological entity (Margulis & Fester, 1991) For instance, a biostimulant from the company Acadian named Stella Maris®, derived from *Ascophyllum nodosum*, showed an increase in plant productivity, fungal, bacterial biodiversity and changed the microbial community structure in the rhizosphere of tomato and pepper plants (Renaut et al., 2019). The same tendency was observed when *A. nodosum* applications increased strawberry root and shoot growth, berry yield, and rhizosphere microbiota diversity and physiological activity (Alam et al., 2013).

Plant growth-promoting bacteria to improve plant performance

The plant growth-promoting bacteria (PGPB) are a diverse group of beneficial bacteria that positively influence plant growth as well as protect plants from several biotic and abiotic stresses through direct and indirect mechanisms (Abhilash et al., 2016). PGPBs are not only used as a tool for sustainable food production without compromising ecosystem services, but also for bioremediation of contaminated and degraded lands and biomass and biofuel production (Abhilash et al., 2016). They can be found in a variety of environments, such as in soil, rhizosphere (commonly known as plant growth-promoting rhizobacteria, PGPR), roots, endosphere, seeds, seaweeds, and many distinct environments (De Souza et al., 2015; Díaz Herrera et al., 2016; Suvega & Arunkumar, 2019).

Specific bacteria traits can directly interact with the plant and its environment, acting as biofertilizers. Some examples of these direct interaction mechanisms include nitrogen fixation, phosphate solubilization, iron sequestration, and modulation of phytohormone levels (cytokinins, gibberellins and auxins)(Glick, 2012). Regarding nitrogen (N) fixation, certain PGPBs can fix atmospheric nitrogen into a form that plants can use, providing a source of essential nitrogen for plant growth. It was demonstrated by Islam et al., (2013), that the inoculation of three nitrogen-fixing bacteria (*Pseudomonas* sp. RFNB3, *Ochrobactrum* sp. RFNB9, and *Novosphingobium* sp. RFNB21) were able to increase plant height (26%) and dry biomass (28%) in red pepper (*Capsicum annuum* L.) when compared with the uninoculated plants. A significant increase in nitrogenase activity was also observed in plant root and rhizosphere soil with all three bacterial inoculations (Islam et al., 2013). Furthermore, the ability of phosphate-solubilizing bacteria (PSB) to dissolve phosphorus (P) is a significant attribute of PGPB. Certain PGPB can solubilize insoluble forms of phosphorus, allowing plants to absorb it more easily as assessed by (Wang et al., 2022). This study reported that *Pseudomonas moraviensis*, *Bacillus safensis*, and *Falsibacillus pallidus* could solubilize P efficiently when they inoculated wheat plants (*Triticum*

aestivum) with those strains. They observed that *P. moraviensis*, *B. safensis* and *F. pallidus* showed a remarkable ability to promote wheat growth, crop productivity and high adaptation to different soil environmental conditions (Wang et al., 2022). Both bacteria and plants require high levels of iron (Fe) for survival. However, the predominant form of iron in nature (ferric ion or Fe⁺³) is only slightly soluble, and together with Fe competition by plants, bacteria and fungi, the amount of available Fe in the soil is meager. Some PGPB can produce phytohormones, siderophores, and organic acids, improve FC-R activity, and/or regulate key Fe-related genes. As reported by Costa (2021), when soybean plants (*Glycine max*) were inoculated with *Sphingobium fuliginis* ZR 1-6 alone or in combination with *Pseudomonas jessenii* ZR 3-8, they improved ferric chelate reductase (FC-R) activity (111 %) and fostered Fe accumulation in trifoliates (144 %), respectively. Many PGPB can also modify phytohormone levels, having the ability to trigger plant's hormonal balance and its response to stress, as described by (Patten & Glick, 2002). As an example, indole-3-acetic acid (IAA) produced by bacteria in plant-microbe interactions can impact both plant growth promotion and root nodulation. A study on canola roots explored the effect of IAA produced by *Pseudomonas putida* GR12-2 on root development, showing that seed inoculation with *P. putida* GR12-2 led to 35-50% longer roots than roots treated with an IAA-deficient mutant or uninoculated roots. When mung bean cuttings were inoculated with an IAA overproducing mutant, a greater number of shorter roots were observed compared to controls. This outcome was attributed to the combined effect of auxins on growth promotion and root elongation inhibition by the phytohormone ethylene (Patten & Glick, 2002).

Plant growth-promoting bacteria can also act by indirect interactions with the plant and its environment with the intention to use the mechanisms employed by biocontrol bacteria for commercial purposes, as an alternative to chemical pesticides. Some examples of indirect mechanisms include the production of enzymes, antibiotics synthesis, production of siderophores, competition between pathogens and nonpathogens PGPBs, the induced systemic resistance (ISR) and ACC deaminase production (Glick, 2012). It is known that enzymes production provides plants protection against some pathogenic fungi because they are able to lyse a portion of the fungi cell walls, as confirmed by Santos et al., (2021) where they observed that the extremophilic bacteria *Stenotrophomonas* sp. AG3 and *Exiguobacterium* sp. S58 have the potential to inhibit the growth of the fungi *Macrophomina phaseolina* *in vitro*. Those bacteria were found to secrete lytic enzymes together with other organic compounds, reducing the disease severity caused by *M. phaseolina* in soybean seedlings (Santos et al., 2021). *Pseudomonas protegens* FD6 produces a variety of antibiotics including 2,4-diacetylphloroglucinol, pyoluteorin, and pyrrolnitrin that can suppress plant pathogens (Zhang et al., 2020). According to Zhang et al., (2020), treatments with the bacterial strain *Pseudomonas protegens* FD6, isolated from the canola rhizosphere, showed a clear effect controlling the fungus *Botrytis cinerea* in tomato fruit. No visible disease lesions were observed at 5 days post inoculation with *P. protegens* FD6, proving that antibiotics synthesis is another strategy used by PGPB to prevent the proliferation of plant pathogens (mostly fungi). On the other hand, a study conducted by Daura-Pich et al., (2020). showed that *Pseudomonas putida* B2017 is not able to synthesize common antibiotics normally produced by *Pseudomonas* spp., but it produces pyoverdine, a siderophore involved in its biocontrol

activity against *Rhizoctonia solani* DSM 63010, *Botrytis aclada* CECT 2851 and *Fusarium oxysporum* H828 (isolated from tomato). Siderophores are iron-binding molecules produced by microorganisms (including bacteria and fungi) with a high affinity for iron, which plays a significant role in the competition for iron and prevention of phytopathogens from acquiring it, limiting their ability to proliferate (Daura-Pich et al., 2020). Some evidence indicates that competition between pathogens and nonpathogens (PGPBs) can also restrict disease incidence and severity (Innerebner et al., 2011). Besides, when plants activate their defense mechanisms in response to infection by a pathogenic agent, the ISR is triggered by some PGPB. ISR-positive plants are known to react faster and more strongly to pathogen attacks by inducing defense mechanisms. Matilla et al., (2010) concluded that *Pseudomonas putida* KT2440 was able to protect the model plant *Arabidopsis thaliana* against infection by the phytopathogen *Pseudomonas syringae* pv. tomato DC3000. The extracellular enzyme haem-peroxidase (PP2561) produced by *P. putida* was found to be important for competitive colonization and essential for the induction of plant systemic resistance (Matilla et al., 2010). Regarding the phytohormone ethylene, some PGPB can also help mitigate plant stress through another mechanism, one of which is lowering the concentration of the plant hormone ethylene. It is widely reported that certain PGPB possess the enzyme ACC deaminase, which breaks down 1-aminocyclopropane-1-carboxylic acid (ACC), the immediate precursor of ethylene, into ammonia and α -ketobutyrate, reducing the level of ethylene inside the plants (Kang et al., 2019). In addition, biofilm production is also considered an indirect mechanism by which some PGPBs produce exopolysaccharides responsible for bacterial adhesion to soil particles or root surfaces, being an important trait. Biofilm is considered a well-organized structure composed of a community of microbial cells in which the cells are embedded in an extracellular matrix (ECM) composed of extracellular polymeric substances (EPSs), in which bacterial cells communicate. EPSs are responsible for the attachment process on surfaces, followed by the formation of the ECM that surrounds and holds cells together, ensuring a biofilm's structural integrity. These plant-associated biofilms can support plant growth, reduce microbial competition, and protect against pathogens and external stresses (Kumar et al., 2020). A study performed by Kasim et al., (2016) suggested that the inoculation with the biofilm-producing *Bacillus amyloquefaciens* improved barley plants growing under salt stress. Srivastava et al., (2020) also indicated that several biofilm-producing PGPR (*Pseudomonas azotoformans* ESR4, *P. poae* ESR6, *P. gessardii* ESR9, *P. cedrina* ESR12, *P. chlororaphis* ESR15, *P. veronii* ESR21, *Stenotrophomonas maltophilia* ESR20, and *Bacillus aryabhatai* ESB6) were able to reduce water-deficit stress and promoted tomato growth, which might be associated with multiple mechanisms such as biofilm formation, production of EPS, synthesis of IAA and ACC deaminase, increased enzymatic and non-enzymatic defense systems, and the improved solubilization of nutrients (Srivastava et al., 2020). In general, plant growth-promoting bacteria (PGPB) can boost plant growth and development through a variety of direct and indirect mechanisms, making them a valuable tool for sustainable agriculture. All these mechanisms demonstrated in laboratory settings are the initial step toward practical use in plants.

Objectives & experimental design

The agriculture sector is facing several challenges worldwide; therefore, it is extremely important to find new strategies to accomplish sustainable agriculture for present and future generations, ensuring food production and security. Overcoming the excessive use of chemical fertilizers in soils with negative impacts on environment and food security has become one of the main topics of research to improve novel and sustainable alternatives for crop production. The main goal of the present study is to develop a pipeline to assess putative biostimulant effects of the seaweed extract, from *Gracilaria gracilis* on two crops namely tomato (*Solanum lycopersicum*) and lettuce (*Lactuca sativa*) and monitoring their impact at specific developmental stages. The specific goals of this project include:

- Identification of bacteria strains in *G. gracilis* extract, and putative characterization as bioinoculants in planta (*Solanum lycopersicum*).
- Evaluate impacts of a range of *G. gracilis* extract concentrations when applied to *Solanum lycopersicum* and select the most effective dose for specific developmental stages.
- Evaluate distinct application methodologies of seaweed extract in planta, soil *versus* spray.
- Evaluation of root/soil microbiota changes after *G. gracilis* treatments.

The experimental set up designed to address these aims is schematically represented in Fig. 2. Two different approaches were followed in this study: (1) *G. gracilis* microbiota application in planta, and (2) *G. gracilis* application in planta. The first approach involved isolating and identifying culturable microbiota from *G. gracilis* extract and screening them for growth-promoting activities. Bacterial strains from *G. gracilis* extract were then inoculated into tomato plants, and the effects on plant growth and phenotypic traits were evaluated.

In the second approach, the biostimulant effects of *G. gracilis* itself on tomato plants at different developmental stages were investigated. This included germination, early and late vegetative stages. Additionally, two extract application methods, soil and spray, were tested in tomato (*Solanum lycopersicum*) and lettuce (*Lactuca sativa*) plants. Furthermore, plant phenotypic analysis was performed to evaluate changes in the growth and development, and possible effects of *G. gracilis* treatments on soil and root culturable microbiota profile were also assessed.

Overall, by evaluating the effects of different application methods and stages of plant development, this study sought to provide insights into the mechanisms underlying the biostimulant effects of *G. gracilis* and its associated microbiota, and to identify potential strategies for their application in agricultural settings.

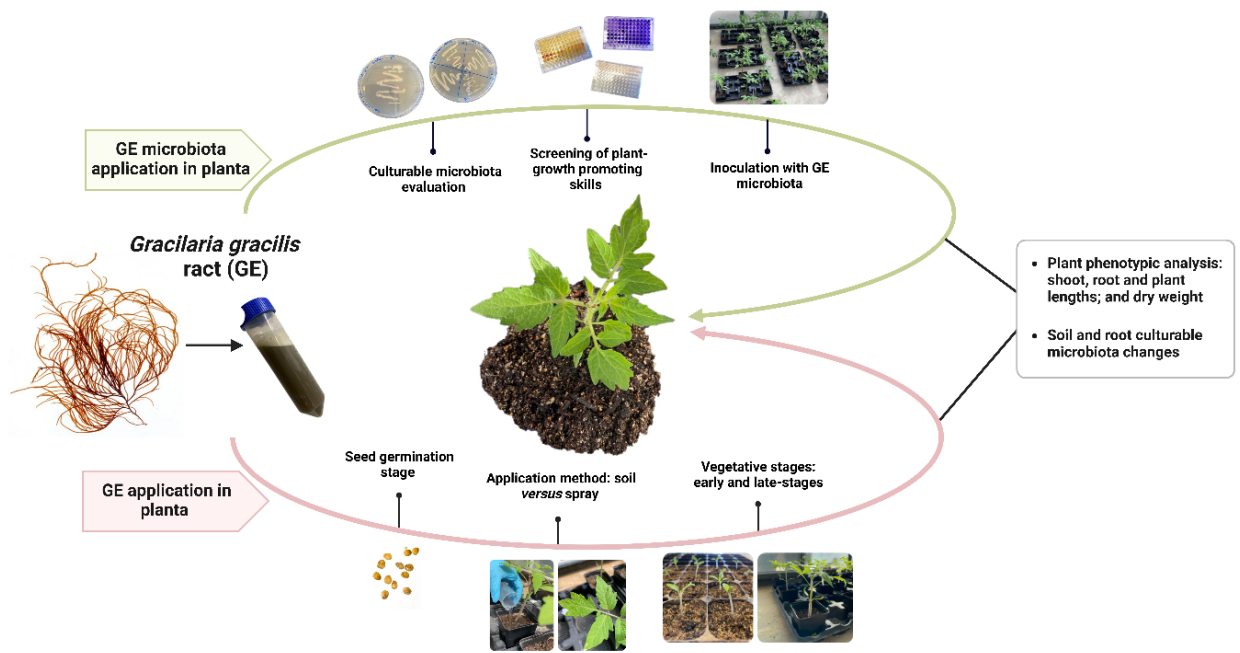


Figure 2. Experimental design with two approaches: (1) *G. gracilis* microbiota application in planta and (2) *G. gracilis* application in planta.

Materials and Methods

The *Gracilaria gracilis* extract

The *Gracilaria gracilis* extract was kindly supplied by the seaweed aquaculture company *Lusalgae*, placed in Figueira da Foz, Portugal. Based on personal communication by the suppliers, seaweed samples from Mondego estuary were hand collected from an Integrated Multi-Trophic Aquaculture (IMTA) system involving cultivation of algae, in an integrated system that allows an eco-sustainable production of fish and other marine organisms enabling to obtain algal biomass as described in (García-Poza et al., 2020). Briefly, after collection, *G. gracilis* samples were washed with distilled water and dried in an air-forced oven at 60 °C for 48 h for subsequent extract preparation. The protocol for preparing an aqueous extract of *G. gracilis* followed the one described by Sousa et al., (2020). In brief, 12 g of milled, dried seaweed mass was wetted with 100 mL of distilled water and placed into a blender. Afterward, the solution was filtered in a Büchner funnel, with a nylon net set, connected to a kitasato flask under vacuum; followed by another vacuum filtration with a Gooch funnel (according to personal communication from suppliers). Finally, the extract was maintained in dark conditions at 4°C for the following experimental studies.

Chemical analysis of the *G. gracilis* extract

The chemical characterization of the seaweed extract was performed within a collaboration with Dr. Kiril Bahcevandziev, at the Soil Laboratory of Escola Superior Agrária de Coimbra with the support of Ing. Rosinda Leonor Pato. The chemical analysis data was kindly given by the company *Lusalgae*.

The trace-elements present in the supernatant of *G. gracilis* extract were quantified by flame atomic absorption spectrophotometry in the PinAAcle 900T Atomic Absorption Spectrometer equipment (Perkin Elmer, Inc., Waltham, MA, USA). The chemical-physical properties of the extract were evaluated by thermogravimetry.

Microbiota characterization of the *G. gracilis* extract

An in-vitro colonization test was performed to investigate the presence of bacteria strains in the pure *G. gracilis* extract. First, four 1/10 seaweed extract dilutions were prepared in 1.5 mL micro-tubes. Then, an LB Petri dish (20 g/L LB Broth Miller's modification with 15 g/L Agar) was divided into four quadrants corresponding to each dilution. In each quadrant was added 50 µL of seaweed extract dilution and the plates were kept at room temperature (RT) to allow the growth. All these procedures were done in proper sterilized conditions to avoid contamination. Additionally, the original material was kept for measurements of total dry weight (DW). Colonies were differentiated by their morphology (margin, color, elevation, texture, and shape), counted, and selected. Then, colonization or culturable microbiota was calculated as colony-forming units (CFUs) mg⁻¹ of sample dry weight (DW). This indicates the number of viable colonies formed per mg of sample. The number of counted colonies by volume, dilutions applied and original mg of DW of samples were corrected/normalized.

For colony isolation, sterile disposable toothpicks were used, hands were disinfected with 70% ethanol between each colony picking, and all procedure was performed in the flow-chamber. LB Petri dishes were divided into several sections, depending on the number of bacteria identified. Then, with a gentle wiping motion, a series of horizontal streaks spread the bacteria across the LB Petri dishes. Finally, the colonies grew at RT. The Petri dishes with the bacteria isolation were grown overnight and, after assessing pure culture, they were preserved as stock in glycerol at -80°C for further studies.

Bacteria identification from *G. gracilis* extract

Pure culture strains were suspended in a sterile microtube with 40-50 µL of distilled sterile water, incubated at 95 °C for 10 min, centrifuged at 12300 rpm for 5 minutes, and used 3-5 µl of clear supernatant in 20 µL PCR reaction. The 799F (5'-AACAGGATTAGATACCCTG-3') and 1392R (5'-GGTTACCTTGTTACGACTT-3') universal pair of primers were used for 16s rRNA gene amplification. The PCR mix was prepared as follows (final volume, 25 µL): 5 µL of extracted DNA, 5.75 µL H₂O, 1.5 µL MgCl, 10 µL PCR buffer, 1.25 µL dNTP, 0.5 µL of each primer, and 0.5 µL DNA polymerase (5 u/ µL). Later, a NZYtaq II 2x Green Master Mix (NZYtech, MB35802) started to be used. A T100 Thermal Cycler from Bio-Rad was used with the following program: initial denaturation at 95°C for 2 min; 30 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s, and elongation at 72°C for 2 min. The final cycle was followed by an extension at 72°C for 7 min. After running a 1% agarose gel, the amount and quality of DNA samples were checked with NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer, and samples were maintained at -20°C for future sequencing. Sequences were kindly provided by GENEWIZ, from Azenta Life Sciences. Strains identification was done using BLAST (Basic Local Alignment Search Tool).

Screening of plant growth promoting activities

For biochemical tests (growth, biofilm, auxins and growth in ACC medium), 5 mL of an overnight growth culture of each strain were prepared in a 15 ml tube with liquid LB Broth (Miller's modification) at 28°C under agitation (160-180 rpm) for 12-16 h. Four strains isolated from pure *G. gracilis* extract were tested. To quantify the growth of each strain, 100 µL of each culture (1:10 dilution) were placed in a 96-well plate and then, an optical density reading was taken at 600 nm, using the ThermoScientific Multiskan, SkyHigh Microplate Spectrophotometer. Three replicates were performed to ensure the consistency of results. The necessary volume to obtain a concentration of 0.05 for each culture was calculated as follows:

$$Volume = \frac{(200\mu L \times 0.05)}{OD_{600\text{ nm}}}$$

Growth in 24h

In order to evaluate the growth of each strain incorporated with the three concentrations of *G. gracilis* extract (0.25%, 0.5% and 1%), 96-well plates were used. In each well, the calculated volume of each culture, 2 µL of each extract concentration (stock solutions: 100%, 50% and 5% of extract) and 200 µL

of LB liquid (for 1 L: 10 g of NaCl, 10 g of Tryptone, and 5 g of yeast extract) were added. A control was also used with LB medium. Three replicates were performed to ensure consistency of results. For the growth evaluation, after 24h growing at 28° under agitation (160-180 rpm), an optical density reading was taken at 600nm, using the ThermoScientific Multiskan, SkyHigh Microplate Spectrophotometer.

Biofilm production

The production of biofilms by bacteria was evaluated using the Crystal Violet assay, following the method described by (Coffey & Anderson, 2014) with minor modifications. The 96-well plates containing the calculated volume of each strain, the extract concentrations and LB medium, prepared as previously described, were incubated for 48 h at 28°C under agitation (160-180 rpm). After incubation, all cultures were removed, and the plates were washed, remaining only solid and stable structures on the plate (biofilm itself). These structures were stained with 200 µL of 0.5% crystal violet (For 100mL: 500 mg C₂₄H₂₈N₃Cl, 25 mL CH₃OH and 75 mL ddH₂O) for 10 minutes, washed with tap water to remove the excess of crystal violet, and dried overnight. Thereafter, glacial acetic acid 30% was added (200 µL) to each well, plates were incubated for 10 min, and OD was measured at 550nm in the ThermoScientific Multiskan, SkyHigh Microplate Spectrophotometer.

Auxins production

The production of auxins by each strain incorporated with the three concentrations of *G. gracilis* extract (0.25%, 0.5% and 1%) was evaluated to investigate if the combination of bacteria and *G. gracilis* extract can enhance the production of auxins. The method described by Ambrosini & Passaglia, (2017) was followed with slight modifications. Briefly, the calculated volume of each culture, 2 µL of each extract concentration (100%, 50% and 5% extract) and 200 µL of an LB tryptophane-supplement medium (0.5 g/L) were added to each well. A control was also used with LB medium. Three replicates were performed to ensure consistency of results. The plates were incubated for 48 h at 28°C, under agitation (160-180 rpm). After, the 96-well plates were centrifuged at 3800 rpm for 50 min in the Eppendorf 5810R centrifuge. Thereafter, 100 µL of the supernatant was collected to fresh 96-well plates to which 100 µL of Salkowski's reagent (For 50 mL: 1 ml (v/v) of 0.5 M FeCl₃ solution to 49 ml of 35% HClO₄) was added to origin a color change as an indicator of auxin's accumulation in the medium. Finally, an optical density reading was taken at 530nm, using the Thermo Scientific Multiskan, SkyHigh Microplate Spectrophotometer. Quantification of auxins (µg/mL) was accomplished by the calibration curve prepared with different standard concentrations of indole-acetic acid.

ACC deaminase production

To evaluate if bacteria have ACC deaminase activity, it must have the ability to grow in *i*-aminocyclopropane-1-carboxylate (ACC) medium, as the only source of nitrogen and carbon. Therefore, the calculated volume of each culture, 2 µL of each extract concentration (100%, 50% and

5% extract) and 200 μL of M9 minimal medium [for 1 L: 100 mL of 10x M9 salt solution with 33.7 mM Na_2HPO_4 , 22.0 mM KH_2PO_4 , and 9.35 mM NH_4Cl ; 1 mL of 1M MgSO_4 and 0.3 mL of 1M CaCl_2 ; 10 mL of 100x trace elements solution (for 1L: 13.4 mM EDTA, 3.1 mM $\text{FeCl}_3\cdot 6\text{H}_2\text{O}$, 0.62 mM ZnCl_2 , 76 μM $\text{CuCl}_2\cdot 2\text{H}_2\text{O}$, 42 μM $\text{CoCl}_2\cdot 2\text{H}_2\text{O}$, 162 μM H_3BO_3 and 8.1 μM $\text{MnCl}_2\cdot 4\text{H}_2\text{O}$) and sterile ddH₂O] supplemented with 3mM ACC were added in each well. A control was also used. Three replicates were performed to ensure consistency of results. The plates were incubated for 72 h at 28°C, under agitation (160-180 rpm). After that time, an optical density reading was taken at 600 nm, using the Thermo Scientific Multiskan, SkyHigh Microplate Spectrophotometer.

Plant material and growing conditions

Commercial tomato (*Solanum lycopersicum*) seeds from the variety “TRES CANTOS” were supplied by Semillas Fitó (Barcelona, Spain) and lettuce (*Lactuca sativa*) seeds from the variety “GRAND RAPIDS” (Flora Lusitana, Lda.) were kindly offered by collaborators from Escola Superior Agrária de Coimbra, Portugal. Seeds were surface disinfected with ethanol 70% for 10 min in agitation and washed two times with sterile distilled water. Tomato seeds were germinated in plastic boxes with water-moistened paper covered with transparent film. After nine days germinating, tomato seedlings at the two-leaf stage were transferred into 0.5 L pots with a single seedling per pot for all the tests, except for the early-stage test where a tray with 0.33 L pots was used. Lettuce seeds were germinated in 0.60% agar Petri dishes for nine days. After that time, lettuce seedlings in the emergence stage were transferred into 0.5 L pots with a single seedling per pot. Germination was performed in a plant growth chamber at $25 \pm 3^\circ\text{C}$ under light conditions for both crops. The soil used for all plant tests contained a mixture of natural soil, substrate and vermiculite (1:1:0,5). Plants were grown in the ITQB NOVA greenhouse, with temperatures varying between 26-30°C, apart from the early-stage test that was performed in a plant growth chamber at $25 \pm 3^\circ\text{C}$ under light conditions. All plants were watered according to the soil requirement.

Plant treatments with *Gracilaria gracilis* extract

***G. gracilis* extract application at distinct tomato developmental stages: Seed germination, early and late vegetative stages**

Tomato seeds, surface disinfected as previously described, were placed in squared Petri dishes (120x120mm) on filter paper moistened with three concentrations of *G. gracilis* extract (0.25%, 0.5% and 1%). For each treatment, two squared Petri dishes were used with 10 seeds per dish. Each dish received 8 ml of the distinct dilutions of *G. gracilis* extract and 8 ml of tap water as a control. All plates were sealed with 3M™ Micropore™ Surgical Tape. Germination was carried out at 29°C in the dark. Five independent germination assays were performed, all in the same experimental conditions. The germination assay was followed daily over five days and on the fifth day root and shoot lengths were measured using the ImageJ software (Schneider et al., 2012) (<https://imagej.nih.gov/ij/download.html>). The germination rate (%) was assessed by the following formula:

$$\text{Germination rate (\%)} = \frac{\text{number of germinated seeds}}{\text{number of total seeds}} \times 100$$

The effects of *G. gracilis* extract were also evaluated at two different vegetative developmental stages of tomato, the early and late-stage. For the early-stage test, when seedlings reached the early-growth developmental stage (first true leaves), three concentrations of *G. gracilis* extract (0.25%, 0.5% and 1%) and a control containing 0% extract concentration (only tap water) were applied in tomato plants by soaking the soil with approximately 26,4 ml. The assay included 15 biological replicates per condition. Here, an additional test condition included the application of 3ml/L of a commercial fertilizer, the Complestal NPK fertilizer 12-4-6 (p/p) with micronutrients [Nitrogen (N) – 12%; Phosphoric anhydride (P₂O₅): 4%; Potassium oxide (K₂O): 6%]. For the late-stage test, when plants achieved three layers of leaves, the same treatments were applied by soaking the soil with 40mL, except from 1% of *G. gracilis* extract and the commercial fertilizer (Complestal NPK fertilizer). The assay included 6 biological replicates per condition.

***G. gracilis* extract application method: soil versus foliar spray**

Two application methods were tested in tomato and lettuce plants to assess the optimal method for the following tests. Three concentrations of *G. gracilis* extract (0.25%, 0.5% and 1%), Complestal NPK fertilizer 12-4-6 (soil application received 3ml/L and spray application received 8ml/L, according to the product label), and a control containing 0% extract concentration (only tap water) were used for this assessment. When plants achieved three layers of leaves (tomato) and seven leaves (lettuce), all the treatments were applied by simply soaking the soil or spraying the leaves. Each plant received 40 ml by soaking the soil or 3 ml by spraying the leaves of *G. gracilis* extract. The same was performed for Complestal NPK fertilizer and control treatments. Before the spraying process, the surface of the potting soil was covered entirely with aluminum foil to avoid leaching into the soil. Then, a second application was performed, using the same conditions previously described. The assay included 6 biological replicates per condition.

Inoculation of tomato plants with *G. gracilis* extract microbiota

When tomato plants achieved three layers of leaves, four bacteria strains GE1_*Bacillus mycoides*, GE2_*Klebsiella oxytoca*, GE3_*Leclercia adecarboxylata* and GE4_*Pseudomonas putida*, isolated from *G. gracilis* extract, were separately inoculated in tomato plants. First, the pre-inoculum was prepared using 5 mL of well-grown overnight culture in liquid LB medium (20g/L LB Broth Miller's modification) for 2 h at 28°C with 150 rpm of agitation. Then, 1 mL of the pre-inoculum was transferred to 400 mL of fresh LB, and it was incubated for 8 h at 30°C with an agitation of 160 rpm. After that, the culture was centrifuged for 7 min at 10000 rpm using an ultracentrifuge (Avanti J30) with the rotor JA-14. The LB was then discarded, and the pellet was resuspended with the same volume of NaCl (0.45%), to keep the osmolarity of the cells. Finally, plants were separately inoculated with 40 mL of each inoculum using

a 50 mL plastic syringe. At the same time, a control was also used, which only received 40 mL of 0.45% NaCl.

Plant phenotypic analysis

Regarding phenotypic analysis, a photographic record of all plants was taken. In addition, shoot, root and plant length were measured for all tests using the ImageJ software (Schneider et al., 2012) (<https://imagej.nih.gov/ij/download.html>). Only for the early-stage test, plants were placed in an incubator at 60°C for two days, and dry weight of the whole plant was quantified.

For the assessment of the best method to apply the extract, phenotypic analysis was performed after 15 days of the second application of *G. gracilis* extract. The phenotypic analysis at distinct tomato developmental stages was performed 23 days after *G. gracilis* treatments for the early-stage test and 15 days after treatments for the late-stage test. For the inoculation of tomato plants with *G. gracilis* microbiota, the analysis was performed 15 days after treatments and consisted in shoot, root and plant lengths.

Soil and root microbiota characterization

Following the soil *versus* foliar spray test, three root samples from each condition were cut and disinfected in micro-tubes: 1000 µL ethanol mixed for 3 min and 1000 µL of water mixed for 3 min (repeated twice). Then, 1000 µL of NaCl was added to keep osmolarity and prevent bacteria from suffering, and the root piece was crushed. Finally, dilutions were done for each biological replica (1:10). Then, Petri dishes were prepared, divided into four quadrants corresponding to each dilution, as previously described. The first tube was reserved to later measure dry weight. In addition, an identical procedure was performed for soil samples, as previously described for each condition. Although, in this case, it was prepared five dilutions because the soil was too concentrated, which could make it challenging to observe and count colonies. When plating, it was not used the first dilution. That tube was reserved to later measure dry weight. Colonies differentiation, microbiota isolation, and bacteria identification were performed as previously described.

Phylogenetic trees

To study the evolutionary history and taxonomical relationships among the isolated bacterial species from the diverse origins, the 16S rRNA gene sequences for 18 newly isolated strains were compared with those in the GenBank database using NCBI BlastN. Two different phylogenetic trees were generated considering two different data origins, root and soil microbiota after treatments with *G. gracilis* extract in tomato plants. The strains used to construct the root phylogenetic tree includes the genus *Bacillus*, *Lysinibacillus*, *Rosellomorea*, *Brevibacterium*, *Peribacillus* and *Paenibacillus*. In the case of soil, the phylogenetic tree includes the genus *Bacillus*, *Cytobacillus*, *Lysinibacillus*, *Priestia* and *Pseudomonas*.

The software ClustalX, a widely used phylogeny tool based on the maximum-likelihood principle, was used for alignment and tree performance. For visualization, the website iTOL v6 (<https://itol.embl.de/>) was used.

Statistical analysis

The statistical analysis was performed using the GraphPad Prism v9.0. software (CA, USA). One-way analysis of variance (ANOVA) was used to determine statistically significant differences between the means of treatments according to Duncan's multiple range test ($p = 0.05$). As a complementary analysis, Shannon Diversity Index (H) and Evenness (E) (Omni Calculator - www.omnicalculator.com/) were used to compare populations from different samples.

Results

Chemical analysis of *Gracilaria gracilis* extract

In the present study, results from the chemical analysis showed the presence of the macronutrients Mg and K in *G. gracilis* extract, 512 ± 102 mg/Kg and 6841 ± 1368 mg/Kg, respectively. Other micronutrients were also identified in the extract, including Fe and Cu with approximately 521 mg/Kg and 14,7 mg/Kg, respectively (Fig. 3). Besides, some heavy metals were also detected at lower concentrations mainly Cd ($<0,02$ mg/Kg) Pb ($0,26\pm 0,05$ mg/Kg) and Hg ($<0,1$) (Fig. 3). K was found to be the most abundant element in *G. gracilis* extract (6841 ± 1368 mg/Kg), one of the most abundant inorganic cation in plant cells, which plays a vital role in many physiological and metabolic processes.

A		B		
Trace elements	Value (mg/Kg)	Parameter	Value (%)	Tolerance
Cadmium (Cd)	$<0,02$	Water content	96,2	$\pm 1,6$
Lead (Pb)	$0,26\pm 0,05$	Dry Matter	1,94	$\pm 1,3$
Copper (Cu)	14,7	Ash	1,86	$\pm 0,09$
Iron (Fe)	521 ± 104			
Magnesium (Mg)	512 ± 102			
Mercury (Hg)	$<0,1$			
Potassium (K)	6841 ± 1368			

Figure 3. Chemical analysis of *Gracilaria gracilis* extract supplied by the company *Lusalgae*. The tables show (A) Determination of trace elements; (B) Determination of chemical-physical properties.

***Gracilaria gracilis* extract microbiota may indicate putative plant growth promoting activities**

Specific bacterial strains isolated from pure *G. gracilis* extract (GE) namely, the GE1_ *Bacillus mycoides*, GE2_ *Klebsiella oxytoca*, GE3_ *Leclercia adecarboxylata* and GE4_ *Pseudomonas putida*, were further characterized based on their putative plant-growth-promoting activities, included growth after 24h, biofilm production, auxins-equivalents production and growth in ACC medium. Biofilm structures own essential activities for land plants, such as facilitating colonization, increasing resistance to specific environmental stresses, suppressing phytopathogens, and antimicrobial tolerance. In addition, many plant-growth-promoting rhizobacteria have been reported to produce auxin, a root growth-promoting hormone. Apart from enhancing plant root growth, this phytohormone also enables microbes to safely enlarge their occupancy and food supply, and regulates a whole repertoire of plant developmental processes. The 1-aminocyclopropane-1-carboxylic acid (ACC) is the main precursor of ethylene. This plant hormone is highly produced when the plant is under stress conditions, inhibiting plant growth and development. PGPR containing ACC deaminase may break down some excess ACC and lower plant ethylene levels, promoting plant growth.

The biofilm and auxins production as well as their ability to growth in ACC were evaluated on a high throughput 96-well plate system with or without the supplementation of three different concentrations of GE (0.25%, 0.5% and 1%) (Fig. 4). Additionally, the growth rate of each strain after 24h was also evaluated in the presence or absence of the seaweed extract. Controls for each biochemical test consisted of sterile media without bacteria.

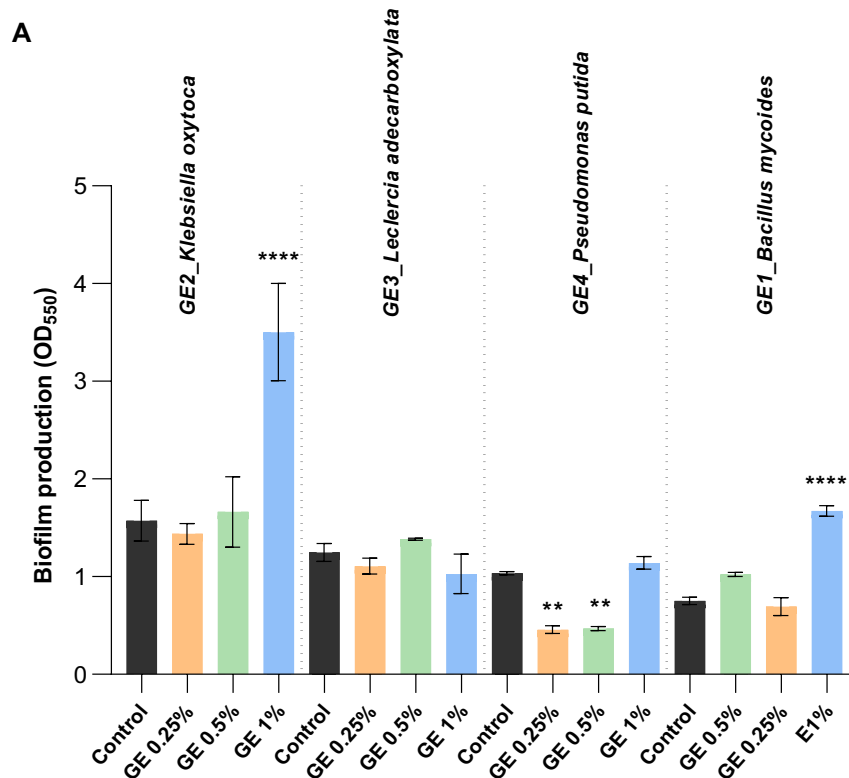
Regarding the evaluation of biofilm production when each bacteria strain was supplemented with different concentrations of GE showed that the strains GE2_ *Klebsiella oxytoca* and GE1_ *Bacillus mycoides* were able to significantly double the production by a factor of 2.2 when supplemented with GE at 1% ($p < 0.0001$) (Fig. 4A). On the other hand, the GE4_ *Pseudomonas putida* decreased by 56% and 55% the biofilm production in the lower concentrations of the extract, 0.25% and 0.5% ($p < 0.01$), respectively. For the GE3_ *Leclercia adecarboxylata* strain the biofilm production was not significantly influenced by the medium supplementation with GE.

Concerning the auxins test, the GE2_ *Klebsiella oxytoca* and GE3_ *Leclercia adecarboxylata* strains were able to significantly improve by 38% ($p < 0.01$) and 35% ($p < 0.001$), respectively, the auxins-equivalents production when the medium was supplemented with the seaweed extract at 1% (Fig. 4B). In addition, the GE1_ *Bacillus mycoides* almost triplicate the auxins-equivalents production at 1%, about 269% ($p < 0.0001$). When 0.25% and 0.5% of GE was added, GE1_ *Bacillus mycoides* could almost double the auxins-equivalents production about 193% ($p < 0.001$) and 208% ($p < 0.0001$), respectively (Fig. 4B). On the contrary, the GE2_ *Klebsiella oxytoca* and GE3_ *Leclercia adecarboxylata* strains did not show significant differences regarding auxins-equivalents production when supplemented with GE at 0.25% and 0.5%. Furthermore, the GE4_ *Pseudomonas putida* strain supplemented with GE at all concentrations significantly decreased auxins-equivalents production ($p < 0.0001$) in comparison with the control (Fig. 4B). Quantification of auxins-equivalents ($\mu\text{g/mL}$) was accomplished by the calibration

curve prepared with 4 different standard concentrations of indole-acetic acid (5, 10, 50 and 100 µg/mL), as previously mentioned (Fig. 5).

To evaluate the growth in ACC medium, the strains under evaluation were cultured in M9 minimal medium with 1-aminocyclopropane-1-carboxylate as the sole carbon and nitrogen source. In Figure 4C, it is observed an improvement of 60% of the growth in ACC medium by GE4_ *Pseudomonas putida* ($p < 0.05$) and an improvement by a factor of 2.3 in relation to GE1_ *Bacillus mycooides* ($p < 0.0001$), when supplemented with GE at 1%. Likewise, GE1_ *Bacillus mycooides* in 0.25% *G. gracilis* extract significantly improved by 70% its growth in ACC medium ($p < 0.05$). (Fig. 4C).

The growth ratio of each strain after 24 hours was evaluated by incorporating GE at three different concentrations (0.5%, 0.5% and 1%). The control represents the untreated growth of strains in LB medium, as previously reported. The results, as seen in Figure 6, indicate that almost all the strains were able to significantly improve their growth when incorporated with 0.25%, 0.5% and 1% of the extract (GE2_ *Klebsiella oxytoca*: 16%, 30% and 85%; GE1_ *Bacillus mycooides*: 239%, 336% and 353%; and GE4_ *Pseudomonas putida*: 32%, 43% and 75%), except from GE3_ *Leclercia adecarboxylata* which decreased by 4% the growth in GE at 0.25% ($p < 0.01$) (Fig. 6).



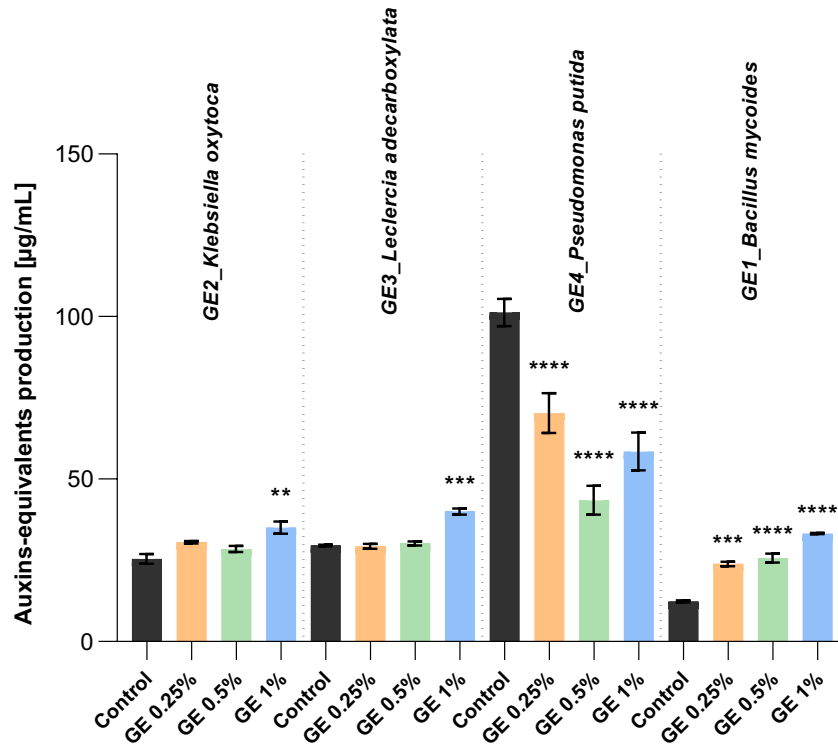
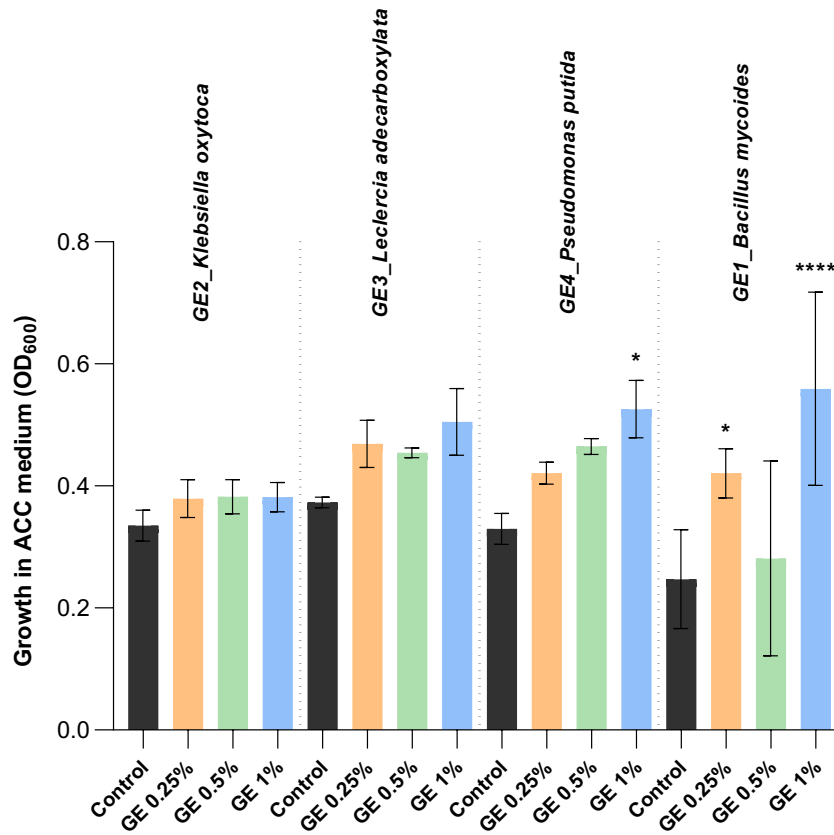
B**C**

Figure 4. Plant growth-promoting activities of specific strains subjected to treatments with different concentrations of GE (0.25%, 0.5% and 1%). The graph bars show the (A) production of biofilm; (B) production of auxins-equivalents; (C) growth recorded in ACC medium as sole nitrogen and carbon source (indicative of ACC deaminase production). Black colored bars represent the control

of each strain (without extract treatment); orange bars represent *G. gracilis* extract at 0.25%; green bars represent *G. gracilis* extract at 0.5%; and blue bars represent *G. gracilis* extract at 1%. This test was prepared with n=3. The sets of data were compared by one-way ANOVA test and 95% confidence intervals (with Šidák's post-tests), wherein asterisks represents a statistically significant difference at p < 0.05, *; p < 0.01, **; p < 0.001, ***; and p < 0.0001, ****; when no statistical marks in the graph it stands for statistically non-significant. Error bars represent s.d.

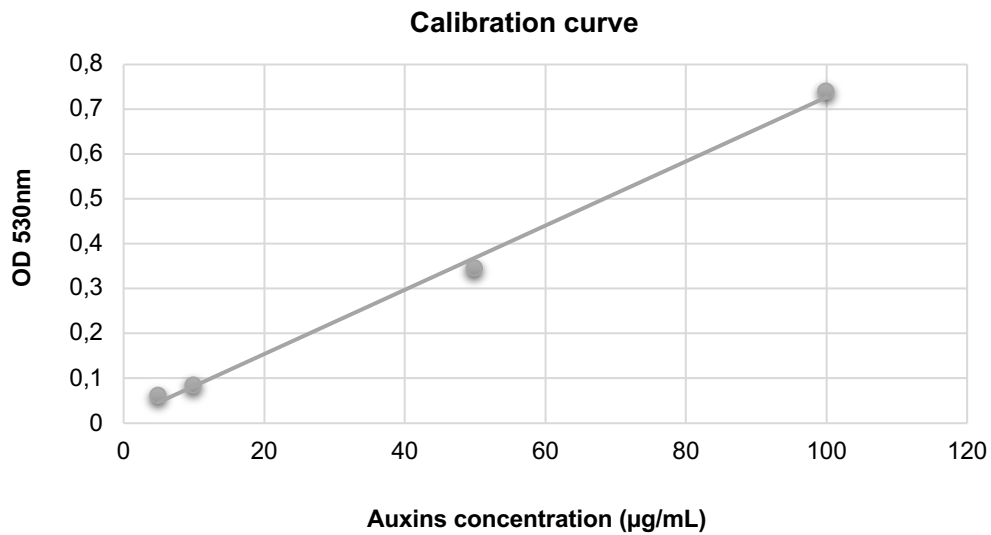


Figure 5. Calibration curve for auxin's quantification (µg/mL). Standard solutions of 5, 10, 50 and 100 µg/mL were used.

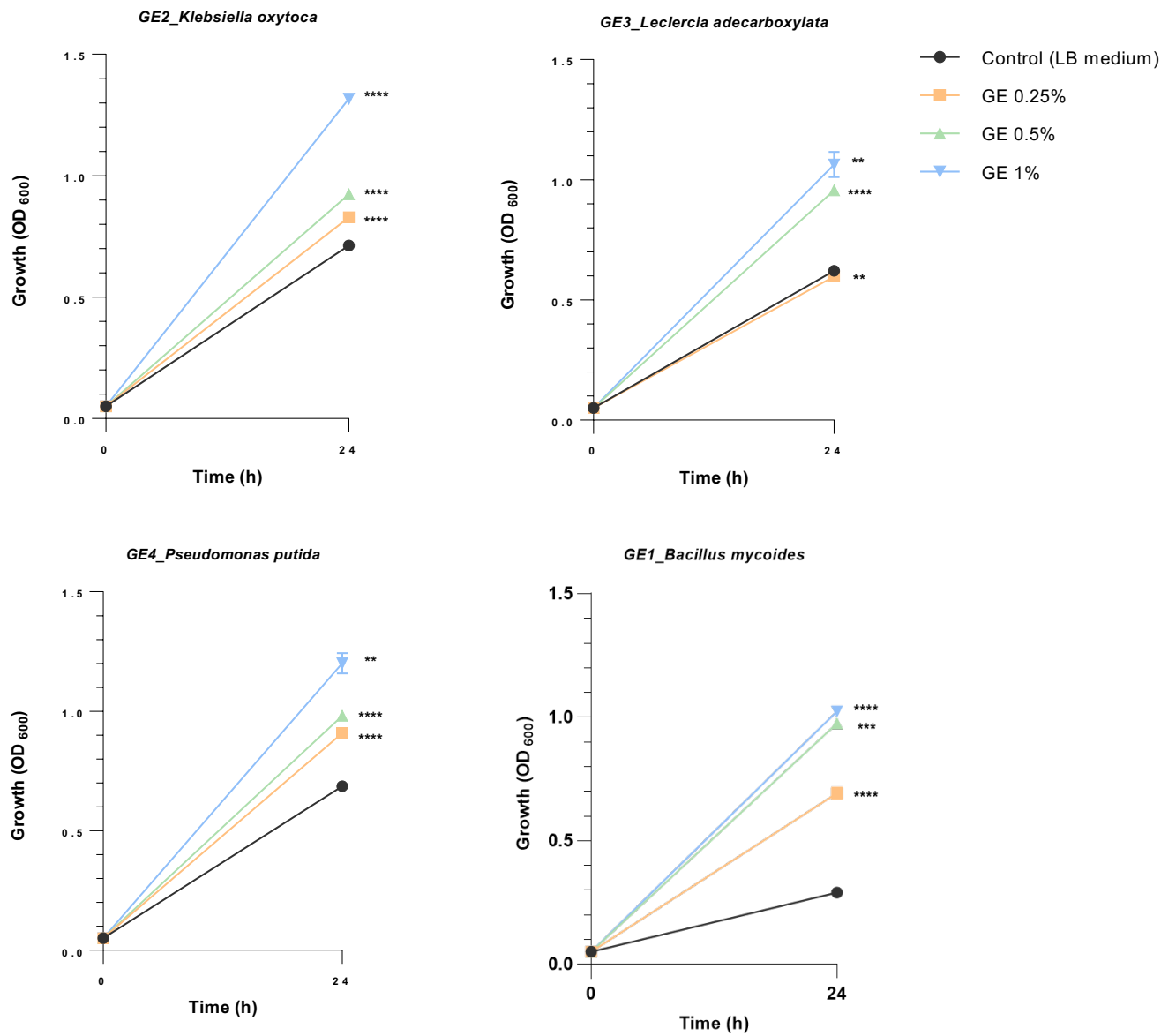


Figure 6. 24h-growth of strains isolated from GE and supplemented with three different concentrations of extract (0.25%, 0.5% and 1%). Dark lines represent the control of each strain (incorporated in LB medium). This test was prepared with n=3. The sets of data were compared by two-tailed Student's t-test (Welch's correction) and 95% confidence intervals, wherein asterisks represents a statistically significant difference at $p < 0.01$, **, $p < 0.001$, ***; and $p < 0.0001$, ****; when no statistical marks in the graph it stands for statistically non-significant. Error bars represent s.d.

***G. gracilis* extract treatment increased seed germination rate**

The germination ratio of tomato seeds treated with *G. gracilis* extract at three different concentrations (0.25%, 0.5% and 1%) was observed daily over five days, counting the germinated and non-germinated seeds. The results, as seen in Figure 7, indicate that the extract can slightly enhance the germination ratio of tomato seeds compared with the control. In addition, seeds treated with *G. gracilis* extract at 0.25% and 1% seem to have the highest (96%) and lowest (94%) germination ratio, respectively.

The effects of distinct concentrations of GE (0.25%, 0.5% and 1%) in the germination stage of tomato seeds were further evaluated (Fig. 8A). At the end of five days, data indicated no significant differences in shoot and root lengths of tomato seedlings compared with the control (Fig. 8B and 8C, respectively). Moreover, it is visible that *G. gracilis* extract at 0.5% slightly improved tomato seedlings' shoot length (Fig. 8B), even though it is not statistically significant. *G. gracilis* extract treatments seem to have a tendency to decrease the root length of tomato plants, although not significant (Fig. 8C).

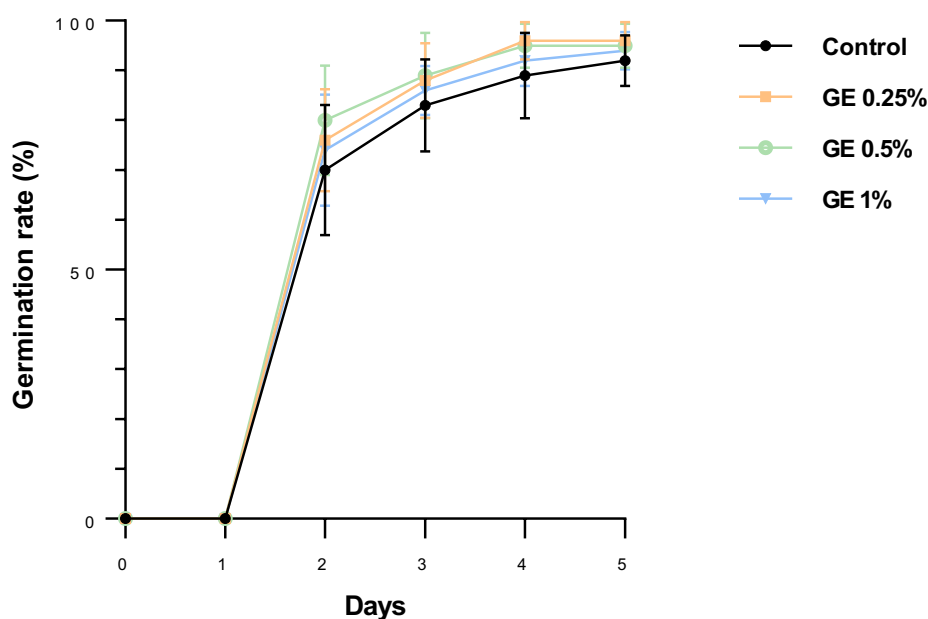


Figure 7. Germination rate (%) of *S. lycopersicum* seeds supplemented with GE (0.25%, 0.5% and 1%), observed daily over a 5-days period. Data showed is representative from five independent experiments (n=20).

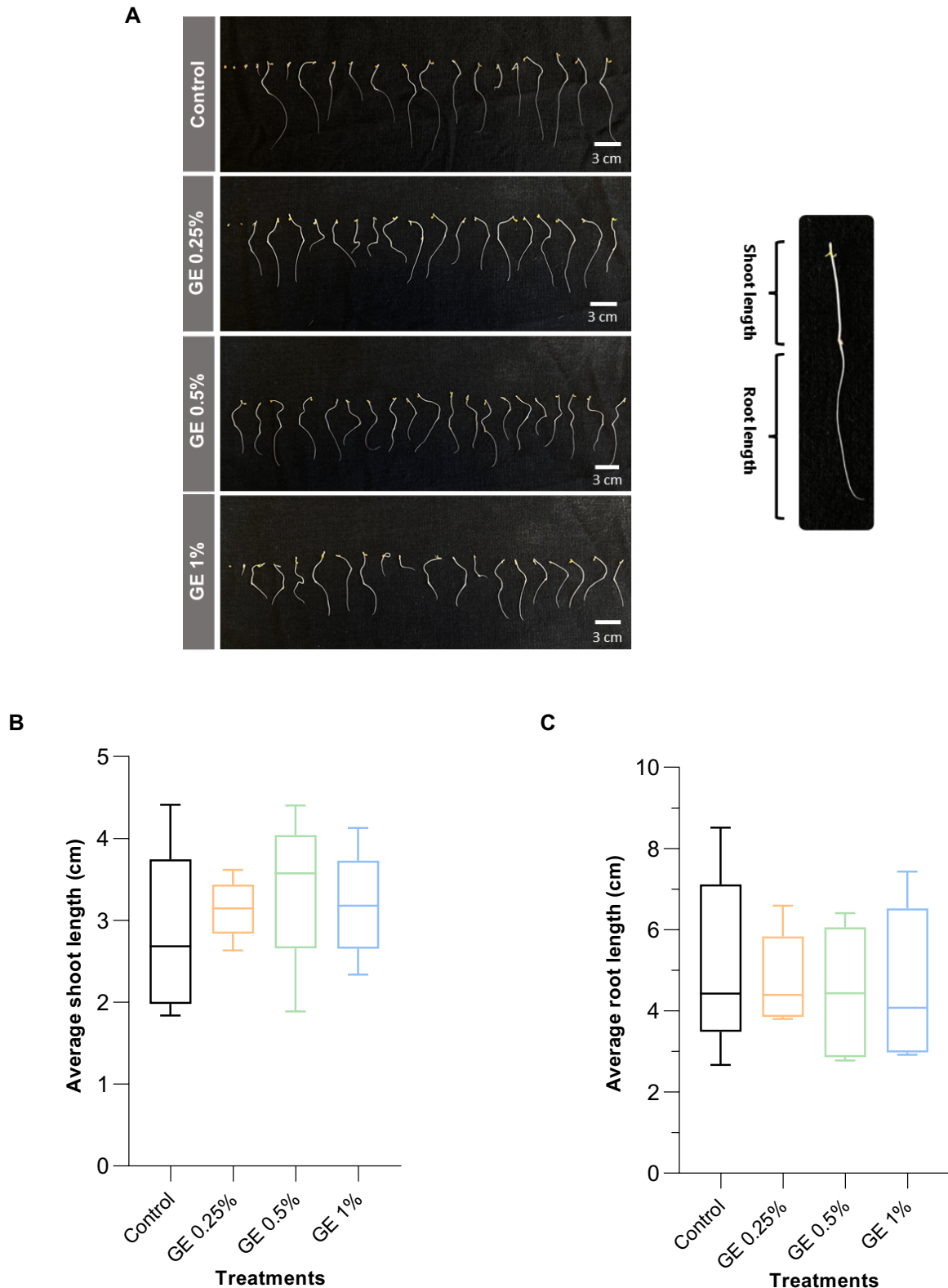


Figure 8. Germination tests with *S. lycopersicum* seeds supplemented with GE at three different concentrations (0.25%, 0.5% and 1%), observed daily over a 5-days period; (A) – Photographic record of *S. lycopersicum* seedlings after treatments; (B) - Shoot length; (C) – Root length. Data showed is representative from five independent experiments (n=20). The sets of data were compared by two-tailed Student's t-test (Welch's correction) and 95% confidence intervals; when no statistical marks in the graph it stands for statistically non-significant. Error bars represent s.d.

***G. gracilis* extract application in soil may be preferable than foliar spray application**

Two application methods, soil and foliar spray, were tested on tomato and lettuce plants to determine the most effective method for the upcoming tests. Additionally, three different concentrations of GE (0.25%, 0.5% and 1%), NPK fertilizer, and control (only tap water) were applied to evaluate the growth within different treatments. This assessment was also performed in lettuce plants since, based on personal communication by the *G. gracilis* extract suppliers, it showed to be beneficial for lettuce growth (Turco et al., 2022).

The application of NPK fertilizer in soil was associated with improved growth of tomato and lettuce plants (Figures 9A and 9C, respectively). Statistics indicate that soil application of NPK fertilizer significantly ($p < 0.001$) improved by 30% the shoot length of tomato plants (Fig. 10A) in comparison with spray application. It was also observed a significant improvement ($p < 0.01$) in the shoot length of control lettuce plants by foliar spray application (Fig. 10A). Regarding root and plant lengths, no significant differences were observed, neither for tomato or lettuce plants and neither for soil or spray application (Fig. 10B and 10C).

In this specific case, planta test made it possible to establish soil application as the best application method for tomato plants, since applying NPK fertilizer by soil application significantly enhanced tomato shoot length, as previously described. For this reason, soil application was selected for the following tests.

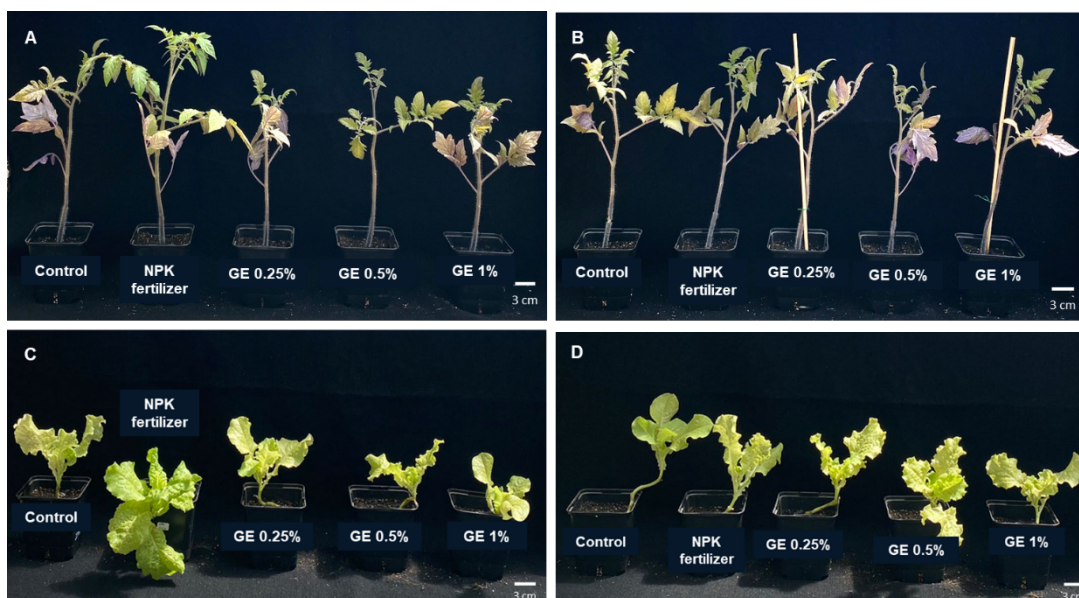


Figure 9. Representative plants treated with NPK fertilizer and GE at three different concentrations (0.25%, 0.5% and 1%) by soil and foliar spray applications ($n=3$). (A) – Soil application in *S. lycopersicum* plants; (B) – Spray application in *S. lycopersicum* plants; (C) – Soil application in *Lactuca sativa* plants; (D) – Spray application in *L. sativa* plants. This test was prepared with $n=6$.

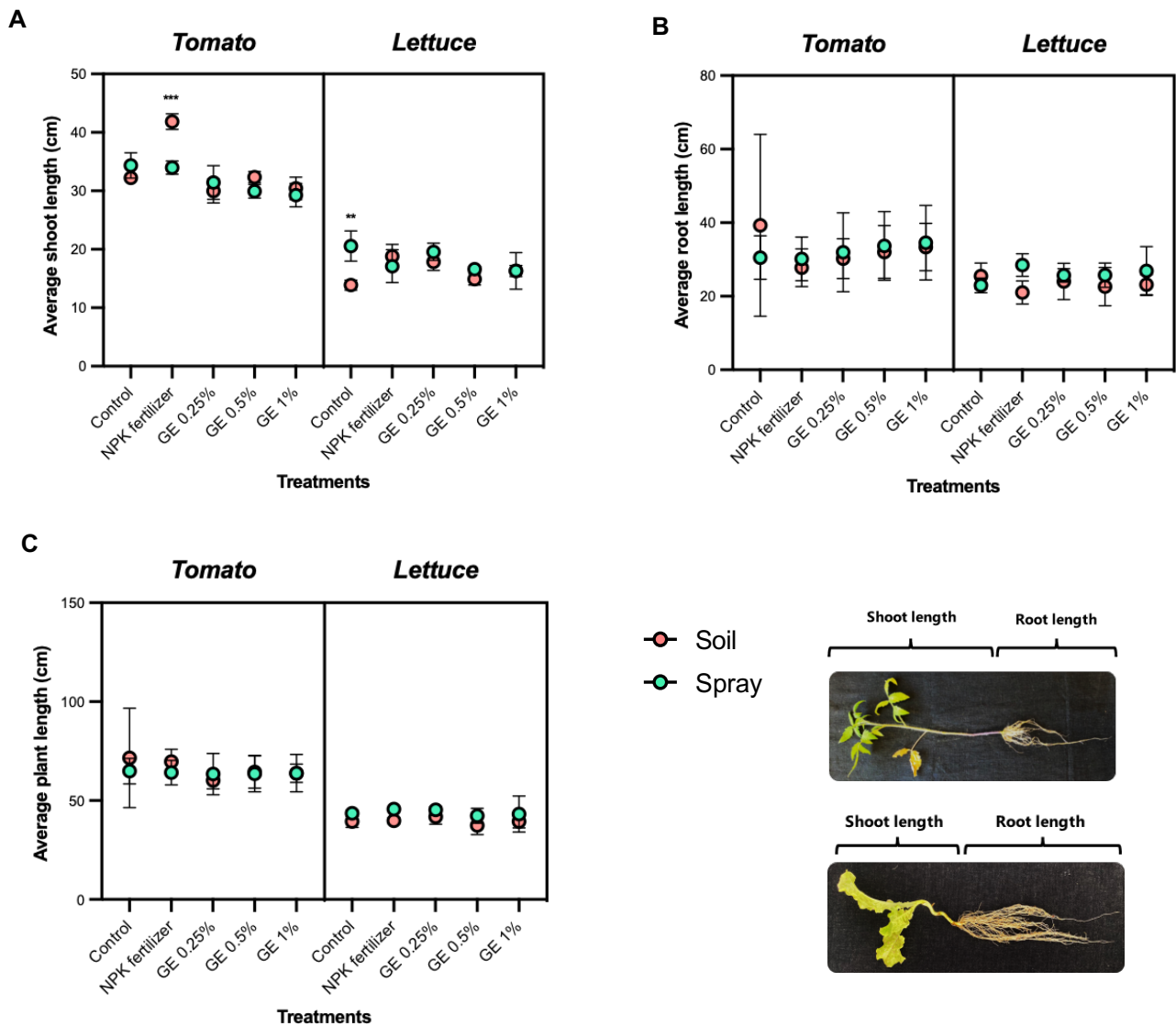


Figure 10. Soil and spray application of NPK fertilizer and GE at three different concentrations (0.25%, 0.5% and 1%) in *S. lycopersicum* and *L. sativa* plants. Each graph represents a different measurement including shoot length (A), root length (B) and plant length (C). This test was prepared with n=6. The sets of data were compared by three-way ANOVA test and 95% confidence intervals (with Tukey's post-tests), wherein asterisks represent a statistically significant difference at $p < 0.01$, ** and $p < 0.001$, ***; when no statistical marks in the graph it stands for statistically non-significant. Error bars represent s.d.

***G.gracilis* extract treatment improved shoot length, plant length and biomass of tomato plants in the early-stage**

The effects of GE in tomato plants at the first true leaves developmental stage were evaluated at three different concentrations: 0.25%, 0.5% and 1%. Apart from that, NPK fertilizer was also applied. The control represents the non-treated plants (only tap water).

The treatment with NPK fertilizer in soil strongly enhanced tomato plants' growth; on the other hand, for the same developmental stage, the treatment with GE did not particularly influenced tomato growth (Fig. 11C). The NPK treatment significantly improved all the growth attributes of tomato plants, including shoot length by 3 times more, plant length by 94% ($p < 0.0001$), and root length by 40% ($p < 0.01$).

The GE treatments were also able to significantly improve by 45% and 54% tomato shoot length at 0.5% and 1%, respectively ($p < 0.001$ and $p < 0.0001$). No significant differences were recorded regarding root length, although a slight improvement is visible after extract treatments (Fig. 11A). The treatment with *G. gracilis* extract, in general, significantly improved the length of tomato plants compared to the non-treated plants (control) (Fig. 11A). In respect to dry weight, a significant increase (by 5 times more) was observed after NPK fertilizer treatment ($p < 0.0001$) and GE treatment at 0.25% by 29% ($p < 0.05$) (Fig. 11B).

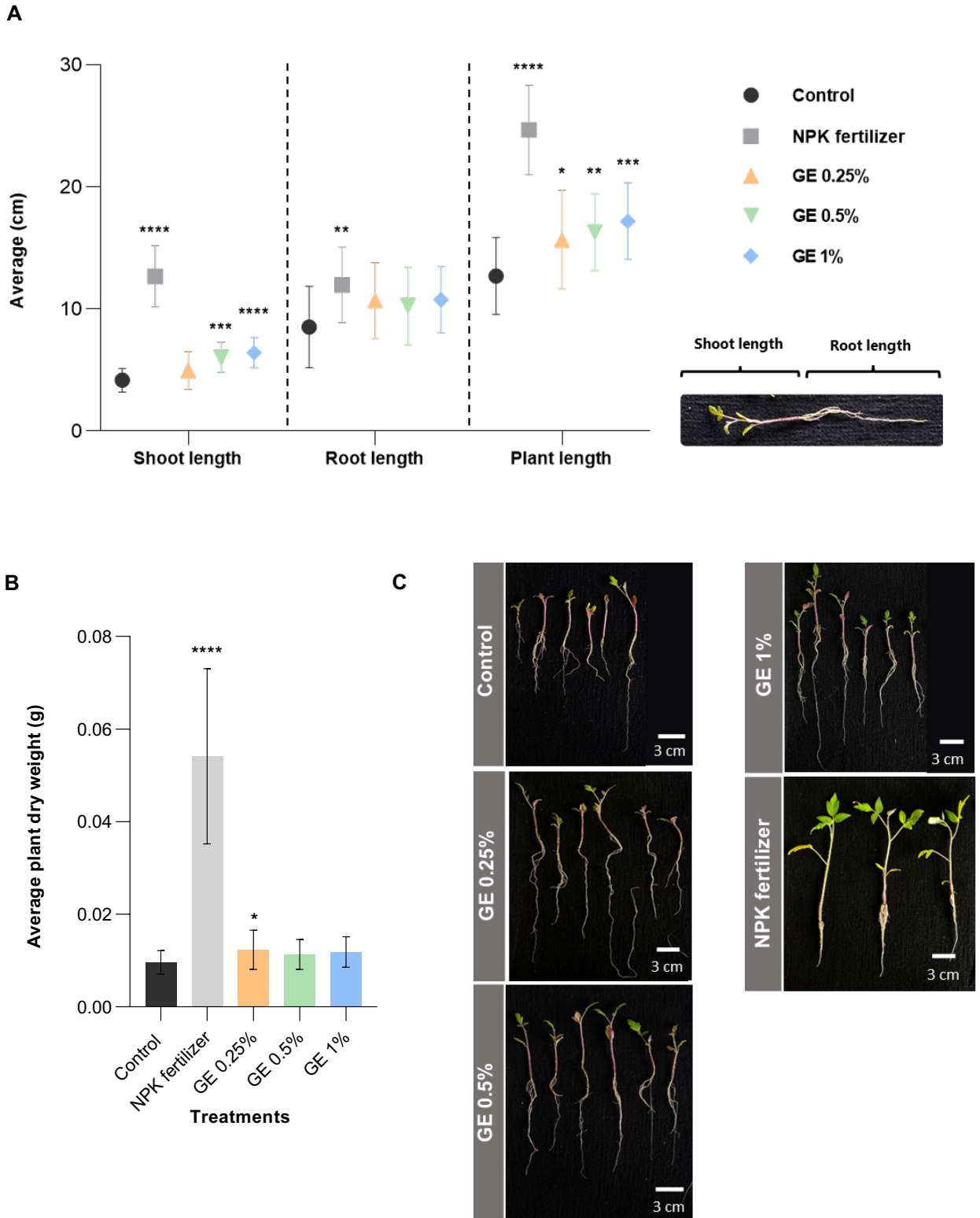


Figure 11. Soil application of NPK fertilizer and GE at three different concentrations (0.25%, 0.5% and 1%) in *S. Lycopersicum* plants in the early-stage. (A) - Shoot length, root length and plant length after treatments; (B) – Average plant dry weight (g) after treatments. (C) - Photographic record of representative *S. lycopersicum* seedlings after treatments. This test was prepared with n=15. The sets of data were compared by two-tailed Student's t-test (Welch's correction) and 95% confidence intervals, wherein asterisks represent a statistically significant difference at $p < 0.05$, *; $p < 0.01$, **; $p < 0.001$, ***; and $p < 0.0001$, ****; when no statistical marks in the graph it stands for statistically non-significant. Error bars represent s.d.

The effects of GE extract in tomato plants were also evaluated in a more advanced vegetative developmental stage (plants with three layers of leaves) at two different concentrations: 0.25% and 0.5%. Here, the treatment with GE at 1% was excluded in a view of having a more economical treatment. The control represents the non-treated plants (only tap water).

After GE treatments at 0.25% and 0.5% it was observed an improvement in tomato plants' growth when compared with the control (Fig.12). Data from the growth attributes (Fig. 13) indicates that GE at 0.5% significantly improved by 14% the shoot length of tomato plants ($p < 0.05$). No significant differences were detected in the root and plant length compared with the control, although a slight increase in root and plant lengths was visible at 0.5% of *G. gracilis* extract.

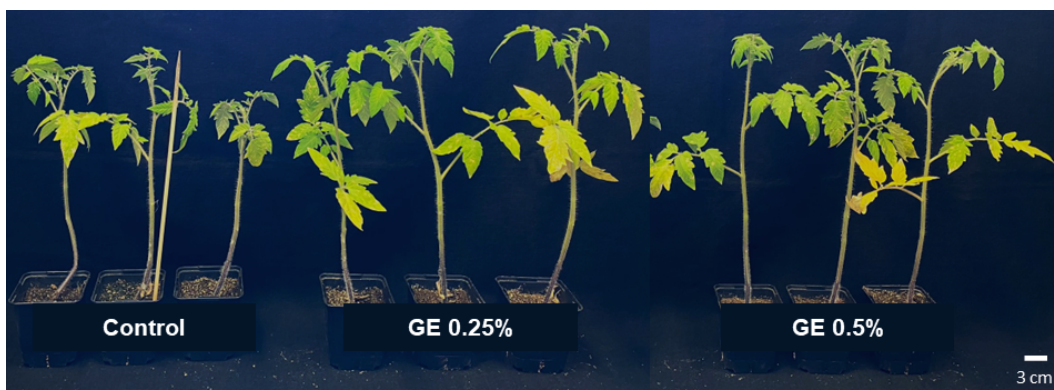


Figure 12. Late-stage test. Representative *S. lycopersicum* plants treated with GE at two different concentrations (0.25%, 0.5% and 1%) by soil application. This test was prepared with n=5.

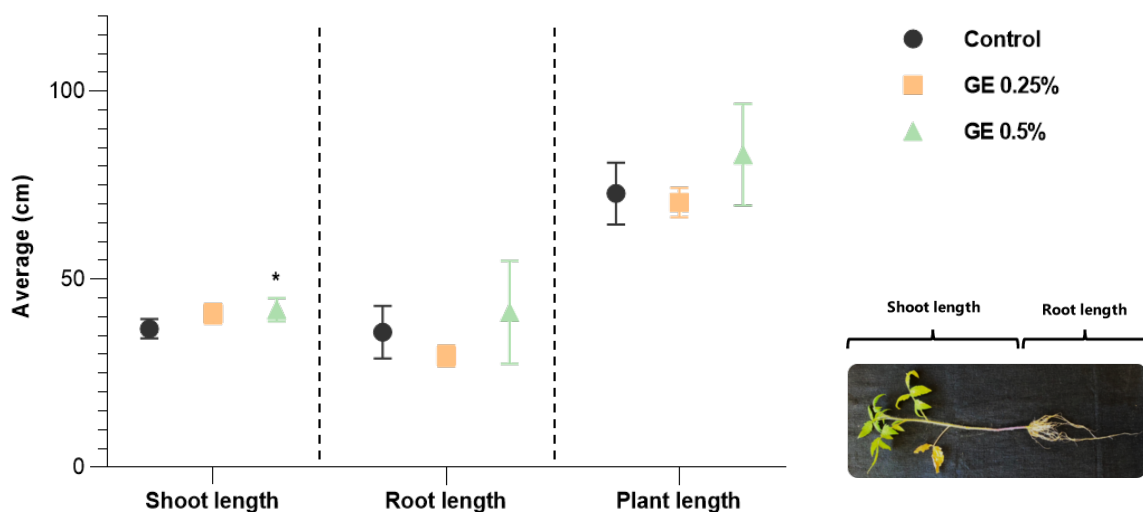


Figure 13. Soil application of GE at two different concentrations (0.25% and 0.5%) in *S. Lycopersicum* plants in the late-stage. The graph represents the shoot length, root length and plant height after treatments. This test was prepared with n=5. The sets of data were compared by two-tailed Student's t-test (Welch's correction) and 95% confidence intervals, wherein asterisks represents a statistically significant difference at $p < 0.05$, *; when no statistical marks in the graph it stands for statistically non-significant. Error bars represent s.d.

***Klebsiella oxytoca* isolated from *G. gracilis* extract seems to increase shoot length of tomato**

Four strains isolated from the *G. gracilis* extract, previously described as PGPB, were individually inoculated in planta, including GE1_*Bacillus mycoides*, GE2_*Klebsiella oxytoca*, GE3_*Leclercia adecarboxylata* and GE4_*Pseudomonas putida*. The effects of individual bacteria strains from GE on tomato plants at the late vegetative stage (plants with three layers of leaves) were evaluated after 15 days of inoculation (Fig. 14 and 15).

In Figure 14, an improvement in tomato plants' growth was observed after inoculation with GE2_*Klebsiella oxytoca* and GE3_*Leclercia adecarboxylata*, when compared with the control. Furthermore, data from the growth attributes (Fig. 15) indicates that GE2_*Klebsiella oxytoca* inoculation significantly improved by 32% the shoot length of tomato plants ($p < 0.01$). Regarding root and plant lengths, no significant differences were detected when compared with the control, although a slight increase in plant length was visible after inoculation with GE2_*Klebsiella oxytoca*.



Figure 14. Plant inoculation test with strains isolated from GE. Photographic record of representative *S. lycopersicum* plants at vegetative late-stage. This test was prepared with $n=5$.

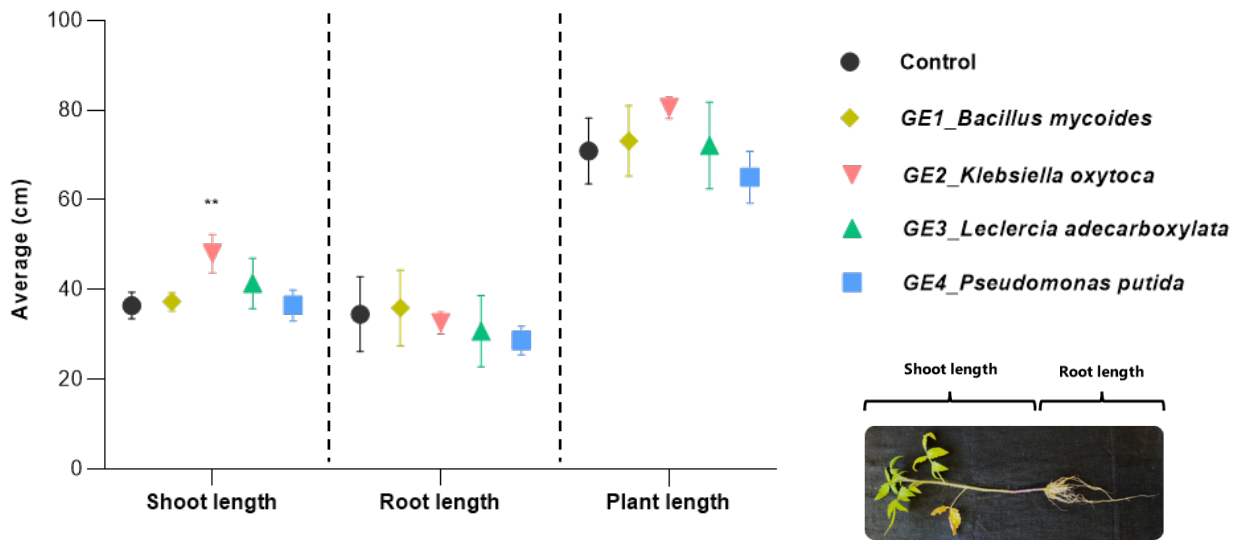


Figure 15. Soil inoculation of GE culturable microbiota in *S. lycopersicum* plants in the late-stage. The graph represents the shoot length, root length and plant height after soil treatments. This test was prepared with n=5. The sets of data were compared by two-tailed Student's t-test (Welch's correction) and 95% confidence intervals, wherein asterisks represent a statistically significant difference at $p < 0.01$, **; when no statistical marks in the graph it stands for statistically non-significant. Error bars represent s.d.

Characterization of bacterial strains from soil and roots after *G. gracilis* treatments: identification, phylogenetic trees, and population distribution

In this study, 50 strains were isolated from soil and roots after soil application of NPK and GE, 25 strains from each origin. In the collection of 25 strains isolated from soil after treatments, a total of 8 species were successfully identified (*Bacillus altitudinis*, *Bacillus mycoides*, *Bacillus cereus*, *Bacillus subtilis*, *Cytobacillus firmus*, *Lysinibacillus sphaericus*, *Priestia megaterium* and *Pseudomonas putida*), 2 strains were only identified by their gender (*Bacillus* sp. and *Pseudomonas* sp.) and only 4 strains could not be identified (Fig. 16A). Additionally, 2 strains were not correctly identified (*Bacillus cereus/toyonensis* and *Lysinibacillus sphaericus/xylanilyticus*). By phylogenetical analysis, it was not possible to assuredly affirm the correct species of those strains (*Bacillus cereus/toyonensis* and *Lysinibacillus sphaericus/xylanilyticus*) because not enough differences were observed.

In the collection of 25 strains isolated from roots after treatments, a total of 11 species were successfully identified (*Bacillus cereus*, *Bacillus mycoides*, *Bacillus safensis*, *Bacillus subtilis*, *Bacillus velezensis*, *Brevibacterium frigoritolerans*, *Lysinibacillus fusiformis*, *Lysinibacillus sphaericus*, *Paenibacillus amylolyticus*, *Peribacillus simplex* and *Rosellomorea marisflavi*), 1 strain was not correctly identified (*Bacillus cereus/Bacillus toyonensis*), and 3 strains could not be identified (Fig. 16B). As previously explained, it was not possible to clearly identify the correct species between *Bacillus cereus* and *Bacillus toyonensis*.

***G. gracilis* extract induced changes in soil and root microbiota profile**

Following the soil vs foliar spray test, where two application methods were tested (Fig. 9 and 10), the overall distribution of culturable microbiota from soil (near the roots) and roots among the different treatments was evaluated from three tomato biological replicas after the first application. Therefore, it was possible to identify patterns of occurrence and prevalence of strains in the populations. It is essential to highlight that population distribution (%) is only from the plants treated in soil, excluding the spray application. The treatments, as previously described, included NPK fertilizer, GE at three different concentrations (0.25%, 0.5% and 1%), and a control (non-treated plants).

Considering the culturable soil microbiota (Fig. 17A), GE induced changes in the soil microbiota profile after treatments. The Shannon diversity index demonstrated that only NPK fertilizer treatments were able to increase the bacterial diversity in the soil population (1,8) when compared with the control (1,59) and GE treatments (1,44, 1,19 and 1,16 for GE at 0.25%, 0.5% and 1%, respectively). Another observation was that *Pseudomonas putida* wasn't found in the control soil; however, after the first application of NPK fertilizer and GE at 0.25% and 0.5%, this PGPB was detected. It is possible that *P. putida* was also present in control but in very low concentrations, so it could not be detected and isolated. After the treatments, it was found so much of *P. putida* that was most likely activated by the treatments, making it easily detected. The same tendency could happen with *Bacillus cereus*, which was only detected in the soil treated with GE at 0.5% and 1%. In addition, several other bacterial strains were possibly activated by GE, such as *Bacillus cereus/toyonensis*, *Bacillus altitudinis*, *Lysinibacillus*

sphaericus and *Pseudomonas* sp.. On the other hand, *Bacillus* sp. and *Bacillus mycoides* were detected in almost all the treatments, including the control.

Furthermore, all treatments showed the capacity to change the culturable microbiota profile in the tomato roots and even the capacity to increase bacterial diversity (Fig. 17B). The Shannon diversity index (H) increased with NPK fertilizer (1,67) and GE at 0.25% (1,84) compared with the control treatment (1,1) meaning that the diversity was enhanced. At higher concentrations, 0.5% and 1%, GE showed a decrease in species diversity in the tomato roots, 1,03 and 0,837, respectively. The relative abundance of individuals in the roots population, also known as Evenness (E), reduced after all treatments. It was also noticed that *Bacillus subtilis* wasn't detected in the control soil; however, after the first application of NPK fertilizer and GE, this strain was in some way activated by the treatments. By contrast, *Brevibacterium frigoritolerans* was detected in all the conditions, including control.

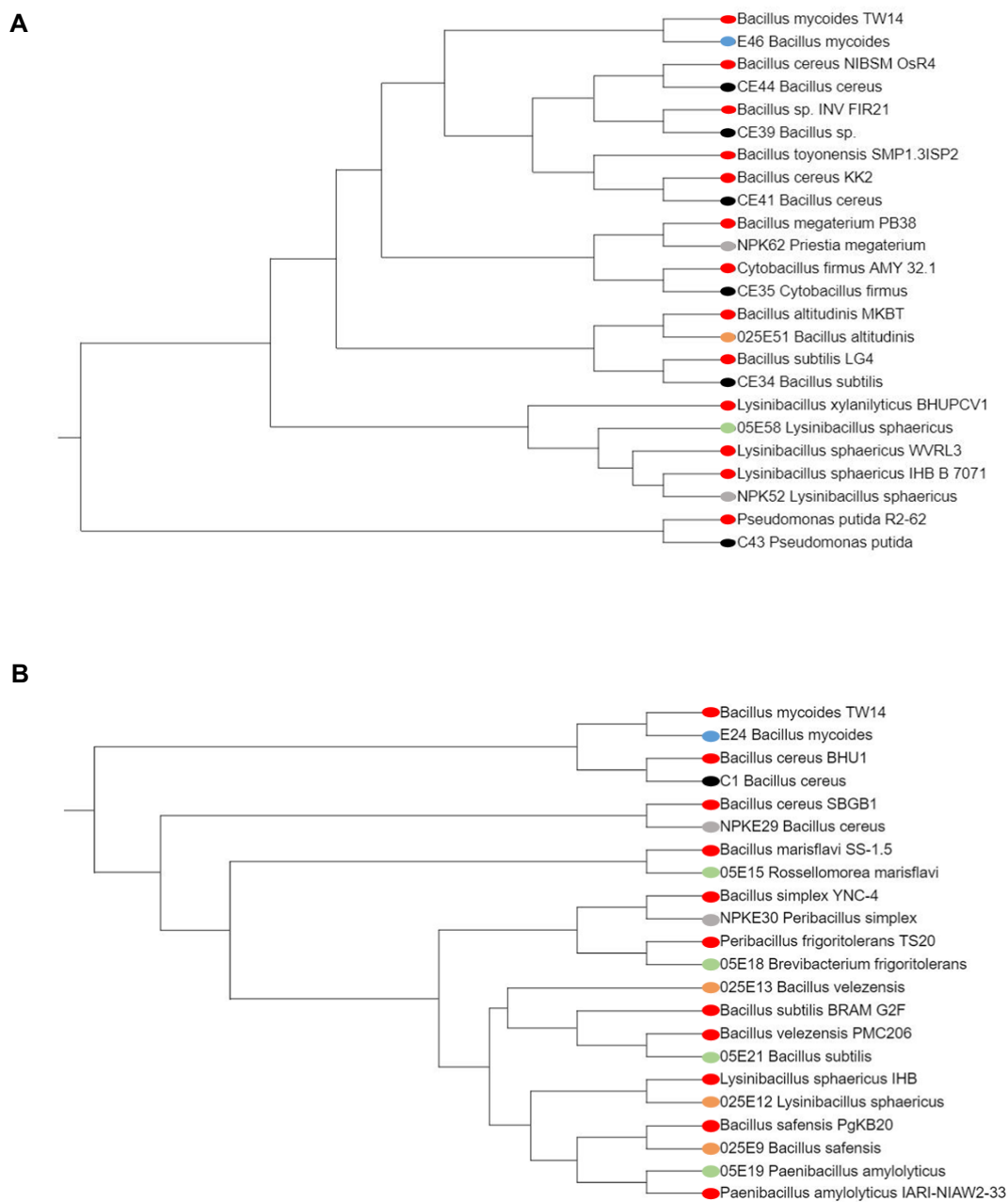
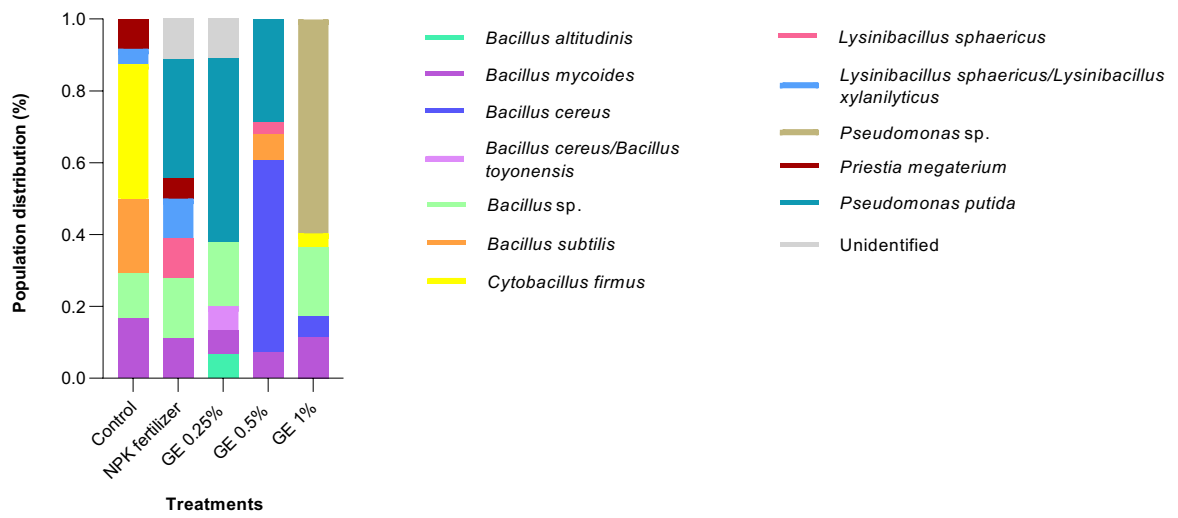


Figure 16. Prevalence of strains isolated from the soil and roots of *S. lycopersicum* plants treated with NPK fertilizer and three different concentrations of GE (0.25%, 0.5% and 1%). Treatments application was performed in soil. This test was prepared with n=3. The phylogenetic trees show the related species used to construct our collection. The tree (A) represents the soil collection and tree (B) represents the roots collection. In both trees, the red labels stand for the control strains determined by BLAST for identified strains; black, for isolates from control treatment; gray, from NPK treatment; orange, from 0.25% *G. gracilis* extract treatment; green, from 0.5% *G. gracilis* extract treatment; and blue from 1% *G. gracilis* extract treatment.

A



B

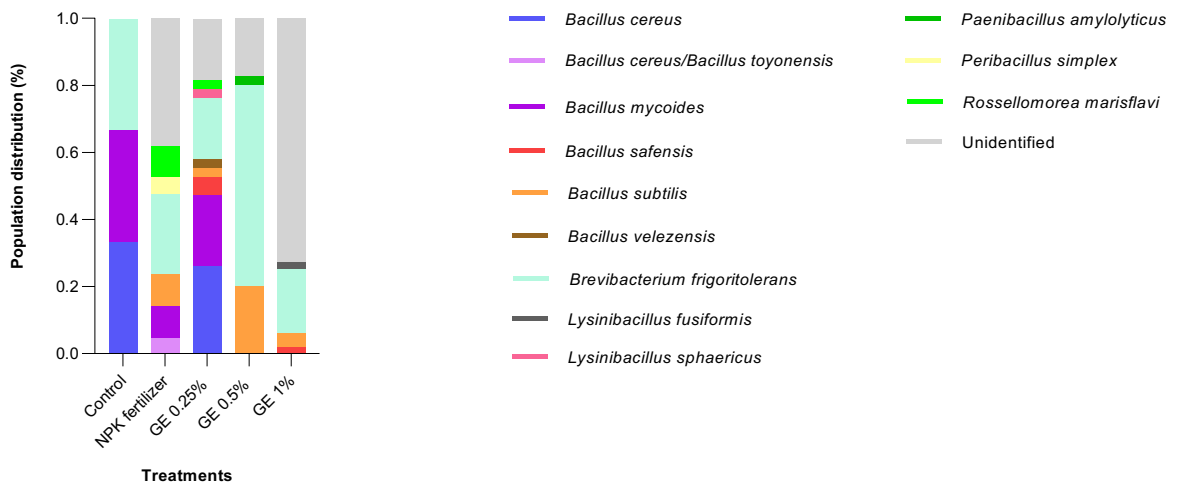


Figure 17. Population distribution (%) of culturable microbiota isolated from soil (A) and roots (B) of *Solanum lycopersicum* plants treated with NPK fertilizer and three different concentrations of GE (0.25%, 0.5% and 1%). Treatments application was performed in soil. This test was prepared with n=3.

Discussion

Seaweed extracts as plant biostimulants: potential uses in agriculture

Considering the ever-increasing world population's demands, food supply has become one of the most important challenges. Conventional agricultural management models overused chemical pesticides and synthetic fertilizers inputs to have a faster and high yield production, harming farms, ecosystems and human health (Godfray et al., 2010). Consequently, we need to implement sustainable and well-established alternatives in agricultural management to ensure safe, healthy, and sustainable food production for the next decades. Plant biostimulants have been widely explored as a sustainable solution to address these challenges. With this in mind, the oceans can be the source of a wide variety of organisms with putative relevance for agricultural management. In particular, seaweeds, have been reported as possible plant biostimulants due to their ability to enhance growth and development through the delivery of essential nutrients, hormones, and other beneficial compounds to plants (Ali et al., 2021a).

In this work, the effect of a seaweed extract produced from the red seaweed *Gracilaria gracilis* was tested in tomato (*Solanum lycopersicum*) and in lettuce (*Lactuca sativa*) plants, two important vegetables with significant economic value. The present study aimed to develop a pipeline to assess possible biostimulant effects of *Gracilaria gracilis* extract in plants, with a particular focus on tomato (*Solanum lycopersicum*), monitoring phenotypic traits at specific developmental stages. Moreover, the present work also aimed to build a comprehensive pipeline to accurately assess and enhance the value of other seaweed extracts.

Before starting the tests, one item to consider was the optimal concentration of seaweed extract to be applied. This can vary depending on the original species but is also affected by the intended application, desired effect, and specific growth stage. Therefore, it's important to determine the optimal concentration of seaweed extract for a particular application through rigorous and well-founded tests. In the present study, three concentrations of *G. gracilis* were tested (0.25%, 0.5% and 1%).

We started by evaluating the impacts of *G. gracilis* extract application at different stages of development, starting by exploring the impact of *G. gracilis* on tomato seed germination. The treatment of tomato seeds with the *G. gracilis* extract induced germination rates (96% and 94% when 0.25% and 1% extract concentrations were applied, respectively) compared to the non-treated seeds (92%). However, regarding shoot and root lengths of tomato seedlings, no significant differences were observed after treatments with *G. gracilis* extract for all tested concentrations. Previous studies have shown that seaweed extracts can increase seed germination rate, which is in agreement with our finding (Mendes et al., 2022; Mireya Hernández-Herrera et al., 2013; Turco et al., 2022). It is known that seaweed extracts are rich in natural plant growth hormones, vitamins, minerals and aminoacids (du Jardin, 2015). These compounds are known to influence seed germination, healthy growth and development of seedlings, providing the necessary energy and nutrients for seed germination and early seedling growth (Leandro et al., 2020). According to Hernández-Herrera et al., (2013), tomato seeds

treated with seaweed extracts made from *Ulva lactuca* and *Padina gymnospora* applied at low concentrations (0.2 %) were able to enhance germination rate, as observed in our study when *G. gracilis* extract was applied at 0.25%. However, seaweed extracts made from *Caulerpa sertularioides* and *Sargassum liebmannii* applied at higher concentrations (1%) negatively impacted tomato seed germination, possibly due to the inhibition of water uptake by seeds (Mireya Hernández-Herrera et al., 2013). The *G. gracilis* extract has also been tested in other plant species e.g., in kale, and in this case, the authors reported both enhanced seed germination and seedlings length when compared with the control (Mendes et al., 2022). The exact mechanisms by which seaweed extracts act on seed germination are still not fully understood. However, it is possible to hypothesize that this enhancement in seed germination rate could be related to small levels of phytohormones (cytokinins, auxins, abscisic acid and gibberellins) present in the *G. gracilis* extract which can activate enzymes involved in seed germination, as well as, micronutrients such as iron, zinc and manganese that can be absorbed by the seeds and promote seed germination and growth (Ali et al., 2021a). Alginates, reported in seaweed extracts, are also known to help break down seed coat and increase its permeability, allowing water and nutrients to enter the seed more easily and promote germination (Tavares et al., 2020). Further chemical and biochemical analysis of the extract may contribute to understanding the biostimulation effects of seaweed extracts on plants.

Also, it can be hypothesized that parameters such as electrical conductivity (EC) and pH of the seaweed extract may influence seed germination and seedlings' growth. Changes in pH and EC of seaweed extracts can affect the bioactivity of extracts, as reported by Hernández-Herrera et al., (2013) and Mendes et al., (2022). According to Mendes et al., (2022), low values of EC are partly related to the absence of salts in the medium, which would favor water seed absorption and thus enhanced germination. Our findings in the germination test, where 0.25% and 1% showed the higher and lower germination rates, respectively, may be related eventually to the intrinsic salt content of *G. gracilis* at higher concentrations, as salt substantially affects germination and early seedling growth of tomato plants and other plant species (Mireya Hernández-Herrera et al., 2013; Reinhardt & Rost, 1995). The effect of salinity on plant growth is associated with osmotic stress, ion toxicity (high concentration of Na⁺ and Cl⁻ ions), and mineral deficiencies, leading to detrimental outcomes (Musyimi et al., 2007). For future studies, the extract's electrical conductivity (EC) and pH values should be considered to ensure the presence of salts and bioactivity.

In another stage, the question was whether the *G. gracilis* extract should be applied directly in soil or by foliar spray. In our hands, the *G. gracilis* extract did not cause drastic changes in tomato or lettuce growth when both application methods were assessed, soil or foliar spray. The NPK fertilizer was also introduced as a positive control since its effects in enhancing plant growth and development are widely reported (Khalofah et al., 2022). These effects agreed with our study, where NPK applied on soil induced the growth of tomato; this result was the basis to continue performing extract application on soil in the upcoming tests. However, in general, all tomato plants, treated or not with the seaweed extract, were not at their best performance, showing yellowish and purplish leaves, and that can possibly be due to the lack of proper nutrients or unfavorable growing conditions, since during this test

the greenhouse temperature was higher than expected during the day and that could negatively impact tomatoes (Alsamir et al., 2017). In any case, seaweed extract applied to soil have been reported to be more effective than foliar spray application in what concerns to tomato plant height (Mireya Hernández-Herrera et al., 2013). The same trend, growth improvement, was demonstrated by Crouch & Van Staden, (1992) when tomato plants were treated with *Ecklonia maxima* extract at 1.0% applied as a soil drench. Hernández-Herrera et al., (2022) also reported an increase in root length, shoot length, fresh weight and root/shoot area of tomato plants when *Padina gymnospora* extract was applied to soil under control and salinity stress conditions. In contrast, Ali et al., (2016) concluded that foliar-spray was an effective method to apply the *A. nodosum* extract on tomato plants since, in this case, plant height and fruit yield were increased when compared to control plants (not treated with the extract). Foliar application of Reabilit® Algas based on the *Kappaphycus alvarezii* and *Sargasum vulgare* (red and brown seaweeds, respectively) in pepper plants was also able to improve growth and agronomic efficiency regarding stomatal conductance and fruit production (Melo et al., 2020). Furthermore, Frioni et al., (2021) proved that between soil and foliar application, foliar application improved water-stress grapevines physiological performances and water use efficiency, but both types of application seemed ineffective in preventing stomatal closure at severe water stress. Overall, the literature suggests that using seaweed extracts through soil and spray applications can improve plant growth and development in both normal and stressful conditions. However, the effectiveness of this approach may differ depending on the species of seaweed used, plant species, and environmental conditions.

Moreover, *G. gracilis* extract was evaluated in the early vegetative stage of tomato plants. Here, was possible to observe that the higher concentrations tested of *G. gracilis* extract treatments (0.5% and 1%) significantly improved shoot length (by 45% and 54% for 0.5% and 1% of extract concentrations, respectively) when compared to *G. gracilis* applied at the lowest concentration tested (0.25%) and to the control seeds. In a more advanced stage, late-stage, *G. gracilis* treatment at 0.5% increased shoot length of tomato plants. From the phenotypic analysis performed, it is plausible to consider that 0.5% extract concentration was the best one, at least in our experimental conditions and for the developmental stages analyzed. These observations, even though still preliminary, are generally aligned with most published research regarding the beneficial effects of seaweed extracts on plant growth. For instance, a study conducted by Hussain et al., (2021) showed that a seaweed extract made from the brown algae *Durvillaea potatorum* and *Ascophyllum nodosum* significantly improved tomato plant growth and productivity. Tomato plants treated with the *Padina gymnospora* extract also showed an increase in root and shoot length, fresh weight, early flowering and enhanced fruit weight and quality under control and salinity stress conditions (Hernández-Herrera et al., 2022). The application of *U. lactuca* extract in tomato showed similar results as it increased root length and plant weight (Hernández-Herrera et al., 2013). Other crop plants have also been reported to be positively influenced by seaweed extracts e.g. pepper (*Capsicum annuum*), maize (*Zea mays*), rice (*Oryza sativa* subsp. *Japonica*), soybean (*Glycine max*) and potato (*Solanum tuberosum*) (Rathore et al., 2009; Prajapati et al., 2016; Basavaraja et al., 2018; Renaut et al., 2019; Melo et al., 2020; Chen et al., 2022). Other studies made by Melo et al., (2020) also reported that the application of Reabilit® Algas (*Kappaphycus alvarezii* and *Sargasum vulgare*) at 0.5% in pepper plants led to enhanced stomatal conductance and fruit production.

In another example, the application of *Ascophyllum nodosum* at 0.5% in tomato plants was able to induce larger root systems, higher concentrations of minerals in the shoots, higher fruit yield and quality, and increased plant height (Ali et al., 2016).

Regarding the chemical analysis of *G. gracilis* extract, potassium (K) was the most abundant element (6841 ± 1368 mg/Kg), one of the most abundant inorganic cations in plant cells that plays a vital role in many physiological and metabolic processes. It is known that potassium plays a critical role in regulating the opening and closing of stomata (affecting water uptake and transpiration in plants), cell elongation, maintaining photosynthesis and cation-anion balances, and enzyme activation. It is also involved in plant protein synthesis, which is essential for many functions, including growth, development, and defense against pests and diseases (Hafsi et al., 2014; Xu et al., 2020). A study conducted by Chrysargyris et al., (2018) examined the effects of foliar application with *Ascophyllum nodosum* extract on lettuce plants grown hydroponically and with K deficiency (at 125 mg/L). They observed that K deficiency reduced plant biomass, photosynthetic rate, leaf stomatal conductance, lettuce potassium content and tissue antioxidant capacity compared to the higher K levels (375 mg/L). Spray applications of *A. nodosum* extract mitigated the effects of K deficiency back to levels of high K content (375 mg/L), increased relative growth of lettuce plants, and also increased the quality of cut lettuce grown in 125 mg · L⁻¹ K conditions., being a possible alternative to the chemical inputs (Chrysargyris et al., 2018). Other macro and micronutrients were found in *G. gracilis* extract, such as Mg, Fe and Cu, which are crucial for plant growth and development. Not all crops indeed have the same nutritional needs, and the ideal quantities of each nutrient may differ based on the soil type, weather patterns, and agricultural techniques. Thus, it is crucial to evaluate the nutrient levels in the soil and identify the precise nutrient demands of the specific crop being cultivated to achieve maximum growth and output.

Besides, in the *G. gracilis* extract used in this study, were detected heavy metals at lower concentrations mainly Cd (<0,02 mg/Kg) Pb ($0,26 \pm 0,05$ mg/Kg) and Hg (<0,1). It is known that seaweed species can accumulate heavy metals in high concentrations, depending on the seaweed species, the location where the seaweed was produced, and the processing method used to produce the seaweed extract (Čmiková et al., 2022). Seaweed extracts-based biostimulants can be obtained through different extraction methods, such as water-based extraction, acid hydrolysis, alkaline hydrolysis, enzyme-assisted extraction, ultrasound-assisted extraction and more. When used as biostimulants (seaweed extracts), the highly concentrated heavy metals from the seaweed extract will remain in the soil or even can be taken up by the crops. By applying seaweed extracts routinely, heavy metals concentrations will increase over time, rendering the land unsuitable for crop production. Therefore, the quality of the seaweed extracts should be well-characterized, considering the heavy metals concentration (and the presence of undesirable compounds) before their use in agriculture to properly define possible uses (Deolu-Ajayi et al., 2022).

Plant improvements induced by seaweeds have been associated with beneficial properties of seaweed extracts, such as their richness in nutrients, vitamins, plant growth hormones (auxins, cytokinins, and gibberellins), and bioactive compounds that can modulate cellular metabolism (Ali et al., 2021a). One

big challenge here is to maintain the biochemical integrity of the seaweed bioactive compounds during extraction procedures, ensuring the biostimulant efficacy (Nanda et al., 2022).

It has been also shown that seaweed extracts can modulate the plant transcriptome e.g. by influencing the expression of specific genes responsible for the endogenous biosynthesis of growth hormones, such as auxin, cytokinin, and gibberellin (Ali et al., 2019). In tomato and sweet pepper plants, changes in gene expression were reported in response to *A. nodosum*, *S. vulgare* and *A. spicifera* seaweed treatments (Ali et al., 2019, 2021b). *A. nodosum*-treated tomato plants by foliar application caused a clear pattern of gene regulation involved in the synthesis of key metabolites. The genes involved in synthesizing these key metabolites acted as activators of signal transduction, which is the process by which cells communicate with one another to coordinate various physiological and developmental processes (Ali et al., 2022). These transcriptomic changes may be related to phenotypic traits such as increased plant height, root length, plant biomass and chlorophyll content (Ali et al., 2022). For future studies, it would be interesting to consider gene expression evaluation as a valuable tool for understanding the underlying mechanisms of seaweed extracts effects on plants.

Seaweed extracts as modulators of soil and roots microbiota

Seaweed extracts have been found to be effective in structuring the microbial communities associated with plants. This can be linked to the fact that seaweed extracts contain a variety of bioactive compounds that can promote the growth and development of beneficial microorganisms in the soil (Renaut et al., 2019; Chen et al., 2022). These microorganisms can then form symbiotic relationships with the plants, providing them with essential nutrients and protection against harmful pathogens. The present study concluded that applying *G. gracilis* extracts to tomato plants can induce dual effects on bacterial diversity and relative abundance in the roots. At lower concentrations, 0.25%, the application of *G. gracilis* was found to increase bacterial diversity in the roots. However, at higher concentrations, such as 0.5% and 1%, the application of the extract was found to decrease bacterial diversity and relative abundance in the roots. In soil, the *G. gracilis* treatments applied at all concentrations reduced the bacterial diversity and relative abundance. This finding may be due to changes in soil pH, nutrient availability, or antimicrobial properties of *G. gracilis* extract, which can affect or even inhibit the growth of certain bacteria in the soil, reducing bacterial diversity and relative abundance metabolism (Hernández-Herrera et al., 2013; Ali et al., 2021a; Mendes et al., 2022). To fully understand the mechanism by which *G. gracilis* extract reduced bacterial diversity in soil, it is essential to analyze the complete composition of the extract, since it is a complex mixture of several bioactive compounds that can exhibit different biological activities. Even though we still don't have this data, *G. gracilis* samples were already send to Dr. Karsten Niehaus lab at CeBiTec (Universität Bielefeld, Germany), who is going to provide us a complete qualitative and quantitative analysis (using Triple Quadrupole GC-MS) of which components are in *G. gracilis* extract. Contrastingly, Renaut et al., (2019) found that the application of *A. nodosum* extract to tomato and pepper plants in greenhouse trials increased parameters related to plant growth, including root, shoot and fruit biomass and a significant increase on the bacterial biodiversity with changes on the microbial community structure in the rhizosphere of both tomato and

pepper plants (Renaut et al., 2019). The same tendency was observed by Chen et al., (2022), where *Sargassum horneri* extract together with a general chemical fertilizer (N-P₂O₅-K₂O:18-20-8) modified the composition of the rhizosphere bacterial community and increased the bacterial diversity and richness of rice plants. These modifications in bacterial diversity and richness could be related to the ability of seaweed extracts to compensate the deficiency of N, P and K in soils (Chen et al., 2022). Some seaweeds, such as *Sargassum horneri*, own the natural polysaccharide alginate known for its ability to chelate with major cations such as Na⁺, Ca²⁺, Mg²⁺, and K⁺. When alginate chelates with these cations, it forms aggregates that are rich in nutrients and improve the crumb structure and capillary activity of soil pores, providing a source of nutrients for soil microorganisms, which can ultimately lead to an increase in soil microbial activity (Yang et al., 2021). Soil microbial diversity can be significantly affected by changes in the soil's availability of carbohydrates and amino-acid carbon sources. These changes have been found to play a major role in shaping the composition of soil microbial communities (Guicciardini et al., 2023). The soil pH was also identified as an important factor affecting soil bacterial diversity and community composition. Changes in soil acidification can be related to both natural and anthropogenic processes, but in crop cultivation, intensive use of nitrogen fertilizers could be the primary reason for the soil pH decrease (Wu et al., 2017). Several studies have reported that microbial community composition is sensitive to short and long-term application of nitrogen (N), phosphorus (P), and potassium (K) fertilizers (Allison & Martiny, 2008; Guicciardini et al., 2023). However, in the present study, the NPK fertilizer treatment enhanced bacterial diversity in tomato plants' soil and roots. Further studies, including more biological and technical replicates, ensuring robustness, will be needed to assess the actual and long-term impact of the NPK treatment. Chen et al., (2022) suggested that adding a small amount of seaweed extract into chemical fertilizer was shown to be a potential strategy to improve soil nutrient levels, rhizosphere bacterial diversity and richness, and increased rice yield and quality. Additional evaluation would be necessary to assess the real impact on the environment and food over time.

Encouraging and preserving the diversity of soil microorganisms can significantly impact the stability and health of agricultural fields. Soil microorganisms, such as bacteria, fungi, and protozoa, play a crucial role in maintaining soil fertility, nutrient cycling, and plant growth. By promoting microbial diversity, farmers can enhance the resilience and resistance of their fields to environmental stresses. Additionally, diverse microbial communities can contribute to reducing the need for synthetic fertilizers and pesticides, which can negatively impact soil health and biodiversity. Overall, prioritizing soil microbial diversity is essential to sustainable agriculture and can help ensure agricultural lands' long-term productivity and health. A recent study conducted by Patel et al., (2023) demonstrated that some of the compounds present in *A. nodosum* extract were able to enhance the production of IAA and other plant-growth promoting activities by *Pseudomonas protegens* CHA0. It is known that the production of phytohormones, such as IAA, is the key mechanism behind growth promotion by PGPR. Supporting this idea, Patel et al., (2023) showed that combined applications of *A. nodosum* extract and *P. protegens* CHA0 greatly improved the growth and tolerance against salinity of pea plants grown in hydroponic conditions. These findings could open new doors in the agricultural sector, providing evidence for the importance of microbiota shaping by seaweed extracts, where specific microbial populations are

selected and promoted for their beneficial effects on plant growth and health. By understanding the mechanisms behind these interactions, it may be possible to develop targeted approaches for sustainably enhancing plant growth and productivity.

The promising role of plant growth-promoting bacteria (PGPBs) in agriculture

In agriculture, plant growth-promoting bacteria (PGPBs) have gained increased popularity, given the need to assume more sustainable ways to increase crop yields and reduce harmful chemicals. Several studies have reported that these beneficial bacteria help plants grow better by promoting nutrient uptake, improving root growth, and protecting them against biotic and abiotic stresses (He et al., 2019; Islam et al., 2016; Kang et al., 2019; Gashash et al., 2022). The present study addresses the effects of a collection of strains isolated from *G. gracilis* in tomato plants. Within the four inoculants tested, GE2_ *Klebsiella oxytoca* showed the greatest improvement in the shoot length of tomato plants compared to the other bacterial strains (GE1_ *Bacillus mycoides*, GE3_ *Leclercia adecarboxylata* and GE4_ *Pseudomonas putida*). Recent research conducted by Khalifa & Aldayel, (2022) demonstrated that in vitro inoculation with LCK121 *Klebsiella oxytoca*, isolated from the rhizosphere of the wild legume *Lotus corniculatus*, significantly increased the root and shoot lengths of barley plants. This strain, LCK121, produced a comparable amount of IAA (16,34 µg/mL), similar to the production of auxins-equivalents by GE2_ *Klebsiella oxytoca* (25,41 µg/mL) in the present study. It is widely known that IAA, is an essential phytohormone for plant growth and development, and enhances cell elongation, cell division, root hair emergence and secondary root growth (Zhao, 2010). This suggests that the enhancing effects of *Klebsiella oxytoca* on tomato shoot length could be related to its ability to produce IAA, which in turn promotes cell elongation and cell division. Kang et al., (2019) also suggested that the IAA- and ACC-deaminase-producing abilities of the bacterial strain MO1 *Leclercia adecarboxylata*, isolated from the rhizosphere of tomato plants, can improve shoot and root lengths of tomato plants under normal and salt stress conditions (Kang et al., 2019) Furthermore, treatments with *Klebsiella oxytoca* isolated from several sampling soil sites in Egypt, had significant effects on the shoot length of potato (*Solanum tuberosum* L.) which belongs to the same family as tomato (*Solanum lycopersicum*) (Elsharkawy et al., 2022).

By harnessing the power of beneficial bacteria like GE2_ *Klebsiella oxytoca*, we may be able to improve crop yields and enhance the sustainability of modern agriculture. It is important to note that the efficacy of microbiota inoculations can depend on various factors, such as the plant species, soil type, environmental conditions, and the specific bacterial strains used. Therefore, while this particular study suggests that GE2_ *Klebsiella oxytoca* was the most effective strain in improving tomato plant shoot length in their specific experimental conditions, further research would be necessary to confirm these findings including: 1) plant growth analysis in terms of photosynthesis, transpiration and plant biomass accumulation rates over time; 2) soil microbial community analysis, such as soil pH, nutrient availability, and organic matter content over time; 3) environmental toxicity assays; 4) crop yield and quality analysis; and 5) genetic analysis to identify the genetic and molecular mechanisms underlying the

beneficial effects of PGPBs on plants (Islam et al., 2016 ;He et al., 2019; Kang et al., 2019; Gashash et al., 2022; Wang, et al., 2022).

Additionally, *G. gracilis* extract was supplemented *in vitro* at three different concentrations to evaluate the PGPB activities of these bacterial strains together with the extract. Almost all the PGPB activities were enhanced by the *G. gracilis* extract, as previously mentioned in the results section. Although additional studies are necessary, these findings propose that combining PGPBs and seaweed-derived biostimulants may have a synergistic effect, resulting in improved plant growth, higher crop yields, and reduced dependence on synthetic fertilizers and pesticides. A study conducted by Ngoroyemoto et al., (2020) explored the effects of a seaweed-derived biostimulant (Kelpak®, made from the kelp *Ecklonia maxima*) on the growth and biochemical composition of *Amaranthus hybridus* L., a leafy vegetable crop, when applied alone or in combination with PGPRs (*Bacillus licheniformis* and *Pseudomonas fluorescens*) (Ngoroyemoto et al., 2020). They found out that when used in combination, it improved the number of leaves and root length of *Amaranthus hybridus* L.. However, other parameters showed similar results to the ones obtained with Kelpak® treatment alone. Consequently, it is essential to conduct more studies to identify more suitable PGPRs that would work synergistically with Kelpak® (Ngoroyemoto et al., 2020). By controlling the power of PGPBs and seaweed biostimulants, we can pave the way for a more sustainable and environmentally friendly future in agriculture.

Conclusions

The present study has revealed some promising results regarding the potential benefits of *G. gracilis* extract and its associated microbiota for plant growth promotion. In addition, it has contributed to developing an experimental pipeline to test seaweed extracts in crops, highlighting some of the challenges of using natural products to enhance plant growth and productivity (Fig. 18).

The isolation of four bacterial strains from *G. gracilis* extract and the demonstration of their plant-growth promoting activities, mainly biofilm, auxins and ACC deaminase production, are significant findings, particularly given the current need for sustainable agricultural practices. Additionally, the discovery that GE2_ *Klebsiella oxytoca* from *G. gracilis* can boost tomato plants is a valuable contribution to the field.

From this study, it was possible to conclude that soil application was preferable to foliar spray, although further tests should be conducted to properly evaluate the best method. In the germination phase of tomato seeds, *G. gracilis* extract enhanced seed germination rate, showing a better improvement at the lowest extract concentration (0.25%), emphasizing the practical implications of this research. Moreover, in more advanced developmental stages, soil application of *G. gracilis* at 0.5% proved to be promising, as it significantly enhanced the shoot length of tomato plants. After *G. gracilis* extract treatment at 0.25%, it was observed an increase in tomato roots microbiota diversity, although soil microbiota diversity decreased after *G. gracilis* extract at all concentrations.

Overall, this study provides important insights into the potential of *G. gracilis* extract and its microbiota as a natural and effective means of enhancing plant growth and productivity. However, further studies are needed to develop a reliable pipeline addressing all the critical steps and challenges of using seaweed extracts for sustainable agriculture.

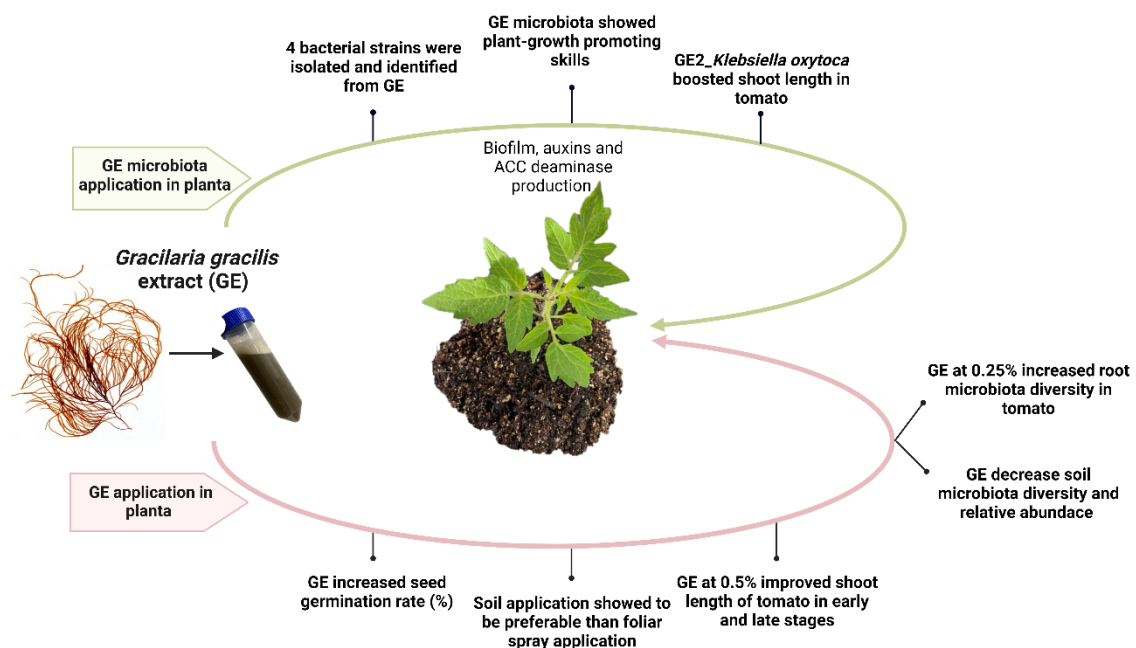


Figure 18. Main conclusions within the two approaches: 1) *G. gracilis* microbiota application in planta and (2) *G. gracilis* application in planta.

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