UNIVERSIDADE D
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SEAWEED POLYSACCHARIDES IN AGRICULTURE

Master Thesis Dissertation within the scope of the Master in Biodiversity and Plant Biotechnology supervised by Professor Dr. Leonel Pereira (University of Coimbra) and by Professor Dr. Kiril Bahcevandziev (Polytechnic Institute of Coimbra) and presented to the Faculty of Sciences and Technology of the University of Coimbra – Department of Sciences of Life

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POLISACARÍDEOS DE MACROALGAS MARINHAS NA AGRICULTURA

Dissertação no âmbito do Mestrado em Biodiversidade e Biotecnologia Vegetal orientada pelo Professor Doutor Leonel Pereira (Universidade de Coimbra) e pelo Professor Doutor Kiril Bahcevandziev (Instituto Politécnico de Coimbra) e apresentada à Faculdade de Ciências e Tecnologia da Universidade de Coimbra – Departamento das Ciências da Vida

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Resumo

Os polissacarídeos de macroalgas marinhas, quando comparados a fertilizantes sintéticos comerciais, demonstraram excelentes resultados nos parâmetros de crescimento de várias plantas. Quando aplicados diretamente no solo ou pulverizados na folhagem, os poli- e oligossacarídeos de macroalgas marinhas conseguem melhorar a germinação das sementes, o vigor das plantas, aumentar a absorção de nutrientes do solo e proteger as plantas contra vários estresses abióticos e bióticos, estimulando a planta a produzir metabolitos secundários e a desencadear as suas vias de defesa. Neste estudo, três polissacarídeos diferentes (alginato, agar e carragenana) foram extraídos de uma macroalga castanha, *Saccorhiza polyschides*, e duas macroalgas vermelhas, *Gracilaria gracilis* e *Chondrus crispus*, respetivamente, com o objetivo de analisar o seu impacto na germinação de sementes, crescimento, desenvolvimento e metabolismo da planta *Brassica napus* L. A estrutura química dos polissacarídeos, o seu perfil mineral e outras propriedades físico-químicas foram avaliadas. A carragenana apresentou os melhores resultados no crescimento das plântulas, percentagem de germinação e na produtividade das nabiças, quando comparada ao alginato e ao agar. A carragenana, extraída do tetrasporófito, dos talos não frutificados e do gametófito feminino de *Chondrus crispus* apresentou 94.67, 92 e 98.67% de germinação de sementes, respetivamente. A λ-carragenana, extraída da geração tetrasporófita de *Chondrus crispus*, apresentou a maior bioatividade e efeito positivo nas nabiças, entre todos os tratamentos. A λ-carragenana mostrou que consegue melhorar o crescimento das plantas, aumentar a sua biomassa e o sistema radicular, aumentar a atividade fotossintética e a absorção de nutrientes essenciais do solo. As bioatividades da carragenana parecem estar relacionadas com o seu nível de sulfatação, em conjunto com o pH e a condutividade da sua solução.

Palavras-chave: Polissacarídeos de macroalgas marinhas, germinação de sementes, metabolismo, bioatividades, nabiça

Abstract

Seaweed polysaccharides, when compared to synthetic commercial fertilizers, have been proven to achieve excellent results in plant growth parameters. When applied to the soil directly or sprayed on the foliage, seaweed poly- and oligosaccharides can improve seed germination, plant vigour, increase the uptake of soil nutrients, and protect plants against several abiotic and biotic stresses, by stimulating a plant to produce secondary metabolites and manage its defence pathways. In this study, three different polysaccharides (alginate, agar and carrageenan) were extracted from one brown seaweed, *Saccorhiza polyschides*, and two red seaweeds, *Gracilaria gracilis* and *Chondrus crispus*, respectively, with aim to analyse their impact on *Brassica napus* L.'s seed germination, plant's growth, development and metabolism. The polysaccharides' chemical structure, mineral profile and other physicochemical properties were assessed. Carrageenan exhibited the best results in seedling growth, germination percentage and in improving the turnip plants' productivity, when compared to alginate and agar. The carrageenan extracted from the tetrasporophyte, non-fructified thalli and female gametophyte of *Chondrus crispus* exhibited 94.67, 92 and 98.67% seed germination, respectively. λ-carrageenan, extracted from the tetrasporophyte generation of *Chondrus crispus*, had the highest bioactivity and positive effect in turnip plants among all treatments. λ-carrageenan has shown that can improve plant growth, increase plant's biomass and root system, enhance photosynthetic activity and essential soil nutrient uptake. Carrageenan's bioactivities appear to be related with their sulphation level, plus the pH and the conductivity of its solution.

Keywords: Seaweed polysaccharides, seed germination, metabolism, bioactivities, turnip greens

General Index

List of publications and scientific communications related directly to this thesis

Published articles

Mamede, M.; Cotas, J.; Bahcevandziev, K.; Pereira, L. Seaweed Polysaccharides in Agriculture: A Next Step towards Sustainability. Appl. Sci. 2023, 13, 6594. https://doi.org/10.3390/app13116594

Submitted articles

Mariana Mamede, João Cotas, Kiril Bahcevandziev, Leonel Pereira. Seaweed Polysaccharides on seed germination of *Brassica napus* L.

Mariana Mamede, João Cotas, Leonel Pereira, Kiril Bahcevandziev. Seaweed polysaccharides biostimulant activity in *Brassica napus* L.: impact analysis

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Abbreviation List

ºC: Degree Celsius

cm: centimetre

DW: Dry weight

EC: Electrical conductivity

EFSA: European Food Safety Authority

EU: European Union

FW: Fresh weight

FTIR-ATR: Fourier-Transform Infrared - Attenuated Total Reflectance

g: gram

h: hour

ι: iota

κ: kappa

λ: lambda

mg: milligram

mL: millilitre

m/v: mass/volume

rpm: Revolutions per minute

v/v: volume/ volume

1. Introduction

Seaweed extracts include a diverse range of bioactive compounds that stimulate and directly boost plant growth and its defensive responses to pathogens (Khan et al., 2009). Some studies have shown that seaweed-based biostimulants can achieve better results in plant growth parameters when compared to commercial fertilizers (Nanda et al., 2022). Plants cultivated in soil treated with seaweed extracts, either applied to the soil directly or sprayed on the foliage, show a wide range of responses. When applied to the soil these extracts can stimulate soil microflora and cause soil water retention and remediation. Seaweed extracts alleviate nutrient deficit in plants and can have a positive impact on the phytohormone balance (Nanda et al., 2022).

Plants serve as biosensors for detecting the presence of bioactive compounds, provided by seaweeds, testing them, and even evaluating their effects, in general. This is an easy way to guarantee that seaweed extracts have a consistent level of bioactivity (Hernández-Herrera et al., 2016). Consequently, seaweed extracts can operate as elicitors in plants by stimulating their defenses, including salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) pathways (Ali et al., 2021). These phytoelicitors can improve crop yield (Craigie, 2011), plant vigor (Craigie, 2011), increase the uptake of soil nutrients (Crouch et al., 1990), provide longer shelf life of the fruits (Craigie, 2011), improve seed germination (Rayorath et al., 2008) and protect plants against several abiotic and biotic stresses such as salinity, drought, temperature (Nanda et al., 2022) and pathogens (Vera et al., 2011).

The active organic compounds responsible for the bioactivity may change depending on the seaweed class and species, as well as the extraction method used. They often include a variety of organic and inorganic bioactive compounds such as polysaccharides (alginate, agar, and carrageenan), polyphenols, phytohormones (auxins, cyto-kinins, and gibberellins), phytohormone-like (betaine), minerals (potassium, phosphorus, calcium, and some trace elements) and other different components (lipids, peptides, glycoproteins, and proteins) (Ali et al., 2021). Seaweed extracts are mainly composed of polysaccharides, sugars known for improving plant growth in a similar way to hormones (Rolland et al., 2002; Sharma et al., 2012) and whose characteristics depend on the species and ecological conditions of the seaweed (Ali et al., 2021; Khan et al., 2009). The extraction procedures have a significant impact on the content of seaweed

extracts. During the extraction process, complex compounds such as polysaccharides are often transformed into oligomers that are extremely bioactive in plants.

In a study related with the influence of oligo-alginates and oligo-carrageenans in the resistance of tobacco plants against Tobacco Mosaic Virus (TMV) (Laporte et al., 2007), was reported that these seaweed oligosaccharides could stimulate the plant's growth and defense against TMV by activating the antioxidant enzyme ascorbate peroxidase (AP), which modulates the level of the antioxidant compound ascorbate (ASC) and docosahexaenoic acid (DHA). In addition, the activation of the defense enzyme phenylalanine ammonia lyase (PAL) led to the activation of the phenylpropanoid pathway and to the synthesis of secondary metabolites with an antiviral activity.

However, there are many challenges in obtaining seaweed extracts without compromising the biochemical integrity of the bioactive compounds and ensuring the efficacy of their biostimulant potential. Some of the methods used are water-based extraction, acid hydrolysis-based extraction, alkaline hydrolysis-based extraction, micro-wave-assisted extraction, ultrasound-assisted extraction, pressurized liquid extraction, enzyme-assisted extraction, and super-critical fluid extraction (Nanda et al., 2022).

It is hypothesized that seaweed polysaccharides' bioactivities are affected by the presence and position of sulphate groups in the molecular chain of the polymers. The degree of sulphation, their concentration and oxidation, all together, have an impact on these bioactivities (Patel et al., 2022). Typically, alginophytes show the lowest sulphate group content, whereas carrageenophytes, the highest (Pacheco et al., 2021).

Though there is still a lack of information about the biochemical diversity of seaweed poly- and oligosaccharides, and their mechanism of action in plant specific activities. All the studies familiar with the influence of seaweed poly- and oligosaccharides bioactivities on plants don't mention the correlation between them and specific components, the structure, or the molecular length of basic monomers (Patel et al., 2022). Therefore, there is an urgent need to characterize seaweed polysaccharides based on their monosaccharide composition and molecular size to understand their potential bioactivity (Pacheco et al., 2021).

1.1. Seaweed Polysaccharides

The chemical structure of the polysaccharides obtained from seaweeds is different depending on the taxonomic group to which they belong, their species, the season when

these seaweeds were harvested, and the respective extraction method. Colloids authorized in the food industry, and widely used worldwide, are alginate (extracted from brown algae), agar and carrageenan (extracted from red algae) (Michel et al., 2006; Rioux et al., 2010; Rodrigues et al., 2015; Zvyagintseva et al., 2003).

1.1.1. Alginate

Alginate (**Figure 1**) is a polysaccharide naturally found in brown seaweed in the form of alginic acid. This anionic polymer is based on monomers of β-D-mannuronic acid (M) and 1,4 α-L-guluronic acid (G) (Haug et al., 1974). Depending on the position of the monomeric units in the chain, the molecular weight of the polymer and the nature of its associated counter ions, the properties of this polysaccharide can differ (Khan et al., 2009; Pereira & Cotas, 2020).

At the level of food certification, the molecular weight of alginate is not considered. However, it is contemplated for good practices in the extractive industry associated with the food industry (Younes et al., 2017).

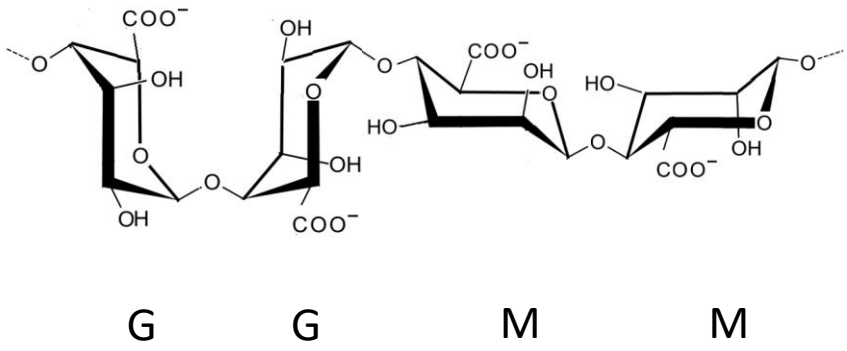

Figure 1 – Chemical structure of alginic acid. G – guluronic acid. M – mannuronic acid. (Pacheco et al., 2021)

The industrial extraction of this polysaccharide involves several steps: washing the seaweed to remove impurities; pre-treatment with heated acid (usually hydrochloric acid for 24 h) to remove pigments, proteins, and lipids (Hernández-Carmona et al., 2013). Next, the solid-liquid extraction takes place, where the solid residue is subjected to an alkaline treatment (sodium carbonate) followed by a centrifugation or filtration process. After this process, hydrochloric acid is added to the liquid extract to precipitate the alginate dis-solved in the solution, in the form of sodium alginate. After precipitation, the

solution with the precipitate is centrifuged/filtered to obtain the precipitated alginate. After-wards, the alginate is dried and milled for later application (Brownlee et al., 2009).

Alginate is classified as a non-organic compound and is approved by the Food and Drug Administration (FDA, USA) and the European Food Safety Authority (EFSA, EU) as a food ingredient (Draget et al., 2002). In this context, the application and labeling of food products containing alginate are regulated according to the European Union Commission Regulation (1333/2008) as E400 (alginic acid), E401 (sodium alginate), E402 (potassium alginate), E403 (ammonium alginate), E404 (calcium alginate) and E405 (propylene glycol alginate) (Younes et al., 2017).

The main characteristics of alginate are its high degree of viscosity and absorption, which makes it possible to thicken food products, such as, jellies, marmalades, sauces (e.g., mayonnaise), syrups and ice cream (Cardozo et al., 2007; Qin et al., 2018).

The FDA has approved alginate for human consumption after toxicological testing. However, the FDA requires evidence of good practices in alginate extraction and the use of alginate at threshold concentrations, which vary according to the type of food product (Draget et al., 2002).

Alginate can be extracted from *Saccorhiza polyschides* (Phaeophyceae). This is an annual opportunistic kelp with a large and flat stipe and characteristic marginal undulated wings near the base. Individuals from this species usually grow up to 3-4 m long. It inhabits the low intertidal and on subtidal rocky reefs of the ocean. This opportunistic seaweed colonizes any vacant space in the sea forest but cannot compete with the local dominant species, such as *Laminaria ochroleuca* and *Saccharina latissima* (Phaeophyceae), which makes it a seaweed with great economic interest (Pacheco et al., 2021). Its distribution is manly United Kingdom, Ireland, France, Spain and Portugal. Despite being an annual seaweed, its biomass reaches its maximum during spring and summer time (Fernández, 2011; Pereira, 2020).

1.1.2. Agar

Agar (**Figure 2**) is a polysaccharide very important industrially extracted mainly from the red seaweeds genus *Gracilaria* and *Gelidium*, from the phylum Rhodophyta. Generally, the industrial extraction method is based on a thermal treatment of the seaweed biomass in an aqueous solution (between 2-4 h at 105-110 °C), for immediate filtration, while the extract is hot (as the agar gels very quickly at 50 °C). After the filtration process,

the extract either gels or is maintained in a viscous solution due to the amount of agar present in the solution. But the gel itself is normally yellowish or brown in color because some of its constituents have been degraded (proteins, monosaccharides). Therefore, the freezing/thawing technique is used to obtain a concentrated agar, with a clear color, as this technique allows the agar to be washed with water, avoiding pre-treatment to reduce impurities during extraction. Finally, the agar obtained is dried in an oven with air circulation and then milled for later application in industry (Brownlee et al., 2009; Kohl et al., 2016; McHugh, 2003).

It is a gel-forming polysaccharide consisting of 70% agarose and 30% agaropectin molecules, composed of residues of (1-4)-3, 6-anhydro-L-galactose and β 9 (1-3)-D-galactose (Hemmingson et al., 1996). To date, there is no evidence that its molecular weight has any significance for food safety and, therefore, it is considered safe regardless of its molecular weight (Mortensen et al., 2016). However, the quality of the agar differs greatly between species be-longing to these two orders. For example, agar extracted from *Gelidium corneum* (Gelidiales, Rhodophyta) is considered more suitable for pharmaceutical applications (Ravishankar & Rao, 2019). On the other hand, agar extracted from *Gracilaria gracilis* (Gracilariales, Rhodophyta) is normally used, almost exclusively, in the food industry. Although, normally this agar has one more step in the industrial extraction system, which consists of an alkaline pre-treatment with sodium hydroxide, to increase the quality of the rheological properties of the agar obtained (McHugh, 2003).

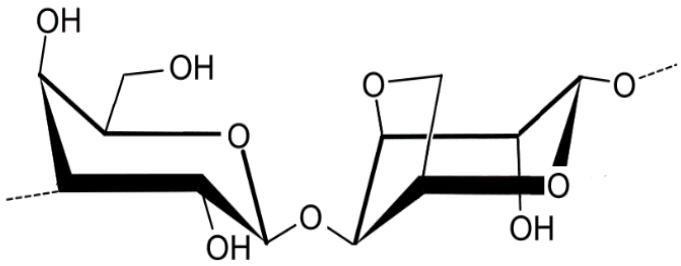

Figure 2 – Chemical structure of agar (Pacheco et al., 2021)

Agar is considered safe for human consumption by regulatory authorities in the United States of America (FDA) and the European Union (EFSA). Despite the inclusion of agar (E406) in the list of approved food additives, its application in food products is

regulated and limited. It is estimated that around 90% of commercialized agar is destined for the food industry (McHugh, 2003).

Gracilaria gracilis is a red seaweed widely used for the extraction of agar all around the world. It is distributed worldwide and inhabits temperate waters at a depth between 0 to 20 m. The variation in life cycle, growth and agar content depends on the environment (water temperature, salinity, dissolved nutrients and other abiotic stresses) (Gioele et al., 2017). Because of the high demand of this seaweed biomass for agar extraction, its natural populations have been decreasing. To protect an extinction and to preserve this significant natural source, seaweed farms are developed. This seaweed has a fast growth rate and an easy vegetive reproduction (Gioele et al., 2017). In 2019, the genus *Gracilaria* had a cultivation production of more than 3.5 million tonnes (fresh weight) worldwide, especially in Asian countries (Cai et al., 2021).

1.1.3. Carrageenan

Carrageenan is extracted from red seaweed of the order Gigartinales. The first historical use of carrageenan was for food purposes and occurred in Ireland (van de Velde & Dr. Gerhard A. De Ruiter, 2002). Carrageenan is a polysaccharide consisting of alternately linked galactose and 3,6-anhydrogalactose units, by alternating α-1,3 and β-1,4 glycosidic bonds, and whose molecular weight (greater than 100 kDa) is required for safe use in food terms (Cohen & Ito, 2002; McKim et al., 2016). In this case, there are three types of carrageenan (**Figure 3**) normally marketed: kappa-carrageenan (κ) (**Figure 3a**), which forms rigid gels with syneresis; iota-carrageenan (ι) (**Figure 3b**), which is characterized by producing elastic and smooth gels; and finally, lambda-carrageenan (λ) (**Figure 3c**), which originates viscous solutions, without ever gelling (McHugh, 2003).

These three types of carrageenan can be extracted from different generations in the life cycle of *Chondrus crispus*. *C. crispus* is a red seaweed commonly known as Irish moss. It has a stipe compressed, narrow, expanding gradually to a flat, repeatedly dichotomously branched frond, in tufts from a discoid holdfast (Pereira, 2020b). This seaweed can reach 15 cm long and some of its individuals are iridescent under water. It is distributed manly in the west coast of Portugal, the Faroe Islands, West Africa, Spain, Canada, USA, as well as in the Bering Sea from Russia to Alaska (Pereira, 2020b). As mentioned before, this seaweed species alternates between two isomorphic life generations that differ in cell wall phycocolloid composition: tetrasporophyte and two

gametophytes, non-fructified thalli and female gametophyte (Carrington et al., 2001). The carrageenan type extracted from *C. crispus'* tetrasporophyte is λ-carrageenan and the carrageenan type extracted from *C. crispus'* non-fructified thalli and female gametophyte is a hybrid κ- and ι-carrageenan (Pereira et al., 2009). Visually, the presence of reproductive structures can differentiate the three generations: tetrasporophytes (presence of tetrasporangia), female gametophytes (presence of cystocarps) and non-fructified thalli (no reproductive structures visible, usually with blue iridescence) (Brown et al., 2004).

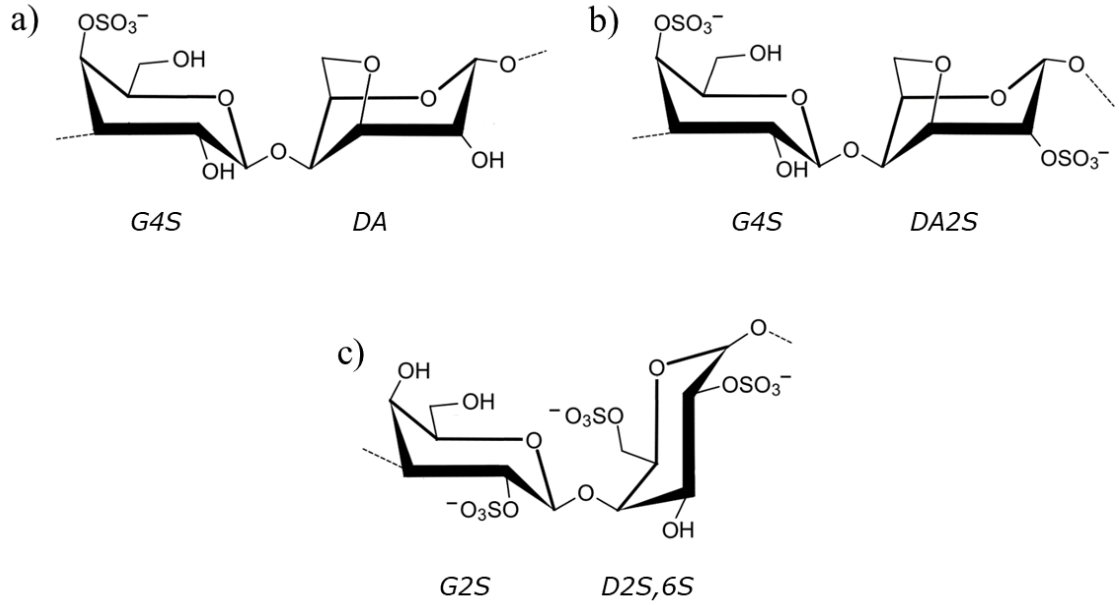

Figure 3 – Chemical structure of the different main types of carrageenan: a) kappa-carrageenan; b) iota-carrageenan; c) lambda-carrageenan (Pacheco et al., 2021)

In the carrageenan extraction industry, pre-treatment of seaweed through a depigmentation step is necessary (with sodium hypochlorite or organic solvent) to obtain a clear color in the final product (Hansen et al., 2000; van de Velde & Dr. Gerhard A. De Ruiter, 2002). The carrageenan extraction step must be carried out in an alkaline (e.g., sodium hydroxide) or aqueous solution. Subsequently, carrageenan can be recovered by alcoholic precipitation, drum drying or precipitation in aqueous potassium chloride and subsequent freezing (as in the case of κ-carrageenan). However, only methanol, ethanol and isopropanol can be used for precipitation and purification of carrageenan (Leandro et al., 2020; Smith et al., 1955; Younes et al., 2018). To ensure food quality and safety,

carrageenan is hot-dried at a temperature above 40 °C in a drying oven with forced ventilation before use (Hansen et al., 2000).

1.2.Seaweed Poly- and Oligosaccharides bioactivities on plants

Unlike phycocolloids in the food area, degraded/hydrolyzed seaweed polysaccharides have been the aim of several studies (**Table 1** and **Table 2**) with promising results, as possible inducers of resistance against pathogens and as biostimulants. With the emerging need to reduce synthetic compounds use in agriculture in the EU, polysaccharides and their oligosaccharides are gaining new scientific interest to serve as alternatives. Seaweed extracts have already demonstrated the potential to promote seed germination, plant vigor and improve cultivars (**Figure 4**) (Di Filippo-Herrera et al., 2019; Hernández Carmona, 2018; Hernández-Herrera et al., 2016; Khan et al., 2009; Nilsun et al., 2006; Silva et al., 2019; Sousa et al., 2020; Vijay Anand et al., 2018). As an advantage, seaweeds do not compete for land space, which allows the exploration of polysaccharides in a sustainable and circular economy way.

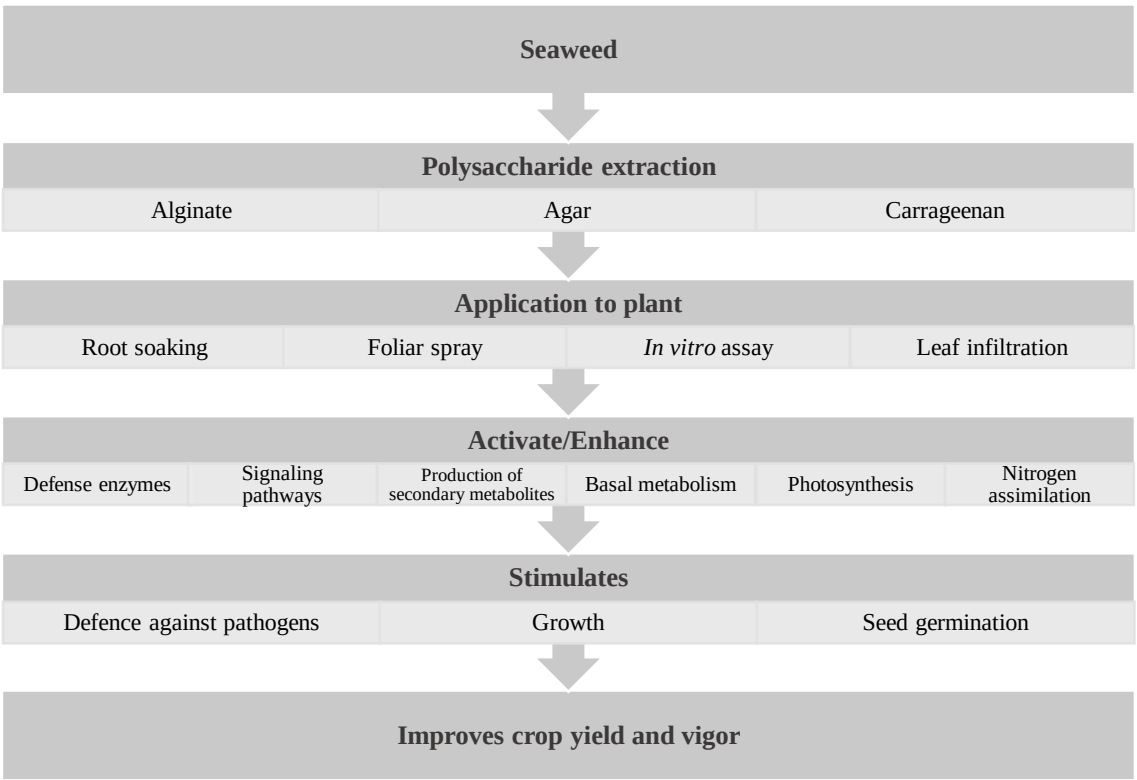

Figure 4 – Schematic representation of seaweed polysaccharides action in plants.

1.2.1. Alginates and Oligo-Alginates

Alginates are biodegradable and non-toxic compounds traditionally used as natural fertilizers due to their superabsorbent or water-retaining properties. The carboxylic acid groups present on the alginic acid chain combined with the metallic ions in the soil, form high-molecular-weight polymers that can absorb moisture and retain large amounts of water. Generally, water retention is a problem in sandy soils. These soils, when watered, dry up easily and drain away valuable nutrients beyond the plant roots. The use of alginates can improve this problem stimulating plant root system development and increase soil microbial activity (Khan et al., 2009; Pereira & Cotas, 2020).

Seaweed alginates and oligo-alginates, produced by enzymatic degradation of alginic acid, were reported to activate defense responses against pathogens (**Table 1**) in wheat plants (Chandía et al., 2004), date palm roots (Bouissil et al., 2020), tomato plants (Dey et al., 2019), olive trees (Salah et al., 2018) and against TMV (Laporte et al., 2007), by regulating defense-responsive signaling pathways. To induce resistance against viral infections, including TMV, alginates activate different defense enzymes such as phenylalanine ammonia-lyase (PAL), peroxidase (POD) and ascorbate peroxidase (AP), which elicit their metabolic pathways and the synthesis of secondary metabolites, like phenolic compounds with antiviral activity (Bouissil et al., 2020; Chandía et al., 2004; Laporte et al., 2007). In tomato plants (**Table 1**), the alginate confers resistance against a fungal infection by inducing antioxidant defense and antifungal pathogenesis-related (PR) protein expression by signaling pathways mediated by salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) (Dey et al., 2019). In an experiment with olive trees (**Table 1**), alginate induced resistance against *Verticillium dahliae* fungal infection by restricting the pathogen's growth and strengthening the host defense metabolism (Salah et al., 2018).

In addition, seaweed alginates and oligo-alginates can stimulate growth, seed germination and shoot elongation in different plant species (Laporte et al., 2007; Pacheco et al., 2021), by enhancing nitrogen assimilation and basal metabolism (González et al., 2013) (**Table 1**).

Therefore, alginates constitute an important source of potential elicitors in plants and a particular interest in agriculture. The chemical characterization of alginates or oligo-alginates and their mechanism to boost plant growth remains unclear.

1.2.2. Agar and Agar-oligosaccharides

Agar is a polysaccharide extracted from the red seaweeds, mainly genus *Gracilaria* and *Gelidium*. Due to the huge availability of biomass from these seaweeds, it represents an excellent choice for commercial cultivation and to study their bioactive potential. Many species of these genus have been evaluated for their antibacterial, antioxidant, antifungal, antiprotozoal, anti-inflammatory, antiviral, cytotoxic, antihypertensive, spermicidal and embryotoxic activities ("Sustainable Global Resources of Seaweeds Volume 2," 2022). The type and number of substituents on the agar structure are crucial elements for the bioactivities' efficacy. Sulphur content generally correlates with activity, which is why active agars are typically sulphated (Torres et al., 2019).

In a study related with seaweeds' carbohydrate polymers as plant growth promoters, agar extracted from two red seaweed, *Gracilaria gracilis* and *Asparagopsis armata*, showed positive results in growth and seed germination of kale plants (Pacheco et al., 2021)

Although there are many studies regarding the bioactivities of agarophytes ("Sustainable Global Resources of Seaweeds Volume 2," 2022), there is still a lack of studies regarding the bioactivities of agar, especially their effect on plants.

1.2.3. Carrageenans and Oligo-Carrageenans

Carrageenans and their oligomers, extracted from various red seaweeds, present a significant source of bioactive substances that activate plants' defense mechanisms and offer resistance against abiotic and biotic stresses. This can be achieved by modulating various physiological and biochemical processes (Shukla et al., 2021). Additionally, carrageenans control several metabolic activities in plants, including cell division, purine and pyrimidine synthesis, assimilation of nitrogen and sulfur, and photosynthesis (Pacheco et al., 2021; Shukla et al., 2016).

Lemonnier-Le Penhuizic et al. (2001) demonstrated that oligosaccharides (of varying molecular weights, but less than 500 Da) of λ-carrageenan act as inducers of embryogenesis. It should be noted that, in this study, both alginate and agar oligosaccharides were also tested, but with less significant results than those obtained by carrageenans. In general, the oligosaccharides obtained from carrageenan promote an increased plant height, greater leaf biomass, better carbon fixation, as well as superior nitrogen assimilation and greater overall plant growth (Shukla et al., 2016), in addition to

promoting plant defenses as elicitors and activating their defense mechanisms against pathogens (Stadnik & Freitas, 2014).

Tobacco plants (Muñoz et al., 2011) and eucalyptus trees (González et al., 2013) treated with commercially available κ-, ι- and λ-carrageenans, showed positive results in their growth. The oligo-carrageenans enhanced photosynthesis, basal metabolism and the synthesis of secondary metabolites such as essential oils and polyphenolic compounds (**Table 2**). In addition, κ-, ι- and λ-carrageenans were reported (**Table 2**) to induce long-term protection against viral, bacterial, and fungal infections at systemic level by activating the phenylalanine ammonia-lyase (PAL) enzyme and enhancing the accumulation of phenylpropanoids with potential antimicrobial activities (Mercier et al., 2001; Vera et al., 2012).

As said previously, the level of sulphation of the polymer is suggested to influence their bioactivity and, therefore, their targeted applications for plant defenses (Patel et al., 2022b). The sulphate group content differs depending on the type of carrageenan: κ-carrageenan has 20-30% of sulphate group content, ι-carrageenan has 28-35% and λ-carrageenan has 32-39% (Cunha & Grenha, 2016; Mercier et al., 2001). Amongst the three carrageenans, λ-carrageenan was considered the most potent elicitor due to its high sulfur content, inducing systemic resistance in plants. Plants treated with λ-carrageenan (**Table 2**), either through leaf infiltration or foliar spray, showed resistance against several pathogens by inducing SA, JA, and ET dependent defense pathways (Le Mire et al., 2019; Mercier et al., 2001; Pettongkhao et al., 2019; Sangha et al., 2010, 2015).

ι-carrageenan, sprayed on leaves, was reported (**Table 2**) to stimulate the growth of tobacco plants by enhancing photosynthesis, basal metabolism, cell cycle as well as ascorbate (ASC) levels and ascorbate peroxidase (AP) enzyme activity (Castro et al., 2012). This oligo-carrageenan can elicit resistance against the moth *Trichoplusia ni* in *Arabidopsis thaliana* by inducing various defense mechanisms, including JA and SA dependent pathways, proteinase inhibitors and an alteration of the products of glycosylate hydrolysis (Sangha et al., 2011).

κ-carrageenan, used in leaf spray treatment, was reported (**Table 2**) to stimulate the growth of chickpea plants, maize plants (Bi et al., 2011) and pine trees (Saucedo et al., 2015) by enhancing the basal metabolism and the production of secondary metabolites. Additionally, κ-carrageenan, applied through leaf infiltration, showed resistance against several pathogens by inducing SA, JA, and ET dependent defense

pathways (Ghannam et al., 2013; Mani & Nagarathnam, 2018; Nagorskaya et al., 2008, 2010).

When compared to λ-carrageenan, κ-and ι-carrageenan showed better results in the growth of the roots and leaves in kale, by inducing the production of indole-3-acetic acid (IAA), responsible for the plant's development (Pacheco et al., 2021).

In sum, carrageenans and oligo-carrageenans can be employed as naturally occurring growth-enhancing, anti-fungal, and anti-viral agents.

Table 1 – Alginates and Oligo-Alginates bioactivities on plants.

Seaweed/Source	Polymer	Plant species	Application method	Bioactivity	Reference
Bifurcaria bifurcata	Alginate	*Phoenix dactylifera* L.	Root soaking	Induces date palm natural defences by enhancing PAL activity and phenolic compounds content.	Bouissil et al. (2020)
Colpomenia peregrina	Alginate	*Brassica oleracea* L.	*In vitro* assay	Stimulates seed germination and growth in kale.	Pacheco et al. (2021)
Commercially available	Sodium alginate	*Solanum lycopersicum* L.	Foliar spray	Resistance against *Alternaria solani* fungal infection in tomato plants by inducing antioxidant defence and antifungal PR protein expression by signalling pathways mediated by ET, JA and SA.	Dey et al. (2019)
Commercially available	Alginate	*Olea europaea* L.	*In vitro* assay	Resistance against *Verticillium dahliae* fungal infection in olive trees by restricting the pathogen's growth and strengthening the host defence metabolism.	Salah et al. (2018)
Fucus spiralis	Alginate	*Phoenix dactylifera* L.	Root soaking	Induces date palm natural defences by enhancing PAL activity and phenolic compounds content.	Bouissil et al. (2020)
Lessonia trabeculata	Oligo-Alginates (Poly-Gu)	*Nicotiana tabacum* L.	Foliar spray	Stimulates growth and induces resistance to TMV in tobacco plants by activating the antioxidant enzyme AP, which modulates the level of the antioxidant compounds ASC and DHA. The activation of the defence enzyme PAL, will lead to the activation of the phenylpropanoid pathway and to the synthesis of secondary metabolites with antiviral activity.	Laporte et al. (2007)

Seaweed/Source	Polymer	Plant species	Application method	Bioactivity	Reference
Lessonia flavicans (formerly *L. vadosa*)	Alginate	*Triticum aestivum* L.	Leaf infiltration	Induces the enzyme activities of PAL and POD.	Chandía et al. (2004)
Lessonia flavicans (formerly *L. vadosa*)	Oligo-Alginates (Poly-Ma)	*Nicotiana tabacum* L.	Foliar spray	Stimulates growth and induces resistance to TMV in tobacco plants by activating the antioxidant enzyme AP, which modulates the level of the antioxidant compounds ASC and DHA. The activation of the defence enzyme PAL, will lead to the activation of the phenylpropanoid pathway and to the synthesis of secondary metabolites with antiviral activity.	Laporte et al. (2007)
Sargassum muticum	Alginate	*Brassica oleracea* L.	*In vitro* assay	Stimulates seed germination and growth in kale.	Pacheco et al. (2021)
Undaria pinnatifida	Alginate	*Brassica oleracea* L.	*In vitro* assay	Stimulates seed germination and growth in kale.	Pacheco et al. (2021)

Table 2 – Carrageenans and Oligo-Carrageenans bioactivities on plants.

Seaweed/Source	Polymer	Plant species	Application method	Bioactivity	Reference
Acanthophora spicifera	λ-carrageenan	*Hevea brasiliensis* L.	Foliar spray	Resistance against *Phytophthora palmivora* fungal infection in *H. brasiliensis* by inducing SA-dependent defence pathways.	Pettongkhao et al. (2019)
Calliblepharis jubata	κ, λ, and ι-carrageenan	*Brassica oleracea* L.	*In vitro* assay	Stimulates seed germination and growth in kale. κ- or ι-carrageenan showed best results.	Pacheco et al. (2021)
Chondracanthus teedei var. *lusitanicus*	κ, λ, and ι-carrageenan	*Brassica oleracea* L.	*In vitro* assay	Stimulates seed germination and growth in kale. κ- or ι-carrageenan showed best results.	Pacheco et al. (2021)
Commercially available	κ, λ, and ι-carrageenan	*Nicotiana tabacum* L.	Foliar spray	Stimulates the growth of tobacco plants by enhancing net photosynthesis and ribulose 1, 5 biphoshphate carboxylase/oxygenase (RuBisCO) activity.	Muñoz et al. (2011)
Commercially available	λ-carrageenan	*Arabidopsis thaliana* L. Heynh.	Foliar spray	Resistance against *Sclerotinia sclerotiorum* fungal infection in *A. thaliana* by inducing JA/ET-dependent defence pathways.	Sangha et al. (2010)
Commercially available	κ, λ, and ι-carrageenan	*Arabidopsis thaliana* L. Heynh.	Foliar spray	Resistance against the moth *Trichoplusia ni* in *A. thaliana* by inducing various defence mechanisms including JA and SA-dependent pathways, proteinase inhibitors and an alteration of the products of glycosylate hydrolysis. ι-carrageenan showed best results.	Sangha et al. (2011)
Commercially available	κ, λ, and ι-carrageenan	*Solanum lycopersicum* cv. Sheyenne	Foliar spray	Resistance against Tomato Chlorotic Dwarf Viroid (TCDVd) by inducing JA-dependent defence pathways. κ- or ι-carrageenan did not have an effect.	Sangha et al. (2015)

Seaweed/Source	Polymer	Plant species	Application method	Bioactivity	Reference
Commercially available	κ-carrageenan	*Pinus radiata*	Foliar spray	Stimulates growth, basal metabolism and increases the level of growth-promoting hormones.	Saucedo et al. (2015)
Commercially available	κ, λ, and ι-carrageenan	*Nicotiana tabacum* L.	Foliar spray	Stimulates the growth of tobacco plants by enhancing photosynthesis, basal metabolism, cell cycle as well as ASC levels and AP activity. ι-carrageenan showed best results.	Castro et al. (2012)
Commercially available	κ, λ, and ι-carrageenan	*Eucalyptus globulus* Labill.	Foliar spray	Stimulates the growth of *Eucalyptus globulus* by enhancing photosynthesis, basal metabolism, total essential oils and polyphenolic compounds with potential antimicrobial activities.	González et al. (2013)
Commercially available	κ, λ, and ι-carrageenan	*Nicotiana tabacum* L.	Foliar spray	Induces long-term protection against viral, bacterial and fungal infections at systemic level in tobacco plants by activating the PAL enzyme and enhancing the accumulation of phenylpropanoids with potential antimicrobial activities.	Vera et al. (2012)
Commercially available	λ-carrageenan	*Triticum aestivum* L.	Foliar spray	Resistance against *Zymoseptoria tritici* fungal infection in wheat plants by inducing SA and JA-dependent defence pathways.	Le Mire et al. (2019)
Commercially available	Carrageenan	*Olea europaea* L.	*In vitro* assay	Resistance against *Verticillium dahliae* fungal infection in olive trees by restricting the pathogen's growth and strengthening the host defence metabolism.	Salah et al. (2018)
Kappaphycopsis cottonii (formerly *Eucheuma cottonii*)	κ-carrageenan	*Nicotiana tabacum* L.	Leaf infiltration	Resistance against *Phytophthora parasitica* in tobacco plants by inducing defence genes encoding sesquiterpene cyclase, chitinase and proteinase inhibitor and triggering the signalling pathways mediated by ET, JA and SA. λ-carrageenan showed best results.	Mercier et al. (2001)

Seaweed/Source	Polymer	Plant species	Application method	Bioactivity	Reference
Eucheuma denticulatum (formerly *Eucheuma spinosum*)	ι-carrageenan	*Nicotiana tabacum* L.	Leaf infiltration	Resistance against *Phytophthora parasitica* in tobacco plants by inducing defence genes encoding sesquiterpene cyclase, chitinase and proteinase inhibitor and triggering the signalling pathways mediated by ET, JA and SA. λ-carrageenan showed best results.	Mercier et al. (2001)
Chondracanthus acicularis (formerly *Gigartina acicularis*)	λ-carrageenan	*Nicotiana tabacum* L.	Leaf infiltration	Resistance against *Phytophthora parasitica* in tobacco plants by inducing defence genes encoding sesquiterpene cyclase, chitinase and proteinase inhibitor and triggering the signalling pathways mediated by ET, JA and SA. λ-carrageenan showed best results.	Mercier et al. (2001)
Gigartina pistillata	λ-carrageenan	*Nicotiana tabacum* L.	Leaf infiltration	Resistance against *Phytophthora parasitica* in tobacco plants by inducing defence genes encoding sesquiterpene cyclase, chitinase and proteinase inhibitor and triggering the signalling pathways mediated by ET, JA and SA. λ-carrageenan showed best results.	Mercier et al. (2001)
Grateloupia turuturu	κ, λ, and ι-carrageenan	*Brassica oleracea* L.	*In vitro* assay	Stimulates seed germination and growth in kale. κ- or ι-carrageenan showed best results.	Pacheco et al. (2021)
Hypnea musciformis	κ-carrageenan	*Cicer arietinum* L. and *Zea mays* L.	Foliar spray or soil drench	Stimulates the growth of chickpea and maize plants by eliciting the production of secondary metabolites. The application by soil drench showed better results than the foliar spray.	Bi et al. (2011)
Hypnea musciformis	κ-carrageenan	*Nicotiana tabacum* L.	Leaf infiltration	Resistance to TMV in tobacco plants by inducing SA, JA and ET-dependent defence pathways.	Ghannam et al. (2013)

Seaweed/Source	Polymer	Plant species	Application method	Bioactivity	Reference
Kappaphycus alvarezii	κ-carrageenan	*Capsicum annuum*	Foliar spray	Resistance against *Colletotrichum gloeosporioides* fungal infection in *C. annuum* by inducing antioxidant defence and antifungal PR protein expression by signalling pathways mediated by ET, JA and SA.	Mani & Nagarathnam (2018)
Schizymenia binderi	Oligo-Carrageenans (Poly-Ga)	*Nicotiana tabacum* L.	Foliar spray	Stimulates growth and induces defence in tobacco plants by activating the antioxidant enzyme AP, which modulates the level of the antioxidant compounds ASC and DHA. The activation of the defence enzyme PAL, will lead to the activation of the phenylpropanoid pathway and to the synthesis of secondary metabolites with antiviral activity.	Laporte et al. (2007)
Tichocarpus crinitus	κ/β-carrageenan	*Nicotiana tabacum* L.	Leaf infiltration	Resistance to TMV in tobacco plants by interfering with the deproteinization and replication in the cells of binding virions, leading to less necrotic lesions on the leaves.	Nagorskaya et al. (2008)
Tichocarpus crinitus	κ/β-carrageenan	*Datura stramonium* L.	Leaf infiltration	Inhibits the development of the Potato Virus X (PVX) infection in *D. stramonium.*	Nagorskaya et al. (2010)

1.3. Turnip

Turnip (*Brassica napus* L.) is a vegetable crop with especially economic importance, mostly cultivated in temperate regions (Australia, Europe, Canada, and northern China) (Batool et al., 2023). It belongs to the genus *Brassica* of the Brassicaceae family (Sun, 2015), one of the most important vegetable families in agriculture, vastly cultivated in the world as oil and vegetable crop, providing edible roots, leaves, stems, buds, flowers and seeds (Rakow, 2004). Natural compounds present in the genus *Brassica* are oil, food, fibres, minerals, vitamins, soluble sugars, phytochemicals like carotenoids, glucosinolates and phenolic compounds. Phenolic compounds have been reported to be involved in protection against various human diseases, such as cancer and cardiovascular problems (Idrees et al., 2019). Moreover, members of the Brassicaceae family can serve as a biomarker of heavy metal pollution by tolerating high levels of cadmium toxicity and bioaccumulating it (Qadir et al., 2004). Turnip is a fast-growing root vegetable that can be harvested almost all year long, depending on the variety and part of interest. In addition, this crop does not need a large cultivation area to grow.

1.4. Objectives

This study aims to understand how polysaccharide solutions of alginate from *S. polyschides*, agar from *G. gracilis* and three types of carrageenan from *C. crispus*, can influence *B. napus* L. seed germination, plants' growth, development, and metabolism.

2. Materials and Methods

2.1. Harvesting and preparation of seaweed biomass for extraction

On the 14th of June 2022 (average air temperature around 24 ºC), different samples of one brown seaweed, *Saccorhiza polyschides*, and two red seaweeds, *Gracilaria gracilis* and *Chondrus crispus*, were collected from tide pools in the intertidal zones of Buarcos Bay, Figueira da Foz (seawater temperature at a maximum of 27 ºC and a minimum of 16 ºC). The seaweed samples were selected and identified according to their morphological characteristics with taxonomic references (Pereira, 2020). All seaweeds were collected with minimal epiphytes or degraded marks and transported in plastic bags in a cool box to the laboratory. Due to carrageenan type variation, the red seaweed, *C. crispus*, was classified and separated according to its life cycle generations (tetrasporophyte, non-fructified thalli and female gametophyte) (**Figure 5c-e**). Afterwards, all the seaweeds were transferred to separate trays and washed with seawater to remove sand, epiphytes, and other detritus from its biomass. Then, transferred again to another separate trays and washed two times with distilled water to remove the salt content of seawater. All washed seaweeds were dried in an air-forced oven (Raypa DAF-135, R. Espinar S.L., Barcelona, Spain) at 60 ºC for 48 h. Dried seaweeds were stored in separate silica bags to reduce moisture, in the dark and at room temperature (23 ºC).

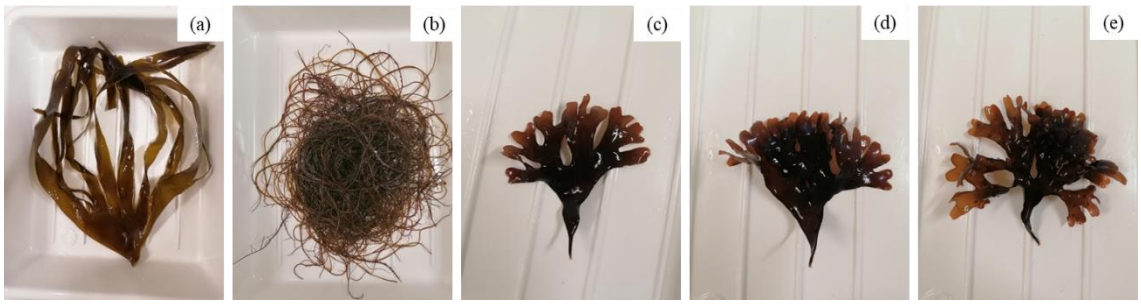

Figure 5 – Washed seaweed of (a) *S. polyschides*, (b) *G. gracilis*, (c) *C. crispus* (tetrasporophyte), (d) *C. crispus* (non-fructified thalli) and (e) *C. crispus* (female gametophyte).

2.2. Polysaccharide extraction

Each type of polysaccharide (alginate, agar and carrageenan) was extracted according to the methods mentioned in **Section 2.2.1.**, **2.2.2.** and **2.2.3**., respectively, and were performed in triplicate. The polysaccharide extraction yield (Y, %) was calculated according to the formula (Wang et al., 2018):

$$Y\,(\%) = \frac{W1}{W2} \times 100 \quad (1)$$

Where, $W1$ is the weight of the dried polysaccharide (g) and $W2$ is the initial weight of the dried seaweed used in the extraction (g).

2.2.1. Alginate

The alginic acid extraction was based on the method described by Sivagnanavelmurugan et al. (2018) with modifications. The dried seaweed (*S. polyschides*, 7 g) (analytical scale: Highland HCB 123, Adam Equipment, UK) was milled (particles <1 cm) with a commercial grinder (TitanMill 300 DuoClean, Cecotec, Valencia, Spain) and then added to a solution of HCl (José Manuel Gomes dos Santos, Portugal) at 1.23% (1:30 v:v) (7 mL of HCl: 203 mL of distilled water per 7 g of dried seaweed) and kept at room temperature (23 ºC) for 24 h. The solution was filtrated, under vacuum (Laborport N820, Lisbon, Portugal), with a Gooch funnel (porosity: G2) and washed with distilled water for 2 or 3 times. The residue obtained was alkali extracted in a 2% sodium carbonate (Fisher Chemicals, Portugal) (90 mL for the initial weight of the dried biomass; 1:30 m:v) and put in the ultrasound machine (ultrasonic cleaner ULTR-3L2-001, IBX instruments, Barcelona, Spain) at 50 ºC for 45 min. The extract was filtrated again, under vacuum, through a cloth filter, with a Gooch funnel (porosity: G2), to remove the residues from the alginate solution. Then a 37% HCl (José Manuel Gomes dos Santos, Portugal) was added to the filtrate for precipitation of the alginic acid (2 mL of 37% of HCl: 30 mL of the final solution). The alginate was washed with ethanol 96% (José Manuel Gomes dos Santos, Portugal) (1:3 v:v) and placed in the cold. The liquid solution was discarded, and the precipitate was dried in an air-force oven (Raypa DAF-135, R. Espinar S.L., Barcelona, Spain) at 60 °C for 48 h.

2.2.2. Agar

Agar extraction was based on the method described by Li et al. (2008) with modifications. The dried seaweed (*G. gracilis*, 15 g) (analytical scale: Highland HCB 123, Adam Equipment, UK) was added to distilled water (600 mL) and placed in an electric pressure cooker (300008IAU, Aigostar, Madrid, Spain) at a temperature of 115 °C with an air pressure of 80 Kpa, for 2 h. The solution obtained was hot filtrated, under vacuum (Laborport N820, Lisbon, Portugal), through a cloth filter supported in a Buchner

funnel. The liquid extract obtained was filtrated again, under vacuum, with a Gooch funnel (porosity: G2). The filtrated solution was solidified at room temperature (23 ºC) and frozen overnight, in a plastic cup. The next day, the agar was unsolidified, washed and purified until it became a white or translucid gel. That gel was dried in an air-forced oven (Raypa DAF-135, R. Espinar S.L., Barcelona, Spain) at 60 ºC for 48 h.

2.2.3. Carrageenan

Carrageenan extraction was based on the method described by Pereira & Van De Velde (2011) with modifications. The dried seaweed (*C. crispus*, 1 g) (analytical scale: Highland HCB 123, Adam Equipment, UK) was milled (particles <1 cm) with a commercial grinder (TitanMill 300 DuoClean, Cecotec, Valencia, Spain) and then pre-treated with an acetone (José Manuel Gomes dos Santos, Portugal):methanol (José Manuel Gomes dos Santos, Portugal) (1:1) solution in a final concentration of 1% (m/v) (final volume: 100 mL; 50 mL acetone: 50 mL methanol) for 16 h at 4 ºC, to eliminate the organic-soluble fraction. The liquid solution obtained was discarded, and the seaweed residues were dried in an air-forced oven (Raypa DAF-135, R. Espinar S.L., Barcelona, Spain) at 60 ºC for about 3-5 min. The dried seaweed was immersed in 150 mL of NaOH (Applichem Panreac, USA) (2%) (1 g of initial seaweed: 150 mL of NaOH solution) in a hot water bath system (GFL 1003, GFL, Burgwedel, Germany) at 85-90 ºC for 3 h. Afterwards, the solution was hot filtrated, under vacuum (Laborport N820, Lisbon, Portugal), through a cloth filter supported in a Buchner funnel. The liquid extract obtained was filtrated again, under vacuum, with a Gooch funnel (porosity: G2). The filtrated solution was evaporated (rotary evaporator: 2600000, Witeg, Germany), under vacuum, to 1/3 of the initial volume. The carrageenan was precipitated by adding twice (1:3) its volume of ethanol 96% and then centrifuged (Christ Universal Junior II, Martin Christ, Osterode/Harz, Germany) for 10 min at 4000 rpm. The precipitate was washed again with ethanol 96% (José Manuel Gomes dos Santos, Portugal) and placed in the cold (4 ºC for 48 h). Finally, the extract was dried in an air-forced oven (Raypa DAF-135, R. Espinar S.L., Barcelona, Spain) at 60 ºC for 48 h.

Due to carrageenan type variation, this method was performed for each sample corresponding to the different life cycle generations (tetrasporophyte, non-fructified thalli and female gametophyte).

2.3. Polysaccharides and polysaccharides' solutions

2.3.1. Preparation of polysaccharide solutions

The dried polysaccharides were milled (particles <0.05 cm), separately, with a commercial grinder (TitanMill 300 DuoClean, Cecotec, Valencia, Spain) and then added distilled water (1 mg/mL), under constant agitation (magnetic stirrer hot plate: H20 series, IBX instruments, Barcelona, Spain) until the complete dissolution of the polysaccharides. Afterwards, each polysaccharide solution was diluted into two solutions with different concentrations, 0.5 mg/mL and 0.25 mg/mL, to be used in **Section 2.4**. The pH (pH meter: 3310 Jenway, Staffordshire, UK), the electrical conductivity (portable conductivity meter: ProfiLine Cond 3310 WTW, Oberbayern, Germany) and the viscosity (DV-E model viscometer, Brookfield, Hadamar-Steinbach, Germany) were determined for each polysaccharide solution, at room temperature (23 ºC). The viscosity measurement was carried out using a spindle S02 with a speed of 100 rpm.

2.3.2. Mineral Characterization

The mineral content was assessed by using flame atomic absorption spectrometry, for the determination of calcium (Ca), magnesium (Mg) and sodium (Na) (Lucas & Sequeira, 1976). To a 25 mL volumetric flask was added 2.5 mL of the polysaccharide solution and 2.5 mL of lanthanum chloride (5%) (Chem-Lab NV, Zedelgem, Belgium), and the volume was adjusted with distilled water (dilution: 1:10). After the necessary dilutions needed to determinate the different elements (1:100 or 1:500) the analysis was carried out on the atomic absorption spectrophotometer (PinAAcle 900T, PerkinElmer, Massachusetts, EUA) equipped with the cathode corresponding to each element.

2.3.3. FTIR-ATR analysis

The Fourier-Transform Infrared Spectroscopy - Attenuated Total Reflection (FTIR-ATR) analysis was based on the protocol described by Pereira et al. (2013). The dried polysaccharides samples obtained from the extraction process in **Chapter 2.2.**, were milled (particles <0.05 cm) with a commercial grinder (TitanMill 300 DuoClean, Cecotec, Valencia, Spain) and subjected to direct analysis (without humidity) (spectrometer: ALPHA II Compact FT-IR Spectrometer, Bruker, Germany) without any further preparation. All spectra obtained are the average of two independent measurements from 400 to 4000 cm^{-1} with 24 scans, each at a resolution of 4 cm^{-1}.

2.4. Seed germination assay

Turnip seeds (Flora Lusitana, Cantanhede, Portugal) were disinfected through emersion for 1 min in a solution of sodium hypochlorite (José Manuel Gomes dos Santos, Portugal) (NaClO) 2% and then for 3 min in distilled water. Sterilized Petri dishes (15 cm x 15 cm) were prepared with cotton and filter paper above and 70 mL of each polysaccharide solution was added. The control was done with addition of distilled water in the same volume. Then, 25 disinfected turnip seeds were sown, on the filter paper, in each Petri dish and incubated (Heraeus B5090E Incubator, Thermo Scientific, Osterode, Germany) at 22 ± 1 °C in darkness, for 10 days. All trials were performed in triplicate. After 4, 7 and 10 days, the number of germinated seeds was counted, and the germination percentage (GP) was calculated according to Hernández-Herrera et al. (2014): GP = (number of germinated seeds/total number of seeds) × 100. From each replica, five random seeds were selected, and the plant growth parameters were evaluated: Aerial part (measured from the hypocotyl base to the apical bud) and radicular length, using a ruler (Shatterless 75 S.50, Molin, Portugal); Fresh weight of the aerial part and radicular part, using an analytical scale (PC2000 Mettler-Toledo, Zurich, Switzerland). The ratios between the growth parameters were also calculated. The ratios aid to understand the influence that the growth parameters have on each other.

2.5. Biostimulant and biofertilizer assay *in vivo*

2.5.1. Experimental conditions

The assay was performed in 5 L black pots (with a diameter of 23 cm at the top, a diameter of 16 cm at the base and 18 cm height), in conditioned substrate (SIRO, Coimbra, Portugal) under greenhouse conditions and with natural photoperiod, at ESAC (Escola Superior Agrária de Coimbra, Portugal). 19 pots were organized in a randomized block design, with 2 turnip seeds (Flora Lusitana, Cantanhede, Portugal) sown in each pot and a plastic bag underneath to prevent water leakage (**Figure 6**). All pots were fertilized with Blaukorn Classic (Blaukorn Classic 12-8-16 (+3+TE), Compo-expert, Portugal) and drip irrigation was used, during 6 min (± 250 mL per pot), 3 times per week.

Figure 6 – Photographic record of the experimental conditions.

The treatments applied in this experiment (**Table 3**) include different solutions obtained from the polysaccharides' extraction of alginate (from *S. polyschides*), agar (from *G. gracilis*) and carrageenan (from three different life cycle generations of *C. crispus*, tetrasporophyte, non-fructified thalli and female gametophyte). As a positive control, was used a commercially leaf fertilizer, "Profertil" (ADP Fertilizantes, Portugal), with 20% (dry matter) based on brown seaweed *Ascophyllum nodosum*, at a concentration of 1.5% (v/v), while as a negative control was used tap water. This assay lasted 63 days (from sowing to plant harvesting) and the treatments were applied two times. The first application was done 31 days after the sowing, when the plants had 3 to 4 grown leaves. The second application was done 10 days after the first application. All the polysaccharides' solutions and the positive control were sprayed on to the foliage (± 3 mL of extract sprayed on each plant; 18 mL per treatment in each application). Each treatment was applied to 6 plants (3 pots with 2 plants each) in 3 repetitions.

Table 3 – Description of the treatments and their concentration, applied in the assay.

Treatment	Concentration
Negative control (Tap water)	-
Positive control ("Profertil")	1,5% (v/v)
Alginate solution	0.50 mg/mL
Agar solution	0.50 mg/mL
Carrageenan (Tetrasporophyte) solution	0.25 m/mL
Carrageenan (Non-fructified thalli) solution	0.50 mg/mL
Carrageenan (Female gametophyte) solution	0.50 mg/mL

2.5.2. Growth parameters of the obtained plant material

The evolution of the plant growth was observed throughout the experiment. Sixty-three days after the sowing, plant material was harvested, washed with tap water, and separated their roots and leaves. The length and fresh weight (FW) of the roots and aerial parts of each sample were measured by using a ruler (Shatterless 75 S.50, Molin, Portugal) and an analytical scale (PC2000 Mettler-Toledo, Zurich, Switzerland), respectively. The number of leaves in each plant sample was counted. Plants were dried in an air-forced oven (Memmert, Büchenbach, Germany) during 3 days at 65 ºC, until the constant weight was reached. Then, each sample was cooled for about 2 h, and the roots and aerial parts dry weight (DW) were separately measured.

2.6. Turnip' physiological and biochemical characterization

All methods described in this chapter were carried out with plant material derived from each treatment separately (**Table 3)** and performed in duplicate.

2.6.1. Dry matter and ashes content

The dry matter and ashes content determination were based on the method described by Cunniff (1997). The dried aerial part of the plant samples obtained in **Section 2.5.2.** were milled (particles <1 mm) with a commercial grinder (electric coffee grinder: KG-39, DeLonghi, Treviso, Italy) and, approximately, 3 g of each sample (analytical scale: AB 204 Mettler-Toledo, Zurich, Switzerland) were placed in crucibles and dried in an air-forced oven (UFB 500, Memmert, Büchenbach, Germany) at 105 °C for 4 h. Then, the samples were placed in a desiccator until the constant weight was reached, being again weighted (analytical scale: AB 204 Mettler-Toledo, Zurich, Switzerland), to calculate the dry matter content.

The dry matter content (DM, g 100 g^{-1} (%) m/m) at 65 ºC was calculated according to the formula (Cunniff, 1997):

$$\text{DM at } 65\ ^{\circ}\text{C } (\%) = \frac{w_1}{w_2} \times 100 \quad (2)$$

Where, w_1 is the weight of the sample dried at 65 ºC (g); w_2 is the weight of the fresh sample (g).

The dry matter content (DM, g 100 g^{-1} (%) m/m) at 105 ºC was calculated according to the formula (Cunniff, 1997):

$$\text{DM at } 105\ ^{o}\text{C } (\%) = \frac{(m_3 - m_1)}{(m_2 - m_1)} \times 100 \quad (3)$$

Where, m_1 is the crucible weight dried at 105 ºC (g); m_2 is the crucible and sample weight dried at 65 ºC (g); m_3 is the crucible and sample weight dried at 105 ºC (g).

To assess the ashes content, the previous samples dried at 105 °C were placed in an incineration muffle (Induzir, Leiria, Portugal) at 480-500 ºC overnight and further cooled in a desiccator and weighted again (analytical scale: AB 204 Mettler-Toledo, Zurich, Switzerland). The ashes content is calculated according to the formulas (Cunniff, 1997):

$$\text{Ashes } (\%\ \text{db}) = \frac{(m_3 - m_1)}{(m_2 - m_1)} \times 100 \quad (4)$$

$$\text{Ashes } (\%\ \text{fb}) = \frac{Ashes(\%\ db) \times (100 - H)}{100} \quad (5)$$

Where, % db is the percentage of dried biomass; % fb is the percentage fresh biomass; m_1 is the crucible weight (g); m_2 is the crucible and sample weight dried at 105 ºC (g); m_3 is the crucible and sample weight incinerated (g).

2.6.2. Total Nitrogen/ Protein

The total nitrogen/ protein content was determined by the Kjeldahl method (Bremner, 1979; Cunniff, 1997). In a Kjeldahl tube, was added approximately 0.5 g (analytical scale: AB 204 Mettler-Toledo, Zurich, Switzerland) of the previously dried matter obtained in **Section 2.6.1.**, and then added a Kjeldahl tablet (Fisher Chemicals, Portugal) and 10 mL of sulfuric acid (Chem-Lab NV, Zedelgem, Belgium). The tubes were then placed into the Kjeldahl digester (Bloc Digest 12 Rat 2, JP Selecta, Lisbon, Portugal) at 400 °C for 2 h, under "hotte". The samples were allowed to cool in the "hotte", and it was added 50 mL of distilled water in each tube and put into the Kjeldahl distiller (VELP Scientifica, Usmate Velate MB, Italy). Concurrently, it was placed 20 mL of boric acid 2% (Chem-Lab NV, Zedelgem, Belgium) in an Erlenmeyer (one for each sample), being further placed into the Kjeldahl distiller as well. During the distillation process, was added to the Kjeldahl tube, 50 mL of sodium hydroxide (NaOH) at 40% (m/v) (Chem-Lab NV, Zedelgem, Belgium). The distilled solution was collected and

titrated with hydrochloric acid (HCl) 0.1 M (Chem-Lab NV, Zedelgem, Belgium). Total nitrogen (N, % m/m_{dry}) was calculated according to the formula (Cunniff, 1997):

$$\mathrm{N}(\%) = \frac{[HCl] \times (V - V0) \times 0.014 \times 100}{m} \quad (6)$$

Where, [HCl] is the hydrochloric acid concentration (M); V is the volume of HCl spent in sample titration (mL); V0 is the volume of HCl spent in control sample titration (mL); m is the sample weight (g); 0.014 is the value (g) of N that reacts with 1ml of HCl 1 mol dm^{-3}.

The total protein content was determined by the multiplication of the protein conversion factor, 6.25, to the total nitrogen content, as described by PortFIR – INSA.

2.6.3. Mineral and Trace Element Characterization

The mineral content was analysed through dry mineralization and assessed by using flame atomic absorption spectrometry, for the determination of copper (Cu), zinc (Zn), manganese (Mn), iron (Fe), calcium (Ca), magnesium (Mg), potassium (K) and sodium (Na) (Lucas & Sequeira, 1976), and molecular absorption spectrometry for the determination of phosphorus (P) (Ribas et al., 1988). With the ashes obtained in **Section 2.6.1.**, it was performed an acid digestion with hydrochloric acid 20% (v/v) (Chem-Lab NV, Zedelgem, Belgium), in a water bath (Memmert, Büchenbach, Germany) at 100 °C for 30 min. Then, the samples were filtrated with a filter paper (cellulose-based ashless types, pore size: 8 µm and diameter of 150 mm, Whatman, Portugal) to a 50 mL volumetric flask, and the volume was adjusted with distilled water (mother liquor). To a 25 mL volumetric flask was added 2.5 mL of the previous solution and 2.5 mL of lanthanum chloride (5%) (Chem-Lab NV, Zedelgem, Belgium), and the volume was adjusted with distilled water (dilution: 1:10). After the necessary dilutions needed to determinate the different elements (1:100 or 1:500) the analysis was carried out on the atomic absorption spectrophotometer (PinAAcle 900T, PerkinElmer, Massachusetts, EUA) equipped with the cathode corresponding to each element. For the phosphorus analysis, was added 2.5 mL of the mother liquor and 5 mL of ammonium molybdate-vanadate solution in nitric medium (Chem-Lab NV, Zedelgem, Belgium) to a 25 mL volumetric flask and adjusted with distilled water. This solution was left overnight at room temperature (23 ºC). The next day, the phosphorus analysis was carried out on the

molecular absorption spectrophotometer (PYE Unicam, SP6-350, Philips, Portugal), at a wavelength of 650 nm.

2.6.4. FTIR-ATR analysis

The FTIR-ATR analysis of each turnip sample was performed as described in **Section 2.3.3**, with the dried plant samples obtained in **Section 2.6.1**.

2.6.5. Pigments content

The detection of pigments was based in the method described by Cotas et al. (2019). This process uses thin-layer chromatography (TLC) to separate and determinate the composition of pigments in methanolic extracts and spectrophotometry for the quantitative and qualitative analysis of those pigments.

2.6.5.1. Thin-layer chromatography (TLC)

The dried samples obtained in **Section 2.6.1.** (0.2 g) (analytical scale: Highland HCB 123, Adam Equipment, UK) were added to 20 mL of acetone (José Manuel Gomes dos Santos, Portugal): methanol (José Manuel Gomes dos Santos, Portugal) (1:1) solution (final volume: 20 mL; 10 mL acetone: 10 mL methanol), under constant agitation (magnetic stirrer hot plate: H20 series, IBX instruments, Barcelona, Spain) for 30 min. The liquid solution was filtrated, under vacuum, with a Gooch funnel (porosity: G2), and then, evaporated (rotary evaporator: 2600000, Witeg, Germany) until all the pigments were all adhered to the surface of the round-bottom flask. The pigments were resuspended again with 2 mL of acetone (José Manuel Gomes dos Santos, Portugal): methanol (José Manuel Gomes dos Santos, Portugal) (1:1) solution, to obtain a concentrated extract. Afterwards, silica gel TLC plate (ALUGRAM Xtra SIL G UV254, Macherey-Nagel, Germany) was activated at 120 ºC for 5 min (air-forced oven: Raypa DAF-135, R. Espinar S.L., Barcelona, Spain) and then 20 µL of each concentrated extract were applied. The plate was developed in a chromatography chamber using a petroleum ether (José Manuel Gomes dos Santos, Portugal): acetone (José Manuel Gomes dos Santos, Portugal) solution (7:3 v/v) as eluent, until the front reached a height of 10 cm. The plate was then removed, and the solvent evaporated at room temperature (23 ºC). The pigments were identified by calculating the retention factor (Rf): Rf=compound migration distance (cm)/distance travelled by the eluent and comparing with the literature.

2.6.5.2. Spectrophotometry

The quantitative and qualitative analyses of the pigments were performed by spectrophotometry. After the TLC and the necessary dilution (1:50) of the extracts, the analysis was carried out on the spectrophotometer (UV-3100PC, VWR, UK), with scanning at 665.2 nm, 652.4 nm, 535 nm and 470 nm. The following formulas were used for the quantification of the pigments (mg/ 100 g) chlorophyll *a* and *b* (Chl *a* and Chl *b*) and carotenoids (Toscano et al., 2023):

$$\text{Chl } a = 16.75 \times A_{665.2} - 9.16 \times A_{652.4} \quad (7)$$

$$\text{Chl } b = 34.09 \times A_{652.4} - 15.28 \times A_{665.2} \quad (8)$$

$$\text{Carotenoids} = \frac{(1000 \times A_{470} - 1.63 \times \text{Chl } a - 104.96 \times \text{Chl } b)}{221} \quad (9)$$

Where, $A_{665.2}$, $A_{652.4}$ and A_{470} is the absorbance of the sample at the wavelength 665.2 nm, 652.4 nm and 470 nm, respectively.

The total of anthocyanins (mg/ 100 g) was calculated according to the formula (Lao & Giusti, 2016):

$$\text{Anthocyanins} = \frac{100 \times A_{535} \times DF \times V}{98.2 \times x} \quad (10)$$

Where, A_{535} is the absorbance of the sample at the wavelength 535 nm; DF is the dilution factor; V is the volume of anthocyanin extract that was made up to after extraction (ml); x is the weight of the dried sample used for extraction (g).

2.7. Substrate characterization

2.7.1. Substrate density

The initial substrate (before the treatments) and final substrates (after the treatments) used for turnip' potting was initially analysed by the apparent compact density method (Soil Improvers and Growing Media – Extraction of Water-Soluble Nutrients and Elements, 2001) to measure the density of the substrate samples. This step is essential to calculate the weight of substrate sample (m_s, g) at 60 mL, used in **Section 2.7.2**. To a 1000 mL plastic graduated cylinder was added the substrate sample without pressing it down. Then, the substrate was compacted by dropping the graduated cylinder 10 times on a 5 mm thick rubber blanket, from a height of about 10 cm. The level of the substrate was marked, and the graduated cylinder was weighted (technical scale: UFB 500, Memmert, Büchenbach, Germany). The apparent compact density of the substrate (Ds, g L^{-1}); was calculated according to the formula (Soil Improvers and Growing Media – Extraction of Water-Soluble Nutrients and Elements, 2001):

$$\mathrm{D_s} = \frac{m_A - m_B}{V} \tag{11}$$

Where, m_A is the weight of the substrate compacted and the graduated cylinder (g); m_B is the weight of the graduated cylinder (g); V is the final volume of the substrate in the graduated cylinder (L).

The weight of substrate sample (m_s, g) at 60 mL, used in **Section 2.7.2** was calculated according to the formula (Soil Improvers and Growing Media – Extraction of Water-Soluble Nutrients and Elements, 2001):

$$\mathrm{m_s} = \frac{D_s \times 60}{1000} \tag{12}$$

Where, D_s is the apparent compact density of the substrate (g L^{-1}).

2.7.2. pH and electrical conductivity

The substrate samples were weighted (technical scale: UFB 500, Memmert, Büchenbach, Germany) to a 500 mL Erlenmeyer, added 300 mL of distilled water, secured the cap and shaken for 1 h at 200 rpm (shaking machine: Rotabit, JP Selecta, Lisbon, Portugal), at room temperature (23 ºC). Afterwards, the pH was determined (pH

meter: 3310 Jenway, Staffordshire, UK) directly from the solution obtained. The electric conductivity (portable conductivity meter: ProfiLine Cond 3310 WTW, Oberbayern, Germany) was determined from the filtrated obtained in **Section 2.7.3**.

2.7.3. Mineral and Trace Element Characterization

The extract obtained in **Section 2.7.2.** was filtrated with filter paper (cellulose-based ashless types, pore size: 8 µm and diameter of 150 mm, Whatman, Portugal), discarding at least the first 10 mL. The rest of the filtrated was added to a 100 mL plastic container and stored at room temperature (23 ºC). The mineral content was assessed by using flame atomic absorption spectrometry, for the determination of copper (Cu), zinc (Zn), manganese (Mn), iron (Fe), calcium (Ca), magnesium (Mg), potassium (K) and sodium (Na) (Lucas & Sequeira, 1976), and molecular absorption spectrometry for the determination of phosphorus (P) (Ribas et al., 1988). To a 25 mL volumetric flask was added 2.5 mL of the previous solution and 2.5 mL of lanthanum chloride (5%) (Chem-Lab NV, Zedelgem, Belgium), and the volume was adjusted with distilled water (dilution: 1:10). After the necessary dilutions needed to determinate the different elements (1:100 or 1:500) the analysis was carried out on the atomic absorption spectrophotometer (PinAAcle 900T, PerkinElmer, Massachusetts, EUA) equipped with the cathode corresponding to each element. The phosphorus analysis was quantified through the colorimetric method of ammonium molybdate in acidic medium and ascorbic acid (final volume: 1000 mL; 800 mL of distilled water: 25 mL of ammonium molybdate in acidic medium: 10 mL of ascorbic acid) (Chem-Lab NV, Zedelgem, Belgium) in a molecular absorption spectrophotometer (PYE Unicam, SP6-350, Philips, Portugal), at a wavelength of 650 nm.

2.7.4. Organic matter content

The substrate samples were placed in separate aluminium trays, weighted, and then dried in an air-forced oven (UFB 500, Memmert, Büchenbach, Germany) at 75 °C for 2 days, until it reached a constant weight. Then, the samples were weighted again and milled, separately, in a soil grinder (FRITSCH GmbH Pulverisette 8, Midland, Canada), through a sieve of 1.5 mm, separating the thin (particles < 1.5 mm) and rough (particles > 1.5 mm) material (Laboratório Químico Agrícola Rebelo da Silva, 1977; Póvoas & Barral, 1992). Approximately, 3 g of each sample (particles < 1.5 mm) (analytical scale: AB 204 Mettler-Toledo, Zurich, Switzerland) were placed in crucibles and dried in an

air-forced oven (UFB 500, Memmert, Büchenbach, Germany) at 105 °C for 4 h. Then, the samples were placed in a desiccator until the constant weight was reached, being again weighted (analytical scale: AB 204 Mettler-Toledo, Zurich, Switzerland). The previous samples dried at 105 °C were placed in an incineration muffle (Induzir, Leiria, Portugal) at 480-500 ºC overnight and further cooled in a desiccator and weighted again (analytical scale: AB 204 Mettler-Toledo, Zurich, Switzerland).

The organic matter content (OM, %) is calculated according to the formula (Laboratório Químico Agrícola Rebelo da Silva, 1977; Póvoas & Barral, 1992):

$$\text{OM } (\%) = \frac{(m_2 - m_3)}{(m_2 - m_1)} \times 100 \quad (13)$$

Where, m_1 is the crucible weight (g); m_2 is the crucible and sample weight dried at 105 ºC (g); m_3 is the crucible and sample weight incinerated (g).

2.7.5. Total Nitrogen

The total nitrogen analysis of each substrate was performed as described in **Section 2.6.2**, with 1 g of the dried samples (at 75 ºC) obtained in **Section 2.7.4**.

The reagent blank test was carried out in parallel with the determinations, by the same procedure as outlined in the **Sections 2.3.1**, **2.3.2**, **2.6.2**, **2.6.3**, **2.6.5.2**, **2.7.2**, **2.7.3**, **2.7.4** and **2.7.5**, using the same quantities of all the reagents as in the determination but omitting the test portion.

2.8. Statistical analysis

The statistical analysis was performed with the software Sigma Plot v.14. Data was checked for normality (Shapiro–Wilk test) and homogeneity (the equal variance test Brown-Forsythe). The Holm-Sidak method was used in the analysis when the normality test was rejected. One-way analysis of variance (ANOVA) was then performed to assess statistically significant differences between each growth parameters of each polysaccharide' solution. The statistical analysis was performed comparing the different treatments, being considered statistically different when p-value was <0.05. The Tukey multiple comparison t-test was used after the rejection of the one-way ANOVA null hypothesis (Holm-Sidak method).

3. Results

3.1. Extraction yield and polysaccharide characterization

In **Figure 7** is represented all the polysaccharides extracted (alginate, agar and carrageenan) before dried. The alginate (**Figure 7a**) and the different carrageenans (**Figure 7c-e**) had a white colour, while agar (**Figure 7b**) had a yellowish colour. The alginate and agar had a more gelatinous and granular texture, whereas the carrageenans were more compact and firmer.

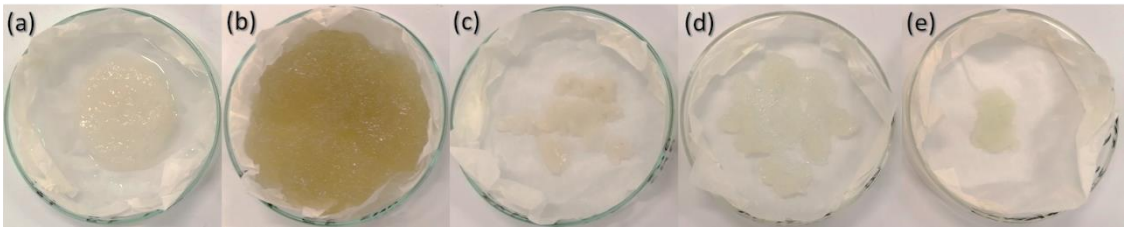

Figure 7 – Photographic record of the extracted polysaccharides: (a) alginate from *S. polyschides*; (b) agar from *G. gracilis*; (c) carrageenan from *C. crispus* (tetrasporophyte); (d) carrageenan from *C. crispus* (non-fructified thalli); (e) carrageenan from *C. crispus* (female gametophyte), before dried.

In **Table 4**, it is demonstrated the yield of the polysaccharide's extractions from the different seaweed species. The alginate sample had 2.33% yield and the agar sample had 17.33%. Among the carrageenan samples, the highest yield was from the non-fructified thalli of *C. crispus*, and the lowest was from the female gametophyte of *C. crispus*, with 23.33% and 10%, respectively.

The three most important elements identified in the mineral profile of the polysaccharide's solutions (**Table 5**) were calcium (Ca), magnesium (Mg) and sodium (Na). Na was the most abundant element in all samples. The highest Na concent was from the alginate solution of *S. polyschides* and the carrageenan solution of *C. c*l (female gametophyte), with 114,.90 mg/L and 108.50 mg/L, respectively. The lowest Na concentration was from the agar solution of *G. gracilis*, with 3.47 mg/L. On the other hand, Mg was the least abundant element in all samples, with the highest concentration of 0.87 mg/L from the carrageenan solution of *C. crispus* (tetrasporophyte), and the lowest concentration of 0.23 mg/L from the carrageenan solution of *C. crispus* (non-fructified thalli). The alginate solution of *S. polyschides* exhibited the highest concentration of Ca, with 2.42 mg/L, and the carrageenan solution of *C. crispus* (female gametophyte) the lowest concentration with 0.58 mg/L.

In the mineral profile represented in **Table 5**, the agar solution of *G. gracilis*, stands out the most, because of the relatively close concentrations between all the elements, contrary of what happens in the other samples.

Table 4 – Extraction yield (% of dry weight) of the polysaccharide's extractions. The extraction yield results are expressed in mean ± standard deviation (n=3). Statistically significant differences found are expressed by letters ($p<0.05$). NI – No Information found in the literature. SP – *S. polyschides*; GG – *G. gracilis*; CC(T) – *C. crispus* (tetrasporophyte); CC(NF) – *C. crispus* (non-fructified thalli); CC(FG) – *C. crispus* (female gametophyte).

Polymer	Seaweed species	Extraction yield (%)	Literature values	Reference
Alginate	SP	2.33 ± 0.00^{b}	NI	NI
Agar	GG	17.33 ± 0.01^{a}	27.00	Pacheco et al. (2021)
Carrageenan	CC(T)	15.33 ± 0.02^{ab}	36.30-58.00	Pereira et al. (2009)
Carrageenan	CC(NF)	23.33 ± 0.05^{a}	29.10-36.00	Pereira et al. (2009)
Carrageenan	CC(FG)	10.00 ± 0.07^{ab}	33.30-36.80	Pereira et al. (2009)

Table 5 – Mineral profile of polysaccharide's solutions (1 mg/mL). The results are expressed in mean ± standard deviation (n=3). Statistically significant differences found among the different samples are expressed by letters ($p<0.05$). SP – *S. polyschides*; GG – *G. gracilis*; CC(T) – *C. crispus* (tetrasporophyte); CC(NF) – *C. crispus* (non-fructified thalli); CC(FG) – *C. crispus* (female gametophyte).

Mineral	SP	GG	CC(T)	CC(NF)	CC(FG)
Ca (mg/L)	2.42 ± 0.19^{a}	0.89 ± 0.04^{ab}	1.13 ± 0.01^{ab}	0.58 ± 0.01^{b}	0.72 ± 0.05^{ab}
Mg (mg/L)	0.53 ± 0.00^{ab}	0.55 ± 0.00^{ab}	0.87 ± 0.05^{a}	0.23 ± 0.00^{b}	0.29 ± 0.00^{ab}
Na (mg/L)	114.90 ± 6.65^{a}	3.47 ± 0.33^{b}	87.57 ± 6.70^{c}	37.98 ± 3.83^{d}	108.50 ± 5.43^{a}

3.2. FTIR-ATR analysis of polysaccharides

The extracted polysaccharides were analysed by FTIR-ATR. This spectroscopic technique allowed the polysaccharide characterization in a rapid, non-destructive manner, demanding low amounts of sample. The obtained spectra were reviewed with bibliographic support (Belattmania et al., 2020; López-Hortas et al., 2023; Pacheco et al., 2021; Pereira et al., 2013; Rashedy et al., 2021).

3.2.1. Alginate

The FTIR-ATR spectra in the range 2000 to 650 cm^{-1} of the alginate extracted from the studied brown seaweed (*S. polyschides*) compared with its commercial counterpart of sodium alginate (A3249 1000, PanReac AppliChem, Germany) are given in **Figure 8** and **Table 6**. Both spectra in displayed the typical alginate absorption bands. The bands between 1730 cm^{-1} and 1710 cm^{-1} only appear in the extracted alginate spectra and were suggested as the carboxylic acid ester form (C=O). Whereas the bands from 1610 cm^{-1} to 1600 cm^{-1}, corresponding to the asymmetric stretching vibration of carboxyl groups (COOH), only appear in the commercial sodium alginate spectra. The peaks at the 1428 cm^{-1} and 1400 cm^{-1}, present in both spectra, were assigned to symmetric stretching vibrations of carboxyl groups (COOH) of alginate. The bands between 1280 cm^{-1} and 1230 cm^{-1} correspond to fucoidan and other sulphated polysaccharides, and its only present in the extracted alginate spectra. In both spectra, the highest peak was detected in the bands 1030 cm^{-1} and 1025 cm^{-1} and directly reflects the typical alginic acid. The intense band at 1027 cm^{-1} in the extracted alginate spectra can indicate that this sample is very rich in guluronic acid. The C-O stretching vibration of uronic acid residues is generally linked to the bands centered around 950 cm^{-1} to 930 cm^{-1}. The anomeric region of carbohydrates was between 806 cm^{-1} and 788 cm^{-1}, attributed to guluronic acids residues and mannuronic acids residues, respectively.

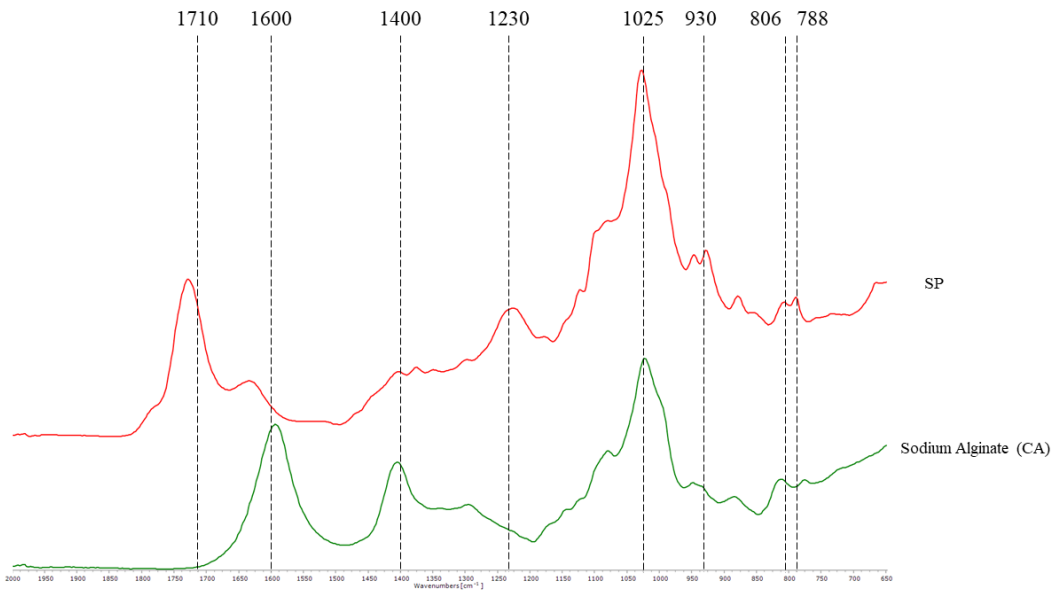

Figure 8 – FTIR-ATR spectra of the brown seaweed polysaccharide (alginate). CA – commercially available. SP – alginate from *S. polyschides*.

Table 6 – FTIR-ATR bands identification and characterization of the brown seaweed polysaccharide (alginate). nd – not detectable. CA – commercially available. SP – *S. polyschides*.

Reference wave number (cm -1)	**Bound**	**Wave number observed (cm -1)**	
		SP	**Sodium Alginate (CA)**
1730-1710	Carboxylic acid ester (C=O)	1728	nd
1610-1600	Asymmetric stretching vibration of carboxyl groups (COOH)	nd	1593
1428-1400	Symmetric stretching vibration of carboxyl groups (COOH)	1403	1405
1280-1230	Sulphated esters (S=O)	1226	nd
1030-1025	Alginic acid (C-O group)	1027	1023
950-930	C-O stretching vibration of uronic acids	946.7	948.1
806	Guluronic acids residues	806.3	812
788	Mannuronic acids residues	789.4	774.7

3.2.2. Agar

The FTIR-ATR spectra in the range 1500 to 650 cm^{-1} of agar extracted from one of the studied red seaweeds (*G. gracilis*) compared with its commercial counterpart (AGAG-00P-500, Labbox Export, Barcelona, Spain) are given in **Figure 9** and **Table 7**. The agar spectra of *G. gracilis* showed lower intensity then the commercially available one. The characteristic band of sulphated esters between 1260 cm^{-1} and 1210 cm^{-1} showed the exact same peak in both spectra at 1247 cm^{-1}. The second band of sulphated esters only appears in the commercial agar at 1113 cm^{-1}. The highest peaks in both spectra are present between 1030-1010 cm^{-1} and are assigned to C-O and C–C stretching vibrations of pyranose ring, common to all polysaccharides. Both in the *G. gracilis* extracted agar and in the commercial agar, the broad band between 975 cm^{-1} and 867 cm^{-1} includes various types of galactoses and 3,6-anhydrogalactoses. The most significant peaks are detected at 975-970 cm^{-1}, 930 cm^{-1}, 900-890 cm^{-1} and 867 cm^{-1}, corresponding to galactose, C–O of 3,6-anhydrogalactose, C–O–SO3 on C2 of 3,6-anhydrogalactose and C–O–SO3 on C6 of galactose, respectively. The peak at 741 cm^{-1} is suggested to be the C-S/C-O-C bending mode in glycosidic linkages of agars and its

present in both spectra. Lastly, the peak at 690 cm^{-1}, very characteristic of agar FTIR-ATR spectra corresponds to 3,6- anhydro-L-galactose.

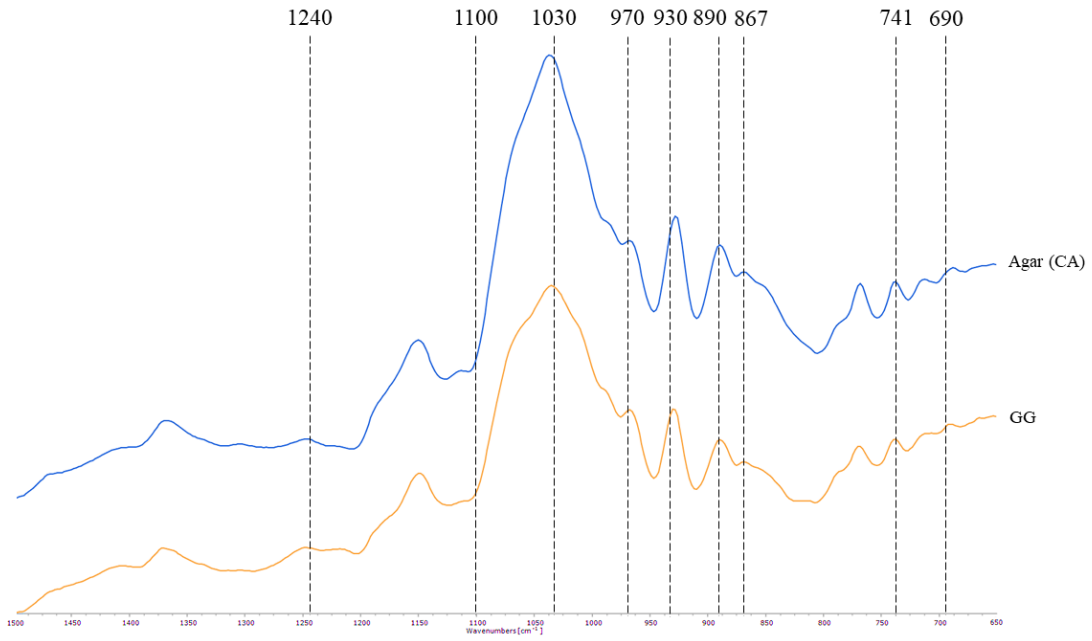

Figure 9 – FTIR-ATR spectra of the red seaweed polysaccharide (agar). CA – commercially available. GG – agar from *G. gracilis*.

Table 7 – FTIR-ATR bands identification and characterization of the red seaweed polysaccharide (agar). sh – shoulder (where peak demonstrate intensity, but not enough to be considered a peak due to the surrounding peak intensities). CA – commercially available. GG – *G. gracilis*.

Reference wave number (cm -1)	**Bound**	**Wave number observed (cm -1)**	
		GG	**Agar (CA)**
1260-1210	Sulphated esters (S=O)	1247	1247
1100	Sulphated esters (S=O)	sh	1113
1030-1010	C-O and C–C stretching vibrations of pyranose ring	1035	1037
975-970	Galactose	967.9	968.1
930	C–O of 3,6-anhydrogalactose	929.6	928.1
900-890	C–O–SO3 on C2 of 3,6-anhydrogalactose	889.2	889.3
867	C–O–SO3 on C6 of galactose	868.8	868.8
845	D-galactose-4-sulfate	sh	sh
790	Characteristic of agar-type in second derivative spectra	sh	sh
741	C-S/C-O-C bending mode in glycosidic linkages of agars	738.7	738.8
690	3,6- anhydro-L-galactose	689	687.8

3.2.3. Carrageenans

The FTIR-ATR spectra in the range 1500 to 650 cm^{-1} of three types of carrageenan extracted from one of the studied red seaweeds (*C. crispus*) compared with their commercial counterparts of kappa-carrageenan and iota-carrageenan (Thermo Scientific, Osterode, Germany) are given in **Figure 10** and **Table 8**.

There is a similarity in the FTIR-ATR peaks of both polysaccharides extracted, agar (**Table 7**) and carrageenan (**Table 8**) from the red seaweeds (*G. gracilis* and *C. crispus*, respectively). What differentiates carrageenans from agar is the presence of a peak at 1070 cm^{-1} and the absence of a peak at 867 cm^{-1}, assigned to C–O of 3,6-anhydrogalactose and C–O–SO3 on C6 of galactose, respectively.

In **Figure 10**, there are four identical spectra, corresponding to the commercial κ-carrageenan, commercial ι-carrageenan and two carrageenans extracted from different

generations of *C. crispus*, non-fructified thalli and female gametophyte. The carrageenan spectra of *C. crispus* (tetrasporophyte) differs from the previous spectra. The similar peaks are around 1210 cm^{-1}, 930 cm^{-1} and 845 cm^{-1}. However, the peaks at 933-928 cm^{-1} and 845 cm^{-1} are almost absent or low in *C. crispus* (tetrasporophyte) spectra. The characteristic band of sulphated esters at 1260-1210 cm^{-1} has the highest intensity in the ι-carrageenan spectra and the lowest intensity in the *C. crispus* (tetrasporophyte) spectra. Whereas the peak at 1070 cm^{-1}, assigned to the C–O of 3,6-anhydrogalactose, has the most intensity in the ι-carrageenan spectra and *C. crispus* (tetrasporophyte), being almost absent in the *C. crispus* (non-fructified thalli and female gametophyte) spectra. In the band between 1030 cm^{-1} and 1010 cm^{-1} was detected the highest peaks in all spectra except the *C. crispus* (tetrasporophyte), which is assigned to C-O and C–C stretching vibrations of pyranose ring, common to all polysaccharides. The peaks at 975-970 cm^{-1} and 690 cm^{-1}, are also absent in the *C. crispus* (tetrasporophyte) spectra but present in all other spectra. The peak at 805 cm^{-1} is characteristic of the ι -carrageenan and its absent in the commercial κ-carrageenan spectra but present in all the spectra of the carrageenans extracted.

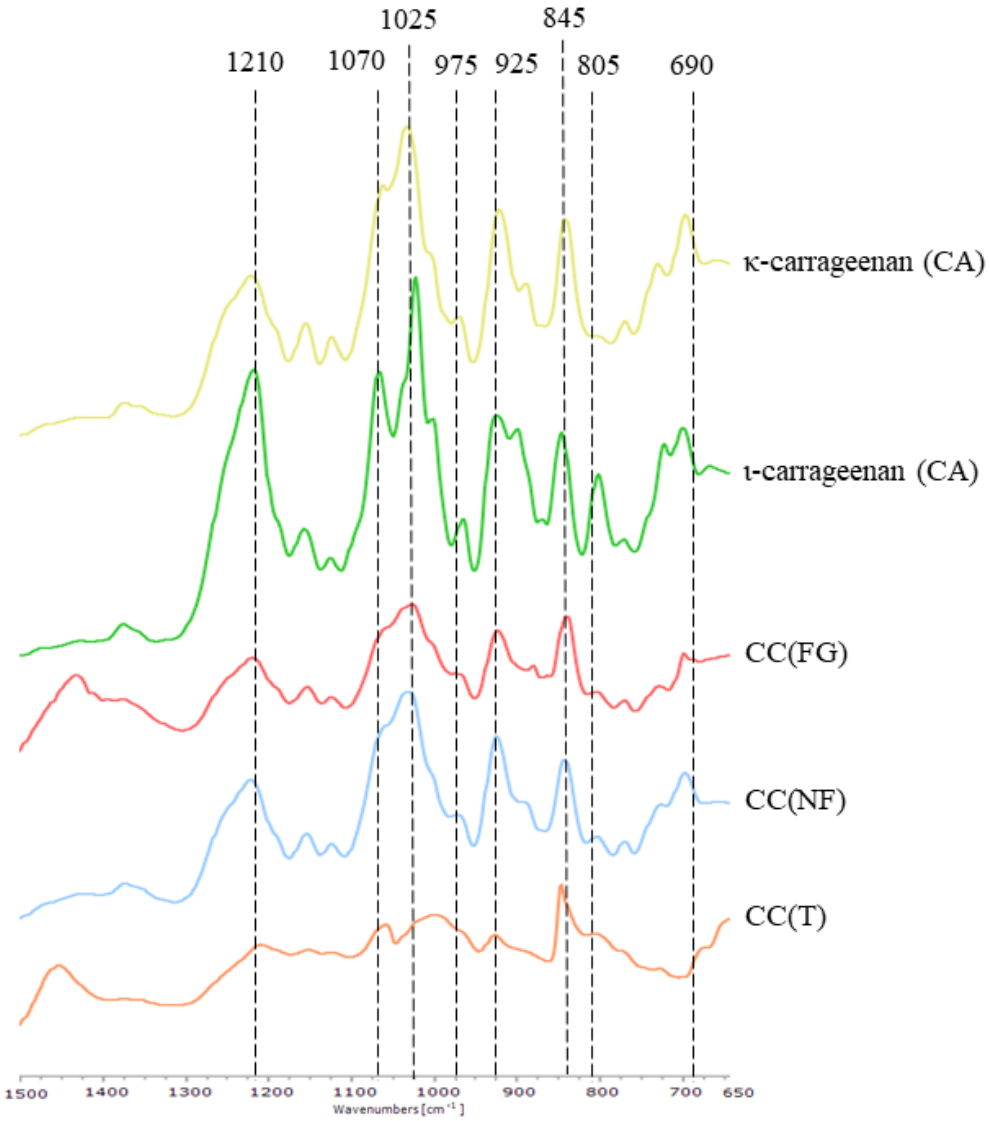

Figure 10 – FTIR-ATR spectra of the red seaweed polysaccharide (carrageenan). CA – commercially available. CC(T) – *C. crispus* (tetrasporophyte); CC(NF) – *C. crispus* (non-fructified thalli); CC(FG) – *C. crispus* (female gametophyte).

Table 8 – FTIR-ATR bands identification and characterization of the red seaweed polysaccharide (carrageenan). nd – not detectable. sh – shoulder. CA – commercially available. CC(T) – *C. crispus* (tetrasporophyte); CC(NF) – *C. crispus* (non-fructified thalli); CC(FG) – *C. crispus* (female gametophyte).

Reference wave number (cm -1)	**Bound**	**Wave number observed (cm -1)**				
		κ-carrageenan (CA)	**ι-carrageenan (CA)**	**CC (FG)**	**CC (NF)**	**CC (T)**
1260-1210	Sulphated esters (S=O)	1222	1219	1220	1222	1209
1100	Sulphated esters (S=O)	sh	sh	nd	nd	nd
1070	C–O of 3,6-anhydrogalactose	1063	1067	sh	sh	1060
1030-1010	C-O and C–C stretching vibrations of pyranose ring	1034	1023	1028	1032	nd
975-970	Galactose	971.3	966.8	972.9	972.7	nd
933-928	C–O of 3,6-anhydrogalactose	922.2	925.2	924.1	925.5	927.8
900-890	C–O–SO3 on C2 of 3,6-anhydrogalactose	890.5	902.4	nd	sh	nd
867	C–O–SO3 on C6 of galactose	nd	sh	nd	nd	nd
845	D-galactose-4-sulfate	842.3	846.5	841	843.3	846.6
830-825	C–O–SO3 on C2 of galactose	nd	nd	nd	nd	nd
820-815	C–O–SO3 on C6 of galactose	nd	nd	nd	nd	nd
805	C–O–SO3 on C2 of 3,6-anhydrogalactose	nd	803.4	805.7	805	805.9
690	3,6- anhydro-L-galactose	697.7	700.9	699.3	698.5	nd

3.3. Seed germination assay

3.3.1. Alginate solutions

The pH of all alginate solutions (**Table 9**) was very similar and close to 7, whereas the electrical conductivity (EC) increased with the concentration (88.30 µS/cm, 177.40 µS/cm and 352 µS/cm, respectively). During the germination assay, the alginate solution with a concentration of 1 mg/mL was very liquid and wasn't absorbed well from the cotton in the petri dishes (**Figure 11d**). This resulted in week seedlings and a dispersal of the aerial and reticular parts.

Table 9 – pH and electrical conductivity (EC) of the alginate solutions used in the seed germination assay of turnip greens . Control – distilled water. SP – *S. polyschides*.

Seaweed	Concentration (mg/mL)	pH	EC (µS/cm)
SP	Control	7.00	1.00
	0.25	6.86	88.30
	0.50	6.84	177.40
	1	6.88	352.00

Measured at room temperature (23.8 ºC).

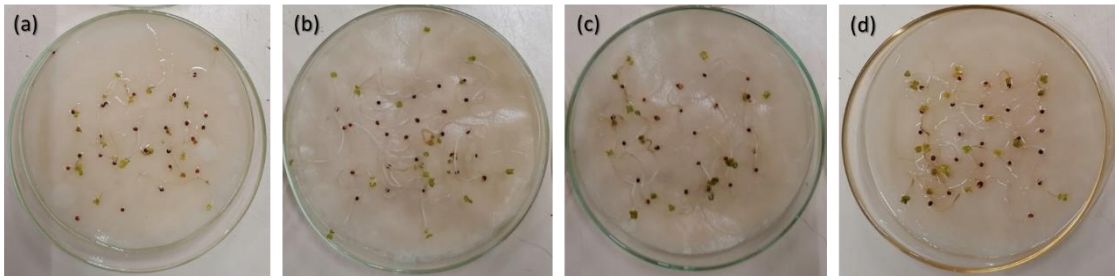

Figure 11 – Photographic record of (a) Control; (b) 0.25 mg/mL solution; (c) 0.50 mg/mL solution; (d) 1 mg/mL solution of alginate from *S. polyschides* in the germination assay of turnip greens .

In **Table 10** is demonstrated the number of germinated seeds observed in each day, the germination percentage and the ratio between the shoot weight and the root weight of the alginate solutions used in the seed germination assay of turnip greens. Regarding the number of germinated seeds, the control solution maintained the same number since day 4, which can indicate that the seeds absorbed all the water needed to germinate since the beginning. The highest germination percentage was observed with the 1 mg/mL alginate solution, and the lowest with the 0.25 mg/mL alginate solution,

with 90.67% and 84%, respectively. Despite the 1 mg/mL alginate solution had the highest germination percentage, it was the solution with the lowest average values regarding the growth parameters, when compared with other alginate solutions (**Figure 12**).

Table 10 – Number of germinated seeds observed in each day, the germination percentage (GP) and the ratio between the shoot weight and the root weight of the alginate solutions used in the seed germination assay of turnip greens . The table presents the average values (n=3). Control – distilled water. SP – *S. polyschides*.

Seaweed	**Concentration (mg/mL)**	**nº germinated seeds (day 4*)**	**nº germinated seeds (day 7*)**	**nº germinated seeds (day 10*)**	**GP (%)**	**Ratio shoot/root weight**
SP	Control	22.00	22.00	22.00	88.00	5.00
	0.25	20.00	21.00	21.00	84.00	3.00
	0.50	21.00	21.33	21.33	85.33	6.17
	1	22.67	22.67	22.67	90.67	4.00

*Day 4: 28/10/2022; Day 7: 31/10/2022; Day 10: 3/11/2022.

In **Figure 12** and **Annex Table 1**, is represented the average values of the growth parameters of the seedlings from the seed germination assay with the alginate solutions, measured after 10 days. The 0.25 mg/mL alginate solution had the highest values at all parameters. Between all alginate solutions, the 0.50 mg/mL concentration presented the most promising parameters, with a similar shoot weight when 0.25 mg/mL was applied, but lower radicular weight and length. Between all concentrations, the ratio between shoot/root weights was higher in seeds treated with 0.50 mg/mL alginate solution (**Table 10** and **Annex Table 2**).

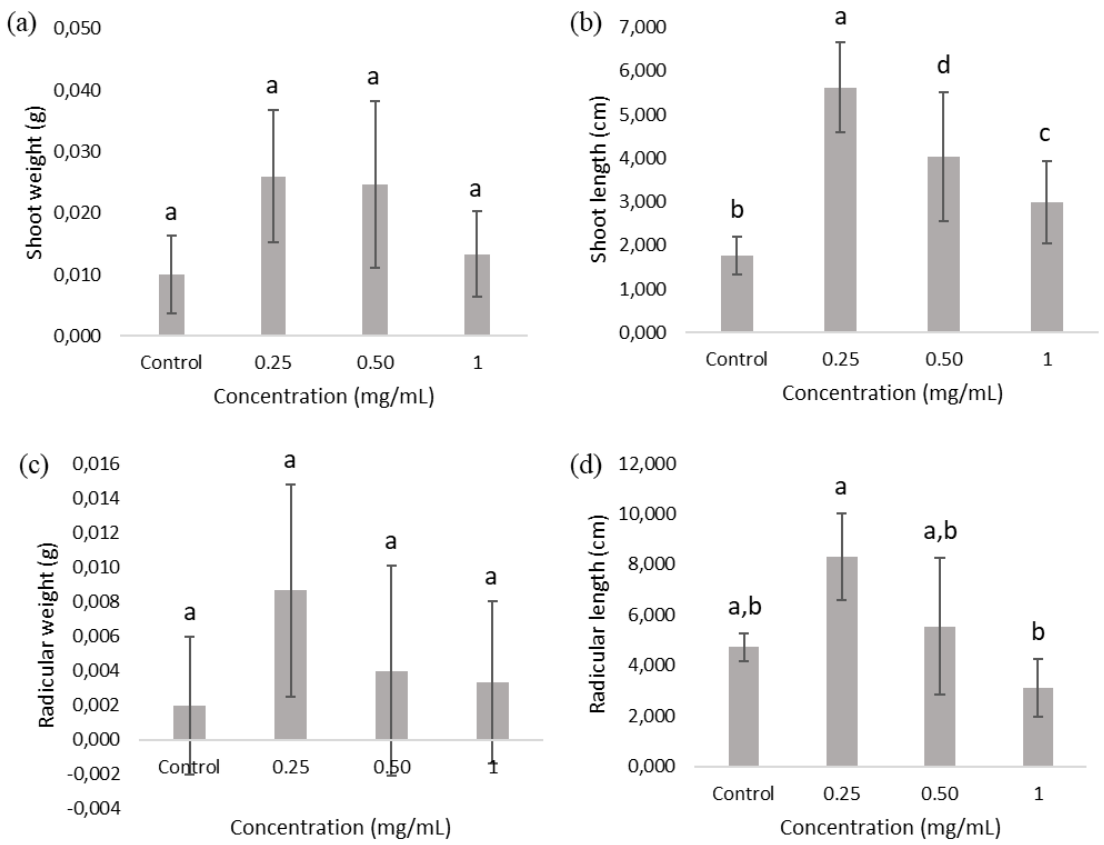

Figure 12 – Growth parameters of the seedlings obtained from the seed germination assay with the alginate solutions, measured after 10 days. (a) Shoot weight; (b) Shoot length; (c) Radicular weight; (d) Radicular length. The graphs present the average values and the standard deviation (n=5). Statistically significant differences found among the different samples are expressed by letters and samples with the same letter do not have statistically significant differences ($p<0.05$). Negative values in y-axis are due to standard deviation calculation. Control – distilled water.

3.3.2. Agar solutions

The pH of all agar solutions (**Table 11**) ranged between 5.52 to 6.07 (control not included), which indicates that the solutions were a little acidic. As good as pH, the electrical conductivity increased with the concentration (19.90 µS/cm, 38.80 µS/cm and 72.40 µS/cm, respectively).

In **Table 12** is demonstrated the number of germinated seeds observed in each day, the germination percentage and the ratio between the shoot weight and the root weight of the agar solutions used in the seed germination assay of turnip greens. The control exhibited the highest germination percentage among all the solutions with 96%. Whereas the 0.50 mg/mL agar solution exhibited the lowest germination with 78.67%.

Table 11 – pH and electrical conductivity (EC) of the agar solutions used in the seed germination assay of turnip greens . Control – distilled water. GG – *G. gracilis*.

Seaweed	Concentration (mg/mL)	pH	EC (µS/cm)
GG	Control	7.00	1.00
	0.25	5.52	19.90
	0.50	5.71	38.80
	1	6.07	72.40

Measured at room temperature (23.8 ºC).

Table 12 – Number of germinated seeds observed in each day, the germination percentage (GP) and the ratio between the shoot weight and the root weight of the agar solutions used in the seed germination assay of turnip greens . The table presents the average values (n=3). Control – distilled water. GG – *G. gracilis*.

Seaweed	Concentration (mg/mL)	nº germinated seeds (day 4*)	nº germinated seeds (day 7*)	nº germinated seeds (day 10*)	GP (%)	Ratio shoot/root weight
GG	Control	20.00	22.00	24.00	96.00	7.00
	0.25	20.33	21.00	21.67	86.67	5.38
	0.50	18.33	19.00	19.67	78.67	8.83
	1	18.67	20.00	21.00	84.00	3.54

*Day 4: 21/10/2022; Day 7: 24/10/2022; Day 10: 27/10/2022.

In **Figure 13** and **Annex Table 1**, is represented the average values of growth parameters of the seedlings from the seed germination assay with the agar solutions, measured after 10 days. Regarding the growth parameters, all solutions exhibit very similar results, nonetheless, excluding the control solution, the 0.25 mg/mL agar solution demonstrated the lowest results in shoot and length weight, whereas the 1 mg/mL agar solution demonstrated the lowest results in radicular length and the highest results in radicular weight. The 0.50 mg/mL agar solution demonstrated the best results in all parameters except in radicular weight, which explains the highest ratio between shoot and root weight (**Table 12** and **Annex Table 2**).

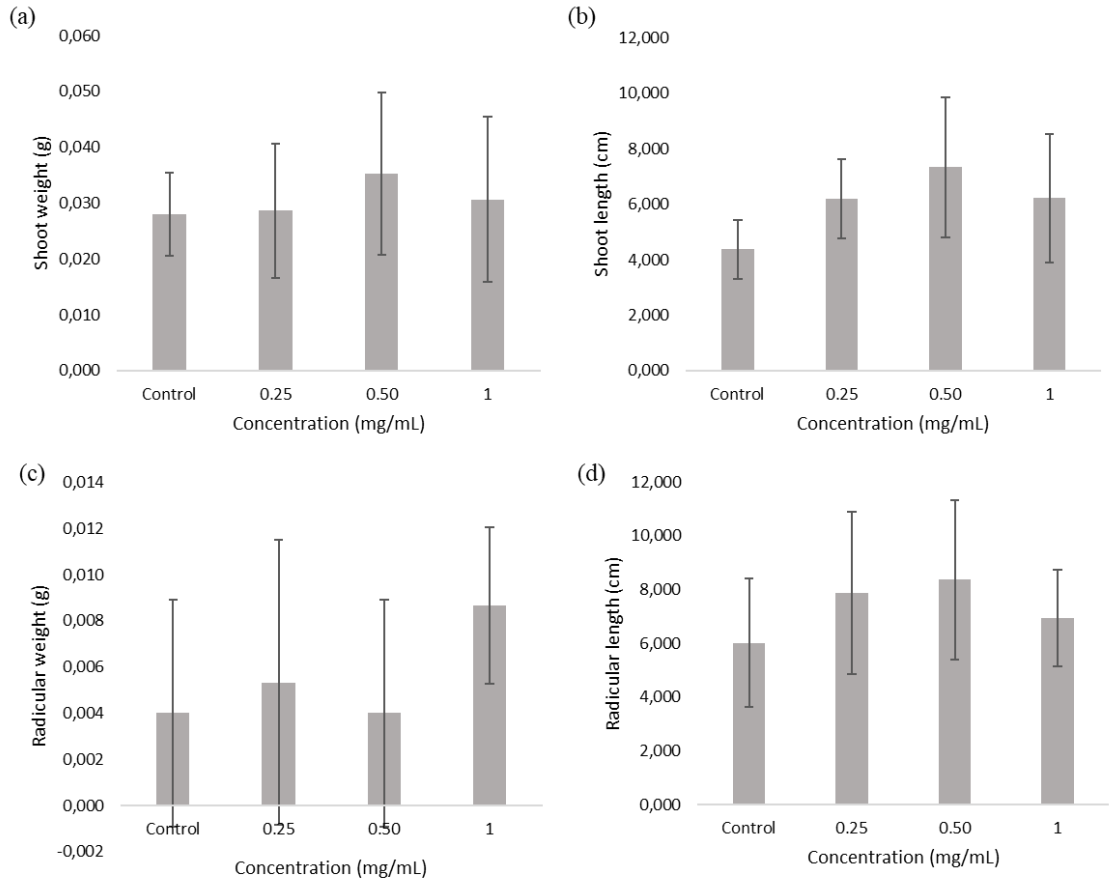

Figure 13 – Growth parameters of the seedlings obtained from the seed germination assay with the agar solutions, measured after 10 days. (a) Shoot weight; (b) Shoot length; (c) Radicular weight; (d) Radicular length. The graphs present the average values and the standard deviation (n=5). There are not statistically significant differences found among the different samples **(**p>0.05**)**. Negative values in y-axis are due to standard deviation calculation. Control – distilled water.

3.3.3. Carrageenan solutions

The pH of all carrageenan solutions (**Table 13**) ranged between 9.02 to 10.08, which indicates that the solutions were alkaline. In all samples, the electrical conductivity increased with the concentration (**Table 13**). The carrageenan solutions of *C. crispus* (tetrasporophyte and non-fructified thalli) had very similar electrical conductivity for the same concentrations, ranging from 60 µS/cm and 260 µS/cm. Whereas the carrageenan solution of *C. crispus* (female gametophyte) had higher electrical conductivity, ranging from 119.60 µS/cm to 452 µS/cm.

Table 13 – pH and electrical conductivity (EC) of the carrageenan solutions used in the seed germination assay of turnip greens. Control – distilled water. CC(T) – *C. crispus* (tetrasporophyte); CC(NF) – *C. crispus* (non-fructified thalli); CC(FG) – *C. crispus* (female gametophyte).

Seaweed	Concentration (mg/mL)	pH	EC (µS/cm)
CC (T)	Control	7.00	1.00
	0.25	9.02	65.50
	0.50	9.23	131.40
	1	9.40	260.00
CC (NF)	Control	7.00	1.00
	0.25	9.29	63.40
	0.50	9.61	132.20
	1	9.77	257.00
CC (FG)	Control	7.00	1.00
	0.25	9.74	119.60
	0.50	9.92	235.00
	1	10.08	452.00

Measured at room temperature (23.8 ºC).

In **Table 14** is demonstrated the number of germinated seeds observed in each day, the germination percentage and the ratio between the shoot weight and the root weight of the carrageenan solutions used in the seed germination assay of turnip greens. Overall, the generation sample with the highest germination percentage in all solutions was the female gametophyte of *C. crispus*. In the samples of *C. crispus* (tetrasporophyte), the solution with the highest and lowest germination percentage was the 0.25 mg/mL with 94.67% and 0.50 mg/mL with 85.33%, respectively. In the samples of *C. crispus* (non-fructified thalli), all solutions had a 92% of germination except the 0.25 mg/mL solution. In the samples of *C. crispus* (female gametophyte), the control solution had a 100% germination, followed by the 0.50 mg/mL solution with 98.67%.

When comparing all polysaccharides' solutions of this study, the solutions of carrageenan extracted from the female gametophyte of *C. crispus* had the most germinated seeds in the first four days of incubation, with around 23 seeds germinated.

Table 14 – Number of germinated seeds observed in each day, the germination percentage (GP) and the ratio between the shoot weight and the root weight of the carrageenan solutions used in the seed germination assay of turnip greens . The table presents the average values (n=3). Control – distilled water. CC(T) – *C. crispus* (tetrasporophyte); CC(NF) – *C. crispus* (non-fructified thalli); CC(FG) – *C. crispus* (female gametophyte).

Seaweed	Concentration (mg/mL)	nº germinated seeds (day 4*)	nº germinated seeds (day 7*)	nº germinated seeds (day 10*)	GP (%)	Ratio shoot/root weight
CC(T)	Control	21.00	21.00	23.00	92.00	1.80
	0.25	20.67	22.33	23.67	94.67	7.14
	0.50	18.67	19.00	21.33	85.33	5.27
	1	21.33	21.33	22.33	89.33	4.67
CC(NF)	Control	21.00	23.00	23.00	92.00	3.33
	0.25	20.67	20.67	21.00	84.00	3.92
	0.50	22.33	22.33	23.00	92.00	5.00
	1	19.67	20.67	23.00	92.00	3.08
CC(FG)	Control	23.00	24.00	25.00	100	3.00
	0.25	23.33	23.33	24.00	96.00	5.57
	0.50	23.00	23.33	24.67	98.67	7.00
	1	23.67	24.33	24.33	97.33	5.88

*For CC(T) and CC(NF): Day 4: 21/10/2022; Day 7: 24/10/2022; Day 10: 27/10/2022. For CC(FG): Day 4: 28/10/2022; Day 7: 31/10/2022; Day 10: 3/11/2022.

In **Figure 14** and **Annex Table 1**, is represented the average values of growth parameters of the seedlings from the seed germination assay with the carrageenan solutions of *C. crispus* (tetrasporophyte), measured after 10 days. Regarding the growth parameters, all solutions exhibit very similar results, nonetheless, the 0.50 mg/mL carrageenan solution demonstrated the highest results in all parameters except the radicular weight, where the control solution had the highest. However, the ratio between shoot and root weight (**Table 14** and **Annex Table 2**) was much greater in the 0.25 mg/mL solution than the other concentrations' solutions.

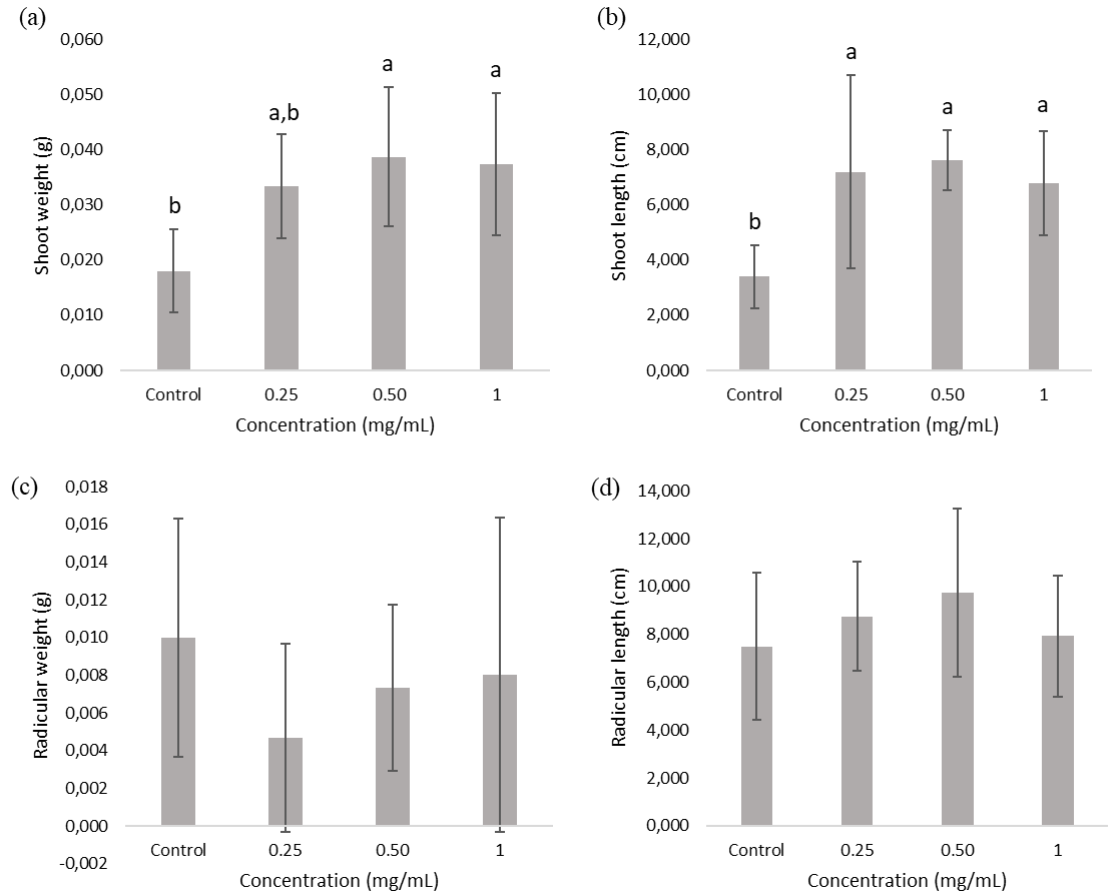

Figure 14 – Growth parameters of the seedlings obtained from the seed germination assay with the carrageenan from the tetrasporophyte of *C. crispus* solution, measured after 10 days. (a) Shoot weight; (b) Shoot length; (c) Radicular weight; (d) Radicular length. The graphs present the average values and the standard deviation (n=5). Statistically significant differences found among the different samples are expressed by letters ($p<0.05$). Negative values in y-axis are due to standard deviation calculation. Control – distilled water.

In **Figure 15** and **Annex Table 1**, is represented the average values of growth parameters of the seedlings from the seed germination assay with the carrageenan solutions of *C. crispus* (non-fructified thalli), measured after 10 days. Regarding the growth parameters, the 1 mg/mL carrageenan solution demonstrated the highest results in all parameters except the radicular weight, where it was identical to the 0.25 mg/mL solution. However, the ratio between shoot and root weight (**Table 14** and **Annex Table 2)** was much greater in the 0.50 mg/mL solution than the other concentrations' solutions.

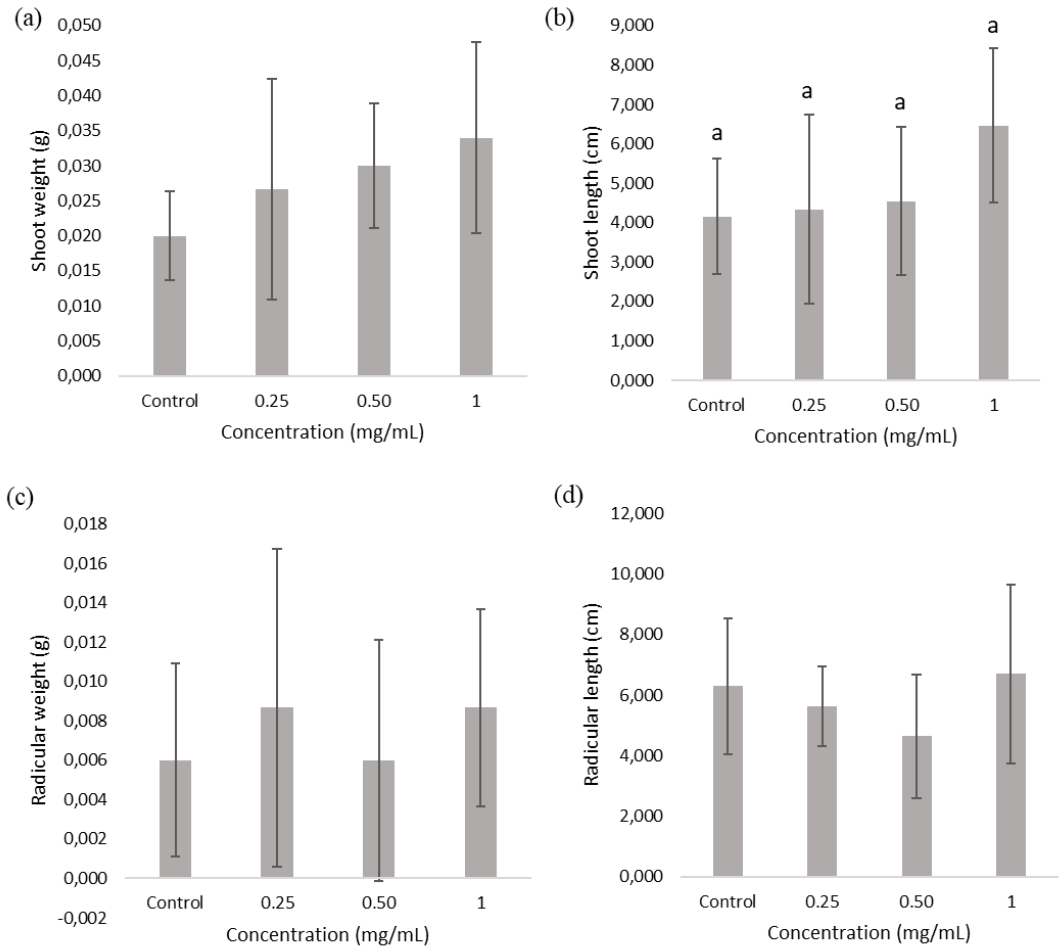

Figure 15 – Growth parameters of the seedlings obtained from the seed germination assay with the carrageenan from the non-fructified thalli of *C. crispus* solution, measured after 10 days. (a) Shoot weight; (b) Shoot length; (c) Radicular weight; (d) Radicular length. The graphs present the average values and the standard deviation (n=5). Samples with the same letter do not have statistically significant differences ($p<0.05$). Negative values in y-axis are due to standard deviation calculation. Control – distilled water.

In **Figure 16** and **Annex Table 1**, is represented the average values of growth parameters of the seedlings from the seed germination assay with the carrageenan solutions of *C. crispus* (female gametophyte), measured after 10 days. Regarding the growth parameters, the highest shoot weight was observed with the 1 mg/mL solution, the highest shoot length was observed with the 0.50 mg/mL solution, the highest radicular weight was observed with the control solution and the highest radicular length with the 0.25 mg/mL solution. The ratio between shoot and root weight (**Table 14** and **Annex Table 2**) was greater in the 0.50 mg/mL solution than the other.

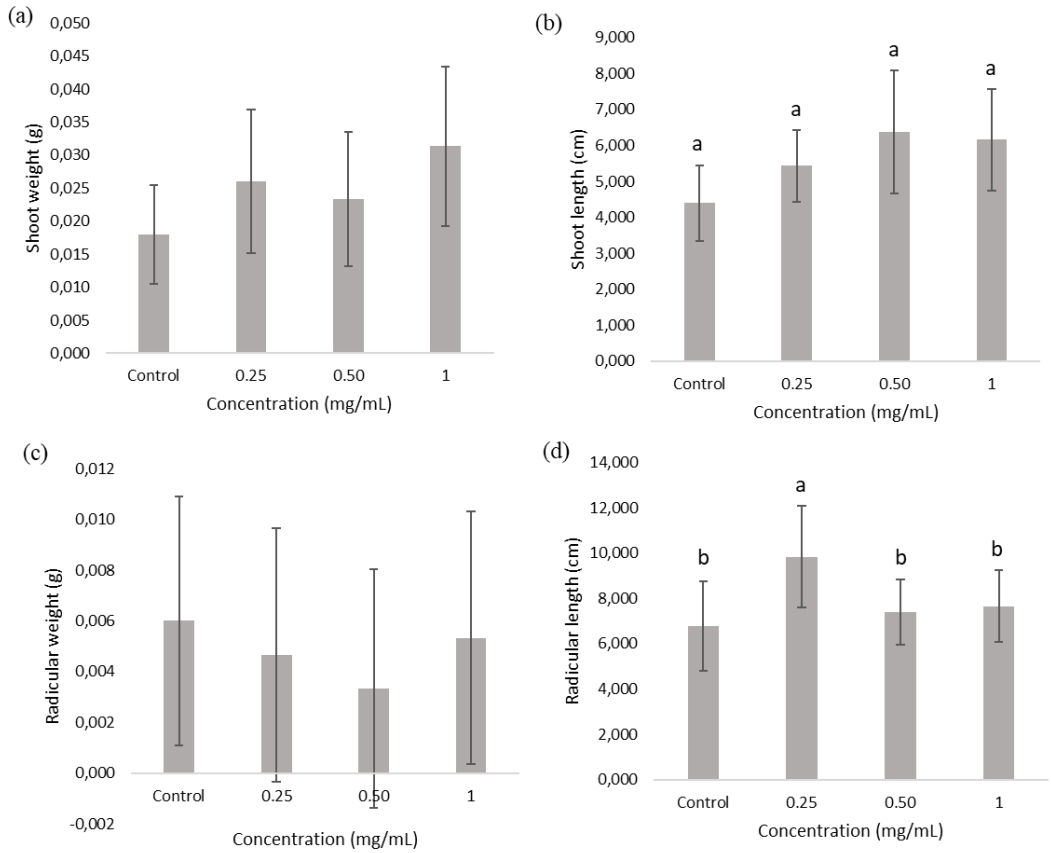

Figure 16 - Growth parameters of the seedlings obtained from the seed germination assay with the carrageenan from the female gametophyte of *C. crispus* solution, measured after 10 days. (a) Shoot weight; (b) Shoot length; (c) Radicular weight; (d) Radicular length. The graphs present the average values and the standard deviation (n=5). Statistically significant differences found among the different samples are expressed by letters and samples with the same letter do not have statistically significant differences ($p<0.05$). Negative values in y-axis are due to standard deviation calculation. Control – distilled water.

3.4. Biostimulant and biofertilizer assay in *Brassica napus* L.

3.4.1. Biochemical characterization of the treatments applied

Following the seed germination assay, the concentration of each polysaccharide's solution selected to be used in this assay is displayed in **Table 15**, as well as their pH, electrical conductivity (EC) and viscosity. The positive control was the treatment with the most neutral pH, with 7.30 **(Table 15)**. The negative control, the alginate solution and the agar solution presented an acid pH of 5.86, 3.70 and 5.83, respectively. The pH of the carrageenan solutions was between 9 and 10, which is considered as alkaline. Regarding the EC, the positive control had the highest values among all treatments, with 1685 µS/cm, whereas the agar solution had the lowest, with 73 µS/cm. Between all carrageenan solutions, EC increased accompanying the pH rise. The viscosity was the highest in the tetrasporophyte solution (the lowest pH and EC). Overall, excluding the negative control, all carrageenan solutions presented the highest viscosity values (10.80 mPa.s for the tetrasporophyte solution and 9 mPa.s for the non-fructified thalli and female gametophyte solutions), and the lowest alginate solution (3.60 mPa.s).

Table 15 – pH, electrical conductivity (EC) and viscosity values of the treatments used in the biostimulant and biofertilizer assay in potted turnip. Negative control – tap water. Positive control – "Profertil".

Treatment	Concentration (mg/mL)	pH	EC (µS/cm)	Viscosity (mPa.s)
Negative control	-	5.86	302	1.00
Positive control	1.5% (v/v)	7.30	1685	5.10
Alginate solution	0.50	3.70	109	3.60
Agar solution	0.50	5.83	73	8.40
Carrageenan (tetrasporophyte) solution	0.25	9.34	100	10.80
Carrageenan (non-fructified thalli) solution	0.50	9.56	184	9.00
Carrageenan (female gametophyte) solution	0.50	9.86	191	9.00

Measured at room temperature (20-22 ºC).

3.4.2. Turnip' morphological parameters

All the potted turnip from each treatment had a similar growth rate **(Figure 17).**

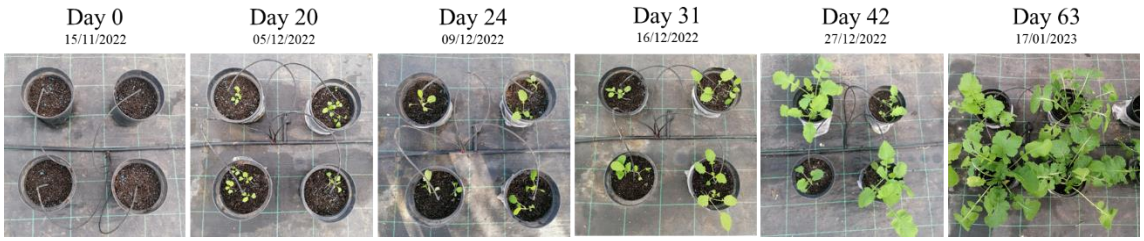

Figure 17 – Photographic record throughout the biostimulant and biofertilizer assay in potted turnip leaves throughout 63 days (from sowing to plant harvesting) treated with an alginate solution of *S. polyschides*.

However, it was possible to observe differences among the treatments (**Figure 19 and Figure 20**). By the end of the experiment (day 63), plants treated with the negative (NC) and positive control (PC) had very reduced size when compared to the other treatments. The samples that presented the most robust turnip leaves development were the ones treated with the carrageenan solutions of *C. crispus* (tetrasporophyte, non-fructified thalli and female gametophyte, CC(T), CC(NF) and CC(FG), respectively). Additionally, at day 42 (the day of the second application), the samples treated with NC and PC started to exhibit various injuries (as holes) on the leaves (**Figure 18a and Figure 18c**). The same was not observed in the other treatments until day 63, and even the number of holes was very reduced when compared to the samples treated with the control treatments. This herbivory activity was caused by an *Agrotis* spp. larvae **(Figure 18b)**.

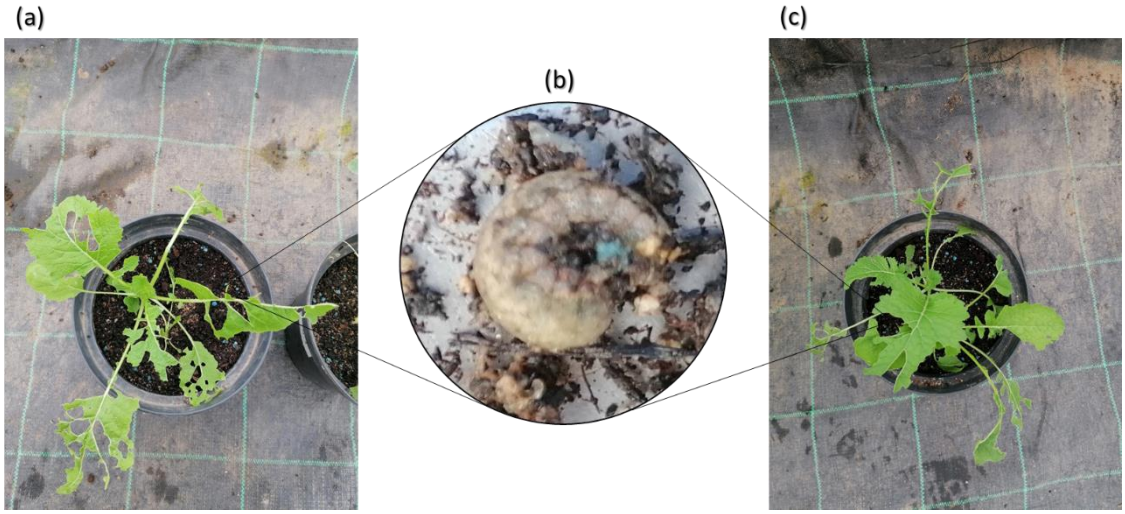

Figure 18 – Photographic record of herbivory activity by (b) *Agrotis* larvae, seen in potted turnip leaves treated with (a) Negative control (tap water) and (c) Positive control ("Profertil"), after 63 days.

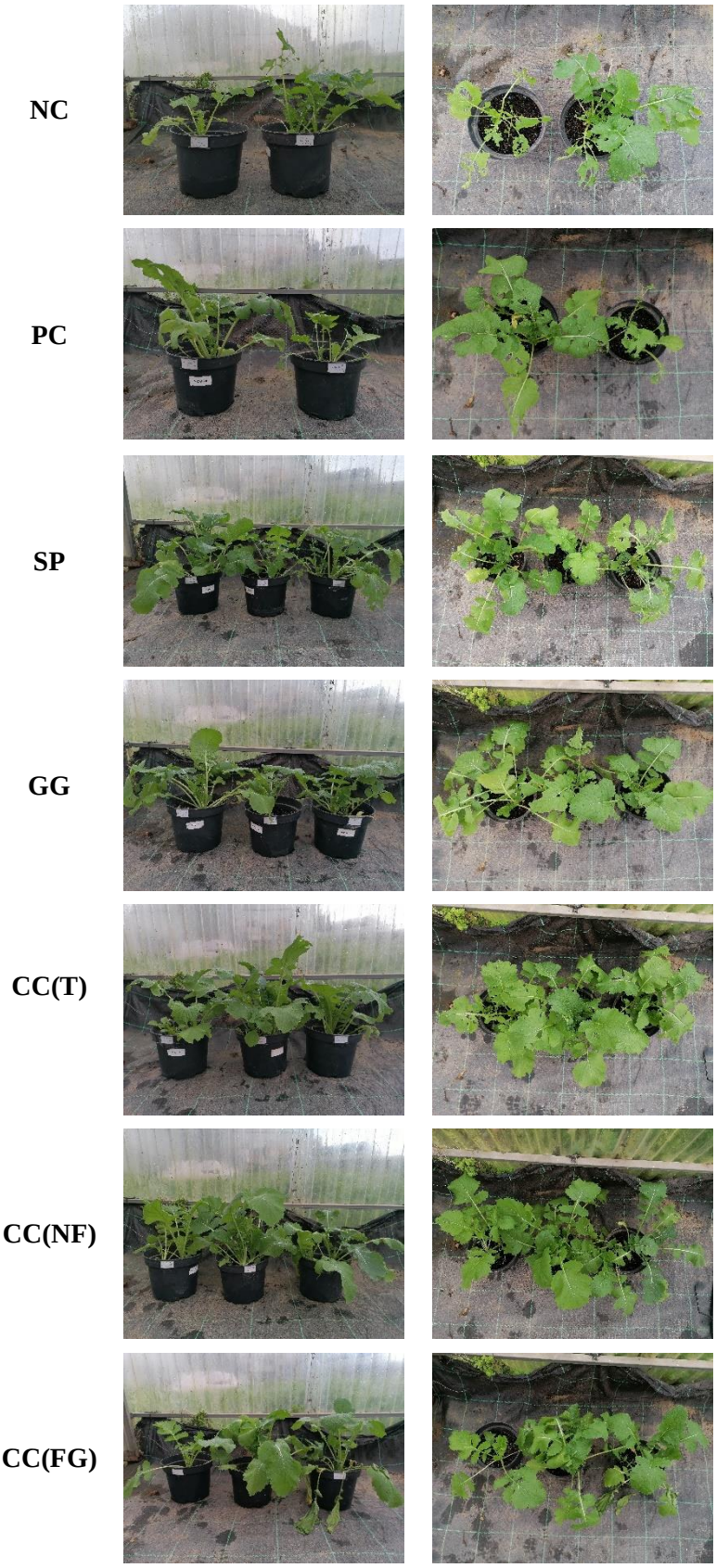

Figure 19 – Photographic record of the potted turnip treated with each polysaccharide solution, after 63 days. NC – negative control; PC – positive control; SP – *S. polyschides*; GG – *G. gracilis*; CC(T) – *C. crispus* (tetrasporophyte); CC(NF) – *C. crispus* (non-fructified thalli); CC(FG) – *C. crispus* (female gametophyte).

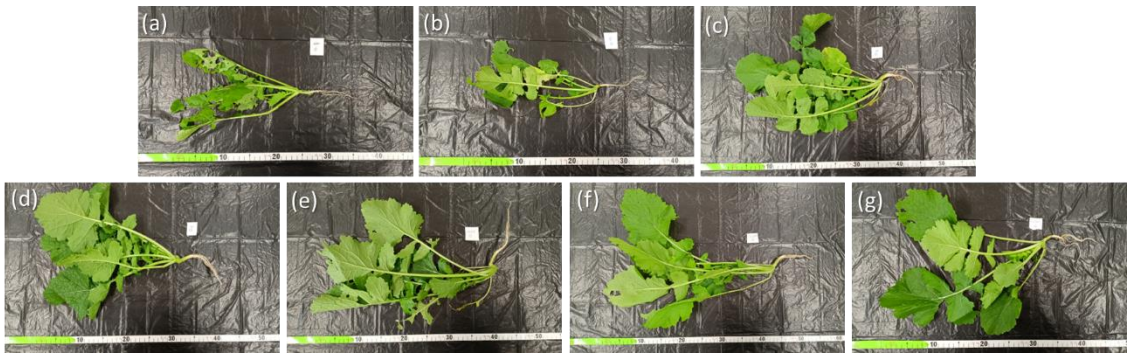

Figure 20 – Photographic record of the turnip leaves obtained from each treatment (a) negative control, (b) positive control, (c) alginate solution of *S. polyschides*, (d) agar solution of *G. gracilis*, (e) carrageenan solution of *C. crispus* (tetrasporophyte), (f) carrageenan solution of *C. crispus* (non-fructified thalli) and (f) carrageenan solution of *C. crispus* (female gametophyte).

After the harvesting of all turnip samples from each treatment, the growth parameters were evaluated (leaf weight and length, root weight and length) (**Figure 21** and **Annex Table 3**). The ratios of the growth parameters were also calculated (**Table 16** and **Annex Table 4**).

In **Figure 21**, are presented the average values of the aerial part weight (**Figure 21a**), leaf length (**Figure 21b**), root weight (**Figure 21c**) and root length (**Figure 21d**) of the fresh turnip from each treatment.

After 63 days, leaf weights, root lengths and root weights (**Figure 21a**, **Figure 21c** and **Figure 21d)**, were not statistically significant ($p>0.05$). Despite this, there was a clear difference between the turnip plants obtained from both control treatments and the polysaccharides treatments. The most robust samples (with the best leaf weight and length) were observed in plants treated with the carrageenan solutions of *C. crispus* (CC(T), CC(NF) and CC(FG)). The samples with the lowest leaf weight (**Figure 21a**) and length (**Figure 21b**) were the control ones, particularly, the PC, with 15.91 g and 26.80 cm, respectively. When compared the turnip plants treated only with the polysaccharides' solutions, turnips that exhibited the best leaf weights were ones treated with the CC(T) solution (40.80 g). The turnips that presented the worst leaf weights were treated with the GG solution (29.53 g). The turnip samples that exhibited the best leaf lengths were treated with the CC(FG) solution (39.98 cm). The worst leaf length was observed in turnip samples treated with the SP solution (35.36 cm).

The heaviest roots were observed in plants treated with the CC(FG) solution (1.06 g), GG solution (0.99 g) and SP solution (0.98 g) (**Figure 21c**). The samples with the

lowest root weight were treated with the control treatments, 0.61 g for the PC and 0.54 g for the NC. On other hand, the samples with the highest root length were treated with the CC(FG) solution (15.80 cm), the CC(NF) solution (15.77 cm) and CC(T) solution (13.76 cm). The lowest root lengths were observed in plants treated with SP (11.72 cm) and GG solutions (11.84 cm), respectively.

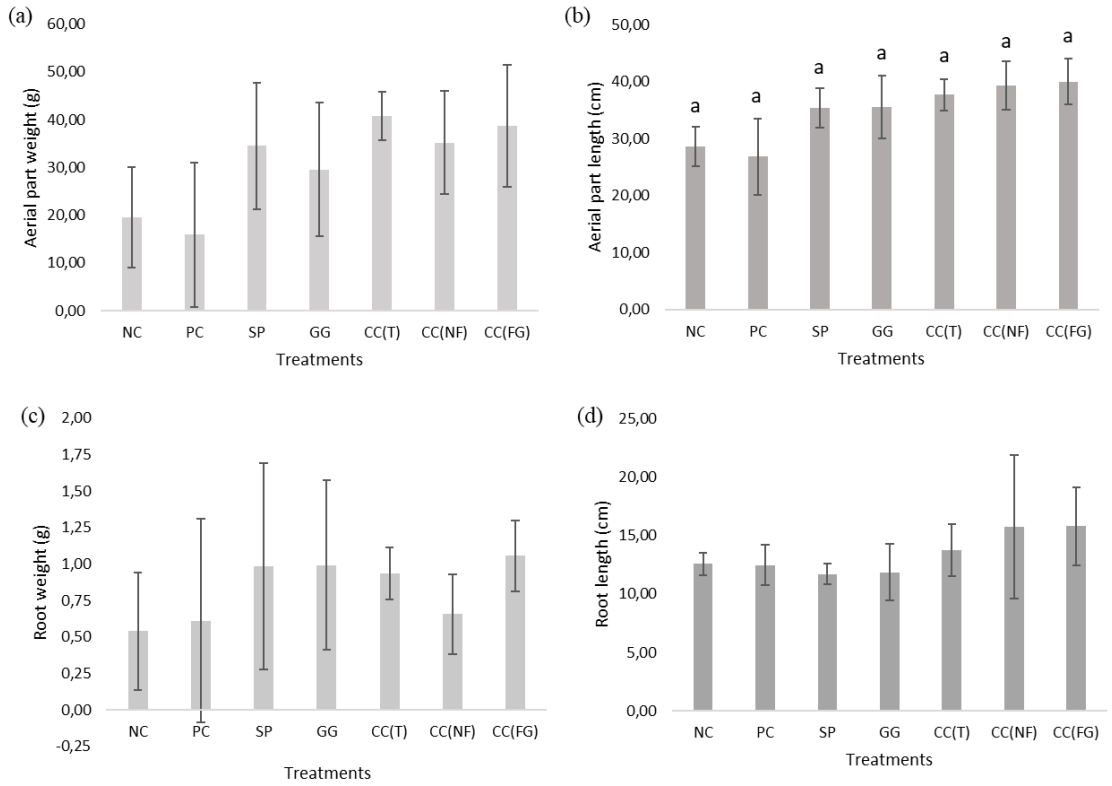

Figure 21 - (a) Aerial part weight, (b) Aerial part length, (c) Root weight and (d) Root length of the fresh turnip leaves from each treatment of the biostimulant and biofertilizer assay, measured after 63 days. The graphs present the average values and the standard deviation (n=3). Samples with the same letter are not statistically different ($p<0.05$). Negative values in y-axis are due to standard deviation calculation. NC – negative control; PC – positive control; SP – *S. polyschides*; GG – *G. gracilis*; CC(T) – *C. crispus* (tetrasporophyte); CC(NF) – *C. crispus* (non-fructified thalli); CC(FG) – *C. crispus* (female gametophyte).

Regarding the leaf number (**Figure 22**), plants with the lowest leaf number were treated with the control solutions, with ± 6 leaves for the NC and ± 7 leaves for the PC. The plants with the highest number of leaves were treated with the CC(T), CC(NF) and CC(FG), with ± 9 leaves.

Regarding the ratios between the growth parameters (**Table 16**), the ratio aerial part weight: root weight was higher in CC(NF) (53.54), CC(T) (43.69) and CC(FG)

(36.62) and lower in PC (26.09) and GG (29.77). Furthermore, the NC and PC had the lowest ratios of aerial part length: root length, with 2.28 and 2.15, respectively.

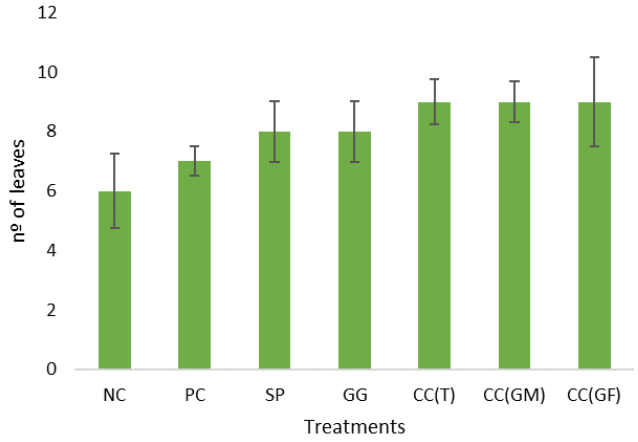

Figure 22 – Number of leaves of the fresh turnip leaves from each treatment of the biostimulant and biofertilizer assay, measured after 63 days. The graphs present the average values and the standard deviation (n=3). There are not statistically significant differences found among the different samples **(**$p<0.05$**)**. NC – negative control; PC – positive control; SP – *S. polyschides*; GG – *G. gracilis*; CC(T) – *C. crispus* (tetrasporophyte); CC(NF) – *C. crispus* (non-fructified thalli); CC(FG) – *C. crispus* (female gametophyte).

Table 16 – Ratios between the aerial part (AP) and root (R) of the fresh turnip from each treatment of the biostimulant and biofertilizer assay, measured after 63 days. NC – negative control; PC – positive control; SP – *S. polyschides*; GG – *G. gracilis*; CC(T) – *C. crispus* (tetrasporophyte); CC(NF) – *C. crispus* (non-fructified thalli); CC(FG) – *C. crispus* (female gametophyte).

Sample	AP length/weight	R length/weight	AP weight/R weight	AP length/R length
NC	1.46	23.27	36.20	2.28
PC	1.68	20.45	26.09	2.15
SP	1.03	11.91	35.04	3.02
GG	1.20	11.94	29.77	3.00
CC(T)	0.92	14.73	43.69	2.74
CC(NF)	1.12	24.01	53.54	2.49
CC(FG)	1.03	14.94	36.62	2.53

3.4.3. Turnip' physiological and biochemical characterization

3.4.3.1. Mineral and trace element characterization

The mineral and trace element characterization of the turnip' edible section (**Table 17**) is critical to understand how the treatments applied to the plants affected their nutritional quality. There were not statistically significant differences among the treatment groups ($p>0.05$).

Regarding the nitrogen (N) content, all treatments had slightly higher percentage when compared to the literature value (3.23%), and the NC had the greatest value among all treatments (5.68%). The NC also had the highest percentage of protein content (35.50%). However, contrary to what happened with the N values, besides the NC, all the other treatments had a similar value compared with the literature (33.33%), ranging from 30.47% with CC(FG), to 33.56% with CC(T). The phosphorus (P) content was slightly higher than cited in the literature (0.75%) in all treatment groups, except in the plants treated with GG (0.73%) and CC(NF) (0.75%) solutions. The calcium (Ca) content was lower in all treatment groups when compared with one from the literature (1.67%). The sodium (Na) content was lower in all treatment groups when compared to the literature (0.67%), except in plants treated with CC(NF), where the Na percentage was higher than all the other treatments (0.90%). Magnesium (Mg) and potassium (K) contents were higher in all treatment groups than cited in the literature (0.17% and 5%, respectively). Regarding the trace elements, zinc (Zn), iron (Fe) and manganese (Mn), excluding copper (Cu), there was a considerable difference between their content in plants treated with NC and the other treatments. For Zn and Mn, all values were slightly lower than found in the literature (87.40% and 98.70%, respectively), except for the NC, that had 118.15% of Zn and 119.75% of Mn. Overall, the NC exhibited the highest values in all mineral and trace elements. It is to consider that there are no published values for Cu and Fe contents.

Table 17 – Mineral and trace element characterization of the turnip leaves within each treatment. The results are expressed in mean ± standard deviation (n=2, Dry weight basis). NI – No Information found in the literature. NC – negative control. PC – positive control. SP – *S. polyschides*; GG – *G. gracilis*; CC(T) – *C. crispus* (tetrasporophyte); CC(NF) – *C. crispus* (non-fructified thalli); CC(FG) – *C. crispus* (female gametophyte).

Treatments	NC	PC	SP	GG	CC(T)	CC(NF)	CC(FG)	Literature values	Reference
Dry matter (%)	5.30 ± 0.00	5.34 ± 0.01	3.42 ± 2.31	7.19 ± 1.68	5.80 ± 0.04	6.66 ± 0.05	6.77 ± 0.43	6.00	PortFIR - INSA
Ashes (%)	22.74 ± 0.02	20.45 ± 0.07	19.52 ± 0.02	19.36 ± 0.28	20.23 ± 0.05	19.47 ± 0.07	18.48 ± 0.10	13.50	PortFIR - INSA
N (%)	5.68 ± 0.05	5.27 ± 0.12	5.23 ± 0.06	4.86 ± 0.01	5.37 ± 0.03	5.16 ± 0.03	4.88 ± 0.05	3.23	Cornforth et al. (1978)
Protein (%)	35.50 ± 0.31	32.91 ± 0.72	32.66 ± 0.34	30.34 ± 0.03	33.56 ± 0.19	32.25 ± 0.19	30.47 ± 0.34	33.33	PortFIR - INSA
P (%)	0.84 ± 0.00	0.87 ± 0.01	0.81 ± 0.01	0.73 ± 0.02	0.80 ± 0.00	0.75 ± 0.01	0.76 ± 0.00	0.75	PortFIR - INSA
Ca (%)	1.46 ± 0.01	1.33 ± 0.01	1.28 ± 0.20	1.23 ± 0.05	1.29 ± 0.00	1.23 ± 0.03	1.20 ± 0.02	1.67	PortFIR - INSA
Mg (%)	0.30 ± 0.01	0.27 ± 0.00	0.23 ± 0.01	0.24 ± 0.00	0.26 ± 0.01	0.28 ± 0.01	0.27 ± 0.01	0.17	PortFIR - INSA
K (%)	8.58 ± 0.16	7.31 ± 0.12	8.23 ± 0.17	7.43 ± 0.29	8.17 ± 0.07	6.88 ± 0.02	7.24 ± 0.17	5.00	PortFIR - INSA
Na (%)	0.39 ± 0.03	0.41 ± 0.00	0.44 ± 0.00	0.34 ± 0.03	0.39 ± 0.10	0.90 ± 0.52	0.36 ± 0.02	0.67	PortFIR - INSA
Cu (mg/kg)	35.25 ± 0.15	36.80 ± 0.50	33.25 ± 0.45	34.70 ± 0.20	35.50 ± 0.80	35.40 ± 1.00	38.00 ± 0.30	NI	NI
Zn (mg/kg)	118.15 ± 1.25	81.00 ± 1.60	77.05 ± 0.15	77.75 ± 1.15	81.10 ± 1.50	77.25 ± 0.15	81.85 ± 0.65	87.40	Cornforth et al. (1978)
Fe (mg/kg)	149.40 ± 1.30	99.35 ± 0.25	93.70 ± 6.80	96.75 ± 0.75	91.50 ± 3.20	94.95 ± 2.45	91.25 ± 1.35	NI	NI
Mn (mg/kg)	119.75 ± 2.15	58.80 ± 2.60	89.50 ± 0.20	78.15 ± 1.25	92.90 ± 2.30	93.25 ± 1.45	71.90± 1.40	98.70	Cornforth et al. (1978)

3.4.3.2. Turnip biochemical characterization

The FTIR-ATR spectra in the range 4000 to 400 cm^{-1} of the turnip within each treatment are given in **Figure 23** and **Table 18**. The spectra of the different treatments are aligned in order of peaks' intensity (from highest to lowest). The obtained spectra was reviewed with bibliographic support (Canteri et al., 2019). All the spectra (**Figure 23**) had similar peaks, except the peak assigned to lignin and phenolic backbone at 1520 cm^{-1}, only present in the negative control spectra. The characteristic peaks of cellulose were present in all spectra around 3280 cm^{-1} and 2921 cm^{-1}. The bands corresponding to pectin's with ester, free carboxyl groups, cellulose and xyloglucan, and proteins, were present in all spectra around 1736 cm^{-1}, 1620 cm^{-1}, 1352-1377 cm^{-1} and 1239 cm^{-1}. The peak with the highest intensity in all spectra was assigned to polysaccharides, sugars and pectin's, at 1020 cm^{-1}. The peak around 825 cm^{-1} was not assigned to any specific bond but had a significant intensity to be mentioned. None of the spectra had a significant peak at 770 cm^{-1} band, corresponding to phenyl groups.

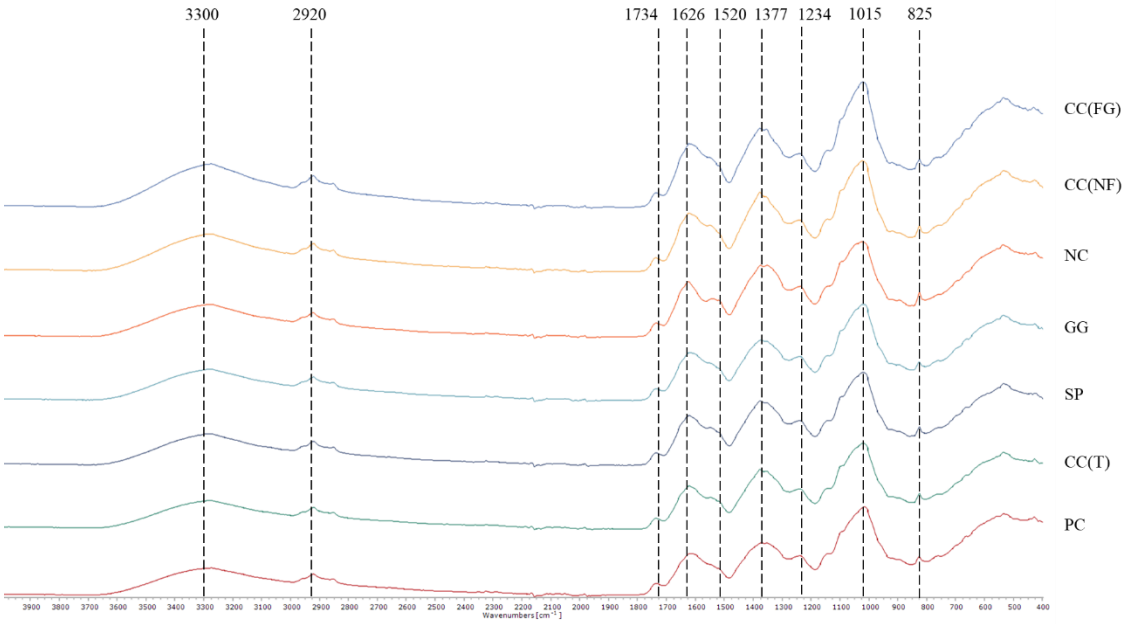

Figure 23 – FTIR-ATR spectra of the turnip leaves within each treatment (Dry basis). NC – negative control. PC – positive control. SP – *S. polyschides*; GG – *G. gracilis*; CC(T) – *C. crispus* (tetrasporophyte); CC(NF) – *C. crispus* (non-fructified thalli); CC(FG) – *C. crispus* (female gametophyte).

Table 18 – FTIR-ATR bands identification and characterization of the turnip within each treatment (Dry basis). nd – not detectable. sh – shoulder. NI – No Information found in the literature. NC – negative control. PC – positive control. SP – *S. polyschides*; GG – *G. gracilis.*; CC(T) – *C. crispus* (tetrasporophyte); CC(NF) – *C. crispus* (non-fructified thalli); CC(FG) – *C. crispus* (female gametophyte).

Reference wave number (cm -1)	**Bound**	**Wave number observed (cm -1)**						
		CC (FG)	**CC (NF)**	**NC**	**GG**	**SP**	**CC (T)**	**PC**
3334	Cellulose	sh	sh	sh	sh	3286	3278	3274
2917	Cellulose	2921	2921	2921	2921	2921	2920	2921
1734	Pectins with ester	1736	1736	1735	1736	1736	1736	1736
1626	Free carboxyl groups	1620	1621	1624	1619	1622	1622	1617
1520	Lignin and phenolic backbone	sh	sh	1540	sh	sh	sh	sh
1371-1314	Cellulose and xyloglucan	1377	1377	1351	1375	1376	1376	1352
1234	Proteins	1240	1240	1238	1239	1239	1239	1238
1015	Polysaccharides, sugars and pectins	1021	1021	1023	1019	1020	1019	1016
825	NI	825.3	825	824.8	825.5	825.2	825.1	825.6
770	Phenyl groups	nd	nd	nd	nd	nd	nd	nd

3.4.3.3. Pigment content

In **Figure 24** is represented a TLC of the methanolic extracts from each treatment sample of turnip. This chromatography separates the different compounds according to their molecular weight. The solvent runs upwards, from non-polar compounds (origin of the pigments) to polar compounds (solvent front). The retention factor (Rf) is used to compare and help identify compounds. The Rf values observed in the different samples are demonstrated in **Table 19**, as well as the comparing Rf values in literature and the assigned pigment. From the absorbance of each sample in the wavelength corresponding to the pigments (chlorophyll *a*, chlorophyll *b*, anthocyanins and carotenoids), it was possible to quantify them (**Table 20** and **Annex Table 5**). Comparing the usual order of the pigments in a TLC and their characteristic colors, the pigments were assigned to each number as seen in **Table 19**.

Samples from CC(T), CC(NF) and CC(FG) moved more than the other samples **(Figure 24)**, therefore had generally higher Rf values (**Table 19**). Additionally, the

pigment marked as “3” in **Figure 24**, corresponding to neoxanthin (**Table 19**), did not appear in the TLC of the NC and PC. On the other hand, the pigment marked as “10”, only appeared in the TLC of the NC, PC, SP and GG. The pigment marked as “8” in **Figure 24**, corresponding to pheophytin *b* (**Table 19**), was very difficult to identify clearly because of its lighter color. It was almost absent in the TLC of the PC, GG and CC(FG). There was not any published information regarding pigments 1, 2 6 and 10.

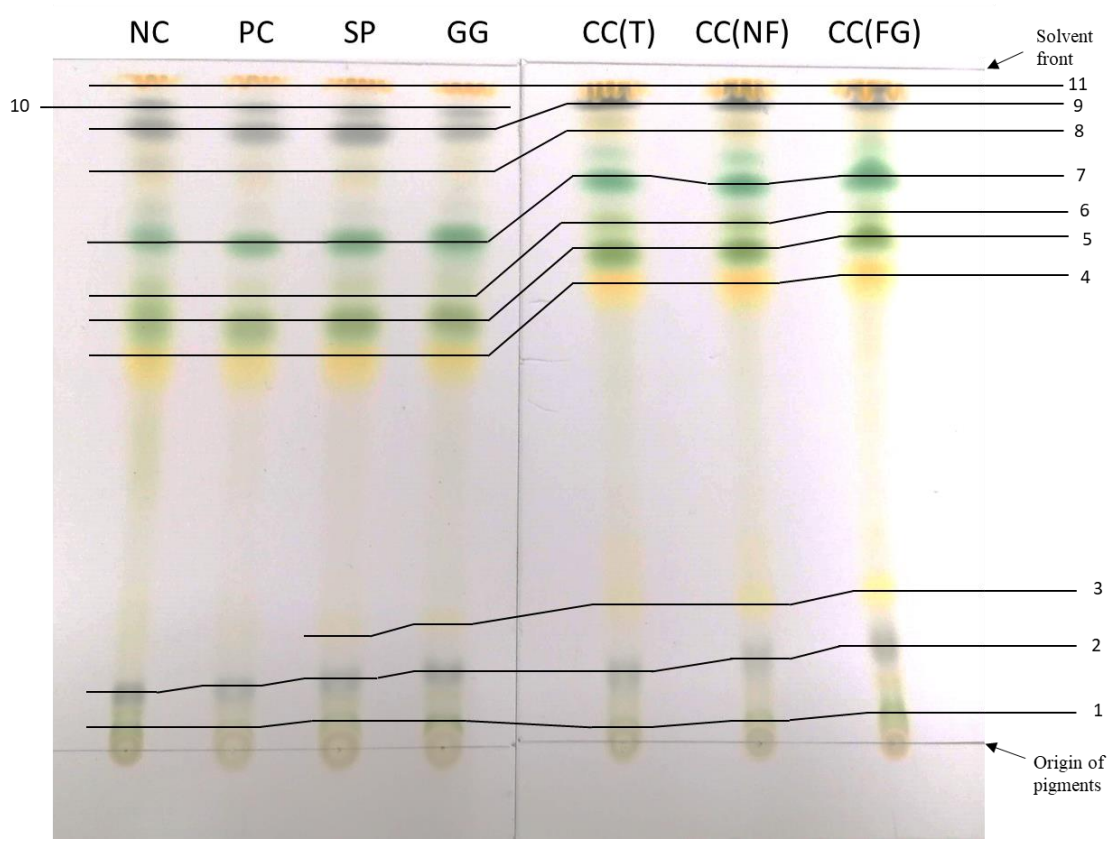

Figure 24 – Thin-layer chromatography of the methanolic extracts from each treatment sample of turnip leaves (Dry basis). NC – negative control. PC – positive control. SP – *S. polyschides*; GG – *G. gracilis*; CC(T) – *C. crispus* (tetrasporophyte); CC(NF) – *C. crispus* (non-fructified thalli); CC(FG) – *C. crispus* (female gametophyte).

Table 19 – Pigments identification from each treatment sample of turnip (Dry basis). Rf – retention factor. NI – No Information found in the literature. NC – negative control. PC – positive control. SP – *S. polyschides*; GG – *G. gracilis*; CC(T) – *C. crispus* (tetrasporophyte); CC(NF) – *C. crispus* (non-fructified thalli); CC(FG) – *C. crispus* (female gametophyte).

Nº *	Visible color	Rf observed							Rf literature	Pigment	Reference
		NC	PC	SP	GG	CC(T)	CC(NF)	CC(FG)			
1	light green	0.02	0.02	0.03	0.03	0.02	0.03	0.04	NI	NI	NI
2	light grey	0.07	0.08	0.09	0.10	0.10	0.12	0.14	NI	NI	NI
3	light yellow	nd	nd	0.16	0.17	0.20	0.20	0.22	0.18	Neoxanthin	Forgacs & Cserhati (2002)
4	bright yellow	0.57	0.57	0.57	0.57	0.68	0.68	0.69	0.15-0.35	Xanthophyll	Quach et al. (2004); Tarragó-Celada & Novell (2019)
5	light green	0.62	0.62	0.62	0.62	0.73	0.73	0.75	0.32-0.42	Chlorophyll *b*	Quach et al. (2004); Tarragó-Celada & Novell (2019)
6	faded green	0.66	nd	0.66	0.66	0.77	0.77	0.79	NI	NI	NI
7	dark green	0.74	0.74	0.74	0.74	0.84	0.83	0.84	0.44-0.59	Chlorophyll *a*	Quach et al. (2004); Tarragó-Celada & Novell (2019)
8	light grey	0.83	nd	0.83	nd	0.91	0.91	nd	0.49	Pheophytin *b*	Quach et al. (2004)
9	dark grey	0.91	0.91	0.91	0.91	0.95	0.95	0.95	0.60	Pheophytin *a*	Quach et al. (2004)
10	light grey	0.95	0.95	0.95	0.95	nd	nd	nd	NI	NI	NI
11	golden	0.99	0.99	0.99	0.99	0.98	0.98	0.98	0.95-0.98	β-carotene	Forgacs & Cserhati (2002); Quach et al. (2004); Tarragó-Celada & Novell (2019)

*Corresponding numbers in Figure 24.

CC(T) had the highest values in all pigments, with 6.916 mg/ 100 g of chlorophyll *a*, 2.301 mg/ 100 g of chlorophyll *b*, 0.016 mg/ 100 g of anthocyanins and 1.448 mg/ 100 g of carotenoids **(Table 20** and **Annex Table 5)**. On the contrary, GG had the lowest values of all pigments except carotenoids, with 4.303 mg/ 100 g of chlorophyll *a*, 1.361 mg/ 100 g of chlorophyll *b*, 0.009 mg/ 100 g of anthocyanins. The NC had the lowest quantity of carotenoids with 0.936 mg/ 100 g. Overall, the samples from *C. crispus* increased the quantity of pigments in turnip plants.

Table 20 – Pigments quantification (mg/ 100 g) from each treatment sample of turnip (Dry basis). NC– negative control. PC – positive control. SP – *S. polyschides*; GG – *G. gracilis*; CC(T) – *C. crispus* (tetrasporophyte); CC(NF) – *C. crispus* (non-fructified thalli); CC(FG) – *C. crispus* (female gametophyte).

Pigments (mg/ 100 g)	**NC**	**PC**	**SP**	**GG**	**CC(T)**	**CC(NF)**	**CC(GF)**
Chlorophyll *a*	4.346	4.458	5.233	4.303	6.916	5.516	5.914
Chlorophyll *b*	1.503	1.399	1.729	1.361	2.301	1.841	1.851
Anthocyanins	0.011	0.010	0.011	0.009	0.016	0.012	0.014
Carotenoids	0.936	1.013	1.231	1.056	1.448	1.230	1.426

3.4.4. Substrate characterization

The initial substrate (negative control) and final substrates (after the treatments) used for turnip' potting were analyzed (**Table 21**, **Figures 25 to 31** and **Annex Table 6)**. There were not found statistically significant differences among the treatment groups ($p>0.05$). Despite, there was observed a clear difference between the NC (initial substrate) and the final substrates. The NC had the highest OM content when compared with the substrates from the rest of the treatments, with 34.97% **(Table 21)**. Contrary, the substrate samples with the lowest OM content were from the treatments with the CC(FG) solution (23.53%) and the GG solution (23.52%). The substrate sample with the highest N content was SP (0.44%) and the lowest N content was CC(GF) (0.37%), CC(T) (0.35%) and CC(NF) (0.33%). When comparing the initial substrate (NC) with the final substrate of each treatment, there was a slightly decrease in the pH (**Figure 25**) but an increase in the

EC value (**Figure 26**). The NC had a neutral pH (6.50) and the other substrates had more acidic pH. The EC of the NC had no saline effects, whereas the other substrates had slightly saline effects, with PC having the highest value (1.50 mS/cm).

All figures (**Figure 27** to **Figure 31)** are divided in fertility classes (very low to very high) according to Laboratório Químico Agrícola Rebelo da Silva (1977). Regarding the P_2O_5 content, NC and CC(NF) demonstrated low levels, with 15.79 mg/L and 18.43 mg/L, respectively. The PC was the only one that exhibited high fertility levels, with 33.21 mg/L. Whereas, the other treatments exhibited moderate levels of soil fertility, with 25.13 mg/L for SP, 24.09 mg/L for GG, 21.23 mg/L for CC(T) and 29.60 mg/L for CC(FG). Considering the CaO and MgO content, all treatments exhibited very high levels, except the NC, that exhibited low and very low levels, with 62.71 mg/L (CaO) and 7.25 mg/L (MgO). The CC(GF) exhibited the highest value for CaO (801.28 mg/L) and MgO (156.68 mg/L). Regarding K_2O content, all treatments showed very high levels, from PC with 922.26 mg/L of K_2O to NC as the lowest with 248.02 mg/L of K_2O.

Table 21 – Apparent compact density (Ds), sample weight (ms) at volume of 60 mL, organic matter (OM) and nitrogen (N) of substrates in pots, where plants were grown and respective treatments. The results are expressed in mean ± standard deviation (n=2). There are not statistically significant differences found among the different samples **(**p>0.05**)**. NC – negative control. PC – positive control. SP – *S. polyschides*; GG – *G. gracilis*; CC(T) – *C. crispus* (tetrasporophyte); CC(NF) – *C. crispus* (non-fructified thalli); CC(FG) – *C. crispus* (female gametophyte).

	Treatments						
Soil sample	**NC**	**PC**	**SP**	**GG**	**CC(T)**	**CC(NF)**	**CC(FG)**
Ds (g/L)	945.19 ± 29.58	804.97 ± 14.01	767.59 ± 10.83	771.10 ± 4.21	836.16 ± 68.54	802.81 ± 4.18	837.85 ± 16.07
ms at 60 mL (g)	56.71 ± 1.77	48.30 ± 0.84	46.06 ± 0.65	46.27 ± 0.25	50.17 ± 4.11	48.17 ± 0.25	50.27 ± 0.96
OM (%)	34.97 ± 1.33	24.17 ± 0.73	27.22 ± 1.89	23.52 ± 0.50	19.54 ± 1.16	27.67 ± 7.40	23.53 ± 0.84
N (%)	0.42 ± 0.01	0.40 ± 0.02	0.44 ± 0.02	0.41 ± 0.02	0.35 ± 0.00	0.33 ± 0.01	0.37 ± 0.03

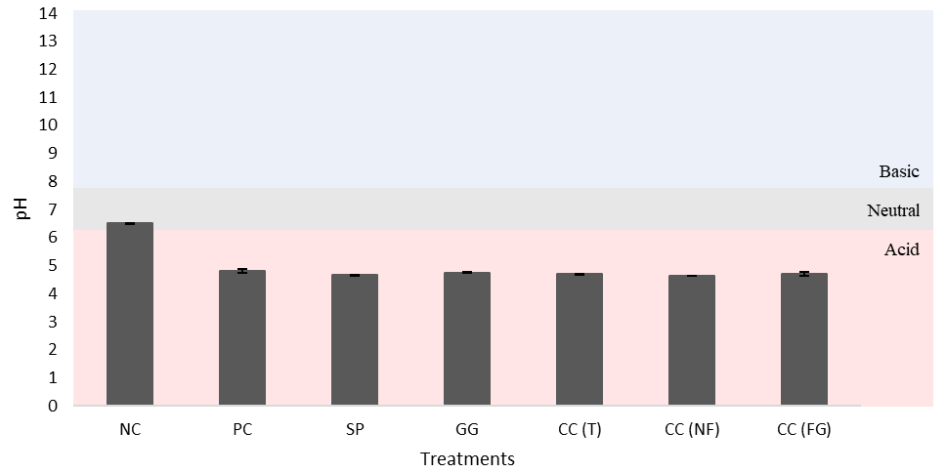

Figure 25 – pH of the substrate samples used for turnip leaves potting of each treatment. NC – negative control. There are not statistically significant differences found among the different samples **(**p>0.05**)**. PC – positive control. SP – *S. polyschides*; GG – *G. gracilis*; CC(T) – *C. crispus* (tetrasporophyte); CC(NF) – *C. crispus* (non-fructified thalli); CC(FG) – *C. crispus* (female gametophyte).

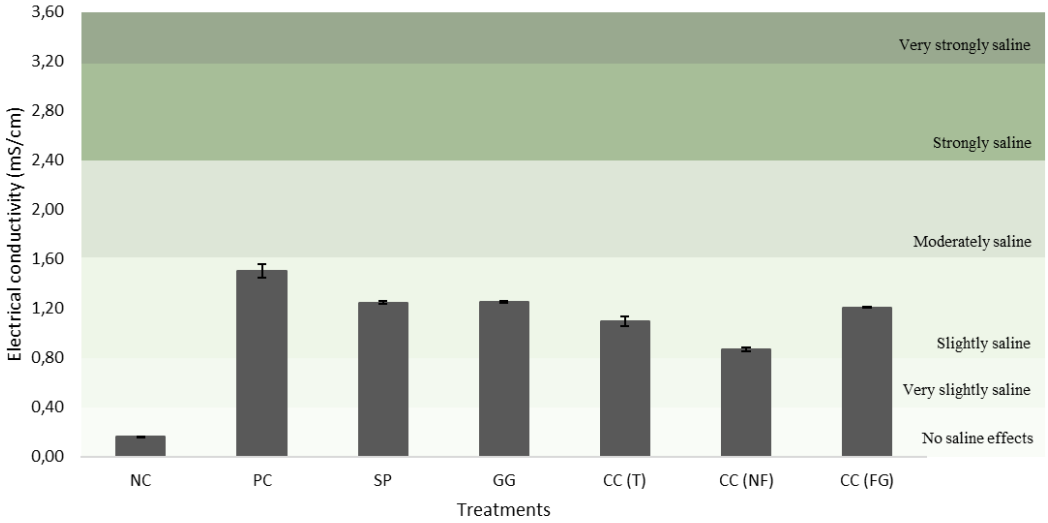

Figure 26 – Electrical conductivity (EC) (mS/cm) of the substrate samples used for turnip leaves potting of each treatment. There are not statistically significant differences found among the different samples **(**p>0.05**)**. NC – negative control. PC – positive control. SP – *S. polyschides*; GG – *G. gracilis*; CC(T) – *C. crispus* (tetrasporophyte); CC(NF) – *C. crispus* (non-fructified thalli); CC(FG) – *C. crispus* (female gametophyte).

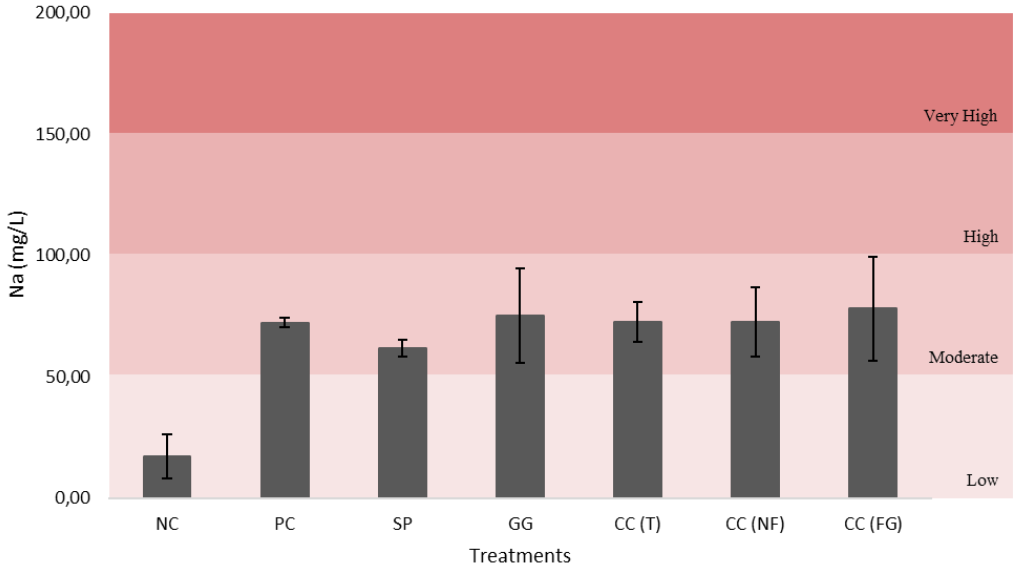

Figure 27 – Sodium (mg/L) of the substrate samples used for turnip leaves potting of each treatment. There are not statistically significant differences found among the different samples **(**p>0.05**)**. NC – negative control. PC – positive control. SP – *S. polyschides*; GG – *G. gracilis*; CC(T) – *C. crispus* (tetrasporophyte); CC(NF) – *C. crispus* (non-fructified thalli); CC(FG) – *C. crispus* (female gametophyte).

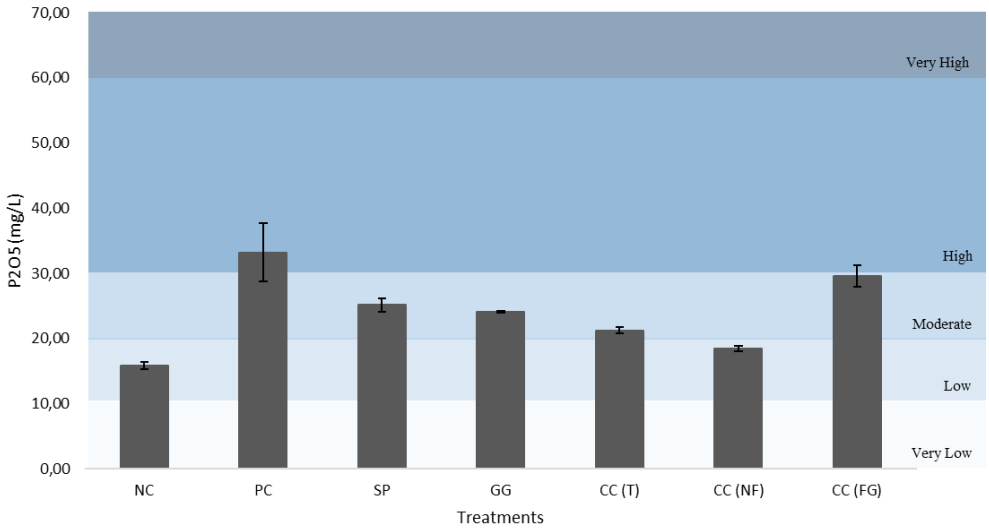

Figure 28 – Phosphorus pentoxide (mg/L) of the substrate samples used for turnip leaves potting of each treatment. There are not statistically significant differences found among the different samples **(**p>0.05**)**. NC – negative control. PC – positive control. SP – *S. polyschides*; GG – *G. gracilis*; CC(T) – *C. crispus* (tetrasporophyte); CC(NF) – *C. crispus* (non-fructified thalli); CC(FG) – *C. crispus* (female gametophyte).

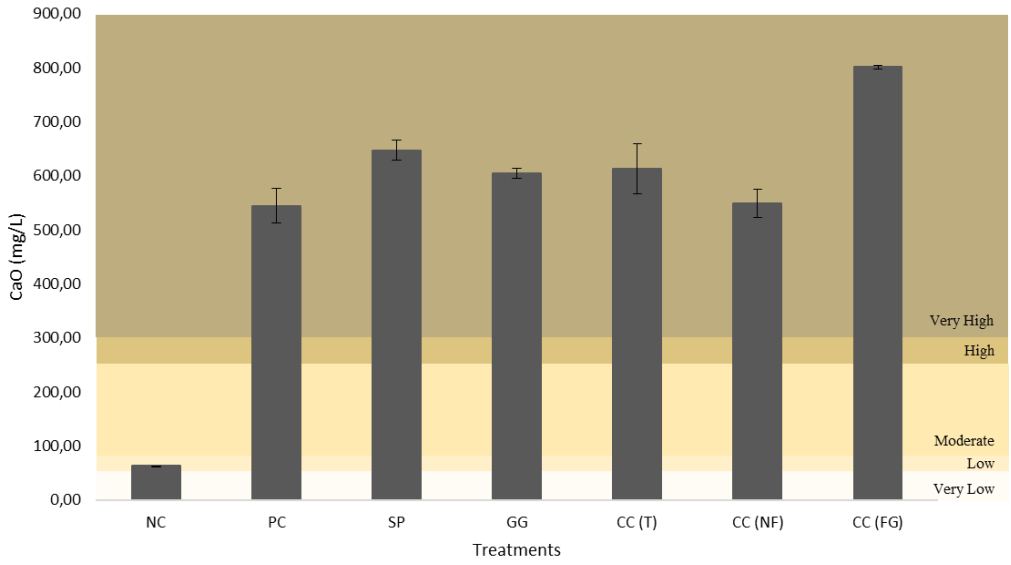

Figure 29 – Calcium oxide (mg/L) of the substrate samples used for turnip leaves potting of each treatment. There are not statistically significant differences found among the different samples **(**p>0.05**)**. NC – negative control. PC – positive control. SP – *S. polyschides*; GG – *G. gracilis*; CC(T) – *C. crispus* (tetrasporophyte); CC(NF) – *C. crispus* (non-fructified thalli); CC(FG) – *C. crispus* (female gametophyte).

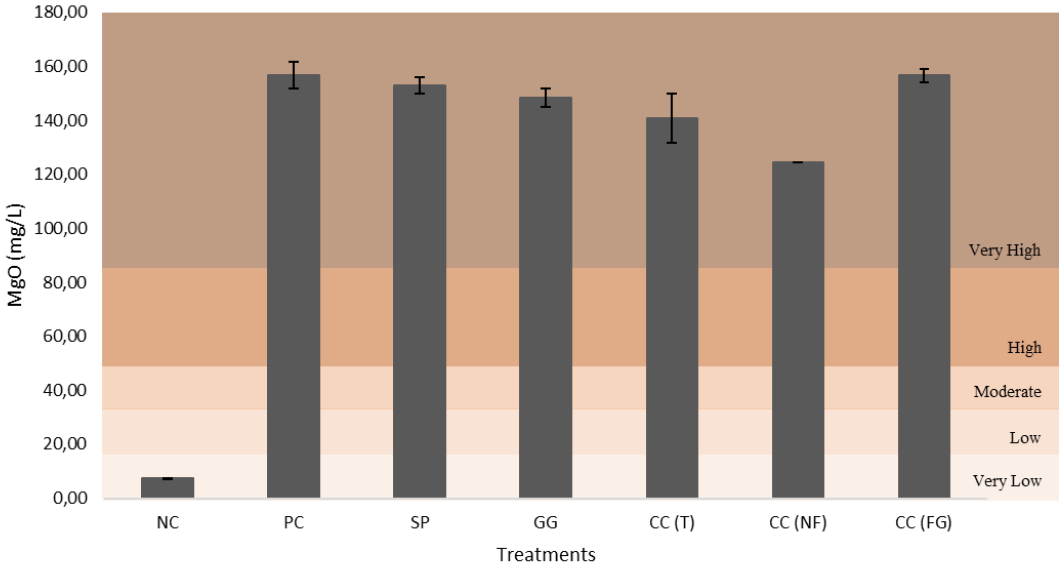

Figure 30 – Magnesium oxide (mg/L) of the substrate samples used for turnip leaves potting of each treatment. There are not statistically significant differences found among the different samples **(**p>0.05**)**. NC – negative control. PC – positive control. SP – *S. polyschides*; GG – *G. gracilis*; CC(T) – *C. crispus* (tetrasporophyte); CC(NF) – *C. crispus* (non-fructified thalli); CC(FG) – *C. crispus* (female gametophyte).

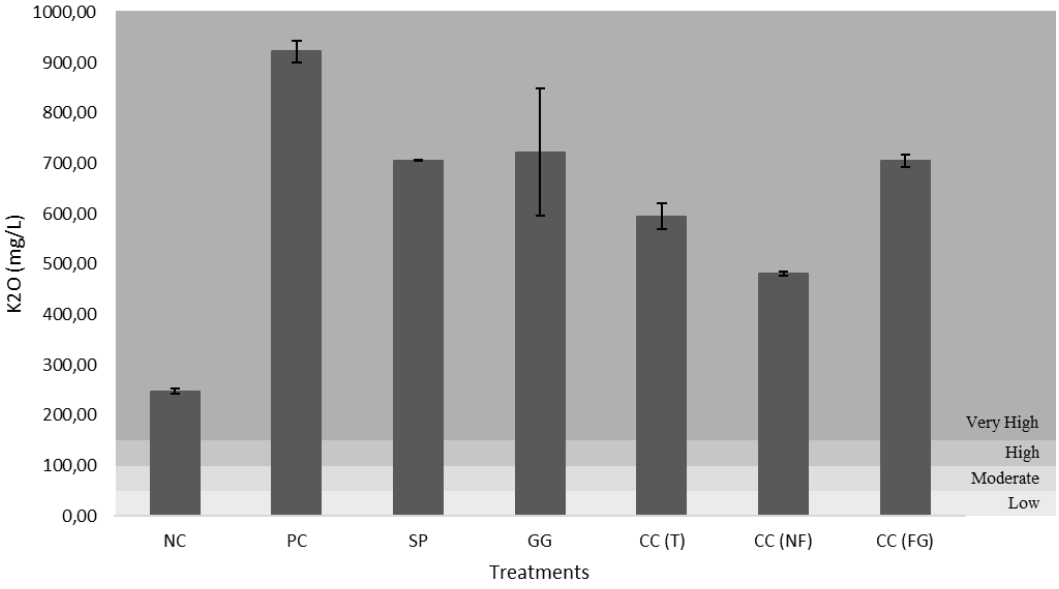

Figure 31 – Potassium oxide (mg/L) of the substrate samples used for turnip leaves potting of each treatment. There are not statistically significant differences found among the different samples **(**p>0.05**)**. NC – negative control. PC – positive control. SP – *S. polyschides*; GG – *G. gracilis*; CC(T) – *C. crispus* (tetrasporophyte); CC(NF) – *C. crispus* (non-fructified thalli); CC(FG) – *C. crispus* (female gametophyte).

4. Discussion

Seaweed polysaccharide characterization is an essential step in the comprehension of their role as plant's biostimulant. By analyzing these compounds structurally and chemically, we can understand their differences and how they will have an impact on the plant's metabolism. The polysaccharide's extraction methods used were based on the literature and optimized for the species used in this study. According to EFSA (Panel on Food Additives and Nutrient Sources added to Food), the color and texture of polysaccharides are important physicochemical properties that guarantee their purity and safety. The polysaccharides observed in **Figure 7** are in accordance with the EFSA propositions for alginate (Younes et al., 2017), agar (Mortensen et al., 2016) and carrageenan (Younes et al., 2018). Regarding the alginate extraction yield (**Table 4**), there is no literature information with the same methodology used in this study. The agar and all extracted carrageenans revealed a lower yield (**Table 4**) when compared to the data found in the literature (Pacheco et al., 2021; Pereira et al., 2009). The polysaccharide's yield can differ depending on the season and location from which the seaweed was harvested, as well as the extraction method used (Cotas et al., 2021). According to some authors, seaweeds synthesize more polysaccharides on spring and in the beginning of the summer (Cotas et al., 2019; Pereira & Mesquita, 2003; Zinoun & Cosson, 1996). In the case of seaweeds with different generations within their life cycle, such as *C. crispus*, the variance in the production of the polysaccharides can be explained by the negative correlation between the seaweed dry weight and the carrageenan content, as well as the hygroscopic properties of carrageenan (Pereira, 2013).

Usually, there is no information, regarding the mineral profile of the polysaccharides' solutions used in the experiments, in the literature. Red seaweeds, like *G. gracilis* and *C. crispus*, generally contain lower, but balanced, concentration of Ca and Mg, when compared to other macroalgae groups (Circuncisão et al., 2018), such as brown seaweed like *S. polyschides* (**Table 5**). Additionally, *S. polyschides* and *C. crispus* generally have higher concentrations of these minerals when compared to *G. gracilis* (Circuncisão et al., 2018), which can explain the values observed in the mineral profile of polysaccharides' solutions in this study (**Table 5**). Comparing all polysaccharide's solutions, the highest Na content was observed in the alginate solution of *S. polyschides*. Brown seaweed cell walls contain sulfated fucans and carboxylate alginic acids that have a chemical affinity to cations, such as Ca^{2+}, K^{+} and Na^{+} (Andrade et al., 2010), which can

explain the higher sodium content of this seaweed polysaccharide' solution (**Table 5**). However, salinity can have a negative effect in the plant's development (Zhao et al., 2020), resulting in week seedlings and a dispersal of the aerial and reticular parts, observed in **Figure 11d**.

The FTIR-ATR analysis of the alginate (**Figure 8**), agar (**Figure 9**) and carrageenan (**Figure 10**) spectra were in accordance with the literature (Belattmania et al., 2020; López-Hortas et al., 2023; Pacheco et al., 2021; Pereira et al., 2013; Rashedy et al., 2021). The similarities in the FTIR-ATR spectra of the two carrageenans extracted from the non-fructified thalli and female gametophyte of *C. crispus*, and both spectra of commercial κ-carrageenan and ι-carrageenan, can indicate a hybrid type of carrageenan present in this seaweed generations. On other hand, the differences observed in the FTIR-ATR spectra related to the carrageenan extracted from the tetrasporophyte of *C. crispus*, when compared to the other spectra, can indicate a totally different type of carrageenan present in this generation (λ-carrageenan). This is supported by the analysis of Pereira (2013).

The pH and electrical conductivity (EC) of the polysaccharides' solutions can affect the seed germination and development (Laghmouchi et al., 2017; Li et al., 2010), therefore their evaluation was important in the discussion of the results. Usually, neutral pH (± 7) is optimal for germination (Li et al., 2010), which was only observed in the alginate solutions (**Table 9**). The agar solutions (**Table 11**) had a pH lower than 7 (acid), around 5 and 6, and the carrageenan solutions (**Table 13**), the pH was higher than 7 (basic), around 9 and 10. According to the literature, carrageenan in solution is stable at a pH of 9. When the solution becomes acid, carrageenan can suffer hydrolysis and lose physical properties. In all polysaccharides' solutions, lower concentrations resulted in lower pH. The EC in all polysaccharides' solutions (**Table 9**, **Table 11** and **Table 13**) was lower than 1000 µS/cm, essential to seed germination (Li et al., 2010). The EC is directly related to salinity (increased salinity will increase the conductivity), which can have a negative impact on plant cell homeostasis, causing a lower water absorbency and compromising metabolic pathways (Kaya et al., 2006; Uçarlı, 2021; Wong & Wong, 1989). These physical parameters can change depending on the seaweed species, where the seaweed was harvested, the extraction method, the pH of the solvent (distilled water) used in preparing the solutions and temperature. The EC of the agar solutions (**Table 11**) was very low when comparing to the alginate solutions (**Table 9**). Since conductivity is

directly related to salinity, this can be explained by the difference in Na concentration of both polysaccharides, as demonstrated previously in the mineral profile of the polysaccharide solutions (**Table 5**). Overall, the carrageenan solutions of *C. crispus* (tetrasporophyte) had the lowest pH, and the carrageenan solutions of *C. crispus* (female gametophyte) had the highest. The EC in the carrageenan solutions of *C. crispus* (tetrasporophyte and non-fructified thalli) were relatively similar when comparing to the same concentration. Whereas the carrageenan solution of *C. crispus* (female gametophyte) had a higher conductivity then the other samples from *C. crispus*. As mentioned before, this solution had higher Na content when compared to the other generations' solutions (**Table 5**). This direct correlation between the salinity (sodium content) and the electrical conductivity of the polysaccharides' solutions was not found in the literature.

Overall, the germination percentage, the shoot weight and shoot length were higher in the assays with the carrageenan solutions (**Table 14**) when compared to other polysaccharides' solutions (alginate and agar) (**Table 10 and Table 12**). This can be explained by the presence and position of sulphate groups in the molecular chain of the polysaccharides. The sulphation degree, their concentration and oxidation can have an impact on the polysaccharides' bioactivities (Patel et al., 2022). Typically, alginophytes, such as *S. polyschides*, show the lowest sulphate group content, whereas carrageenophytes, the highest (Pacheco et al., 2021). As said previously, Lemonnier-Le Penhuizic et al. (2001) demonstrated that oligosaccharides of λ-carrageenan can act as inducers of embryogenesis. In this study, both alginate and agar oligosaccharides were also tested, but with less significant results than those obtained by carrageenans. Several studies have demonstrated the effects of carrageenans in plants (Mamede et al., 2023). Carrageenans stimulate plant growth by enhancing the basal metabolism, including cell division, purine and pyrimidine synthesis, assimilation of nitrogen and sulfur, and photosynthesis (Pacheco et al., 2021; Shukla et al., 2016), and the production of secondary metabolites, such as essential oils and polyphenolic compounds. When compared to λ-carrageenan, κ-and ι-carrageenan showed better results in the growth of the roots and leaves in kale, by inducing the production of indole-3-acetic acid (IAA), responsible for the plant's development (Pacheco et al., 2021). However, contrary to previous literature, there were not significant differences in the germination of *B. napus* L. seeds among the samples from *C. crispus*, tetrasporophyte (λ-carrageenan) and non-

fructified thalli and female gametophyte (hybrid κ-and ι-carrageenan). Thus, not justifying the separation of this seaweed species, *C. crispus*, into generations, for their usage in seed germination.

Following the seed germination assay, the concentration of each polysaccharide's solution selected to be used in the biostimulant and fertilizer assay in adult turnip plants is displayed in **Table 3** and **Table 15**. These concentrations were selected according to the results of the seed germination assay, especially the seedlings weight and the ratio shoot weight/root weight.

In agriculture, the determination of the pH and electrical conductivity (EC) of biostimulant treatments applied to crops, can help to anticipate the crop's yield, quality and pathogen resistance (Khan et al., 2021). The typical pH for an alginate in solution is between 2.0 and 3.5 (Younes et al., 2017), since the carboxylate groups in the alginate backbone become protonated and form hydrogen bonds, which makes the solution more acidic. The pH of the carrageenan solutions is usually between 8 and 11 (Younes et al., 2018). Results presented in **Table 15** are in accordance with the previous statements. When compared to the other treatments, the positive control exhibited an increased EC, which can be explained by the composition of the extract that this commercial leaf fertilizer is made off. As mentioned before, the positive control was a solution of "Profertil" (ADP Fertilizantes, Portugal), containing 20% (dry matter) of the seaweed *A. nodosum*, at a concentration of 1.5% (v/v). The most abundant elements in *A. nodosum*, are potassium, sodium and calcium (Lorenzo et al., 2017). These elements can increase the salinity of the solution, therefore, increase the EC. In addition, viscosity is considered among the most important physical properties used to assess the gelling capability of polysaccharides (Kaidi et al., 2022). This property depends on the degree of polymerization, temperature, concentration, molecular weight and the presence of polyvalent metal cations in the polysaccharide structure (Younes et al., 2017). According to EFSA (Panel on Food Additives and Nutrient Sources added to Food), the viscosity of alginate solutions can vary from 4 to 1000 mPa.s but can be affected when the solution has a pH lower than 4 (Younes et al., 2017), which explains the slight decrease in viscosity of the alginate solution (**Table 15**). Additionally, the viscosity of carrageenan solutions should not be less than 5 mPa.s (Younes et al., 2018). There is no information in literature regarding the pH, EC and viscosity of agar solutions.

The application of these polysaccharide solutions to turnip plants was very effective improving plant growth, increased development of plant's biomass and root system, enhance photosynthetic activity, essential nutrient uptake and soil quality. Polysaccharides, such as alginate, agar and carrageenan, act as elicitors to enhance plant's metabolism and resistance against environmental stresses (Jamiołkowska, 2020). When polysaccharides' solutions are sprayed on to the foliage, the plant's cell wall reacts quickly to this interaction and binds with these molecules to induce local resistance. Usually, the plant's pathogen- or pattern-recognition receptors (PRRs), recognize pathogen-associated molecular patterns (PAMPs), or in the case of non-pathogen related molecules, microbe-associated molecular patterns (MAMPs), which is more common in agricultural practices. This recognition triggers a complex chain of defense responses called PAMP-triggered immunity, pathogen-triggered immunity, or pattern-triggered immunity (PTI) (Bigeard et al., 2015; Jamiołkowska, 2020). In plants, these defense mechanisms are the first line of local defense against biotic and abiotic stresses, restraining, for example, pathogen activity, often resistant to chemical pesticides. This defense response can also trigger an induced systemic resistance (ISR) or systemic acquired resistance (SAR), making the plant less susceptible to a subsequent pathogen attack (Jamiołkowska, 2020). In this study, the activity of SAR was clearly observed when the turnip plants treated with the polysaccharide's solutions showed signs of possible inhibitory effects against a pathogen (e.g., *Agrotis* larvae) more than the controls (**Figure 18**). *Agrotis* is a genus of moths from the Noctuidae family, that usually attack turnip plants. This type of larvae remains hiden during the day and emerges at night to feed, becoming a major agricultural pest (Smit, 1964). Thus, polysaccharides' solutions, used in this work, can have an indirect inhibitory effect against this type of pathogen.

As seen in other studies (Mamede et al., 2023), during this resistance process, the plant can have a biochemical response related with the production of phytoalexins and pathogenesis-related (PR) enzymes, such as phenylalanine ammonia-lyase (PAL), peroxidase (POD) and ascorbate peroxidase (AP), by signaling pathways mediated by salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) (Ali et al., 2018). This reaction elicits the plant's metabolic pathways and the synthesis of secondary metabolites, like phenolic compounds with antiviral activity. These biochemical responses trigger other morphological responses, related with nutrient uptake and consequently, growth and development.

In this study, the turnip plants demonstrated clear differences in growth parameters among the treatments (**Figure 19**). The turnip plants treated with the polysaccharides' solutions exhibited the best results in both leaf weight and length, when compared to the negative (tap water) and positive controls ("Profertil") (**Figure 21** and **Figure 22**). Samples that produced the best results were the carrageenan solutions of *C. crispus*, particularly the tetrasporophyte generation, with higher ratio aerial part weight: length (**Table 16**).

Ratios make an association between the development of the aerial part and the roots. A lower aerial part weight: root weight ratio indicated that the turnip plants had spent more energy on root biomass growth than aerial part, whereas a greater of aerial part weight: root weight ratio indicated that the plants had spent more energy on leaves biomass (**Figure 20e-g**). However, as observed in the negative and positive controls, the lowest aerial part length: root length ratios, indicated that the turnip plants developed more roots than the aerial part (**Figure 20a-b**). However, despite these ratios, major root biomass development, instead of the leaves (as observed in NC and PC), does not necessarily resulted in a better root system and more efficient absorption of the nutrients available in the soil. As demonstrated in **Figure 20**, roots from plants treated with polysaccharides' solutions (**Figure 20c-g**), were more robust and more branched than the ones obtained from the control treatments (**Figure 20a-b**).

A developed root system influences the plant nutrient uptake. Plant's roots absorb nutrients from the soil and transport them throughout the plant to support life activities. To maintain their growth and development, plants need substantial quantities of macronutrients and micronutrients. Lack of nutrients in a soil can result in leaf chlorosis, reduced development, and even plant death. However, the excess of nutrients in the soil can also have harmful effects on plants, affecting the plant nutrient uptake, such as oxidative stress production, cell damage, and growth inhibition (Thye et al., 2022). Plants require 16 essential elements to survive, such as carbon (C), hydrogen (H), oxygen (O), nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sulfur (S); and trace elements, such as iron (Fe), zinc (Zn), manganese (Mn), copper (Cu), boron (B), molybdenum (Mo), and chlorine (Cl). These elements can be supplied directly from the soil minerals and soil organic matter or by organic or inorganic fertilizers, when applied (Uchida, 2000). The mineral profile of the turnip from each treatment was compared with the reference values of PortFIR (Plataforma Portuguesa de Informação Alimentar) as

safety and quality standard of turnip greens for human consumption in Portugal (**Table 17**). The differences between the treatment groups and the literature values can be related to genetic differences and environmental factors. The mineral and trace element contents of each treatment can affect the percentage of the dry matter and ashes. Low ash content usually indicates that the plant is denser, therefore this should be taken into consideration when analyzing the mineral percentage (Uchida, 2000). For instance, N improves the quality and quantity of dry matter in leafy vegetables, such as turnip (Uchida, 2000). Amino acids, which are the building blocks of proteins, are created when N is joined with C, H, O, and S. Therefore, there is a direct correlation between the protein content and the nitrogen content. N is required for all enzymatic processes in plants and for photosynthesis (Uchida, 2000). Phosphorus plays a major role in photosynthesis (as storage and transfer of energy), in cell division and root development. P can increase the quality of various crops and has been demonstrated to lower the incidence of disease in some plants (Uchida, 2000). Calcium (Ca) is responsible for formation of the cell wall membrane and its plasticity. Ca can act as a detoxifying agent by neutralizing organic acids in plants and assists in improving crop yields by reducing soil acidity (Uchida, 2000). Moreover, magnesium is actively involved in photosynthesis and assists the movement of sugars within a plant (Uchida, 2000). On other hand, potassium is an enzyme activator that promotes metabolism which is vital for plant growth and controls the opening and closing of leaf stomata, regulating the plant water uptake. Like P, K can increase the quality of various crops and has been demonstrated to lower the incidence of disease in some plants (Uchida, 2000). Overall, Zn, Fe, Mn and Cu are essential elements in several plant enzyme systems involved in photosynthesis and plant's metabolism (Uchida, 2000).

As said previously, the turnip plants treated with the negative control exhibited the highest values of all mineral and trace elements (**Table 17**). However, these samples showed the worst results in plant growth (leaf weight and length) (**Figure 19** and **Figure 21**) and were heavily affected by the herbivory activity of *Agrotis* larvae (**Figure 18**). In this case, plants were prevented to use their mineral resources in metabolic processes such as photosynthesis, enzymatic activity, cell division, root development, and more (as said previously), as a defense response. This behavior could also indicate a survival mechanism since this turnip plants had to maintain their mineral content and not spend too much energy in metabolic processes and help them to survive abiotic and biotic

stresses. On other hand, the turnips treated with the polysaccharides' solutions did not exhibit growth deficiencies or were affected by herbivory activity (**Figure 19**). Additionally, they had very similar mineral content compared with the literature standard for human consumption in Portugal (**Table 17**), which indicates that these solutions are not toxic and can even improve the nutritional quality of turnip greens.

In addition to mineral and trace element characterization, analyzing the cell wall and its components can help us understand and characterize the effects the treatments groups had on the turnip plants, since plants' cell wall has a big role on their metabolic processes. FTIR-ATR has been used for fast cell wall characterization (Canteri et al., 2019), however, due to the complexity and variability of the cell wall composition, it is not always possible to assign exactly each FTIR-ATR band to its respective functional chemical group or compound. The obtained spectra (**Figure 23**) were compared with bibliographic supported data (Canteri et al., 2019). Contrary to other samples, the negative control was the only one to exhibit a peak in the lignin and phenolic backbone area at 1520 cm^{-1} (**Table 18**). Numerous studies have reported a variation in the quantity of lignin and other polyphenols when plants were under stressed environment (Šamec et al., 2021). Polyphenols play a crucial role in plant–environmental interactions and can indicate when a certain plant have been exposed under abiotic stress, which was the case of the turnips from the negative control.

Apart from the mineral and cell wall characterization of the turnip plants, the detection and quantification of their pigments is a crucial step in further understanding the effects of the treatment groups in these plants during the experiment, especially in photosynthetic activity. Overall, the Rf values observed were greater than the Rf values found in the literature (**Table 19**), except in the case of neoxanthin and β-carotene. This difference could be related to oxidation of the pigments, the type of silica plate used, the eluent, the plant species, and the quantity of the solution applied to the TLC. The absence of pigment marked as "10" in the *C. crispus*' TLC (**Figure 24**) could be explained by the pigment entrainment in the end of the silica plate, not allowing to differentiate the pigments clearly. Overall, the turnip treated with the carrageenans extracted from *C. crispus* exhibited the greatest pigment content among all treatments (**Table 20**). The increase in pigments, such as chlorophyll, can indicate an increase in photosynthetic activity, and consequently, an increase in growth and development.

Soil/substrate is an extremely complex and important ecosystem that directly influences plants' growth and development. The soil density (Ds) could be influenced by several physical and chemical properties, such as organic matter, texture, minerals, and porosity. This information is essential for soil management and the understanding of the best farming technics (Chaudhari et al., 2013). The Ds of all samples had very high standard deviation (**Table 21**), which could be explained by the variation of porosity in the different replicates made, since its property was very difficult to control in the used method. In addition, organic matter (OM) and nitrogen (N) content had big influence on plant's growth. Soils with high content of OM and N usually enhance the photosynthetic processes and consequently the development of the plant (Henneron et al., 2020). However, their availability in the soil does not imply their absorption by the plant's roots and their use in plant's photosynthesis. As shown in our results the turnip from the negative control did not take advantage of the availability of the OM and N content present in the substrate.

The EC is directly related with salinity, so it was expected a similarity between it (**Figure 26**) and the sodium quantity in the substrates (**Figure 27**). Soil salinity can have a negative effect on the plant's development (Zhao et al., 2020). Hence, the moderate sodium content of the substrate samples in the treatments was ideal for the turnip plants' productivity (**Figure 27**). For a soil/substrate to be considered fertile, it must have enough levels of various nutrients, such as N, P, K, Ca, and Mg, that may restrict plant development, as well as enough organic matter to hold onto water and nutrients. Low concentrations of one or more of these nutrients might lower plant production (Furey & Tilman, 2021). When comparing the initial substrate (negative control) with the final substrates (after the treatments), all soil samples where the turnip plants were treated with the polysaccharides' solutions exhibited very high fertility levels, contrary to what happened in the initial substrate (**Figure 27** to **Figure 31**). The polysaccharides' solutions, applied to the aerial part of the turnip plants, increased the soil fertility. Some studies (Furey & Tilman, 2021) have reported that the improvement of the plant's metabolism and development can influence the soil quality by positive feedback (when the plant exhibits an increase in nutrient content and growth, the soil usually becomes more fertile). The increased nutrient content in plant biomass is returned to the soil/substrate when the plant tissue decomposes, increasing like that a soil fertility.

The application of polysaccharide solutions to turnip plants was very efficient in, improving plant growth (**Figure 19**), increased plant's biomass and root system (**Figure 20, Figure 21** and **Figure 22**), enhanced photosynthetic activity (**Table 20**), essential nutrient uptake (**Table 17**) and soil quality (**Figure 27** to **Figure 31**), when compared to the positive and negative control. Turnip plants treated with the carrageenan solutions of *C. crispus* presented the best results in improved crop's productivity than in plants treated with alginate (*S. polyschides*) and agar solutions (*G. gracilis*). This was particularly noticeable in turnip plants treated with the carrageenan solutions of *C. crispus* (tetrasporophyte). The type of carrageenan extracted from this generation of *C. crispus* is λ-carrageenan, which is usually more sulphated (32-39% of sulphate group) than κ-carrageenan (20-30% of sulphate group) and ι-carrageenan (28-35% of sulphate group), that is a hybrid type of carrageenan extracted from the non-fructified thalli and female gametophyte of *C. crispus* (Cunha et al., 2016; Mercier et al., 2001). The degree of sulphation can directly influence the bioactivity of the polysaccharides. Typically, alginophytes, such as *S. polyschides*, show the lowest sulphate group content, whereas carrageenophytes, the highest (Pacheco et al., 2021), which was supported by this study. λ-carrageenan was the polysaccharide that had the most bioactivity and positive effect in turnip plants.

There are many different sulphated compounds present in plants that play a major role in their metabolic processes, influencing the plant's development and its stress responses (Kopriva et al., 2019). For example, glucosinolates is a group of sulfated secondary metabolites limited to the order Capparales, including the Brassicaceae family, that are responsible for their protection against pathogens by inducing defense pathways (Koprivova & Kopriva, 2016). The relation between the polysaccharides' sulphation degree and the bioactivities observed in this study could be in some way related to the interaction of sulphate groups and the plant's metabolism, by enhancing the sulfur content in turnip plants and triggering the activity of SAR (Künstler et al., 2020).

5. Conclusion

In a planet with increased demand for new and greener alternatives for the agricultural practices, seaweed based-biostimulants gain an important role as non-synthetic fertilizers. Seaweed based-biostimulants (seaweed extracts with biostimulant activities) can substitute the synthetic compounds present in commercial stimulants and fertilizers, used in agriculture to improve crop yield and vigor. As an advantage, seaweeds do not compete for land space, which allows the exploration of polysaccharides in a sustainable and circular economy way.

The polysaccharide (carrageenan) extracted from *C. crispus* exhibited the best results, firstly, in the seedling growth and germination percentage, and afterwards, in turnip plants' growth, development and metabolism, when compared to alginate from *S. polyschides* and agar from *G. gracilis*.

The polysaccharides chemical structures, their physicochemical characteristics (pH and electrical conductivity) and mineral profile, were crucial for the germination of turnip seeds and respective growth of the plantlets.

The application of polysaccharide solutions to turnip plants was very efficient in improving their growth, increase the plant's biomass and root system, enhance photosynthetic activity, essential nutrient uptake, and soil quality, when compared to a commercial seaweed leaf fertilizer ("Profertil"). Contrary, to what was observed in the seed germination assay (where there were not significant differences found that justified the separation of *C. crispus* into generations), the turnip plants treated with the carrageenan solutions of *C. crispus* (tetrasporophyte) exhibited the best results. λ-carrageenan, which is the type of carrageenan extracted from the tetrasporophyte generation of *C. crispus*, was the polysaccharide that had the most bioactivity and positive effect by improving turnip plants growth, biomass and root system, enhancing the quantity of photosynthetic pigments and nutrients, and soil nutrient uptake. Therefore, the separation of *C. crispus* seaweeds into different life cycle generations, and use their extracts, could be extremely beneficial to increase the productivity of agricultural crops.

The bioinsecticide potential of the seaweed polysaccharides observed in this study should be further analyzed, as well as the polyphenol profile and the sulfur content of the treated turnip plants, to understand the full potential of the seaweed polysaccharides in the agriculture sector.

6. References

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Annex

Annex Table 1 – Growth parameters of the seedlings from the seed germination assay with different polysaccharides' solutions. The results are expressed in mean ± standard deviation (n=5). Statistically significant differences found among the different samples are expressed by letters and samples with the same letter are not statistically different ($p<0.05$). Control – distilled water. SP – *S. polyschides*; GG – *G. gracilis*; CC(T) – *C. crispus* (tetrasporophyte); CC(NF) – *C. crispus* (non-fructified thalli); CC(FG) – *C. crispus* (female gametophyte).

Polymer	Seaweed	Concentration (mg/mL)	Shoot weight (g)	Shoot length (cm)	Radicular weight (g)	Radicular length (cm)
Alginate	SP	Control	0.010 ± 0.006^{a}	1.760 ± 0.441^{b}	0.002 ± 0.004^{a}	4.720 ± 0.567^{ab}
		0.25	0.026 ± 0.011^{a}	5.620 ± 1.030^{a}	0.009 ± 0.006^{a}	8.313 ± 1.725^{a}
		0.50	0.025 ± 0.014^{a}	4.040 ± 1.474^{d}	0.004 ± 0.006^{a}	5.547 ± 2.718^{ab}
		1	0.013 ± 0.007^{a}	2.987 ± 0.950^{c}	0.003 ± 0.005^{a}	3.120 ± 1.133^{b}
Agar	GG	Control	0.028 ± 0.007	4.380 ± 1.059	0.004 ± 0.005	6.020 ± 2.389
		0.25	$0,029 \pm 0.012$	6.207 ± 1.431	0.005 ± 0.006	7.880 ± 3.019
		0.50	0.035 ± 0.015	7.327 ± 2.532	0.004 ± 0.005	8.367 ± 2.957
		1	0.031 ± 0.015	6.213 ± 2.323	0.009 ± 0.003	6.947 ± 1.790
Carrageenan	CC(T)	Control	0.018 ± 0.007^{b}	3.400 ± 1.135^{b}	0.010 ± 0.006	7.500 ± 3.066
		0.25	0.033 ± 0.009^{ab}	7.200 ± 3.506^{a}	0.005 ± 0.005	8.753 ± 2.286
		0.50	0.039 ± 0.013^{a}	7.627 ± 1.096^{a}	0.007 ± 0.004	9.740 ± 3.507
		1	0.037 ± 0.013^{a}	6.793 ± 1.893^{a}	0.008 ± 0.008	7.920 ± 2.534
	CC(NF)	Control	0.020 ± 0.006	4.160 ± 1.457^{a}	0.006 ± 0.005	6.300 ± 2.249
		0.25	0.027 ± 0.016	4.340 ± 2.390^{a}	0.009 ± 0.008	5.640 ± 1.314
		0.50	0.030 ± 0.009	4.547 ± 1.887^{a}	0.006 ± 0.006	4.640 ± 2.039
		1	0.034 ± 0.014	6.467 ± 1.959^{a}	0.009 ± 0.005	6.700 ± 2.953
	CC(FG)	Control	0.018 ± 0.007	4.400 ± 1.049^{a}	0.006 ± 0.005	6.780 ± 1.987^{b}
		0.25	0.026 ± 0.011	5.433 ± 0.996^{a}	0.005 ± 0.005	9.840 ± 2.226^{a}
		0.50	0.023 ± 0.010	6.380 ± 1.712^{a}	0.003 ± 0.005	7.400 ± 1.443^{b}
		1	0.031 ± 0.012	6.153 ± 1.405^{a}	0.005 ± 0.005	7.653 ± 1.589^{b}

Annex Table 2 – Ratios between the growth parameters of the seedlings from the seed germination assay with different polysaccharides' solutions. Control – distilled water. SP – *S. polyschides*; GG – *G. gracilis*; CC(T) – *C. crispus* (tetrasporophyte); CC(NF) – *C. crispus* (non-fructified thalli); CC(FG) – *C. crispus* (female gametophyte).

Polymer	Seaweed	Concentration (mg/mL)	Ratio shoot length/weight	Ratio root length/weight	Ratio shoot/root weight	Ratio shoot/root length
Alginate	SP	Control	176.00	2360.00	5.00	0.37
		0.25	216.15	959.23	3.00	0.68
		0.50	163.78	1386.67	6.17	0.73
		1	224.00	936.00	4.00	0.96
Agar	GG	Control	156.43	1505.00	7.00	0.73
		0.25	216.51	1477.50	5.38	0.79
		0.50	207.36	2091.67	8.83	0.88
		1	202.61	801.54	3.54	0.89
Carrageenan	CC(T)	Control	188.89	750.00	1.80	0.45
		0.25	216.00	1875.71	7.14	0.82
		0.50	197.24	1328.18	5.27	0.78
		1	181.96	990.00	4.67	0.86
	CC(NF)	Control	208.00	1050.00	3.33	0.66
		0.25	162.75	650.77	3.08	0.77
		0.50	151.56	773.33	5.00	0.98
		1	190.20	773.08	3.92	0.97
	CC(FG)	Control	244.44	1130.00	3.00	0.65
		0.25	208.97	2108.57	5.57	0.55
		0.50	273.43	2220.00	7.00	0.86
		1	196.38	1435.00	5.88	0.80

Annex Table 3 – Growth parameters of the fresh and dried turnip leaves from each treatment of the biostimulant and biofertilizer assay, measured after 63 days. The results are expressed in mean ± standard deviation (n=3). Samples with the same letter are not statistically different ($p<0.05$). AP – aerial part. R – root. NC – negative control; PC – positive control; SP – *S. polyschides*; GG – *G. gracilis*; CC(T) – *C. crispus* (tetrasporophyte); CC(NF) – *C. crispus* (non-fructified thalli); CC(FG) – *C. crispus* (female gametophyte).

Sample	AP fresh weight (g)	AP dry weight (g)	AP fresh length (cm)	R fresh weight (g)	R dry weight (g)	R fresh length (cm)	nº leaves
NC	19.55 ± 10.51	2.31 ± 1.44	28.63 ± 3.41[a]	0.54 ± 0.40	0.09 ± 0.07	12.57 ± 0.94	5.67 ± 1.25
PC	15.91 ± 15.15	2.97 ± 2.14	26.80 ± 6.70[a]	0.61 ± 0.70	0.08 ± 0.11	12.48 ± 1.71	6.50 ± 0.50
SP	34.48 ± 13.26	3.05 ± 1.27	35.36 ± 3.41[a]	0.98 ± 0.70	0.12 ± 0.10	11.72 ± 0.89	7.60 ± 1.02
GG	29.53 ± 13.99	3.08 ± 0.98	35.54 ± 5.51[a]	0.99 ± 0.58	0.13 ± 0.09	11.84 ± 2.42	8.40 ± 1.02
CC(T)	40.80 ± 5.11	3.47 ± 0.41	37.70 ± 2.77[a]	0.93 ± 0.18	0.10 ± 0.03	13.76 ± 2.24	8.80 ± 0.75
CC(NF)	35.16 ± 10.79	3.08 ± 1.07	39.27 ± 4.28[a]	0.66 ± 0.27	0.08 ± 0.05	15.77 ± 6.13	8.83 ± 0.69
CC(FG)	38.73 ± 12.75	3.50 ± 1.17	39.98 ± 4.00[a]	1.06 ± 0.24	0.14 ± 0.04	15.80 ± 3.33	8.50 ± 1.50

Annex Table 4 – Ratios between the aerial part (AP) and root (R) of the fresh turnip leaves from each treatment of the biostimulant and biofertilizer assay, measured after 63 days. NC – negative control; PC – positive control; SP – *S. polyschides*; GG – *G. gracilis*; CC(T) – *C. crispus* (tetrasporophyte); CC(NF) – *C. crispus* (non-fructified thalli); CC(FG) – *C. crispus* (female gametophyte).

Sample	AP length/weight	R length/weight	AP weight/R weight	AP length/R length
NC	1.46	23.27	36.20	2.28
PC	1.68	20.45	26.09	2.15
SP	1.03	11.91	35.04	3.02
GG	1.20	11.94	29.77	3.00
CC(T)	0.92	14.73	43.69	2.74
CC(NF)	1.12	24.01	53.54	2.49
CC(FG)	1.03	14.94	36.62	2.53

Annex Table 5 – Total pigments quantification (T) from each treatment sample of turnip leaves (Dry basis), with the absorbance (Abs.) of the sample at the corresponding wavelength. NC– negative control. PC – positive control. SP – *S. polyschides*; GG – *G. gracilis*; CC(T) – *C. crispus* (tetrasporophyte); CC(NF) – *C. crispus* (non-fructified thalli); CC(FG) – *C. crispus* (female gametophyte).

Pigments	Wave length (nm)	NC		PC		SP		GG		CC(T)		CC(NF)		CC(FG)	
		Abs.	T (mg/ 100 g)	Abs.	T (mg/ 100 g)	Abs.	T (mg/ 100 g)	Abs.	T (mg/ 100 g)	Abs.	T (mg/ 100 g)	Abs.	T (mg/ 100 g)	Abs.	T (mg/ 100 g)
Chlorophyll *a*	665.2	0.3757	4.346	0.3823	4.458	0.4506	5.233	0.3692	4.303	0.5959	6.916	0.4754	5.516	0.5071	5.914
Chlorophyll *b*	652.4	0.2125	1.503	0.2124	1.399	0.2527	1.729	0.2054	1.361	0.3346	2.301	0.2671	1.841	0.2816	1.851
Anthocyanins	535	0.0522	0.011	0.0510	0.010	0.0549	0.011	0.0445	0.009	0.0768	0.016	0.0589	0.012	0.0665	0.014
Carotenoids	470	0.3717	0.936	0.3779	1.013	0.4621	1.231	0.3832	1.056	0.5728	1.448	0.4741	1.230	0.519	1.426

Annex Table 6 – pH, electrical conductivity (EC) and mineral characterization of the substrates used for turnip leaves potting of each treatment. The results are expressed in mean ± standard deviation (n=2). There are not statistically significant differences found among the different samples **(**$p>0.05$**)**. NC – negative control. PC – positive control. SP – *S. polyschides*; GG – *G. gracilis*; CC(T) – *C. crispus* (tetrasporophyte); CC(NF) – *C. crispus* (non-fructified thalli); CC(FG) – *C. crispus* (female gametophyte).

Soil sample	NC	PC	SP	GG	CC(T)	CC(NF)	CC(FG)
pH (1/5, v/v)	6.50 ± 0.01	4.82 ± 0.07	4.66 ± 0.02	4.76 ± 0.00	4.70 ± 0.00	4.65 ± 0.00	4.71 ± 0.08
EC at 25 ºC (1/5, v/v)	0.16 ± 0.00	1.50 ± 0.05	1.25 ± 0.01	1.26 ± 0.01	1.09 ± 0.04	0.87 ± 0.01	1.21 ± 0.00
P (mg/L)	6.88 ± 0.25	14.48 ± 1.96	10.96 ± 0.45	10.50 ± 0.08	9.26 ± 0.22	8.03 ± 0.18	12.91 ± 0.72
P_2O_5 (mg/L)	15.79 ±0.58	33.21 ± 4.51	25.13 ± 1.04	24.09 ± 0.18	21.23 ± 0.51	18.43 ± 0.40	29.60 ± 1.66
Ca (mg/L)	44.83 ± 0.38	389.50 ± 22.50	463.00 ± 13.50	432.50 ± 6.50	438.50 ± 33.50	392.50 ± 18.50	572.75 ± 2.25
CaO (mg/L)	62.71 ± 0.52	544.91 ± 31.48	647.74 ± 18.89	605.07 ± 9.09	613.46 ± 46.87	549.11 ± 25.88	801.28 ± 3.15
Mg (mg/L)	4.38 ± 0.17	94.50 ± 3.00	92.25 ± 1.75	89.50 ± 2.00	85.00 ± 5.50	75.00 ± 0.00	94.50 ± 1.50
MgO (mg/L)	7.25 ± 0.29	156.68 ± 4.97	152.95 ± 2.90	148.39 ± 3.32	140.93 ± 9.12	124.35 ± 0.00	156.68 ± 2.49
K (mg/L)	206.00 ± 4.75	766.00 ± 17.75	585.75 ± 1.00	599.50 ± 105.25	494.00 ± 20.75	399.00 ± 3.25	585.50 ± 9.75
K_2O (mg/L)	248.02 ± 5.72	922.26 ± 21.37	705.24 ± 1.20	721.80 ± 126. 72	594.78 ± 24.98	480.40 ± 3.91	704.94 ± 11.74
Na (mg/L)	17.25 ± 9.00	72.25 ± 2.00	61.75 ± 3.50	75.25 ± 19.50	72.50 ± 8.25	72.50 ± 14.25	78.00 ± 21.25