Pedro Henrique Queiroz Pereira dos Santos

BIOTECHNOLOGICAL EVALUATION OF SEAWEEDS AS BIO-FERTILIZER.

Master´s degree in Biodiversity and Biotechnology

July/2016

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University of Coimbra

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Dissertation submitted to the Department of Life Sciences of University of Coimbra to comply with the degree of Master in Plant Biodiversity and Biotechnology, hold under the scientific guidance of Dr. Leonel Pereira and Dr. Jorge Canhoto.

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Index of Abbreviation

2,4-D	2,4-Dichlorophenoxyacetic acid
4-Cl-IAA	4-Chloroindole-3-acetic acid
AUX1	Auxin transporter protein 1
CAE	Crude Aqueous Extract
CO^2	Carbon dioxide
E400	Alginic acid
E401	Sodium alginate
E402	Potassium alginate
E403	Ammonium alginate
E404	Calcium alginate
GP	Germination percentage
HCl	Hydrochloric acid
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
n-PAT	non-Polar auxin transport
O^2	Oxygen
PAA	Indole-3-propionic acid
PAT	Polar auxin transport
PDAB	p–dimethylaminobenzaldehyde
PGR	Plant growth regulator
Picloram	Amino-3,5,4,6-trichloropicolinic acid
PIN	Proteins for auxin efflux carriers
SA	Standard Addition
SAE	Serial aqueous extract
SC	Standard Curve
SESE	Seaweeds Evaporated Serial Extraction
SHE	Serial hexane extract
SLF	Seaweed liquid fertilizer
SME	Methanol serial extract
SSE	Seaweed Serial Extraction

SVI	Seedling vigor index
TCA	Trichloroacetic acid
Trp-DP	Tryptophan-dependent pathway
Trp-IP	Tryptophan-independent pathway
θfc	Field capacity

Abstract

The seaweeds contain several molecules and compounds which assist in the evolution of plants. The present study evaluates the effect of water extracts of the brown seaweeds *Fucus vesiculosus* and *Saccorhiza polyschides* as seaweed liquid fertilizer (SLF) on germination, growth, development and yield of tomato plants (*Solanum lycopersicum*).

Different concentrations (5%, 10%, 15%, 20% and 25%) of SLF were prepared using distilled water and applied to evaluate their efficiency as plant growth regulator and the inhibiting (toxic) concentration.

The seedlings treated with different concentration of SLF were monitored for various parameters such as radicle length, plumule length, total length, seedling vigor index and germination percentage, whereas the tomato plants were monitored root length, shoot length, total length, number of leaves, flowers and fruits.

At the same time, had as objective to relate the presence and the role of the plant hormone auxin (indole-3-acetic acid or IAA) in the seaweed liquid fertilizer. Both species shown to have plant hormones, being *Fucus vesiculosus* the holder of higher concentrations: 331µg/mL, while *Saccorhiza polyschides* presented 80.314µg/mL.

The study showed that the concentration required for optimum plant growth varies depending on their stage of development. In the germination test, applying 10 to 15% of SLF there was an increase in all parameters but an inhibition of nearly 50% in the plants treated with extracts of 25% SLF.

Plants in a greenhouse, in a higher stage of development, when treated with 10% extracts to 25% increase the number of leaves by about 50% and plants treated with extracts from 20 to 25% accelerated fruit development, thus obtaining a better yield.

Seaweeds are natural resources that when employed in a controlled way, are not harmful to plants and ever more, due to diverse scientific studies, this organisms are increasingly gaining importance and acceptance for use in sustainable crops.

Beyond, seaweeds are fundamental because belonging to the basis of the food chain, thus, should be utilized responsibly and based on biology studies that do not influence the environment where were harvested.

Key words: Seaweed liquid fertilizer (SLF); Crop Yield; Bioassays; Plant hormones; Indole-3-acetic acid; Sustainable crops.

Resumo

As algas possuem várias moléculas e compostos que auxiliam na evolução das plantas. O presente estudo avalia o efeito de extratos aquosos de algas castanhas (*Fucus vesiculosus* e *Saccorhiza polyschides*) como fertilizantes líquidos na germinação, crescimento, desenvolvimento e produtividade do tomateiro (*Solanum lycopersicum*).

Diferentes concentrações (5%,10%,15%,20% e 25%) de extratos aquosos de algas foram preparados utilizando água destilada e aplicada para avaliar a sua eficiência como regulador de crescimento vegetal e a concentração em que ocorre inibição (toxicidade).

As plântulas tratadas com diferentes concentrações de extratos de algas foram monitorizadas em vários parâmetros, tais como comprimento radicular, comprimento da plúmula , comprimento total, índice de vigor da plântula e percentagem de germinação; enquanto que para as plantas de tomateiro foram monitorados o comprimento das raízes, comprimento da parte aérea, comprimento total, número de folhas, flores e frutos.

Ao mesmo tempo, teve como objetivo relacionar a presença e o papel da hormona vegetal auxina (ácido indol-3-acético) nos fertilizantes à base de algas. As duas espécies de algas mostraram possuir a hormona citada, sendo *Fucus vesiculosus* a que apresentou uma concentração mais elevada: 331 µg/mL, enquanto *Saccorhiza polyschides* teve uma concentração de 80,314 µg/mL.

O estudo mostrou que a concentração necessária para um crescimento ótimo da planta varia de acordo com seu estágio de desenvlvimento. No teste de germinação, a aplicação de 10% a 15% de extrato de algas provoca um aumento em todos os parâmetros e uma inibição de aproximadamente 50% nos extratos de 25% de algas.

As plantas em estufa, que estão em uma fase mais avançada de desenvolvimento, quando tratadas com 10% a 25% de extratos de algas, aumentam o número de folhas em cerca de 50%, e as plantas tratadas com extratos de 20% a 25% aceleram o desenvolvimento dos frutos, obtendo-se um melhor rendimento na produção.

As algas são recursos naturais que, quando utilizadas de forma controlada, não são prejudiciais para as plantas e cada vez mais, devido a diversos estudos científicos, estes organismos estão a ganhar cada vez mais importância e aceitação para uso em culturas sustentáveis.

Além disso, as algas são seres fundamentais porque pertencentem à base da cadeia alimentar, assim, devem ser utilizadas de forma responsável e com base em estudos biológicos que não influenciem o meio ambiente em que foram colhidas.

Palavras chave: Fertilizantes à base de algas; Rendimento de culturas agrícolas, Bioensaios, Hormonas Vegetais, Ácido indol-3-acético; Agricultura Sustentável.

1- Introduction

Seaweeds and Ecological Importance

The seaweeds are macroscopic algae commonly found in rocky shores during low tide, and have great notoriety in the vast marine ecosystem. They exhibit wide variety of colors, shapes and sizes. Some species can reach values higher than 50 meters, and form true aquatic forests, known as kelps.

It has been estimated that there are about 9,000 species of macroalgae [1] broadly classified into three main groups based on their pigmentation (for example, Ochrophyta (Phaeophyceae), Rhodophyta, and Chlorophyta; or the brown, red, and green algae, respectively). But the distinction between the three groups involves more than the visible color. In addition to the pigmentation, they differ considerably in many structural and biochemical features including photosynthetic pigments, storage compounds, composition of cell walls, presence or absence of flagella.

The current groups of seaweeds originated through different evolutionary processes (primary endosymbiosis for green and red algae, secondary endosymbiosis for brown algae) and for this reason they are now classified in different kingdoms (green algae and red algae in the Kingdom Plantae, and Brown algae in the Kingdom Chromista)[2].

Like plants and some bacteria, seaweeds use the energy of sunlight to convert in chemical energy. The seaweeds capture light energy through photosynthesis and convert inorganic substances into simple sugars using the captured energy.

Photosynthetic algae must be considered the true survivors among the Plantae as they have survived dramatic changes in climatic conditions from a hot, CO_2^- and methane-rich nitrogen atmosphere to presently occupy virtually all niches on Earth from tropics to the cold, dry, frozen deserts of Antarctic. The key requirement is there be liquid water, even if only intermittently. Gradually, they produced the oxygen necessary to support animal life and, perhaps more importantly, participated in the evolution of macroalgae and the eukaryotic plants upon which we depend for our survival [3].

About 90% of the species of marine plants are algae and about 50% of the global photosynthesis is algae derived [4]. Thus every second of time, each molecule of oxygen we inhale comes from algae and algae reuses every molecule of carbon dioxide we exhale [5].

Seaweeds are fundamental to the food chain of all aquatic ecosystems. As primary producers they produce oxygen and organic compounds which serve as the basic trophic level or food for many other living beings. In addition, seaweeds assure spaces in the environment in which sustain and shelter a great biological diversity.

The seaweeds are normally exposed to different variations of environmental factors throughout the water column, such as the concentration of dissolved gases, luminous intensity, mineral concentration (salinity and nutrients), temperature, ultraviolet light, several pollutants, pathogens (fungi, viruses and bacteria) and predators [6].

From these variations described and the high level of nutrients absorbed, seaweeds can synthesize compounds associated with the cell wall and intercellular spaces that, unlike primary metabolites, do not have a direct role in their survival. These compounds are known as secondary metabolites, and are produced by processing and converting by the primary metabolites of seaweeds.

The seaweeds are considered as a source of bioactive compounds as they are able to produce a great variety of secondary metabolites characterized by a broad spectrum of biological activities. Compounds with antioxidant, antiviral, antifungal, and antimicrobial activities have been detected in brown, red, and green algae [7, 8]. There are numerous reports of compounds derived from macroalgae with a broad range of biological activities, such as antibacterial [9], antivirals [10], antitumorals [11], anticoagulant [12], and antifouling [13], antihelminthic and antifungal [14].

Seaweeds contain high amounts of carbohydrates, proteins and minerals [15-19]. Mineral content in seaweeds is generally higher than that of land plants and animal products (8-40%) [20,21]. This wide range in mineral content, not found in edible land plants, is related to factors such as seaweed phylum, geographical origin, and seasonal environmental and physiological variations [18]. Ash content was higher

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in brown (30,1-39,3%) than in red seaweeds (20.6-21.1%) and all the seaweeds contained sulphate, ranging from 1.3 to 5-9% [22].

Members of the brown seaweeds (Ochrophyta, Phaeophyceae) produce uronates (alginates) and other sulphated polysaccharides (e.g.,fucoidan and laminaran). The different phycocolloids used in food industry as natural additives (European codes of phycocolloids) are: alginic acid—E400, sodium alginate—E401, potassium alginate—E402, ammonium alginate—E403, calcium alginate—E404, propylene glycol alginate—E405 [23].

Utilization and Benefits of Seaweeds

Seaweeds are used in many maritime countries as a source of food, for industrial applications and fertilizer [21, 24-25]. It was reported recently that seaweeds formed an important resource for humans in pre-historic times, about 14,000 before present [26].

Interest in agricultural uses of seaweeds is increasing rapidly as judged by the number of related publications appearing since 1950. The number of species involved is small, but the volumes of biomass can be sizeable [27].

The benefits of seaweeds as sources of organic matter and fertilizer nutrients have led to their use as soil conditioners for centuries [28-30].

Numerous studies have revealed a wide range of beneficial effects of seaweed extract applications on plants, such as early seed germination and establishment, improved crop performance and yield, elevated resistance to biotic and abiotic stress, and enhanced postharvest shelf-life of perishable products [31-34].

According to Newton (1951) [35], the earliest reference to seaweed manure is in the second half of the first century when the Roman Columella recommended that cabbages be transplanted at the sixth leaf stage and their roots be mulched and manured with seaweed.

A method, published more than 150 years ago, for compressing seaweeds or marine plants into a compact, transportable form indicates the value placed on seaweed manure and the need to transport the product over long distances [36]. An alkaline seaweed extract was produced almost a century ago in recognition of the fact that it was uneconomical to transport seaweeds more than a few kilometers from the coastline for use as manure [37].

A major advance in the utilization of seaweeds in agriculture resulted from research conducted during World War II on new sources of fiber. The British government focused research efforts to develop local sources of fiber, and a biochemist, Dr. Reginald F. Milton moved to Birmingham where he purchased property with a large garden and glasshouse, and set up a small laboratory to investigate methods for liquefying kelp for use as a fertilizer. By 1947, he had succeeded in making a liquid product. His method, based on a hot pressurized alkaline process, was patented and formed the basis for the Maxicrop process [38]. During that period, Milton became acquainted with W. A. (Tony) Stephenson, an accountant, who was making a career change by moving to the countryside to indulge his gardening hobby. Thus, each could test the various interactions of the early liquefied seaweed products in his own plot. The chemistry worked for both men and, one night in 1949 over a bottle of brandy, the name Maxicrop was born [39].

Stephenson soon realized that an additional product line was important to his business so he undertook to market seaweed meal for stock feed and for manure (name Neptune's Bounty). By 1952, he had formed the company Maxicrop Limited to sell both the liquid seaweed extract (previously sold by Plant Productivity Ltd.) and the meal products. The combined businesses permitted him to support a year-round sales staff to further develop the market [27].

The collection and uses of seaweeds in Portugal was described in the fourteenth century, since as an ancient activity, and the harvesting of kelp, which is still done in the north, were regulated in 1308 by King D. Dinis. This use was constant until the twentieth century in which the lack of Japanese Agar, during World War II, allowed the emergence of an Agar Portuguese industry, due to the abundance and quality of Portuguese seaweeds. However, due to an unfavorable international

conjecture led to the disappearance of this industry, leaving today only one company (Iberagar- Luso-Spanish Society of Marie Colloids, SA) [40].

Algae harvesting in the region between the Minho and Douro River was from the Middle Age until the mid-twentieth century, an economic and social activity with great significance, as illustrated in figure 1. Today, the use of seaweed as fertilizer is mostly restricted to the northern area, particularly in agricultural fields of Póvoa de Varzim and Viana do Castelo [41].

Figure 1. Veloso Salgado oil painting depicts a typical harvesting of the "Sargaço". In "Ilustração Portugueza" magazine (may, 26th 1913) [200].

The two main mixtures of algae used as fertilizer are traditionally the "Moliço" and "Sargaço". Moliço is a mixture of seaweeds and plants harvested in the Ria de Aveiro. This mixture contains mainly *Ulva, Gracilaria* and *Lola*, and marine plants belonging to the genus *Zostera, Ruppia and Potamogeton*. Sargaço (also called "argaço and limos") is the seaweed mixture (*Saccorhiza, Laminaria, Fucus, Codium, Palmaria, Gelidium* and *Chondrus*) that grow on the rocks of the littoral zone. The traditional harvesting of "Sargaço" was the seaweed collection on the beach, at low tide [41].

Based on the yearbook of Food and Agriculture Organization (FAO, 2012) on the state of fisheries and aquaculture worldwide [42], the world production of

seaweed grew from 1,097,998 tons in 2011 to 1,107,381 t in 2012. Portugal follows the pattern and also increased the production of 461 to 801 tons in the same period.

Currently the list of companies that provide algae extracts as fertilizers is varied, but the diversity of algae that part is small, being mostly composed of brown algae of the species *Ascophyllum nodosum.*

Brown Seaweeds

Brown seaweeds are aquatic photosynthetic organisms belonging to the Domain Eukarya and the Kingdom Chromista (brown algae). They are included in the phylum Ochrophyta (or Heterokontophyta), class Phaeophyceae; their pigments are the chlorophylls a, c, and carotenoids, dominated by fucoxanthin (Figure 2), responsible for the brownish color [41, 43].

Brown seaweeds are the second most abundant group of seaweeds comprising about 2,000 species which reach their maximum biomass levels on the rocky shores of the temperate zones. They are the type most commonly used in agriculture [28].

The Phaeophyceae, or brown algae, derive their characteristic color from the large amounts of the carotenoid fucoxanthin in their chloroplasts as well as from any phaeophycean tannins that might be present. The chloroplasts also have chlorophylls A, C_1, and C_2. Most of the Phaeophyceae grow in the intertidal belt and the upper littoral region. They dominate these regions in colder waters, particularly in the Northern Hemisphere, where the number of phaeophycean species is less than that of the Rhodophyta, but the number of phaeophycean is much greater. In the tropics, the only place where large numbers of Phaeophyceae are found is the ´´Sargaço´´ Sea of the Atlantic [44].

The Phaeophyceae has an ancient lineage, the estimate of its Origen is from 150 [45] and 200 million years ago [46]. Probably evolved from an organism in the Phaeothamniophyceae, which have motile cells similar to those in the Phaeophyceae,

but lack the characteristic unilocular and plurilocular sporangia of the Phaeophyceae [47].

Brown seaweeds have frequently been used as bio-indicators for metal pollution in seawater, because of their high accumulation capacity. Analyses of seaweeds have indicated the possibility of getting a qualitative picture of the metal pollution in different areas [48-53].

The cell walls are generally composed of at least two layers, with cellulose making up the main structural skeleton [54]. The amorphous component of the cell wall is made up of alginic acid and fucoidin (Figure 2), whereas the mucilage and cuticle are composed primarily of alginic acid [55, 56].

The long-term storage product is laminarin (Figure 2), a β-1,3 linked glucan. The sugar alcohol, D-mannitol is, however, the accumulation product of photosynthesis [44].

In a number of brown algae, the mannitol (Figure 2) concentration in the cell increases or decreases as the salinity of the surrounding medium increases or decreases [57]. This osmoregulatory mechanism prevents the cells from bursting in hypotonic media or shrinking in hypertonic media. The increase in mannitol concentration occurs in the dark as well as in the light, showing that photosynthesis is not involved in the process [44].

Tannins are phenolic compounds of secondary metabolism and have great ecological and economic value. Exhibit water solubility and molecular weight between 500 and 3000 Da, having the ability to form water insoluble complexes with proteins and alkaloids [58]. The tannins are non-glycosidic (do not contain sugars), bind proteins, have strong reducing action, and are astringent to the taste. They are readily oxidized in air, resulting in the formation of a black pigment, phycophaein, giving dried brown algae their characteristic black color [44]. Phlorotannins (phaeophycean tannins) (Figure 2) are stored physodes in the cytoplasm of many brown algae. Phlorotannins are formed by Golgi in the perinuclear area of the cell by polymerization of phloroglucinol (1,3,5-tri-hydroxybenzene) (Figure 2) through the acetate-malonate pathway [59,60] .

The phlorotannin content of brown algae varies from 1% to 15% of dry mass. Phlorotannins occur at high levels in brown algae of the temperate and tropical Atlantic, whereas levels are low (less than 2% of dry mass) in the tropical Pacific Ocean and Indo-Pacific [61]. In temperate areas, the fucoids (Fucales) have high concentrations of phlorotannins whereas kelps (Laminariales) have low concentrations of phlorotannins. The same species of brown alga will have higher phlorotannin content when deprived of nitrogen [62]. Phlorotannins have been postulated to function in deterring grazing by herbivores, absorbing ultraviolet radiation, and serving as a component of cell walls [63].
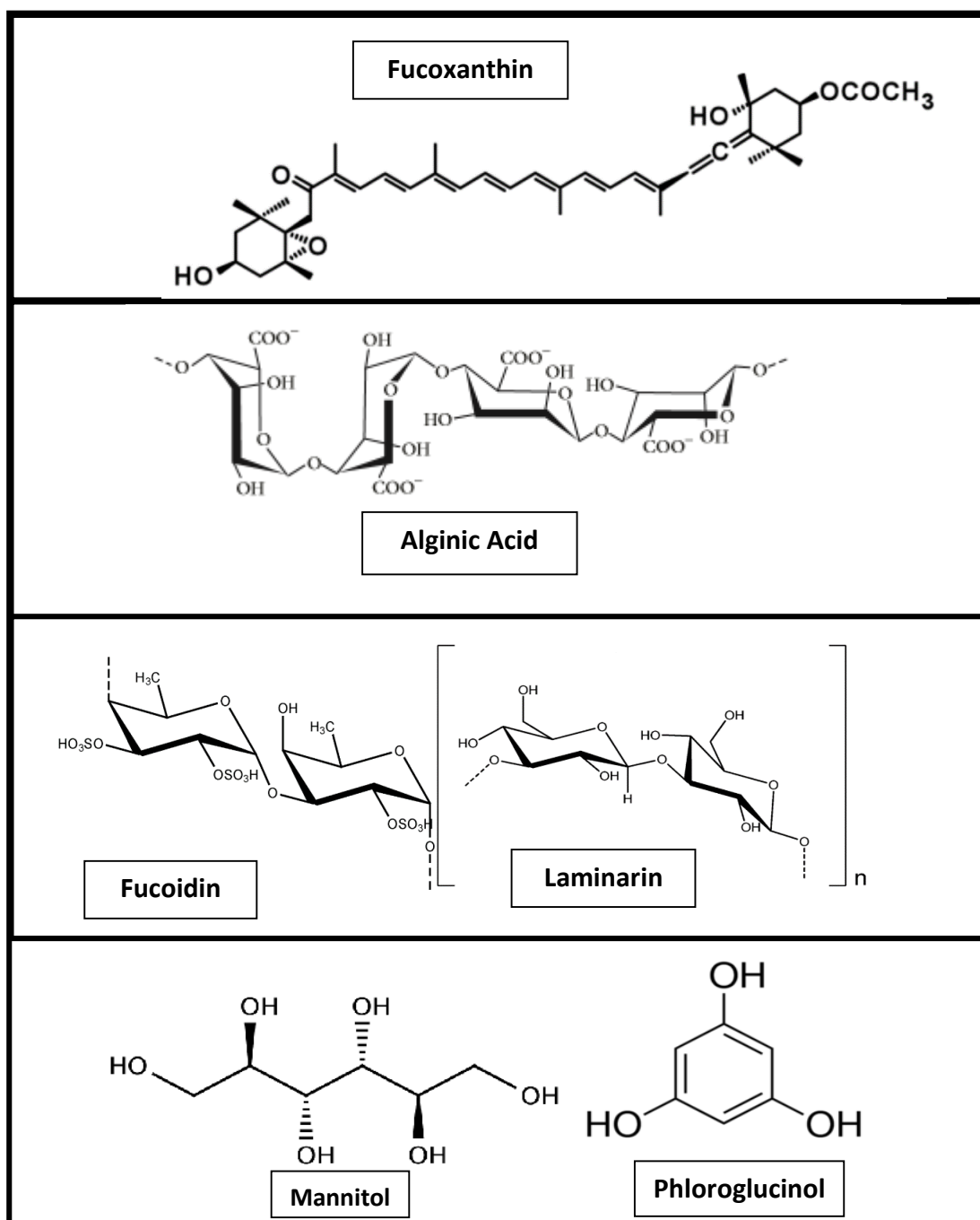
Figure 2. Chemical structure of some Pheophyceae compounds. From Pereira (2013) [23], DrugBank [201], Trc-Canada [216], ResearchGate [217].

Plant Hormones in Seaweeds.

The term "hormone" comes originally from the Greek and is used in animal physiology to denote a chemical messenger. Its original use in plant physiology was derived from the mammalian concept of a hormone. This involves a localized site of synthesis, transport in the bloodstream to a target tissue, and the control of a physiological response in the target tissue via the concentration of the hormone [64]. But it is now clear that plant hormone does not fulfill the requirements of a hormone in the mammalian sense.

The synthesis of plant hormones may be localized (as occurs for animal hormones), but may also occur in a wide range of tissues, or cells within tissues. While they may be transported and have their action at a distance this is not always the case. They may also act in the tissue in which they are synthesized or even within the same cell [64]. They can act on the tissue in which they are synthesized and even the same cell, as often happens with ethylene. Also, unlike animal hormones, plant hormones are not usually produced in specific tissues, analogous to animal glands [65].

The plant hormones (or phytohormones) are a group of natural compounds in plants with an ability to affect physiological processes at concentrations far below those where either nutrients or vitamins would affect these processes [66].

A plant growth regulator (PGR) is a term commonly used in the agrochemical industry to distinguish synthetic plant growth regulators from the endogenous ones [27]. It is now recognized that many of the common higher plant hormones, such as: auxins, cytokinins, gibberellins, abscisic acid, and ethylene occurs in algae including brown seaweeds [67-69].

At present, through current studies and new methods offered by biotechnology, understanding the mechanisms of phytohormones in plant development is becoming accurate. Some chemical structures of plant hormones are illustrated in Figure 3.
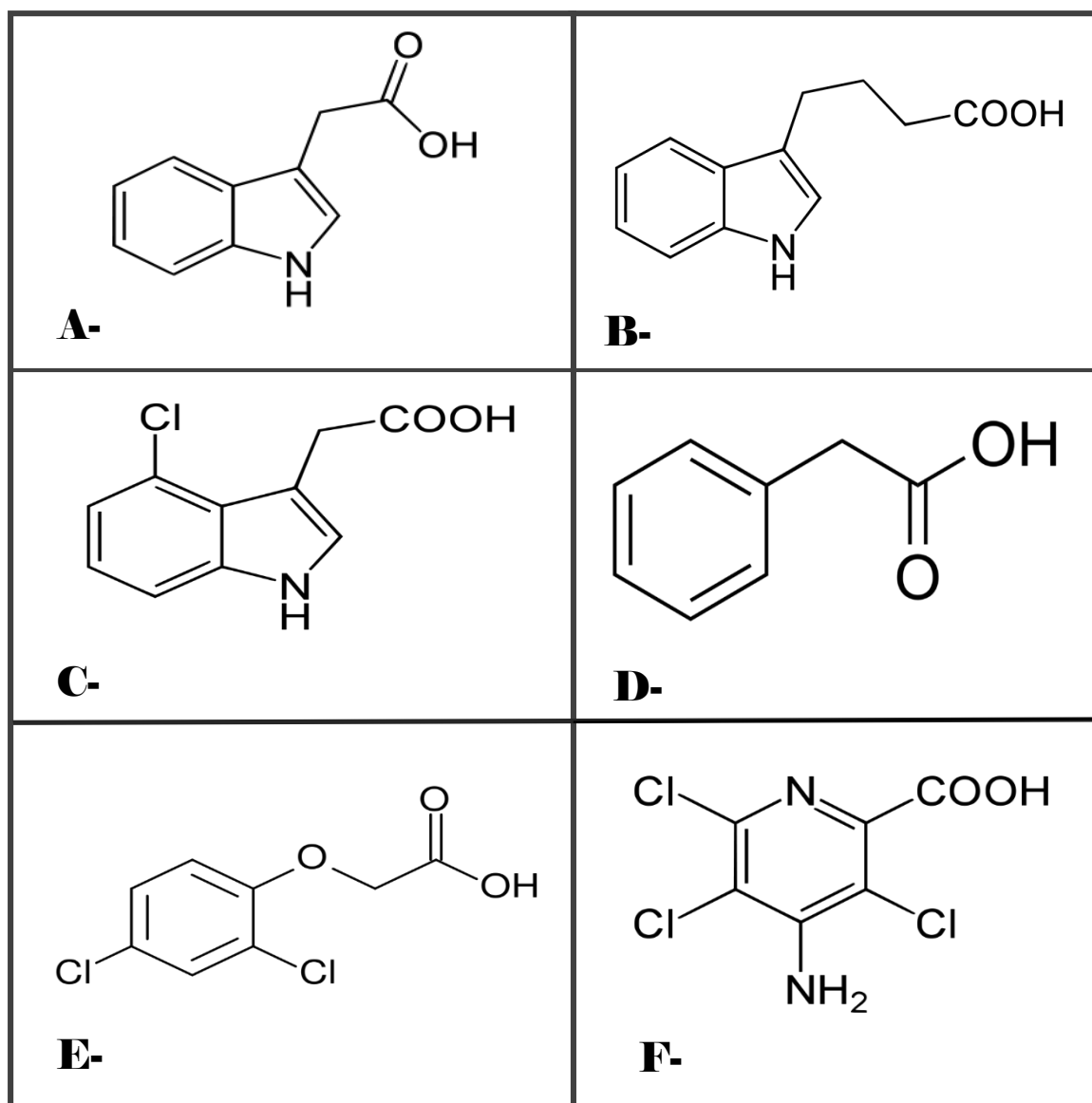
Figure 3. Chemical structure of Indoles. Features for an aromatic ring and a carboxylic acid group. Native auxins: (A) Indol-3-acetic acid (IAA); (B) indole-3-butyric acid (IBA); (C) 2-phenylacetic acid (PAA) ; 4-Chloroindole-3-acetic acid (4-CI-IAA). Synthetic auxins: (E) 2,4-diclophenoxyacetic acid (2,4-D); (F) amino-3,5,6-trichloropicolinic acid. From Canhoto (2010) [65], Caeiro (2015) [156].

Auxin

The history of auxin began in the late 19th century when Charles and Francis Darwin were studying the phototropic responses of canary grass coleoptiles. They demonstrated the existence of an "influence" which moved from the tip of the coleoptile to the region below where it controlled bending [70]. This discovery of the first plant hormone, later termed auxin (from the Greek 'auxein', which means to grow), was followed several decades later by the identification of auxin as indole-3-acetic acid (IAA) [71,72].

The first report of auxin in marine algae was in Van Overbeek, 1940 [73], but a conclusive identification of IAA, however, remained questionable until the 1970s although Avena coleoptile bioassays revealed several indoletype compounds released by alkaline hydrolysis of several seaweeds including *Fucus vesiculosus* [74].

Auxins are defined as a family of plant growth regulators involved in several physiological mechanisms, namely tropistic growth, apical dominance, embryo and fruit development or acquisition of cell totipotency [75]. Some of this mechanisms are illustrated in Figure 4.

While IAA seems to be the most physiologically important form of auxin, other natural forms exist, such as indole-3-butyric acid (IBA),2-phenylacetic acid (PAA) and 4-chloroindole-3-acetic acid (4-Cl-IAA) [76]. But these auxins are less studied and their mechanisms of action are thought to be similar to those of IAA [77].

There are also commonly known and useful synthetic auxins, such as 1-naphthaleneacetic acid (1-NAA), 2,4-dichlorophenoxyacetic acid (2,4-D) and 4-amino-3,5,6-trichloropicolinic acid (picloram) are also regularly used to mimic auxins or as herbicides [78]. The natural and synthetic auxins differ in their effective concentrations, metabolic stability and transport properties [79,80].
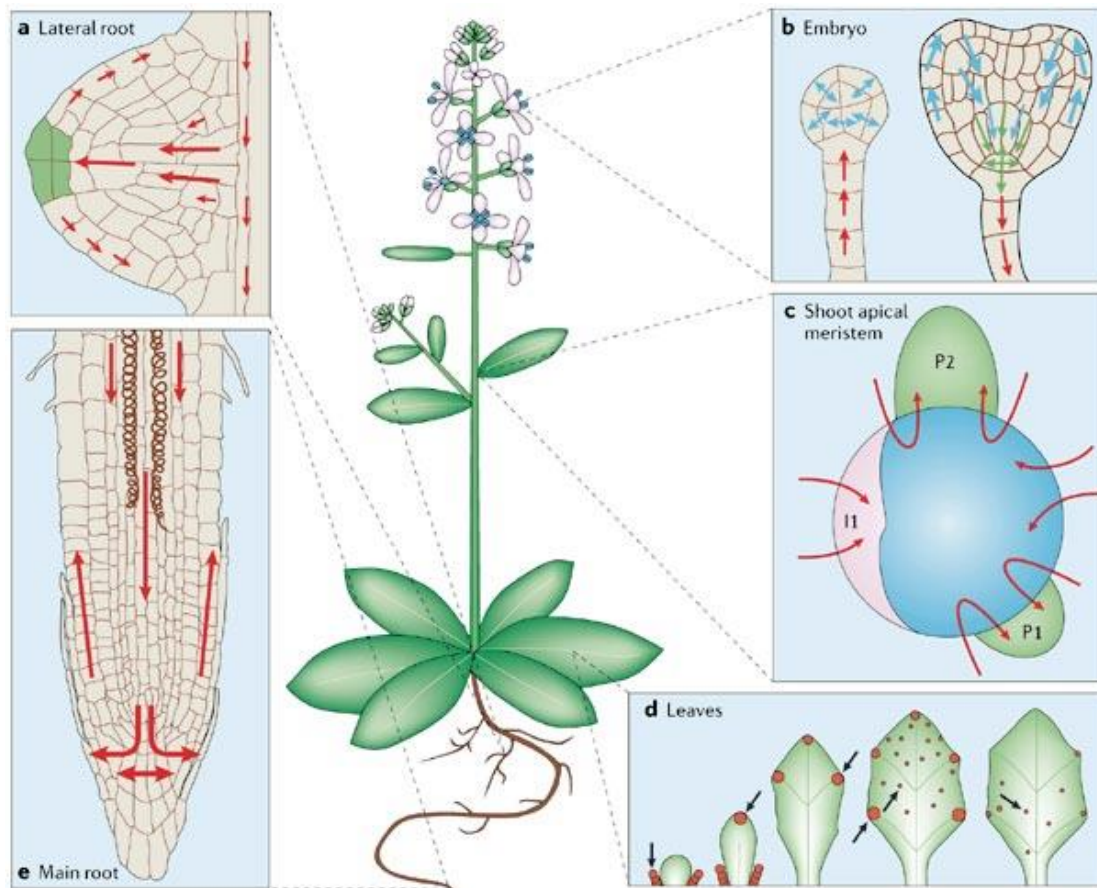
Figure 4. Some mechanisms of auxin in plant development. Teale *et* al (2006) [228].

Although virtually all plant tissues appear to be capable of synthesizing auxin, most is normally produced in young developing parts of plant such as the shoot apex, emerging leaves and developing seeds [81,82]. The details of auxin biosynthesis and its regulation remain largely elusive, but it is known that both tryptophan and indole can serve as precursors [83]. Little is known about the Trp-independent pathway (Trp-IP), but indole-3-glycerol or indole are the likely precursors [84]. The Trp-dependent pathway (Trp-DP) is better known, with several pathways being postulated, namely the indole-3-acetamide acid, indole-3-pyruvic acid, tryptamine and indole-3-acetadoxime, and with several genes putatively identified as involved in these biochemical pathways. As Trp is synthetized in the chloroplast it is likely that this organelle is closely related with the Trp-dependent pathway [85].

From places of synthesis, auxin is redistributed throughout all the plant body where it is required for a variety of developmental processes such as cell division and elongation, lateral root formation, apical dominance, leaf and flower development and tropic responses to environmental stimuli [66].

The mechanism by which auxin is transported throughout the plant has for a long time been of great interest to plant biologists. Based on the physiological, molecular and biochemical data collected so far, we know that auxin distribution throughout a plant is conducted through two physiologically distinct and spatially separated transport pathways: a fast, non-polar transport in the phloem and a slower, cell-to-cell polar auxin transport in various tissues [86].

Non-Polar Auxin Transport (nPAT).

Experiments with radioactively labeled auxin established the existence of phloem transport [87]. Labeled auxin or tryptophan (precursor of IAA), when applied directly to source leaves were rapidly loaded into the phloem and passively translocated along a concentration gradient. This mass transport occurs relatively fast, with the molecules moving at the velocity of 5 to 20 cm/h. In contrast to the phloem, xylem does not seem to play any important role in long-distance auxin movement pathway. Only traces of endogenous IAA were found in this tissue [88]. Auxin, together with other metabolites that are transported in the phloem sap, is gradually unloaded to the different sink organs and tissues where it is redistributed further by regulated, short-distance PAT. The connection between phloem-based transport and PAT has been established by experiments in pea, which showed that labeled auxin, which was transported within the phloem, was later detected in the PAT system [89].

Polar Auxin Transport.

The identification of substances that can inhibit auxin flow [90, 91] established that cellular auxin efflux is crucial for auxin transport and provided tools for further studies into the physiological importance of this process. Studies using these inhibitors combined with auxin transport experiments led, in the middle 1970s, to the

formulation of the chemiosmotic hypothesis, which proposed a mechanism by which auxin could move from cell to cell. It postulated that auxin is transported into and out of the cell through the action of specific carrier proteins [92, 93].

In the fairly acidic environment of the cell wall (pH 5.5), some IAA exists in its protonated form (IAAH). Such a neutral, lipophilic molecule is able to pass the plasma membrane via simple diffusion. In the more alkaline cytosolic environment (pH 7.0) most IAA undergoes deprotonation giving rise to polar IAA- anions. Charged, deprotonated IAA- cannot easily depart from the cell, which consequently leads to the accumulation of IAA molecules inside the cell. IAA- can leave the cell only by active efflux, presumably mediated by specific efflux carriers. An asymmetric, cellular distribution of these carriers within each cell would explain the unidirectional (polar) feature of auxin flow [86]. In addition, the existence of specific auxin influx facilitating the uptake of IAA into cells was also proposed and demonstrated [94].

This classical chemiosmotic model (Figure 5) was strengthened 30 years later by the identification and characterization of proteins for auxin efflux carriers (PIN family) and auxin influx carriers (AUX1 family) [95-97].

The influx carriers are less characterized than the PIN proteins, as the auxin cellular influx is greatly influenced by the ionization state of IAA (a weak acid). However, heterologous expression studies of the AUX1 genes on *Xenopus oocytes* have shown a pH-driven, saturable intake of IAA, confirming the importance of these carriers in the auxin transport [98].

The PIN efflux carriers are substantively more studied, in both biochemical characteristics and physiologic importance, particularly in plant models such as *A. thaliana*, for which eight PIN genes are known [99]. In terms of function, PIN proteins seem to act as secondary gradient-driven transporters, acting cooperatively with ATP –binding primary transporters [100].

The interaction between these two types of transporters, as well as the intra and extracellular chemical environment, specifically the pH, leads to an asymmetric transport of auxins to specific cells, generally referred as auxin polar transport, forming the auxin gradients needed for biological responses [101].
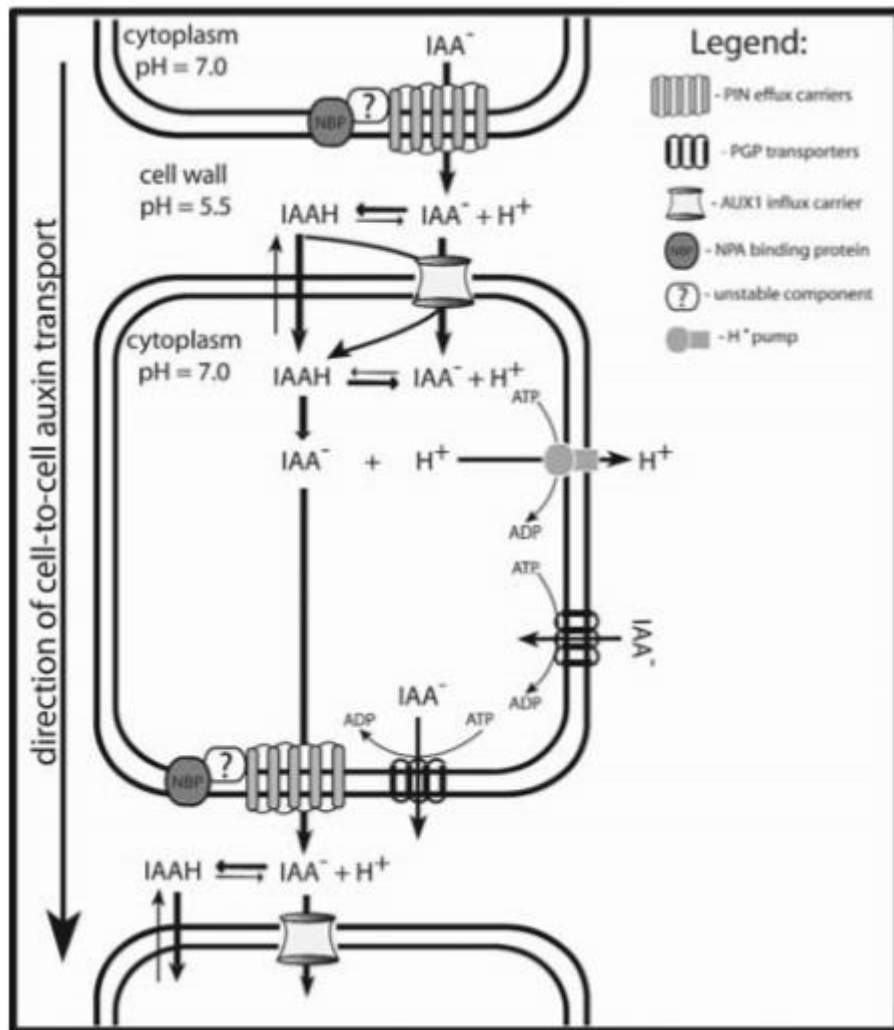
Figure 5. Model for polar auxin transport (the chemiosmotic hypothesis) [92,93].

POLYSACCHARIDES

Seaweeds contain many different polysaccharides, which chemical structure relates to the corresponding taxonomic classification of algae and their cell structure [102, 103]. Sulfated polysaccharides inhibit activity of many bacterial species as well as viruses [104]. Seaweed-derived polysaccharides are effective and non-toxic antioxidants [105]. Beyond, it can act as prebiotics (substances that stimulate the growth of beneficial bacteria in the digestive track) and exert growth-promoting and health improving effects [106]. Also, many of them are soluble dietary fibers which have positive effect on digestive track of animals (i.e. alginic acid) [107]. The contents of polysaccharides show seasonal variations. The total level of these compounds in seaweeds is up to 76% of dry weight [108]. Among brown algal polysaccharides, alginates, fucoidan and laminaran are presented and characterize these organisms.

Uronates (Alginates)

"Alginate" is the term usually used for the salts of alginic acid, although this is also commonly used to refer to all the derivatives of alginic acid and to alginic acid itself. Some authors use the term "algin" (i.e. the name given by E.C.C. Stanford to alginic acid by the time of its discovery, in the 1880´s [109]. Alginates are absent in terrestrial plants. They can be extracted from brown seaweeds, in which they constitute up to 47% of dry biomass [110].

Alginic acid is present in the cell walls of brown seaweeds, where it is partially responsible for their flexibility. In this context, brown seaweeds that grow in more turbulent conditions usually have higher alginate content than those in calmer waters [111].

Chemically, alginates are linear copolymers of β-D-mannuronic acid (M) and α-L-guluronic acid (G) (1 → 4)-linked residues, arranged either in heteropolymeric (MG) and/or homopolymeric (M or G) blocks (Figure 2) [112-114]. Alginates extracted from different sources differ in their M and G ratios, as well as on the length of each block. It is noted that more than 200 distinct alginates are presently produced [115].

Importantly, mannuronic acid residues establish β-(1 → 4) linkages, while guluronic acid forms α-(1 → 4) linkages. As a consequence, M-block segments have a linear and flexible conformation whereas the G-block segments cause a folded and rigid structural conformation, which is responsible for a pronounced stiffness of the polymer [116]. It is accepted that only G-blocks participate in the gel formation and hence, their length is a main factor affecting the functional properties of the gels [117].

Ionic cross-linking with divalent ions (e.g. calcium) is the most common method of obtaining hydrogels from an aqueous alginate solution, in a model that is termed "egg- box" [118,119]. In this model, the divalent cations are trapped in a stable, continuous and thermo-irreversible, three dimensional networks, allowing interaction with COO-groups of guluronate residues, of two adjacent G-block polymers chains (junctions). This results in a gel structure [119].

Algins/alginates are commercially available in both acid and salt forms. These are typically extracted by treating the seaweeds with aqueous alkali solutions (NaOH) [120] that converts all the alginate to the sodium salt. Later the salt is dissolved in water and separated from the seaweed residue by filtration [111, 121, 122]. The alginate salt can be transformed into alginic acid by treatment with dilute HCl [115].

About 30 years ago, almost all extraction of alginates took place in Europe, USA and Japan. This picture is now changing since the emergence of producers in China in the 1980´s [123]. Initially, this production was limited to low cost (low quality) alginate for the internal, industrial markets produced from locally cultivated *Saccharina japonica*. In the 1990´s, Chinese producers were competing in western, industrial markets to sell alginates, primarily based on low cost [124].

Alginates have several commercial applications based on their thickening, gelling, emulsifier and stabilizing abilities. They are used in the food industry for improving the textural quality of numerous products such as salad dressing, ice-cream, beer, jelly and lactic drinks, but also in cosmetics, pharmaceuticals, textiles and painting industries [125, 126].

Moreover, due to its outstanding properties in terms of biocompatibility, biodegradability, non-antigenicity and chelating abilities, the use of alginates in a

variety of biomedical applications (e.g. tissue engineering, drug delivery and in some formulations of preventing gastric reflux) is growing [115].

The binding capacity of alginates also includes cholesterol/lipids that are then eliminated from the digestive system and result in hypocholesterolemic and hypolipidemic responses, as well as an antihypertension effect [127]. Furthermore, and since alginates bind to divalent metallic ions, heavy metals taken into the human body are gelated or rendered insoluble in the intestines and cannot be absorbed into the body tissue [128].

Additional biological properties of alginates that might potentiate their applications in the future include their antibacterial activity [129], anticancer [125], antitoxic effects on hepatitis [128], prevention of obesity and diabetes [130].

Fucoidan

Fucoidans are a complex series of sulfated polysaccharides found widely in the cell walls of brown seaweeds, where they are thought to play a protective role against the effects of desiccation [129]. These polysaccharides were first isolated by Kylin in 1913 and named as "fucoidin". Presently, they are mainly named according to the IUPAC terminology (fucoidans), although other terms such as fucans, fucosans, fucose containing polymers or sulfated fucans have also been adopted [131].

For the majority of algal sources, the chemical composition of fucoidans is mainly composed of fucose and sulfate, together with minor amounts of distinct molecules, that can vary from monosaccharides (i.e. mannose, glucose, galactose, xylose,etc.), acidic monosaccharides, acetyl groups to proteins [131].

Fucus vesiculosus is the seaweed most enriched in fucoidans (up to 20% on a dry weight basis). This polysaccharide was first believed to comprise a linear structure mainly composed of (1→2)-linked 4-O-sulfated fucopyranose residues [132], but later Patankar et al. [133] rebuilt its structure model and established that the backbone of

this fucoidan was a fucose polymer, bonded through α-(1→3) with a sulfate group, substituted at C-4, in several fucose residues and with branched fucose (linked to fucose) moieties appearing in every 2-3 residues. More recently, Chevolot et al. [134] reported that the fucoidan from *F. vesiculosus* (and *Ascophyllum nodosum*) have a core disaccharide motif of Type II containing sulfate at the 2-position of the 3-linked fucose and sulfate groups on the 2- and 3-positions of the 4-linked fucose [135].

The extraction method for fucoidans can be quite simple. A native extraction with hot water can result in a good method, but an acid extraction or a combined hot acidic extraction with ethanol precipitation is the most commonly applied method [131, 136]. The variation of extraction methods is known to result in the extraction of structurally distinct fucoidans [131, 136].

The commercial importance of fucoidans is presently much lower than that of seaweed hydrocolloids; however, these polysaccharides are attracting considerable attention because of the growing market for them as bioactive polysaccharides in wide areas of applications [137]. More recently, anticoagulant and antithrombotic activities are the most studied effects of fucoidans. Commonly, the anticoagulant activity of fucoidans is mediated through the activation of thrombin inhibitors, although direct thrombin inhibition and competitive binding of fibrinogen to block thrombin's actions are also possible [137, 138].

Laminaran

Laminaran is one of the major polysaccharides found in brown algae. Their content can represent up to 32-35% of dry weight, with variations occurring between growth seasons of the seaweed [129, 139]. It has a chemical structure consisting of β(1→3)-linked glucose in the main chain and random β(1→6)-linked side-chains [140].

Laminaran can occur in soluble or insoluble forms, being the first totally soluble in cold water while the second can be solubilized with hot water [141]. The solubility is influenced by branching, with better solubility being observed for higher branched polymers [141]. Laminarans contain polymeric chains of two types, i.e, the G-chains

which are built only of Glcp residues and the M-chains, with 1-O-substituted, D-mannitol residue at the terminal reducing end [128]. Laminarans from different seaweeds may vary with regard to their structural features, such as the M:G ratio, degree of branching and molecular weight [139, 141]

Laminarin is a dietary fibre and can act as a prebiotic. Also, it has antiviral and antibacterial properties [140]. Antioxidative activity of laminarin depends on its molecular weight and chemical structure [131].

There are even feasible applications of laminarans in the agricultural field. These polysaccharides have been shown to provide protection against pathogens due to the stimulation of specific metabolic pathways of plants which result in the expression of specific compounds known to trigger the defense responses of plants to pathogens [139].

Fucus vesiculosus

The alga *Fucus vesiculosus* was first described by Carolus Linnaeus (1753) in his book *Species plantarum* [142]. In Portugal this seaweed has several common names in Portuguese, such as ´´trambolho´´, ´´estalos´´, ´´esgalhota´´, ´´bagão´´, ´´limbo-bexiga´´ [143] and ´´bodelha´´ [144]. *F. vesiculosus* classification is described in table I.

Table I. Scientific classification of *Fucus vesiculosus*.	
Empire	Eukaryota
Kingdom	Chromista
Phylum	Ochrophyta
Class	Phaeophyceae
Subclass	Fucophycidae
Order	Fucales
Family	Fucaceae

F. vesiculosus varies in color from olive green to olive brown to reddish brown to almost black and is typically about 40cm in length (although fronds can grow longer). It attaches to rocky substrates by means of a small disk-shaped holdfast. *F. vesiculosus* is characterized by the small nearly spherical gas-filled vesicles (bladders) which look like bubblewrap and occur in pairs one on either side of an obvious central midrib running along the center of the strap-like frond (Figure 6) [145].

The flattened, branching fronds can grow from one to two meters in length, and the air-filled bladders which keep the seaweed floating upright in its rocky anchorages increase its ability to photosynthesize. Typically it grows gregariously, forming dense mats of long ribbons up to one meter long and five centimeters across [145].

The appearance of *F. vesiculosus* varies depending on the environmental conditions in which it occurs; in more sheltered areas there are many air bladders, whereas there are fewer in more exposed conditions. Also, in small plants, air bladders may be entirely absent. In exposed areas, it is beneficial for *F. vesiculosus* to lack bladders, as this decreases the potential for severe damage, and minimizes the risk of it being detached and swept away [41,147].

F. vesiculosus is found on the coasts of the North Sea, the western Baltic Sea, and the Atlantic and Pacific Oceans. It occurs around the coastline of Greenland, Britain, Ireland, Norway, the Atlantic coast of France, Spain and Morocco, and the

Atlantic coasts of Canada and the United States from Hudson Bay to North Carolina [145]. The distribution is illustrated in the figure 7.

Figure 6. *Fucus vesiculosus* specimen.
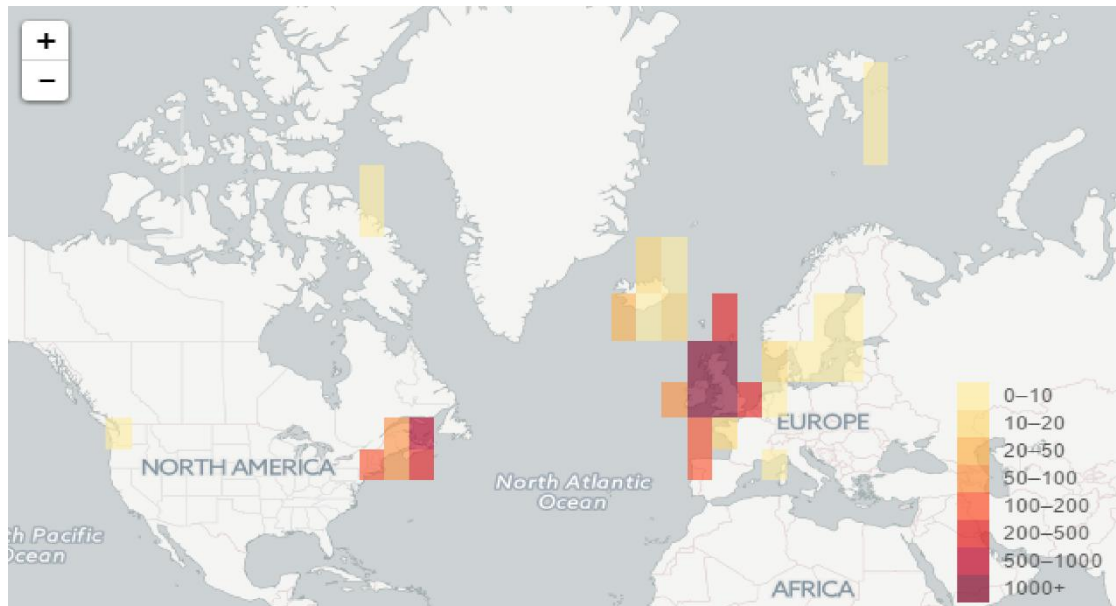
Figure 7. *Fucus vesiculosus* distribution in number of records. From Ocean Biogeographic Information System [146].

Saccorhiza polyschides

The first binary nomenclature applied to the species was Fucus polyschides [148] until be accepted by the current scientific name [149] as shown in table II. In Portugal it has popular portuguese names such as ´´carocha´´, ´´caixeira´´, ´´cintas´´, ´´golfe´´ or ´´gulfo´´, ´´limo-correia´´, ´´limo-corriola´´ [150].

Table II. Scientific classification of *Saccorhiza polyschides*.	
Empire	Eukaryota
Kingdom	Chromista
Phylum	Ochrophyta
Class	Phaeophyceae
Subclass	Fucophycidae
Order	Tilopteridales
Family	Phyllariaceae

The life cycle of *S. polyshides* is deter-mined by alternation of generations. The bo-dy in remarkable phase (diploid) is called sporophyte while another phase microscopy, the gametophyte [151].

The sporophyte has the prostrate stem dark brown color to pale, clearly differentiated blade, stipe and bulb with apteros (fastener). The blade has membranous-leathery texture divided without central nervure. The stipe is flat while its base is twisted [43, 152]. The species is an annual, and very fast growing. It is opportunistic and colonizes available hard substrata in the sublittoral [41,153]. *S.polyschides* distribution is shown in figure 7.
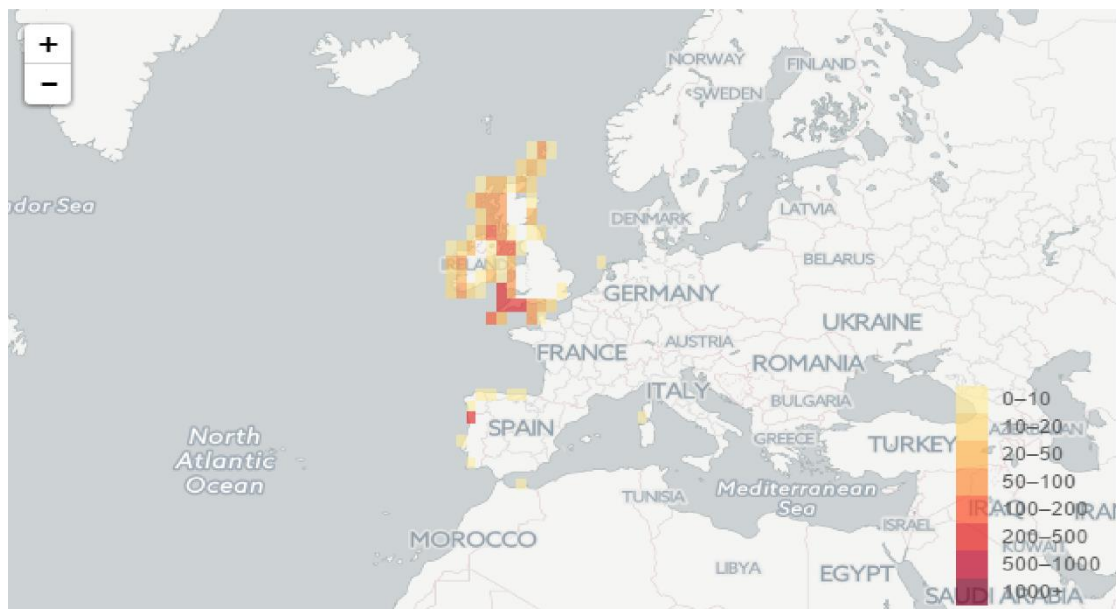
Figure 8. *Saccorhiza polyschides* distribution in number of records. From Ocean Biogeographic Information System [146].
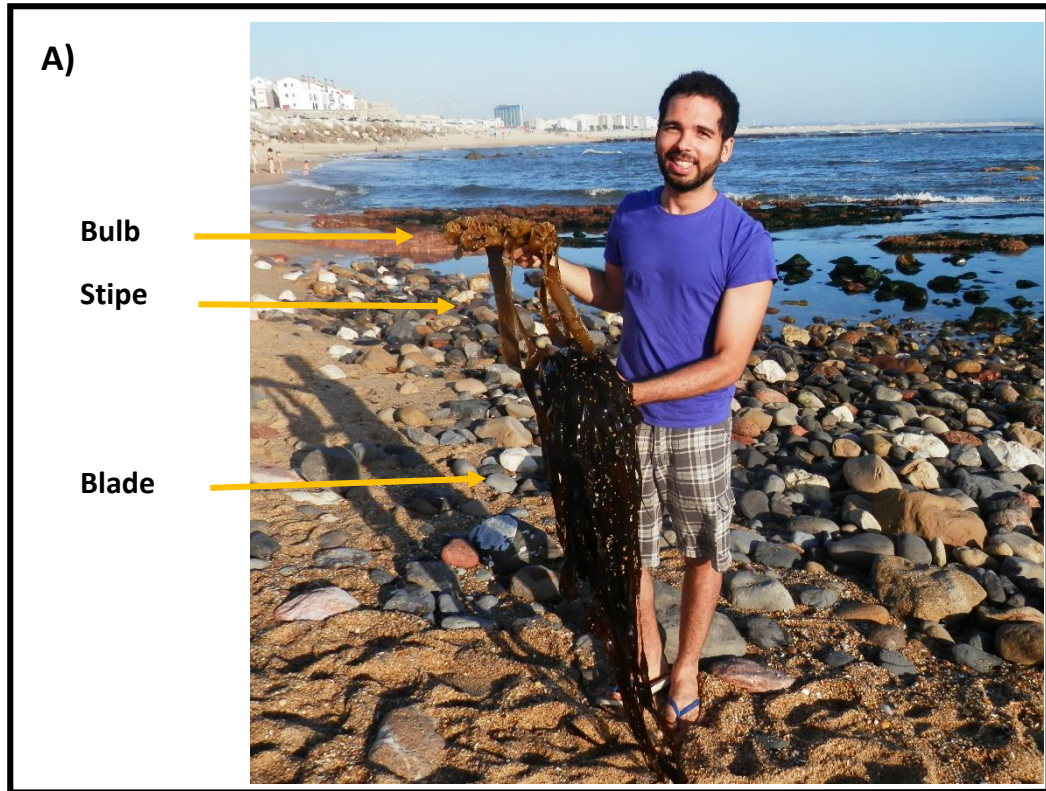
Figure 9. The adult sporophyte of *S. polyschides* in site harvest showing the distinctive morphological features of the species. A) Blade, stipe and bulb are visible.

Both species have a high percentage of carbohydrates, while the protein level is moderate and have low lipid concentration, as shown in table III. As already mentioned, the concentration of minerals in this seaweeds are higher than land plants, and is also described in table IV.

Table III. Nutritional composition of the seaweed used in the extracts. Percentage of dry weight [124, 154].

Nutritional Composition (%)		
Element	*Fucus vesiculosus*	*Saccorhiza polyshides*
Carbohydrates	46,8	45,6
Proteins	8,5	14,4
Lipids	1,9	1,1
Ash	22	28,15

Table IV. Mineral composition of the seaweed used in the extracts. Values in milligrams per ten gram of dried seaweed [124, 150, 154].

Mineral composition (mg/10g)		
Elemento	**Fucus vesiculosus**	**Saccorhiza polyshides**
Na	245-547	519
K	250-432	765,4
P	31	23,2
Ca	72-94	91,1
Mg	67-100	79,7
I	1,5	1
Fe	0,4-1	0,79
Zn	0,37	0,65
Mn	0,5	0,08
Cu	0,05	0,03

Objectives

This study aims to use techniques of biotechnology in the implementation and evaluation of a sustainable management project that use seaweed liquid fertilizer in a useful tomato crop, also enhancing the natural marine resources.

Algae compounds have great importance in many areas but are still underutilized. An example is the non-use of large biomass of algae that consistently appear in the marine ecosystem in compounds to be used in agriculture.

Portugal (continent) has a rib of about 830 kilometers with availability of algae harvesting from several centuries. However, currently alternative and sustainable methods of agricultural production are little encouraged and are hardly developed.

Extracts of algae are organic, biodegradable and stand out in sustainable agriculture. The algae used (Phaeophyceae), based on previously mentioned studies, have enough compounds to nurture and develop the plants. Many of these studies are related to plant growth regulators, and thus these compounds are featured in the work.

In addition to the benefits already described, the use of algae in agriculture has had a growing popularity, not only because of the benefits of organic farming but also to the effects of chemicals in the environment.

Products related to the algae are diverse and these organisms have great prominence in the aquatic ecosystem. Thus, studies are required for their correct application to ensure the least possible impact on the system they inhabit.

Then, intended to evaluate the potential of seaweed liquid fertilizers, through its acting in plant development, observing the real benefits and prospects of seaweeds compounds as agricultural fertilizer. For this, tests will be made with tomato (*Solanum lycopersicum*) using extracts from *Fucus vesiculosus* and *Saccorhiza polyschides*.

2- Material and Methods

Material harvest

The seaweeds used in this study were _Fucus vesiculosus_ and _Saccorhiza polyschides_ (Heterokontophyta, Phaeophyceae) and were collected from the Buarcos Bay, coastal area of Figueira da Foz, Portugal. The first seaweed (_F.vesiculosus_) was collected from the Mondego estuary and the other from intertidal zone at low tide in September 2015. The collection sites are shown in figure

Seaweed species were collected by hand and washed with seawater to remove debris, adhering sand particles and epiphytes. The samples were transported to the macro algae laboratory at University of Coimbra in plastic bags, and washed with tap water to remove surface salt.
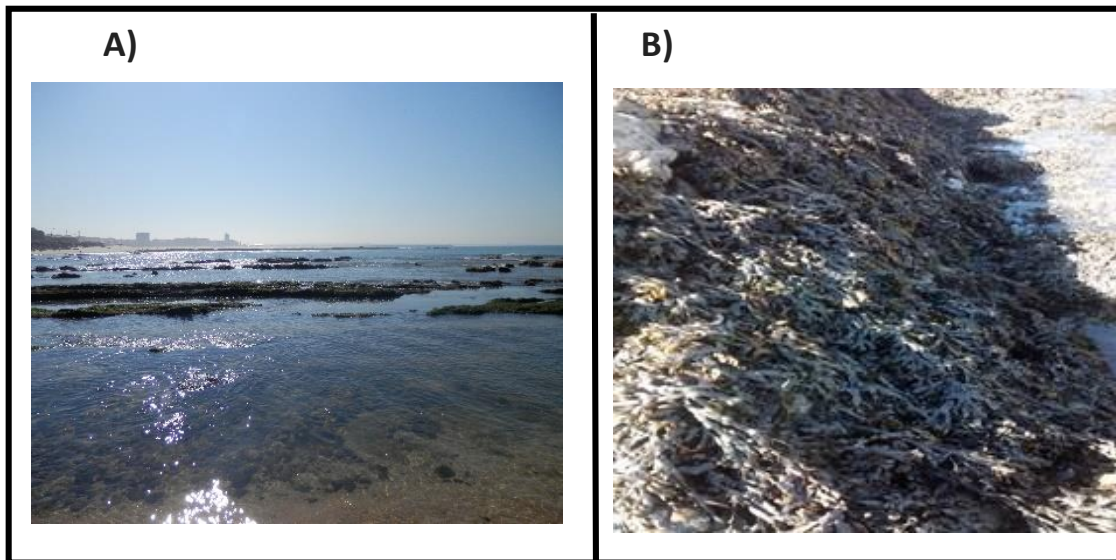
Figure 10. Harvest sites (Portugal). A) Intertidal zone of Buarcos Bay. B) Mondego estuary, with a large amount of _Fucus vesiculosus_.

The crude seaweed extracts were obtained from fresh seaweeds. The samples were then cut with scissors aid and triturated with distilled water to reduce the particles. Half a litter of distilled water is poured into a blender with 300 g of algae. The material was then filtered through a sieve and what goes through the filter paper is stored in airtight bottles in a refrigerator at 4 °C and the remainder excluded. All material obtained was considered a 100% extract concentrate which will be diluted to obtain the different concentrations.

Another part of the seaweeds were, after washing in a laboratory, taken drying. For this, each specie was placed in petri dishes, and stored in an oven at 60 °C for 48 h. After treatment, the dry seaweeds were ground to fine powder by electric grinder. The entire milled sample was stored in sealed jars and protected from light.

Seaweeds Serial Extraction

The standard bioassay as described by Carvalho (2013) [157] was used in order to acquire different compounds of seaweeds. The extraction is a sequenced extraction procedure where the sample used is the same from the beginning to the end of the process. For the procedure, the initial amount of algae (milled) to be extracted is weighed to begin work.

The method takes three extracts, composed of non-polar substances, polar and polysaccharides. The extraction is sequential with a ratio of 1 g of seaweed to 20 mL of hexane and also to methanol, both at room temperature. The aqueous extraction is, however, prepared in the ratio of 1:100 and 100 °C.

In the first step of the extraction, algae and the hexane is added in a goblet which is stirred with magnet for 20 min. After this time, vacuum filtration is used to aid silica filter funnel with porosity G3 and G4 coupe kitasato flask.

Under vacuum, the solvent with nonpolar compounds are transferred to the kitasato and stored, as the initial sample is retained on the silica filter. The process is repeated until the solution becomes translucent (about three times), because pigments are an indicator of organic compounds in the solvent.

After the last extraction with hexane, the sample contained in the filter is placed in another goblet for initiating the extraction with methanol. The second extraction with the aid methanol for extracting non-polar compounds is carried out under the same conditions of the previous and the solvent obtained, stored with their identification.

The aqueous extract obtained is sequenced through the material retained on the last extraction filter with methanol. This aqueous extraction sequenced obtains

polysaccharide and include distilled water as the solvent, which is previously prepared in a goblet and is treated in the heating plate to the temperature of about 100 °C. After reaching temperature, the sample is added and held at room temperature for two hours.

Samples in hexane and methanol are treated in the rotary evaporator (vacuum trunk and water bath) concentrating the material and for the solvent does not interfere in plant development. These materials are referred to Seaweeds Evaporated Serial Extraction (SESE).

The dry seaweed, Seaweed Serial Extraction (SSE) and Seaweeds Evaporated Serial Extraction (SESE) were used at measurement of the wavelength on spectrophotometer to quantify indole compounds by Ehrlich Reaction.

Oat coleoptile cylinder bioassay

The bioassay was developed based on Nitsch and Nitsch (1965) [158] and conducted at the Laboratory of Plant Biotechnology, Departmet of Life Sciences at the University of Coimbra. This test for auxin with oat coleoptile is used as a sensitive method for quantification of indol-3-acetic acid, through the study and development of the oat coleoptile in different concentration of the plant hormone.

The oat seeds are germinated in the dark for 3 days. When the coleoptiles reach about 2.5 cm in length, they are selected in order to keep the same size between them. After, each sample is observed by a magnifying glass for cutting sections of 4 mm, always with a distance of 3 mm from its apex.

The coleoptiles are then placed in a petri dish with distilled water to avoid dehydration, because the number of samples to be cut are large, very accurate and it takes some time for the preparation of all sections. When completed the cut, all these sections of the coleoptiles are floated on glass distilled water containing 1 mg / $MnSO_4$. H_2O for 3 h.

After the period (3 h), ten sections are placed in 2mL of the solutions to be tested, which should contain 2% sucrose plus a buffer at pH 5 (K_2HPO_4 1.794mg/L + citric acid monohydrate 1.019 mg/L). The pH was adjusted to 5.8 (using KOH or HCl solutions).

The auxin concentrations tested were 10, 20, 30, 40 and 50 µg/mL, while the algae sample (extract applied on the plants) diluted 20% with K_2HPO_4. Under these conditions, the samples are incubated in the dark at 25 °C for 20 h and are then measured, again with the assist of a magnifying glass. From the different and controlled auxin concentration tested, can relate the coleoptile growth and the plant hormone concentration of the seaweed liquid fertilizer.

Ehrlich Reaction

The indole content (auxin) in the seaweed extracts was performed based on Antony and Street (1970) [155], optimized by Caeiro (2015) [156] and took place at the Laboratory of Plant Biotechnology, Department of Life Sciences, University of Coimbra.

The amount of indole (the molecular structure is shown in figure 11) in seaweeds is obtained by a colorimetric reaction (Ehrlich reaction) which occurs in a compatible sensitivity between a linear concentration of compounds and its absorbance (Beer-Lambert law). This experiment is sensitive and has a limit of detection of 2 µg/mL and 50 µg/mL. Therefore, it is often necessary previous dilution of the material to be quantified.

For preparation of the reaction, 2 g of p–dimethylaminobenzaldehyde (PDAB) (the molecular structure is shown in figure 12) were dissolved in 100 mL of Hydrochloric acid (HCl) 2.5 M. The preparation of Trichloroacetic acid 100 % (w:v) (TCA) is made with distilled water at ratio of 500 g to 227 mL.

Figure 11. 3D Molecular Structure of indole [159].

Figure 12. 2D Molecular Structure of PDAB [190].
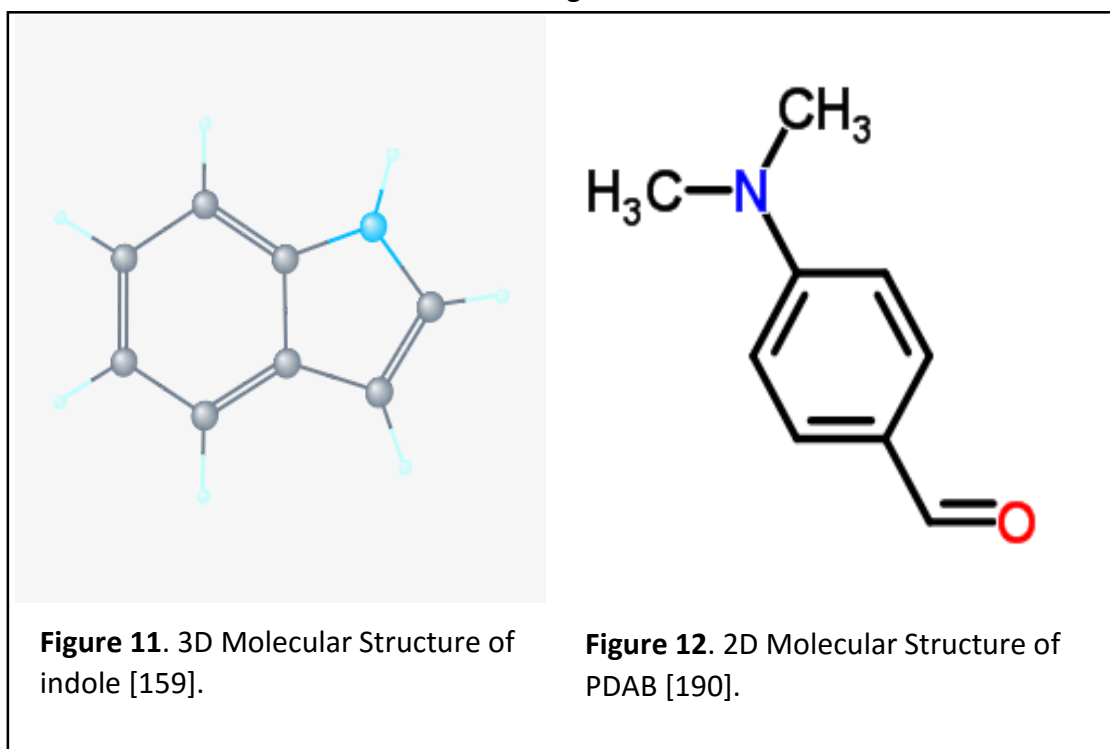
The seaweed was grounded in a sterilized mortar with 2 ml of K-phosphate buffer 0.01 M (pH 6.0) and centrifuged (4800 g; 20 min). After centrifugation, the supernatant was used for the quantification.

When mixing 1 mL of the extract to be analysed, 2 mL of the solution of TCA and 2 mL of PDAB, the reaction starts and it is necessary 30 minutes to make the analysis. Throughout the half hour the sample is kept in the dark to avoid degradation of the compounds, whereas indole compounds are photosensitive.

After the specified time, the color intensity in each sample is analyzed in a Jenway 7305 spectrometer at 530 nm.
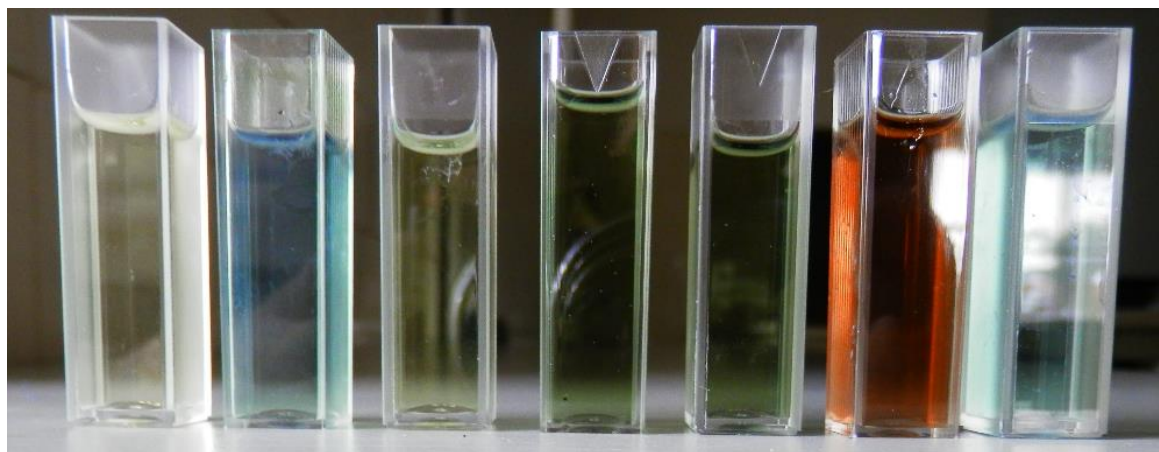
Figure 13. Samples after Ehrlich reaction.

The auxin quantification assay was carried with the Macerated, Crude Aqueous Extract (CAE) and serial extract from *Fucus vesiculosus* and *Saccorhiza polyschides*. The samples were diluted and analysed (Standard Curve) but the CAE of *S. polyschides* did not fully agree with the method. Therefore, required new assay with controlled addition of auxin (Standard Addition).

Tomato Germination

The tomato germination bioassay measures the total seed germination when exposed to different concentrations of seaweed liquid fertilizer. The test was conducted at the Laboratory of Plant Biotechnology, Department of Life Sciences at the University of Coimbra.

Experiments were conducted using tomato seeds (*Solanum lycopersicum*) from "Flora Lusitana, Lda". Before treatment with SLF, tomato seeds were surface-sterilized in 7% calcium hypochlorite solution for 10 min and subsequently triple-rinsed in sterile (autoclaving) distilled water prior to soaking in the specific seaweed extract.

In laminar flow hood, 30 tomato seeds were placed on a Whatman No 5 filter paper in sterilized Petri dishes and then treated with 10 mL distilled water (control) or different seaweed extract: 5%, 10%, 15%, 20% and 25% (with 3 repetitions). The plates were incubated at 25 ±1 °C and 16-h light/8-h dark regime. Germination was observed daily over a period of 8 days according to methods of the Association of Official Seed Analysts [199].

Measured variables included germination percentage (GP), seedling vigor index (SVI), plumule length, radicle length and total plant height of tomato seedlings. Parameters were calculated as follows: GP=(number of germinated seeds/total number of seeds)×100. SVI was determined according to Orchard (1977) [202] by the following formula: SVI= seedling length (cm)×germination percentage). After 14 days, the SLF effects on seed germination and growth of tomato seedlings were measured with string and ruler.

Greenhouse Growth Bioassay

The bioassay was developed in the greenhouse at Botanical Garden of University of Coimbra. Tomato plants at 10 weeks were arranged in plastic pots with 14 cm diameter and 26 cm depth under 14-h light regime at 25°C and dark regime at 18°C in sterilized soil peat moss (*Biohazard*). Five plants were randomly selected for the assay.

Potted plants were grown for seventeen weeks and treated with water (control) or SLF. To determine the amount of water or seaweed extract applied to the plants was measured field capacity of the substrate (expressed symbolically as θfc). The plants were treated every other day with θfc 60% (1600 mL).

The morphological characteristics were evaluated weekly. Measured variables included root length, shoot length, total length, and the number of flowers and fruits.

Statistical analyses

In the case of homogeneity of variances, the data was analyzed with one-way analysis of variance (ANOVA) and, where necessary, the means were compared by Tukey test ($p < 0.05$).

3. Results

Oat Coleoptile Cylinder Bioassay

The crude aqueous extract was tested using coleoptile cylinder bioassay to affirm and quantify the auxin concentration. The final size of the coleoptile treated with extract from *Fucus vesiculosus* was 5 cm, and the auxin concentration result was 97µg/mL, while in *Saccorhiza polyschides* 4.6 cm and 42 µg/mL, respectively. The development or not of the coleoptile is shown in the figure 13, while the standard curve that relates growth of the coleoptile with different auxin concentrations are shown in figure 14.
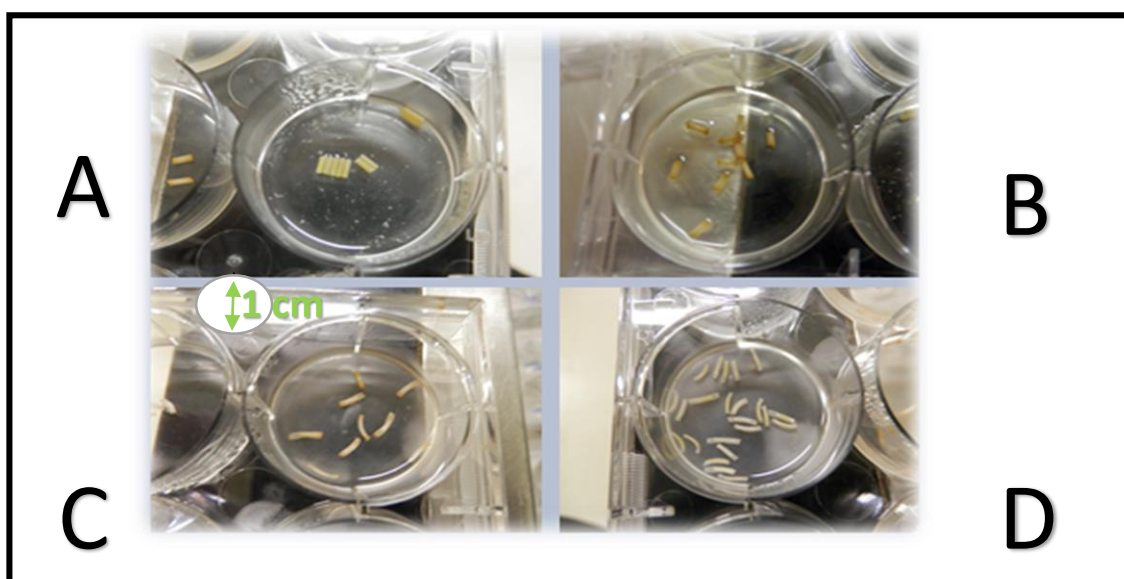
Figure 14. Coleoptiles soaked with solutions to be tested. A) Seaweed serial extraction with hexane. B) Seaweed serial extraction with methanol. C) *Fucus vesiculosus* crude extract. D) *Saccorhiza polyschides* crude extract.

One second assay was developed using the serial aqueous extract and the final size of the coleoptile treated with extract from *Fucus vesiculosus* was 5,50 cm and the auxin concentration result was 148,72µg/mL while in *Saccorhiza polyschides* 5,56 cm and 156,20µg/mL., respectively. The development of the coleoptiles are shown in the figure 15, while the standard curve that relates growth of the coleoptile with different auxin concentrations are shown in figure 16.
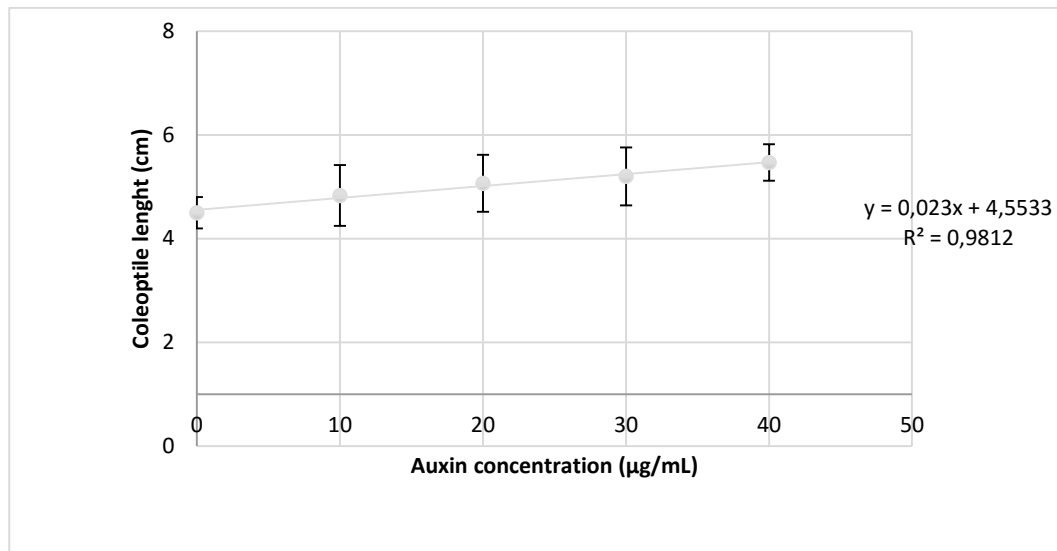
Figure 15. Oat's coleoptile development at different auxin concentrations. Bio-assay for testing the crude aqueous extract.
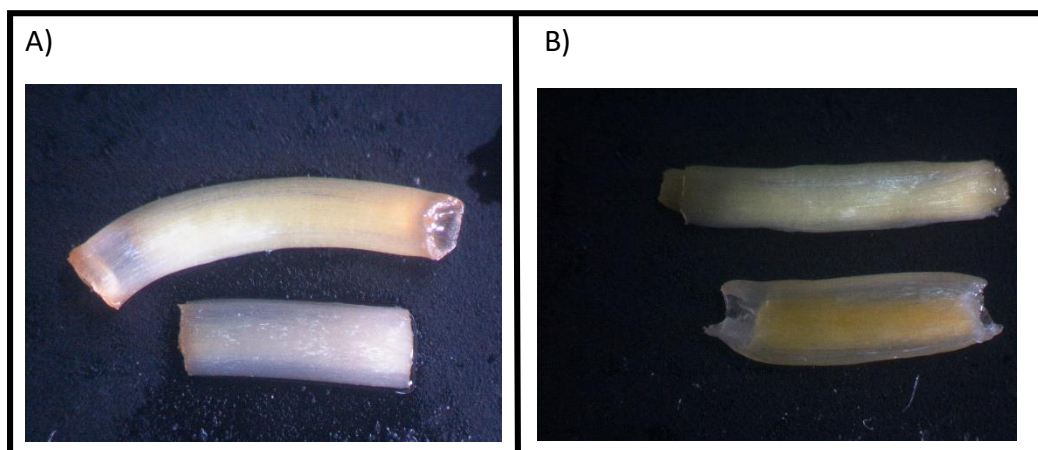
Figure 16. Oat coleoptile elongation assay. A) Oat coleoptile after treatment with *Fucus vesiculosus* (above) and control (below); B) Oat coleoptile after treatment with *Saccorhiza polyschides* (above) and control (below).
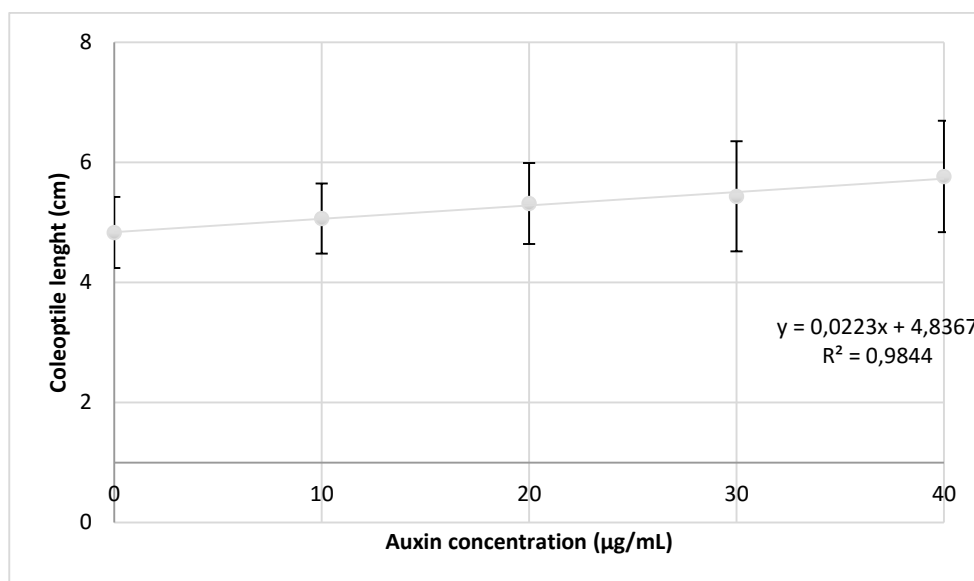
Figure 17. Oat's coleoptile development at different auxin concentrations. Bioassay for testing serial aqueous extract.

Ehrlich Reaction

The auxin quantification assay showed a higher amount of indole compounds for *Fucus vesiculosus*. The values of the quantification of the plant hormones for *F. vesiculosus* are shown in the table V.

Table V. Auxin quantifying by Ehrlich reaction.

Algae	Analytic solution	Value (average ± SD)	Unit
Fucus vesiculosus	Crude Aqueous Extract	331± 0.008	µg/mL
	Macerated	5524 ± 2.038	µg/g
Saccorhiza polyschides	Crude Aqueous Extract	80.314 ± 40.593	µg/mL
	Macerated	407 ± 0.155	µg/g

After this first assay, the quantification of auxin was performed on extracts of serial extraction, that is, the extract obtained from hexane, methanol and distilled water. The first serial quantification assay was done with 5g of *Fucus vesiculosus*. The *Saccorhiza polyschides* assay has been optimized for 1g. While the macerated extract of the two algae could be quantified, the crude aqueous extract of *Sacccorhiza polyschides* had to be calibrated for being so diluted and the colorimetric method is little sensitive to the extract.
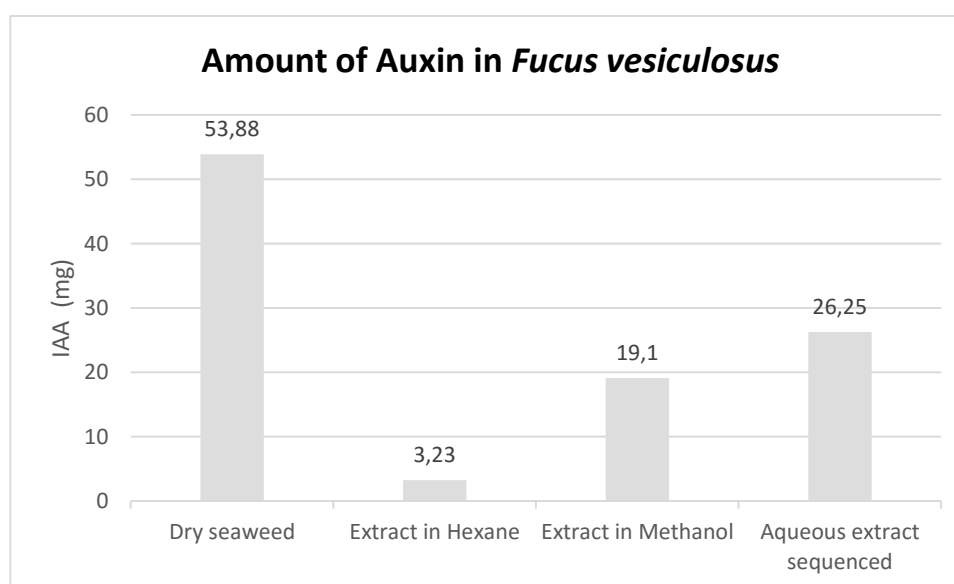
Figure 18. Amount of IAA in different extractions from 5 g of seaweed.

Saccorhiza polyschides showed hyper quantification. Thus, to confirm the solvent that interfered with the reaction was developed a Standard addition using hexane or methanol as control. Different auxin concentrations were tested. The values of the quantification of the plant hormones for *S. polyschides* are shown in the table VI.

The auxin concentration in the first Serial Hexane Extract (SHE) of *S. polyschides* was 103,85µg by standard curve and 108,26µg by standard addition, while *F. vesiculosus* 16,85µg and 32,33µg, respectively.

The auxin concentration in the first Serial Methanol Extract (SME) of *S. polyschides* was 129,78µg by standard curve and 82,78µg by standard addition, while *F. vesiculosus* 129,85µg and 188,65µg, respectively.

The auxin concentration in the Serial Aqueous Extract (SAE) of *S.polyschides* was 156,20µg and *F. vesiculosus* 52,2µg, both assays performed by standard curve.

Table VI. Auxin quantifying in 1g of *Saccorhiza polyschides*.

Algae	Analytic solution	Value (average ± SD)	Unit
Saccorhiza polyschides	Macerated	407 ± 0.155	µg/g
	Serial Hexane Extract	103,85	µg/ml
	Serial Methanol Extract	129,78	µg/ml
	Serial Aqueous Extract	156,20	µg/ml
	Amount	346	µg

Rotary Evaporator

The amount of auxin in 1 mL of the SESE of *S.polyschides* was 740,77µg, and *F. vesiculosus* was concentrated in 443,41µg. The amount of auxin in 1 mL of the the SME of *S.polyschides* was 188,65µg, and *F. vesiculosus* concentrated in 617,81µg.

Tomato Germination

The germination test using tomato seeds showed a morphological difference between different concentrations was treated with the seaweeds. The results obtained when the plants are treated with the crude aqueous extract made from *F.vesiculosus* area shown in the figure 18.
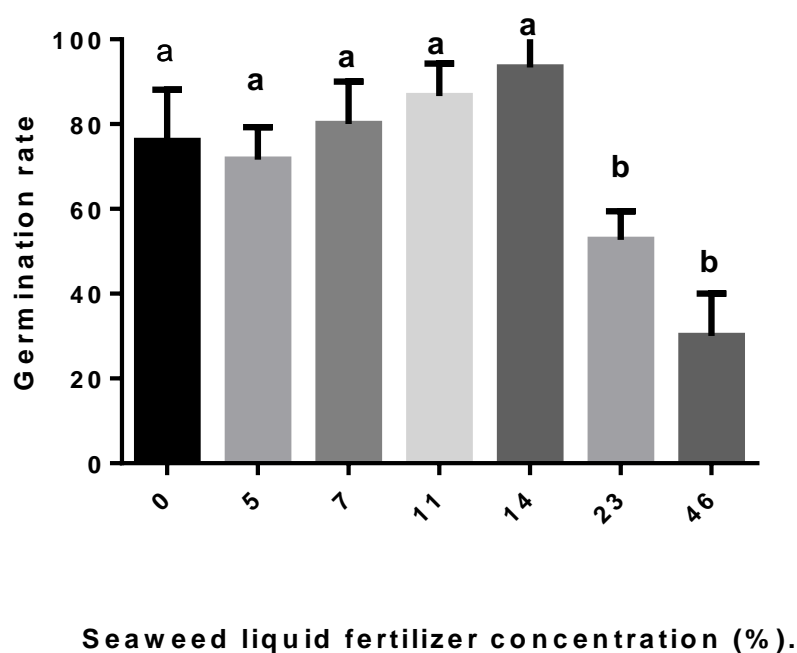
Figure 19. Tomato germination after treatment with *Fucus vesiculosus* extract.

The first germination assay was developed using *Fucus vesiculosus* and showed improved germination rate when apply 14% of SLF. As the concentration increases there is an inhibition in germination rate. When the seeds are watered with a concentration of 23% of the seaweed, there is an inhibition of germination by fifty percent.

The germination assay using the seaweed *Saccorhiza polyschides* showed no significant enhanced germination rate. And when the seeds are watered with a concentration of 27% of the seaweed, there is an inhibition of germination by fifty percent. The germination rate obtained in the assay when the plants are treated with the crude aqueous extract made from S.polyschides are shown in the figure 19.

Seaweed liquid fertilizer concentration (%).
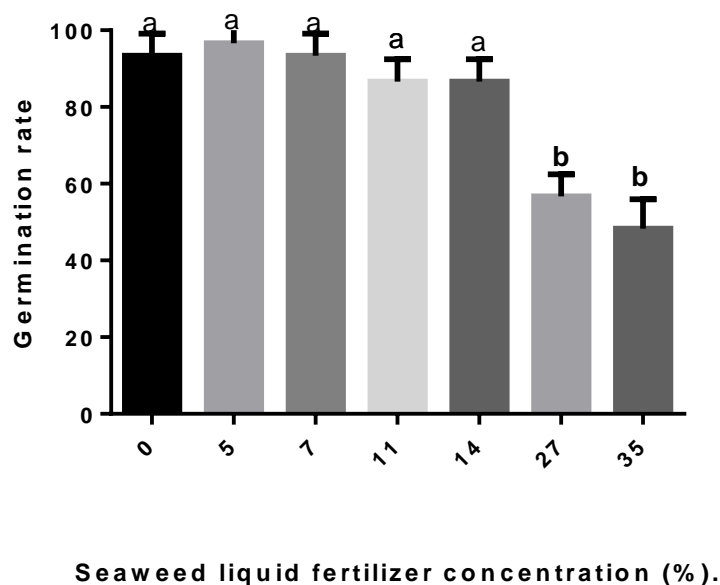
Figure 20. Tomato germination after treatment with *Saccorhiza polyschides* extract.

After these two bioassays (considered preliminary), another was developed for testing the germination with application of the both seaweed extracts.

The germination assay was also developed by applying a mixture of the both seaweeds, *F.vesiculosus* and *S.polyschides*. In this assay the concentrations used of the seaweed liquid fertilizer was 5%, 10%, 15%, 20% and 25%. The germination of the tomato seeds were monitored over 8 days and the results obtained are shown in table VII.

Table VII. Germinated tomato seeds over days.

Day	Number of Germinated Seeds					
	Control	Seaweed liquid fertilizer concentration				
		5%	10%	15%	20%	25%
1	----------	----------	----------	---------	----------	----------
2	----------	----------	----------	----------	----------	----------
3	2	----------	----------	----------	----------	----------
4	7	3	----------	----------	---------	----------
5	10	8	3	2	2	2
6	17	13	10	9	7	7
7	20	16	15	16	13	11
8	23	22	25	27	25	17

Day	Number of Germinated Seeds					
	Control	Seaweed liquid fertilizer concentration				
		5%	10%	15%	20%	25%
1	----------	----------	----------	---------	----------	----------
2	----------	----------	----------	----------	----------	----------
3	5	----------	----------	----------	----------	----------
4	10	4	1	2	2	----------
5	16	9	4	4	3	2
6	20	13	12	10	9	7
7	23	16	21	18	17	13
8	25	21	27	26	24	18

Day	Number of Germinated Seeds					
	Control	Seaweed liquid fertilizer concentration				
		5%	10%	15%	20%	25%
1	-----------	-----------	-----------	----------	-----------	-----------
2	-----------	-----------	-----------	-----------	-----------	-----------
3	2	-----------	-----------	-----------	----------	-----------
4	8	4	-----------	-----------	-----------	-----------
5	13	7	5	7	4	2
6	19	15	10	13	9	8
7	23	17	17	22	16	15
8	24	20	26	27	23	15

At the end of the assay (14 days), the following parameters were measured and are described in the table VII.

Table VIII. Effects of different concentrations of seaweed liquid fertilizer treatment in the germination of tomato seedlings.

PARAMETERS	CONTROL	SEAWEED LIQUID FERTILIZER (%)				
		5	10	15	20	25
GERMINATION PERCENTAGE (GP)	80 ± 3.3	70 ± 3.3	86,7 ± 3.3	88,9 ± 2	80 ± 3.3	55,6 ± 5
TOTAL PLANT LENGTH (CM)	7.3 ± 0.7	7.0 ± 0.7	8.7 ± 0.4	9.1 ± 0.6	9.3 ± 0.8	10.1 ± 0.7
PLUMULE LENGTH (CM)	3,1 ± 0.4	3.0 ± 0.4	3.7 ± 0.3	4.0 ± 0.2	4.0 ± 0.3	4.6 ± 0.4
RADICLE LENGTH (CM)	4,2 ± 0.5	4.0 ± 0.6	5.0 ± 0.4	5.2 ± 0.5	5.3 ± 0.5	5.6 ± 0.4
SEEDLING VIGOR INDEX (SVI)	584 ± 2.3	490 ± 2.3	754.3 ± 1.3	809 ± 1.2	744 ± 2.6	561.6 ± 3.5

The figure 20 illustrates after 14 days, the seedlings of tomato after treatment with 10% of seaweed liquid fertilizer.

Figure 21. Seedlings of tomatoes treated with 10% SLF.

Greenhouse Bioassay

A seaweed liquid fertilizer made from *Fucus vesiculosus* and *Saccorhiza polyschides* were applied to evaluate their effect as seaweed biofertilizer on growth and production of *Solanum lycopersicum.*

Tomato plants treated with 5% SLF showed no significant difference in the vegetative structures with control. However, with a concentration between 10% and 15% of SLF there is a response with a higher plant growth in the number of leaves and root length. The reproductive structures of plants treated with SLF showed differences both in the number of flowers as well as fruit in all concentrations tested. The results are shown in the following table.

Table IX. Effects of different concentrations of seaweed liquid fertilizer treatment on the growth parameters of tomato plants after 17 weeks. The number of repetitions of each concentration and control were 5 plants.

PARAMETERS	CONTROL	SEAWEED LIQUID FERTILIZER (%)				
		5	10	15	20	25
TOTAL PLANT LENGTH (CM)	101,8	101,2	133,2	142	149,2	135,2
SHOOT LENGTH (CM)	73.8 ± 15.2	71.2 ± 8.1	87.6 ± 5.0	101.8 ± 5.8	107.6 ± 14.4	92.2 ± 10.5
ROOT LENGTH (CM)	28 ± 2.6	30 ± 6.2	45.6 ± 8.1	40.2 ± 10.0	41.6 ± 7.4	43 ± 4.7
NUMBER OF LEAVES	19.4 ± 4.6	28.2 ± 2.4	48,6 ± 9.9	40.6 ± 4.7	41.6 ± 7.9	46 ± 11.6
NUMBER OF FLOWERS	24	52	48	29	65	68
NUMBER OF FRUITS	0	0	5	6	54	51

The first parameter evaluated was shoot length. Control plants had an average of 73.8 cm, which was even higher than the plants treated with 5% SLF (71.2 cm). A comparison between the two plants is shown in figure 21. However, increasing concentration of SLF (15%), the average length of the shoot length reached 101.8 cm.

Tomato plants treated with 10 until 25% by SLF drew special attention by the number of leaves per plant: 48.6± 9.9 and 40.6± 4.7, respectively (see figure 22). Furthermore this treatment (between 10% and 25%) stimulate root elongation of plants. All tomato plants treated with SLF increased the number of flowers. But what draws more attention is the amount of fruit on the plants treated with 20 and 25% of

SLF: 54 and 51, respectively. Fruits of tomato plants treated with 25% of seaweed liquid fertilizer are shown in figure 23.

Figure 22. Comparison between control plant (left) and treated with 5% of SLF.

Figure 24. Tomatoes of plants treated with 25% SLF

Figure 23. Tomato plant treated with 15% SLF.

4. Discussion

Seaweeds have been used since antiquity either directly or in composted form as a soil amendment to improve the productivity of crops in coastal regions (35, 160-162). The earliest reference to seaweed manure is in the second half of the first century when the Roman Columella recommended that cabbages be transplanted at the sixth leaf stage and their roots be mulched and manured with seaweed. Pre-Roman Britons also added seaweed to soil as manure. Depending on regional practices, seaweeds were mixed directly with sand or soil, or composted with straw, peat, or other organic wastes for later use (38,163).

The observed benefits to the growth, health and yields of plant crops were traditionally attributed to the supply of essential nutrients and to improved soil texture and water holding capacity. The ability of liquefied seaweed extracts to maintain enriched trace metal mixtures (Cu, Co, Zn, Mn, Fe, Ni as well as Mo and B) in a soluble form for application to soils or for use as foliar sprays was clearly understood in 1962 (164).

Over the decades of 60 and 70 a large number of reviews have shown the variety of responses of plants growing in soil treated with seaweed fertilizer (165-169) and realize that mineral nutrients are not sufficient to provide the improvements observed in plants. Francki (170) suggested that increased trace element supply could explain some of the beneficial effects of using seaweed preparations.

Drought, salinity and extreme temperatures can reduce the yield of crops and restrict agricultural production. These abiotic factors are manifested as osmotic stress and cause secondary effects like oxidative stress, leading to an accumulation of reactive oxygen species (ROS) such as the superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) [220].These are known to damage DNA, lipids, carbohydrates, and proteins and also cause aberrant cell signaling [221].

Marine algae can serve as an important source of plant defense elicitors [222]. Plants protect themselves against pathogen invasion by the perception of signal molecules called elicitors which include a wide variety of molecules such as oligo and polysaccharides, peptides, proteins, and lipids, often found in the cell wall of attacking

pathogens [223]. A variety of polysaccharides present in algal extracts include effective elicitors of plant defense against plant diseases [54].

For exemple, a seaweed extract, Kelpak 66, is reported to reduce root damage from nematode (*Meloidogyne incognita*) predation in tomatoes whether applied to the foliage or as a soil drench in a 1:500 dilution [224].

In view of the low rates of application needed to elicit a physiological response it was suggested that organic compounds, rather than mineral elements, are responsible for yield increases. Loss of stimulatory action after ashing seaweed extract supported this view [171]. Ashing the sample removes nitrogen from seaweed, lowering the concentration of indole compounds, such as auxin, which also may reduce the stimulatory effect.

Over the years, some authors have shown that seaweed products contain certain Plant Hormones (172-176). But a conclusive identification (of IAA), however, remained questionable until the 1970s although *Avena coleoptile* bioassays revealed several *indoletype* compounds released by alkaline hydrolysis of several seaweeds including *Fucus vesiculosus* and *Ascophyllum nodosum* (74).

Hence it was developed an assay for the confirmation of bioactivity and quantification of auxin in algae extracts.

Evidence for the presence of Auxin in Seaweeds.

The procedure that was conducted with oat coleoptile showed a low sensitivity (42 µg/mL was the lowest quantification). The proportionally range was good, although the relationship between growth and auxin concentration.

The specificity for auxin is reasonable, because certain substances such as arginine, glutamic acid, methionine, glutathione, etc. also stimulate to some extent the growth of coleoptile sections.

The results reflect the care taken in cutting the sections exactly where they should be cut and of exactly the same length. But the reliability can prove the existence of indole compounds in the brown seaweeds.

Extracts prepared from hexane and methanol showed no bioactivity for coleoptile treatment and instead inhibited growth, probably due to the toxicity of the solvents (Figure 15 – A and B).

Based on the growth of oat coleoptile treated with macerated and crude aqueous extract, the procedure proved the existence of auxin in the brown seaweeds: *Fucus vesiculosus* and *Saccorhiza polyschides*. And confirmed, in conjunction with Tarakhovskaya et al (2007), Provasoli, L and Carlucci (1974), Stirk, W.A. and van Staden (1997), Basu et al (2002), Polevoi et al (2003), the presence of auxin in Phaeophyceae.

The coleoptile cylinder biotest corroborates with Bügeln and Craigie (1971) the existence of indoles in *F. vesiculosus* and beyond, shows the presence also in *S. polyschides*.

Ehrlich Reaction

As mentioned, the first test was unable to complete the existence of indoles in toxic organic solvents to plant tissues, as was the case of hexane and matanol. However, the existing variety of bioactive compounds in algae led to the investigation of different extraction methods of their compounds.

Bioactive compounds are sensitive to extraction techniques based on heat or solvent use. Nowadays, marine algae researchers have been working toward the development of novel techniques that are more efficient in terms of yield, time, cost, and more important, are environmentally friendly [181].

There are different methods that can be used to obtain these compounds. Initially, a suitable extraction technique should be selected. For extraction, various mechanical, chemical, biological methods can be used. The choice of the appropriate method should depend on several factors, for example, what type of seaweeds will undergo extraction process, what type of bioactive compounds is going to be obtained (oils, polysaccharides, nutritional elements, dietary fibers, etc.). A key issue is the selection of the appropriate solvent for the extraction. The type of solvent (regarding its polarity) will lead to the extraction of different groups of compounds,which will have various final use. Extraction methodology involves the use of single solvents, select binary solvent mixtures, and time-based extractions [182,183].

Studies about the extraction of active compounds from natural products have attracted special attention in the recent years. Nowadays, the main challenge is to optimize conditions for the extraction of bioactive components (high yield and activity) from seaweeds [183].

The use of liquid seaweed fertilizer or biostimulants for different crops aims to substitute the commercial chemical fertilizers and to reduce the cost of production. Liquid fertilizers derived from seaweeds through extraction method are found to be superior to chemical fertilizers because of high level of organic matter, micro and macroelements, vitamins, fatty acids, and growth regulators [184]. Agricultural biostimulants include diverse formulations of compounds, substances, and other products, such as microorganisms, trace elements, enzymes, plant growth regulators (PGRs), and macroalgal extracts that are applied to plants or soils to regulate and enhance the crop's physiological processes, thus making them more efficient. According to European Biostimulant Industry Council (EBIC) "Plant biostimulants contain substance(s) and/or micro-organisms whose function when applied to plants or the rhizosphere is to stimulate natural processes to enhance/benefit nutrient uptake, nutrient efficiency, tolerance to abiotic stress, and crop quality." [185].

Generally, production methods of seaweed extracts can be divided into three groups: biological method (i.e., enzymatic degradation method), chemical hydrolysis method (i.e., organic, inorganic solvents), and physical extraction method (i.e., high pressure and cold process, supercritical fluid extraction) [186].

Various extractants can be used to release soluble compounds from the algal matrix. The basic procedure for large-scale samples is to extract algal biomass with organic solvents or water. According to the literature, organic solvents are used for the extraction of compounds, which possess, for example, antioxidant, antiviral, and antimicrobial properties; however, water is used as a solvent for the extraction of compounds that can be applied as components of liquid fertilizers in plants cultivation. However, in this case the detailed composition of water extracts (content of biologically active compounds–for example, plant growth-promoting substances like auxins, gibberellins, and cytokinins) is rarely determined [187].

The extraction with organic solvents are the most frequently used methods for extraction, because of their simplicity in operation, relative safety, and potencial for scaling up to industrial process [183]. The disadvantages of these methods are that high volumes of solvents are used and the duration of extraction is long. These techniques often produce low extraction yields of bioactive compounds and present low selectivity [182]. In the case of chemical hydrolysis with organic extractant, the most appropriate type of solvent should be chosen in order to extract a given group of compounds. Therefore, screening of organic extracts from marine algae is a common approach to identify bioactive compounds [188].

Thus, following extraction based on Carvalho (2013) [157], which uses chemical hydrolysis method for extracting polysaccharides, polar and nonpolar compounds. Therefore, with this method the concentration of indole compounds in the different extracts can be evaluate by Ehrlich Reaction.

Ehrlich's reagent is a chemical test to presumptively identify indoles. In the basis of this reaction is the ability colorless indole in the presence of a hydrochloric acid to form with paradimethylaminobenzaldehyde (PDAB) compound red. It is named after Nobel Prize winner Paul Ehrlich who used it to medicine [189].

The extracts used for quantification of indole compounds by Ehrich reaction were: Serial hexane extract (SHE), Serial Methanol Extract (SME), Serial Aqueous Extract (SAE), Crude Aqueous Extract (CAE), Macerated Extract (ME) and Concentrated Extract (CE).

Although the present study is optimized assay to quantify auxins, the original bioassay was developed for quantification of several indoles (as indolamine), and thus, interference could occur in the total quantification. However, the method showed a linearity range similar to the described when tested with buffered stock solutions of IAA.

The assay confirms the presence of indole compounds in seaweed extracts. The macerated samples have a higher concentration of indoles: 5,524 mg per gram of dry seaweed to *Fucus vesiculosus* and 0.407 mg per gram of dry seaweed to *Saccorhiza polyschides*.

The values are lower than reported by Kingman and Moore [191], when they determined the presence of 50 mg per gram of dry extract from the seaweed *Asccophyllum nodosum* commercial preparation. However, the values are higher than reported by Sanderson et al [192], when was estimate that one gram of dried Maxicrop powder contains $6.63 \pm 0.29 \mu g$ IAA.

The crude aqueous extract, or the extract applied to the plants itself, have a low concentration of indole compounds, as they are more dilute so as not to interfere with other plant development parameters, such as growth inhibition.

The serial extracts for both seaweeds were also performed by Ehrlich reaction and it was expected that the sum of the compounds among the three solvents did not exceed the total value of macerated seaweed. And this is what happened to *F. vesiculosus*, as shown in the figure 19. But the S. polyschides seaweed did not answer the expected way, ie, the sum of the serial extracts exceeded the limit of the macerated extract.

Besides the bioassay be a presumptive test for indole compounds and optimized for auxins, what may have happened is that the standard curve method interfere with the quantification when the solvents are different from distilled water. Therefore, is necessary optimization with new method. For this, a new assay using the standard addition is required with their respective solvents (hexane and methanol).

Thus, to confirm the solvent that interfered with the reaction was developed a Standard addition using hexane and methanol as control. Different auxin

concentrations were tested. Thereby it could be making sure if the previous assay was genuine. This time was used 1g of each algae.

This time the values did not exceed the amount of indoles of dry extracts. But what caught attention was to high concentration of the compounds in Saccorhiza polyschides SHE (table VI).

The most likely is that other compounds are interfering with quantification. The use of hexane in the extraction aims to extract liposoluble compounds, such as fucoxanthin present in macroalgae, and not water soluble compounds such as the case of the indoles.

Fucoxanthin has remarkable biological properties based on its unique molecular structure similar to neoxanthin, dinoxanthin, and peridinin, which is different from that of other carotenoids such as β-carotene and astaxanthin. Fucoxanthin has an unusual allenic bond and some oxygenic functional groups such as epoxy, hydroxyl, carbonyl and carboxyl moieties in its molecule (Figure 2) that contribute to its unique structure [193, 194]. The allenic bond was found mainly in carotenoids such as fucoxanthin, which was the first allenic carotenoid found in brown seaweeds [195], and was responsible for the higher antioxidant [196]. Fucoxanthin is one of the most abundant carotenoids accounting for >10% of estimated total natural production of carotenoids [197].

It is to remember, as already mentioned, that fucoxanthin is responsible for the brown color of Pheophyceae, and that just as there was overestimation in the *S. polyschides* hexane extract, their natural color is a brown darker compared to the seaweed *F. vesiculosus*.

There are significant challenges associated with the development of seaweeds-based products due to varying level of bioactive content, in this case, the indole compunds. This variation in the concentration of auxins found in this work may be due species are from different orders (Fucales and Tilopteridales), thus having clear morphological differences and different compounds. Also *S. polyschides* is an annual seaweed, as opposed to the perennial *F. vesiculosus.*

Another explanation for the variation of the compound are the environment which they grow and develop. As it has been previously mentiones that an alga was collected in the intertidal zone and the other (*F.vesiculosus*) with higher levels of indole compund in the estuary zone, which has influences from both the sea and the Mondego River.

Despite having bioactive compound of interest, *Saccorhiza polyschides* grows quickly, may not have enough time to develop large amount of bioactive compounds, and it agrees with a lower rate of indole compound found in this work.

In temperate waters the brown alga Saccorhiza polyschides will grow to a size of 2 m or more in less than two months [226], and single species weighing as much as 22 kg have been reported [227].

Although few studies about the variation of auxins in seaweeds and *S.polyschides* in general, van Hal et al., (2014) [218] has a study with a similar algae (*Laminaria digitata*) and shows the large variation in the amount of compounds that occours according to the time they were collected. In this case, during the period of June-September the monosaccharides content of seaweed ranged from 5% to 31% for mannitol over a whole year.

Moreover, *Saccorhiza polyschides* is an opportunistic seaweed, thus take advantages exploiting pollution peaks and form large areas in a short time which ultimately inhibit the growth of other species around which require sunlight to develop. This large amount of algae that appears seasonally in beaches (bloom) could be used to fertilize crops, but for now are decomposing on the beaches to be wasted.

The utilization of seaweeds as natural fertilizers in agricultural crops has been getting more space and visibility. The benefits of using seaweeds is evident, however the concentration of fertilizer extract varies depending on the seaweed used and the plant to be applied.

To evaluate the in vivo effect of applying plants in algae extracts, has been developed bioassays (in greenhouse and germination test that demonstrate better results than when not using these natural resources.

The obtained results show that the development stage of the plant to be fertilized with seaweed extract is critical to know the appropriate concentration. According to Reinhardt and Rost (1995) [218], most plants are more sensitive to salinity during germination and seedling growth. Perhaps also because their seeds already possess greater amount of endosperm and no longer require much additional nutrients, but plant hormones in low concentrations. In more advanced stages of development need more auxin concentration and in addition, seaweed fertilizer compounds.

Statistically significant differences were observed for shoot length, root length, number of leaves and fruits in response to application of different concentrations of the seaweed extracts under study.

Concentration of seaweed liquid fertilizer of 10% until 15% should stimulate root elongation of tomato plants and accumulation of plant biomass. This suggests that SLF assistance in crop development and promotes increased growth compared with control plants, which agree with *van Staden and others (1994)* [204]. They claim that in their studies the number of flowers and seeds per plant head increased (as much of 50% over the control).

Several studies shown that biofertilizer from seaweeds in general are capable of affecting root development by both improving lateral root formation [205-206] and increasing total volume of the root system [207-209].

An improved root system when SLF is applied to plants was already argued by Crouch and others (1992)[210]and the response could be influenced by endogenous auxins. Seaweed extracts improve nutrient uptake by roots, resulting in root systems with improved water and nutrient efficiency, thereby causing enhanced general plant growth and vigor [211].

According to agricultural statistics of Portugal [212], in 2015 about 1300 tons of tomatoes were produced. The present study shows that agricultural crops (such as tomatoes), may be fertilized with natural extracts and the yield in production is greater when applied extracts with 20 to 25% of the algae extract.

The results of this study agree with a variety of researches which claim that seaweed biofertilizer triggers early flowering and fruit set in a number of crop plants [213-215]. And this was not considered to be a stress response [210].

Tomato plants treated with SLF showed no signs of disease, either in a decrease or total loss of tomatoes, agreeing with Fritig et al. (1998) on the paper biotic stress resistance of algae extracts. They claim that seaweeds stimulate natural defense responses in plants and are involved in the induction of genes encoding various pathogenesis–related (PR) proteins with antimicrobial properties

5. Conclusion and future perspectives

The present research highlights the efficiency and yield of SLF obtained from the Phaeophyceae (*Fucus vesiculosus* and *Saccorhiza polyschides*). This seaweeds exhibited great promise as a source of biofertilizer for raising crops. Plants treated with this natural growth stimulator showed a raised germination percentage, seedling vigor index, plant length, number of flowers and fruits.

Due to the rapidly increasing world population and occurrence of biotic and abiotic stresses (likely due to climate change) the food production is challenged. Therefore, tools of plant biotechnology should be more active to alleviate this problems developing sustainable and natural fertilizer.

The industries based on seaweeds have the potential to contribute to the sustainable development of ocean resources utilizing seaweeds as biofertilizer.

Unlike natural fertilizers (SLF), the synthetic adversely affect the soil chemistry and become less affable for plant growth. In addition to it, the use of these unnatural products is very polemic. Many countries (including in the European Union) are against the use of chemical herbicides/fertilizers since it does not possess attested studies (especially in the long term) that are not harmful to human health.

Seaweeds liquid fertilizers are considered an organic form input as they are environmentally friend and safe for the health of animals and humans. The application of SLF at appropriate rates in different developments of agricultural crops is crucial in order to obtain optimal beneficial effect on plant growth, yield and is found to be more promising in possessing fertilization activity due their use as plant biofertilizer. This influence is explained on the basis of containing low rates of Plant Hormones, such as Auxins, proved in this scientific research.

6- Attachment

1) Oat coleoptile bioassay.
1.1- Crude extract.

Treatment	0	10	20	30	40	Fucus 20%	Saccorhiza 20%
	0,5	0,76	0,5	0,5	0,4	0,5	0,6
	0,5	0,5	0,5	0,5	0,6	0,5	0,5
	0,4	0,5	0,5	0,5	0,6	0,5	0,5
	0,4	0,5	0,5	0,5	0,5	0,5	0,4
	0,4	0,4	0,5	0,5	0,5	0,5	0,4
	0,4	0,4	0,4	0,5	0,5	0,5	0,4
	0,4	0,4	0,4	0,4	0,5	0,5	0,4
	0,4	0,4	0,4	0,4	0,5	0,5	0,4
	0,4	0,4	0,4	0,4	0,5	0,5	0,4
	0,4	0,4	0,4	0,4	0,5	0,5	0,4
	0,4	0,6	0,5	0,6	0,6	0,5	0,5
	0,4	0,5	0,5	0,5	0,6	0,5	0,5
	0,5	0,6	0,6	0,4	0,6	0,5	0,5
	0,5	0,6	0,6	0,6	0,5	0,5	0,5
	0,5	0,5	0,5	0,5	0,5	0,5	0,5
	0,5	0,4	0,6	0,7	0,5	0,5	0,5
	0,5	0,6	0,6	0,6	0,6	0,5	0,5
	0,5	0,5	0,6	0,5	0,7	0,5	0,5
	0,5	0,6	0,6	0,7	0,7	0,5	0,5
	0,5	0,6	0,5	0,6	0,5	0,5	0,4
	0,5	0,5	0,5	0,6	0,7	0,5	0,4
	0,5	0,4	0,5	0,6	0,5	0,5	0,4
	0,5	0,5	0,6	0,5	0,6		
	0,4	0,4	0,5	0,5	0,5		
	0,4	0,4	0,5	0,5	0,5		
	0,4	0,4	0,5	0,5	0,5		
	0,5	0,5	0,5	0,5	0,5		
	0,5	0,5	0,5	0,5	0,7		
	0,4	0,5	0,5	0,5	0,5		
	0,4	0,5	0,5	0,6	0,5		
	0,45	0,46	0,51	0,53	0,55		

Calibration

Concentration		0	10	20	30	40
	1	4,20	4,40	4,50	4,60	5,10
	2	4,80	5,50	5,60	5,70	5,80
	3	4,50	4,60	5,10	5,30	5,50
Average		4,50	4,83	5,07	5,20	5,47
SD		0,30	0,59	0,55	0,56	0,35

Fucus 20%	Saccorhiza 20%
0,50	0,475
0,50	0,44
0,50	0,46
[IAA]	0,4575
19,43478	8,5652174
97,1739	**42,82609 µg/mL**

1.1- Serial aqueous extract.

0	10	20	30	40	Fucus EA	Saccorhiza EA
4	4,5	5	5	6	6	4,5
5	5	5	5,5	5	6	5,5
5	5,5	4	5	5,5	6	6
5	6	5,5	5,5	5	6	6
5	5	5,5	7	5	5	4,5
5	5	6	5	6	5	5
5	5	5	5,5	7,5	4	6
5	5	6	6	5	5	6
5	5	5	5	5	6	6
6	5,5	5,5	4	7	6	6
5	5	5,5	6	6	5	6
5	5	5	7,5	6,5	5	6
4	5,5	7	4	4	5	5
4	4	7	6	4	5	5
4	6	6	5	6	6	5
5	6	5,5	5	5	6	5
5	5	4,5	4	7	6	6
5	4,5	5	6,5	5	6	6
5	5	4,5	5	5	6	5
6	6	5,5	4	7	6	5
4	5	4,5	6	5	6	5,5
4	4	5	5,5	6,5	6	6
4	5	5,5	5	7	5,5	6
4	4,5	5	5,5	5	5	6
5	6	6	5	6	5	5
5	4	5	7	6	5,5	6

5	4,5	5	5	6	5	5
5	5	5,5	5	6,5	5	5
5	5	4,5	5,5	7	5	6
6	5,5	5,5	7	5,5	6	6
4,833333333	5,066666667	5,316666667	5,433333333	5,766666667	5,5	5,533333333	média
0,5920935	0,583292281	0,67572864	0,916640543	0,925997443	0,557086015	0,5403277	SD
					29,74439462	31,23916293	µg
					148,7219731	**156,1958146**	µg/ml

2) Ehrlich reaction.

2.1- *Fucus vesiculosus*.

2.1.1-**Macerated.**

	Initial Volume	1	2	3	Average	Cf	Ci ($\mu g/ml$)	µg	µg/g	mg/g
F10	0,1	0,261858	0,261513	0,254784	0,259385	21,26105	1063,05242	6378,315	3189,157	3,189157
F5	0,2	0,544578	0,548586	0,546293	0,546486	44,79391	1119,847773	6719,087	6719,087	6,719087
F10	0,1	0,281483	0,282648	0,283042	0,282391	23,1468	1157,339971	6944,04	6944,04	6,94404
F5	0,2	0,463528	0,470758	0,476775	0,470354	38,55358	963,8395928	5783,038	5783,038	5,783038
F10	0,1	0,261428	0,261882	0,262302	0,261871	21,46483	1073,24139	6439,448	6439,448	6,439448

2.1.2- **Crude aqueous extract.**

	Inicial volume	1	2	3	Average	Cf	Ci ($\mu g/ml$)	C (mg/ml)
F5	0,2	0,160544	0,161267	0,162301	0,161371	13,2271	330,6776	0,330678
F10	0,1	0,078363	0,07885	0,079472	0,078895	6,466803	323,3402	0,32334
F5	0,2	0,171535	0,171792	0,172686	0,172004	14,09872	352,4679	0,352468
F10	0,1	0,081789	0,08344	0,083665	0,082965	6,800396	340,0198	0,34002

2.2-*Saccorhiza polyschides*.

2.2.1-**Macerated.**

initial Volume	1	2	3	Average	Cf	Ci ($\mu g/ml$)	µg	µg/g	mg/g
0,2	0,173247	0,17303	0,1744	0,173559	14,22615	355,6537	2133,922	1066,961	1,066961
0,1	0,023815	0,02336	0,024181	0,023786	1,94964	97,48201	584,892	292,446	0,292446
0,1	0,013267	0,014083	0,014812	0,014054	1,151961	57,59806	345,5884	345,5884	0,345588
0,1	0,023251	0,02363	0,024241	0,023707	1,943232	97,16161	582,9696	582,9696	0,58297

Média: 0,407 ±0,154692

2.2.2-Crude aqueous extract.

Adição		Absorvância 1	2	3	Média		Ci ± SD	
0	0	0,103	0,104	0,103	0,103183	Cf		
2µg/ml	2	0,488			0,487561	2,543742	80,31µg/mL	40,59
5µg/ml	5	0,493	0,496	0,497	0,495443	1,138922	±40,59	
10µg/ml	10	0,936	0,938	0,940	0,938096	1,136201		
20µg/ml	20		1,058	1,061	1,059206	Média:		
						1,606288		

2.3- Serial assay.

Sample	Absorbance			Average	SD	Cf	Ci	Qe	Qe (mg)	Average (mg)	SD
	Dry seaweed										
1	0,374	0,375	0,376	0,375	0,0008165	30,7377	1536,885	53790,98	53,79098		
2	0,369	0,371	0,371	0,370	0,00094281	30,35519	1517,76	53121,58	53,12158		
3	0,381	0,382	0,382	0,382	0,0004714	31,28415	1564,208	54747,27	54,74727	53,88	0,817
	Extract in hexane										
1	0,006	0,007	0,008	0,007	0,0008165	0,57377	28,68852	2868,852	2,868852		
2	0,009	0,010	0,011	0,010	0,0008165	0,819672	40,98361	4098,361	4,098361		
3	0,006	0,007	0,007	0,007	0,0008165	0,546448	27,3224	2732,24	2,73224	3,23	0,614
	Extract in methanol										
1	0,043	0,044	0,044	0,044	0,0004714	3,579235	178,9617	17896,17	17,89617		
2	0,046	0,046	0,048	0,047	0,00094281	3,825137	191,2568	19125,68	19,12568		
3	0,048	0,05	0,051	0,050	0,00124722	4,071038	203,5519	20355,19	20,35519	19,12	1,004

2.4- Extract inHexane.

Auxin addition (µg)	Absorbance			Average
0				0
5	0,09	0,09	0,09	0,09
10	0,16	0,16	0,16	0,16
20	0,26	0,26	0,26	0,26
30	0,33	0,33	0,33	0,33
40	0,46	0,46	0,46	0,46

$y = 0,0117x$
$R^2 = 0,973$
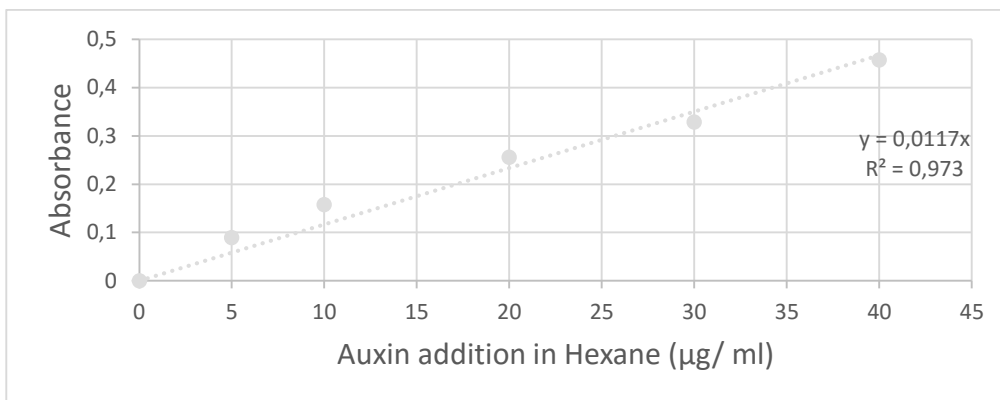
2.4.1-*Fucus vesiculosus.*

	1	2	3	Média	SD	Cf	**Ci**	Qe	Qe (mg)
-extracto not evaporated	0,007	0,01	0,00667	0,007889	0,00149897	0,674264	**16,8566**	1685,66	1,6
-extract evaporated	0,206047488	0,20741	0,20858	0,207343	0,00103297	17,72162	**443,0406**	1329,122	1,3

2.4.2- *Saccorhiza polyschides*.

	1	2	3	Média	SD	Cf	**Ci**	Qe	Qe (mg)
-extract not evaporated	0,025	0,026	0,025	0,025333	0,00057735	2,165242	**108,2621**	10826,21	10,8
-extract evaporated	0,433158111	0,43367	0,43324	0,433354	0,00027319	37,03882	**740,7764**	2222,329	2,2

2.5. Extract in methanol.

	Absorbance			Average	SD
Adittion	1	2	3		
0	0,038	0,039	0,04	0,039	0,001
5	0,129	0,13	0,131	0,13	0,001
10	0,216	0,216	0,218	0,216667	0,001155
15	0,288	0,289	0,29	0,289	0,001
20	0,342	0,342	0,345	0,343	0,001732

$y = 0,0153x + 0,0501$
$R^2 = 0,9903$
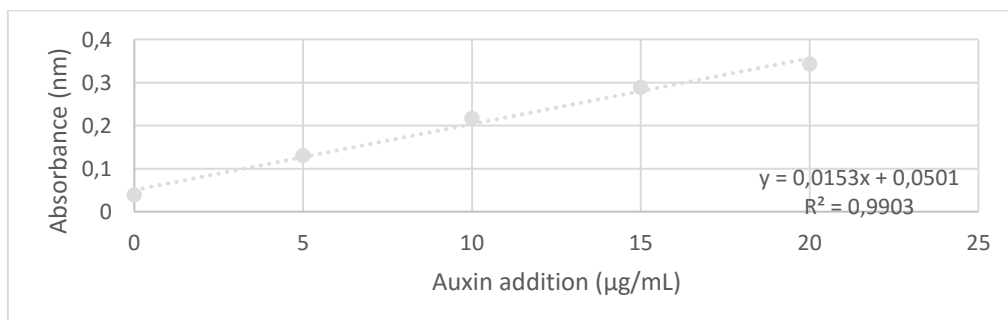
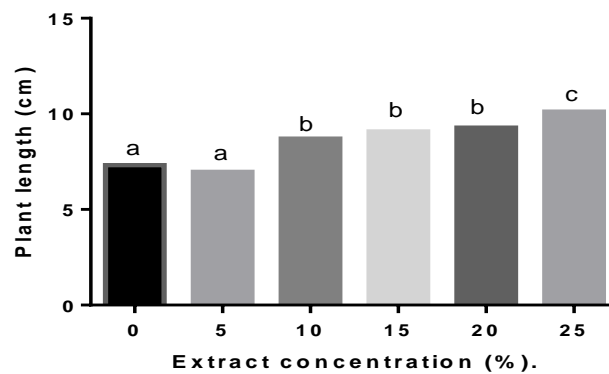|	1	2	3	Average	SD	Cf	**Ci**	Qe	Qe (mg)
-Saccorhiza	0,025	0,026	0,025	0,025333	0,000577	1,655773	**82,78867**	8278,867	8,2
Methanol 50%	0,031	0,031	0,033	0,031667	0,001155	2,595628	129,7814	12978,14	12,97814
-Fucus	0,043667	0,046667	0,049667	0,046667	0,002449	3,050109	**152,5054**	15250,54	15,2
Methanol 30%	0,566123	0,567197	0,568136	0,567152	0,000823	37,06877	**617,8128**	1853,438	18,3

3- Germination assay.

3.1-

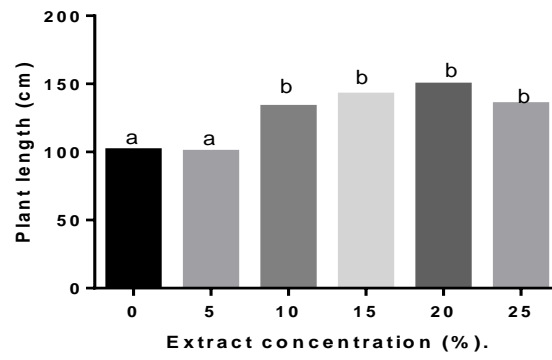Tomato Germination using seaweed liquid fertilizer.
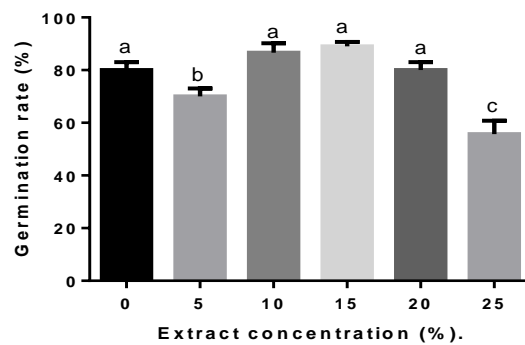
3.2-

Tomato plant length after SLF application.

4. Greenhouse bioassay.

Tomato plant length after SLF application.

Figure: Bar chart showing plant length (cm) on the y-axis (0 to 200) versus extract concentration (%) on the x-axis (0, 5, 10, 15, 20, 25). Bars labeled: 0 (a), 5 (a), 10 (b), 15 (b), 20 (b), 25 (b).

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