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Seaweed Carbohydrates

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3.1 Introduction

The seaweeds (macroalgae) are a large and diverse group of marine and freshwater organisms. Currently, approximately 10 000 species of seaweeds have been identified and classified into three groups based on their pigmentation: brown (phylum *Ochrophyta*), green (phylum *Chlorophyta*) and red (phylum *Rhodophyta*) [1]. Seaweed diversity can be subcategorized on the basis of a broad range of characteristics such as morphological, ultrastructural, biochemical, physiological and ecological features [2–4].

Seaweeds are consistently exposed to both biotic and abiotic stresses in their natural marine environments (e.g. salinity, temperature, pathogens, nutrient starvation, radiation or combination of light and oxygen concentration). These stresses exert an influence on the seaweed’s physiology that leads to the production of unique metabolites for the plant to survive and thrive [2]. Some of these metabolites include fatty acids, sterols, carotenoids, vitamins, proteins, minerals and carbohydrates [5–9]. This plethora of bioactive compounds are attractive for commercial exploitation in several areas of interest such as human and animal nutrition, cosmetics and plant biostimulants (PBs) [10–12]. Almost 21 million tonnes of seaweeds are utilized worldwide, of which less than 800 000 tonnes are being harvested from the wild and the remaining 94% produced by aquaculture. An estimation of the wholesale value of the global annual seaweed production ranged between US\$10.1 and US\$16.1 billion in 2012 [13], with some projecting that the market will reach US\$17.6 billion by 2021 [14].

Seaweed extracts are prominent in the PB market, representing the fastest growing PB product category [15, 16]. The effects of seaweed extracts on plants has been reviewed [12, 17–19] with some of the key PB effects reported to include: improved plant vigour, root development, enhanced chlorophyll synthesis, earlier flowering, fruit set and uniformity of fruit, delay of senescence, or abiotic stress tolerance. In the literature, there are numerous studies investigating potential modes of action for seaweed extract PBs in various model plants and crops [20–35]. However, it is important to note that seaweed extract PBs are not a homogenous category of products. Seaweed extract PBs vary depending on the family and species of seaweed used for manufacture (e.g. brown, green, or red) [17], the source of the seaweed raw material (e.g. season harvested, geographical location, sheltered or exposed shoreline, water temperature and salinity) [36, 37] and the process used for manufacture/extraction (e.g. micronization, cell burst, ultrasound, extraction under acidic, aqueous or alkaline conditions, enzymatic hydrolysis or fermentation) [25, 38–40]. These three variables significantly contribute to the chemical composition and physicochemical properties of seaweed extracts.

Seaweeds, especially brown seaweeds, are recognized as a rich source of carbohydrates with high biodiversity serving numerous biological applications. These seaweed-derived carbohydrates are broadly classified into different classes depending upon their chemical diversity, namely, fucoidan, alginate, carrageenan, ulvan, laminarin, cellulose/hemicellulose. The composition and sequence analysis of seaweed carbohydrates are still challenging tasks because of the complexity of their structures and their heterogeneity. There are a number of parameters that define/characterize a carbohydrate, such as the molecular mass. The molecular mass of carbohydrates allows them to be categorized as polysaccharide, oligosaccharide, disaccharide or monosaccharide. Other parameters include the nature of building units which can be the same sugar or a combination of different sugars (e.g. homopolysaccharide or heteropolysaccharide); the type of glycosidic bond; the amount of substitutions and their positions or the molecular geometry (e.g. linear or highly branched). All these parameters control very important structural characteristics that confer distinct physicochemical and bioactive properties [8]. Further structural differentiation may also occur depending on the extraction and purification procedures, resulting from the use of different solvents, specific hydrolytic enzymes and experimental conditions such as pH, temperature, time or pressure [41, 42].

Many seaweed carbohydrates function as either a structural component of the cell wall or as storage molecules in the plastids. Larger storage polysaccharides in seaweeds serve as a photosynthetic reserve (e.g. laminarin in brown seaweed) [43]. Other low molecular weight storage compounds such as mannitol or sucrose play a significant role in the seaweed life cycle as osmoprotectants [44, 45]. Most seaweed cells are surrounded by a polysaccharide-rich cell wall/extracellular matrix which has an important structural function and also regulates development and innate immunity [46]. Several reviews of seaweed carbohydrates showed that many are confined to a particular taxonomic group [2, 3, 8]. Conversely, some seaweed classes may be very enriched in a particular carbohydrate (Table 3.1). Seaweed carbohydrates undergo structural changes during development and life cycle. For example, cell wall composition depends on the cell type, time of year and various environmental factors [37, 47–50].

Plants possess an efficient and multifaceted immune system that effectively protects them from a wide range of different phytopathogenic microorganisms such as bacteria, viruses

Table 3.1 Major storage and cell-wall carbohydrates present in different seaweed classes.

		<i>Ochrophyta</i> (Brown seaweed)	<i>Chlorophyta</i> (Green seaweed)	<i>Rhodophyta</i> (Red seaweed)
Storage carbohydrate	Photosynthetic reserve	Laminarin	Starch Inulin	Floridean starch
	Low molecular weight compounds	Mannitol	Sucrose	Mannitol Floridoside Isofloridoside
Cell wall carbohydrates	Crystalline polysaccharides	Cellulose	Cellulose	Cellulose (1 → 4)-β-D-mannan (1 → 4)-β-D-xylan (1 → 3)-β-D-xylan
	Hemicelluloses	Sulphated xyloglucan Sulphated xylo-fucoglucuronan (1 → 3)-β-glucan	Xyloglucan Mannans Glucuronan (1 → 3)-β-glucan	Glucomannan Sulphated MLG (1 → 3),(1 → 4)-β-D-xylan
	Matrix carboxylic polysaccharides	Alginates	Ulvans	—
	Matrix sulphated polysaccharides	Homofucans (Fucoidans)	Ulvans	Agars Carrageenans

Source: Adapted from [2], [3] and [8].

and fungi [51, 52]. Defence signals can be systemically emitted to activate a broad array of defence responses in the non-colonized organs of a plant locally infected by a microbe, infested by an herbivore or even stimulated by a chemical compound. Defence signals can also be primed for rapid activation after a localized perception of beneficial saprophytic microorganisms. The two main types of systemic resistance, Systemic Acquired Resistance (SAR) and Induced Systemic Resistance (ISR), are dependent on SA signalling or the JA and ET signalling pathway, respectively. However, both SAR and ISR phenomena converge downstream since they are controlled by the same transcriptional regulator NPR1 [53].

The main and evolutionary older element of the plant immune system is based on the external recognition of molecules, known as elicitors, whose chemical pattern/structure is generally conserved. They are perceived by plant cell surface receptors to induce a local or systemically expressed resistance. Those elicitors isolated from infectious agents or non-pathogenic microorganisms are designated Pathogen-Associated Molecular Patterns (PAMPs) or MAMPs (Microbe-Associated Molecular Patterns), respectively. These are molecules essential for the overall fitness of microbes [52], such as chitin or different glucans present in fungal/oomycete cell walls. Many different types of MAMPs have been described; they can be glycoproteins, specific carbohydrate structures or lipids [54, 55]. Some endogenous molecules also activate the plant innate immune system when they are released into the extracellular space from their normal location due to damage; these molecules are referred to as Damage-Associated Molecular Patterns (DAMPs) [56]. For the recognition of the D/M/PAMPs, a large diversity of membrane-bound or

soluble pattern recognition receptors (PRRs) with a lectin domain have been identified in plants (Figure 3.1). However, only a limited number of them have been functionally characterized [57].

Due to their sessile lifestyle, plants are not only subjected to biotic stresses but also to a multitude of abiotic stress factors (e.g. drought, heat, or salinity). Abiotic stresses are one of the key challenges for plant growth and agricultural productivity in arable lands, with estimated annual loss of billions of dollars [58, 59]. The acclimation of plants to abiotic stress is a complex and coordinated response involving hundreds of genes that interact with various environmental factors throughout the life cycle of the plant [60, 61]. Interestingly, several of the plant PRRs have been reported to act both upon biotic and abiotic stresses [62–65].

Many carbohydrates are involved in plant immunity as signalling molecules in a manner similar to cytokines/hormones in mammals [55, 66]. This has led to the ‘sweet-immunity’ and ‘sugar-enhanced defence’ concepts [67]. Hence, as they interact with diurnal changes, abiotic and biotic stresses, and hormone signalling, carbohydrates are considered essential players for the coordination of plant metabolism with growth, development and responses to stresses [68]. Over the past 20 years, several papers have described the PB activity of some polysaccharides and oligosaccharides purified from seaweeds such as alginates, carrageenan or laminarins [23, 55, 66, 69]. However, the information on the PB effects of seaweed carbohydrates on plant systems is still rather limited. To date the PRRs for some marine carbohydrates such as fucoidan or alginate have not been identified. The remainder of this chapter will discuss the advancements in this field of research focusing on the mechanisms through which seaweed carbohydrates can promote plant growth and induce plant stress tolerance, resulting in increased plant productivity.

3.2 Fucoïdan from Brown Algae

3.2.1 Detailed Description of Chemical Composition and Structure of Fucoïdan

Fucoïdians from brown macroalgae are polysaccharides composed of a backbone of L-fucose with varying degrees of sulfate substitutions. Although L-fucose can make up more than 50% of the monosaccharide structure, many fucoïdians contain small amounts of other monosaccharides, including D-glucose, D-galactose, D-mannose, D-xylose, D-glucuronic acid and also acetyl groups [8, 70]. These minor components can be found to be an integral part of the α -L-fucose backbone structure [71, 72] or linked to fucose residues as side branches [73]. Reported molecular weight for these polysaccharides vary between 5.87 and 1600 kDa [74–76].

The highly variable fucoïdan structures found in different brown seaweed taxonomical orders (e.g. Fucales, Laminariales or Chordariales) can be classified into two main groups [70, 73]. One group includes fucoïdians isolated from *Ascophyllum nodosum*, *Fucus vesiculosus*, *Fucus serratus* and other *Fucus* species that have a backbone composed of repeating (1 \rightarrow 3) and (1 \rightarrow 4)-linked α -L-fucose residues (Figure 3.2a). The sulfate groups from *A. nodosum* and *F. vesiculosus* are typically located at the C-2 position of the (1 \rightarrow 3)-linked unit and on the C-2 and C-3 positions of the (1 \rightarrow 4)-linked residue. However, L-fucose residues from *F. serratus* may be substituted with sulfate on C-2 and C-4, although some terminal fucose residues may be non-sulfated [77].

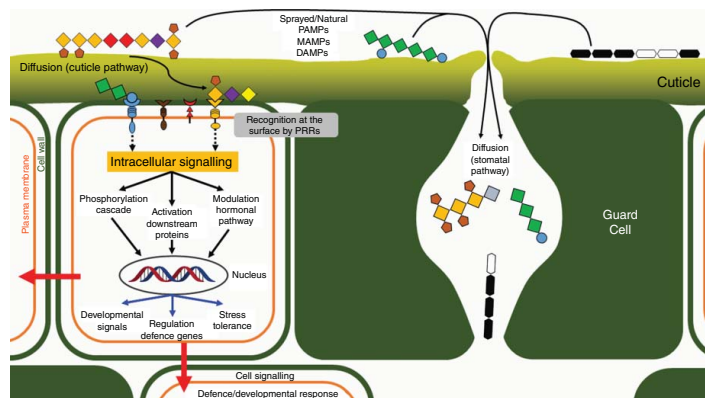


Figure 3.1 Model of PB effects of natural/sprayed carbohydrates at the leaf surface. Small carbohydrate molecules can penetrate through the hydrophobic cuticle to reach plant cells. Bigger molecules can also enter the leaf along the surfaces of the stomatal pores. Depending on their structure and composition, carbohydrates can behave as D/M/PAMPs and be perceived by membrane bound PRRs with lectin domains. Then, an intracellular signalling cascade is initiated in plants which includes downstream protein phosphorylation, transcription factor activation, or modulation of hormonal pathways, ultimately leading to activation of stress-responsive or developmental signal-responsive genes. Local defences are followed by the production of mobile signals that are transported via xylem and prime distal plant parts. Source: Adapted from [55] and [57].

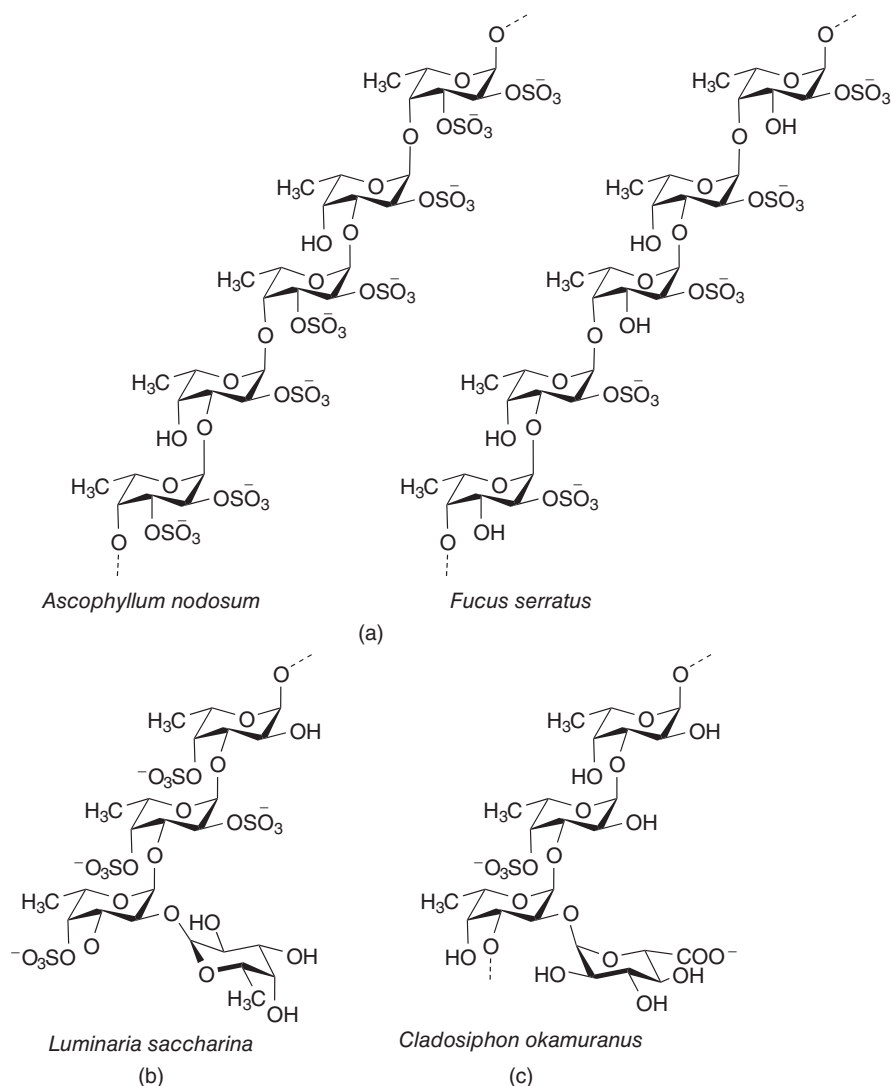


Figure 3.2 Schematic representation of the different structures of fucoidans from (a) Fucales, (b) Laminariales, and (c) Chordariales. Source: Redrafted from [73].

A second group includes several fucoidans extracted from *Laminaria saccharina*, *Laminaria digitata*, *Cladosiphon okamuranus*, or *Chorda filum* that are mainly composed of α -(1 \rightarrow 3)-linked L-fucose units. Fucoidans isolated from *L. saccharina* and *C. filum* are usually sulfated at both C-2 and C-4, or at C-4 alone with some fucose residues being 2-O-acetylated (Figure 3.2b). In contrast, fucoidans from *C. okamuranus* showed some sulfate substitution at the C-4 position of L-fucose residues with D-glucuronic acid residues linked to the C-2 positions of the non-sulfated fucose residues (Figure 3.2c) [73].

Previous reviews have illustrated that the classical treatment with dilute acid (e.g. HCl) at ambient or slightly elevated temperature is still a preferred first step in extraction protocols

for isolating fucoidan from different types of brown seaweeds. However, it is important to mention that different extraction parameters such as acid concentration, time or temperature can influence the yield, structural integrity or composition analysis of the fucoidan polysaccharides [73, 78].

3.2.2 Experimental Methods for Chemical Characterization of Fucoidan

Because of the complexity of chemical compositions and structures of fucoidans from brown seaweeds, there is no commercially available method that can directly quantify the actual amount of fucoidan from a crude extract. Chemical methods of analysis can give partial information on their composition, and a multiple-technique approach is necessary to quantify the presence of different monosaccharides or sulfate in fucoidans. In general, care must be taken to set up appropriate methods of analysis followed by scrutiny of the results. One of the more traditional methods of determining fucoidan, expressed as amount of L-fucose, is the cysteine-sulfuric method. In this technique the backbone of fucoidan is hydrolysed with concentrated sulfuric acid at an elevated temperature and the generated L-fucose reacts with cysteine to form a coloured complex [79]. Among the methods for monosaccharide analysis, acidic hydrolysis with TFA and further HPAEC-PAD analysis is the major method used [76, 80, 81]. Alternatively, the separation and quantification of monosaccharides of the acid hydrolysed fucoidans can be done by GC/GLC-MS. However, prior to GC/GLC-MS analysis, desulfation is required along with the production of methylated alditol acetates [76, 82, 83]. Enzymes catalysing partial cleavage of fucoidans (e.g. fucoidanases and fucosidases) have been proposed to be useful tools for simplifying the structure of fucoidan and reducing the difficulty of analytical works [73]. However, only a handful of fucoidanases/fucosidases have been biochemically characterized in detail and the commercially available recombinant enzymes are still expensive [84].

The barium chloride-gelatine method is the routine method to determine the sulfate content of fucoidans after employing an acidic hydrolysis [85]. Because the Dodgson's gelatine reagent requires great care in preparation, a less time consuming turbidometric method based on agarose-barium reagent can be used [78, 86]. FTIR can also provide some information about the substitution pattern and position of sulfate groups. Specific absorption bands such as the S—O stretching and C—S—O bond are typical of fucoidans and thus can act as a qualitative tool [87].

Knowing the structure of fucoidan requires not only the determination of its monosaccharide composition and sulfate content, but also the molecular weight, sulfation pattern, degree of branching, isomer position and the anomeric configuration of each of its glycosidic bonds. A simple, fast and reliable HPLC-RID method can be used to determine the molecular weight of fucoidans [76, 88]. Because fucoidan polysaccharides are usually heterogeneous and branched, usually only partial information on their structures can be obtained by ^1H and ^{13}C NMR spectroscopy. In order to obtain interpretable NMR spectra, some chemical modifications are applied to fucoidan extracts (e.g. acid hydrolysis, deacetylation and desulfation) [83, 89, 90]. MALDI-MS and ESI-MS are analytical methods of high sensitivity and selectivity that can give information about the sugar linkages, monosaccharide content, or sulfation pattern. By using a recent advanced analytical technology that combines autohydrolysis, ESI-MS and MALDI-MS techniques, structurally different fucoidans have been deeply characterized by Anastyuk et al. [91].

3.2.3 Fucoidan PB Activity and Potential Applications

The literature about the biological activities of fucoidans is extremely large. In recent years, this sulfated carbohydrate from brown seaweed has been the subject of many studies in the field of biomedicine and nutraceuticals due to its reported anticoagulant, anti-inflammatory, antitumor or immunomodulatory activities [73, 78]. However, there is scant research on the application of fucoidan extracts on plants, with those available being focused on elicitation of plant defence system. For example, highly sulfated fucoidan oligosaccharides produced by enzymatic hydrolysis from the brown algae *Pelvetia canaliculata* and applied at a dosage rate of 0.2 mg ml⁻¹ to tobacco cell suspensions induced a release of H₂O₂ followed by stimulation of PAL and LOX enzymatic activities [92]. Furthermore, tobacco leaves treated with these fucoidan oligosaccharides induced the systemic accumulation of SA, the phytoalexin scopoletin and several PR proteins [92]. Consistently, fucoidan oligosaccharides infiltrated the mature leaves, strongly stimulated both local and systemic resistance to tobacco mosaic virus (TMV) for doses as low as 0.002 mg ml⁻¹. Likewise, by using transgenic plants unable to accumulate SA, it was determined that this phytohormone was required for the establishment of oligofucan-induced resistance [92]. Similarly, tobacco plants sprayed with native fucoidan from *Lessonia vadosa* and the partially depolymerized fucoidan fraction at a rate of 0.5 mg ml⁻¹ showed a significant activation of PAL, LOX and GST defence enzyme activities. Interestingly, the fucoidan polysaccharide with an average molecular weight of 320 kDa presented comparable activity as the 32 kDa fucoidan fraction [93]. Fucoidan extracted from *Fucus evanescens* applied at a concentration of 1 mg ml⁻¹ directly to the leaves of tobacco coinoculated with TMV was found to delay the development of the virus-induced infection. The effectiveness of fucoidan in reducing the spread of infection was also found to be cultivar dependant. Interestingly, the induced resistance against TMV was suppressed by actinomycin D (an antibiotic that inhibits RNA synthesis on the DNA template), indicating that the fucoidan eliciting activity worked through a genetic mechanism [94, 95]. Finally, the same fucoidan fraction purified from *F. evanescens* significantly reduced the level of infection induced by potato virus X (PVX) after priming *Datura stramonium* leaves 24 hours before inoculation. Fucoidan was also seen to increase the protein-synthesizing capability of cells, provoking stimulation of intracellular lytic processes that lead to destruction of virus particles [96].

3.3 Alginate from Brown Algae

3.3.1 Detailed Description of Chemical Composition and Structure of Alginate

Alginate is the main polysaccharide found in the cell wall of brown seaweeds and can make up 40% of the biomass [8]. It is a linear anionic polysaccharide, which consists of binary copolymers of the uronic acids β -D-mannuronic acid (M) and α -L-guluronic acid (G) units bound via β -(1 \rightarrow 4) or α -(1 \rightarrow 4) linkages. The molecular weight of alginate can vary widely between 50 and 100 000 kDa [97]. The structure of the alginate varies according to the arrangement of the M and G units into either homogenous section (MM or GG) or heterogeneous sections (MG). The block types and their respective chair conformations are shown in Figure 3.3. The chemical structure of alginate is typically described by the frequencies of monads (M or G), dyads (MM, GG, MG or GM) or triads (blocks containing

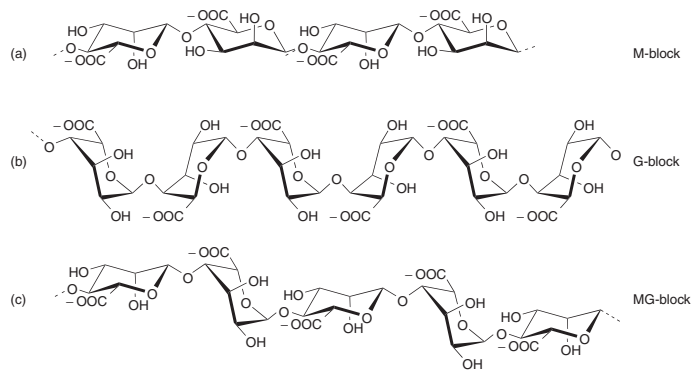


Figure 3.3 Schematic representation of the principal alginate block structures in a chair conformation (a) M block, (b) G-block and (c) MG-block.

three monomer units). In addition, commercial alginate is traditionally characterized by the ratio of mannuronic to guluronic acid (M/G). *Lessonia trabeculata* and *Laminaria hyperborea* have the highest fraction of guluronic acid (M/G ratio of <1), while lowest proportions of guluronic acid are observed in *Macrocystis pyrifera*, *A. nodosum*, or *Laminaria japonica* (all with M/G ratios of >1.5). Alginate chemical arrangement and content can also depend on the harvest season, location or the age and part of the brown seaweed used [98, 99]. The overall M/G ratio or the relative proportion of the three types of blocks (MM, GG, MG) is a crucial factor that determines the physical properties of the alginate. For example, solubility of alginate in acid depends on the proportion of MG blocks present [97].

One important property of alginates for their isolation from seaweed is their ability to form gels in the presence of certain divalent cations, such as Ca^{2+} or Ba^{2+} . The mechanism for the alginate gelation involves the interaction between the divalent cations and the carboxylic groups of two adjacent polymer chains containing GG units (Figure 3.3b), adopting a structure that resembles an 'egg-box' [98, 100]. Hence, the stoichiometry of alginate with the chelating cation (dependant on M/G ratio) or the molecular weight of the alginate polymer influence the gelling properties of this polysaccharide [97, 99].

Alginate is generally industrially extracted by mixing the brown seaweed with NaCO_3 at elevated temperatures. In this process, water-insoluble mixed salts of alginic acid from algal cell wall matrix are converted to water-soluble salts (e.g. sodium alginate). To remove potential contaminants or impurities, such as polyphenols, pigments, proteins, or other carbohydrates, an acid pre-treatment or ethanol washing steps can be used. Calcium chloride is then added to the sodium alginate solution to form insoluble calcium alginate which can easily be separated by sieving or filtering. Precipitated calcium alginate can be converted to soluble alginic acid by acid treatment, which is separated from the solution by decantation or centrifugation. The resulting alginic acid can also be reconverted by alkaline neutralization to any of the commercial forms of alginate (e.g. sodium alginate, potassium alginate, ammonium alginate, etc.), which are now ready to be dried and milled [98, 101]. Biological methods of extraction involve enzymes where alginate is the substrate and the alginate oligosaccharides are the desired compounds [102]. Other methods include the use of pressure, ultrasound, microwaves, radiation, solvent extraction and supercritical CO_2 [103, 104]. However, a lot of these methods are more applicable for removing potential contaminants for laboratory analysis and small-scale production rather than industrial extraction.

3.3.2 Experimental Methods for Chemical Characterization of Alginate

Initial analysis of alginate can involve thin layer chromatography (TLC) which is an economical and quick method making it ideal as a screening technique for crude extracts [105]. Some procedures for the estimation of alginates are based on specific reactions of uronic acids. These reactions involve certain initial hydrolysis step of alginate samples. For example, a traditional method involves hydrolysing the samples in concentrated sulfuric acid at 100 °C before using meta-hydroxydiphenyl as a colour forming reagent in order to detect the corresponding uronic acids [106]. While this method is quick and inexpensive the presence of neutral sugars can cause interferences. Therefore, some modifications have been proposed to minimize this problem [107]. Although these spectrophotometric methods have found wide application, it is evident that the presence of other carbohydrates

containing uronic acids in the sample (e.g. D-glucuronic acid in fucoidans) will inevitably give overestimated results of alginate assays. Direct measurement of the total content of alginate without hydrolysis using CE has been reported [108]. This method is based on sample pretreatment to separate the alginic acid from other ingredients in antacid formulation before quantification by CE. HPAEC-PAD is fast becoming the go-to method for determining monosaccharide composition, M/G ratio and total alginate amount. However, it is important to mention that uronic acids showed different stability at low pH values and high temperatures which made accurate determination difficult [109].

HPLC coupled to RID, MALLS or viscometry are analytical techniques frequently used to obtain information on molecular weight distributions of alginates [110]. Consideration of different column types can also allow the effective and sensitive routine analysis of alginate formulations [111]. Information on the M/G ratio, monomer conformation and linkage position can be obtained by ^1H and ^{13}C NMR spectroscopy. Although these techniques are considered the reference method for characterization of the alginate structure, depolymerized samples using a stepwise hydrolysis are required [103, 112, 113]. Other techniques for investigating the M/G ratio include CD and FTIR [114], with neither technique requiring preparatory hydrolysis. NIR in combination with chemometrics is also a rapid non-destructive method that can give information on alginate content or M/G ratio in brown algal biomass [113, 114]. Finally ESI-MS and MALDI-MS both may give information about DP and sequence of alginate oligosaccharides and are frequently used to analyse the action of alginate lyases [110, 115, 116].

3.3.3 Alginate PB Activity and Potential Applications

Investigation of alginate as a potential plant elicitor typically first involves the depolymerisation of alginate using chemical hydrolysis or by enzymatic means with alginate lyases. This research, which began in earnest 25 years ago, mainly investigated the plant growth promoting activities of 1.8 kDa alginate oligosaccharides prepared with a bacterial alginate lyase [117]. Natsume et al. [118] generated, purified and characterized different alginate oligosaccharides mixtures using an alginate lyase from *Alteromonas macleodii* and results showed that trisaccharides from the lysate had the highest root growth-promoting activity in a barley bioassay. The effects of oligosaccharides on the promotion of root growth in lettuce, rice and carrots were also compared to the growth promotion effects of the polysaccharides [119, 120]. In general, it was found that the alginate polysaccharide had little or no root growth promoting activity while the alginate oligosaccharides with DP4 to DP6 were very effective at stimulating root growth. However, this enhanced root promotion activity in both rice and carrots plants was mainly confined to the guluronic acid-rich alginate oligosaccharides [120]. Enhanced germination and root growth of maize seeds was seen following a soaking application of an alginate oligosaccharide with an average molecular weight of 1445 Da. The highest activities of enzymes associated with enhanced germination, such as α and β -amylase or protease, were also observed after applying these alginate oligosaccharides at 0.75 mg ml^{-1} [121]. These growth- (in particular root) promoting traits of alginate oligosaccharides may be due to an auxin-eliciting type response in the plants. When applied to rice, alginate oligosaccharides with DP2 to DP4 induced the expression of the auxin-related genes, namely *OsYUCCA1*, *OsYUCCA5*, *OsIAA11* and *OsPIN1*, resulting in an increase of 37.8% in IAA concentration in roots [122]. Similarly, a recent study

showed that $100 \mu\text{g ml}^{-1}$ calcium alginate submicroparticles significantly promoted seed germination and seedling growth of wheat by influencing IAA synthesis and metabolism [123]. A novel growth-promoting mode of action may involve the induction of NO in the root system of wheat by alginate oligosaccharides with DP2 to DP4. This NO generation was linked to the up-regulation of the gene expression and enzyme activity of NR at the post-transcriptional level [124].

The role of alginate oligosaccharides as an elicitor-like substance was proven in plant cell suspensions. It was reported that autoclaved alginate, which was degraded to fragments below 100 kDa, promoted chitinase production in *Wasabia japonica* cells [125]. Guluronic-acid-rich alginate oligosaccharides below 2 kDa stimulated the release of the indole alkaloid ajmalicine by *Catharanthus roseus* protoplasts [126]. Akimoto et al. compared the effect of alginate 1n oligosaccharides and oligogalacturonic acid [127]. All of them promoted the accumulation of defence enzymes or secondary metabolites (e.g. 5'-phosphodiesterase, chitinases, or ajmalicine) in the suspension culture of *W. japonica* or *C. roseus* cells [127]. Mannuronic acid-rich alginate oligosaccharides with DP20 generated by chemical hydrolysis were successful in helping the plant defence system against TMV through the activation of the enzymes AOX and PAL ascorbate peroxidase [128]. Also, mannuronic acid-rich alginate oligosaccharides from the brown seaweed *L. vadosa* were found to be a better elicitor of PAL and POD in wheat plants than oligosaccharides derived from a polyguluronic acid fraction [129]. Alginate oligosaccharides of DP6 have also been implicated in eliciting PAL and the accumulation of phytoalexins in soybean, enhancing antimicrobial activity on *Pseudomonas aeruginosa* [130]. Similarly, alginate oligosaccharides with DP values between 4.2 and 11.4 were found to induce the enzyme activities of PAL, POD and CAT in rice plant cells for protection of the plant against *Magnaporthe grisea* reducing the disease index damage of the plant from 10% to 17% [131].

The application of radiation is an additional method for the hydrolysis of alginate and obtaining bioactive formulations. Alginate oligosaccharides of 10 kDa produced by depolymerisation by gamma-ray radiation was applied to rice through a hydroponic system where it enhanced growth with the optimum application at 20–50 ppm. The same alginate was also applied as a foliar spray on tea, carrot, peanut and cabbage at concentrations from 20 to 100 ppm, leading to increases of productivity of 15–60% with respect to control [132]. Alginate oligosaccharides derived from this production method have proven successful in stimulating growth in poppy and fennel plants [133, 134]. There were also increases in photosynthetic pigments, proline, NR activity or secondary metabolites (e.g. essential oils or morphine and codeine for fennel and poppy plants, respectively). In maize plants it was found that 15 kDa alginate oligosaccharides, depolymerized by radiation and H_2O_2 , stimulated growth promotion traits such as grain dry weight and yield when sprayed at a rate of 100 ppm [135]. Irradiated sodium alginate applied at a concentration of 20–120 mg l^{-1} on *Artemisia annua* L. improved the growth attributes significantly, photosynthetic and flowering capabilities, NR and CA enzymatic activities or artemisinin content of the plant [136, 137], while irradiated alginate applied at a rate of 100 mg l^{-1} on mint also had beneficial effects on these very same parameters [138]. Further studies with irradiated alginate applied at concentrations from 20 to 80 mg l^{-1} on lemongrass also revealed similar beneficial effects on growth, biochemical and quality parameters [139, 140]. Enhanced N, P and K were also seen when irradiated alginate was sprayed at a rate of 80 mg l^{-1} on *A. annua*

[141, 142], suggesting a more efficient absorption and utilization of mineral nutrients in plants. Corresponding outcomes in terms of growth-promotion, photosynthetic and biochemical parameters were also seen with irradiated alginate applied on *C. roseus* [143], *Eucalyptus citriodora* Hook. [144], mint [145] and *Trigonella foenum-graecum* L. [146]. The advantageous effects of irradiated alginate on yield quality were likewise seen with spearmint [147] and vetiver [148].

Similar to other seaweed carbohydrates, there appears to be more research related to growth and biotic stress than abiotic stress for alginate. Alginate oligosaccharides have demonstrated an enhancement of plant tolerance to abiotic stressors such as drought [116, 149] salinity [150, 151] and heavy metals [152]. With regard to drought stress, exogenous alginate oligosaccharides DP2-DP6 recovered growth suppression of wheat from water deficit induced with PEG [116]. Wheat plants sprayed with 1000 mg l^{-1} of alginate oligosaccharides displayed an increased plant growth and biomass, improved relative water content, enhanced POD and SOD enzymes activities, decreased lipid peroxidation and upregulated expression of genes involved in the ABA signalling pathway [116]. Similarly, a recent study suggested that alginate oligosaccharides with a molecular weight of 4 and 8 kDa may induce some antioxidant enzyme synthetic genes involved in the ABA signalling pathway by stimulating ABA synthesis to induce expression of drought resistance genes and to improve the capacity of drought resistance in cucumber [149]. Alginate oligosaccharides DP2-DP6 stimulated root elongation and relieved salt stress in *Brassica campestris* L., enhancing also SOD and POD enzymatic activities [150]. A coating of 32 kDa alginate oligosaccharides of *Eucomis autumnalis* bulbs alleviated negative effects of salt stress, limiting the accumulation of toxic Cl^- and Na^+ ions in leaf tissues and increasing leaf levels of L-ascorbic acid and antioxidant activity [153]. Finally, the positive effects of alginate oligosaccharides in alleviating cadmium toxicity were reflected in wheat plants by increased biomass, chlorophyll content, photosynthetic rate or enhanced SOD, POD and CAT enzyme activities or decreased lipid peroxidation [8].

3.4 Carrageenan from Red Algae

3.4.1 Detailed Description of Chemical Composition and Structure of Carrageenan

Carrageenan is extracted from red seaweed species that grow along the coast of north America and Europe, making up between 30% and 80% of the cell wall constituents. These concentrations are influenced by season, species and growth conditions of red seaweeds [154]. Carrageenan is a diverse group of linear, partially hydrophilic, polysaccharides made up of ammonium, calcium, magnesium, potassium and sodium sulfate esters of alternating 3-linked- α -D-galactose and 4-linked- β -D-galactose or 4-linked-(3 \rightarrow 6)-anhydro-D-galactose units. Besides galactose and sulfate, additional substituents such as pyruvate and xylose groups have been observed. Thus, commercial carrageenans are usually heterogeneous polymers with an average molecular weight ranging between 100 and 1000 kDa [8].

The main differences which influence the properties of carrageenan are the number and position of ester sulfate groups as well as the content of the (3 \rightarrow 6)-anhydro-D-galactose units (Figure 3.4). These structural variations will affect the water solubility or gel strength of carrageenan. For example, water solubility is essentially correlated to the

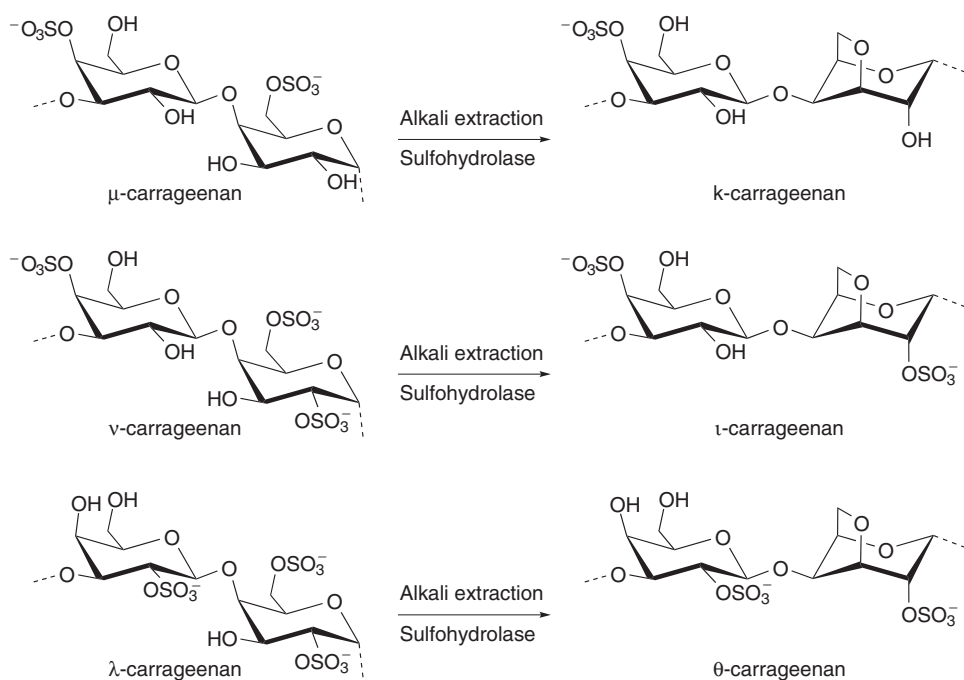


Figure 3.4 Schematic representation of the different structures of carrageenan. Source: Redrafted from [155].

levels of sulfate groups and their associated cations [156]. Carrageenans can contain between 15% and 40% of sulfate esters [157] and are traditionally split into six basic structures: kappa (κ), iota (i), theta (θ), mu (μ), nu (ν) and lambda (λ) [155]. As we can observe in the Figure 3.4, μ -, ν - and λ -carrageenans may be precursors of κ -, i - and θ -carrageenans after alkali extraction at high temperatures or the action of a sulfohydrolase [158, 159]. κ -Carrageenan is predominantly obtained by chemical extraction of the tropical seaweed *Kappaphycus alvarezii* and is composed of alternating D-galactose-4-sulfate and (3,6)-anhydro-D-galactose units. i -Carrageenan differs from this by the addition of sulfate groups in the position 2 of the 3,6-anhydro-D-galactose units and is usually extracted from *Eucheuma denticulatum*. λ -Carrageenan contains little or no (3 \rightarrow 6)-anhydro-D-galactose units but a significant amount of sulfate, alternating units of (1 \rightarrow 3)-D-galactose-2-sulfate and (1 \rightarrow 4)-D-galactose-(2 \rightarrow 6)-disulfate. This type of carrageenan, commonly isolated from different species of the *Gigartina* and *Chondrus* genera, shows stronger hydrophilic properties and does not gel [8].

Even though different carrageenans are soluble in hot water, commercial extraction generally takes place in an alkaline solution at elevated temperatures. This step removes some sulfate groups and increases the formation of 3,6-anhydrogalactose units, generating a stronger gel [8]. After the extraction step, the carrageenan fraction can be separated from the seaweed residue by centrifugation and/or filtering. Carrageenan can be concentrated by adding KCl or alcohol to form a precipitate which can then undergo a freeze/thaw and ground cycle. Once carrageenan is gelled in the presence of potassium, the interaction

between sulfate groups and potassium ions helps to prevent hydrolysis reactions [160]. Because solutions of carrageenan have a lower viscosity in an acid medium ($\text{pH} < 4.3$), an alternative extraction protocol proposed the use of acid pre-treatment to increase the rate of extraction followed by alkaline extraction with $\text{Ca}(\text{OH})_2$ at a temperature above 90°C for up to 24 hours [161].

3.4.2 Experimental Methods for Chemical Characterization of Carrageenan

Spectrophotometry, NMR, MS, SEC, GLC and HPAEC-PAD are popular analytical techniques for determining the concentration, molecular weight distribution and chemical structure of carrageenan [155]. The spectrophotometric method based on the cationic dye methylene blue is considered the most advantageous colorimetric method for the determination of total carrageenan so far [162]. The common method of total acid hydrolysis for investigating monosaccharide composition in carrageenan is complicated by the fact that it results in the complete degradation of 3,6-anhydro-galactose and its 2-O-methyl ester. However, 4-methylmorpholine-borane complex can be added to prevent the acidic degradation of these monosaccharides before being determined by GLC or HPAEC-PAD [163]. The official method for determining the sulfate content of carrageenan is based on the selective hydrolysis of the sulfate ester by acid and subsequent selective precipitation of the sulfate ions as barium sulfate [164].

Size exclusion chromatography in conjunction with various detector systems has also proved useful in determining molecular weight distributions of carrageenan [159]. NMR spectroscopy, both ^1H and ^{13}C , is one of the standard tools for the determination of the chemical structure of carrageenan samples [165]. Although they are fast and suitable for a quantitative approximation of the different types of carrageenan, both NMR techniques require high-purity samples. Finally, MALDI-MS and ESI-MS are powerful and sensitive tools for determining the molecular weight and level of sulfation of carrageenan [166–168].

3.4.3 Carrageenan PB Activities and Potential Applications

Shukla et al. recently provided a thorough account of the effects that carrageenan and carrageenan oligosaccharides can have when applied to plants in terms of growth promotion and defence [169].

Although there are quite a number of studies that have investigated the role of carrageenan on plant growth promotion, much of them focused on carrageenan oligosaccharides produced by acid hydrolysis, enzyme degradation or with radiation. Muñoz et al. described in a patent application a method for stimulating carbon fixation in plants by applying an aqueous solution of carrageenan oligosaccharides produced by acid hydrolysis [170]. Using 10 kDa oligosaccharides obtained from commercial λ -, κ - and ι -carrageenan by acid hydrolysis, Castro et al. found an increase in leaf biomass and height in tobacco plants when these oligosaccharides were sprayed at a rate of 1 mg ml^{-1} [171]. There was also an increase in chlorophyll content, RuBisCO activity and PSII quantum efficiency [171]. An increase in plant height, chlorophyll content and RuBisCO activity was also seen when commercial carrageenans hydrolysed in acid producing 10 kDa carrageenan oligosaccharides were applied to *Pinus radiata* [172]. Likewise, a foliar spray of irradiated carrageenan at 80 mg l^{-1} significantly improved the growth attributes, quality parameters

and yield of essential oil of a fennel crop [173]. Interestingly, when that carrageenan was not hydrolysed with radiation, no beneficial effect on the plant growth parameters were observed [173]. Similarly, oligo-carrageenan applied at a rate of 1 mg ml^{-1} to *Eucalyptus globulus* trees resulted in an increase in height and trunk diameter as well as the content of α -cellulose and total essential oils [174]. That bioactive effect was associated with the carrageenan type, with κ -carrageenan the most successful PB treatment tested [174]. In a follow-up study, the same research group observed how 10 kDa carrageenan oligosaccharides, applied once again to eucalyptus trees, caused an increase in NADPH, ascorbate and glutathione synthesis or thioredoxin reductase activity, leading to activation of photosynthesis, basal metabolism and plant growth [175]. Further analysis revealed an effect by these carrageenan oligosaccharides on endogenous plant hormone levels including higher concentrations of the auxin IAA, the gibberellin GA_3 and the cytokinin trans-zeatin as well as a decrease in the level of the brassinosteroid epi-brassinolide [176]. Similarly, Saucedo et al. corroborated these changes on phytohormonal levels when oligo-carrageenans were applied to pine trees, increasing endogenous levels of IAA and GA_3 [172]. In terms of linking carrageenan molecular weight to growth promoting activities, a trend associated with size and application procedure was observed in pak choi plants (*Brassica napus* var. chinensis) treated with different irradiated carrageenan oligosaccharides [177]. While the growth promoting effect of the different oligosaccharides followed the order of $5 \text{ kDa} > 3 \text{ kDa} > 1 \text{ kDa}$ using hydroponics application, the reverse was observed in the order of $1 \text{ kDa} > 3 \text{ kDa} > 5 \text{ kDa}$ when treatments were applied by foliar spraying [177]. Conversely, a high molecular weight crude κ -carrageenan extract also improved different plant growth parameters in chickpea and maize as well as promoting early flowering [178]. Likewise, the spray application of unmodified commercial ι -carrageenan significantly improved the height, number of leaves and inflorescences in *Verbena bonariensis* [179].

There have also been numerous reports about the role of carrageenan against plant pathogens including viroids, viruses, bacteria and fungi. In general, these sulfated carbohydrates have been shown to induce accumulation of secondary metabolites associated with plant disease resistance such as phytoalexins [110]. λ -Carrageenan elicited resistance against the viroid TCDVd, which can cause more than 25 diseases in plants. When three-week-old tomato plants were spray-treated with different carrageenan at 1 g l^{-1} and inoculated with TCDVd after 48 hours, only λ -carrageenan resulted in a significant suppression of disease symptoms including viroid concentrations in the infected shoots [180]. Proteome analysis of λ -carrageenan treated plants revealed that 16 tomato proteins were differentially expressed including JA related genes, allene oxide synthase and *LOX*, which were all up-regulated [180]. λ -Carrageenan was also shown to be effective in protecting tobacco plants against the pathogen *Phytophthora parasitica* var. *nicotianae* when infiltrated in the tobacco leaves at concentrations from 0.1 to 1 mg ml^{-1} [181]. The authors found that λ -carrageenan was the most effective of all carrageenan in triggering signalling and defence responses [181]. This highly sulfated carbohydrate induced the accumulation of SA in leaves and caused increased expression of antifungal chitinases and a type 2 proteinase inhibitor with antipathogenic activity along with the expression of *LOX*, whose gene expression led to JA biosynthesis [181]. Interestingly, this study also found that polymerization and the level of sulfation can determine the bioactivity of the carrageenan used [181]. This phenomenon was also seen by Sangha et al. when they investigated the role of different carrageenans on the fungal pathogen *Sclerotinia sclerotiorum*

in *Arabidopsis thaliana* [182]. This report showed that λ -carrageenan induced differential resistance to the pathogen by increasing the expression of JA related genes AOS, PDF1.2 and PR3 and the *in planta* oxalate oxidase activity [182]. This last defence mechanism is remarkable because *S. sclerotiorum* requires the secretion of oxalic acid to infect the plant by suppressing the oxidative burst of the host plant. Furthermore, in contrast to Mercier et al., the λ -carrageenan induced resistance was proven to be independent of SA through the use of a SA-deficient plant mutant [181].

Carrageenans have well-known antiviral activities and the link with their structural features was explored in great detail by Ghosh et al. [183]. According to Nagorskaya et al., it is possible that carrageenans may suppress the effects of TMV by inhibiting the binding of viral particles into the host cell [184]. Likewise, it has been observed that κ/β -carrageenans stimulated a number of lytic processes in *D. stramonium*, preventing the intracellular accumulation and translocation of PVX particles [185]. κ -carrageenan extracted from the red alga *Hypnea musciformis* was also able to reduce the TMV infection in tobacco plants [186]. Molecular analysis suggested that this carbohydrate modulated the defence response by inducing the crosstalk between the SA and JA-dependent signalling pathways [186]. The importance of the size of the carrageenan when eliciting the plants defences against TMV was demonstrated by Vera et al. [23]. While *t*-carrageenan being applied at concentrations of 1 mg ml⁻¹ did not protect tobacco plants, *t*-carrageenan oligosaccharides offered a 79% reduction in necrotic lesions at the same concentration [23]. Further evidence of the effect of molecular weight of carrageenan on plant bioactivity was put forward by Kalitnik et al. [187]. From high molecular weight carrageenan (250–400 kDa), different hydrolysis techniques were used to generate carrageenan fractions with molecular weight values ranging from 1.2 to 4.3 kDa [187]. The antiviral activities against TMV of the higher molecular weight carrageenan were significantly higher than the low molecular weight counterparts [187]. Furthermore, they observed differences in the antiviral activity according to the method of depolymerisation, where low molecular weight products from enzymatic depolymerisation performed the worst [187]. Similar depolymerisation techniques were used on four different κ -carrageenans to study the antioxidant effects of these extracts. It was shown that κ -carrageenan oligosaccharides degraded by different methods had different antioxidant effects. The antioxidant activity of hydrolysates was also significantly affected by the DP or the content of carboxyl groups and sulfate groups [188]. Although this last study shows the direct antioxidant activity *in vitro* of carrageenan oligosaccharides of various sizes rather than their eliciting effects when applied to plants, these results confirm the correlation between chemical activity and structure. In addition, it was observed how the treatment of plants with various carrageenan modulated the resistance of *Arabidopsis thaliana* to herbivory. In particular, *t*-carrageenan seemed to elicit significant resistance to cabbage looper (*Trichoplusia ni*) by inducing JA and SA-dependent pathways or an alteration of the products of glucosinolate hydrolysis [182]. Taken together, these results showed that the degree of sulfation of the polysaccharide chain may well mediate this effect [182]. The ability of commercial κ -carrageenan to increase the resistance of basil plants to the parasitic plant *Cuscuta campestris* was also recently proved. In this study, foliar treatment of carrageenan induced beneficial effects on plants such as growth stimulation, activation of the phenylpropanoid pathway or decrease of parasite infestation [189].

Much less work has been carried out concerning carrageenan and abiotic stress. A recent study by Singh et al. looked at γ -ray irradiated carrageenan oligosaccharides which were

sprayed on lemongrass plants subjected to drought stress [190]. Foliar application of this oligosaccharide fraction with molecular weight values ranging from 20 to 100 kDa at concentrations between 80 and 120 mg ml⁻¹ augmented the plant water status parameters under stress and unstressed conditions at both growth stages [190]. Enhancement in osmotic adjustment under water stress conditions was attributed to the endogenous accumulation of soluble sugars, proline and inorganic ions [190]. A reduction of electrolyte leakage in treated plants during drought stress was concomitant to an increase in the activities of CAT and POD enzymes [190]. In terms of growth promotion, the irradiated carrageenan oligosaccharides caused an enhancement of essential oils yield and quality along with the accumulation of nutrients (N and P) in both stressed and unstressed conditions [190]. Outcomes of this work shared many similarities with the growth-promoting and biotic stress response (as mentioned in previous paragraph). The gelling properties of carrageenan can also be exploited to alleviate drought stress in wheat seeds by forming a hydrogel that will physically retain water [191]. However, any protection here was as a consequence of a physical barrier rather than eliciting the plants own defences [191]. Finally, carrageenan oligosaccharides appear to act as signal molecules enhancing the induction of microspore embryogenesis of *Brassica oleracea* var. *italica*. The induction efficiency increased as the number of sulfate substituents on the glycosidic backbone and the signalling mode of action was likely common to heat shock or oxidative stresses [192].

3.5 Ulvan from Green Algae

3.5.1 Detailed Description of Chemical Composition and Structure of Ulvan

Ulvan is a water-soluble polysaccharide that is found in green seaweed, namely *Ulva* and *Enteromorpha*, and has been reported to make up between 8% and 29% of the algal dry weight [193]. Like fucoidan, ulvan is a sulfated polysaccharide composed of neutral sugars (L-rhamnose, D-xylose and D-glucose) and uronic acids such as D-glucuronic acid and iduronic acid [194] connected by α - and β - (1 \rightarrow 4) glycosidic bonds [195]. However, low proportions of galactose, glucose and protein were also generally found in ulvan [196]. Ulvan structure can alternate between two major repeating disaccharides composed of sulfated rhamnose and uronic acids. They are also designated ulvanobiuronic acid A and B units. The main difference between these units is the presence of D-glucuronic acid in A which is replaced by L-iduronic acid in B (Figure 3.5). From the monosaccharide composition analysis, L-rhamnose seems to be the most abundant sugar (17–45%), followed by uronic acids (6.5–19%), D-xylose (2–12%) and D-glucose (0.5–6.4%). Most of the sulfate groups are located in position three of the L-rhamnose units and their contents range from 16% to 23% [193]. It is accepted that ulvan is composed of two major macromolecular populations, identified as a high molecular weight fraction (300–1200 kDa) and a medium molecular weight fraction (85–180 kDa), with the high molecular weight fraction being the most abundant [196, 197].

Because a decrease in seaweed particle size can increase the yield of extraction, the first steps involve material washing prior to thermal drying and grinding [197]. Interfering substances such as pigments, lipids and polyphenolic compounds can then be removed through the use of organic solvents or supercritical extraction [176, 198]. As a hydrophilic polysaccharide, ulvan can be effectively extracted in water. Some studies reported that water extractions at hot temperatures in the range of 80–100 °C are more efficient to

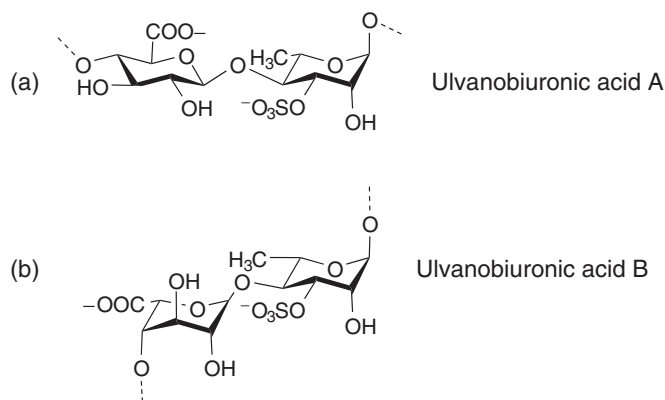


Figure 3.5 Schematic representation of the main units of ulvans: (a) ulvanobiuronic acid A and (b) ulvanobiuronic acid B. Source: Redrafted from [8].

obtain high molecular weight species [197]. Alkaline and acidic extraction conditions in combination with calcium chelating agents have also been used for more efficient extraction. Ulvan, like alginate, is able to gel in a reaction mechanism mediated by calcium ions. Thus, these chelating agents facilitate ulvan extraction disrupting chemical bonds formed by ulvan in the presence of calcium ions within the cell wall of green seaweed [199, 200]. Finally, water-soluble polysaccharides can be purified by precipitation with organic solvents before being dried for long term storage [194, 201]. Anion exchange chromatography and gel permeation chromatography can also be employed for further purification of crude ulvan extraction fractions [202]. Overall, yield of extraction of ulvan can vary between 1.2% and 27.5%. However, the maximum extraction efficiency can be in the range of 70% [193, 196, 197].

3.5.2 Experimental Methods for Chemical Characterization of Ulvan

Extraction conditions or seaweed seasonal variation can influence the chemical composition and the molecular weight of ulvan [176, 203]. While it is possible to determine the uronic acid and sulfate content by colorimetric methods [204, 205], a complete monosaccharide composition of ulvan needs to be analysed by HPAEC-PAD or GLC after acidic hydrolysis [203, 204]. However, the establishment of an accurate ulvan sugar composition is complicated by the strength of the glycosidic linkages of ulvanobiuronic units and the labile nature of iduronic acid in acidic conditions. To overcome these analytical limitations, an alternative methodology of combining mild acid hydrolysis with enzymatic degradation followed by HPAEC-PAD detection has been proposed [206]. Molecular weight distribution and structure of ulvans can be determined by using techniques mentioned for other sulfated polysaccharides such as SEC, CD, FTIR, NMR, or ESI-MS [200, 202, 205, 207].

3.5.3 Ulvan PB Activities and Potential Applications

The use of ulvan and ulvan oligosaccharides, in particular those extracted from green algae of the genus *Ulva* or *Enteromorpha*, as elicitors of mechanisms for nitrogen absorption and protein synthesis have been proposed [208]. This US patent described how a single ulvan

treatment application at low concentrations ($0.1\text{--}1\text{ g l}^{-1}$) stimulated the expression of GS and GDH in model legume *Medicago truncatula* [208]. Double application resulted in an overexpression of the genes encoding the ammonium transporter or the enzymes NR, GS and GDH [208]. In peas and maize, improvement in the root protein content was seen in conjunction with the stimulation of the root biomass which further translated into a 7% increase in yield per hectare for maize [208]. An additional nitrate accumulation in the leaves was also reported in lettuce following ulvan treatment [208].

Application of ulvan extracts on plants tends to focus on biotic stress, stimulating defence responses against pathogens. In an early study, Cluzet et al. reported on the elicitor effect of a crude *Ulva* spp. extract on *M. truncatula* [209]. When infiltrated into plant tissues or sprayed onto the leaves, this ulvan extract induced the expression of the defence-related marker gene PR10 without provoking necrosis [209]. A broad range of defence-related transcripts was found to be up-regulated, notably genes involved in the biosynthesis of phytoalexins [209]. In contrast, the expression of primary metabolism-related genes did not change significantly [209]. In a further report, a pre-treatment of ulvan extracted from *Ulva fasciata* at 0.2 mg ml^{-1} on rice and wheat cells amplified the oxidative burst induced by chitin oligosaccharides or a chitosan polysaccharide by up to five times [210]. This priming activity corresponded to a 45% reduction in infection by *Blumeria graminis* [210].

Jaulneau et al. illustrated how a sulfated polysaccharide extracted from *Ulva* spp. can be a potential reservoir of elicitors which acts through a JA signalling pathway, but not an SA-dependent pathway, in plants belonging to three botanic families: *Nicotiana tabacum* (Solanaceae), *A. thaliana* (Brassicaceae) and *M. truncatula* (Fabaceae) [211]. 400–600 kDa ulvan fractions particularly enriched in rhamnose, uronic acid and sulfate were the most effective at inducing JA-related genes such as NtLOX1 or PDF1.2 [211]. The same research group also showed how a similar ulvan extract could protect *Phaseolus vulgaris*, *Vitis vinifera* and *Cucumis sativus* against powdery mildew [212]. This bioactive effect increased as ulvan concentration and number of spray applications increased [212]. Likewise, de Freitas et al. observed that Arabidopsis plants sprayed with hydrolysed ulvan fractions extracted from *U. fasciata* decreased the severity of infection by phytopathogens *Alternaria brassicicola* and *Colletotrichum higginsianum* [213]. Ulvan-treated plants also increased NADPH oxidase and H_2O_2 levels along with enhanced activities of enzymes associated with removing ROS [213]. However, this induced resistance did not seem to be directly affected by the degree of ulvan sulfation [213]. Another strikingly-related study reported that an ulvan extract that was applied at a concentration of 10 mg ml^{-1} reduced the disease severity of *Colletotrichum gloeosporioides* by 66% in apple trees [214]. While this effect was found to be cultivar specific, there was an overlap with other studies about bioactive effects of ulvan as enhanced POD levels were also found [214]. In addition, ulvan oligosaccharides DP2 were found to be more effective than ulvan polysaccharides at protecting apple fruits from *Penicillium expansum* and *Botrytis cinerea* [214]. Both treatments triggered a transient accumulation of H_2O_2 as well as the activation of antioxidant-related enzymes. These treatments also increased the activities of enzymes involved in phenylpropanoid metabolism and the levels of lignin and phenolic compounds. Moreover, since an *in vitro* assay showed that the ulvan extracts had no effect on fungal development, it was assumed that the protection was due to their elicitor activity [215].

In terms of abiotic stress, an US patent described ulvan extracts from green seaweed of the genus *Ulva* or *Enteromorpha* as activators in plants defence against biotic and abiotic stress [211]. This ulvan-derived treatment applied at a concentration range between 1 and 10 g l^{-1} to leaves or roots of maize plants growing under heat and hydric stress significantly enhanced the biomass of treated plants with respect to control [211].

3.6 Laminarin from Brown Algae

3.6.1 Detailed Description of Chemical Composition and Structure of Laminarin

Laminarin was first isolated by Schmiedeberg in 1885. It is usually a linear polysaccharide made up of glucose units linked by β -(1 \rightarrow 3) linkages and β -(1 \rightarrow 6) or β -(1 \rightarrow 2) intrachain branching [8, 216, 217]. The terminal reducing ends of laminarin chains can either be a glucose residue (G type) or a D-mannitol residue (M type) (Figure 3.6). The ratio of each type of chain depends on the seaweed species [163]. Its average molecular weight is approximately 5 kDa, showing a DP that usually ranges between 20 and 33 units [8, 74, 163, 218]. The DP has a marked effect on the solubility of laminarin in water. While highly branched

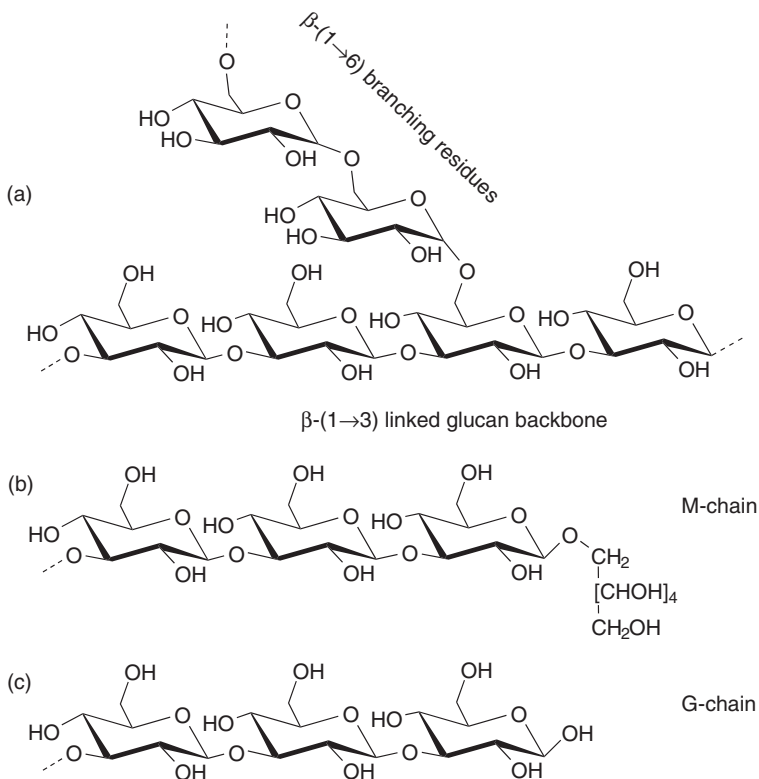


Figure 3.6 Schematic representation of the laminarin structure: (a) β -(1 \rightarrow 3) linked glucan backbone with β -(1 \rightarrow 6) intrachain branching, (b) M-chain and (c) G-chain.

laminarins are soluble in cold water, a lower extent of branching requires warm water for full solubility [216, 219].

Laminarin is the principal storage polysaccharide of brown seaweeds [2, 3, 8] and can represent up to 35% of the algal dry weight [220]. This is evidenced by the gradual disappearance of laminarin from tissues of *Laminaria* during the winter. Zvyagintseva et al. used the brown seaweeds *Laminaria cichorioides*, *L. japonica* and *F. evanescens* to illustrate how the species along with its location can affect laminarin content and structure [221]. Environmental factors such as temperature, depth of the immersion and nutritive salts in the water can also affect laminarin composition [163, 217]. For example, a decrease in the level of nitrite and nitrate in the water has been known to stimulate the synthesis of laminarin in the seaweed [222].

Across the literature laminarin has been reported to be extracted from fresh weed or extracted following a drying step. Generally, it is cut, ground or blended to enhance extraction efficiency. Laminarin extraction is complicated by potential co-extractions with other seaweed polysaccharides. It can be isolated along with fucoidan in hot water after adding calcium chloride to prevent the further extraction of alginate and can be precipitated at this point with ethanol. Additionally, dialysis can be used to remove salts and polyphenols can be removed through the use of an SPE column [217]. Both acidic and basic methods of laminarin extraction have been reported, although acidic extractions seem to be more frequently used [223].

3.6.2 Experimental Methods for Chemical Characterization of Laminarin

Early methods of quantitative determination of laminarin involved hydrolysis with sulfuric acid to leave glucose as the sole reducing carbohydrate. However, this method is complicated due to the presence of other carbohydrates such as alginate and fucoidan that can also produce reducing sugars [224]. More modern methods of detection generally revolved around molecular weight determination using FAB-MS and MALDI-MS [163], HPSEC-MALLS [74], GC-MS [225] and ESI-MS [217]. A new simple, efficient, cold water extraction protocol coupled to a new quantitative LC-MS method was recently developed [226]. Laminarin was determined in 9 out of 12 brown seaweed species, and its expected typical molar mass distribution of 2–7 kDa was confirmed. NMR and FT-IR methodologies have been also used to analyse different water-soluble polysaccharides from brown and red seaweeds, including laminarins [227]. Laminarin can be detected through enzymatic methods by using the β -glucosidase enzyme [228]. It should be noted that the detection of laminarin will depend of the extraction and purification methods used [229].

3.6.3 Laminarin PB Activities and Potential Applications

In terms of human and animal health, laminarin has been widely reported to have biological activities such as anti-tumour, anti-inflammatory, anti-coagulant and anti-oxidant effects along with possessing beneficial effects for gut micro flora [217, 220]. Rioux and Turgeon provide a short summary of the research undertaken in this area [8]. As a result of these effects, laminarin is available commercially as a natural human health product and as an additive to animal feed.

β -glucan oligosaccharides have long been known to elicit the plants own defence responses including the production of phytoalexins, antimicrobial proteins and ROS

[230], for example in plants such as rice [231], grapevine [232–235], tobacco [236], alfalfa [237], soybean [238] and *A. thaliana* [239]. However, these responses are typically associated with biotic stress and fall outside the definition of PBs according to Du Jardin [15]. Nevertheless, as mentioned previously, there can be an overlap between stimulating typical biotic stress mechanisms and plant responses to abiotic stress [62–65, 240, 241]. For example, fungal β -glucan elicitors were able to cause a rapid increase in cytosolic Ca^{2+} concentration in soybean [238]. This elicitor effect of laminarin on calcium signalling is relevant because different reports described how the rise of free cytosolic Ca^{2+} concentration is correlated to many physiological stimuli such as light, touch, pathogenic elicitor, phytohormones and abiotic stresses including high salinity, cold and drought [242, 243]. Many changes in environmental conditions and hormones are mediated by MAPK (mitogen-activated protein kinase) cascades in plants. These kinases play various roles in intra- and extra-cellular signalling by transferring the information from sensors to responses and can work as mediators of various biotic and abiotic stresses in plants [244]. Interestingly, MKK1 was found to be activated by laminarin or H_2O_2 in Arabidopsis protoplasts but not by salt, cold and heat stress conditions [245]. The chemical sulfation of laminarin was shown to be able to elicit a wider array of defence responses in tobacco and Arabidopsis than the native compound. Interestingly, this artificial sulfated laminarin was an inducer of the SA signalling pathway [239]. A further study suggested two distinct perception systems for laminarin and sulfated laminarin [246]. Moreover, the same sulfated laminarin demonstrated its efficiency in protecting a susceptible grapevine cultivar against downy mildew [247].

Laminarin can also provide significant levels of abiotic stress tolerance to plants. Wu et al. observed in Arabidopsis seedlings growing in agar medium with 25 mg l^{-1} of laminarin that it had a significant promotion of plant growth and tolerance to heat and salt stress by modulating the chloroplast antioxidant system [248]. The transcriptome analysis also indicated that a number of genes associated with abiotic stress tolerance were affected by laminarin treatment, including genes associated with high temperatures, light, salinity and water deficit. Another report investigated the effect of the molecular weight of β -glucan elicitors on the induction of defence responses [249]. Curdolan oligosaccharides (a water-insoluble linear beta-1,3-glucan) with low DP [2–10] induced a different response pattern than for high DP [25–40] laminarin in tobacco cells. These responses included hydrogen peroxide burst, stomatal closure or protection against TMV. Stomatal movement is recognized as one of the most sensitive responses to biotic and abiotic stresses [250] and the same stomatal closure was also observed after applying laminarin DP13 in grapevine [251]. Thus, laminarin is one of a few exogenous carbohydrate elicitors reported, along with oligogalacturonides and chitosans [252, 253], to induce significant stomatal control.

3.7 Cellulose and Hemicellulose Derived Oligosaccharides

3.7.1 Detailed Description of Chemical Composition and Structure of Cellulose and Hemicellulose Oligosaccharides

Cellulose is the most abundant organic substance on the earth, consisting of chains of β -(1 \rightarrow 4)-linked glucose residues. It is a crystalline polysaccharide existing as two forms: α -cellulose has one-chain triclinic structure, while β -cellulose has two-chain monoclinic structure [254]. Even though cellulose is a fibrous, tough and water-insoluble polymer that

plays an essential role in maintaining the structure of cell walls in higher plants [255], this polysaccharide has a porous web-like structure in algae [256]. Cellulose is found in many types of seaweed [2, 3] but it remains a relatively under-researched polysaccharide in terms of its potential applications in crops as plant PBs. Hemicellulose is also found in the plant cell walls of green, red and brown seaweeds, although it is more of a mixture of xyloglucans, xylans, mannans and glucuronans that are linked by β -(1 \rightarrow 4) and β -(1 \rightarrow 3) glycosidic bonds [8].

Cellulose can be extracted from seaweed by first bleaching the material with NaCl dissolved in an acid medium [257]. Then the mixture is diluted, heated and washed until neutrality before being filtered. NaOH is added to the remaining pulp and stored at 60 °C in a water-bath overnight. At this point, hemicelluloses can be separated by acidification followed by precipitation with ethanol [258]. Once again, the final cellulose residue is washed until neutrality, filtered and dried at room temperature. Cellulose and hemicellulose enriched fractions can be also obtained as a surplus by-product from the alginate extraction process [259].

3.7.2 Experimental Methods for Chemical Characterization of Cellulose and Hemicellulose Oligosaccharides

One of the most common methods for cellulose and hemicellulose compositional analysis involves hydrolysis (through acid or enzymatic means) to the monosaccharide units. While the amount of glucose can be determined spectrophotometrically, a complete monosaccharide analysis is usually evaluated by HPAEC-PAD or GLC [259–261]. Additionally, there is abundant literature using methods such as FTIR, XRD, NMR or electron microscopy for investigating the structure of cellulose and hemicellulose fractions [259, 262].

3.7.3 Cellulose and Hemicellulose Oligosaccharides PB Activity and Potential Applications

The accumulation of cellulose and hemicellulose is a cell wall reinforcement mechanism involved in plant development and response to external stresses. In addition, the plant cell wall acts as a source of signalling molecules to alert the plant immune system in the presence of potential harmful microbial pathogens. As one of the earliest structural barriers pathogens encounter, cellulose and hemicellulose are largely targeted by microbial cell-wall degrading enzymes (e.g. cellulases) to weaken the plant cell wall structure and facilitate pathogen entry. As an evolutionary response to microbial attacks, plants may have adapted to recognize cellulose and hemicellulose oligomers as a warning signal or DAMP and activate downstream defence mechanisms (Figure 3.1). This hypothesis is supported by the recent identification of cellulose-derived oligomers as DAMPs in *A. thaliana* [263]. These oligomers triggered similar signalling cascades to those activated by well-known elicitors such as chitoooligosaccharides and oligogalacturonides [263]. Overall, Arabidopsis plants treated with cellulose oligomers showed less cellular damage following infection with *Pseudomonas syringae* [263]. Intracellular effects included a rapid and transient increase in Ca^{2+} , crucial to the pathogen defence and activation of MAP kinases. Cellulose-derived oligomers can also contribute to abiotic stress tolerance as seen when an oligosaccharide complex containing cellobiose, glucose β -(1 \rightarrow 4) dimer that makes up the

cellulose polymer, provided freezing tolerance in winter wheat by acting as a signalling molecule to increase the cell's sensitivity to ABA [264].

3.8 Conclusions

Macro-algae provide a rich and unique source of carbohydrate polymers which have significantly undiscovered potentials. It is likely that their unique properties as a PB for land plants is due to their origin in the harsh marine environments in which they thrive. Unravelling the carbohydrate chemical characteristics which elicit discrete plant responses, represents a significant challenge, but has potential to deliver significant benefits for next-generation PBs. Current research on the mode of action of macroalgal carbohydrate PBs is promising and suggests that they represent a toolbox to enhance plant growth and productivity in stressed environments. A deficit in the chemical characterization of the macro-algal carbohydrates makes it difficult to assign effects to specific chemical/structural features such as that used in a classical chemical biology approach to discovery. Nonetheless, this deficit provides significant opportunity to further enhance the efficacy and knowledge of macro-algal carbohydrates as plant PBs.

Abbreviations

ABA	abscisic acid
AOX	ascorbate peroxidase
CA	carbonic anhydrase
CAT	catalase
CD	circular dichroism
CE	capillary electrophoresis
DAMP	damage-associated molecular pattern
DP	degree of polymerization
ET	ethylene
ESI-MS	electrospray ionization mass spectrometry
FAB-MS	fast atom bombardment mass spectrometry
FTIR	Fourier-transform infrared spectroscopy
GA3	gibberellin A3
GC-MS	gas chromatography-mass spectrometry
GDH	glutamine dehydrogenase
GLC	gas liquid chromatography
GS	glutamine synthetase
GST	glutathione-S-transferase
HPAEC-PAD	high-performance anion exchange chromatography pulsed amperometric detection
HPLC-RID	high-performance liquid chromatography-refractive index detector
IAA	indole 3-acetic acid
ISR	induced systemic resistance
JA	jasmonic acid
kDa	kilo Dalton

LC-MS	liquid chromatography-mass spectrometry
LOX	lipoxygenase
MALDI-MS	matrix-assisted laser desorption/ionization mass spectrometry
MALLS	multiple angle laser light scattering
MAMP	microbe-associated molecular pattern
MAP	mitogen-activated protein
MAPK	mitogen-activated protein kinase
MS	mass spectrometry
NIR	near-infrared spectroscopy
NMR	nuclear magnetic resonance
NR	nitrate reductase
PAMP	pathogen-associated molecular patterns
PAL	phenylalanine ammonia lyase
PB	plant biostimulant
PEG	polyethylene glycol
POD	peroxidase
PR	pathogenesis-related
PRR	pattern recognition receptors
PSII	photosystem II
PVX	potato virus X
RID	refraction index detector
ROS	reactive oxygen species
RuBisCO	ribulose-1,5-bisphosphate carboxylase/oxygenase
SA	salicylic acid
SEC	size exclusion chromatography
SAR	systemic acquired resistance
SOD	superoxide dismutase
SPE	solid phase extraction
TCDVd	tomato chlorotic dwarf viroid
TMV	tobacco mosaic virus
US	United States
XRD	x-ray diffraction

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