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## Journal Pre-proof

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**Oligoalginates from *Padina pavonica* obtained by chemical and mechanical fractionation differentially induce resistance of date palm vitroplants to biotic and abiotic stresses**

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**Abstract**

Alginate and/or oligoalginates are known as plant defense stimulators that become a novel alternative to agrochemicals. The elicitor effects of alginate and oligoalginates derived from

the brown algae *Padina pavoniva* on the date palm vitroplants was tested. Firstly, alginate (SAPP) was extracted and the structure was characterized by ATR-FTIR, GC/MS and  $^1\text{H}$ -/ $^{13}\text{C}$ -NMR. The M/G ratio was 0.8 indicating that G units were abundant in the alginate polymer. Secondly, alginates depolymerization was performed using ball milling as mechanical depolymerization (OABM) and acid hydrolysis as chemical fractionation (G+M, G and M). The expression level of four plant defense marker genes PAL, SOD, PR1 and DHN in date palm vitroplants after 48 h treatments with alginate (SAPP), dried algae powder treated by ball milling (PPBM), and oligoalginates were investigated. The four genes were up-regulated differentially, according to the elicitor treatment. The highest transcription levels of PAL (phenylalanine ammonia-lyase), PR1 (Pathogenesis-related protein 1), SOD (superoxydes dismutases) and DHN (dehydrin) (~11, 7, 5 and 4-fold respectively more than obtained in control vitroplants) were observed in the vitroplants treated with OABM followed by oligoalginates obtained by chemical hydrolysis. The lowest transcription levels were observed in both SAPP and PPPM treated plants. OABM treated vitroplants showed increased resistance to salt, drought and fungal contamination. These findings open up promising prospects for a new depolymerization method of alginate as a more efficient tool to induce a defense response in plants against abiotic and biotic stresses.

## Keywords

Alginate; ball milling; oligoalginates; elicitor; Date palm; stress; *Padina*

### 1. Introduction

Micropropagation has been usually used to produce commercially a large number of plants from a single specimen [1, 2]. However, two main problems hampered tissue culture technology. Firstly, microbial contamination that can dominate plant tissues, affects growth and shoot proliferation [3]. Secondly, poor survival plants are observed after transfer to soil due to the sudden changes in light, temperature and relative humidity [4]. To cope with these

harmful factors, such resistance can be activated by plant application with various agents. PGPR, hormones, minerals, proteins or polysaccharides are widely reported to stimulate plant immunity [5]. Algal polysaccharides such as carrageenan, fucan, laminarin and alginate derived from macroalgae were successfully proved as a plant's protector as well as growth-promoting agents [6,7,8,9,10]. Alginates are heteropolysaccharides from brown macroalgae. They are defined based on  $\beta$ -D-mannuronic (M) and  $\alpha$ -L-guluronic (G) acids (1,4) linked. These monomers can be arranged on homogenous (MM or GG) and heterogeneous (M/G) blocks. The proportion of these blocks determines the biological and physicochemical properties of these anionic polysaccharides [11]. Thanks to their nontoxicity and biodegradability, alginates or oligoalginates have been demonstrated as plant growth stimulators and found to enhance seed germination and seedling growth of maize [12] and wheat [13]. Moreover, they were reported to promote root and shoot growth of several plants such as barley [14], rice and peanut [15]. Besides, alginate-derived polysaccharides were proved to mitigate the negative effects of abiotic stress including heavy metals [16], high temperature [17], drought [18, 19] and salinity [20, 21]. Further research demonstrated that those biopolymers are effective elicitors that improve plant resistance to several pathogens by stimulating the expression of the genes involved in salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) dependent signaling pathways [9]. A foliar spray containing oligoalginates was applied to *Arabidopsis thaliana* leading to a decrease of disease symptoms provoked by Pst DC3000 by inducing the early accumulation of nitric oxide (NO), ROS and the transcript level of PR1 [22]. Similarly, Dey et al., [23] demonstrated that tomato leaves sprayed with oligoalginates showed higher resistance to *Alternaria solani* by regulating the antioxidant enzymes and PR proteins.

Date palm tissue culture for large-scale multiplication has gained industrial relevance [24]. Bacterial (e.g. *Bacillus*, *Pseudomonas*, *Staphylococcus*, *Proteus*) and fungal (e.g. *Alternaria*

*alternate*, *Aspergillus niger*, *Penicillium spp.* contaminations of cultures [24, 25] and high mortality during acclimatization [27] are still unresolved obstacles affecting the subsequent steps of date palm cultivation.

To evaluate the elicitor effect of oligoalginates from *Padina pavonica*, a brown macroalgae from Tunisian coasts, oligomers were produced with acid and mechanocatalytic hydrolysis and then tested on date palm vitroplants subjected to abiotic and biotic stresses.

## 2. Materials and methods

### 2.1. Extraction of alginate from *Padina pavonica* (SAPP)

*Padina pavonica* was collected from Mahdia-Tunisia seawater in (35.410995, 11.041932) in August 2021. Algae were washed with tap water before sun-drying at ambient temperature. The dry algal biomass was then ground using an electric blender. Alginates were extracted according to Hentati et al., [28]. Fifty grams of algae powder were soaked twice in 700 mL of acetone and then twice with 300 mL of methanol during 24 h at room temperature under constant agitation. Subsequently, biomass was dried in oven at 50°C for 24h. Twenty-five grams of depigmented powder were treated twice with HCl (0.1 M, pH: 2, 500 mL) for 2h at 60°C under constant agitation. The insoluble fraction was collected by centrifugation (10,000×g, 10 min, 20°C) and washed with ultra-pure water before extraction with sodium carbonate (3% (w/v), pH = 11, 500 mL) at 60 °C for 2 h under stirring. After centrifugation (10,000×g, 15 min, 4°C) polysaccharides were precipitated by three volumes of cold ethanol 96%. Sodium alginate collected by centrifugation (12,000×g, 30 min, 4°C) was dissolved in ultra-pure water and acidified by HCl (6M) at pH value comprised between 1.5 and 3. Precipitated alginic acid collected by centrifugation (5,000×g, 20 min, 4°C) was solubilized in ultra-pure water and neutralized with NaOH (1 M). It was then precipitated again with three volumes of cold ethanol 96% and collected by centrifugation (12,000×g, 30 min, 4°C). Finally, sodium alginate (SAPP) was solubilized in ultra-pure water and then freeze-dried.

## 2.2. Acid hydrolysis of alginate

Partial acid depolymerization of *Padina pavonica* alginate was performed according to Fenoradosoa et al., [29]. Briefly one gram of sodium alginate was dissolved in 100 mL of ultra-pure water and then heated at reflux with 3 mL of HCl 3 M for 20 min. After centrifugation (3,000×g, 20 min, 20°C), the supernatant was neutralized with NaOH (1M) and subsequently 100 mL of ethanol 96% were added. The precipitate was collected by centrifugation (10,000×g, 20 min, 20°C), dissolved in ultra-pure water and then freeze-dried (fraction G+M). The pellet of the first centrifugation was dissolved in 100 mL of HCl 0.3M and heated at reflux for 2h. The suspension was centrifuged (10,000×g, 20 min, 20°C) and the insoluble fraction was neutralized and then the pH was adjusted to 2.85. After centrifugation (10,000×g, 20 min, 20°C), the soluble fraction was neutralized, dialyzed against ultra-pure water and finally freeze-dried (fraction M). The pellet was dissolved in ultra-pure water, neutralized, dialyzed and freeze-dried (fraction G).

## 2.3. Acid-Assisted Ball Milling depolymerization of *Padina pavonica* and its alginate

Acid-assisted ball milling depolymerization of dried *Padina pavonica* powder (PPP) or of SAPP was performed according to Boissou et al., [30]. Samples were impregnated with acid. Typically, 1 g of PPP or SAPP was suspended in 15 mL of acetone containing 4 wt % (0,45mmol) H<sub>2</sub>SO<sub>4</sub>. The suspension was shaken for 1 hour. The solvent was then removed under vacuum at 40°C, affording a powder. The acid impregnated PPP or SAPP was immediately introduced into a planetary ball mill (Retsch PM 100) and was milled for the desired time. The two obtained samples were named hereafter PPBM and OABM for acid-assisted ball milling depolymerization of PPP and SAPP respectively.

## 2.4. Hydrolysis Yield

To determine the yield of the degradation process, the weight of the used alginate and obtained hydrolysis product were determined and the yield of the process was calculated

using the following equation (1)

$$(1) \% \text{ yield} = \frac{W_p}{W_a} \text{ with } W_p : \text{ weight of the obtained hydrolysate and } W_a : \text{ weight of used alginate}$$

### 2.5. Molecular Weight Analysis by HPSEC-MALLS High-pressure

Average molecular weights of SAPP, OABM, M, G and G+M blocks were determined by high pressure size exclusion chromatography (HPSEC) comprising on-line detectors including a multi-angle laser light scattering (MALLS) filled with a He-Ne laser at 690 nm and a K5 cell (50  $\mu$ l) (HELEOSII, Wyatt Technology Corp., USA) equipped with 18 measuring diodes and calibrated with Toluene and BSA, and a differential refractive index (DRI) (RID10 A Shimadzu, Japan). Columns (OHPAK 80+ and 806 HQ columns (Shodex)) were eluted with LiNO<sub>3</sub> (0.1M) at 0.5 ml.min<sup>-1</sup>. Samples (10 g/L) were solubilized in 0.1 M LiNO<sub>3</sub> for 24h at room temperature under stirring. Solutions were then filtrated (0.45  $\mu$ m filter) and injected in a 100  $\mu$ L full loop. The collected results were analyzed on the basis of a dn/dc of 0.15 mL/g. The collected data were analyzed using the Astra 6.1 software package. The number average degree of polymerization (DP<sub>n</sub>) and the weight-average degree of polymerization (DP<sub>w</sub>) values were estimated using equation (2) [31].

$$(2) DP_n = \frac{M_n}{198}; DP_w = \frac{M_w}{198}$$

Where M<sub>n</sub> and M<sub>w</sub> are the number-average molecular mass and the weight-average molecular mass of alginate or oligoalginates respectively and 198 g/mol represents the repeat unit in the sodium salt form.

### 2.6. NMR spectroscopy analyses of SAPP, OABM, M, G and G+M fractions

Samples were dissolved in D<sub>2</sub>O and lyophilized to replace exchangeable protons with deuterium (two times). <sup>13</sup>C and <sup>1</sup>H NMR spectra were recorded with a Bruker Avance 400 spectrometer operating at a frequency of 100.618 MHz for <sup>13</sup>C and 400.13 MHz for <sup>1</sup>H. Samples were solubilized in D<sub>2</sub>O at a temperature of 353 K for the polysaccharide and



residual signal of the solvent was used as internal standard: HOD at 4.25 ppm at 353 K.  $^{13}\text{C}$  spectra were recorded using  $90^\circ$  pulses, 20,000 Hz spectral width, 65,536 data points, 1.638 s acquisition time, 1 s relaxation delay and between 8192 and 16,834 scans.  $^1\text{H}$  spectra were recorded with a 4006 Hz spectral width, 32,768 data points, 4.089 s acquisition times, 0.1 s relaxation delays and 16 scans.

On the bases of Grasdalen method [32], the areas of A, B and C signals were used to calculate the individual blocks of mannuronic and guluronic acids (FG and FM), the homopolymeric (FGG and FMM) and heteropolymeric blocks (FCM and FMG) as reported by equations (3) and (4).

$$(3) \quad FG = \frac{A}{B+C}, FGG = \frac{C}{B+C}, FG + FM = 1$$

$$(4) \quad FGM = FMG = FG - FGG, FMM = FM - FMG$$

Where A, B and C are the relative areas at 4.96–5.18 ppm (H-1 of G), 4.57–4.82 ppm (H-5 of G and H-1 of M) and 4.38–4.55 ppm (H-3 of G) respectively.

The block distributions in alginate or oligoalginate were estimated by the calculation of the parameter  $\eta$  (equation 5)

$$(5) \quad \eta = \frac{FGM}{FG * FM}$$

## 2.7. FTIR analyses

The IR spectra were recorded using VERTEX 70 FTIR instrument with ATR A225 diamante (Bruker, Palaiseau, France) at room temperature in the wavenumber range of 600-4,000  $\text{cm}^{-1}$  at 4  $\text{cm}^{-1}$  resolution. The acquisition and the processing of spectra have been carried out using the “Spectrum” software.

## 2.8. Biochemical characterization of SAPP and PPBM

Total carbohydrate content was determined by the phenol and sulfuric acid assays with glucose as standard [33]. Neutral sugar and uronic acid contents were quantified using

respectively the sulfuric resorcinol method with glucose as standard [34] and the m-hydroxydiphenyl (MHDP) assay with glucuronic acid as standard [35]. Protein concentrations were determined according to Bradford method with bovine serum albumin as standard [36].

### 2.9. Elicitor treatment

Date palm vitroplants var. Khnizi at the end of rooting stage were obtained by *in vitro* vegetative multiplication and kindly provided by the commercial laboratory “Green Biotechnology”, Sfax, Tunisia. For elicitor treatments SAPP, PALM, PPBM, G+M, G or M fractions at final concentration of 1 mg/mL were added to the ½ MS medium [37]. After 48 h vitroplants were harvested and stored at -80°C for further uses.

### 2.10. Extraction of RNA, synthesis of cDNA and Real-time quantitative PCR

Total RNA extraction was performed on the fresh vitroplant of date palm (100 mg) with the plant-RNA mini kit (Qiagen, USA) in accordance with the manufacturer's instruction. Five µg of total RNA were used to synthesize cDNA using PrimeScript™ RT Reagent Kit with gDNA Eraser (Perfect Real Time) (Takara, Kyoto, Japan) according to the manufacturer's procedure. The real-time qPCR was conducted for PAL (phenylalanine ammonia-lyase), PR1 (Pathogenesis-related protein 1) SOD (superoxydes dismutases) and DHN (dehydrin) (Table 1). The amplification reactions were performed in 10 µL final volumes containing 5 µL 2x TB green (Takara), 0.4 µL dye, 1 µL primer-pair mix (0.5/ 0.5 µM for forward and reverse primers), 1 µL cDNA, and 3 µL RNase-free water. Reactions were carried out in StepOnePlus™ PCR cycler (Applied Biosystems, Foster City, USA). Thermal cycling conditions were performed as described by Drira et al., [6]. Transcript abundance of each selected gene was normalized to the housekeeping gene EF- 1a and was determined with the  $2^{-\Delta\Delta Ct}$  method [38].

**Table 1:** Specific primers used for qRT-PCR.

Gene	Description	Primer (F = forward; R = reverse)
<b>DHN</b>	Environmental stress-inducible protein TAS14	F 5'-GGACGAAGGAAGAAGGGATT-3' R 5'-ATTCCTGGTCCTGGACAACA-3'
<b>SOD</b>	Superoxide dismutase	F 5'-GTCAATGCAATCAGGTGGTG-3' R 5'-CGTTCTCAAGACAAGCCACA-3'
<b>PAL</b>	Phenylalanine ammonia-lyase	F 5'-CTTTTGCGAGGTCATGCAGG-3' R 5'-TTTGTGAGTCAAGTGGTCGG-3'
<b>PR1</b>	Pathogenesis-related protein 1	F 5'-ATGTCCCTATCAGCCATGC-3' R 5'-GAACATGTTCTCGCCGTAGG-3'
<b>EF-a</b>	Elongation factor 1-alpha	F 5'-TTCCACTGTGGAAGTTTCGAG-3' R 5'-CCGAATTGACCAGCTTCAA-3'

### 2.11. Stress treatments

After 48 of elicitor treatments, vitroplants were transferred in liquid MS medium supplemented with 200 mM NaCl for salt stress, 200 mM mannitol for drought stress or inoculated with  $10^6$  of *Aspergillus niger* for biotic stress and incubated for 4 additional weeks.

### 2.12. Measurement of chlorophyll content

Fresh leaves (100 mg) of untreated and treated date palm with OABM were collected and chlorophyll was extracted using 80% acetone. After centrifugation (10,000xg, 10 min, 4°C). The  $A_{645}$  and  $A_{663}$  of the supernatants were measured and the total chlorophyll content in each sample, were calculated according to Drira et al. [39].

### 2.13. Leaf disease severity measurement

The leaf disease severity (LDS) of vitroplants infected by *Aspergillus niger* was estimated using the ImageJ software (<https://imagej.net/ij/>) and calculated as following (equation 6) :

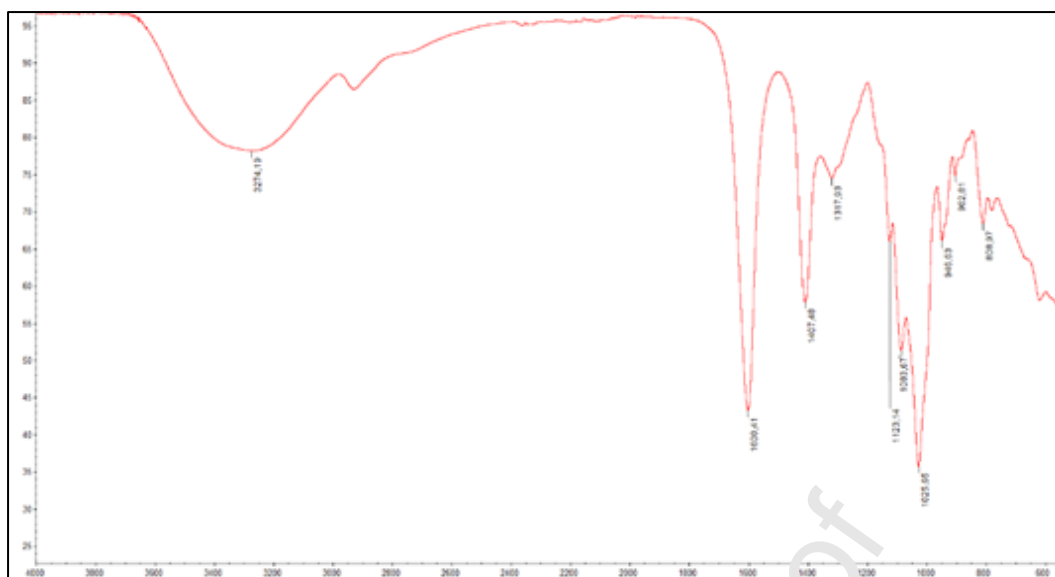
$$(6) \% LDS = \frac{\text{Diseased leaf area}}{\text{Entire leaf area}} * 100$$

### 3. Results and discussion

#### 3.1. Structural features of sodium alginates of *Padina pavonica* (SAPP)

Extraction yield of alginate depend on the algae species, age, season and ecological habitat. The extraction yield of SAPP (around 10.8% ± 0.9 (w/w)) is near to those achieved in different species such as *Sargassum turbinarioides* (10%) [29], *Cystoseira sedoides* (11%) and *Fucus guiryi* (13.6%) [40]. Since alginates are polyuronic polymers [11], the total sugars (56.68%) is mainly composed by uronic acids (48.18%) with minors amount of proteins (0.73%).

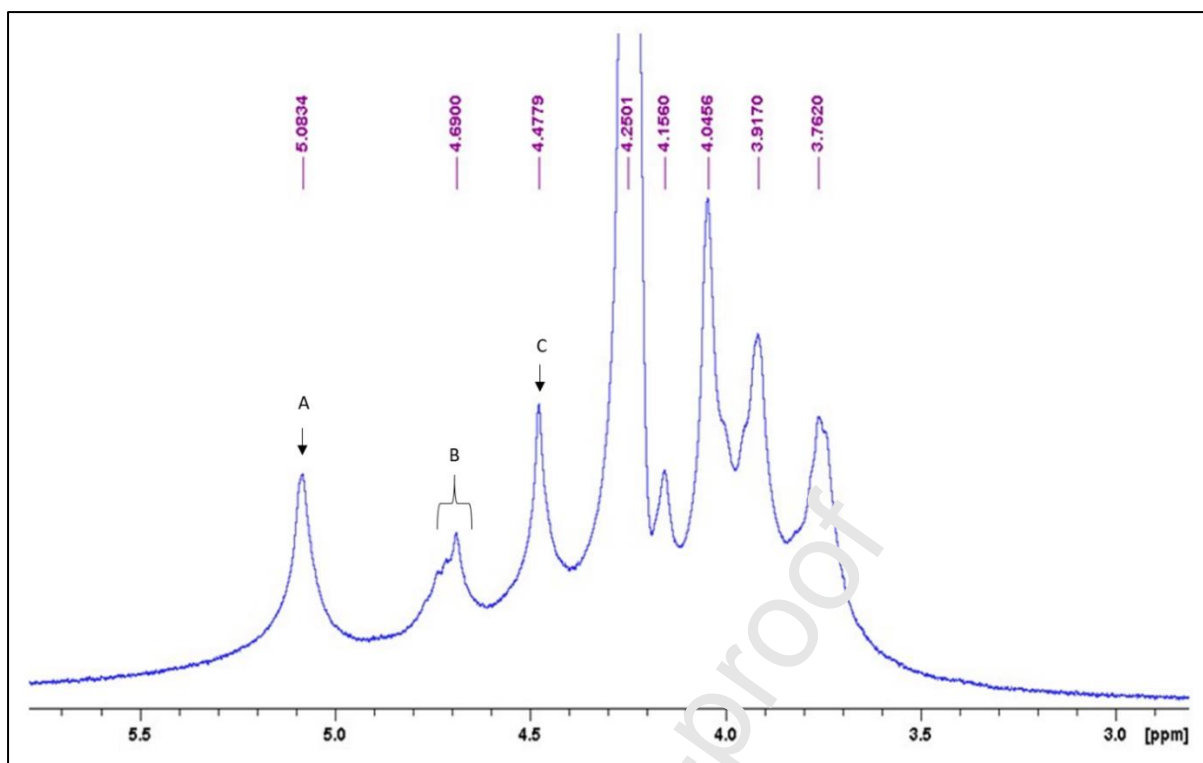
FTIR spectroscopy was used to partially characterize the functional groups of SAPP. Fig. 1, showed clearly the fingerprint of alginates that is observed in many studies [28, 40, 41]. In fact, SAPP displayed several characteristic signals at 3274, 1600, 1317, 1123, 1083, 1025, 946, 902 and 808 cm<sup>-1</sup>. According to Fencadosoa et al., [29], Hentati et al, [28] and Hentati et al., [11], the band at 3274 cm<sup>-1</sup> was assigned to hydrogen bond O-H stretching vibrations. The signal observed at 1600 cm<sup>-1</sup> could be attributed to carboxylate O–C–O asymmetric stretching vibrations. Peaks at 1317, 1123 cm<sup>-1</sup> could be assigned respectively to the deformation of (O-C-H) groups and to the elongation vibration of the (C-O) groups of pyranose rings. In addition, the bands at 1083 and 1025 cm<sup>-1</sup> correspond to the units of guluronic and mannuronic acids respectively. The signal at 946 cm<sup>-1</sup> may be attributed to the C–O stretching vibration of uronic acids. More peaks at 902 and 808 cm<sup>-1</sup> could be assigned to C-O stretching vibrations of alginate backbone (Fig.1).



**Figure 1:** FT-IR spectra of SAPP

The molecular mass distribution of SAPP was determined using SEC-MALLS analysis. A weight average molecular weight ( $M_w$ ) calculated at 340 kDa with high polymer chain length was considerably higher than those obtained for other alginates obtained from brown algae such as *Ascophyllum nodosum*, *Fucus vesiculosus* and *Saccharina longicuris* which have  $M_w$  values ranging from 106 and 177 kDa [42].

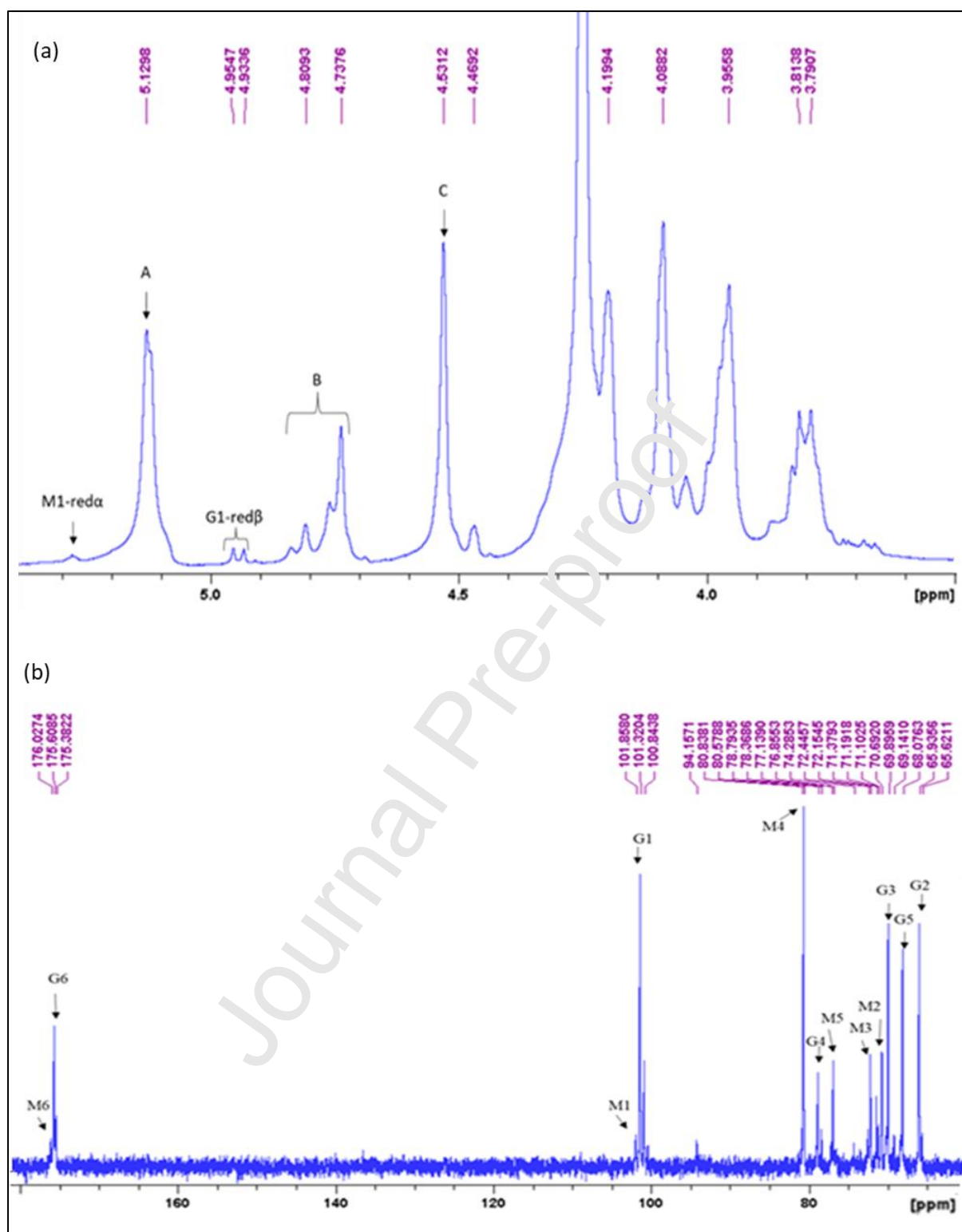
For detailed block structure of the SAPP,  $^1\text{H-NMR}$  spectroscopy was used and presented in Fig. 2. The values of FG, FCG, FM, FMM and FGM or FMG were respectively 0.554, 0.573, 0.446, 0.427 and 0.019. Moreover, SAPP had a low M/G ratio of 0.805 ( $<1$ ) indicating that G units were abundant in the alginate polymer. Thus, the parameter  $\eta = 0.08$  ( $<1$ ) demonstrated a high quantity of GG homopolymeric blocks. By contrast, the M/G ratio of some other *Padina* alginates such as *Padina gymnospora* and *Padina antillarum* were 1.75( $>1$ ) and 1.85( $>1$ ) respectively [43]. The variability of data obtained from the same species could be explained by the influence of environment on the composition of alginates [43].



**Figure 2:** <sup>1</sup>H-NMR spectra of SAPP

### 3.2. Structural features of oligoalginates (OABM) obtained by acid assisted ball milling

Ball milling in the presence of an acid catalyst (H<sub>2</sub>SO<sub>4</sub>) was considered as a promising tool for particle size reduction of many bioorganic material such as polysaccharides especially cellulose [30] and chitin [44]. However, mechanical treatment of alginate was never demonstrated. Alginate acid-assisted ball milling depolymerization of SAPP was verified by <sup>1</sup>H-NMR. There were some different chemical shifts of signals compared to the <sup>1</sup>H-NMR profile of native alginate (Fig 2 and 3a). An increase in signal intensities of the terminal reducing end was observed at around 5.27 ppm and 4.95 ppm which represent M1-red $\alpha$  and G1-red $\beta$  respectively (Fig 3a). <sup>13</sup>C-NMR was also performed to get insight about the monomer composition and the structure of the chain ends of OABM (Fig. 3b).



**Figure 3:**  $^1\text{H-NMR}$  (a) and  $^{13}\text{C-NMR}$  (b) spectra of OABM

The  $^{13}\text{C-NMR}$  can be divided into three main regions: pyranose (60-90 ppm), anomeric (90-110 ppm) and carboxyl (172-180 ppm) carbon signal. As shown in Fig. 3b, signals in the

anomeric region, corresponding to the C-1 of mannuronic and guluronic acids were assigned at 101.85 ppm (M1) and 101.32 ppm (G1) respectively. Other chemical shifts are listed in supplemental table [32]. It's worth to note that chemical shifts at 94.15, 69.14 and 74.28 ppm could be attributed to C1, C5 and C3 reducing end respectively [45].

OABM was structurally characterized by the ratio M/G (0.33) < 1 indicating that mechanical depredated alginate exhibited higher amounts of monomer G (FG=0.75) and diad GG (FGG=0.72) than the monomer M (FM= 0.25) and diad MM (FMM=0.22). The value of FMG or FGM was 0.03. Interestingly, we note that the acid-assisted ball milling depolymerization gives higher values for the G block and lower values for MG blocks (75% and 3% respectively) compared to the native alginate (60 % and 19 % respectively). It seems that the mechanical hydrolyses may affect the MG blocks.

The parameter  $\eta$  (0.18<1) which completes the description of the OABM sequence reveals the abundance of homopolymeric blocks.

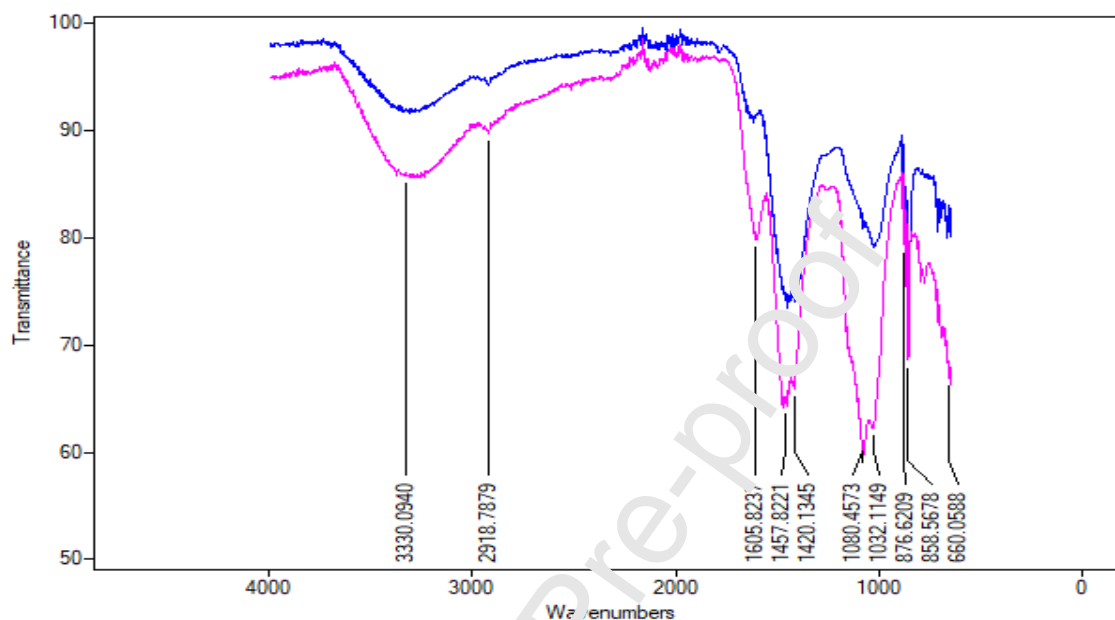
Interestingly, based on the SEC-MALLS analyses, OABM was characterized by a low Mw and Mn ( $4.3 \pm 0.28$  and  $3.43 \pm 0.47$  kDa respectively). A low polydisperse character was deduced by a polydispersity index < 2 ( $\mathcal{D} = 1.25$ ). In addition, the calculated degree of polymerization ( $DP_n = 1732$ ) confirms that ball milling could be an effective method for alginate depolymerization which produces homogeneous hydrolysates composed of oligomers with short chain lengths.

### 3.3. Physicochemical characterization of PPBM

After ball milling treatment of *Padina pavonica* powder, the chemical analysis of PPBM demonstrated its richness in total sugar (31% (w/w)) mainly composed by neutral carbohydrates (26.15 (w/w)) and lower content of uronic acids (12.29 % (w/w)) with the presence of minor amount of proteins (1% (w/w)). Those results were close to those obtained with untreated *Padina pavonica* [39]. Moreover, it can be seen in Fig. 4 that the general



profile FTIR spectra of untreated and treated algae powder was similar. The two bands at 1080 and 876  $\text{cm}^{-1}$  are more prominent in the PPBM spectra which are characteristic to the guluronic and mannuronic acids respectively. This might be attributed to the alginate depolymerization after mechanocatalytic treatment of the dried algae.



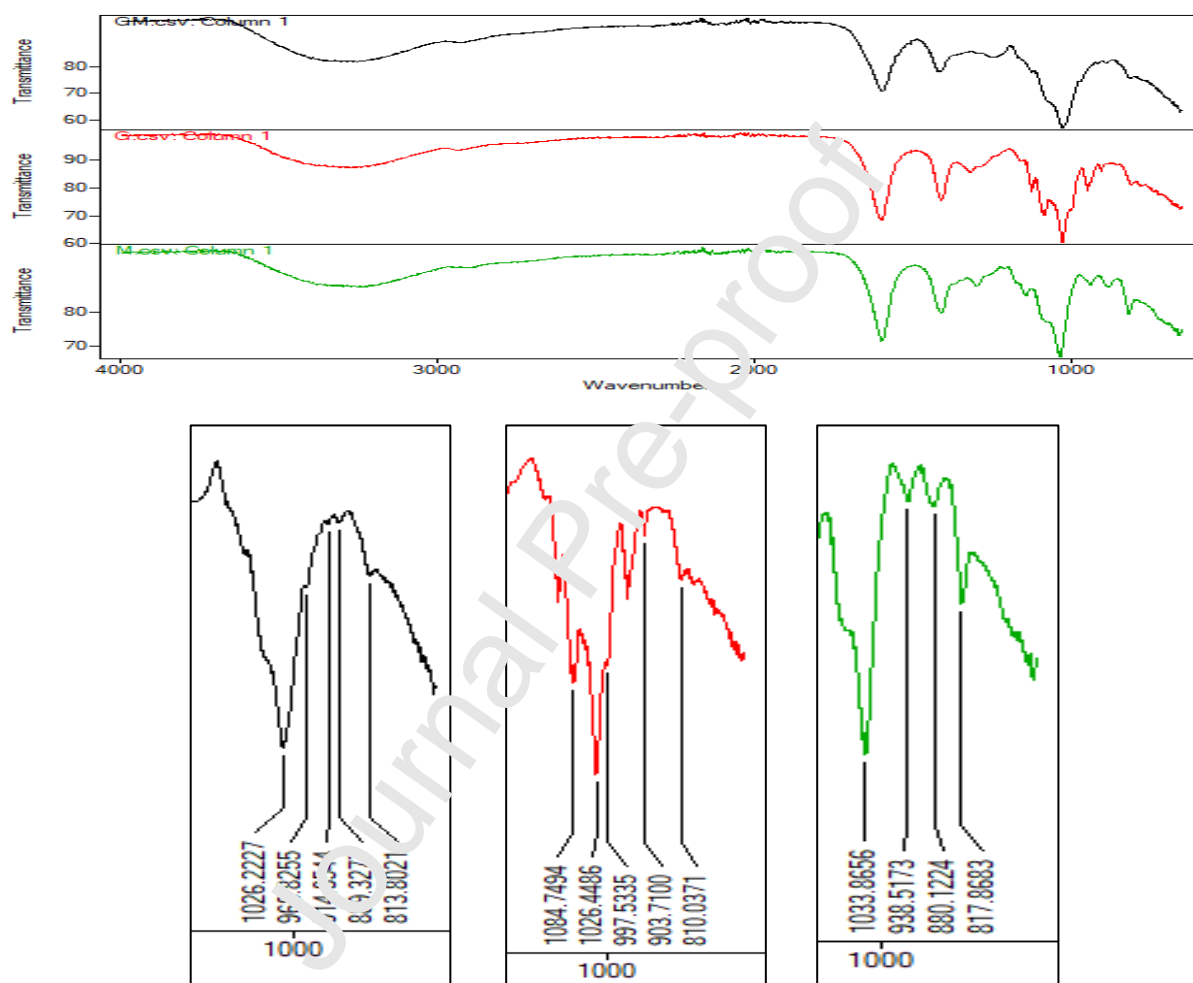
**Figure 4:** FTIR spectra of untreated (Blue spectra) and ball milling treated (Pink spectra) dried *Padina pavonica*

### 3.4. Structural characterization of G+M, G and M fractions obtained by HCl hydrolysis

#### 3.4.1. FTIR analyses

Spectroscopic analysis was performed to confirm the depolymerization process of alginates and the results are presented in Fig. 5. The partially degraded alginates: G+M, G and M present different characteristics in the fingerprint region ( $950\text{--}750\text{ cm}^{-1}$ ) which is related to uronic acids vibration [46, 47, 48]. The FTIR spectrum of the heteropolymeric fraction (G+M) look similar to the SAPP but changed slightly in the fingerprint region and showed specific bands at 968 and 813  $\text{cm}^{-1}$ . The spectrum of M fraction contained a band at 938  $\text{cm}^{-1}$

corresponding to the C–O stretching vibration of uronic acids. An additional band was detected at  $880\text{ cm}^{-1}$  assigned to  $\beta$ -D-mannopyranuronic C1–H deformation vibration.  $817\text{ cm}^{-1}$  signal which is characteristic of mannuronic acid was also observed. The spectrum of G fraction contained three signals at 1084, 903, 810 and  $779\text{ cm}^{-1}$  assigned respectively to the deformation vibrations of C-O, C-OH, C-CH and O-CH of guluronic acid.



**Figure 5:** FTIR analysis of the fractions G+M (black spectra), G (red spectra) and M (Green spectra)

### 3.4.2. Molecular weight distribution

Oligoalginates fractions obtained by chemical hydrolysis were analyzed by SEC–MALLS to determine their molecular weights and their DP. As shown in Table 2, the average molecular weight ( $M_w$ ) of G+M, G ( $DP_n = 29.89$ ) and M ( $DP_n = 20.4$ ) were 16.6, 9.87 and 7.53 kDa

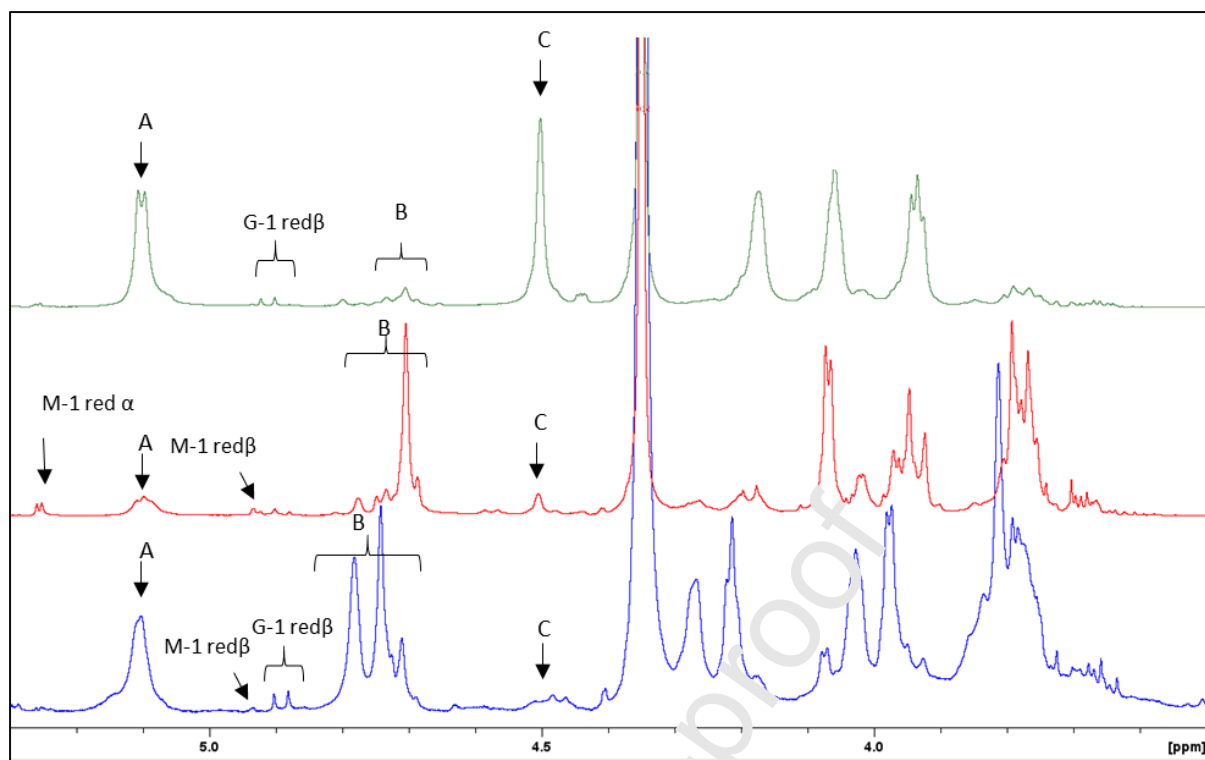
respectively, indicating that G-blocks, M blocks and M+G-blocks are well fractionated. The high polydispersity of G+M fraction ( $\bar{D}$ ) prevents calculation of its DP<sub>n</sub>/w. The Mw distribution of G+M fraction is highly polydisperse with a polydispersity index ( $\bar{D}$ ) 11.09 (>2), while a monodisperse distribution was estimated the in the case of G and M fractions with calculated  $\bar{D}$  less than 2.

**Table 2:** SEC-MALLS results of G+M, G and M fractions

Fraction	Yields (%)	Mn (kDa)	Mw (KDa)	$\bar{D}$	DP <sub>n</sub>	DP <sub>w</sub>
G+M	9.98±0.08	1.5±0.64	16.6±0.55	11.09		
G	51.72±0.49	5.92±0.45	9.87±0.25	1.67	29.89	49.84
M	12.97±0.12	4.04±0.33	7.53±0.25	1.86	20.4	38.03

### 3.4.3. <sup>1</sup>H-NMR analysis of G+M, G and M fractions

Compared to the <sup>1</sup>H-NMR spectrum of the native alginate SAPP (Fig. 2), Fig.6 showed an increase in signals intensities for G reducing end (G-1 redβ) at 4.85 and 4.95 ppm for G and G+M fractions. Only a slight increase in the M reducing end (M-1 redβ) at around 4.95 ppm for the G+M fraction was detected. In the case of M fraction, an increase in signals intensities for M reducing end M-1 redα and M-1 redβ was observed at around 5.25 and 4.95 ppm respectively. Moreover, remarkable decline in internal H<sub>M-1</sub>+H<sub>GM-5</sub> (signal B) and H<sub>G-5</sub> (signal C) for G and G+M fractions respectively was measured. The <sup>1</sup>H-NMR spectrum of M fraction showed an important decrease in the both internal signal intensities of HG-1 (signal A) and HG-5 (signal C) respectively (Fig.6). Similar <sup>1</sup>H-NMR spectra profiles were obtained after chemical hydrolysates alginates extracted from *Cystoseira trinode*, *Cystoseira myrica*, *Sargassum dentifolium*, *Sargassum asperifolium* and *Sargassum latifolium* [49].



**Figure 6:**  $^1\text{H-NMR}$  spectra of G fraction (green spectra), M fraction (red spectra) and G+M spectra (bleu spectra)

The results of the chemical fractionation for the SAPP are summarized in Table 3. In agreement with the FTIR analysis (Fig. 5), the obtained fractions varied in terms of their monomeric block distributions. In fact, fraction M showed the highest MM content, while the highest content in GG was observed in G fractions. The measured M/G values confirmed that G+M and G fractions were rich with G units ( $M/G < 1$ ), whereas in the M fraction ( $M/G > 1$ ) the M units were abundant. Moreover, the block distribution of M and G fractions were homogenous with calculating  $\eta$  parameter ( $< 1$ ) 0.45 and 0.15 respectively. In the case of M+G fraction the parameter  $\eta$  ( $> 1$ ) demonstrates a high distribution of heteropolymeric blocks (Table 3).

**Table 3:** Distribution of monomers and dyads in G+M, G and M fractions

Fraction	FG	FM	FGG	FMM	FMG	M/G	$\eta$
G+M	0.9	0.1	0.0056	0.0	0.89	0.11	9.88
G	0.98	0.02	0.95	0.0	0.03	0.02	0.15
M	0.14	0.86	0.084	0.776	0.056	6.14	0.45

Conclusively, the two different methods of alginate depolymerization generate alginate oligosaccharides with different blocks distribution, Mw and DP. Interestingly, compared to the fraction obtained by acid hydrolysis, OABM has the much higher yield (71.5 %) after mechanical degradation. This suggests that ball milling assisted depolymerization could be an effective method for potential industrial use.

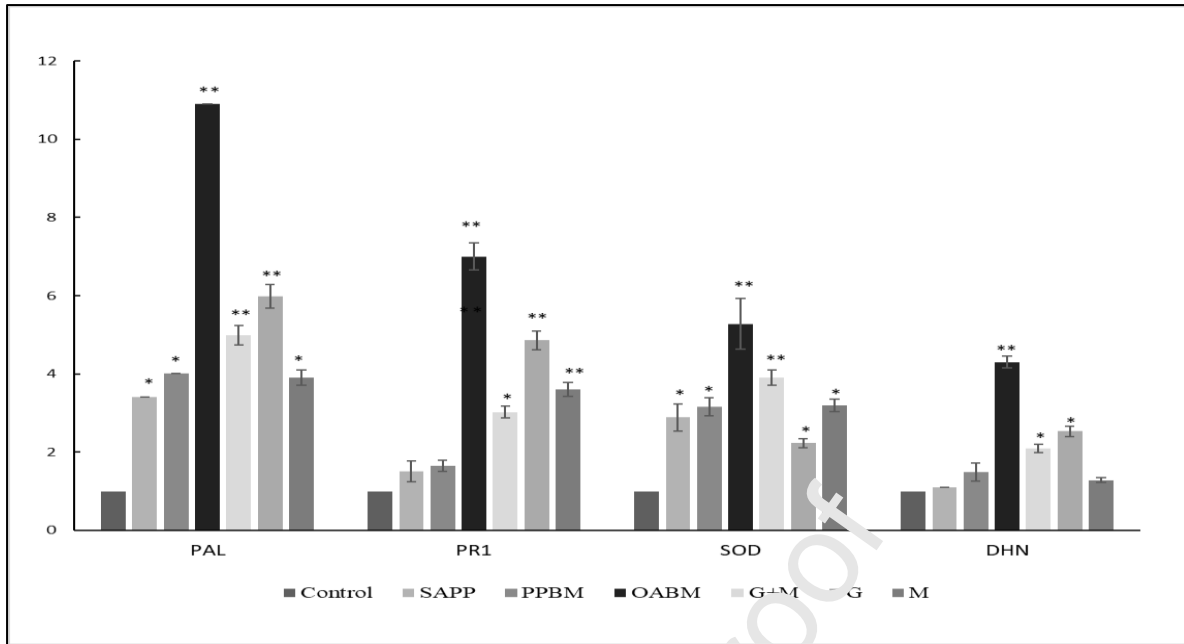
### 3.5. Effect of SAPP, OABM, PPBAM, G+M, G and M treatments on the induction of plant defense gene expression

To investigate whether SAPP and their derivatives activated date palm vitroplants resistance to diverse environmental stresses, we the expression levels of PAL, SOD, PR1 and DHN was monitored. PAL is considered as an efficient marker, which involved in the plant cells defense response to ROS generated under environmental stresses [50]. SOD is the first important line of antioxidant enzyme defense systems against ROS [51]. DHN play key role in plant adaptation to abiotic and biotic stresses [52] and PR1 serves as molecular indicator in the case of pathogen attack [53].

The relative expression levels of all the analyzed genes were normalized with the elongation factor (EF-a) gene. Compared to those of untreated date palm vitroplants, all the studied genes were activated differentially in regard to the elicitor treatment. Interestingly, the highest transcription levels were observed in the vitroplants treated with oligoalginates

obtained with mechanical depolymerization (OABM) followed by those obtained by chemical hydrolysis. The up-regulated transcription levels were similar in both PPBM and SAPP treated plants (Fig.7).

A large number of studies support that differential elicitor responses of alginates and their derivatives may be associated with their structural characteristics. In fact, the molecular weight, the M/G ratio and the degree of polymerization were key factors affecting their biological activities [8, 54]. For example, oligoalginates fractions obtained by  $\gamma$ -irradiation with the lowest  $M_w$  (1-3 kDa) exhibited the highest increased leaf biomass of flower vitroplants [55]. Likewise, oligoalginates with the lower degree of polymerization values obtained with an alginate lyase, mitigate the negative effects of salt stress in *Brassica campestris* by the stimulation of the superoxide dismutase (SOD) and guaiacol peroxidase (POD) activities [56]. In contrast with our results, M fraction obtained with chemical depolymerization was proved to be more active than G one to stimulate defense response against tobacco mosaic virus in tobacco [57]. Results from this study, revealed clearly that there is a close relationship between oligosaccharides structure and the elicitor activity. The structural modification of SAPP by reducing the  $M_w$  and DP may improve their functionality. As suggested by many authors, the low  $M_w$  and DP may help the oligosaccharides to bind plant cell receptors and facilitate the stimulation of certain signal transduction pathways [58].



**Figure 7:** Relative expression level of defense-related genes PAL, PR1, SOD, WRKY in date palm vitroplants after 48h of treatments with SAPP, PPBM, OABM, G+M, G, and M fractions. Transcript abundance of each selected gene is expressed relative to control and untreated plants. Data were analyzed from two independent Real-Time PCR runs. \* indicates significant differences ( $p < 0.01$ ) and \*\* indicates very significant differences ( $p < 0.001$ ) relative to control untreated plants (Student's t-test).

### 3.6. Phenotypic and physiological analyzes of vitroplants treated by OABM under abiotic and biotic stresses

Beside transcriptional analysis, tolerance of vitroplants treated with oligoalginates (OABM) was investigated under salt, drought and fungal contaminations. In the absence of stress, similar phenotypes, fresh weight, root length and chlorophyll content were observed between control and treated vitroplants under normal conditions (MS), indicating that OABM had no detrimental effect on growth (Fig. 8a, Fig.9). Four weeks of salt stress applications led to the shoot and root growth inhibition and development of chlorotic leaves in control plants, whereas growth inhibition and chlorophyll content were less pronounced in treated

vitroplants (Fig. 8b, Fig.9). Likewise, Salachna et al [20] reported that oligoalginates improve morphological features, physiological and biochemical parameters of *Eucomis autumnalis* under salt stress application. In fact, those authors suggested that oligoalginates may activate plant stress adaptation mechanisms via the stimulation of antioxidants such as a high accumulation of L-ascorbic acid.

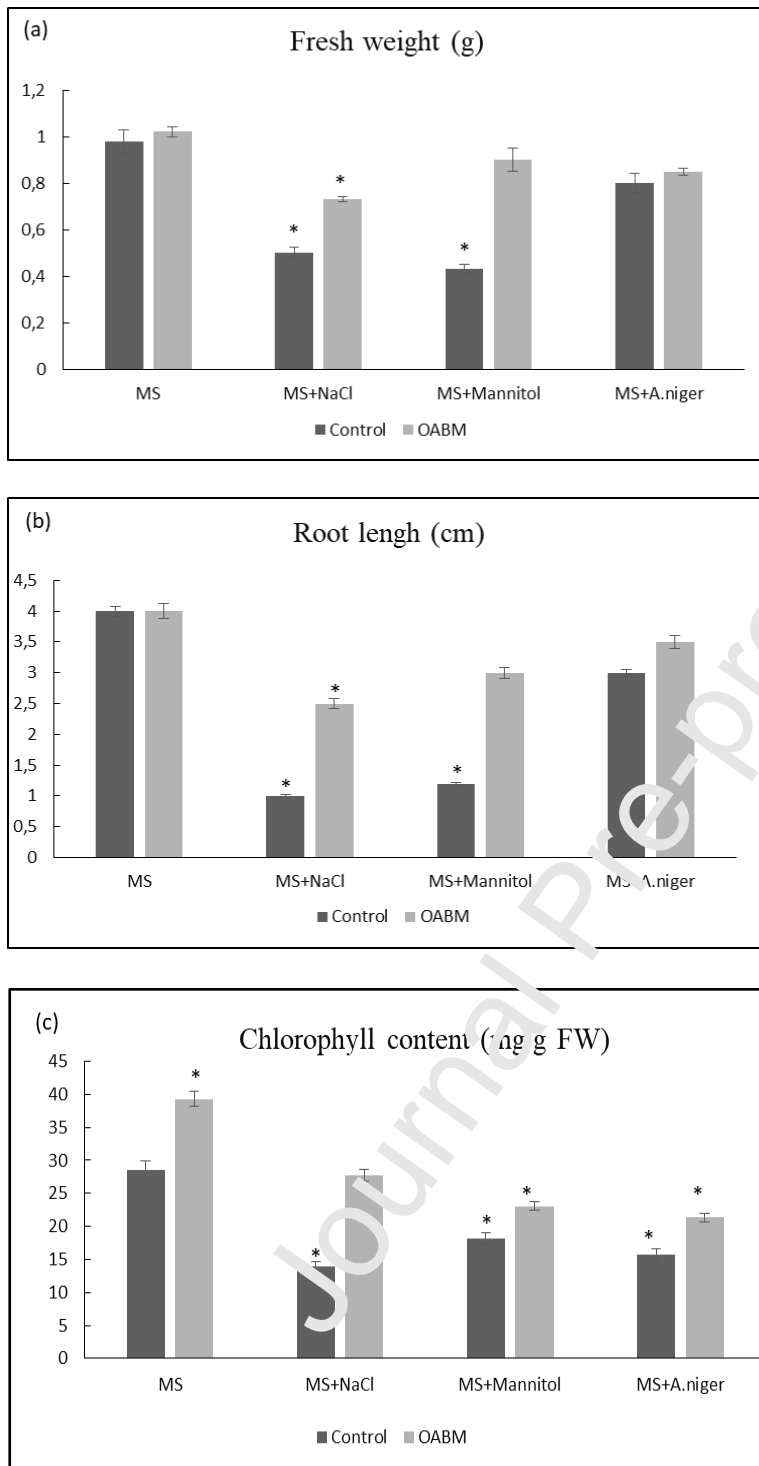
Under drought stress, treated OABM plants revealed phenotypic tolerance associated with the apparition of secondary roots (Fig. 8b). In addition, fresh weight, root length and chlorophyll content values were strongly decreased in control vitroplants, whereas these values were less pronounced in OABM treated vitroplants (Fig. 9). Those results were similar to those observed by Liu et al., [19] which demonstrated that application of oligoalginates in barley plants confers an increased root length and fresh weight compared to the PEG plants under dehydration status.

Fungal contamination is one of the major causes of *in vitro* culture losses that led finally to the death of tissues [59]. The genus *Aspergillus* is the most abundant contaminant which is identified in date palm tissue culture growth media [60]. The Fig 8b demonstrated that the addition of OABM in MS medium enhanced the resistance of vitroplants to the *Aspergillus niger* expressed by a minor necrosis area in treated plant (LDS=10%) compared to the untreated ones (LDS=30%) (Fig 8a).





**Figure 8:** Phenotypic analyzes of untreated date palm vitroplants (a) and OABM treated plants under abiotic (salt, drought) and biotic (fungal contamination) stresses.



**Figure 9:** Physiological parameters in untreated and OABM treated plants date palm vitroplants (a) Fresh weight (b) Root length and (c) Chlorophyll content under abiotic (salt, drought) and biotic (fungal contamination) stresses. \* indicates significant differences ( $p < 0.05$ ) relative to control untreated plants (Student's t-test).

## Conclusion

Oligosaccharides were generated by using two different degradation methods of sodium alginate (SAPP): mechanocatalytic depolymerization (OABM) and hydrochloric acid (G, M and G + M fractions) in order to compare their elicitor activity on date palm with the native alginate (SAPP). We demonstrated that OABM with homogenous distribution of blocks, low Mw and DP showed the highest capacity to induce defense genes expression levels. Moreover, OABM treated vitroplants were able to mitigate the negative effects of salt, drought and fungal contamination. Based on those results, the products of ball milling depolymerization of alginate oligosaccharides could act as novel environment-friendly elicitors agents that can efficiently protect vitroplants of date palm against abiotic and biotic stresses for successful micropropagation process.

## Author Contributions

Conceptualization, S.A., I.F. and M.E.; methodology, M.D., F.E., R.D., F.B. and J.S.G.; software, M.D., S.D., P.M.; validation, F.I. and A.S.; formal analysis, M.D., J.E., P.M. and I.F.; investigation, M.D.; resources, I.F., A.S. and P.M.; writing—original draft preparation, D.M., F.I. and A.S.; writing—review and editing, M.D., S.A. and I.F.; supervision, F.I.; project administration, I.F. and A.S.; funding acquisition, I.F.; All authors have read and agreed to the published version of the manuscript.

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## Declaration of competing interest

The authors declare that there is no conflict of interest and give their informed consent. No financial or other interests influenced the outcome of the research.

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### Conflicts of Interest

The authors declare no conflict of interest

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## Highlights

- Extraction and characterization of sodium alginate from *Padina pavonica*
- Generation of oligoalginates with two different methods: ball milling catalytic and hydrochloric acid hydrolysis
- Oligoalginates obtained by mechanocatalytic hydrolyze confer a better tolerance of date palm vitroplants against abiotic and biotic stresses

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