

Moroccan seaweed polysaccharides elicit defense response and induce protection against *Botrytis cinerea* in tomato plants

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Abstract

Eight Polysaccharides Enriched Extracts (PEEs) obtained from Moroccan seaweeds were selected to test their effectiveness on the natural defense of tomato plants. Firstly, we examined the effect of the 8 PEEs at 4 concentrations (0.02, 0.05, 0.1 and 0.2 mg. mL⁻¹) with 3 application methods (T1: root irrigation, T2: foliar spray, and T3: combining the 2 methods T1 and T2) on protein content and plant defense enzyme Phenylalanine Ammonia-lyase (PAL) on tomato plants in absence of a pathogen. In the second part, we analyzed the ability of PEEs to induce protection against *Botrytis cinerea*, causative agent of gray mold, by testing PEEs on detached tomato leaves. Results showed that the PEEs obtained from *Codium tomentosum*, *Ulva rigida*, and *Bifurcaria bifurcata* at 0.1 mg. mL⁻¹ and *Gelidium crinale*, *Schizymenia dubyi* and *Fucus spiralis* at 0.02 mg. mL⁻¹ were the best treatments that significantly stimulated protein content and PAL activity in tomato plants with the three application methods. The same extracts at the same concentrations, in addition of PEEs from *G. pistillata* at 0.02 mg. mL⁻¹ were the treatments having the greatest inhibitory effect on the lesion diameter of *B. cinerea* in detached leaves when compared to the control. The principal component analysis showed a correlation between PAL content and the reduction of lesion diameter. The comparison of the application method in our study did not show differences. These findings confirmed that these algal polysaccharides treatments could be a promising method to reduce dependency on synthetic fungicides. The presence of uronic acid and sulfated groups in the extracts could explain the elicitation mechanism induced in plant cells.

Keywords: Tomato plants, PEEs, seaweeds, plant defense, PAL, *Botrytis cinerea*

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INTRODUCTION

The phytopathogenic fungus *Botrytis cinerea* Pers. is one of the most extensively studied necrotrophic fungal pathogen, it has a wide host range including more than 200 species of crops (Williamson *et al.*, 2007; Elad *et al.*, 2016). It is the causative agent of gray mould in tomato and many other economically important crops, such as pepper, eggplant, grape, lettuce and raspberry. It is a devastating disease that causes losses of \$ 10 billion to \$ 100 billion annually because it reduces crop yields before harvest or results in postharvest waste and spoilage (De Vega *et al.*, 2021). As a result, *B. cinerea* has been considered the second most serious phytopathogen worldwide (Dean *et al.*, 2012). Under conditions of high humidity coupled with low temperature, the typical disease symptoms can be visualized on tomato fruit and every part of the tomato plant infected with the pathogen through wounds after pruning and harvesting or through direct penetration (Williamson *et al.*, 2007; Elad *et al.*, 2007; Romanazzi *et al.*, 2014). For several reasons, it is difficult to control *B. cinerea*; it has a wide host range, various modes of attack and asexual and sexual stages to survive under favorable or unfavorable conditions (Fillinger and Elad, 2016). The forms of resistance of *B. cinerea* are sclerotia, which are essential for survival in an unfavorable environment, and the asexual spores are conidia, which are easily dispersed by wind or water (Brandhoff

et al., 2017). Over past decades, the control of gray rot caused by *B. cinerea* has depended mainly on the application of synthetic fungicides (Adnan *et al.*, 2018). However, two main problems are encountered with the application of fungicides. On one hand, *Botrytis* tends to change constantly during generations and developed certain resistance to several fungicides, including anilopyrimidines dicarboximides, benzamide, fenhexamid, diethofencarb, procymidone, pyrimethanil, and hydroxylanilide (Myresiotis *et al.*, 2007; Mosbach *et al.*, 2017; Adnan *et al.*, 2019). Additionally, the abuse and excessive use of fungicides have caused environmental pollution and disruption of ecological ecosystems, as well as a danger to human beings, especially their toxicological residues in tomato fruit (Abbey *et al.*, 2019; Rosero-Hernández *et al.*, 2019). In order to overcome the obstacles resulting from the chemical fungicides, there is an urgent need for alternative measures that could safely and effectively control gray mold disease.

Alternatively, sustainable strategies of stimulation of the natural plant defense can also be achieved by the application of natural elicitors which can be derived from natural compounds (Battacharyya *et al.*, 2015). Plant species have developed an innate immune system with two levels of pathogen recognition (Jones et Dangl, 2006). Levels change in terms of location, pattern recognition and development of the defense response. The first level

of recognition is established in the plasma membrane or apoplastic space, plants activate their resistance mechanisms against pathogens by recognizing pathogen-associated molecular patterns (PAMPs) through membrane receptors called 'Pattern Recognition Receptors' (PRR) (Jones et Takemoto, 2004; Jones et Dangl, 2006; Dodds et Rathjen, 2010; Claverie et al., 2016). This recognition leads to the activation of downstream signaling events such as oxidative explosion, nitric oxide production, callose deposition, which ultimately will trigger a defense response (Boller et al., 2009). The second level of recognition occurs in the cytoplasm through repeated leucine-rich receptors (NB-LRRs) at the nucleotide binding site that detect pathogen effector proteins (Jones et Dangl, 2006; Boller et al., 2009; Dodds et al., 2010).

Elicitors are those compounds recognized as PAMPs and these trigger the induction of the expression of genes involved in defense responses. Marine organisms can produce elicitors, such as polysaccharides and seaweeds that represent a promising resource of bioactive substances (Vera et al., 2011). For many years, beneficial effects of spraying seaweed extracts on crop plants have been observed, and there is an increasing interest in oligo- and polysaccharides in plant immunity. Sulfated polysaccharides have gained attention in crop protection for their roles as priming agents and elicitors that act as signaling molecules (Shukla et al., 2016; Shukla et al., 2019). Ulvan from green seaweed, laminarin isolated from brown algae as well as carrageenan from red seaweed, and their oligomers derivatives, are extensively studied due to their capacity to enhance various plants defenses (El Modafar et al., 2012; Abouraïch et al., 2015; de Freitas et al., 2015; Ben Salah et al., 2018; Aitougouinane et al., 2020; Shulka et al., 2021).

In this context, the present work aim is to investigate the use of polysaccharides extracted from Moroccan seaweeds as elicitors in the control of *B. cinerea* in tomato. In the first experiment, we investigated the ability of 8 Polysaccharides Enriched Extracts (PEEs) at different concentrations to induce the phenylpropanoid defense pathway, in particular, phenylalanine ammonia-lyase (PAL) activity in the leaves of tomato seedlings in the absence of the pathogen. In the second part, we tested the effectiveness of the selected 8 PEEs at adequate

concentrations on the reduction of the severity of gray rot disease induced by the pathogen *B. cinerea* using detached leaves of tomato plants.

MATERIALS AND METHODS

Preparation of PEEs

Eight Polysaccharides Enriched Extracts (PEEs) were screened from previous studies for their common beneficial effect on plant growth (Mzibra et al., 2020). These polysaccharides were extracted from 2 green seaweeds (*Codium tomentosum* and *Ulva rigida*), 2 brown seaweeds (*Bifurcaria bifurcata* and *Fucus spiralis*) and 4 red seaweeds (*Chondracanthus acicularis*, *Gelidium crinale*, *Gigartina pistillata* and *Schizymenia dubyi*). All these species of seaweed were collected from Moroccan seashore during August 2016 (Table 1) and Polysaccharide's extraction had been carried out using the conventional hot water method under neutral conditions, then precipitated with ethanol, as previously described (Mzibra et al., 2018).

Characterization of extracted Polysaccharides by Fourier transform infrared spectroscopy

The ATR-FTIR spectra of the polysaccharide was measured in the range of 400 - 4000 cm^{-1} using a Perkin Elmer FT-IR Spectrometer Spectrum Two Universal ATR. Each spectrum was obtained by adding 16 consecutive scans with a resolution of 4 cm^{-1} . The FTIR spectra were applied to highlight the presence of some characteristic vibration bands related to the organic functional groups of the polysaccharide material.

Plant treatment for detection of enzyme activity and phenylpropanoid compounds

Plant culture and treatment

Tomato plants were cultivated for 4 weeks under controlled conditions (26 °C, photoperiod of 16:8 h at an irradiance of 240 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$), and treated one time with 100 ml of distilled water (control), or sterile PEEs solutions at 4 concentrations (0.02; 0.05; 0.1 and 0.2 mg mL^{-1}). Three application methods were used for each treatment of PEEs, T1: foliar spray, T2: root irrigation, and T3: Foliar spray + root irrigation. In the spray application, plants were sprayed in the upper and lower

Table 1: Collection site locations of the studied seaweeds

Seaweed	Location	GPS Point	Date of Collection
Chlorophyta			
<i>Codium tomentosum</i>	Guy Ville—El Harhoura	33°56'11" N, 6°56'45" W	03 August 2016
<i>Ulva rigida</i>	Ouled Hmimoun—Mohammedia	33°40'34" N, 7°26'55" W	03 August 2016
Phaeophyta			
<i>Bifurcaria bifurcata</i>	Sidi Bouzid	33°13' 2952" N, 8°33'19 " W	17 August 2016
<i>Fucus spiralis</i>	Sidi Daoui	33°15' 35,11" N,8°30'8,75" W	17 August 2016
Rhodophyta			
<i>Chondracanthus acicularis</i>	El Jadida beach	33°14'564" N, 8°29'4" W	17 August 2016
<i>Gelidium crinale</i>	El Jadida beach	33°14'564" N, 8°29'41" W	17 August 2016
<i>Gigartina pistillata</i>	Skhirat beach	33°52'10"N, 7°3'32" W	03 August 2016
<i>Schizymenia dubyi</i>	Ain Sebaa—Casablanca	33°37'14" N, 7°32'9"W	03 August 2016

faces of all leaves. 24 hours after the treatment, leaves were collected from the lower, middle and upper parts of each plant, pooled and frozen in liquid nitrogen for enzymatic analyzes. Five replications of plants were tested for each treatment of PEEs.

Preparation of protein extracts

Protein extraction was done according to the method described by El Modafar *et al.*, (2001). Tomato leaves (100 mg) were milled in 1 ml extraction buffer: 100 mM Tris-HCl pH 8.5 containing 14 mM β -mercaptoethanol (w/v). The homogenate was then centrifuged at 13,000 g for 30 min and the supernatant constitutes the enzymatic extract. Protein concentration was determined using bovine serum albumin (BSA) as a standard, according to Bradford (1976). Samples were incubated at room temperature for 30 min, then the absorbance was measured at 595 nm.

Determination of Phenylalanine Ammonia-lyase (PAL) activity

PAL activity was determined according to the technique described by El Modafar *et al.*, (2001), with some modifications. The reaction mixture consisted of 100 μ l of enzymatic extract, 1 ml of 100 mM Tris-HCl buffer, pH 8.5, and 200 μ l of 100 mM L-phenylalanine. After incubation at 40 °C for 60 minutes, the reaction was stopped by addition of 250 μ l of 5N HCl. The formed cinnamic acid was extracted twice by 2 ml of diethyl ether. After evaporation of the ether, the residue was suspended in 500 μ l of methanol and the absorbance was determined at 290 nm. In the same way, a standard curve was made with cinnamic acid under the same experimental conditions.

Plant treatment and disease evaluation

The causal pathogen of *Botrytis cinerea*

The strain of *Botrytis cinerea* used in this study was obtained from sporulated isolates developed on tomato stems collected in November 2018 from a greenhouse of Tomato production farm located in Belfaa, Chtouka Ait Baha, Agadir, Morocco. The stems were placed in plastic bags and transported to the laboratory (green biotechnology laboratory of MAScIR Foundation, Rabat, Morocco). A mycelium fragment was removed in sterile conditions, placed on a PDA (Potato Dextrose Agar) medium and incubated in a humid chamber at 23 °C. Subsequently, successive subcultures of mycelium were made from the borders of young colonies in order to purify the isolated strain.

The virulence of the isolated strain of *B. cinerea* was studied according to the method of Wang *et al.* (1986), by inoculation of 2 months aged leaves detached from tomato plants. The leaves were placed in Petri dishes containing tissue filter papers moistened with sterile distilled water to maintain a humid atmosphere. A 5 mm mycelial explant from an actively growing colony of *B. cinerea* on PDA was placed centrally on the upper surface of each leaf and incubated in a humid chamber at 23 °C until sporulation.

Effect of PEEs treatment on gray mold on detached tomato leaves

Evaluation of the effect of PEEs on the severity of *B. cinerea* was studied on detached leaves from tomato plants aged 2 months according to the technique of Aziz *et al.* (2003). Each leaf was soaked in 10 mL of PEEs solutions at different concentrations (0.02; 0.05; 0.1 and 0.2 mg. mL⁻¹) or sterile distilled water for control leaves, and put in Petri dishes containing wet filter papers at the rate of 3 leaves per dish. After 24 hours, *B. cinerea* infection was carried out on each leaf by depositing 5 μ L of conidia suspension prepared beforehand, then the leaves were incubated at 23 °C. Spore suspension was prepared by adding 10 ml of sterile distilled water containing 0.05% (w / v) Tween 80 on a 10-days old *B. cinerea* culture and the suspension was then collected in a sterile tube and adjusted to a concentration of 5 x 10⁵ conidia mL⁻¹ using a Malassez cell. Quantification of disease development was measured as the average diameter (two perpendicular diameters) of lesions formed after 7 days of infection.

Statistical analysis

For protein and PAL content, comparison of multiple means was determined by analysis of variance ANOVA using the LSD test of IBM SPSS statistics 22 (SPSS InC., Chicago, IL, USA). Comparison of diameter lesions on detached leaves and the principal component analysis (PCA) of the correlation table obtained between variables including PEEs, Protein content and PAL activity were carried out using R software version 3.5, package: MixOmics, FactoMineR, Factoextra, and FactoMineR.

RESULTS

FTIR Analysis

FTIR spectroscopy is one of the important analytical techniques widely used to study molecular structures and conformations of macromolecules to identify vibrations between different atoms in molecules. The spectrum obtained between 400 and 4000 cm⁻¹ could be used to analyze the structural characteristics of polysaccharides, including glucosidic bonds and functional groups (Chen *et al.*, 2021).

The FTIR spectra of polysaccharide standards (alginic acid and laminarin) and the eight seaweed polysaccharides in the range 350 - 4000 cm⁻¹ are presented in figure 1. Each peak was assigned a functional group. Common to all polysaccharide standards and seaweed samples, two bands appeared in the 4000-2000 cm⁻¹ region of the FTIR spectra: a broad band centred between 3500-3200 cm⁻¹ assigned to hydrogen bonded O-H stretching vibrations and a weak signal at 2900-2800 cm⁻¹ due to C-H stretching vibrations (Fernando *et al.*, 2017). In addition, the medium to strong IR absorption bands at 1700-1600 cm⁻¹ due to the carboxylate O-C-O bond stretching vibrations (Fernando *et al.*, 2017) which is within the spectral range for uronic acid (Elnahas *et al.*, 2017), this band was also common for all polysaccharides.

The broadened peak between 1280-1120 cm^{-1} indicates the existence of a significant amount of sulfate in the polysaccharides (S=O stretching) (Gómez-Ordóñez *et al.*, 2011; Fernando *et al.*, 2017; Chen *et al.*, 2021), this intense absorption in this spectral region was commonly observed in extracts obtained from *U. rigida*, *Co. tomentosum*, *F. spiralis*, *S. dubyi*, *B. bifurcata* and alginic acid

as a standard. The IR band between 890-800 cm^{-1} , shows the C-O-S bonding vibration and further confirms the presence of a sulfate group (Gómez-Ordóñez *et al.*, 2011; Fernando *et al.*, 2017; Cardoso *et al.*, 2019), it was present in PEEs of *U. rigida*, *F. spiralis*, *G. pistillata*, *S. dubyi*, *B. bifurcata* and Laminarin as a standard.

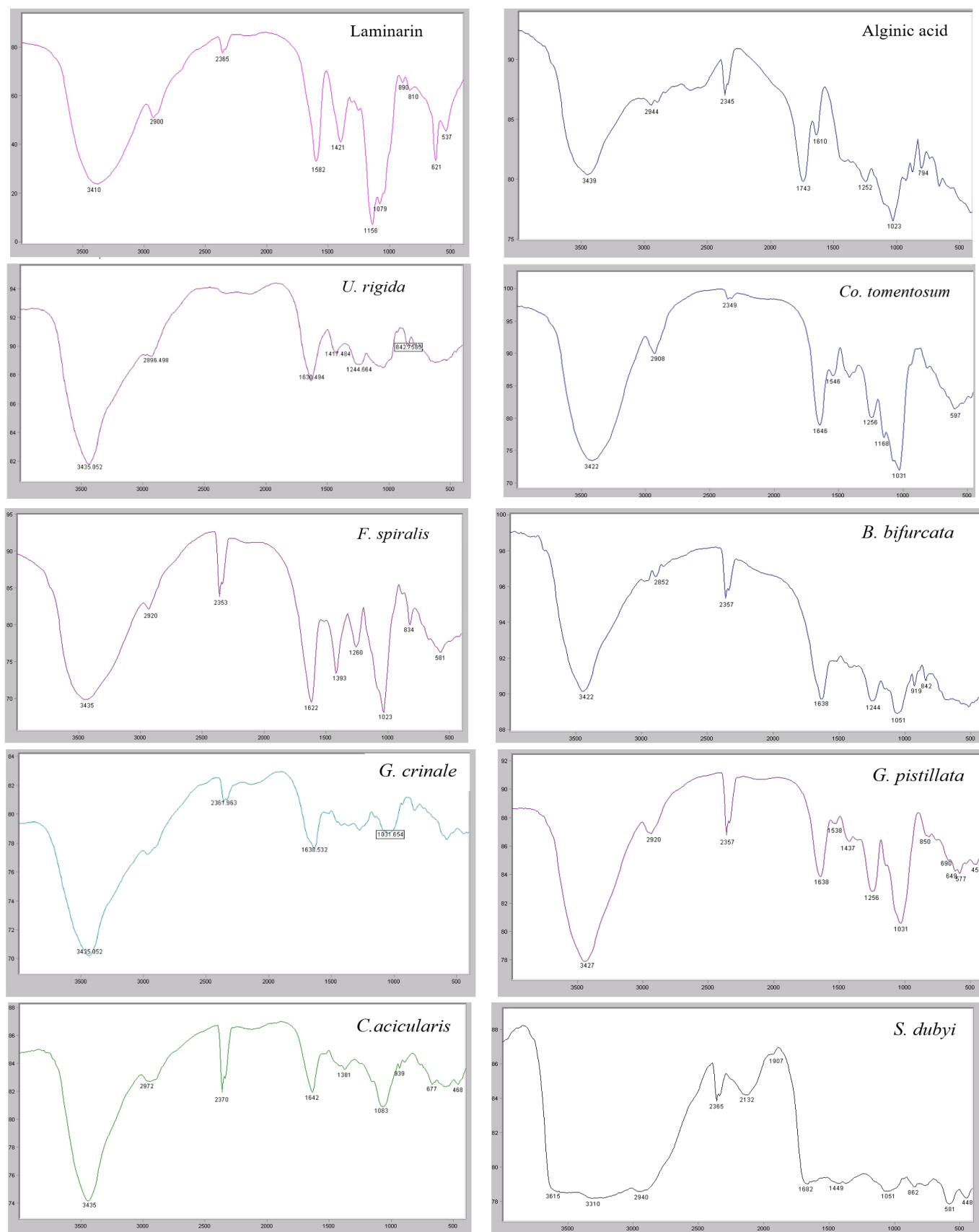


Figure 1: FTIR spectrum of extracts of the seaweed species obtained from green seaweed *Codium tomentosum*, *Ulva rigida*, red seaweeds: *Bifurcaria bifurcata* and *Fucus spiralis* and brown seaweeds: *Chondracanthus acicularis*, *Gelidium crinale*, *Gigartina pistillata*, *Schizymenia dubyi*

PEEs effect on protein content and PAL activity

The effect of Polysaccharides Enriched Extracts (PEEs) on plant proteins content was presented in figure 2. The significant increase was shown in the 3 modes of application of PEEs obtained from *U. rigida* applied at concentrations 0.05 and 0.1 mg. mL⁻¹ (Figure 2-A), *B. bifurcata* (0.1 and 0.2 mg. mL⁻¹), *F. spiralis* (0.02 mg. mL⁻¹) (Figure 2-B) and *G. crinale* (0.02; 0.05 and 0.1 mg. mL⁻¹) (Figure 2-C). However, PEEs obtained from *Co. tomentosum* and *S. dubyi* affected proteins content by all concentrations used in this assay (0.02; 0.05; 0.1 and 0.2 mg. mL⁻¹). Furthermore, the differentiation in application mode, particularly on

some parts of tomato plant, improved significantly protein contents, as for the root irrigation of PEEs obtained from *U. rigida* at 0.02 mg. mL⁻¹ (Figure 2-A), *F. spiralis* (0.05 and 0.1 mg. mL⁻¹) (Figure 2-B), *G. pistillata* and *G. crinale* at 0.2 mg. mL⁻¹ (Figure 2-C). Also, foliar application (T2) of PEEs obtained from *U. rigida* at 0.02 mg. mL⁻¹ (Figure 2-A), *G. crinale* at 0.2 mg. mL⁻¹ and *C. acicularis* at 0.05 mg. mL⁻¹ (Figure 2-C) induced a significant increase in protein content of tomato plants.

Regarding the effect of PEEs biostimulants on plant PAL activity, the results illustrated in figure 3 showed a significant increase in PAL activity in plants treated with

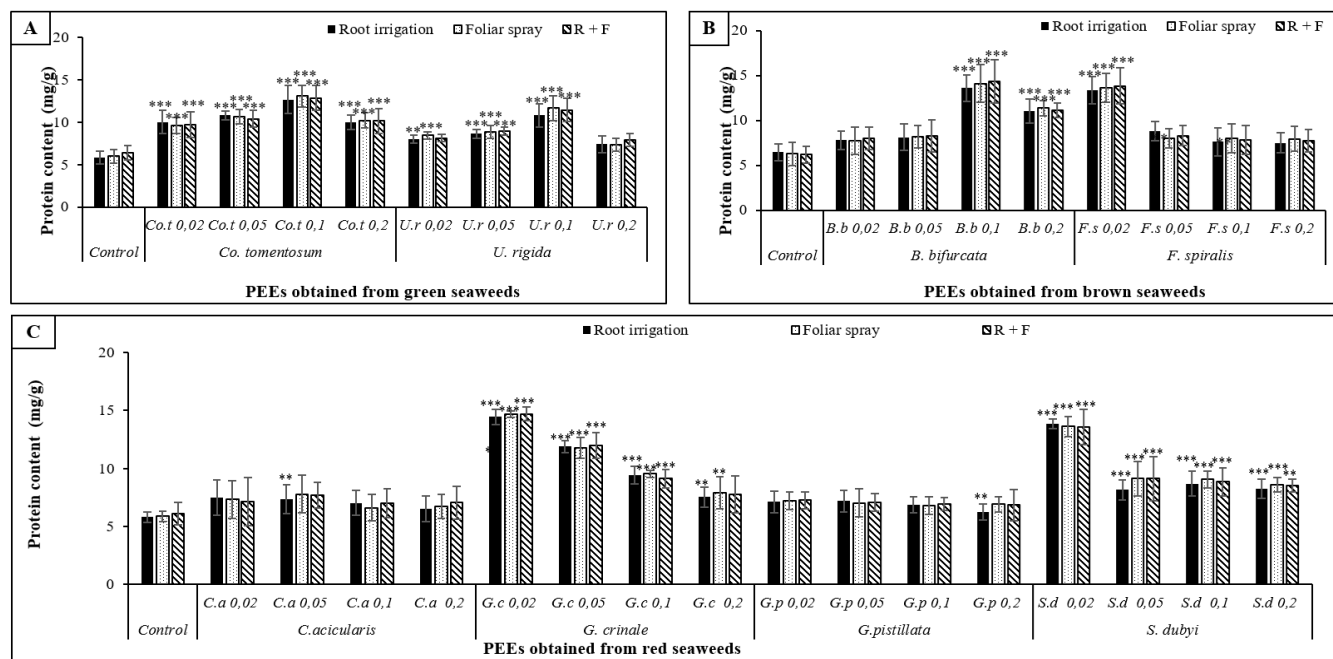


Figure 2: Protein content in control tomato plants and in plants treated with 8 Polysaccharides Enriched Extracts (PEEs) obtained from green seaweed (A): *Codium tomentosum*, *Ulva rigida*, brown seaweeds (B): *Bifurcaria bifurcata* and *Fucus spiralis* and red seaweeds (C): *Chondracanthus acicularis*, *Gelidium crinale*, *Gigartina pistillata*, *Schizymenia dubyi*, at 4 concentrations (0.02, 0.05, 0.1 and 0.2 mg mL⁻¹). Protein content is expressed as micrograms of protein per per 1 g of leaves fresh weight. Data are expressed as mean ± SE of three replicates. Bars indicate the standard errors. Significant difference compared to the control treatment by ANOVA analysis ($p \leq 0.05$) * $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$**

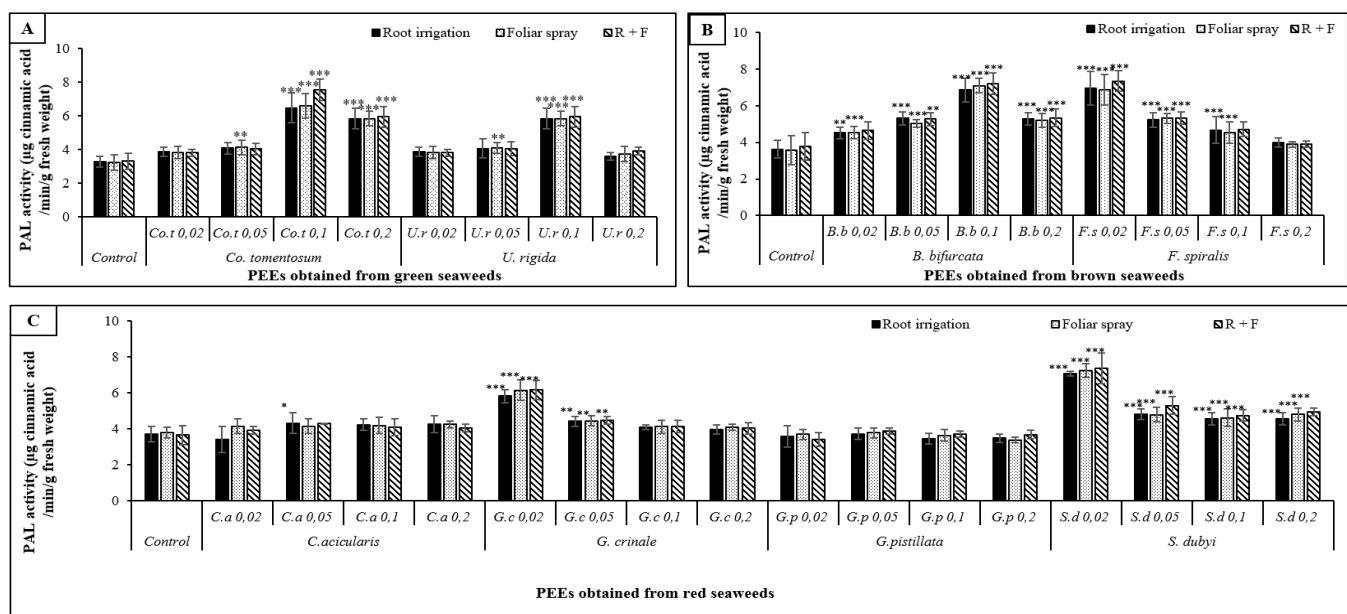


Figure 3: PAL activity in control tomato plants and in plants treated with 8 Polysaccharides Enriched Extracts (PEEs) obtained from (A): *Codium tomentosum*, *Ulva rigida*, brown seaweeds (B): *Bifurcaria bifurcata* and *Fucus spiralis* and red seaweeds (C): *Chondracanthus acicularis*, *Gelidium crinale*, *Gigartina pistillata*, *Schizymenia dubyi*, at 4 concentrations (0.02, 0.05, 0.1 and 0.2 mg. mL⁻¹). PAL activity is expressed as micrograms of cinnamic acid per minutes per 1 g of leaves fresh weight. Data are expressed as mean ± SE of three replicates. Bars indicate the standard errors. Significant difference compared to the control treatment by ANOVA analysis ($p \leq 0.05$) * $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$**

the three application methods of PEEs obtained from *Co. tomentosum* at 0,2 and 0,1 mg. mL⁻¹, *U. rigida* at 0,1 mg. mL⁻¹, *B. bifurcata* at 0.05; 0.1 and 0.2 mg. mL⁻¹, *F. spiralis* at 0.02 and 0.05 mg. mL⁻¹, *G. crinale* at 0.02 and 0.05 mg. mL⁻¹ and *S. dubyi* at all concentrations. In addition, this significant increase was also shown by the root irrigation by PEEs obtained from *B. bifurcata*, *F. spiralis* and *C. acicularis* at 0.1, 0.02 and 0.05 mg. mL⁻¹, respectively. However, PAL activity was significantly stimu-

lated with the foliar application of PEEs obtained from *Co. tomentosum* and *U. rigida* at 0.05 mg. mL⁻¹, *B. bifurcata* at 0.02 mg. mL⁻¹ and *F. spiralis* at 0.1 mg. mL⁻¹.

To conclude, the PEEs obtained from *Co. tomentosum* and *U. rigida*, and *B. bifurcata* at 0.1 mg. mL⁻¹ and PEEs obtained from *G. crinale*, *S. dubyi* and *F. spiralis* at 0.02 mg. mL⁻¹ were the best treatments inducing significant stimulation of protein content and significant elicitation of PAL activity in tomato plants with the tree application methods.

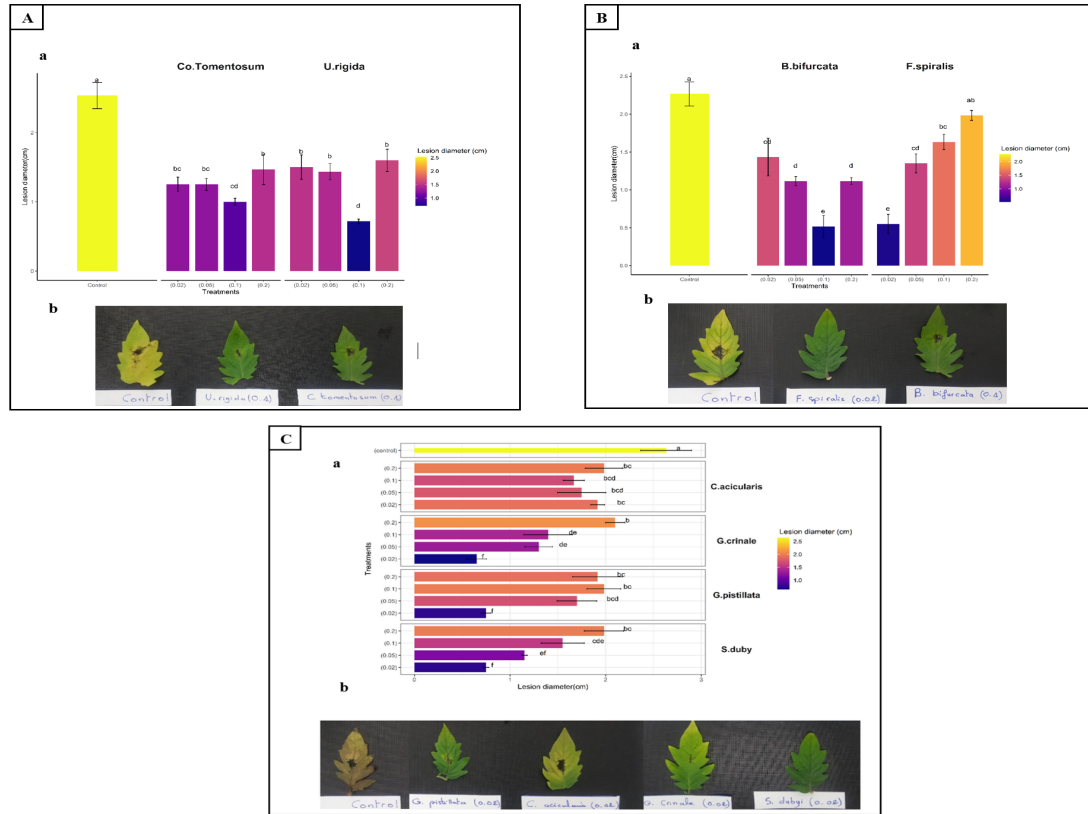


Figure 4: The effect of Polysaccharides Enriched Extracts (PEEs) obtained from green seaweed (A) *Codium tomentosum*, *Ulva rigida*, red seaweeds (B): *Bifurcaria bifurcata* and *Fucus spiralis* and brown seaweeds (C): *Chondracanthus acicularis*, *Gelidium crinale*, *Gigartina pistillata*, *Schizymenia dubyi* on detached tomato leaves after 7 days infection by *B. cinerea*. a: The average lesion diameter (two perpendicular diameters) after 7 days of *B. cinerea* inoculation of detached tomato leaves treated by PEEs at 4 concentrations (0.02, 0.05, 0.1 and 0.2 mg. mL⁻¹). Data were obtained from three biological replicates. b: Lesion development of *B. cinerea* infection on tomato leaves treated by PEEs showing the significant inhibitory effect compared to control leaves

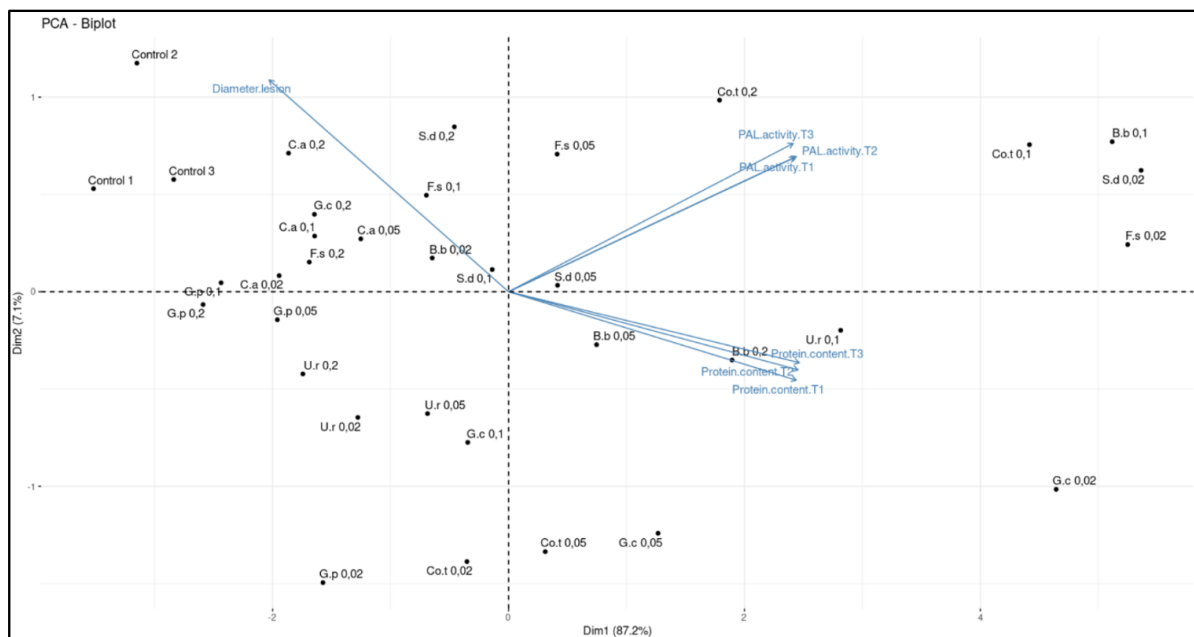


Figure 5: Principal component analysis between the following variables: Protein content at the three application methods (T1, T2 and T3), PAL activity at the three application methods (T1, T2 and T3) and Diameter lesion of *B. cinerea*

PEEs effect on gray mold on detached tomato leaves

As shown in figure 4 (A, B and C), the pathogenic effect caused by *B. cinerea* on detached tomato leaves were found in all treatments using different concentrations of PEEs (0.02; 0.05; 0.1 and 0.2 mg. mL⁻¹). However, there is significant differences in the diameter lesions between leaves treated by PEEs and control leaves ($p < 0.05$). Compared to control leaves, the inhibition effect on the gray mold was especially remarkable with 71, 6 and 77,0 %, using 0.1 mg. mL⁻¹ of PEEs obtained from *Co. tomentosum*, *U. rigida* and *B. bifurcata*, respectively. Additionally, the smallest concentration of PEEs (0.02 mg. mL⁻¹) obtained from *G. crinale*, *G. pistillata*, *S. dubyi* and *F. spiralis* had also decreased the lesion diameter by 75; 71; 71 and 76 %, respectively.

DISCUSSION

Research to reduce the application of chemical pesticides in agriculture through the discovery of new natural compounds is urgently needed in order to respond to the increasing demand for pesticide-free food and to meet the needs of sustainable agriculture (Lykogianni *et al.*, 2021). The use of algal polysaccharides in agriculture has been widely reported as elicitors of natural plant defense (Agarwal *et al.*, 2021). In this study, we have studied the effect of eight polysaccharides extracts obtained from Moroccan seaweeds at four concentrations with three different applications methods on protein content and PAL activity in tomato plants. In the second assay, the eight PEEs were tested for inhibitory activity in tomato detached leaves against the aggressive plant pathogen *Botrytis cinerea*, which causes gray mold rot in fresh horticultural crops, resulting in heavy economical losses worldwide.

Our results showed that the PEEs obtained from *Co. tomentosum*, *U. rigida*, and *B. bifurcata* at 0.1 mg. mL⁻¹ and *G. crinale*, *S. dubyi* and *F. spiralis* at 0.02 mg. mL⁻¹ were the best treatments by significantly stimulating protein content and PAL activity in tomato plants with the three application methods (Figure 2 and Figure 3). The same extracts at the same concentrations, in addition of PEEs from *G. pistillata* at 0.02 mg. mL⁻¹, were the treatments having the greatest inhibitory effect on the lesion diameter of *B. cinerea* in detached leaves when compared to the control (Figure 4). Our results corroborated those reported by Zhang *et al.*, (2015), who reported that seaweeds alginoligosaccharides could induce the enzyme activity of PAL in rice plants as defense against *Magnaporthe grisea*. Similarly, the treatment of olive and tomato plants by Ulvan extracted from *Ulva lactuca* significantly induced PAL activities against *Verticillium wilt* and *lycopersicum*, respectively (Ben Salah *et al.*, 2018). Likewise, polysaccharides extracted from microalga have the same effect. Rachidi *et al.*, (2021) reported that The PAL activity have been improved in tomato plants leaves treated by polysaccharides extracted from several microalgae such as *P. triocnutum*, *Desmodesmus* sp., *P. triocnutum* and *Porphyridium* sp.

The analysis of the PEEs from the studied seaweeds was previously published and revealed the presence of high sugar content and sulfated groups in the extracts (Mzibra *et al.*, 2018). Infra-red analysis revealed the footprint of some known polysaccharides extracted from the green, brown and red seaweed species with the presence of sulfated groups. FTIR analysis confirmed the presence of uronic acids in the composition of the eight polysaccharides. Molecules containing uronic acid and sulfate groups are known to trigger plant defense responses against pathogens (De Borba *et al.*, 2021; Limaet *al.*, 2021), this finding reinforces the proposition that the recognition of the seaweed PEEs by tomato plant may be associated with uronic acid residues and sulfate groups.

Such polysaccharides can be recognized by plant cells as PAMPs that activate their resistance mechanisms against pathogens. In this sense, Aitouguinane (2020) indicated that fucoidans isolated from Moroccan *Bifurcaria bifurcata* and *Fucus spiralis* induced natural defense in the roots of date palm by the significant increase in PAL activity, phenols and lignin in roots. Interestingly, this elicitor effect seems to be linked to the sulfated groups in the extracted polysaccharide.

Resistance reactions induced in tomato leaves as a result of PEEs applications could be explained by the induction of secondary metabolism PAL, which is considered as an important enzyme related to defense reactions in plants under stress and the key element in the lignification process that converts the phenylalanine to trans-cinnamic acid, the first step in the general phenylpropanoid way (Reichert *et al.*, 2009). This enzyme is frequently studied in plant defense research known to correlate with the occurrence of induced resistance. Indeed, PAL activity is accompanied by the biosynthesis of active metabolites such as Phytoalexins, lignins, flavonoids, phenolics compounds and salicylic acid leading to plant defense against microbial phytopathogens (Hsieh *et al.*, 2010; Battacharyya *et al.*, 2015).

The PCA represented in the figure 5 showed the correlation between PAL content and the reduction of lesion diameter. The only exception contradicting this explanation was shown with the *G. pistillata* extract, which significantly inhibited the lesion diameter of *B. cinerea* without stimulating the PAL activity. In our previous studies, the PEE of the red seaweed *G. pistillata* was the only extract which inhibited tomato seed germination (Mzibra *et al.*, 2020). Accordingly, this may be explained by the fact that this extract contains some bioactive compounds which decrease the lesions of *Botrytis* without affecting PAL activity and needs further investigation.

Algae polysaccharides and oligo-saccharides are applied mainly by foliar spray, root soaking or drench and leaf infiltration (Shukla *et al.*, 2021). The comparison of the application method in our study did not show differences as presented in figure 4. The best treatment at the identified concentration increased the PAL activity and reduced the lesion diameter of the fungal pathogen regardless the application mode.

CONCLUSION

The results from this study showed that the application of PEEs obtained from *Co. tomentosum*, *U. rigida*, and *B. bifurcata* at 0.1 mg. mL⁻¹ and *G. crinale*, *S. dubyi* and *F. spiralis* at 0.02 mg. mL⁻¹ may be able to modulate antioxidant enzymes and activate the phenolic metabolism pathway leading to an effective control of gray mold in tomato plants. This study demonstrates that those algae polysaccharides can be an abundant, renewable and safety source of elicitors, which suggests that these treatments could partially replace the use of synthetic fungicides, until further studies to test these natural algal polysaccharides on large-scale conditions are successfully conducted. The presence of uronic acid and sulfated groups in the extracts could explain the elicitation mechanism induced in plant cells.

REFERENCES

- Abbey, J.A.; Percival, D.; Abbey, L.; Asiedu, S.K.; Prithiviraj, B.; Schilder, A. (2019). Biofungicides as alternative to synthetic fungicide control of grey mould (*Botrytis cinerea*)—prospects and challenges. *Biocontrol. Sci. Technol.*, 29: 207–228.
- Abouraïcha, E.; El Alaoui-Talibi, Z.; El Boutachfai, R.; Petit, E.; Courtois, B.; Courtois, J.; El Modafar, C. (2015). Induction of natural defense and protection against *Penicillium expansum* and *Botrytis cinerea* in apple fruit in response to bioelicitors isolated from green algae. *Sci. Hortic.*, 181: 121–128.
- Adnan, M.; Hamada, M.; Li, G.; Luo, C. (2018). Detection and molecular characterization of resistance to the dicarboximide and benzamide fungicides in *Botrytis cinerea* from tomato in Hubei Province, China. *Plant Dis.*, 102: 1299–1306.
- Adnan, M.; Hamada, M.S.; Hahn, M.; Li, G.-Q.; Luo, C.-X. (2019). Fungicide resistance of *Botrytis cinerea* from strawberry to procymidone and zoxamide in Hubei, China. *Phytopathol. Res.*, 1: 1–12.
- Agarwal, P. K., Dangariya, M., & Agarwal, P. (2021). Seaweed extracts: Potential biodegradable, environmentally friendly resources for regulating plant defence. *Algal Research*, 58: 102363.
- Aitouguinane M, Bouissil S, Mouhoub A, Rchid H, Fendri I, Abdelkafi S, Ould El-Hadj MD, Boual Z, Dubessay P, Gardarin C, Michaud P, El Alaoui-Talibi Z, El Modafar C, Pierre G, Delattre C. (2020). Induction of Natural Defenses in Tomato Seedlings by Using Alginate and Oligoalginates Derivatives Extracted from Moroccan Brown Algae. *Marine Drugs*, 18: 521.
- Aziz A, Poinsot B, Daire X, Adrian M, Bézier A, Lambert B, Joubert JM, Pugin A (2003). Laminarin elicits defense responses in grapevine and induces protection against *Botrytis cinerea* and *Plasmopora viticola*. *Molecular Plant-Microbe Interactions*, 16: 1118–1128.
- Battacharyya, D.; Babgohari, M.Z.; Rathor, P.; Prithiviraj, B. (2015). Seaweed extracts as biostimulants in horticulture. *Sci. Hortic.* (Amsterdam), 196: 39–48.
- Ben Salah, I., Aghrouss, S., Douira, A., Aissam, S., El Alaoui-Talibi, Z., Filali-Maltouf, A., & El Modafar, C. (2018). Seaweed polysaccharides as bio-elicitors of natural defenses in olive trees against verticillium wilt of olive. *Journal of Plant Interactions*, 13: 248–255.
- Boller, T. & He, S. Y. (2009). Innate immunity in plants: An arms race between pattern recognition receptors in plants and effectors in microbial pathogens. *Science*, 324: 742–744.
- Bradford, M.M., (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal. Biochem.*, 72: 248–254.
- Brandhoff, B., Simon, A., Dornieden, A., Schumacher, J. (2017). Regulation of conidiation in *Botrytis cinerea* involves the light-responsive transcriptional regulators *bclt3* and *bcreg1*. *Current Genetics*, 63: 931–949.
- Cardoso, I., Cotas, J., Rodrigues, A., Ferreira, D., Osório, N., & Pereira, L. (2019). Extraction and analysis of compounds with antibacterial potential from the red alga *Grateloupia turuturu*. *Journal of Marine Science and Engineering*, 7: 220.
- Chen, S., Sathuvan, M., Zhang, X., Zhang, W., Tang, S., Liu, Y., & Cheong, K. L. (2021). Characterization of polysaccharides from different species of brown seaweed using saccharide mapping and chromatographic analysis. *BMC chemistry*, 15: 1–11.
- De Borba, M. C., Velho, A. C., Maia-Grondard, A., Baltenweck, R., Magnin-Robert, M., Randoux, B., & Siah, A. (2021). The Algal Polysaccharide Ulvan Induces Resistance in Wheat Against *Zymoseptoria tritici* Without Major Alteration of Leaf Metabolome. *Frontiers in Plant Science*, 12.
- de Freitas, M.B.; Ferreira, L.G.; Hawerth, C.; Duarte, M.E.; Nosedá, M.D.; Stadnik, M.J. (2015). Ulvans induce resistance against plant pathogenic fungi independently of their sulfation degree. *Carbohydr. Polym.*, 133: 384–390.
- De Vega, D., Holden, N., Hedley, P. E., Morris, J., Luna, E., & Newton, A. (2021). Chitosan primes plant defence mechanisms against *Botrytis cinerea*, including expression of Avr9/Cf-9 rapidly elicited genes. *Plant, Cell & Environment*, 44: 290–303.
- Dean, R., et al. (2012). The top 10 fungal pathogens in molecular plant pathology. *Molecular Plant Pathology*, 13: 414–430.
- Dodds, P. N. & Rathjen, J. P. (2010). Plant immunity: Towards an integrated view of plant-pathogen interactions. *Nat. Rev. Genet.*, 11: 539–548.
- El Modafar, C., Tantaoui, A., & El Boustani, E. S. (2001). Differential induction of phenylalanine ammonia-lyase activity in date palm roots in response to inoculation with *Fusarium oxysporum* f. sp. *albodinis* and to elicitation with fungal wall elicitor. *Journal of Plant Physiology*, 158: 715–722.
- El Modafar, C.; Elgadda, M.; El Boutachfai, R.; Abouraïcha, E.; Zehhar, N.; Petit, E.; El Alaoui-Talibi, Z.; Courtois, B.; Courtois, J. (2012). Induction of natural defence accompanied by salicylic acid-dependant systemic acquired resistance in tomato seedlings in response to bioelicitors isolated from green algae. *Sci. Hortic.*, 138: 55–63.
- Elad, Y.; Pertot, I.; Prado, A.M.C.; Stewart, A. (2016). Plant hosts of *Botrytis* spp. In *Botrytis—The Fungus, the Pathogen and Its Management in Agricultural Systems*; Springer: pp. 413–486.
- Elad, Y.; Williamson, B.; Tudzynski, P.; Delen, N. (2007). *Botrytis* spp. and diseases they cause in agricultural systems—an introduction. In *Botrytis: Biology, Pathology and Control*; Springer: Berlin/Heidelberg, Germany, 2007; pp. 1–8.
- Elnahas, M. O., Amin, M. A., Hussein, M., Shanbhag, V. C., Ali, A. E., & Wall, J. D. (2017). Isolation, characterization and bioactivities of an extracellular polysaccharide produced from *Streptomyces* sp. MOE6. *Molecules*, 22: 1396.
- Fernando, I. P., Sanjeeva, K. K. A., Samarakoon, W., Lee, W. W., Kim, H. S., Kim, E. A., ... & Jeon, Y. J. (2017). FTIR characterization and antioxidant activity of water-soluble crude polysaccharides of Sri Lankan marine algae.
- Fillinger, S., Elad, Y. (2016). *Botrytis - the Fungus, the Pathogen and its Management in Agricultural Systems*. Pub. Springer, New York.
- Gómez-Ordóñez, E., & Rupérez, P. (2011). FTIR-ATR spectroscopy as a tool for polysaccharide identification in edible brown and red seaweeds. *Food hydrocolloids*, 25: 1514–1520.
- Hao, Q., Deng, J., Yang, F., Wang, G., Xiao, Y., & Xiao, X. (2020). Biological Control of Tomato Gray Mold Caused by *Botrytis Cinerea* with the Entomopathogenic Fungus *Metarhizium Anisopliae*. *Pathogens*, 9: 213.
- Hsieh, L., Yeh, C., Pan, H., Cheng, C., Yang, C., & Lee, P. (2010). Cloning and expression of a phenylalanine ammonia-lyase gene (BoPAL2) from *Bambusa oldhamii* in *Escherichia coli* and *Pichia pastoris*. *Protein Expression and Purification*, 71: 224–230.
- Hua, L.; Yong, C.; Zhanquan, Z.; Boqiang, L.; Guozheng, Q.; Shiping, T. (2018). Pathogenic mechanisms and control strategies of *Botrytis cinerea* causing post-harvest decay in fruits and vegetables. *Food Qual. Saf.*, 2: 111–119.

- Lima, C. P. C. D., Oster, A. H., Cavalcanti, F. R., Paula, R. C. M. D., & Feitosa, J. P. A. (2021). Induction of defense in apples by sulfated and deacetylated chichá gum. *Polímeros*, 31.
- Lykogianni, M., Bempelou, E., Karamaouna, F., & Aliferis, K. A. (2021). Do pesticides promote or hinder sustainability in agriculture? The challenge of sustainable use of pesticides in modern agriculture. *Science of the Total Environment*, 148625.
- Modafar, C. E. *et al.* (2012). Induction of natural defence accompanied by salicylic acid-dependant systemic acquired resistance in tomato seedlings in response to bioelicitors isolated from green algae. *Sci. Hortic.* (Amsterdam), 138: 55–63.
- Mosbach, A.; Edel, D.; Farmer, A.D.; Widdison, S.; Barchietto, T.; Dietrich, R.A.; Corran, A.; Scalliet, G. (2017). Anilinopyrimidine resistance in *Botrytis cinerea* is linked to mitochondrial function. *Front. Microbiol.*, 8: 2361.
- Myresiotis, C.; Karaoglanidis, G.; Tzavella-Klonari, K. (2007). Resistance of *Botrytis cinerea* isolates from vegetable crops to anilinopyrimidine, phenylpyrrole, hydroxyanilide, benzimidazole, and dicarboximide fungicides. *Plant Dis.*, 91: 407–413.
- Mzibra, A., Aasfar, A., Benhima, R., Khouloud, M., Boulif, R., Douira, A., Bamouh, A., Kadmiri, I. M. (2021). Biostimulants derived from Moroccan seaweeds: seed germination metabolomics and growth promotion of tomato plant. *Journal of Plant Growth Regulation*, 40: 353-370.
- Mzibra, A., Aasfar, A., El Arroussi, H., Khouloud, M., Dhiba, D., Kadmiri, I. M., & Bamouh, A. (2018). Polysaccharides extracted from Moroccan seaweed: A promising source of tomato plant growth promoters. *Journal of applied phycology*, 30: 2953-2962.
- Nandiyanto, A. B. D., Oktiani, R., & Ragadhita, R. (2019). How to read and interpret FTIR spectroscopy of organic material. *Indonesian Journal of Science and Technology*, 4: 97-118.
- Nürnberg, T. & Scheel, D. (2001). Signal transmission in the plant immune response. *Trends Plant Sci.*, 6: 372–379.
- Rachidi, F., Benhima, R., Kasmi, Y., Sbabou, L., & El Arroussi, H. (2021). Evaluation of microalgae polysaccharides as biostimulants of tomato plant defense using metabolomics and biochemical approaches. *Scientific reports*, 11: 1-16.
- Reichert, A. I., He, X. Z., & Dixon, R. A. (2009). Phenylalanine ammonia-lyase (PAL) from tobacco (*Nicotiana tabacum*): Characterization of the four tobacco PAL genes and active heterotetrameric enzymes. *Biochemical Journal*, 424: 233–242.
- Romanazzi, G., & Feliziani, E. (2014). *Botrytis cinerea* (gray mold). In *Postharvest decay* (pp. 131-146). Academic Press.
- Rosero-Hernández, E.D.; Moraga, J.; Collado, I.G.; Echeverri, F. (2019). Natural compounds that modulate the development of the fungus *Botrytis cinerea* and protect *Solanum lycopersicum*. *Plants*, 8: 111.
- Shakeel, Q.; Lyu, A.; Zhang, J.; Wu, M.; Li, G.; Hsiang, T.; Yang, L. (2018). Biocontrol of *Aspergillus flavus* on peanut kernels using *Streptomyces yanglinensis* 3–10. *Front. Microbiol.*, 9: 1049.
- Shukla, P.S.; Borza, T.; Critchley, A.T.; Prithiviraj, B. (2016). Carrageenans from Red Seaweeds as Promoters of Growth and Elicitors of Defense Response in Plants. *Front. Mar. Sci.*, 3: 1–9.
- Shukla, P.S.; Borza, T.; Critchley, A.T.; Prithiviraj, B. (2021). Seaweed-Based Compounds and Products for Sustainable Protection against Plant Pathogens. *Mar. Drugs*, 19: 59.
- Shukla, P.S.; Mantin, E.G.; Adil, M.; Bajpai, S.; Critchley, A.T.; Prithiviraj, B. (2019). *Ascophyllum nodosum*-based biostimulants: Sustainable applications in agriculture for the stimulation of plant growth, stress tolerance, and disease management. *Front. Plant. Sci.*, 10: 655.
- Vera, J.; Castro, J.; González, A.; Barrientos, H.; Matsuhira, B.; Arce, P.; Zuñiga, G.; Moenne, A. (2011). Long-term protection against tobacco mosaic virus induced by the marine alga oligo-sulphated-galactan Poly-Ga in tobacco plants. *Mol. Plant. Pathol.*, 12: 437–447.
- Wang Z.N., Coley-Smith J.R. (1986). Studies some characteristics of dicarboximide-resistant isolates of *Botrytis cinerea* from protected lettuce. *Plant Pathol.*, 35: 544-550.
- Weiberg, A., *et al.* (2013). Fungal small RNAs suppress plant immunity by hijacking host RNA interference pathways. *Science* (New York, N.Y.), 342: 118–123.
- Williamson B, Tudzynski B, Tudzynski P, Van Kan JAL. (2007). *Botrytis cinerea*: The cause of grey mould disease. *Mol. Plant Pathol.*, 8: 561–580.
- Zhang, S. *et al.* (2015). Elicitor activity of alginoligosaccharide and its potential application in protection of rice plant (*Oryza sativa* L.) against *Magnaporthe oryzae*. *Biotechnol. Biotechnol. Equip.*, 29: 1–7.