horticulturae

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A New Promising Plant Defense Stimulator Derived from a By-Product of Agar Extraction from *Gelidium sesquipedale*

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Abstract: Stimulation of plant defenses by elicitors is an alternative strategy to reduce pesticide use. In this study, we examined the elicitor properties of a by-product of the industrial extraction of agar from the red alga *Gelidium sesquipedale*. Agar extraction process leads to the formation of an alkaline residue which is poorly valorized. This by-product has been analyzed for its chemical composition. It contains 44% minerals and, among the organic compounds, sugars are the most represented and encompass 12.5% of the dry matter. When sprayed on tomato plants, this by-product enhanced the levels of defense markers such as peroxidase or phenylalanine ammonia lyase activities. Furthermore, this treatment increased the expression levels of the pathogenesis-related gene, *PR9* encoding peroxidase. A field trial conducted on grapevine revealed that spraying treatment with this by-product resulted in a reduction of the macroscopic disease symptoms induced by *Plasmospora viticola*, with 40 to 60% efficacy. These results indicate that this agar extraction by-product could be used as a plant defense stimulator.

Keywords: tomato; grapevine; elicitor; plant defense; by-product; *Gelidium sesquipedale*

Citation: Koçi, R.; Dupuy, F.; Lebbar, S.; Gloaguen, V.; Faugeron Girard, C. A New Promising Plant Defense Stimulator Derived from a By-Product of Agar Extraction from *Gelidium sesquipedale*. *Horticulturae* **2022**, *8*, 958. https://doi.org/10.3390/horticulturae8100958

Academic Editors: Hillary Righini, Roberta Roberti, Stefania Galletti and Jiri Gruz

Received: 24 August 2022
Accepted: 13 October 2022
Published: 16 October 2022

1. Introduction

Plant pathogens are responsible for significant reductions in crop yields. Large quantities of pesticides are being used to reduce their impact. However, an international study published in 2017 revealed that pesticides are responsible for the death of 5000–20,000 and the poisoning of 500,000 to a million people [1]. Despite their controversial side effects, 2.6 million tons of pesticides have been used worldwide in 2020 [2]. In addition, these chemicals might alter the quality of water and soil and could be harmful to farmers. Furthermore, pesticide residues are frequently found in commercialized plants. In order to reduce the use of pesticides, an alternative strategy can be adopted consisting in improving the natural defenses of plants against their pathogens. Indeed, plants naturally defend themselves from pathogen attacks by reinforcing their natural barriers or by developing direct attacks against the pathogens. Plants benefit from passive as well as active defense mechanisms. Passive mechanisms are permanent and consist of a series of physical barriers like cuticles or cell walls [3], in addition to constitutively produced molecules like phenolic compounds, known for their antimicrobial properties [4]. However, pathogens are able to produce cell wall-degrading enzymes (cutinases, polygalacturonases, etc.) that weaken this barrier of the plant cell [5]. In case of failure of the passive defenses, plants build a series of active defenses in response to pathogen aggression. Active defenses include a hypersensitive response (HR), leading to an auto-destruction of the damaged cells, and a local acquired resistance (LAR), consisting in isolating the cells close to the damage site. LAR can later become a SAR (systemic acquired resistance), a resistance that involves the whole plant, thanks to the production of PR proteins (pathogenesis related proteins), which are targeted towards specific pathogens [3]. The responses to the pathogen occur in

the following order: recognition of the elicitor (plant defense stimulator, PDS), signaling to other cells, and defensive reactions. The initial event is, therefore, the recognition of a signal of pathogen attack, for example the recognition of pathogen molecules, called microbe-associated molecular patterns (MAMPs), or also of molecules released from the damaged host cells, called the damage-associated molecular patters (DAMPs). The recognition process directly implies specific receptors called PRRs (patterns recognition receptors). These are usually receptor-like kinases (RLKs) or receptor-like proteins (RLPs) which harbor an extracellular domain recognizing MAMPs/DAMPs. Among MAMPs, bacterial flagellin, peptidoglycans, lipopolysaccharides, fungal chitin, oomycete β-glucans or toxins and degradation enzymes like endopolyglucanases can be mentioned. On the other hand, DAMPs are endogenous molecules, for example oligogalacturonides, which are released from the walls of the damaged cells. The recognition of MAMPs/DAMPs gives place to the aforementioned plant defense mechanisms like the production of Pathogenesis-Related (PR) proteins and phytoalexins, HR activation and cell wall thickening. For example, deposition of callose and lignification render the plant cell wall more resistant to pathogen attack [5]. These plant defenses might be induced by elicitors defined as compounds which mimic either MAMPs or DAMPs. Therefore, elicitors could be used to protect the plant from pathogen attacks [6].

An elicitor is usually a molecule produced by a pathogen, but hormonal and abiotic elicitors (some metals for example) have also been described [7]. Moreover, some environmental stresses, like excess light, hyperosmolarity, drought, thermal variations, etc., can induce defense responses [7]. Among elicitor compounds, various types have been characterized, including hormonal source elicitors, carbohydrate-containing polymers, like (glyco)peptides and (glyco)proteins, and algae-derived elicitors. Hormonal elicitors are phytohormones that are implicated in the signaling of defense responses, for example salicylic acid [8,9]. Carbohydrate elicitors are secreted by microorganisms or derived from the cell walls of fungi, bacteria or from the host plants themselves [8,9]. Also, many carbohydrate-based polymers whose structures are close to those present in the pathogens or the plant have been found to act as elicitors. Among them xyloglucans, oligogalacturonides, chitin or chitosan oligomers and ramified β-(1,3)-(1,6)-glucans, exhibit elicitor activities in different plant species and trigger pathogen defense responses. Seaweed polysaccharides such as fucans, laminarin, or carrageenans have also been described as plant elicitors [10]. We have shown in a previous work that an extract from the red alga *Gelidium sesquipedale* contained some original molecules, such as glycerol-galactosides, endowed with elicitor activities [11,12]. However, this extract was obtained at a laboratory scale. As *G. sesquipedale* is used at the industrial level for the production of agar, large volumes of by-products are generated which might potentially contain molecules exhibiting biological activities. We describe in the present work, the chemical characterization and the determination of the principal components of a by-product of the industrial agar extraction from *G. sesquipedale*. Then, the biological activities of this extract were assessed, in controlled conditions, on tomato plantlets by measuring some defense responses such as peroxidase or phenylalanine ammonia lyase (PAL) activities. Transcription levels of some PR protein genes were also determined. Furthermore, the capacity of this by-product to protect plants from pathogens was evaluated in a field trial conducted on grapevine infected by *Plasmopara viticola* (downy mildew grapevine disease).

2. Materials and Methods

2.1. By-Product Origin, Production and Preparation

The agar by-product was obtained from the industrial treatment of *G. sesquipedale*. Algae harvested from the Moroccan seashore during summer 2019 were processed by the SETEXAM company (Kenitra, Morocco). The extraction process comprises an alkaline treatment of the dried algae with a 5% NaOH solution during 1 h. Algae are then separated from the alkaline solution. The recovered solution is analyzed and its NaOH content is readjusted, if necessary, in order to be used for the treatment of a new batch of alga. This

step is repeated 35 times (i.e., 35 batches of alga). The final alkaline solution, recovered *in loco*, neutralized to pH = 7 with 1% sulfuric acid, concentrated and finally freeze-dried, constitutes the lyophilizate, called SL35, which was used in the present study.

2.2. Elemental Analyses

Samples of SL35 were calcined at 800 °C for 8 h and around 250 mg batches of the resulting residue were weighed and digested as described by Nguyen et al. [13]. Elements, except P, C, H, N and S, were analyzed by MP-AES as in [13]. C, H, N and S were quantified by CHNS as described by Bascle et al. [14]. The digested samples were also quantified for their phosphorus content using the LCK349^{TM} and LCK350^{TM} kits (Hach$^{©}$, Düsseldorf, Germany).

2.3. Organic Compounds Assays

2.3.1. Carbohydrate Assays

The method of Dubois et al. [15] was used for the quantification of neutral sugars. Galactose and galacturonic acid were used for the calibrating curve with respective ranges of 0–100 and 0–200 µg mL^{-1}. Uronic acids were quantified by the method of Blumenkrantz and Asboe-Hansen [16] using meta-hydroxydiphenyl as reagent. The calibrating curve was made with galactose and galacturonate as standards with respective ranges of 0–500 and 0–100 µg mL^{-1}.

2.3.2. Protein Assay

Protein concentration was determined as described by Bradford et al. [17] using bovine serum albumin as a standard.

2.3.3. Phenolic Compounds Assay

To quantify the total amount of phenolic compounds the method from Piló-Veloso et al. [18] was used with a calibration curve of gallic acid from 0 to 100 µg mL^{-1}.

2.4. Greenhouse Experiments

The experiments were conducted as described by Faugeron-Girard et al. [19] except that the present treatment was done with the lyophilized agar extraction by-product, SL35.

Briefly, tomato plants (*Marmande* variety), at least 1 month old, cultured in greenhouse conditions were treated three times at days 1, 3 and 5 by foliar spraying. SL35 was applied in solution with a surfactant (Tween$^{®}$80 0.05%, Thermo Fisher 76870 Kandel, Germany). The concentration range of SL35 was from 10 to 1000 mg L^{-1} and 0.05% Tween$^{®}$80 was applied as a control. Plants were inoculated on the 8th day by infiltration of leaves with conidial suspensions of *Botrytis cinerea* (UBOC 117017). The leaves above the point of inoculation were collected and subjected to biochemical analyses.

2.5. Plant Defense Activities

Peroxidase activity was determined as described by Faugeron-Girard et al. [19]. Briefly, foliar samples were ground in presence of 100 mM phosphate buffer (pH = 7). Then, extracts were assayed in a 250 mM acetate buffer (pH = 4.4) containing 500 µM 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid and 250 µM H_2O_2. The reaction was followed spectrophotometrically at λ = 412 nm.

Phenylalanine ammonia lyase (PAL) activity was determined as described by Francini et al. [20]. Extraction was done in 100 mM borate buffer (pH = 8.8) containing 14 mM β-mercaptoethanol. Assay medium consisted of 1 mL of the borate buffer mixed with 200 µL of 100 mM phenylalanine and the formation of *trans*-cinnamic acid at room temperature was deduced from absorbance of the medium at λ = 290 nm.

Callose content of tomato leaves was determined as described by Hirano et al. [21]. Samples from 15 different plants treated the same way were dried and ground together and 3 replicas were created by weighing 30 mg of the mixed powder obtained from the 15 plants. Phenolic compounds were removed by addition of polyvinylpyrrolidone followed with

ethanol washings until no visible green color could be seen. The remaining pellet was resuspended in 1 M NaOH to extract callose. A calibration curve was prepared with a 15 µg mL^{-1} β-1,3-glucan (Megazyme/Libios) solution dissolved in 1 M NaOH (concentration range: 0–15 µg mL^{-1}). Callose was measured using aniline blue chromophore in 1 M glycine buffer (pH = 9.5).

2.6. Quantification of Defense Gene Expression by Real Time q-PCR

The experiment was carried out as described by Grassot et al. [22] with slight modifications. Here, samples consisted of 3 tomato foliar disks of 1 cm diameter. A NanoDrop One (ThermoFisher Scientific™, F67403 Illkirch, France) was used to measure RNA amounts and quality was assessed with RNA easy kit (Qiagen). Reverse transcription was effectuated with a Thermo Scientific *TM* RevertAid First Strand cDNA Synthesis Kit using 1 µg of RNA. Finally, 50 ng of cDNA were used with 10 nM of primers in each well of the PCR 96-well plates. The analyzed genes were *PR2*, *PR8*, *PR9*, *PR14*, *PR15* and *PAL*, with *β-actin* as housekeeping reference gene. The sequences of the primers used are presented in Table 1.

Table 1. List of primers used in real time q-PCR experiments on tomato plants (*Solanum lycopersicum*). F: Forward; R: Reverse.

Gene	References	Primers in the Corresponding Gene
PR2b	-	F: CCGTTGGAAACGAAGTTGAT R: TCATCAGCATGGCCAAAATA
PR8	[23]	F: TGC AGG AAC ATT CAC TGG AG R: TAA CGT TGT GGC ATG ATG GT
PR9	[24] [25]	F: GCTTTGTCAGGGGTTGTGAT R: TGCATCT CTAGCAACCAACG
PR14	[26]	F: CTCCATGCCTCCCTTATCTTC R: CATGCTGTCTTTCGATCCG
PR15	[27]	F: GGGCTAAATCCACCTCA R: GGCACCACGAACATCTC
PAL	[28]	F: CTTTGATGCAGAAGCTGAGACA R: TCGTCCTCGAAAGCTACAATCT
β-actin	[23]	F: AGG CAC ACA GGT GTT ATG GT R: AGC AAC TCG AAG CTC ATT GT

DNA quantification was possible though the SYBR GREEN method. To calculate the expression of the genes of interest we chose to set their expression relative to the reference gene based on threshold values (Ct) using the ΔΔCt method (fold gene expression = $2^{-\Delta\Delta Ct}$). The formula ($-1/2^{-\Delta\Delta Ct}$) was applied to values between 0 and 1, in order to optimize their graphical representation. For ΔΔCt relative quantification, only Ct(s) below 35 were taken into consideration.

2.7. Field Trial on Grapevine against P. viticola Artificially Inoculated

The tested plants belong to *Vitis vinifera* species (Merlot variety) cultivated at Sainte-Livrade sur Lot (47), France. Plants were watered twice a day. The trial was organized in 25 plots (with 7 plants per elementary plot), randomly disposed, and including 4 replicas of each treatment. The treatments consisted in spraying the products over the leaves at 7-to-10-day intervals. The treatment dates are mentioned in Table 2. The different treatments were: not-treated control (NTC), a positive control as IODUS 2 CULTURES SPECIALISEES® with laminarin as the active molecule applied at 90 g active ingredient per ha, and the by-product SL35 applied at different doses: 5 g SL35 per ha (SL35x1), 35 g SL35 per ha (SL35x7), and 70 g SL35 per ha (SL35x14). All SL35 solutions were done in 0.05% (*w*/*v*) Tween 80. Artificial contamination was performed on May 28 at the beginning of the flowering (BBCH 61). An inoculum was prepared with frozen grapevine leaves infected by *P. viticola*, harvested in 2019. It was applied by spraying on ten leaves belonging to a grapevine plant positioned at the end of each plot. This plant was further excluded

from the rating of the symptoms. To evaluate the progression of the disease, 100 leaves from the same canopy level (similar age) per plot or 50 bunches per plot were used for each rating. The severity was defined as the average surface of leaves or bunches showing symptoms. The incidence was determined as the average percentage of leaves or bunches showing symptoms.

Table 2. Treatment schedule: dates of application and vegetative development stage of the grapevine plants at each time of application.

Dates	Developmental Stage
5 May	First leaf unfolded (BBCH 11)
12 May	Inflorescences clearly visible (BBCH 53)
20 May	Inflorescences fully developed; flowers separating (BBCH 57)
27 May	Beginning of flowering: 10% of flowerhoods fallen (BBCH 61)
8 June	Fruit set: young fruits beginning to swell, remains of flowers lost (BBCH71)
15 June	Pea-sized berries, hanging bunches (BBCH 75)
24 June	Berries beginning to touch (BBCH 77)

2.8. Statistical Analyses

For the biochemical experiments 15 samples per treatment were used. Callose quantification was realized on 3 analytical replicates, each one using the material collected on 15 plants. Real time qPCR was independently realized on 5 biological replicas for each treatment. Statistical analyses were carried out with the Past free software (version 2.17c). The one-way ANOVA or the Kruskal Wallis tests were used (noted on each figure). p values under 0.05 were considered as significant.

For the field trials, the statistical significances of differences among treatments were estimated using one-way ANOVA followed by the Newman and Keuls test ($p < 0.05$) and the Kurtosis analysis.

3. Results

3.1. SL35 Composition

Chemical analyses were done to determine the contents of the major compounds of the SL35 by-product. Mineral compounds represent on the average 44% (±1.7) of the dry mass (Table 3). Na and K are the most important in quantity with respectively 140 (± 9) and 40 (± 0.7) mg g^{-1} DW. Elemental analysis revealed that carbon is quite abundant with 170 (±7.4) mg g^{-1} DW. Among organic compounds, sugars were the most represented since total sugar content was estimated to 125 (±0.2) mg g^{-1} DW. Comparatively, proteins and phenolic compounds were less present as their contents were respectively 7 to 16 times lower compared to the sugar content. Also, the pH value of the SL35 solution measured at a final concentration of 25 mg DW mL^{-1} was 7.0.

3.2. Biological Activity of SL35: Effect of the Concentration on the Stimulation of Tomato Plant Defenses

To evaluate the effect of the treatment on a model plant (tomato), enzymatic activities related to defense mechanisms, peroxidase and PAL specific activity, and callose content were measured. Peroxidase is involved in hydrogen peroxide detoxification and in the synthesis of lignin (*PR9*), PAL transforms phenylalanine into *trans*-cinnamic acid, a precursor of phenolic compounds and, therefore, contributes to the cell wall thickening. Phenolic compounds also possess antimicrobial activity, while callose is one of the main contributors to cell wall thickening.

When tomato plantlets were pre-treated with SL35, a significant increase in peroxidase specific activity was observed for SL35 concentrations of 50 and 1000 mg L^{-1} as compared with control (Figure 1). A tendency to increase was also observed with 200 mg L^{-1} despite the fact that this increase is not significant. A concentration of 10 mg L^{-1} seems not to be sufficient to stimulate this activity.

Table 3. Chemical composition of SL35 (average ± standard deviation, n = 3). CHNS: elemental analysis; DW: Dry Weight; MP-AES: Microwave Plasma Atomic Emission Spectroscopy.

Types of Components	Analysis Method	CONTENTS
Mineral content	calcination	440 ± 17 mg g−1 DW
Na	MP-AES	140 ± 9 mg g−1 DW
K	MP-AES	40.5 ± 0.7 mg g−1 DW
Si	MP-AES	0.48 ± 0.0 mg g−1 DW
Ca	MP-AES	0.43 ± 0.1 mg g−1 DW
Elemental analysis		
C	CHNS	170 ± 7.4 mg g−1 DW
H	CHNS	27.6 ± 1.5 mg g−1 DW
N	CHNS	24 ± 1 mg g−1 DW
S	CHNS	86 ± 6.4 mg g−1 DW
P	LCK349TM and LCK350TM kits	4.3 ± 0.1 mg g−1 DW
Organic compounds		
Total sugars	Colorimetry	125.4 ± 0.2 mg g−1 DW
Uronic acids *	Colorimetry	9.3 ± 0.2%
Proteins	Colorimetry	16.9 ± 2 mg g−1 DW
Phenolic compounds	Colorimetry	7.7 ± mg g−1 DW

* Expressed as % of total sugars.

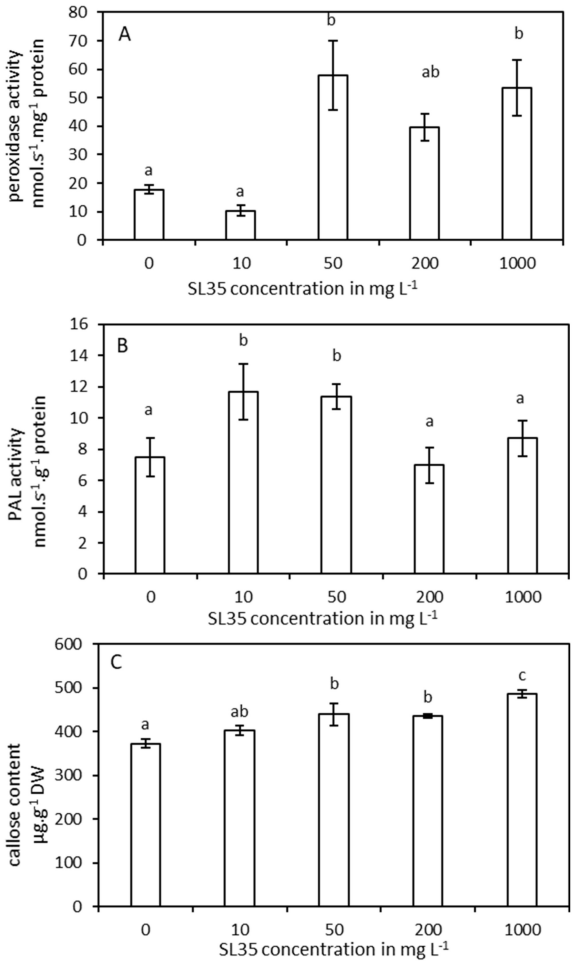

Figure 1. Peroxidase (**A**) and PAL (**B**) specific activities and callose content (**C**) of tomato leaves after treatment with increasing concentration of SL35 diluted in 0.05% Tween 80 (days 1, 3 and 5) and inoculation with *B. cinerea* at day 8. All samples were collected at day 15 (mean ± SD; n = 15 for peroxidase and PAL activities and n = 3 for callose contents). No letters in common between treatments indicate significant differences ($p < 0.05$; one-way ANOVA).

PAL specific activities were also significantly more important in plants treated with 10 or 50 mg SL35 L^{-1} as compared with control. An increase in SL35 concentration of sprayed solution at 200 and 1000 mg L^{-1} seemed not to increase PAL specific activities which were close to control values. Data suggest that concentrations over 200 mg L^{-1} are supra-optimal for the PAL stimulation.

Treatments of tomato plants with SL35 concentrations of 50, 200 and 1000 mg L^{-1} significantly increased callose contents above control.

Finally, SL35 concentration of 50 mg L^{-1} seemed to be the optimal concentration to induce an increase of all the three defense markers: peroxidase and PAL specific activities, together with callose contents.

3.3. Biological Activities of SL35 in Presence or Not of Pathogen: Comparison with Commercial Standard

The elicitor activity of SL35 was compared with that one of laminarin (Vacciplant®; Goëmar laboratories, Saint Malo 35400, France) which is a known PDS, containing a polysaccharide composed of (1–3)-β-D-glucan with β-(1–6) branching [29]. It was applied by spraying a solution at a concentration of 300 mg L^{-1}. The SL35 concentration (50 mg L^{-1}) was chosen as the lowest concentration leading to a significant difference with control (see Figure 1). Then, a part of the tomato plants was inoculated with *B. cinerea* while another part was not inoculated.

As seen in Figure 2, peroxidase activity was not significantly increased in presence of laminarin, whether the plants were inoculated or not. However, a small increase of peroxidase activities was obtained in presence of laminarin as compared with control. Even if the SL35 treatment induced a lower peroxidase activity in non-inoculated plants in comparison with laminarin-treated plants, this activity was significantly higher in the inoculated specimens as compared with control and plants treated with laminarin. The profile of PAL activities was quite different. In absence of inoculation, the laminarin treatment seemed to significantly reduce the PAL activity. The peak value of activity probably occurred before the 7^{th} day after the last laminarin treatment. According to previous works on suspended tobacco cells treated with laminarin, the peak of activity was reached 4 h after treatment [30]. Laminarin treatment of the inoculated plants induced a significant increase of PAL activity in comparison with inoculated plants devoid of laminarin treatment. Besides, SL35 treatment significantly stimulated PAL activity in non-inoculated plants in comparison with their control (non-inoculated and SL35-free plants). Finally, inoculation by itself did not lead to any difference with non-inoculated control specimens.

For the callose contents, the laminarin treatment induced a significant difference with the control in the case of non-inoculated plants: 284 ± 5 μg g^{-1} compared with 207 ± 2 μg g^{-1} respectively. Nevertheless, no statistical difference was observed in the inoculated counterpart, 315 ± 17 μg g^{-1} for plants treated with laminarin vs. 298 ± 16 μg g^{-1} for the control ones. However, a difference appeared between the inoculated plants and the non-inoculated control ones sprayed with water. The same goes for SL35, where there was a statistical difference in non-inoculated samples (SL35-treated plants compared with water-treated control) with a higher value at 318 ± 14 μg g^{-1}. However, inoculated samples did not present any notable difference with the control, since only 286 ± 21μg g^{-1} of callose was found in the former samples. The statistical difference between inoculated SL35 and non-inoculated water control persists, as does the difference between inoculated and non-inoculated controls. These data give the information that pathogen, elicitor, or a combination of both, stimulate callose formation at the same level since all of these treatments led to statistical differences with the non-inoculated water control but not between themselves. It might be possible that callose accumulation reach a maximum value which cannot be increased even if other stimuli are perceived.

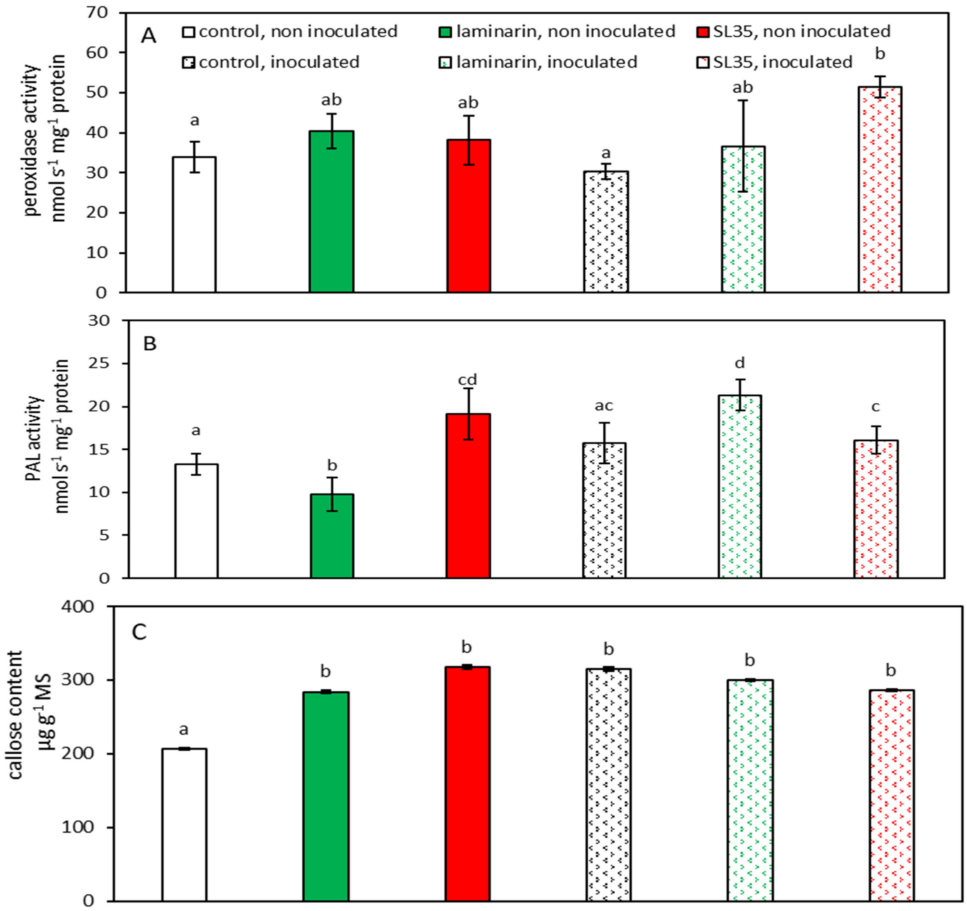

Figure 2. Peroxidase (**A**) and PAL (**B**) specific activities and callose content (**C**) determined in tomato leaves after treatment with SL35 (50 mg L^{-1}) or laminarin (days 1, 3 and 5). At day 8, inoculation by *B. cinerea* was applied or not (non-inoculated). All samples were collected at day 15. Peroxidase values are means of 15 samples ± SE and Kruskal Wallis test was performed. PAL values are means of 15 samples ± SD and one-way ANOVA was performed. Callose values are mean of 3 samples ± SD and one-way ANOVA was performed. Different letters indicate significant differences ($p < 0.05$).

3.4. Evaluation of Defense Responses by Quantification of Gene Expression

Gene expression in relation to some PR proteins and PAL was quantified in tomato plantlet leaves one day following inoculation (day 9) after pre-treatment with laminarin (300 mg L^{-1}) or SL35 (50 mg L^{-1}) comparatively to a control treated with water. The same experiment was done on plantlets without inoculation (harvested on day 9). Relative expressions refer to the appropriate water controls (non-inoculated water-treated plants for the inoculation-free treatments and inoculated water-treated plantlets for the treatments with inoculation).

For non-inoculated plants, no induction was recorded for all of the tested genes in comparison with the positive control (laminarin treatment). Moreover, a significant 5-fold repression of the *PR9* gene was observed relative to the control (Figure 3). For the inoculated samples, laminarin significantly induced increased expression of *PR2*, *PR8* and *PR15*, respectively 7.7, 3.4 and 5.6-fold. A slight increase for the *PR9* gene expression was visible (2.1-fold), but the difference was not sufficient to generate a statistical significance. Expression of the other *PR* or *PAL* genes did not show any significant difference.

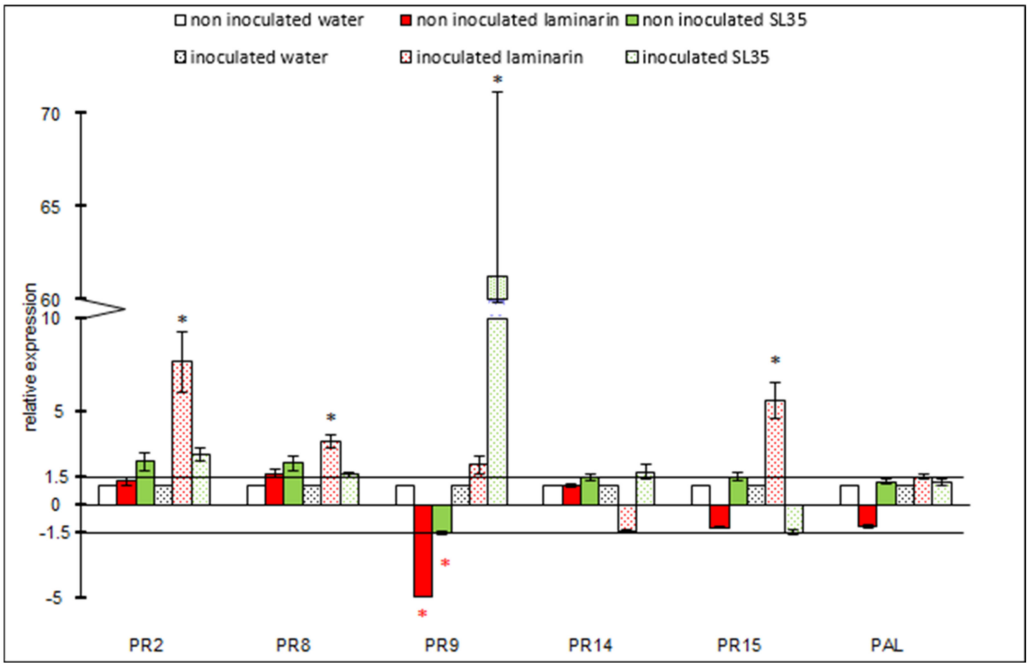

Figure 3. Relative expression levels of some genes encoding PR proteins and PAL in tomato leaves subjected to SL35 or laminarin treatment by foliar spraying (days 1, 3 and 5). Plants were infiltrated with *B. cinerea* conidia at day 8 (dotted histograms). All samples were harvested at day 9. Gene expressions were calculated relative to the appropriate water controls (non-inoculated water for non-inoculated laminarin and non-inoculated SL35, inoculated water for inoculated laminarin and inoculated SL35). Values are means of at least 5 independent samples ± SE. One-way ANOVA was performed to evaluate statistical differences. Only relative expression values out of the interval (+1.5, −1.5), were taken in consideration. * Means significant statistical differences in comparison with controls ($p < 0.05$).

When tomato plantlets were treated with SL35 without inoculation, *PR2* and *PR8* genes were overexpressed 3.1 and 2.2-fold respectively, but no significant difference was observed. *PR14*, *PR15* and *PAL* genes showed almost no increased expression. *PR9* gene expression seemed to be significantly reduced (by 1.5-fold) by the SL35 treatment compared with control. The same tendency was observed for laminarin treatment as described above. For this gene, inoculation seemed to increase the level of expression as compared with the plantlets treated with either laminarin or SL35. Indeed, the induction by SL35 was quite high, reaching 61.3-fold over control. On the other hand, for *PR2*, *PR8* and *PR14* genes, the expression showed a tendency to increase (respectively 2.7, 1.6 and 1.8-fold as compared with control). Again, *PR15* and PAL did not show any significant expression change, whatever the plant treatment.

When SL35 was compared with laminarin, known as an inducer of defense genes, the expression profiles followed the same tendency for the *PR2*, *PR8* and *PR9* genes, i.e., an increase in expression, *PR9* being the only significantly overexpressed of these three genes in SL35-treated plants. Opposite results were obtained for *PR9* in non-inoculated plants as compared with inoculated ones. The only stronger effect of laminarin was observed with *PR15* which presented a significant induction although no significant effect was observed with SL35 treatment.

3.5. Field Trial on Grapevine against P. viticola Artificially Inoculated

The preliminary work performed in greenhouse on tomato as model plant, allowed to assess the elicitor activity and the optimal concentration of SL35. The field trial was conducted on the pathosystem grapevine-*P. viticola* instead of tomato-*B. cinerea* because of

the economic importance of grapevine in France. The long distance of the experimental field from the laboratory did not allow to carry out the analysis on gene expression.

The field trial was localized in Sainte-Livrade sur Lot (France, 47), during the year 2020. The SL35 capacity to protect grapevine from *P. viticola* was assessed in comparison with a commercial laminarin (IODUS 2 CEREALES®, Goëmar laboratories, Saint Malo 35400, France). The control condition corresponded to treatment with water. The concentrations of the sprayed solutions of SL35 were 50 mg SL35 L^{-1} named SL35x1, 350 mg SL35 L^{-1} named SL35x7 and 700 mg SL35 L^{-1} named SL35x14. These choices were based on the previous greenhouse experiments in which the lowest concentration leading to a significant increase of tomato defense markers was 50 mg L^{-1} (Figure 1). While the two other concentrations, 350 mg L^{-1} and 700 mg L^{-1}, were chosen because field treatments are known to generate losses in applied product (drift during spraying, or rainwater leaching, depending on weather conditions). The treatments consisted in spraying the products over the leaves at 7-to-10-day intervals. An artificial contamination with spores of *P. viticola* was done on 28 May, one day after the 4th application of the treatments since the disease did not occur naturally.

Figure 4 shows the results recorded on 4 June, 7 days after the artificial inoculation (for grapes), on 18 June, 21 days after artificial inoculation (for leaf incidence) and on 30 June, 33 days after inoculation (for leaf severity). For leaf incidence, even if a decrease in incidence was observed on plants treated with laminarin and with SL35 (six treatments done), no significant difference was observed whatever the concentration (Figure 4A).

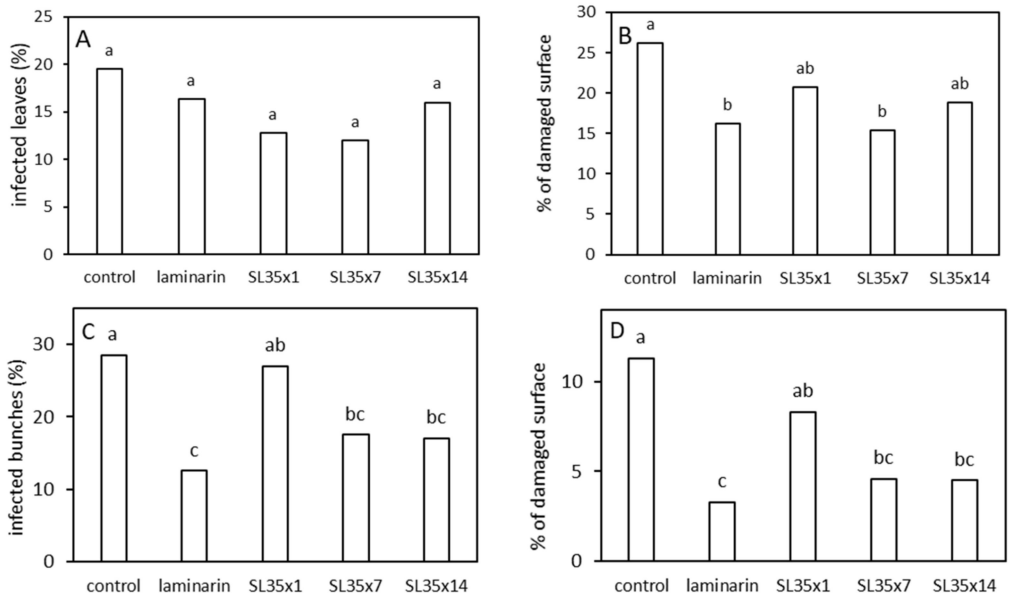

Figure 4. Effects of SL35 treatments on incidence (**A**) of downy mildew on grapevine (field trial, notation 18 June 2020) expressed as % of infected leaves and on severity (**B**) expressed as % of damaged surface (notation 30 June 2020). The same parameters were measured on bunches: incidence (**C**) and severity (**D**) (notation 4 June 2020). SL35 was applied at different concentrations (50 mg L^{-1} for SL35x1, 350 mg L^{-1} for SL35x7, 700 mg L^{-1} for SL35x14) beginning from May 5 and repeated at 7-12-day intervals (see Table 2 for details). Control corresponds to water treatment. Laminarin corresponds to application of IODUS 2 CEREALES®. Means followed by the same letter do not significantly differ ($p < 0.05$, one-way ANOVA followed by Newman and Keuls test and Kurtosis analysis).

However, the control showed an advanced stage of the disease in terms of percentage of infected leaves (19.5% on June 18). On 30 June (seven treatments done and 33 days after the artificial inoculation), a significant reduction in severity was recorded with both laminarin and SL35x7 which showed efficacies of 38% and 41%, respectively (Figure 4B;

see also Table S1). Treatments with SL35x1 and SL35x14 could possibly decrease the severity of the disease but these results were not significantly different from the control which showed a severity of 26.2%, compared with 20.7% and 18.8% for SL35x1 and SL35x14, respectively. Taken together, the results obtained during this field trial showed that 350 mg L^{-1} was an optimal concentration of SL35, able to reduce the symptoms of mildew on grapevine and that this concentration was statistically as efficient as the reference used in this study (laminarin, applied as IODUS 2 CEREALES).

Downy mildew symptoms were also observed on sampled bunches of grapes. They were significantly less important with SL35x7 and SL35x14 treatments as compared with control, showing an efficacy to reduce the disease symptoms around 38% (Figure 4C). This activity was equivalent to the laminarin treatment. The same profile was observed for damaged surface of bunches and again SL35x1 seemed to be insufficient to reduce the disease symptoms, even if a slight, but not significant, reduction was observed as compared with control.

4. Discussion

Agar extraction from red algae such as *G. sesquipedale* is a source of large amounts of by-products which are poorly valorized. We have previously shown that alkaline treatment of this seaweed could extract some original components such as low molecular weight carbohydrates. In this study, the alkaline solution obtained from the industrial process was recovered, neutralized and dried. Afterwards, it was analyzed for its chemical composition. It is characterized by a quite high content of minerals representing 44% of DW, Na being the main element (140 mg g^{-1} DW) which could be explained by the NaOH solution used for alkaline treatment. K content reached 40 mg g^{-1} DW probably due to the marine provenience of the alga, NaCl and KCl being very present salts of seawater. The high level of the S element (86 $\pm$ 6.4 mg g^{-1} DW) might be due to the neutralization of the by-product by sulfuric acid before lyophilization. Among organic compounds, carbohydrates are the most represented, their content reached 125.4 mg g^{-1} DW. They could be extracted from algal cell wall during the alkaline treatment; the cell wall is known to be rich in polysaccharides such as agar (a galactan) and cellulose (in lower quantity) [31]. Among them, uronic acids represent only 9.3%. This value might also be overestimated because of the important concentration in salts of the by-product which interfere with the colorimetric assay. Nevertheless, previous studies showed the presence of uronic acids (a little more than 20%) in the sugar composition of polysaccharides extracted from red algae of the *Gelidium* genus [32,33]. Protein and phenolic compounds are minor components, representing 16.9 and 7.7 mg g^{-1} DW, respectively.

The ability of SL35 to induce defense responses in plant was firstly studied in controlled conditions on tomato plantlets. A significant three-fold increase in peroxidase specific activity was observed for concentrations of 50 and 1000 mg SL35 L^{-1} (Figure 1) as compared with the control. This biomarker is often used to evaluate the plant response and 2-3-fold increases in specific activity have previously been described [19,34,35]. Indeed, peroxidase is involved in hydrogen peroxide detoxification and in the synthesis of lignin (*PR9*). PAL-specific activities also increased, by about a factor of two, when tomato plants were treated with 10 or 50 mg SL35 L^{-1} as compared with the control. The same trend has already been observed, for example, in soybean treated with algino-oligosaccharides [36]. As PAL transforms phenylalanine into *trans*-cinnamic acid, a precursor of phenolic compounds, this enzyme contributes to the cell wall thickening. The phenolic compounds also possess antimicrobial activity. Concerning callose, the minimum concentration of SL35 needed to significantly induce an increase in callose content is 50 mg L^{-1}. Induction of callose deposition has also been described as a plant defense against pathogens [37] since it can reinforce the plant cell wall structure and so diminish the ability of some pathogens to attack plant cells [38]. From data presented in Figure 1, it can be concluded that SL35 can induce the plant defense responses, notably the three biomarkers chosen in this study (peroxidase and PAL activities and callose content) at an optimal concentration of 50 mg L^{-1}.

In the literature, elicitors are able to induce plant defense responses even if the plant is not subjected to a pathogen attack [34,36]. Laminarin, used here as a positive control, did not induce any significant increase in PAL and peroxidase activities in absence of pathogen; only callose formation was stimulated in comparison with the control. PAL activity significantly increased after pathogen inoculation in plants treated with laminarin, exemplifying the priming process already described for this elicitor [39]. Unfortunately, to our knowledge, data on peroxidase activity in plants treated with laminarin are not available so far in the literature. In the case of SL35, the increased response of defense biomarkers was confirmed with PAL and callose levels, even if the plants were not first inoculated; concerning peroxidase, even if a slight increase was observed, it was not significant in comparison with control. Concerning this activity, the presence of the pathogen seems to be necessary to fully express the potential of SL35. Therefore, SL35 treatment seemed to be efficient, also as a preventive tool designed to help the plant to resist the pathogen attack.

When biological activities are considered at the molecular level, i.e., the regulation of expression of defenses genes (some PR proteins and PAL-encoding genes), a strong increase in *PR2* gene expression was observed after laminarin treatment, but only after inoculation. This result is equivalent to previously reported data [30,40]. Since this gene encodes a β-1,3-glucanase, this activation could be implied in the destruction of the fungus cell wall. SL35 treatment was also able to induce an activation of the *PR2* gene after inoculation, even if our results are not statistically significant.

PR8 encodes a type III chitinase, which is responsible for the hydrolysis of chitin, a polysaccharide of fungal cell walls. Concerning *PR8* gene expression in laminarin-treated plants, the profile was equivalent to what was observed with *PR2* gene. Indeed, Xin et al. [41] have reported an increase of total chitinase activities in green tea leaves two days after laminarin spraying. And, more recently, Borba et al. [40] signaled a significant increase in wheat chitinase activity after laminarin treatment. SL35 was also able to induce an increased expression of the *PR8* gene, whether the plants were inoculated or not.

The *PR9* group peroxidases are specific enzymes that only intervene in cell wall thickening [42,43]. The expression profile for this PR group is similar in laminarin- and SL35-treated tomato plants, i.e., both treatments led to a statistically significant repression in absence of pathogen inoculation, while presenting an induction when plants were first inoculated. This induction is strongly marked and statistically different from control in presence of SL35. Despite of that, many studies did report increases in H_2O_2 content consecutive to laminarin treatment [44,45] but, to our knowledge, the peroxidase (H_2O_2 detoxifier) gene expression has not been recorded yet after treatment with laminarin. However, a closely comparable result has been reported with poplar cells treated with laminarin [46]. The authors observed a peak of expression of the peroxidase gene 10 h after treatment, as revealed by Northern blot analysis. Since our assay was done 24 h after inoculation (day 9), the maximal expression might not be revealed. The marked expression induced by SL35 in presence of pathogen may explain the increase observed in peroxidase activity at day 15, which was 10 days after the last treatment (Figure 2).

PR14 encodes lipid transfer proteins implicated in the reinforcement of cutin and might also have a direct effect on the pathogen by inducing its membrane permeabilization [47]. The weak induction of *PR14* by SL35 treatment of tomato plants after inoculation with the pathogen needs to be confirmed.

PR15 encodes oxalate oxidase-type proteins. Expression of this gene has been found to increase in tomatoes infected with *B. cinerea* strains [27]. The only treatment which induced a significant increase of the *PR15* gene expression is laminarin after inoculation with the pathogen. Data concerning this *PR* do not allow us to deduce any effect of SL35 on the expression of its gene, but we cannot exclude that oxidative stress might be a defense response of the plant. A quantification of the H_2O_2 content of leaf tissues might give an answer to this question.

The PAL gene expression after laminarin treatment presents a profile suggesting a priming process according to the enzymatic activity shown in Figure 2, despite an absence of significant results about the expression of this gene. SL35 treatments do not induce any change in PAL gene expression. Keeping in consideration the enzymatic activities assayed at day 15, maybe the timelapse for the characterization of the gene expression, i.e., day 9, was not optimal.

To assess the efficiency of SL35 to stimulate the defense mechanisms of plant cells in order to make the plant more resistant to pathogen attack, a field trial conducted on grapevine artificially inoculated with *P. viticola* was designed. Laminarin treatment was efficient to reduce the severity of the symptoms on leaves and on bunches with an efficacy of 38 and 71%, respectively. The SL35x7 treatment showed a similar activity (59% on bunches and 41% on leaves). These effects could be explained by the results showing the activation of some markers of plant defense implied either in cell wall reinforcement (callose, peroxidase, PAL), or in the direct attack against the pathogenic fungus (*PR2* and *PR8*). The optimal spray concentration of SL35 estimated in field conditions was 350 mg L^{-1}. This is seven-fold higher than during the experiment in controlled conditions, but the field conditions might reduce the biological activity, due to partial drift during spraying, or leaching by rainwater. This optimal concentration led to an equivalent dose of 88 g of SL35 per ha which is in line with agricultural practices. Since downy mildew symptoms have been not fully eliminated by SL35 treatment, the use of synthetic pesticide could be useful under strong pathogen pressure. However, the integration of SL35 with synthetic products at low doses could be considered in integrated pest management strategies. In order to progress in the comprehension of the biological activity of SL35, a more detailed chemical composition has to be established to ascertain which component(s) is (are) responsible for the stimulation of the plant defenses.

5. Conclusions

The results of this study show that a raw alkaline extract of the red alga *G. sesquipedale* might be considered as a new promising plant defense stimulator. Further studies will be focused on the evaluation of the properties of some components of this crude extract, in particular carbohydrates, since oligosaccharides have been shown to elicit plant defense responses. These data would be of interest in the comprehension of the mode of action of this seaweed-originated by-product and could provide enhanced value to the economic sector of agarophytes.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae8100958/s1, Table S1: Photographs of vine plants taken on 23 June 2020 showing the symptoms of infection by Plasmopara viticola on grapes and leaves.

Author Contributions: Conceptualization, C.F.G., V.G., S.L., F.D. and R.K.; methodology, C.F.G., V.G., S.L., F.D. and R.K.; resources, S.L. and C.F.G.; investigation, C.F.G., S.L., F.D. and R.K.; writing—original draft preparation, R.K. and C.F.G.; writing—review and editing: C.F.G., V.G., S.L., F.D. and R.K.; funding acquisition, S.L., C.F.G. and V.G. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by SETEXAM (Morocco).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: The authors acknowledge M. Guilloton for his help in manuscript editing and ANADIAG S.A.S. company which have carried out the field trial on grapevine.

Conflicts of Interest: SETEXAM collected the seaweed, supplied the industrial by-product and approved the publication of the results presented herein.

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