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Abstract:

Laminaran, a β-(1→3)-glucan extracted from *Laminaria digitata*, is a known elicitor of plant defenses, but provides only low level of disease control in vineyard trials. In this context, laminaran was partly hydrophobized by grafting from 1.6 to 7.6 lauryl chains to the native saccharidic chain and the impact of sulfation of the hydrophobized glucans was studied. The activity of the different synthetized laminaran derivatives as antimicrobial agents against *Plasmopara viticola,* the causal agent of grape downy mildew, and as elicitors of defense reactions *in planta*, was evaluated. Our results showed that acylation imparts an antimicrobial activity to laminaran which is related to the degree of acylation, **AL3**, with 7.6 lauryl chains, being the most effective derivative. Sulfation of the acylated laminarans did not further increase the antimicrobial activity. Our results also demonstrated that the efficacy of **AL3**

against *Plasmopara viticola* was most likely due to the direct antimicrobial activity of the lauryl chains rather than to an elicitation of plant defenses.

Keywords: Laminaran; β-(1→3)-Glucans; Acylation; Sulfation; Grapevine; Induced resistance.

1. Introduction

β-(1→3)-Glucans, a large class of polysaccharides, are widely distributed among fungi, microorganisms and brown algae in which they act as carbon and energy resources (Stone & Clarke, 1992). Modern research related on this family of natural compounds originates from ancient observations on health benefits (Vetvicka, Vannucci, Sima, & Richter, 2019). Nowadays, those glucans and derivatives thereof find more rational applications as antitumor, anti-inflammatory, anti-coagulative or anti-oxidative agents (Descroix, Ferrières, Jamois, Yvin, & Plusquellec, 2006; Kuda, Yano, Matsuda, & Nishizawa, 2005; Legentil et al., 2015; Miao, Ishai-Michaeli, Peretz, & Vlodavsky, 1995; Neyrinck, Mouson, & Delzenne, 2007). Considering plant health potential, Klarzynski and collaborators gave first evidences that a laminaran extracted from *Laminaria digitata*, a linear β-(1→3)-glucan with only little branching, was able to elicit immunity in tobacco through the production of reactive oxygen species (ROS), expression of defense genes and accumulation of salicylic acid (Klarzynski et al., 2000). This elicitation of defenses led to induced resistance against tobacco mosaic virus. In 2003, Aziz *et al*. demonstrated that laminaran, deprived of antimicrobial activity, and applied to grapevine leaves, significantly reduced infection due to two important grapevine pathogens: *Botrytis cinerea* (agent of grey mold) and the oomycete *Plasmopara viticola* (*Pv,* agent of downy mildew) in greenhouse trials (Aziz et al., 2003). This resistance was associated with the expression of an array of defense reactions including early events such as

H_2O_2 production, expression of defense genes and accumulation of stilbene phytoalexins. Such an activation of the defense mechanisms was assigned to the close structural similarity between β-(1→3)-glucans from pathogenic microorganisms and the ones from algae, the latter being thus sensed as a danger signal by the plant.

Nevertheless, while laminaran succeeded in eliciting defense reactions in grapevine, protection against *Pv* was unsatisfactory because of inconsistency in the vineyard trials, and absence of protection in vineyard trials under real conditions (X. Daire, unpublished). This could be first attributed to a low penetration rate of hydrophilic compounds into the leaf. Indeed, leaves are covered by a cuticle made up of polyester of hydroxylated fatty acids and waxes which form a hydrophobic barrier preventing water losses. Interestingly, we have recently demonstrated that an ethoxylated surfactant significantly helps the penetration of such highly polar carbohydrates through abaxial surfaces of plant leaves (Paris et al., 2016). Secondly, the important variability of the biological results could also be due to the fact that laminaran acts solely as an elicitor of plant defense mechanisms and does not have any toxic effect on oomycete. Recently, we have shown that a plant extract with a dual mode of action (elicitor of grapevine defenses and antimicrobial) was a good candidate for ecofriendly control of grape downy mildew (Krzyzaniaka, Trouvelot, et al., 2018). Indeed, if one mode of action is ineffective (e.g., low-responsiveness of the plant to induced resistance), the other one may take the lead and keep on protecting the plant.

In this context, modulation of both the hydrophilic/hydrophobic balance of laminaran [MW≈4 000 $g.mol^{-1}$, linear (Klarzynski et al., 2000), see Supplementary information] extracted from brown seaweeds, and its toxicity against *Pv* were considered. Such controlled hydrophobization of seaweed laminaran was addressed though partial masking of hydroxyl functions by acylation with long chain aliphatic groups (Fig. 1). Introduction of aliphatic groups can result in better interactions with the lipophilic cuticle and surfactant used as

formulating agent, thus favoring penetration of the bioactive carbohydrate into plant leaves, and then increasing its bioavailability. Lauric acid was chosen as a model aliphatic chain to be grafted on laminaran. It is indeed known to drastically reduce powdery mildew infection rate in barley (Walters, Walker, & Walker, 2003) and we could expect a similar activity against *Pv*. These modifications may interestingly impart new antimicrobial properties to laminaran. Three derivatives characterized by different degree of branching were prepared and their protective effect against grapevine downy mildew evaluated. Plant defense reactions elicited by these modified laminarans were also investigated. Since persulfation of laminaran is known to increase biological effects (Menard, 2004; Trouvelot et al., 2008), the hydrophobized laminarans were similarly functionalized (Fig. 1) and further tested.

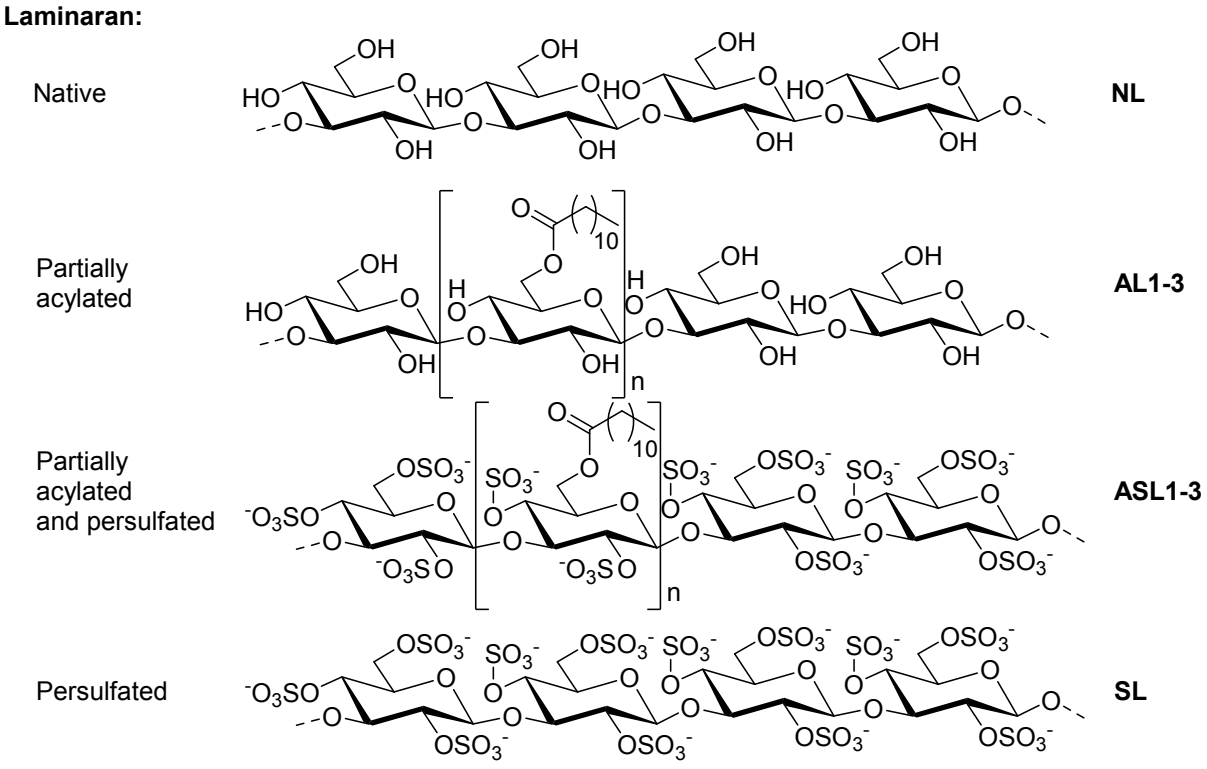


Fig. 1. Structures of native and partially acylated laminarans, and sulfated derivatives thereof.

2. Material and methods

2.1. Chemistry

90 *2.1.1. Materials*

91 Laminaran and persulfated laminaran (MW ≈ 4 000 g.mol^{-1}, Average degree of
92 polymerization: 25, see MS spectrum in supplementary material) used in this study were
93 kindly supplied by Goëmar. ^{1}H, ^{13}C, NMR spectra were recorded with a Brüker ARX 400
94 spectrometer at 400 MHz, and 100 MHz, respectively. Chemical shifts are given in δ units
95 (ppm). Coupling constants *J* were calculated in Hertz (Hz). Abbreviations were used to
96 precise signal multiplicity: s (singulet), ls (large singulet), d (doublet), t (triplet), m
97 (multiplet), dd (double doublet)...

98

99 *2.1.2. General procedure for lauroylation of laminaran*

100 Laminaran was suspended in dry dimethylacetamide and heated at 105 °C until complete
101 solubilization. Triethylamine, lauroyl chloride and *N*,*N*-dimethyl-4-aminopyridine (DMAP)
102 were then added and the solution stirred for 3 h at 105 °C. After cooling at room temperature,
103 ethanol or deionized water was added to the mixture to precipitate the acylated laminaran. It
104 was then concentrated by centrifugation (9000 rpm for 30 min) and the resulting precipitate
105 was freeze-dried. The resulting solid was washed with ethyl acetate to remove excess
106 triethylamine and DMAP. It was then freeze-dried again and stored at 4 °C until use.

107 *2.1.2.1. Lauroylation of laminaran to afford* **AL1**

108 **AL1** was obtained according to the general procedure above using laminaran (40 g, 10
109 mmol), triethylamine (20 mL, 0.143 mol), lauroyl chloride (11.9 mL, 51.5 mmol) and DMAP
110 (3 g, 24.6 mmol) in dry dimethylacetamide (400 mL). After 3 h stirring at 105 °C, 1 L of
111 ethanol was added to the cooled mixture to precipitate the acylated laminaran. After
112 treatments, a beige powder of the desired product **AL1** (24.25 g, 5.3 mmol) was recovered in
113 53% yield. The calculated number of acyl chains was 1.6. ^{1}H NMR (400 MHz, DMSO-*d*6) δ
114 5.38-4.75 (ls, 25H, OH), 4.74-4.56 (ls, 25H, OH), 4.51 (d, *J* = 7.6 Hz, 25H, H-1), 4.23-4.11

(m, 1.6H, H-6a-Lauryl), 4.11-4.00 (m, 1.6H, H-6b-Lauryl), 3.77-3.57 (m, 23.4H, H-6a), 3.57-2.87 (m, 123.4H, H-2, H-3, H-4, H-5, H-6b), 2.36-2.23 (m, 3.2H, $COCH_2$), 1.59-1.38 (m, 3.2H, $COCH_2CH_2$), 1.32-1.19 (m, 25.6H, $(CH_2)_8$), 0.84 (t, J = 6.6 Hz, 4.8H, CH_3).

2.1.2.2. Lauroylation of laminaran to afford **AL2**

AL2 was obtained according to the general procedure described above using laminaran (20 g, 5 mmol), triethylamine (10 mL, 0.072 mol), lauroyl chloride (10.7 mL, 46.3 mmol) and DMAP (1.5 g, 12.3 mmol) in dry dimethylacetamide (200 mL). After 3 h stirring at 105 °C, 850 mL of deionized water were added to the cooled mixture to precipitate the acylated laminaran. It was then concentrated by centrifugation (9000 rpm for 25 mn) and resuspended in a 125 mL mixture of ethanol and dichloromethane (50:50, v/v). Deionized water (350 mL) was added to precipitate the acylated laminaran again. It was once again concentrated by centrifugation (9000 rpm for 25 mn). These steps were repeated twice before freeze-drying the resulting solid, which was then washed with ethyl acetate to remove excess triethylamine and DMAP. It was then freeze-dried again and stored at 4 °C until use. A beige powder was isolated (9.40 g, 34%, number of acyl chains: 4.6). ^{1}H NMR (400 MHz, DMSO-*d*6) δ 5.28-4.8 (ls, 25H, OH), 4.72-4.55 (ls, 25H, OH), 4.50 (d, J = 7.4 Hz, 25H, H-1), 4.35-4.23 (m, 4.6H, H-6a-Lauryl), 4.35-4.23 (m, 4.6H, H-6a-Lauryl), 4.13-3.97 (m, 4.6H, H-6b-Lauryl), 3.78-3.58 (m, 20.4H, H-6a), 3.57-3.35 (m, 45.4H, H-6b, H-3), 3.34-2.98 (m, 75H, H-2, H-4, H-5), 2.35-2.18 (m, 9.2H, $COCH_2$), 1.60-1.40 (m, 9.2H, $COCH_2CH_2$), 1.34-1.15 (m, 73.6H, $(CH_2)_8$), 0.84 (t, J = 6.6 Hz, 13.8H, CH_3).

2.1.2.3. Lauroylation of laminaran to afford **AL3**

AL3 was obtained according to the general procedure described above using laminaran (50 g, 12.5 mmol), triethylamine (20 mL, 0.143 mol), lauroyl chloride (35.6 mL, 153.9 mmol) and DMAP (3 g, 24.6 mmol) in dry dimethylacetamide (500 mL). After 3 h stirring at 105 °C, 1 L of deionized water was added to the mixture to precipitate the acylated laminaran. It was then

concentrated by centrifugation (9000 rpm for 25 mn) and resuspended in a 300 mL mixture of ethanol and dichloromethane (50:50v v/v). Deionized water (1 L) was added to precipitate the acylated laminaran. It was again concentrated by centrifugation (9000 rpm for 25 mn). Those steps were repeated twice before freeze-drying the resulting solid, which was then washed with ethyl acetate to remove excess triethylamine and DMAP. It was then freeze-dried again and stored at 4 °C until use. The product is purified on a reverse-phase chromatography column using a water/acetonitrile mixture (gradient from 90:10 to 50:50) giving a beige powder (30.0 g, 31%, number of acyl chains: 7.6). ^{1}H NMR (400 MHz, DMSO-*d*6) δ 5.30-4.8 (ls, 25H, OH), 4.72-4.55 (ls, 25H, OH), 4.49 (d, *J* = 7.4 Hz, 25H, H-1), 4.31-4.22 (m, 7.6H, H-6a-Lauryl), 4.13-3.97 (m, 7.6H, H-6b-Lauryl), 3.77-3.57 (m, 17.4H, H-6a), 3.57-3.00 (m, 117.4H, H-2, H-3, H-4, H-5, H-6b), 2.35-2.20 (m, 15.2H, $COCH_2$), 1.57-1.36 (m, 15.2H, $COCH_2CH_2$), 1.34-1.13 (m, 121.6H, $(CH_2)_8$), 0.84 (t, *J* = 6.6 Hz, 22.8H, CH_3).

2.1.3. General procedure for sulfation of lauroyl laminaran

Acylated laminaran (1 equiv.) and dry pyridine (124 equiv.) were suspended in dry dimethylformamide under nitrogen. The mixture was heated at 60 °C under vigorous stirring and SO_3-pyridine complex (54 equiv.) was added. The solution was further stirred at 60 °C for 2 h, and overnight at room temperature. Precipitation of the product was obtained by adding absolute ethanol (20 mL). After filtration, the solid residue was washed three times with absolute ethanol. The product was then suspended in warm deionized water (5 mL), and neutralized with 2 mol.L^{-1} sodium hydroxide solution. Excessive pyridine was removed by coevaporation with toluene under reduced pressure, and the product was freeze-dried and stored at 4 °C until use.

2.1.3.1. Sulfation of lauroyl laminaran **ASL1**

Sulfation of **AL1** (0.5 g, 0.110 mmol) was performed according to general procedure using dry pyridine (1.10 mL, 13.6 mmol) and SO_3-pyridine complex (0.94 g, 5.9 mmol) in dry dimethylformamide (20 mL). A white powder (1.16 g, 87%, number of acyl chains: 1.5) was obtained. ^{1}H NMR (400 MHz, D_2O+CD_3OD) δ 5.18-4.69 (m, 25H, H-1), 4.75-3.49 (m, 150H, H-2, H-3, H-4, H-5, H-6), 2.14 (t, *J* = 7.6 Hz, 3H, $COCH_2$), 1.64-1.48 (m, 3H, $COCH_2CH_2$), 1.36-1.16 (m, 24H, $(CH_2)_8$), 0.84 (t, *J* = 7.0 Hz, 4.5H, CH_3).

2.1.3.2. Sulfation of lauroyl laminaran **ASL2**

Sulfation of **AL2** (0.5 g, 0.089 mmol) was performed according to general procedure using dry pyridine (0.87 mL, 11.1 mmol) and SO_3-pyridine complex (0.76 g, 4.8 mmol) in dry dimethylformamide (20 mL). A white powder (0.77 g, 71%, number of acyl chains: 3.6) was obtained. ^{1}H NMR (400 MHz, D_2O) δ 5.01-4.37 (m, 25H, H-1), 4.32-3.36 (m, 150H, H-2, H-3, H-4, H-5, H-6), 2.40-2.27 (m, 7.2H, $COCH_2$), 1.61-1.41 (m, 7.2H, $COCH_2CH_2$), 1.29-1.05 (m, 57.6H, $(CH_2)_8$), 0.85-0.70 (m, 10.8H, CH_3).

2.1.3.3. Sulfation of lauroyl laminaran **ASL3**

Sulfation of **AL3** (0.5 g, 0.075 mmol) was performed according to general procedure using dry pyridine (0.74 mL, 9.3 mmol) and SO_3-pyridine complex (0.65 g, 4.1 mmol) in dry dimethylformamide (20 mL). A white powder (0.70 g, 77%, number of acyl chains: 10) was obtained. ^{1}H NMR (400 MHz, D_2O) δ 5.38-4.82 (m, 25H, H-1), 4.75-3.83 (m, 150H, H-2, H-3, H-4, H-5, H-6), 2.55-2.33 (m, 20H, $COCH_2$), 1.77-1.53 (m, 20H, $COCH_2CH_2$), 1.46-1.18 (m, 160H, $(CH_2)_8$), 0.99-0.79 (m, 30H, CH_3).

2.2. Biological material, protection assays and analyses

2.2.1. Grapevine plant production and treatments

The grapevine cultivar used was *V. vinifera* L. cv. Marselan (Cabernet sauvignon × Grenache), which is susceptible to *P. viticola (Pv)*. Grapevine plants were obtained from

189 herbaceous cuttings placed in individual pots (10x10x8 cm) containing a mixture of blond
190 peat and perlite (3:1v/v). They were grown in a greenhouse at a temperature of 24 and 18 °C
191 (day and night, respectively) with a photoperiod of 16 h of light and at a relative humidity
192 (RH) of 70 ± 10 % until they developed six leaves. Plants were watered with a fertilizing
193 solution (0.25 % Topfert2 solution NPK 10-10-10 + oligonutrients, Plantin, France).
194 Polysaccharides were used at a concentration of 1 $g.L^{-1}$ unless otherwise mentioned. For
195 protection assays, the compounds were dissolved in Dehscofix CO 125 (Huntsmann), an
196 ethoxylated castor oil used at 0.1% (v/v).
197 Treatments were sprayed onto upper and lower leaf surfaces of the second and third youngest
198 fully expanded leaves, until run-off point (Steimetz et al., 2012). Pots were arranged in a
199 randomized block design in the glasshouse. Unless otherwise mentioned, controls were
200 sprayed without surfactant.
201
202 *2.2.2. Protection assays against* Pv
203 At 48 hours post treatment (hpt), leaves were inoculated with *Pv* by spraying a freshly
204 prepared sporangia suspension at 1.10^4 sporangia mL^{-1} (Khiook et al., 2013). Disease severity
205 was visually assessed 6 days post inoculation by measuring the leaf area covered by
206 sporulation. Results are given in % of sporulating leaf area (% SA). For each experiment,
207 efficacy rates were calculated as:
208 [1 – (% SA treatment/% SA reference)] x 100, where the reference can be either the water or
209 the adjuvant treatment.
210
211 *2.2.3. Grapevine cell supensions*
212 Grapevine (*Vitis vinifera* L. cv. Gamay) cell suspensions were cultivated in Nitsch–Nitsch
213 medium (Nitsch & Nitsch, 1969) on a rotary shaker (125 rpm) at 25 °C and under continuous

light. They were subcultured every 7 days by transferring 10 mL of cell suspensions into 90 mL of new culture medium. Seven-day-old cultures were diluted twice in Nitsch–Nitsch medium 24 h prior to use for all experiments. For early signaling events (ROS and MAPK) and resveratrol analyses, cells were prepared and sampled as previously described by (Krzyzaniaka, Negrela, et al., 2018).

2.2.4. Defense-Related Responses Assessment

2.2.4.1. In grapevine suspension cells

H_2O_2 production: H_2O_2 production was measured in cell suspension as described by (Gauthier et al., 2014). Measurements were carried out using a luminol chemiluminescence assay with a luminometer (Lumat LB 9507, Berthold, Evry, France). Two hundred and fifty microliters of cell suspension were added to 300 µL of H50 medium (50 mM HEPES, 175 mM mannitol, 5 mM $CaCl_2$, 0.5 mM K_2SO_4; pH 8.5), and 50 µL of 0.3 mM luminol. Relative luminescence was recorded within a 10 s period and was converted in nmol of H_2O_2 per gram of FWC, after the establishment of a H_2O_2 reference range with untreated cell suspension.

Detection of MAPKs by western blot analyses: Protein extraction and western blot analyses were performed as previously described (Poinssot et al., 2003). Briefly, aliquots of treated cells were collected, as described above, at 0, 15, 30, 60 min post-treatment (pt). After protein extraction, aliquots containing 15 µg of protein per sample were solubilized in Laemmli buffer and submitted to 10% SDS-PAGE, before transfer to nitrocellulose membrane (Hybond ECL, Amersham biosciences, Munchen, Germany) for western blotting. Phosphorylated MAPKs were detected with an antibody raised against a synthetic phosphor-thr202/tyr204 peptide of human phosphorylated extracellular regulated protein kinase 1/2 (α-perk1/2, Cell Signaling, Danvers, MA, United States). Probing and detection were carried out by an ECL western detection kit (Amersham biosciences, Little Chalfont, United Kingdom).

2.2.4.2. In grapevine foliar tissues

Localization of H_2O_2 in treated leaves: Leaves from treated plants and inoculated or not with *Pv* were harvested at different post-inoculation time points (12, 24, 48, or 72 hpi), and treated as described elsewhere (Trouvelot et al., 2008). H_2O_2 was visualized as a reddish brown coloration after treatment with diaminobenzidine (DAB).

Localization of callose in treated leaves: Leaf disks (7 mm in diameter) were harvested at different times and incubated overnight in absolute methanol at room temperature. The samples were then transferred to chloral hydrate solution (2.5 $g.mL^{-1}$) overnight at room temperature to soften and clear the tissues. Cleared samples were incubated in 0.05% aniline blue (in 0.1 M phosphate buffer, pH 8.0) overnight at room temperature. They were then mounted on microscope slides in the staining solution, with the abaxial surface uppermost. Callose production was observed by epifluorescence microscopy under UV (λ_{exc} 340 nm, λ_{em} 380 nm, stop filter LP 430 nm) as it fluoresces blue under these conditions.

Quantification of stilbenes in leaves: Frozen leaves (0.5 g fresh weight) were ground in liquid nitrogen and directly extracted overnight in 1.5 mL MeOH in 15 mL Falcon tubes. After centrifugation at 4 000 g for 10 min, part of the supernatant (1mL) was transferred to Eppendorf tubes and recentrifuged (17,500 g for 10 min). Ten µL aliquots were then analyzed by HPLC using a Beckman System Gold chromatography system equipped with a diode array detector Model 168 and a Beckman 507 sample injector as previously described (Krzyzaniaka, Negrela, et al., 2018). Quantification of piceid (Rt 11.37 min), *trans*-resveratrol (Rt 13.58), epsilon-viniferin (Rt 17.62 min), delta-viniferin (Rt 19.32 min), and pterostilbene (Rt 21.26 min) was performed with standard calibration curves using peak areas at 310 nm.

2.2.5. Evaluation of toxicity towards Pv

To assess the effect of treatments on the ability of *Pv* sporangia to release zoospores, *in vitro* experiments were managed as described by (Krzyzaniaka, Negrela, et al., 2018).

2.2.6. Scanning Electron Microscopy (SEM)

Observations of leaf surface were made by SEM. At 24 hpi and 6 dpi, 0.5 cm^2 leaf squares were excised from treated and inoculated leaves. The leaf surface was then characterized by cryo-scanning electron micrographs (cryo-SEMs) taken using a Hitachi (SU 8230) scanning electron microscope equipped with Quroum PP3000 t cryo attachment. Prior to imaging, leaf samples were rapidly frozen in liquid nitrogen in order to fix them. Thereafter they were sublimated at -90 °C for 5 min and coated with a thin platinum layer by sputtering at 5 mA for 10 s.

2.2.7. Statistical analysis

In order to compare values for infection levels, analysis of variance was performed with the statistical program PAST (Hammer, Harper, & Ryan, 2001) using the One-Way ANOVA test to detect significant differences ($p < 0,05$) between treatments followed by Tukey's test for pairwise comparisons.

3. Results and discussion

*3.1. Chemical synthesis of modified laminarans **AL** and **ASL***

In order to (i) facilitate the penetration of laminaran through the leaf cuticle, and (ii) possibly enhance the laminaran biological activity, a lipid chain was grafted to the more reactive position *O*-6 of the polysaccharide. Lauryl chains were selected for their ability to interact with lipidic layers such as cuticle and also for their antimicrobial potential (Walters et al., 2003). Practically, in order to produce **AL** compounds with modulated hydrophilic/lipophilic

balances, acylation reactions of the used native laminaran (**NL**), a linear polysaccharide (Klarzynski et al., 2000) with a molecular weight of 4 000 g.mol^{-1} (see Supplementary information), were performed in dimethylacetamide (DMAc) using triethylamine as a base and *N*,*N*'-dimethylaminopyridine (DMAP) as a catalyst, in the presence of increasing equivalents of lauroyl chloride (Table 1). The desired conjugates were precipitated by adding ethanol or water depending of the degree of branching.

The efficiency of the acylation reaction was evaluated thanks to ^{1}H NMR. For the three conditions used, the presence of an acyl group on the polysaccharide was confirmed by the downfield shift of some H-6 protons from 3.63 ppm to 4.10 ppm, correlated with the electron-withdrawing nature of the acyl chain. The percentage of acylation was then calculated by setting first the integration of the more downfield proton to one. The ratio 100*1/(1+y), with y the integration of the upfield protons, gave the percentage of acylation. The number of aliphatic chains on the polysaccharide could then be deduced by applying this percentage to the total number of primary position, set by convention to 25. As an example, for **AL2**, setting up the integration of the downfield H-6 to 1, lead to an integration value of 4.4 for the other primary protons (Fig. 2A). The percentage of acylation reached then 18.4%, meaning an average of 4.6 lauryl chain per laminaran. The evaluation of this degree of substitution was corroborated considering the integration of the CH_2 functions at 1.23 ppm, signal which corresponds to 16 protons for one acyl chain (Fig. 2B).

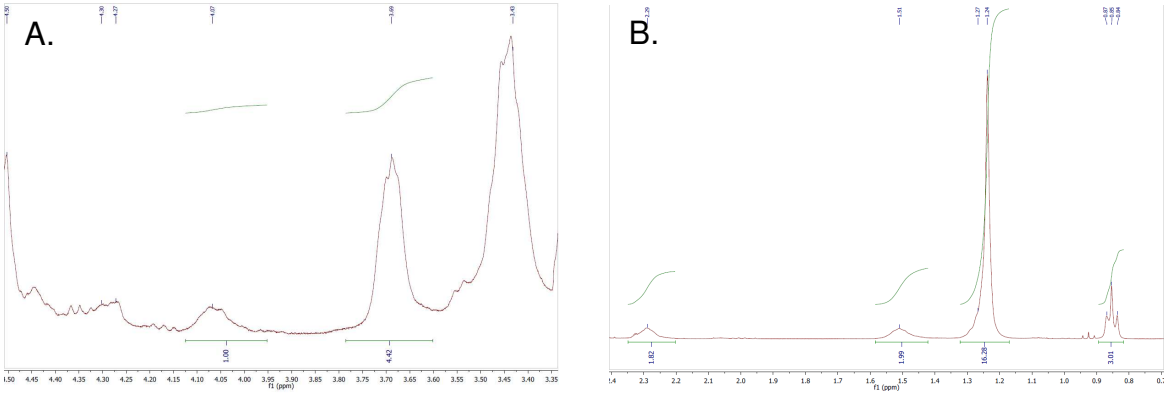
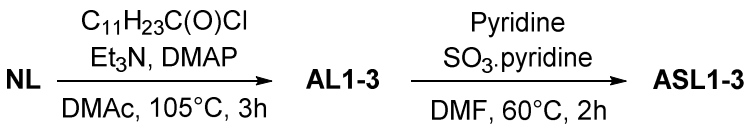


Figure 2. ^{1}H NMR spectrum of **AL2** in the area of the H-6 (2A) and the lauryl chain (2B)

Thanks to this calculation, the presence of 1.6, 4.6, and 7.6 chains of lauryl per polysaccharidic chain was established for the samples **AL1**, **AL2**, and **AL3**, respectively (Table 1). After precipitating with water and removing the remaining acylating reagent, yields in isolated **AL** compounds reached an average of 30%.

Table 1. Number of acyl chains and yields obtained after acylation of **NL**.



Entry	Lauroyl chloride (equiv.)	Product	Number of acyl chains (for one laminaran chain)	Yield (%)
1	5.1	**AL1**	1.6	53
3	9.3	**AL2**	4.6	34
4	12.3	**AL3**	7.6	31

As persulfation of laminaran is known to effectively elicit defense mechanisms in plants (Menard, 2004; Trouvelot et al., 2008), **AL** derivatives were subsequently reacted with excess sulfur trioxide in pyridine to afford the desired **ASL1**, **ASL2** and **ASL3,** in 87%, 71%, and

77% yields, respectively. This step also had the advantage to ensure the products solubility in water solution, especially for **AL2** and **AL3** which bear more lipid chains. In addition, the conditions of sulfation were mild enough to avoid any hydrolysis of the ester functions. It was confirmed thanks to ^{1}H NMR. The protons corresponding to the acyl chains could be found between 2.60 and 0.8 ppm. The average amounts of branching were deduced from the ratio between the polysaccharide scaffold (4.70 to 3.76 ppm) that integrate for 150 protons (All protons minus the anomeric ones) and the methyl group at 0.80 ppm. It reached 1.5, 3.6 and 10 for **ASL1**, **ASL2** and **ASL3,** respectively (see S.I.). Such discrepancy when compared with the starting acylated laminarans could be explained by the method of purification chosen.

*3.2. Assessment of the efficacy of **AL1-3** and **ASL1-3** against* Pv

The second phase of this study was then dedicated to determine the biological activity of hydrophobized laminarans on grapevine plants. The protection against the pathogenic agent *Pv* was first investigated. Aqueous solutions of modified laminarans were prepared with an adjuvant, Dehscofix CO 125 and sprayed onto plant leaves. This surfactant was chosen as it is the best penetration enhancer for saccharides into grapevine leaves (Paris et al., 2016). In addition to promoting the penetration of native laminaran, this additive was also beneficial for the solubilization of modified laminarans having a large number of acyl chains (**AL2**, **AL3**). It has no protecting effect against *Pv* (Fig. 3).

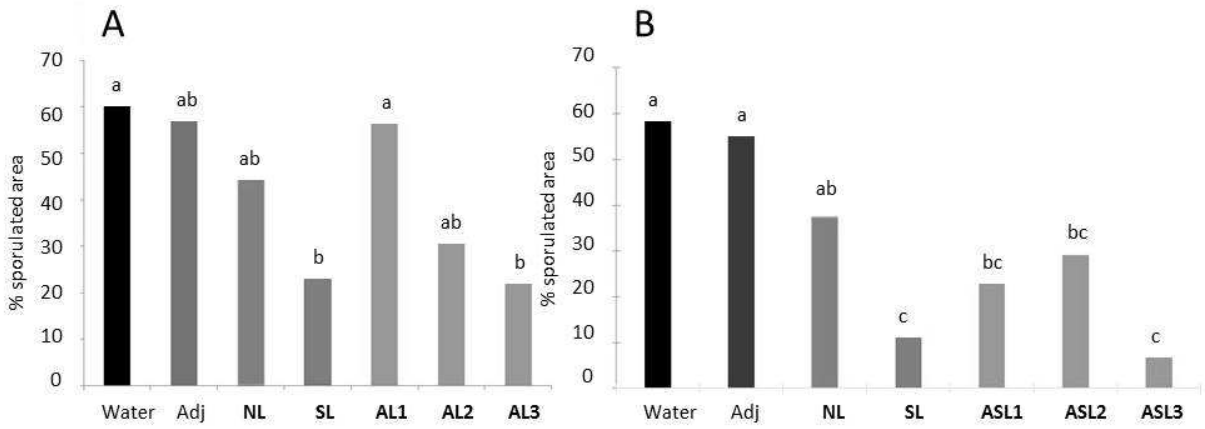


Fig 3. Efficacy of acylated laminarans (1 g.L^{-1}) (A), and acylated and sulfated laminarans (B), against grapevine downy mildew. Values are the means of 3 independent experiments. A and B correspond to two different sets of experiments. Treatments with a same letter are not significantly different. Adj, adjuvant; **NL**, native laminaran; **SL**, sulfated laminaran; **AL1-3**, acylated laminarans; **ASL1-3**, acylated sulfated laminarans.

Using the partially acylated laminarans **AL1-3**, increased protection against *Pv* was obtained by increasing the degree of grafting (Fig. 3A). While **AL1** (1.6 chain) exhibited less activity than **NL**, and was similar to water control, the significant protection observed with **AL3** (6.3 chains) reached a level comparable with that of **SL**. The persulfated and partially acylated derivatives **ASL1-3** were also evaluated for their protection efficacy (Fig. 3B). As expected, persulfation of the different **ALs** drastically reduced the sporulation of *Pv* as compared to **NL**. However **ASL3**, the more effective acylated and sulfated compound, did not exhibit higher activity than **LS**.

As **AL3** was easier to synthesize than **ASL3** and showed the most promising activity among the acylated laminarans assessed for protection against *Pv*, a dose-response experiment was further performed. **AL3** had no effect at 0.5 g.L^{-1} and below, whereas it achieved a disease reduction of 65% at 1 g.L^{-1} and of nearly 95% at 2 g.L^{-1} (data not shown). We then compared the efficacy against *Pv* of **AL3** and lauric acid at the same concentration (1 g.L^{-1}), given that,

as mentioned above, lauric acid has been reported to harbor anti-microbial properties. Under our conditions, **AL3** turned out to be much more effective than lauric acid, which did not show any efficacy in plant protection against *Pv* (Fig. 4).

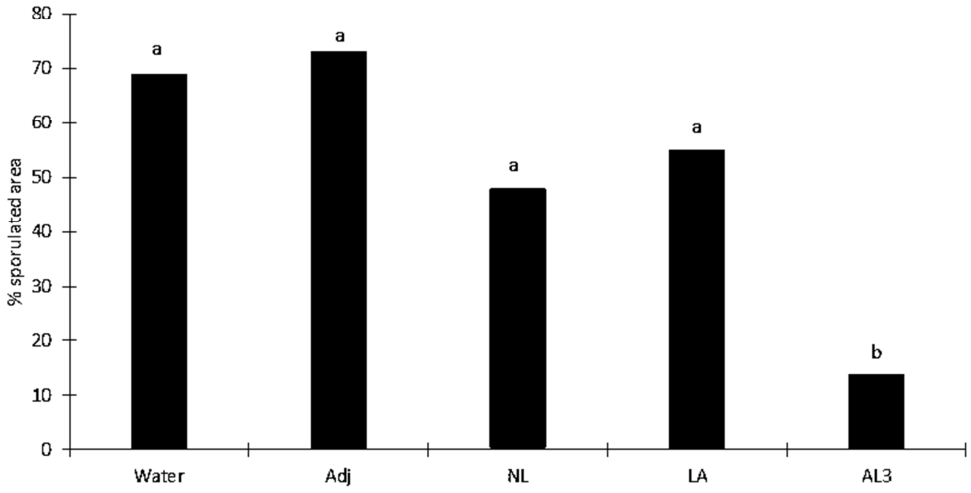


Fig 4. Comparison of the effect of **AL3** with natural laminaran and lauric acid on the protection against *Pv*. Values are from one representative experiment out of two. Treatments with a same letter are not significantly different. Adj, adjuvant; **NL**, natural laminaran; **LA**, lauric acid.

3.3. Toxicity of acylated laminarans to zoospore of Pv

In order to better understand the mechanism of action of the acylated laminaran, the less acylated **AL1** and **ASL1** were compared to the more acylated ones **AL3** and **ASL3** with respect to their *in vitro* antimicrobial properties on a zoospore suspension of *Pv*. The efficacy of the new compounds was also compared with that of native laminaran (**NL**), persulfated laminaran (**LS**) and lauric acid (**LA**) (Fig. 5). Unlike the **NL**, all compounds affected the number of *Pv* zoospores in suspension, and this effect was dose-dependent. Increasing the number of lauryl chains on the laminaran led again to a higher toxicity with both families of

compounds. Persulfation did not enhance activity of the acylated laminarans as the biological effect of acylated and sulfated laminarans on zoospores was not better than that of the acylated ones. Moreover, **ASL** efficacy did not overcome that of persulfated laminaran **SL** itself. Interestingly, pure lauric acid (**LA**) had strong impact on mobility of *Pv* spores, even at 0.01 $g.L^{-1}$ concentration. This result confirmed the wide antimicrobial spectrum of **LA**, previously shown to be effective against plant pathogenic fungi unrelated to the *Pv* (Walters et al., 2003). This active concentration should be however related to the lower molecular mass of **LA** compared to that of polysaccharides. Best results were obtained with the laminaran grafted with 7.6 lauryl chains (**AL3**) that showed high toxicity, from the 0.10 $g.L^{-1}$ concentration. The similarity of *in vitro* efficacy of **LA** and **AL3** suggests that **AL3** activity relies on the lauryl chains. Scanning electronic microscopy observations (S.I., Fig. S1) of the leaf surface showed that *Pv* zoospores, probably affected in their mobility, could not reach the stomata, their site of infection, when plants were treated by **AL3**. This observation directly confirmed that **AL3** is toxic to *Pv* in planta. However, one cannot rule out the possibility that the elicitation of grapevine defenses can reinforce this direct protective activity.

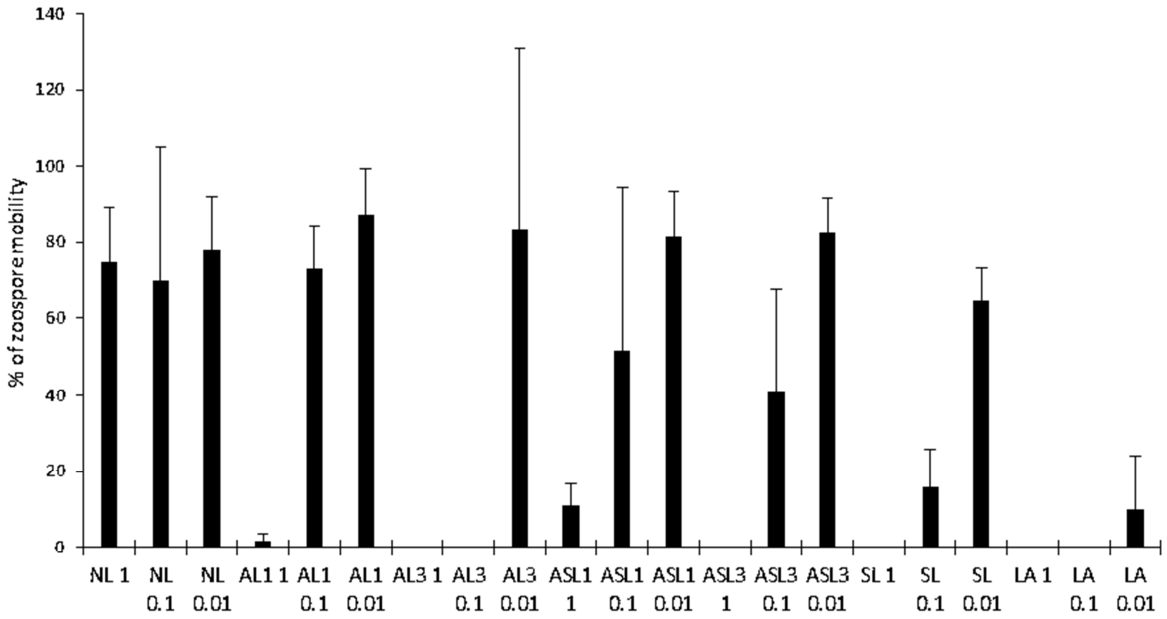


Fig. 5. Effect of new laminaran derivatives on *Pv* zoospores mobility *in vitro*. Values are expressed as the % of zoospore mobility relatively to the water control and are the means of two independent experiments. Compounds were applied at 0.01; 0.1 and 1 $g.L^{-1}$. Mobility was measured 1 hour after treatment. **NL**, native laminaran; **AL1** and **AL3**, acylated laminarans; **ASL1** and **ASL3**, acylated sulfated laminarans; **SL**, persulfated laminaran; **LA**, lauric acid.

*3.4. Evaluation of **AL3** as an elicitor*

As **AL3** showed the most promising activity in both protection and toxicity against *Pv*, it was further investigated as potential plant defense elicitor. For this purpose, grapevine cell suspensions were used to assess and compare the eliciting activity of **AL3**, **NL** and **LA** regarding typical grapevine defense events, i. e. MAP kinase activation, H_2O_2 and stilbene production in cell suspension. Grapevine stilbenes are represented by resveratrol and its derivatives, which are the more documented antimicrobial defense products of this plant species (Adrian et al., 2012). **AL3** and **LA** actually induced MAP kinase activation from 5 mn to 60 mn after treatment, with an identical pattern (a unique band at 49 kD). This pattern differed from the one of **NL**, with 2 bands at 49 kD and 45 kD (Fig. 6). That **AL3** and **LA**

shared a similar pattern suggested that **AL3** was rather perceived by the plant cell *via* its lauryl chains than *via* its glucan backbone. Moreover, **AL3** and **LA** turned out to be poor elicitors of H_2O_2 production in cell suspension in comparison with **NL** (Table 2). To complete these investigations, we searched for H_2O_2, stilbene and callose production *in planta*. The results are summarized in Table 2. While **NL** induced weak H_2O_2 production and callose deposition (S.I., Fig. S2), neither **AL3** nor **LA** induced such reactions.

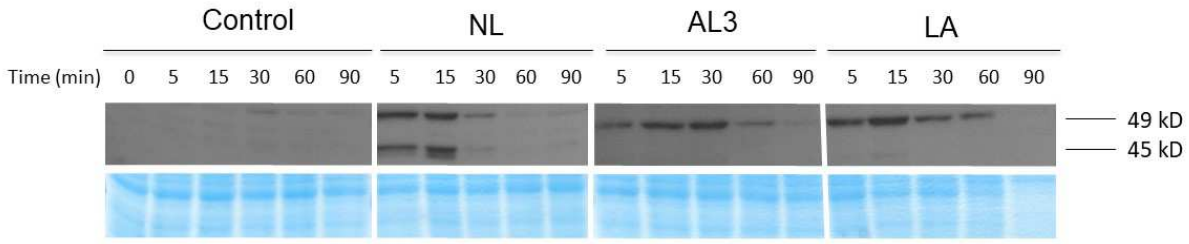


Fig. 6. Time-course study of the activation of mitogen-activated protein kinases (MAPK) of grapevine cells treated with water (control), native laminaran (**NL**), acylated laminaran 3 (**AL3**) or lauric acid (**LA**). Line 1: Western blot analysis of grapevine cell phosphorylated MAPK (49 and 45 kD). Line 2: Polyacrylamide gel electrophoresis stained with coomassie blue as loading control. Results are from one representative experiment out of two.

Table 2. Grapevine defense responses triggered by native laminaran (**NL**), lauric acid (**LA**) or acylated laminaran (**AL3**) treatments.

		NL	**LA**	**AL3**
Cell suspension	MAPK	++	+	+
	H_2O_2	+	-	-
In planta	H_2O_2	+	-	-
	Stilbenes	-	-	-
	Callose	+/- (in stomata)	-	-

On the whole, **AL3** elicitor activity was weak but significant in cell suspensions and null *in planta*. A typical elicitor enters the leaf and reaches its cellular target to subsequently trigger defense reactions. As a consequence, its protective effect is not affected by rinsing off the leaves with water once it has entered the leaf tissues (Thuerig, Felix, Binder, Boller, & Tamm, 2005). However, we have observed that protection achieved by **AL3** was completely abolished after washing (data not shown). This led us to suggest that **AL3** penetration rate into the leaf is probably very low and that it acts mainly as an antimicrobial compound that remains on the surface of the leaf and not as an elicitor of plant defense reactions.

That **AL3** was found more effective than **LA** is interesting but intriguing. It is possible that **LA**, owing to its low molecular weight, gets quickly into the cuticle which is mainly constituted of fatty acids, and is then no longer available at the leaf surface to kill the pathogen inoculated subsequently. The hydrophilic moiety of **AL3** would prevent such a penetration, allowing the protective effect to last longer.

4. Conclusion

The aim of our study was to improve the protective effect of laminaran against an aggressive pathogen by grafting an antimicrobial lipophilic chain on position *O*-6 of part of the glucosyl residues. It is part of the current trend to formulate biobased active compounds in the field of agriculture. For this purpose, lauryl chains were grafted on laminaran and the subsequent compounds, characterized by different acylating ratio, were further persulfated. While sulfation did not show major improvement on the action against *Pv*, the simple acylation can be beneficial to the biological activity induced by laminaran. Also it was shown that protection against *Pv* was improved by increasing the number of lauryl chains on linear β-(1→3)-glucan. Best protection against *Pv* was obtained with laminaran grafted with a

456 minimum of 7 lauryl chains. However, this result was not correlated with the elicitation of
457 plant defense events like H_2O_2, stilbene or callose. Interestingly significant direct anti-
458 oomycete effect was observed for most of the synthesized acylated compounds. This is likely
459 due to the anti-microbial property of the lauryl chains. It would be of interest to define the
460 antimicrobial spectrum of these compounds, and to determine if they are effective against
461 other important plant pathogens, such as those responsible for powdery mildew which
462 develops externally. For practical purposes, attention should be paid to develop an optimized
463 formulation in order to improve their solubilization and their retention on the leaf surface for
464 an enhanced efficacy.
465
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471
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