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1 **Hydrophobized laminarans as new biocompatible anti-oomycete**
2 **compounds for grapevine protection**

3

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13

14 **Abstract:**

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

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

27

28 **Keywords:** Laminaran; β -(1 \rightarrow 3)-Glucans; Acylation; Sulfation; Grapevine; Induced
29 resistance.

30

31 **1. Introduction**

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

50 H₂O₂ production, expression of defense genes and accumulation of stilbene phytoalexins.
51 Such an activation of the defense mechanisms was assigned to the close structural similarity
52 between β-(1→3)-glucans from pathogenic microorganisms and the ones from algae, the
53 latter being thus sensed as a danger signal by the plant.

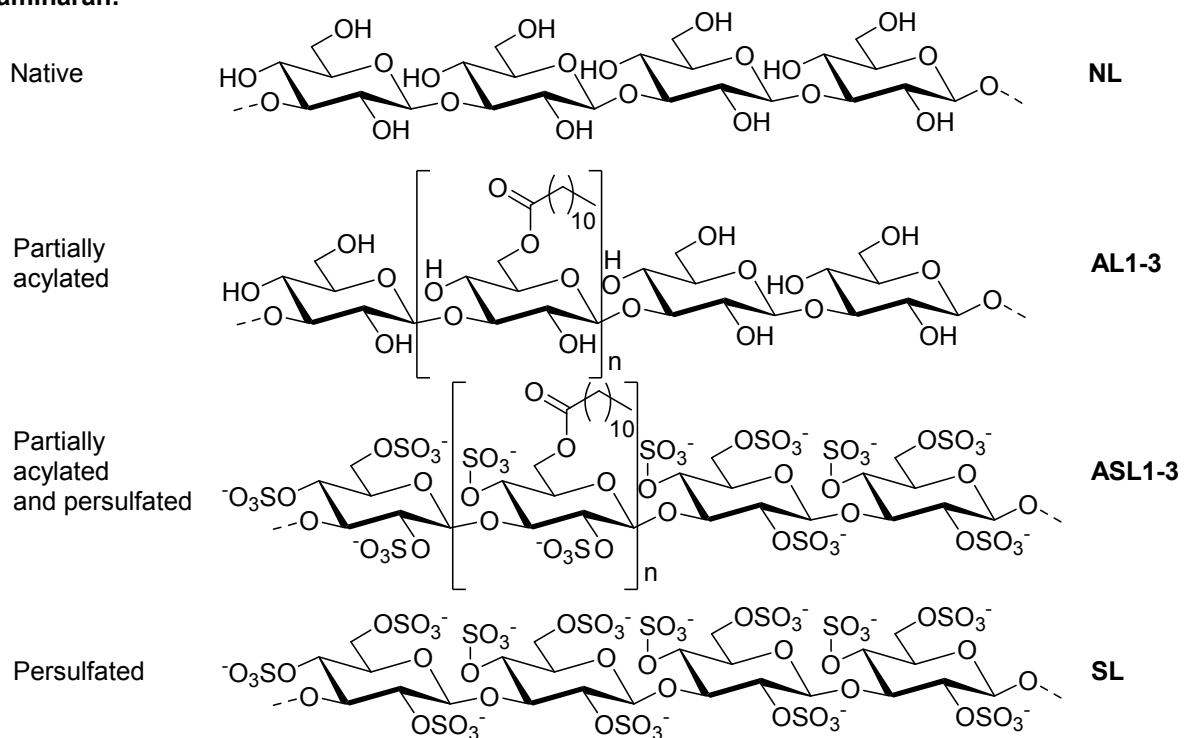
54 Nevertheless, while laminaran succeeded in eliciting defense reactions in grapevine,
55 protection against *Pv* was unsatisfactory because of inconsistency in the vineyard trials, and
56 absence of protection in vineyard trials under real conditions (X. Daire, unpublished). This
57 could be first attributed to a low penetration rate of hydrophilic compounds into the leaf.
58 Indeed, leaves are covered by a cuticle made up of polyester of hydroxylated fatty acids and
59 waxes which form a hydrophobic barrier preventing water losses. Interestingly, we have
60 recently demonstrated that an ethoxylated surfactant significantly helps the penetration of
61 such highly polar carbohydrates through abaxial surfaces of plant leaves (Paris et al., 2016).

62 Secondly, the important variability of the biological results could also be due to the fact that
63 laminaran acts solely as an elicitor of plant defense mechanisms and does not have any toxic
64 effect on oomycete. Recently, we have shown that a plant extract with a dual mode of action
65 (elicitor of grapevine defenses and antimicrobial) was a good candidate for ecofriendly
66 control of grape downy mildew (Krzyszaniaka, Trouvelot, et al., 2018). Indeed, if one mode of
67 action is ineffective (e.g., low-responsiveness of the plant to induced resistance), the other one
68 may take the lead and keep on protecting the plant.

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

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

Laminaran:



85

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

87

88 **2. Material and methods**

89 **2.1. Chemistry**

90 *2.1.1. Materials*

91 Laminaran and persulfated laminaran (MW \approx 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. ¹H, ¹³C, 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. ¹H 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

115 (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-
116 2.87 (m, 123.4H, H-2, H-3, H-4, H-5, H-6b), 2.36-2.23 (m, 3.2H, COCH₂), 1.59-1.38 (m,
117 3.2H, COCH₂CH₂), 1.32-1.19 (m, 25.6H, (CH₂)₈), 0.84 (t, *J* = 6.6 Hz, 4.8H, CH₃).

118 2.1.2.2. *Lauroylation of laminaran to afford AL2*

119 **AL2** was obtained according to the general procedure described above using laminaran (20 g,
120 5 mmol), triethylamine (10 mL, 0.072 mol), lauroyl chloride (10.7 mL, 46.3 mmol) and
121 DMAP (1.5 g, 12.3 mmol) in dry dimethylacetamide (200 mL). After 3 h stirring at 105 °C,
122 850 mL of deionized water were added to the cooled mixture to precipitate the acylated
123 laminaran. It was then concentrated by centrifugation (9000 rpm for 25 mn) and resuspended
124 in a 125 mL mixture of ethanol and dichloromethane (50:50, v/v). Deionized water (350 mL)
125 was added to precipitate the acylated laminaran again. It was once again concentrated by
126 centrifugation (9000 rpm for 25 mn). These steps were repeated twice before freeze-drying
127 the resulting solid, which was then washed with ethyl acetate to remove excess triethylamine
128 and DMAP. It was then freeze-dried again and stored at 4 °C until use. A beige powder was
129 isolated (9.40 g, 34%, number of acyl chains: 4.6). ¹H NMR (400 MHz, DMSO-*d*₆) δ 5.28-
130 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,
131 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),
132 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,
133 H-5), 2.35-2.18 (m, 9.2H, COCH₂), 1.60-1.40 (m, 9.2H, COCH₂CH₂), 1.34-1.15 (m, 73.6H,
134 (CH₂)₈), 0.84 (t, *J* = 6.6 Hz, 13.8H, CH₃).

135 2.1.2.3. *Lauroylation of laminaran to afford AL3*

136 **AL3** was obtained according to the general procedure described above using laminaran (50 g,
137 12.5 mmol), triethylamine (20 mL, 0.143 mol), lauroyl chloride (35.6 mL, 153.9 mmol) and
138 DMAP (3 g, 24.6 mmol) in dry dimethylacetamide (500 mL). After 3 h stirring at 105 °C, 1 L
139 of deionized water was added to the mixture to precipitate the acylated laminaran. It was then

140 concentrated by centrifugation (9000 rpm for 25 mn) and resuspended in a 300 mL mixture of
141 ethanol and dichloromethane (50:50v v/v). Deionized water (1 L) was added to precipitate the
142 acylated laminaran. It was again concentrated by centrifugation (9000 rpm for 25 mn). Those
143 steps were repeated twice before freeze-drying the resulting solid, which was then washed
144 with ethyl acetate to remove excess triethylamine and DMAP. It was then freeze-dried again
145 and stored at 4 °C until use. The product is purified on a reverse-phase chromatography
146 column using a water/acetonitrile mixture (gradient from 90:10 to 50:50) giving a beige
147 powder (30.0 g, 31%, number of acyl chains: 7.6). ¹H NMR (400 MHz, DMSO-*d*₆) δ 5.30-4.8
148 (1s, 25H, OH), 4.72-4.55 (1s, 25H, OH), 4.49 (d, *J* = 7.4 Hz, 25H, H-1), 4.31-4.22 (m, 7.6H,
149 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,
150 117.4H, H-2, H-3, H-4, H-5, H-6b), 2.35-2.20 (m, 15.2H, COCH₂), 1.57-1.36 (m, 15.2H,
151 COCH₂CH₂), 1.34-1.13 (m, 121.6H, (CH₂)₈), 0.84 (t, *J* = 6.6 Hz, 22.8H, CH₃).

152

153 *2.1.3. General procedure for sulfation of lauroyl laminaran*

154 Acylated laminaran (1 equiv.) and dry pyridine (124 equiv.) were suspended in dry
155 dimethylformamide under nitrogen. The mixture was heated at 60 °C under vigorous stirring
156 and SO₃-pyridine complex (54 equiv.) was added. The solution was further stirred at 60 °C
157 for 2 h, and overnight at room temperature. Precipitation of the product was obtained by
158 adding absolute ethanol (20 mL). After filtration, the solid residue was washed three times
159 with absolute ethanol. The product was then suspended in warm deionized water (5 mL), and
160 neutralized with 2 mol.L⁻¹ sodium hydroxide solution. Excessive pyridine was removed by
161 coevaporation with toluene under reduced pressure, and the product was freeze-dried and
162 stored at 4 °C until use.

163 *2.1.3.1. Sulfation of lauroyl laminaran ASL1*

164 Sulfation of **AL1** (0.5 g, 0.110 mmol) was performed according to general procedure using
165 dry pyridine (1.10 mL, 13.6 mmol) and SO₃-pyridine complex (0.94 g, 5.9 mmol) in dry
166 dimethylformamide (20 mL). A white powder (1.16 g, 87%, number of acyl chains: 1.5) was
167 obtained. ¹H NMR (400 MHz, D₂O+CD₃OD) δ 5.18-4.69 (m, 25H, H-1), 4.75-3.49 (m, 150H,
168 H-2, H-3, H-4, H-5, H-6), 2.14 (t, *J* = 7.6 Hz, 3H, COCH₂), 1.64-1.48 (m, 3H, COCH₂CH₂),
169 1.36-1.16 (m, 24H, (CH₂)₈), 0.84 (t, *J* = 7.0 Hz, 4.5H, CH₃).

170 2.1.3.2. Sulfation of lauroyl laminaran **ASL2**

171 Sulfation of **AL2** (0.5 g, 0.089 mmol) was performed according to general procedure using
172 dry pyridine (0.87 mL, 11.1 mmol) and SO₃-pyridine complex (0.76 g, 4.8 mmol) in dry
173 dimethylformamide (20 mL). A white powder (0.77 g, 71%, number of acyl chains: 3.6) was
174 obtained. ¹H NMR (400 MHz, D₂O) δ 5.01-4.37 (m, 25H, H-1), 4.32-3.36 (m, 150H, H-2, H-
175 3, H-4, H-5, H-6), 2.40-2.27 (m, 7.2H, COCH₂), 1.61-1.41 (m, 7.2H, COCH₂CH₂), 1.29-1.05
176 (m, 57.6H, (CH₂)₈), 0.85-0.70 (m, 10.8H, CH₃).

177 2.1.3.3. Sulfation of lauroyl laminaran **ASL3**

178 Sulfation of **AL3** (0.5 g, 0.075 mmol) was performed according to general procedure using
179 dry pyridine (0.74 mL, 9.3 mmol) and SO₃-pyridine complex (0.65 g, 4.1 mmol) in dry
180 dimethylformamide (20 mL). A white powder (0.70 g, 77%, number of acyl chains: 10) was
181 obtained. ¹H NMR (400 MHz, D₂O) δ 5.38-4.82 (m, 25H, H-1), 4.75-3.83 (m, 150H, H-2, H-
182 3, H-4, H-5, H-6), 2.55-2.33 (m, 20H, COCH₂), 1.77-1.53 (m, 20H, COCH₂CH₂), 1.46-1.18
183 (m, 160H, (CH₂)₈), 0.99-0.79 (m, 30H, CH₃).

184

185 2.2. Biological material, protection assays and analyses

186 2.2.1. Grapevine plant production and treatments

187 The grapevine cultivar used was *V. vinifera* L. cv. Marselan (Cabernet sauvignon ×
188 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 $[1 - (\% \text{ SA treatment} / \% \text{ SA reference})] \times 100$, where the reference can be either the water or
209 the adjuvant treatment.

210

211 2.2.3. *Grapevine cell suspensions*

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

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

219

220 *2.2.4. Defense-Related Responses Assessment*

221 *2.2.4.1. In grapevine suspension cells*

222 **H₂O₂ production:** H₂O₂ production was measured in cell suspension as described by
223 (Gauthier et al., 2014). Measurements were carried out using a luminol chemiluminescence
224 assay with a luminometer (Lumat LB 9507, Berthold, Evry, France). Two hundred and fifty
225 microliters of cell suspension were added to 300 µL of H50 medium (50 mM HEPES, 175
226 mM mannitol, 5 mM CaCl₂, 0.5 mM K₂SO₄; pH 8.5), and 50 µL of 0.3 mM luminol. Relative
227 luminescence was recorded within a 10 s period and was converted in nmol of H₂O₂ per gram
228 of FWC, after the establishment of a H₂O₂ reference range with untreated cell suspension.

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

239 2.2.4.2. *In grapevine foliar tissues*

240 **Localization of H₂O₂ in treated leaves:** Leaves from treated plants and inoculated or not
241 with *Pv* were harvested at different post-inoculation time points (12, 24, 48, or 72 hpi), and
242 treated as described elsewhere (Trouvelot et al., 2008). H₂O₂ was visualized as a reddish
243 brown coloration after treatment with diaminobenzidine (DAB).

244 **Localization of callose in treated leaves:** Leaf disks (7 mm in diameter) were harvested at
245 different times and incubated overnight in absolute methanol at room temperature. The
246 samples were then transferred to chloral hydrate solution (2.5 g.mL⁻¹) overnight at room
247 temperature to soften and clear the tissues. Cleared samples were incubated in 0.05% aniline
248 blue (in 0.1 M phosphate buffer, pH 8.0) overnight at room temperature. They were then
249 mounted on microscope slides in the staining solution, with the abaxial surface uppermost.
250 Callose production was observed by epifluorescence microscopy under UV (λ_{exc} 340 nm, λ_{em}
251 380 nm, stop filter LP 430 nm) as it fluoresces blue under these conditions.

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

261

262 2.2.5. *Evaluation of toxicity towards Pv*

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

265

266 2.2.6. Scanning Electron Microscopy (SEM)

267 Observations of leaf surface were made by SEM. At 24 hpi and 6 dpi, 0.5 cm² leaf squares
268 were excised from treated and inoculated leaves. The leaf surface was then characterized by
269 cryo-scanning electron micrographs (cryo-SEMs) taken using a Hitachi (SU 8230) scanning
270 electron microscope equipped with Quorum PP3000 t cryo attachment. Prior to imaging, leaf
271 samples were rapidly frozen in liquid nitrogen in order to fix them. Thereafter they were
272 sublimated at -90 °C for 5 min and coated with a thin platinum layer by sputtering at 5 mA for
273 10 s.

274

275 2.2.7. Statistical analysis

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

280

281 3. Results and discussion

282 3.1. Chemical synthesis of modified laminarans **AL** and **ASL**

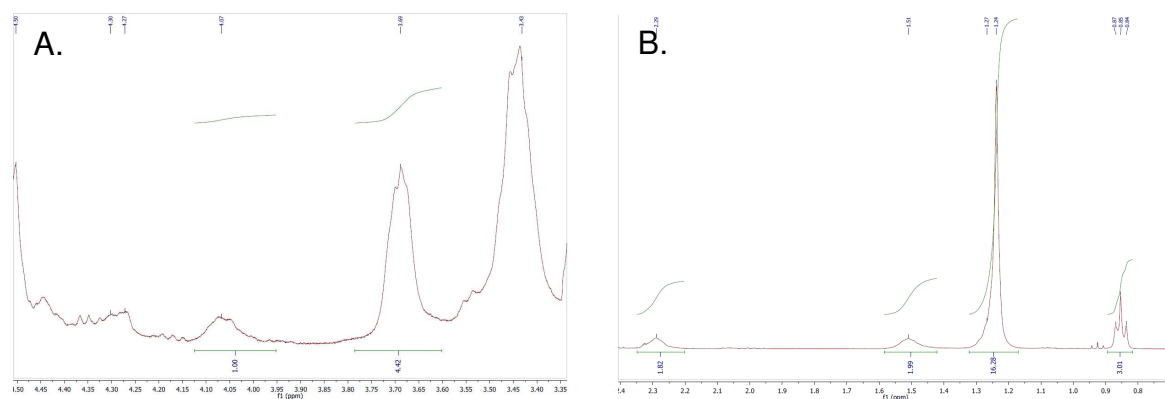
283 In order to (i) facilitate the penetration of laminaran through the leaf cuticle, and (ii) possibly
284 enhance the laminaran biological activity, a lipid chain was grafted to the more reactive
285 position O-6 of the polysaccharide. Lauryl chains were selected for their ability to interact
286 with lipidic layers such as cuticle and also for their antimicrobial potential (Walters et al.,
287 2003). Practically, in order to produce **AL** compounds with modulated hydrophilic/lipophilic

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

294

295 The efficiency of the acylation reaction was evaluated thanks to ¹H NMR. For the three
296 conditions used, the presence of an acyl group on the polysaccharide was confirmed by the
297 downfield shift of some H-6 protons from 3.63 ppm to 4.10 ppm, correlated with the electron-
298 withdrawing nature of the acyl chain. The percentage of acylation was then calculated by
299 setting first the integration of the more downfield proton to one. The ratio $100 \cdot 1 / (1 + y)$, with y
300 the integration of the upfield protons, gave the percentage of acylation. The number of
301 aliphatic chains on the polysaccharide could then be deduced by applying this percentage to
302 the total number of primary position, set by convention to 25. As an example, for **AL2**, setting
303 up the integration of the downfield H-6 to 1, lead to an integration value of 4.4 for the other
304 primary protons (Fig. 2A). The percentage of acylation reached then 18.4%, meaning an
305 average of 4.6 lauryl chain per laminaran. The evaluation of this degree of substitution was
306 corroborated considering the integration of the CH₂ functions at 1.23 ppm, signal which
307 corresponds to 16 protons for one acyl chain (Fig. 2B).

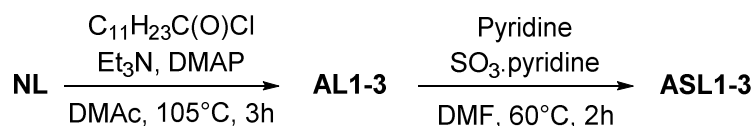
308



309
310 **Figure 2.** ^1H NMR spectrum of **AL2** in the area of the H-6 (2A) and the lauryl chain (2B)

311
312 Thanks to this calculation, the presence of 1.6, 4.6, and 7.6 chains of lauryl per
313 polysaccharidic chain was established for the samples **AL1**, **AL2**, and **AL3**, respectively
314 (Table 1). After precipitating with water and removing the remaining acylating reagent, yields
315 in isolated **AL** compounds reached an average of 30%.

316
317 **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

319
320 As persulfation of laminaran is known to effectively elicit defense mechanisms in plants
321 (Menard, 2004; Trouvelot et al., 2008), **AL** derivatives were subsequently reacted with excess
322 sulfur trioxide in pyridine to afford the desired **ASL1**, **ASL2** and **ASL3**, in 87%, 71%, and

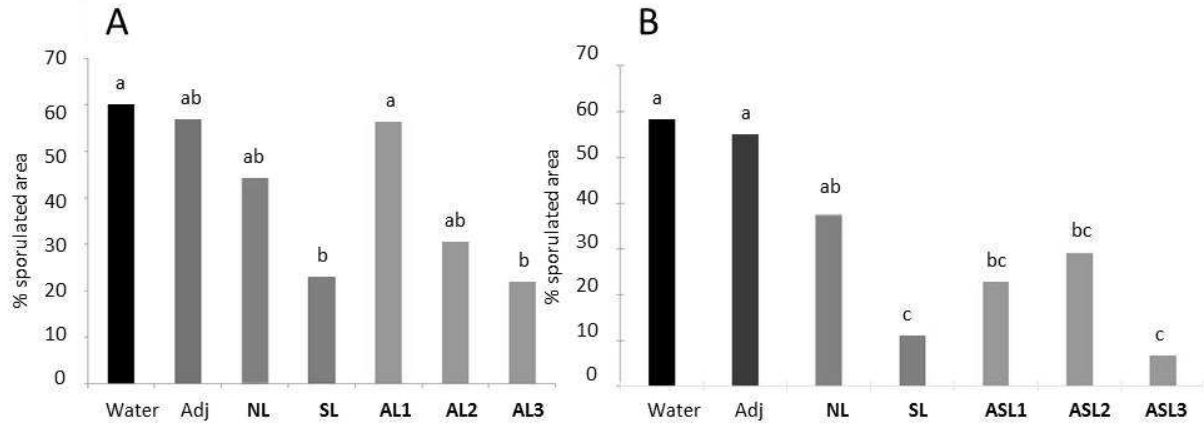
323 77% yields, respectively. This step also had the advantage to ensure the products solubility in
324 water solution, especially for **AL2** and **AL3** which bear more lipid chains. In addition, the
325 conditions of sulfation were mild enough to avoid any hydrolysis of the ester functions. It was
326 confirmed thanks to ¹H NMR. The protons corresponding to the acyl chains could be found
327 between 2.60 and 0.8 ppm. The average amounts of branching were deduced from the ratio
328 between the polysaccharide scaffold (4.70 to 3.76 ppm) that integrate for 150 protons (All
329 protons minus the anomeric ones) and the methyl group at 0.80 ppm. It reached 1.5, 3.6 and
330 10 for **ASL1**, **ASL2** and **ASL3**, respectively (see S.I.). Such discrepancy when compared with
331 the starting acylated laminarans could be explained by the method of purification chosen.

332

333 *3.2. Assessment of the efficacy of ALI-3 and ASLI-3 against Pv*

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

342



343

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

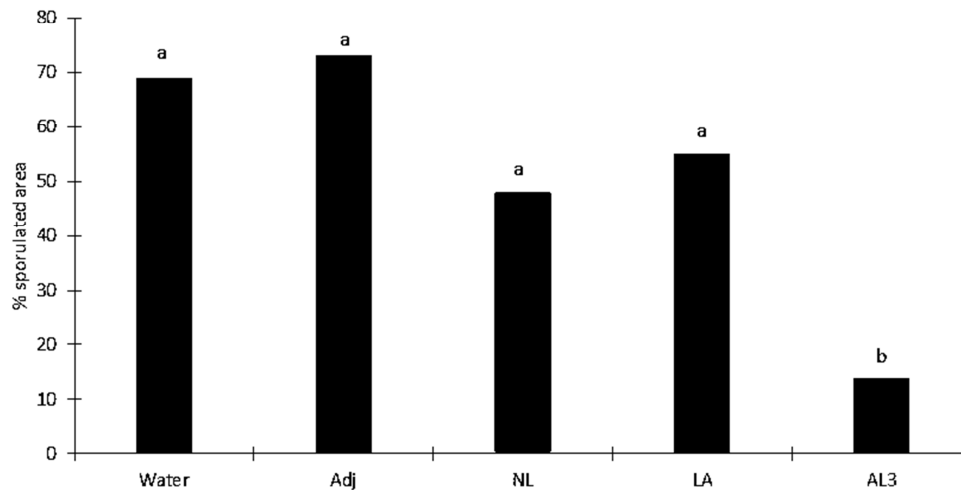
349

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

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

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

366



367

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

372

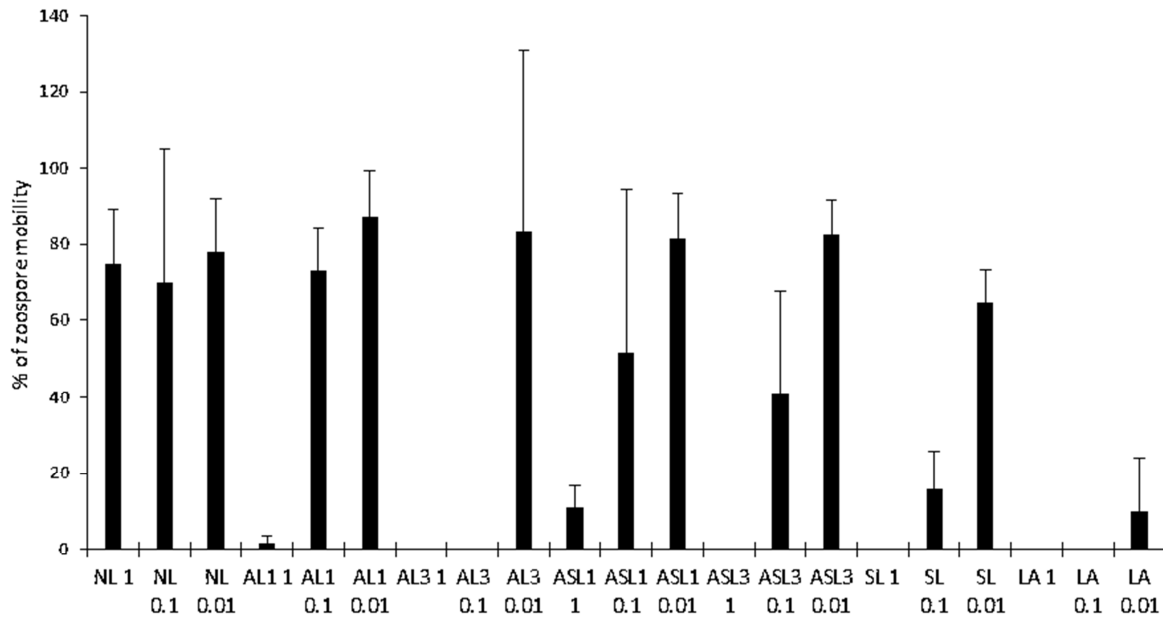
373 3.3. Toxicity of acylated laminarans to zoospore of *Pv*

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

381 compounds. Persulfation did not enhance activity of the acylated laminarans as the biological
382 effect of acylated and sulfated laminarans on zoospores was not better than that of the
383 acylated ones. Moreover, **ASL** efficacy did not overcome that of persulfated laminaran **SL**
384 itself. Interestingly, pure lauric acid (**LA**) had strong impact on mobility of *Pv* spores, even at
385 0.01 g.L⁻¹ concentration. This result confirmed the wide antimicrobial spectrum of **LA**,
386 previously shown to be effective against plant pathogenic fungi unrelated to the *Pv* (Walters
387 et al., 2003). This active concentration should be however related to the lower molecular mass
388 of **LA** compared to that of polysaccharides. Best results were obtained with the laminaran
389 grafted with 7.6 lauryl chains (**AL3**) that showed high toxicity, from the 0.10 g.L⁻¹
390 concentration. The similarity of *in vitro* efficacy of **LA** and **AL3** suggests that **AL3** activity
391 relies on the lauryl chains. Scanning electronic microscopy observations (S.I., Fig. S1) of the
392 leaf surface showed that *Pv* zoospores, probably affected in their mobility, could not reach the
393 stomata, their site of infection, when plants were treated by **AL3**. This observation directly
394 confirmed that **AL3** is toxic to *Pv* in planta. However, one cannot rule out the possibility that
395 the elicitation of grapevine defenses can reinforce this direct protective activity.

396

397



398

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

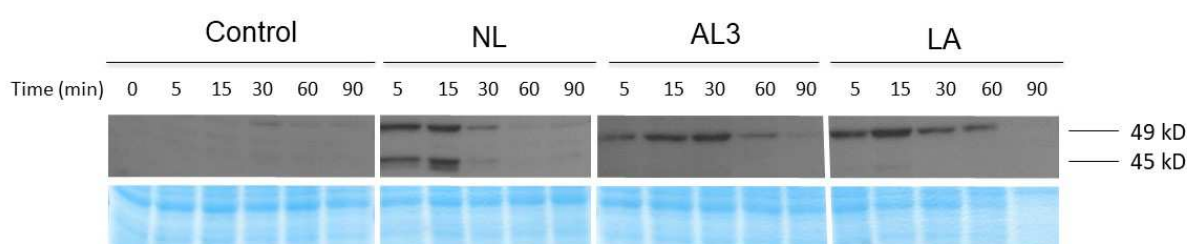
404

405 3.4. Evaluation of *AL3* as an elicitor

406 As **AL3** showed the most promising activity in both protection and toxicity against *Pv*, it was
 407 further investigated as potential plant defense elicitor. For this purpose, grapevine cell
 408 suspensions were used to assess and compare the eliciting activity of **AL3**, **NL** and **LA**
 409 regarding typical grapevine defense events, i. e. MAP kinase activation, H₂O₂ and stilbene
 410 production in cell suspension. Grapevine stilbenes are represented by resveratrol and its
 411 derivatives, which are the more documented antimicrobial defense products of this plant
 412 species (Adrian et al., 2012). **AL3** and **LA** actually induced MAP kinase activation from 5 mn
 413 to 60 mn after treatment, with an identical pattern (a unique band at 49 kD). This pattern
 414 differed from the one of **NL**, with 2 bands at 49 kD and 45 kD (Fig. 6). That **AL3** and **LA**

415 shared a similar pattern suggested that **AL3** was rather perceived by the plant cell *via* its
 416 lauryl chains than *via* its glucan backbone. Moreover, **AL3** and **LA** turned out to be poor
 417 elicitors of H₂O₂ production in cell suspension in comparison with **NL** (Table 2). To complete
 418 these investigations, we searched for H₂O₂, stilbene and callose production *in planta*. The
 419 results are summarized in Table 2. While **NL** induced weak H₂O₂ production and callose
 420 deposition (S.I., Fig. S2), neither **AL3** nor **LA** induced such reactions.

421



422

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

428

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

		NL	LA	AL3
Cell	MAPK	++	+	+
suspension	H ₂ O ₂	+	-	-
<i>In planta</i>	H ₂ O ₂	+	-	-
	Stilbenes	-	-	-
	Callose	+/- (in stomata)	-	-

431

432 On the whole, **AL3** elicitor activity was weak but significant in cell suspensions and null *in*
433 *planta*. A typical elicitor enters the leaf and reaches its cellular target to subsequently trigger
434 defense reactions. As a consequence, its protective effect is not affected by rinsing off the
435 leaves with water once it has entered the leaf tissues (Thuerig, Felix, Binder, Boller, & Tamm,
436 2005). However, we have observed that protection achieved by **AL3** was completely
437 abolished after washing (data not shown). This led us to suggest that **AL3** penetration rate
438 into the leaf is probably very low and that it acts mainly as an antimicrobial compound that
439 remains on the surface of the leaf and not as an elicitor of plant defense reactions.

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

445

446 **4. Conclusion**

447 The aim of our study was to improve the protective effect of laminaran against an aggressive
448 pathogen by grafting an antimicrobial lipophilic chain on position *O*-6 of part of the glucosyl
449 residues. It is part of the current trend to formulate biobased active compounds in the field of
450 agriculture. For this purpose, lauryl chains were grafted on laminaran and the subsequent
451 compounds, characterized by different acylating ratio, were further persulfated. While
452 sulfation did not show major improvement on the action against *Pv*, the simple acylation can
453 be beneficial to the biological activity induced by laminaran. Also it was shown that
454 protection against *Pv* was improved by increasing the number of lauryl chains on linear β -
455 (1 \rightarrow 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 H₂O₂, 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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