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## **Inducing Plant Defense Reactions in Tobacco Plants with Phenolic-Rich Extracts from Red Maple Leaves: A Characterization of Main Active Ingredients**

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### ► **To cite this version:**

Elodie Peghaire, Samar Hamdache, Antonin Galien, Mohamad Sleiman, Alexandra ter Halle, et al.. Inducing Plant Defense Reactions in Tobacco Plants with Phenolic-Rich Extracts from Red Maple Leaves: A Characterization of Main Active Ingredients. *Forests*, 2020, 11 (6), pp.705. <10.3390/f11060705>. <hal-02920408>

**HAL Id: hal-02920408**

**<https://hal.science/hal-02920408v1>**

Submitted on 12 Nov 2020

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HAL Authorization

1 **Title:** Inducing plant defence reactions in tobacco plants with phenolic-rich extracts from red  
2 maple leaves: a characterization of main active ingredients

3

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19

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22 **Keywords:** alkaline hydrolysis, defence reactions, gallotanins, red maple leaf extract, tobacco

23

24

25

26 **Abstract**

27

28 Red maple leaf extracts (RME) were tested for their plant defence inducer (PDI) properties.  
29 Two extracts were obtained and compared by different approaches: RME1 using ethanol-  
30 water (30-70%, v/v, 0.5% HCl 1N) and RME2 using pure water. Both extracts titrated at 1.9  
31 g/L in polyphenols and infiltrated into tobacco leaves efficiently induced hypersensitive  
32 reaction-like lesions and topical accumulation of auto-fluorescent compounds noted under UV  
33 and scopoletin titration assays. The antimicrobial marker *PR1*,  $\beta$ -1,3-glucanase *PR2*, chitinase  
34 *PR3*, and osmotin *PR5* target genes were all upregulated in tobacco leaves following RME1  
35 treatment. The alkaline hydrolysis of RME1 and RME2 combined with HPLC titration of  
36 gallic acid revealed that gallate functions were present in both extracts at levels comprised  
37 between 185 and 318 mg.L<sup>-1</sup>. HPLC-HR-MS analyses and glucose assay identified four  
38 gallate derivatives consisting of a glucose core linked to 5, 6, 7 and 8 gallate groups. These  
39 four galloyl glucoses possessed around 46% of total gallate functions. Their higher  
40 concentration in RME suggested that they may contribute significantly to PDI activity. These  
41 findings define the friendly galloyl glucose as a PDI and highlight a relevant methodology for  
42 combining plant assays and chemistry process to their potential quantification in crude natural  
43 extracts.

## 44 **1. Introduction**

45 In the context of sustainable development, agriculture is incorporating more eco-  
46 friendly alternatives to limit the use of chemical pesticides and regulate pest management.  
47 Increasing the natural resistance of plants is one favoured line of research, notably using  
48 biological substances that can stimulate plant immunity [1,2]. A complex array of immune  
49 response is triggered as early as plant detect pests [3,4]. The detection of pathogen- or plant-  
50 derived elicitors lead to the activation of numerous biochemical and molecular events in plant  
51 cells which prevent pathogen development [5,6]. The reactive oxygen species (ROS)  
52 production causes a hypersensitive reaction (HR) leading to topical cell death that restrict the  
53 systemic spread of the pathogen [7,8]. Surrounding tissues will acquire local resistance  
54 (named LAR) thanks to phytoalexin biosynthesis, cell wall and/or cuticle reinforcement with  
55 phenylpropanoid compounds, callose deposition, defence enzymes and pathogenesis-related  
56 (PR) proteins synthesis [9,10]. The whole plant will be mobilized with the systemic acquired  
57 resistance (SAR) undertaken by salicylic acid which allows uninfected distal parts of the plant  
58 to respond more effectively to subsequent infection [11,12].

59 The non-host resistance strategy involved therefore the local and systemic production of  
60 defence compounds with antimicrobial properties to counter pathogen development. Phenolic  
61 compounds are plant secondary metabolites preformed (named phytoanticipins) or induced in  
62 the plant after biotic attacks (named phytoalexins) and constitute inbuilt antibiotic chemical  
63 barriers to a wide range of potential pests and pathogens [13-16]. Our group developed the  
64 biotechnology concept consisting of extracting polyphenols (PPs) from biomass and  
65 reapplying them to plants to intentionally protect them against pathogens. This way, we  
66 showed that plant PP-rich extracts could trigger their own plant defence reactions. In  
67 particular, the grape marc extracts enriched in PPs were first demonstrated as playing the role  
68 of plant defence inducer (PDI) in tobacco [17-20]. Later on, we evidenced the elicitation

69 properties of alkyl gallates on whole tobacco plants and cell suspensions [21]. These simple  
70 phenols could induce early perception events on plasma membrane, potential hypersensitive  
71 reactions and PR-related downstream defence responses in tobacco. Supporting this idea, we  
72 initiated a research to find enriched-polyphenol extracts able to stimulate the plant immunity.  
73 Developing new natural substances from low-value raw materials while developing  
74 sustainable concepts in plant protection is a major challenge at this time. In this context,  
75 plants represent inexhaustible supplies of biomolecules that might serve in disease  
76 management and leaves of trees constitute an important available biomass that contain various  
77 class of polyphenols [22-25].

78 The present work is focused on red maple (*Acer rubrum*) trees largely distributed in Europe  
79 decorating in various public parks and gardens. Their leaves are enriched in PPs and  
80 numerous phenolic compounds have been identified in aerial parts of *Acer* species, among  
81 them gallate derivatives and gallotannins [26-32]. Here, our objective was to determine which  
82 PPs could be responsible for the PDI properties of red maple leaves extracts. With this goal,  
83 we extracted PPs from red maple leaves using two environmental friendly solvents (water and  
84 ethanol/water) and hydrolyzed them to destroy the gallate functions. Hydrolyzed and non-  
85 hydrolyzed extracts were infiltrated into tobacco leaves to compare their PDI activity. Based  
86 on these results and on UPLC-HR-MS-MS analyses, potential candidates are proposed.

87

## 88 2. Materials and Methods

### 89 2.1. Plant materials

90  
91 Fresh red maple leaves (*Acer rubrum*) were collected on trees in Auvergne, France, in  
92 September 2017. Leaves were dried in an oven (30°C), pulverized using a waring blender and  
93 stored at room temperature until further use. The biological activity of red maple leaf extracts  
94 was assayed on 2-months old tobacco plants (*Nicotiana tabacum* L. var. Samsun NN).  
95 Tobacco plants were grown in a greenhouse under controlled conditions (22+/- 5°C with a 16  
96 h photoperiod).

97

### 98 2.2. Tobacco treatments

99 Polyphenolic extracts (50 µL) were infiltrated on foliar tissue using a plastic syringe until the  
100 solution was spread across a 1-2cm<sup>2</sup> leaf area. The three most mature leaves showing no signs  
101 of aging were infiltrated on each tobacco plant. Leaves were infiltrated with acidic water (pH  
102 adjusted to 3.5 with acetic acid) for negative control. Macroscopic symptoms were examined  
103 under bright light and UV light (at 312 nm). For scopoletin quantification, leaves were  
104 infiltrated with 1 mL polyphenolic extracts on 20 distinct areas spread across the limb. For  
105 RNA analysis, tobacco leaves were sprayed onto both adaxial and abaxial faces of the three  
106 leaves with a fine atomizer (2 mL per leaf).

107

### 108 2.3. Total polyphenols extraction and quantification

109 Red maple leaf extracts (RME) were produced from the dried raw material. Two extraction  
110 protocols were used. Pulverised powder was grounded in liquid nitrogen and resuspended in  
111 acidic ethanol solvent (30% v/v, 0.5% HCl 1N) for RME1 or in pure water for RME2. The  
112 mixture in acidic ethanol-water solvent was incubated for 2h at 20°C, while the mixture in  
113 pure water was incubated at 70°C for 4h. After centrifugation at 9000 rpm for 20 min at 4°C

114 supernatants were lyophilized. The dried materials were resuspended in water. The aqueous  
115 resuspended compounds were centrifuged at 9000 rpm for 10 min to remove impurities and  
116 provide supernatants from the RME1 and RME2. Total phenolic content was determined by  
117 the Folin-Ciocalteu colorimetric method as described by Emmons and Peterson (2001) [33].  
118 Data were expressed as  $\text{mg}\cdot\text{g}^{-1}$  gallic acid equivalent using a standard curve of this standard.

119

#### 120 *2.4.Chemicals*

121 All chemicals reagents - scopoletin, pentagalloyl glucose (1,2,3,4,6-Penta-O-galloyl- $\beta$ -D-  
122 glucopyranose), gallic acid, ethanol, acetonitrile, methanol, Folin-Ciocalteu phenol reagent  
123 (2M) - were purchased from Sigma-Aldrich (Sigma-Aldrich Inc., Germany), they were the  
124 best grade available and used without further purification.

125

#### 126 *2.5.Scopoletin assay*

127 Scopoletin was extracted according to the modified ultrasound-assisted extraction protocol  
128 described by Chen et al. (2013) [34]. Tobacco leaves (2g) were grounded in liquid nitrogen  
129 and resuspended in anhydrous methanol (2 mL, containing 0.5% ascorbic acid). The mixture  
130 was immediately transferred to the ultrasonic apparatus and extracted at room temperature for  
131 2h. Following sonication, the solution was centrifuged at 9000 rpm at 20°C and the  
132 supernatant was cleaned-up (50 $\mu$ -filters) before HPLC analysis. The scopoletin quantities are  
133 the mean of biological replicates (3 plants, 3 leaves per plant) and presented as ng  
134 scopoletin/g FW.

135

#### 136 *2.6.Semi-quantitative real-time RT-PCR*

137 Leaf tissues (200 mg) were grounded in liquid nitrogen and RNA extraction was performed  
138 according to the manufacturer's instructions (RNeasy® Plant Mini Kit, Qiagen). RNA

139 received two treatments with DNase (RNase-Free DNase Set, Qiagen) and kept at -80°C.  
140 Purified RNAs were quantified by NanoDrop™ 2000 spectrophotometer (Thermo Fisher  
141 Scientific) and the RNA concentration was measured using the Agilent 2200 Tape Station and  
142 the RNA ScreenTape kit (Agilent Technologies). First-strand cDNA was synthesized from 1  
143 µg of total RNA with Euroscript Reverse Transcriptase (Eurogentec, France) according to the  
144 manufacturer's instructions. PCR reactions were prepared using the qPCR kit manufacturer's  
145 protocol. The cDNA concentration used produced a threshold value ( $C_T$ ) of between 15 and  
146 30 cycles. Amplification specificity was checked by melting-curve analysis. The relative  
147 quantity ( $Q_R$ ) of PR gene transcripts using EF-1α gene as internal standard was calculated  
148 with the  $\delta$ - $\delta$  mathematical model. QPCR data were expressed as the threshold cycle ( $C_t$ )  
149 values normalized to EF-1α and calculated using the  $2^{-\Delta\Delta C_t}$  method following standard  
150 protocols [35]. For every PR gene analyzed, three independent biological replicates were run,  
151 and every run was carried out at least in triplicate. Primers and amplicon sizes were given in  
152 Benouaret et al. (2015) [20].

153

#### 154 *2.7.HPLC-UV and UPLC-HRMS analyses*

155 UV-vis spectra were recorded using a Varian Cary 3 spectrophotometer in a 1-cm quartz cell.  
156 Analysis of RME1 and RME2 were performed with liquid chromatography (Alliance Waters  
157 HPLC) using a Waters 2695 separation module and a Waters 2998 photodiode array detector.  
158 HPLC-UV separation was conducted using a Phenomenex reversed phase column C<sub>18</sub> grafted  
159 silica, (100 mm length, 2.1 mm i.d. 1.7 µm particle size) and a binary solvent system  
160 composed of acetonitrile (solvent A) and water containing 0.1% orthophosphoric acid  
161 (solvent B) at a flow rate of 0.2 ml min<sup>-1</sup>. The initial composition 90% A and 10% B was  
162 maintained for 4 min, then solvent B was linearly increased to 25% in 4 min, and to 40% in  
163 22 min, to finish at 95% in 5 min. The identification of active constituents was performed

164 using high resolution mass spectrometry (HRMS) with an Orbitrap Q-Exactive  
165 (ThermoScientific) and an ultra-high-performance liquid chromatography (UPLC) instrument,  
166 the Ultimate 3000 RSLC (ThermoScientific). Analyses were carried out in both negative and  
167 positive electrospray modes (ESI<sup>+</sup> and ESI<sup>-</sup>). UPLC separations were performed using the  
168 same column and elution gradient as previously indicated. Identification of compounds was  
169 based on structural elucidation of mass spectra and the use of accurate mass determination  
170 was obtained with Orbitrap high resolution. MS-MS was done by the HCD technique (35 eV).  
171 Scopoletin was titrated by HPLC-fluorescence. Separation was achieved using 30% of solvent  
172 A and 70% of solvent B at a flow rate of 0.2 ml min<sup>-1</sup>. The excitation wavelength was set at  
173 340 nm and the emission wavelength at 440 nm. The concentration of the authentic scopoletin  
174 in the extracts was obtained by comparing the peak area with that of reference solutions.

175

#### 176 *2.8. Alkaline hydrolysis of RME1 and RME2*

177 RME1 and RME2 (12 mL) titrated at 0.19% in PPs were deoxygenated by argon purging for  
178 15 min prior to the addition of 60 mg of Sodium Hydroxide (NaOH) used to adjust the pH at  
179 11.5. Then the mixture was heated at 60°C for 4h30, under continuous argon flux. At the end  
180 of the experiment, the solution was let to cool down for several minutes, neutralized by the  
181 addition of 150 µL of Chloride Hydroxide (HCl) and then left to air. The final pH was  
182 between 2 and 3.

183

#### 184 *2.9. Glucose quantification*

185 Glucose measurements were recorded for both RME and h-RME (600 µL) after pH  
186 readjustment to 7.8 as water negative control. The assay was calibrated with a set of glucose  
187 concentrations. GOD-POD reagent (4 mL) was added to each sample, mixed by pipetting and

188 incubated in the dark for 10 min. The absorbance was recorded at 503 nm on a Varian Cary 3  
189 spectrophotometer. Glucose concentration was calculated using the calibration curve.

190

### 191 2.10. Statistical analysis

192 Statistical analysis was performed using the statistical software R 3.2.5 ([https://cran.r-](https://cran.r-project.org/https://cran.r-project.org/)  
193 [project.org/https://cran.r-project.org/](https://cran.r-project.org/https://cran.r-project.org/)). For all statistical comparisons across different  
194 treatments, the normality (Shapiro-Wilk) and the homogeneity of variances (Bartlett test)  
195 were verified. To identify any significant differences among treatments, statistical  
196 comparisons were made across the different conditions with the Kruskal-Wallis test followed  
197 by Dunn's test as well as Bonferroni correction.

198

199

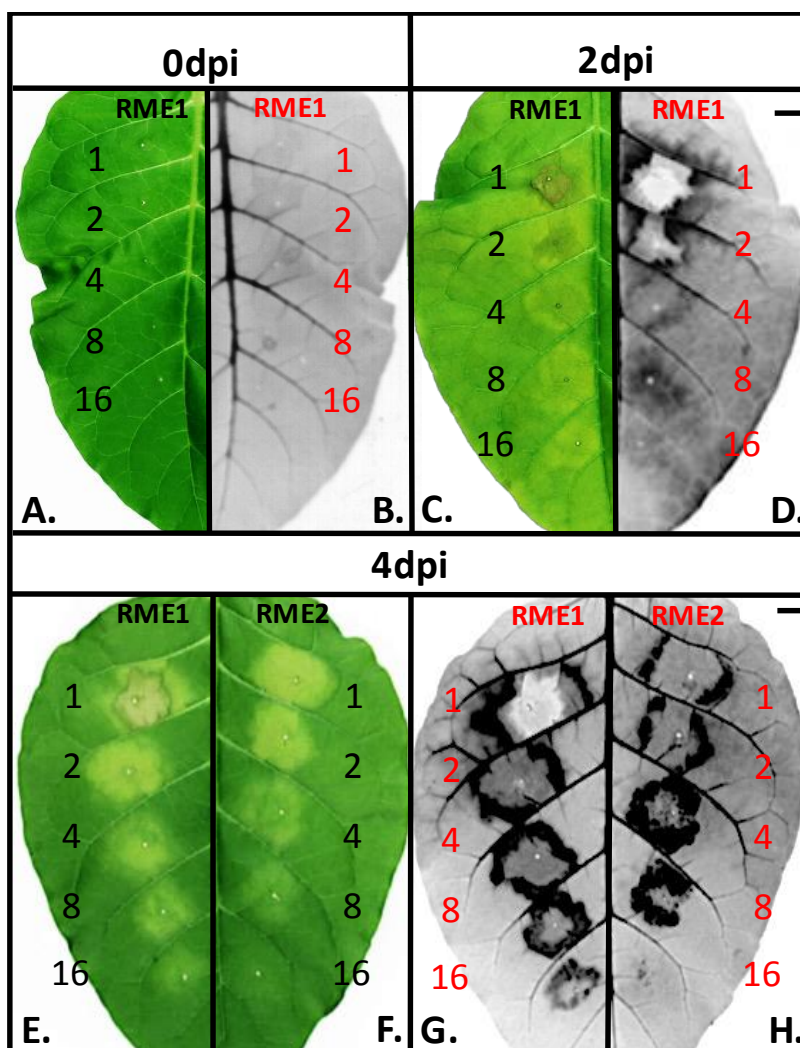
## 200 3. Results and Discussion

201

### 202 3.1. Plant defence inducer (PDI) activity of enriched-polyphenol red maple extracts (RME)

203 PDI activity of red maple hydroalcoholic (RME1) and water (RME2) leaf extracts  
204 were investigated using the HR-like reaction assays used previously for defence reaction  
205 explorations [17, 19-21]. Figure 1 shows the kinetic of macroscopic changes in symptoms  
206 induced after RME1 infiltration on the adaxial face of tobacco leaves that was exposed under  
207 bright light (Figure 1A, C, E) and UV light (Figure 1B, D, G). The extend of symptoms are  
208 shown for a range of RME1-PP concentrations (0.19% PP diluted 1 to 16 fold). The highest  
209 PP titer (0.19% PP) was chosen because it was provoked high defence levels in tobacco after  
210 infiltration of grape marc extracts [19,20]. The RME1-0.19% PP concentration clearly  
211 induced changes in the tobacco limb. The bright light examination of infiltrated tobacco  
212 leaves showed a topical brownish zone at 2 days post-infiltration (dpi) that rapidly became

213 necrotic at 4 dpi. Lower RME1-PP concentration (dilution 2) attenuated the infiltrated injured  
 214 areas and a more restricted necrotic zone was visible at 4 dpi. The more diluted RME1  
 215 (dilution 4 to 16) infiltration led to the spread of light damaging zone with chlorotic tissues.  
 216 UV examination ( $\lambda=312$  nm) of infiltrated tobacco leaves revealed fluorescent areas  
 217 surrounding or within the infiltration zones linked to the RME1-PP concentration, suggesting  
 218 the recruitment of phytoalexins.  
 219

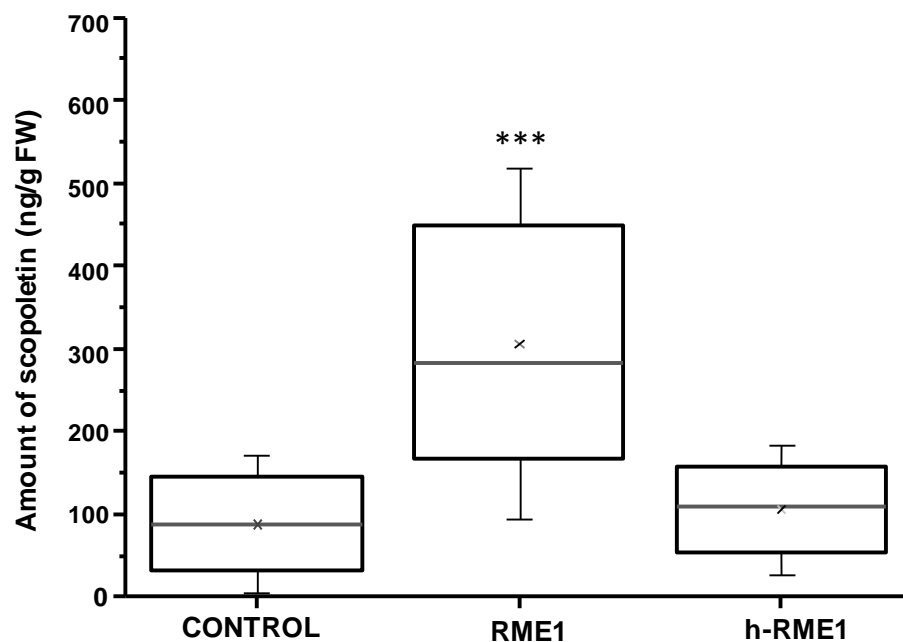


220  
 221 Figure 1: Macroscopic symptoms induced in tobacco leaves by RME1 and RME2 infiltrations  
 222 at 0 dpi, 2 dpi and 4 dpi observed under bright light (A,C,E,F) and UV light (B,D,G,H).  
 223 Tobacco leaves were infiltrated with a range of PP concentrations: 0.19% PP concentration  
 224 (1) was diluted twice (2), 4 fold (4), 8 fold (8) and 16 fold (16). Bar 1.5 cm

225 RME2 infiltration induced similar phenotypic symptoms at 4 dpi on tobacco leaves  
226 (Figure 1 F, H) but reduced the extent of damage. RME2 did not induce necrotic area at  
227 0.19% PP concentration and the low PP concentration (dilution 16) remained symptomless  
228 with no chlorotic zone or fluorescent areas detected suggesting the lower potential of RME2  
229 to induce HR-like reactions.

230 We further investigated the RME1 ability to induce phytoalexin production and  
231 defence-related gene expression. We monitored the formation of scopoletin, a phytoalexin  
232 known to be involved in the activation of defence mechanism. The quantification of  
233 scopoletin by HPLC reveal an over-accumulation in RME1-infiltrated tobacco leaves  
234 reaching  $307 \pm 138$  ng scopoletin/gFW. This was significantly higher at 3.5-fold (p-value <  
235 0,001) than for the control leaves (Figure 2). Control leaves were infiltrated with acidic water  
236 and remained symptomless (data not shown). Additionally, RME1 did not show any natural  
237 auto-fluorescence (Figure 1B).

238

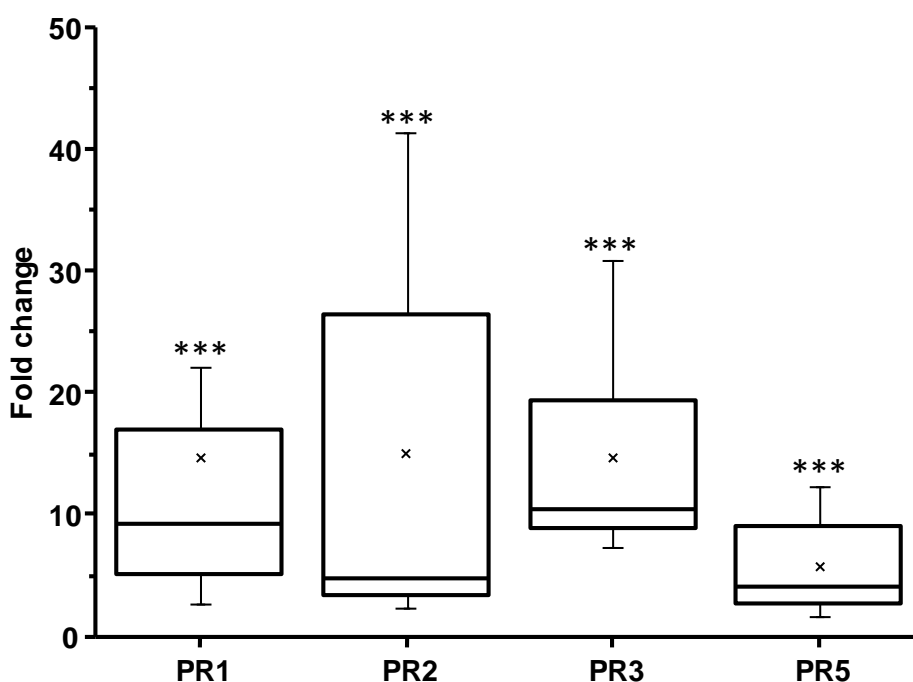


239

240 Figure 2: Scopoletin accumulation in tobacco leaves after infiltration of 0.095% PP  
241 concentration of RME1 before (RME1) and after alkaline hydrolysis (h-RME1). Leaves were  
242 infiltrated with RME1 or h-RME1 on 20 distinct areas and scopoletin quantification was  
243 measured at 4dpi by HPLC. Each experiment was performed in triplicate (3 leaves per plant,  
244 3 plants). Asterisks indicate significant differences compared with the control (\*\*\*)  $P < 0.001$ .

245

246 Transcript levels of defence-related genes were assessed by quantitative real-time PCR.  
247 Figure 3 shows the fold change ratio of transcript levels of four PR target genes in RME1-  
248 sprayed tobacco leaves at 4 days post-treatment. RME1 led to high PR transcript  
249 accumulation:  $17 \pm 9$ -fold for the antimicrobial marker *PR1*,  $15 \pm 7$ -fold for  $\beta$ -1,3-glucanase  
250 *PR2*,  $14 \pm 3$ -fold for chitinase *PR3*, and  $5 \pm 1$ -fold for osmotin *PR5* (on average, with p-value  $<$   
251  $0,001$  for all comparisons). RME1 should activate the SAR pathway by inducing expression  
252 of SAR related genes i.e. *PR1*, *PR2*, *PR3* and *PR5* that are induced by SA [20, 36]. The  
253 underlying processes triggered by RME1 are basically identical to the one induced by grape  
254 marc extracts. The PP-rich grape marc extracts were able to elicit HR, LAR and SAR  
255 responses in tobacco [17,19,20] and both water- and hydroalcoholic PP-rich grape extracts  
256 were active in inducing plant defence reactions [19]. Based on these data, we focused on PPs  
257 to further characterize the active ingredients responsible for these properties.



258

259 Figure 3: *PR* transcript accumulation in tobacco leaves 4 days after RME1-spaying.

260 Transcripts were quantified by real-time RT-PCR in treated leaves. Values are expressed

261 relative to control (acidic water treatment) values. Each experiment was performed in

262 triplicate (2 leaves per plant, 3 plants). Asterisks indicate significant differences compared

263 with the control (\*\*\*)  $P < 0.001$ .

264

265

### 266 3.2.HPLC-UV fingerprints and UPLC-HR-MS analysis of RME1 and RME2

267 In order to identify the chemical compounds responsible for the PDI properties, we

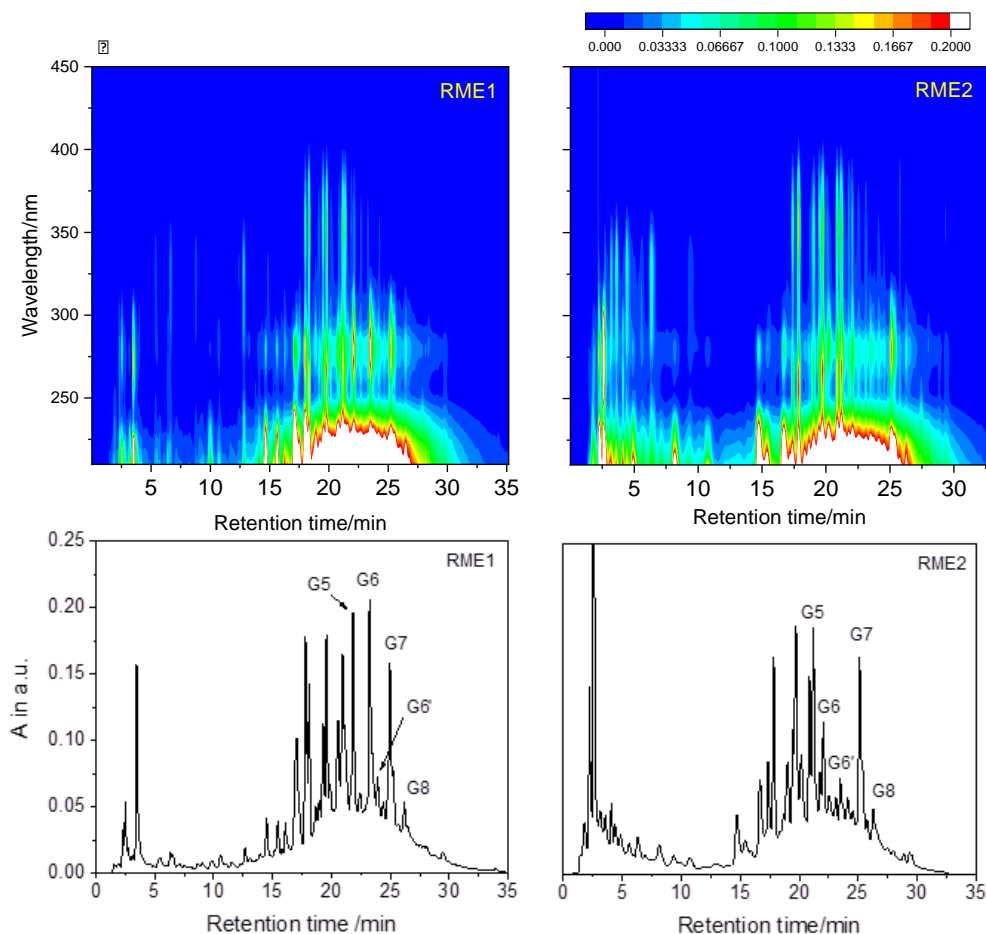
268 performed comparative HPLC fingerprints of RME1 and RME2. HPLC-UV chromatograms

269 of RME1 and RME2 are shown in Figure 4. The absorbing components were mainly eluted

270 between 2 and 4 min and after 15 min. Some constituents had absorption maxima at 275 nm

271 and 350 nm while other at 280 nm. RME1 and RME2 showed similar fingerprints but

272 differences in peak intensities. In particular, RME1 displayed higher peaks for molecules  
273 eluted after 21 min. As RME2 exhibited weaker PDI properties than RME1, we supposed that  
274 these molecules could be active components and focused on these compounds.  
275



276  
277 Figure 4: HPLC-UV chromatograms of aqueous extracts RME1 (A) and RME2 (B) prepared  
278 at 0.19% in polyphenols. Top figures relate to 2D spectra while bottom figures relate to  
279 chromatograms extracted at 278 nm.

280  
281 RME1 was further analyzed by UPLC-HR-MS in negative electrospray (Figure SI-1).  
282 The five main components detected eluted after 21 min and were labelled G5-G8 (Figure 4).  
283 Their UV, MS and MS-MS spectra are given in SI (Figures SI-2 to SI-5). They all exhibited  
284 the same absorption spectrum ( $\lambda = 218$  and 280 nm) (Figure SI-2A, SI-3A, SI-4A and SI-5A).

285 The MS spectrum of G5 displayed two peaks at  $m/z = 469.0531$  and  $939.1143$  (Figure SI-2B).  
286 Based on the accurate masses, the first one corresponded to  $z=2$ ,  $[M-2H]^{-2}$  and the latter one  
287 to  $z=1$ ,  $[M-H]^{-1}$ , giving  $C_{41}H_{32}O_{26}$  ( $\Delta ppm = 4.9$ ) for the chemical formula of the neutral  
288 molecule. The MS-MS on ion 469 yielded two fragments at  $m/z = 169.0139$  and  $125.0238$   
289 (Figure SI-2C). These ions corresponded to  $C_7H_5O_5$  and  $C_6H_5O_3$  and to deprotonated gallic  
290 acid and trihydroxybenzene, with the latter likely generated by decarboxylation of gallic acid.  
291 The chemical formula of G5 was consistent with a hexose coupled to 5 gallic acid functions to  
292 form a pentagalate hexose. In this case, the formula would be  $C_6H_{12}O_6 + 5 \times (C_7H_6O_5 - H_2O) =$   
293  $C_{41}H_{32}O_{26}$  because each gallate function is obtained by elimination of  $H_2O$ . To confirm this,  
294 we injected the commercial 1,2,3,4,6-penta-O-galloyl- $\beta$ -D-glucopyranose in which the hexose  
295 is a glucose. This compound showed the same retention time in HPLC, the same HR-MS and  
296 MS-MS spectra and the same absorption spectrum as G5. However, the structure of the  
297 hexose was however not firmly established at this stage. Further experiments, as listed below,  
298 were required to fully confirm this.

299 G6 and G6' had the same MS and MS-MS spectra (Figure SI-3B and C). Only their  
300 retention times differed which is consistent with two isomeric compounds. In agreement with  
301 the chemical formula  $C_{48}H_{36}O_{30}$  ( $\Delta ppm = 4.1$ ) for the neutral molecules, two peaks were  
302 detected at  $m/z = 545.0593$  ( $z=2$ ) and  $1091.1252$  ( $z=1$ ) for G6 and G6'. The MS-MS on the  
303 ion 545 yielded fragments at  $m/z = 469.0537$ ,  $169.0139$  and  $125.0238$  (Figure SI-3C). G7 and  
304 G8 peaked at  $m/z = 621.0652$  ( $z=2$ ) and  $1243.1362$  ( $z=1$ ) and at  $m/z = 697.0717$  ( $z=2$ ) and  
305  $1395.1481$  ( $z=1$ ), respectively (Figure SI-4 and SI-5 B), corresponding to  $C_{55}H_{40}O_{34}$  ( $\Delta ppm =$   
306  $3.7$ ) and  $C_{62}H_{44}O_{38}$  ( $\Delta ppm = 3.6$ ) and the same fragments in MS-MS as G6 and G6' (Figure  
307 SI-4 and SI-5 C). In comparison with G5, compounds G6, G7 and G8 are likely hexa, hepta  
308 and octagalloyl glucose derivatives, respectively. As glucose contains only 5 OH functions  
309 and can only be linked to five gallic acids, the other gallic groups are evidently linked to OH

310 functions of gallate in a depside fashion. Hexa- and hepta-galloyl glucoses have previously  
311 been described [26,27]. Other galloyl glucoses with 1 or 3 gallate units which were identified  
312 in Acer species [24,32] were not found in our samples. We did not detect either methyl  
313 gallate [30] and ethyl gallate.

314

### 315 *3.3. Quantification of gallate functions by alkaline hydrolysis*

316 As the comparative HPLC analyses of water- and hydroalcoholic-RME revealed that  
317 the organic solvent offered more extractable gallate derivatives and RME1 was more potent  
318 than RME2 in the induction of HR-like reactions, we predicted that gallate derivatives were  
319 involved in PDI activity. To titrate the gallate functions, we conducted alkaline hydrolysis of  
320 RME1 and RME2 in order to convert gallate functions in gallic acid and ensure they were  
321 easily quantifiable. The protocol used involved heating the basic solutions in the absence of  
322 oxygen to avoid oxidation of the phenolic functions. The hydrolysis was first tested on pure  
323 ethyl gallate. The yield of gallic acid recovery was of 60%. The same protocol was  
324 subsequently used for RME1 and RME2. HPLC fingerprints of hydrolyzed RME1 and RME2  
325 confirmed the full elimination of G5-G8 and the formation of gallic acid. Using gallic acid as  
326 a reference in HPLC, we could determine that gallate functions accounted for 318 mg.L<sup>-1</sup> in  
327 RME1 and for 185 mg.L<sup>-1</sup> after correction for the yield of gallic acid recovering.

328

329 Using the GOD-POD method, we confirm the release of glucose following basic  
330 hydrolysis. Glucose was quantified in the solutions of extracts titrated at 0.19% of  
331 polyphenols. Absorbance values of 503 nm before and after hydrolysis indicated that the  
332 amount of formed glucose was equal to 29 mg.L<sup>-1</sup> in RME1.

333

### 334 *3.4. Quantification of gallotanins in RME1*

335 Gallate functions linked to a carbohydrate form the class of PPs named gallotanins.  
 336 The amount of the gallotanin G5 (five gallate moieties linked to a glucose sugar) was  
 337 determined using the commercial pentagalloyl glucose as a reference. This was equal to 37.9  
 338 mg.L<sup>-1</sup> in RME1 and to 12 mg.L<sup>-1</sup> in RME2 at 0.19% in PPs. In G5-G8, the absorbing  
 339 moieties are the gallate functions and as the light absorption property is additive, the  
 340 absorption coefficient,  $\epsilon$ , is expected to be linked to the number of gallate functions in all our  
 341 structures. With this in mind, we took the corrected G5 coefficient to determine the number of  
 342 gallate functions for G6-G8. This finally gave the following concentrations of galloyl  
 343 glucoses: 45 mg.L<sup>-1</sup> for G6+G6', 62 mg.L<sup>-1</sup> for G7 and 13 mg.L<sup>-1</sup> for G8 in RME1 and 7  
 344 mg.L<sup>-1</sup> for G6+G6', 62 mg.L<sup>-1</sup> for G7 and 9 mg.L<sup>-1</sup> for G8 in RME2.

345 From these values, the amount of glucose contained in G5-G8 in RME1 can be  
 346 calculated according to :

$$347 \text{ Amount of glucose} = M_{\text{glucose}} \times (m_{G5}/M_{G5} + m_{G6+G6'}/M_{G6} + m_{G7}/M_{G7} + m_{G8}/M_{G8})$$

348 where  $M_{\text{glucose}}$ ,  $M_{G5}$ ,  $M_{G6}$ ,  $M_{G7}$ ,  $M_{G8}$  are the molecular mass of glucose, G5, G6, G7 and G8,  
 349 respectively and  $m_{G5}$ ,  $m_{G6+G6'}$ ,  $m_{G7}$  and  $m_{G8}$ , the concentrations in mg/L of G5, G6, G7 and  
 350 G8. We then arrived at:

$$351 \text{ Amount of glucose} = 180 \times (m_{G5}/940 + m_{G6+G6'}/1092 + m_{G7}/1244 + m_{G8}/1396) = 25 \text{ mg.L}^{-1}.$$

352 This is very similar to the value of 29 mg.L<sup>-1</sup> found in the GOD-POD quantification of  
 353 glucose and confirms the assignment of G5 to pentagalloyl glucose.

354 Moreover, the amount of gallate functions can be also calculated using the  
 355 relationship:

$$356 \text{ Amount of gallate} = M_{\text{gallic acid}} \times (m_{G5} \times 5/M_{G5} + m_{G6+G6'} \times 6/M_{G6} + m_{G7} \times 7/M_{G7} + m_{G8} \times 8/M_{G8})$$

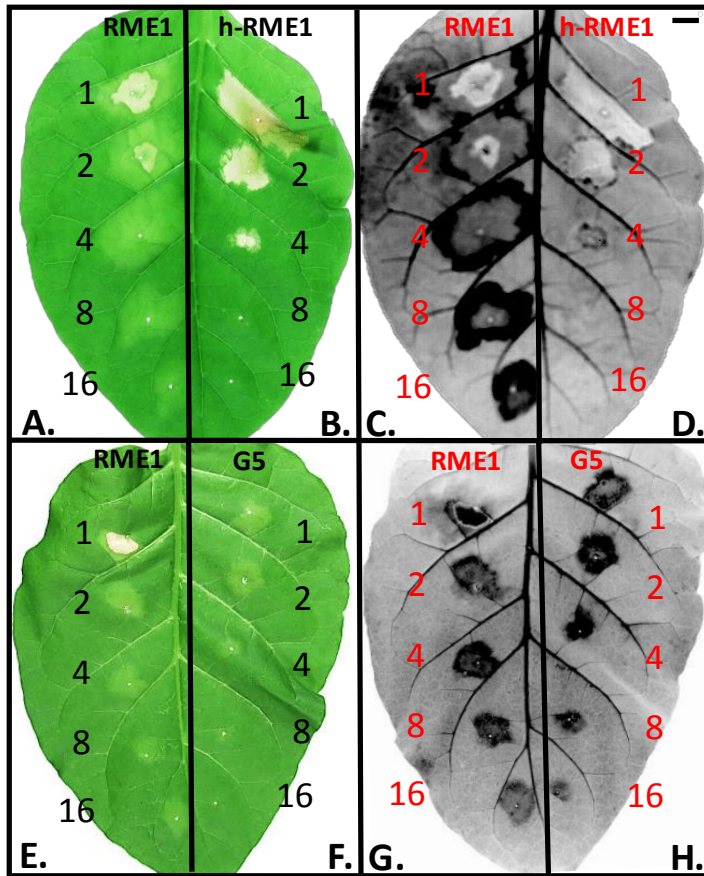
357 where  $M_{\text{gallic acid}}$  is the molecular mass of gallic acid.

358 We found 148 mg.L<sup>-1</sup>. This corresponds to 46% of the total gallate functions obtained by  
359 basic hydrolysis of RME1. In the case of RME2, we found 85 mg.L<sup>-1</sup> of gallate from the  
360 same calculation, i.e. also to 46% of total gallate functions.

361

### 362 *3.5. Suppression of topical symptoms induced by alkaline hydrolysed RME1*

363 To investigate the involvement of gallotanins in RME1-PDI activity, we looked at the  
364 comparative deployment of macroscopic symptoms on tobacco leaves at 4 dpi after  
365 infiltration of RME1 before and after hydrolysis occurred (RME1 and h-RME1, respectively).  
366 Tobacco leaves showed different levels of sensitivity to RME1 and h-RME1 (Figure 5 A-D).  
367 The h-RME1 provoked large and marked necrotic symptoms when infiltrated at the 0.19%  
368 PPs and 4- and 8- fold diluted h-RME1-PP concentrations. No distinct chlorotic zones were  
369 observed for lower h-RME1-PP concentrations (Figure 5B). The h-RME1 also failed to  
370 produce auto-fluorescent compounds within surrounding necrotic zones regardless of the h-  
371 RME1-PP concentrations (Figure 5D). These data clearly show that h-RME did not display  
372 PDI activity. We ascertain the symptomless action of gallic acid produced as a result of RME  
373 hydrolysis (Figure SI-6) and suggest that necrotic tissues observed after h-RME1 infiltration  
374 should be the result of toxicity symptoms induced by the h-RME cocktail of molecules.



375

376 Figure 5: Macroscopic symptoms induced in tobacco leaves by RME1 infiltration at 4dpi

377 before (RME1 in A,C,E,G) and after alkaline hydrolysis (h-RME1 in B,D) and pentagalloyl

378 gallate infiltration (G5 in F,H). Tobacco leaves were infiltrated with a range of PP

379 concentrations: 0.19% PP concentration (1) diluted twice (2), 4 fold (4), 8 fold (8) and 16 fold

380 (16). G5 in F,H was infiltrated at  $148 \text{ mg.L}^{-1}$  (1) and diluted following the same range.

381 Tobacco leaves were examined under bright light (A, B, E, F) and UV light (C,D,G,H). Bar

382 1.5 cm

383 To validate the HR-like reactions assay, we monitored the phytoalexin accumulation  
384 in tobacco leaves. Figure 2 shows the ratio of fluorescent scopoletin production in leaves  
385 induced at 4 dpi in response to RME1 versus control (acidified water) and h-RME1  
386 infiltrations. Since fluorescence never appeared within dead tissues, the experiment was  
387 conducted with the 2-fold diluted RME1-PP concentration that induced restricted necrotic  
388 zones. The h-RME1 infiltrated leaves produced  $105\pm 51$  ng scopoletin/gFW that was 2.9 fold  
389 lower than for the RME1-infiltrated conditions. The amount of scopoletin produced in  
390 tobacco leaves after h-RME1 infiltration was similar to the amount produced in the control  
391 leaves. These data clearly evidenced that h-RME1 was not able to induce local plant defence  
392 reactions in tobacco leaves meaning that alkaline hydrolysis which suppress gallate functions  
393 suppress PDI activity as well.

394

### 395 *3.6. PDI activity of pentagalloyl glucose*

396 The ability of the gallotannins to induce HR-like reactions was tested on tobacco leaves.  
397 Since pentagalloyl glucose (G5) was the main RME1-gallate derivative and is readily  
398 available commercially, it was infiltrated into tobacco leaves in the range  $148 \text{ mg.L}^{-1}$  -  $9.25$   
399  $\text{mg.L}^{-1}$ , with the highest concentration corresponding to the amount of G5+G6+G6'+G7+G8  
400 found in RME1. Figure 5 displays comparative RME1/G5-induced macroscopic symptoms.  
401 The infiltrated tissues were observed at 4 dpi under bright (E, F) and UV light (G, H). The  
402 G5-infiltrated zone developed dose-dependent chlorotic and auto-fluorescent areas showing  
403 that this gallotannin was bioactive and could efficiently trigger PDI activity. However, G5  
404 appears less effective than RME1 at the tested concentrations. Three hypotheses can be  
405 postulated: (i) the PDI activity was not only caused by G5-G8 but also by the other galloyl  
406 esters that are present at  $170 \text{ mg.L}^{-1}$  in RME. (ii) the PDI activity could be modulated by the  
407 content of gallate functions within the G5-G8 molecules. The G5//G6/G7/G8 potential to

408 induce macroscopic symptoms should be comparatively investigated. (iii) RME1 could also  
409 contain others PDI active ingredients not identified herein and the cocktail of biomolecules in  
410 RME1 could maximize the PDI activity.

411

### 412 3.7. *Acer leaf extracts and gallotannins as PDI*

413 The PDI activity of RME involved hypersensitive reaction-like lesions, accumulation  
414 of scopoletin, and the overexpression of the antimicrobial *PR1*,  $\alpha$ -1,3-glucanase *PR2*,  
415 chitinase *PR3*, and osmotin *PR5* encoding genes. The crude extract induced expression of the  
416 set of PR that are induced by salicylic acid (SA) and should then activate the SAR pathway  
417 [20,36]. The crude extracts are enriched in gallotannins that appear to be the prominent RME  
418 active ingredients. Tannins are ubiquitous chemical defence components in plants and act as  
419 plant antioxidants. Structurally, the high content of aromatic hydroxyl groups provides free-  
420 radical scavengers to module cell redox balance [37]. Tannin accumulation is correlated with  
421 antimicrobial properties and resistance against pathogens [38]. The present work  
422 demonstrates that pentagallates and hydrolysable tannins as evidenced here, could participate  
423 in the activation of plant defences in tobacco. A previous report has shown that exogenous  
424 application of ellagitannin, i.e. the 1-0-galloyl-2,3;4,6-bis-hexahydroxydiphenoyl- $\beta$ -D-  
425 glucopyranose elicits plant defence responses on strawberry and lemon plants leading to  
426 systemic protection against the virulent pathogen M11 and *Xanthomonas*, respectively [39].  
427 Phenolics other than galloylglucoses have been involved in induction of plant defence  
428 reactions. The mediator of SAR pathway, SA, is the most ubiquitous phenolic that acts  
429 downstream of elicitor recognition [9-12]. Interestingly, our group reported the PDI properties  
430 of alkyl gallates which activate the SAR pathway upon exogenous treatment of tobacco plants  
431 [21]. Since alkyl gallates and gallotannins were both inducers of the SAR pathway, it suggests  
432 that the galloyl functions could play the central role in the activation of plant defence

433 reactions. It should therefore be determined whether galloyl compounds directly participate in  
434 the activation of plant defence as either inducers or mediators of the response. An indirect  
435 action of the galloyl compounds through the modulation of events such as the redox potential  
436 cannot be ruled out.

437         A wide range of structurally different compounds have been shown to have the ability  
438 to induce plant defence reactions. The non-specific elicitors are structurally diverse  
439 compounds such as proteins, peptides, oligosaccharides, lipids. Most of them are derived from  
440 plants or pathogen cell surfaces [40]. Here we propose the use of natural substances from low-  
441 value raw materials provided by red maple (*Acer rubrum*) trees which are widespread  
442 deciduous trees through Eastern North America and cultivated in Europe as ornamental trees.  
443 The galloyl ester groups and the  $\beta$ -D-glucose galloyl derivatives reviewed by Haddock et al.  
444 (1982) are abundant in many plant families [23]. The wide distribution of these gallate  
445 derivatives across plants constitutes a rather advantageous lead for the development of the  
446 galloyl-enriched PDI [41].

447

448

#### 449 **4. Conclusions**

450         The paper describes an original, strong and reliable chemical methodology to detect  
451 the galloyl-active ingredients from a complex mixture of biomolecules. Discovered here as  
452 bioactive ingredients in RME and easily quantifiable by chemical methodology, these natural  
453 molecules could offer a tremendous tool to screen plant or crude by-products extracts with  
454 potential PDI activity. Future investigations will define the most suitable and abundant  
455 galloyl bioproducts and the optimum efficiency for controlling the incidence of diseases in  
456 crops.

457

458 **Supplementary Materials:** a graphical abstract, a supporting information file (11 pages; 6  
459 figures): Figure SI-1 : UPLC-HR-MS chromatogram of RME1 extract. Upper view for UV  
460 detection and bottom view for TIC detection. Figure SI-2: UPLC-HR- MS data for  
461 pentagallate glucose (G5). Figure SI-3: UPLC-HR-MS data for hexagallate glucose (G6 and  
462 G6'). Figure SI-4: UPLC-HR-MS data for heptagallate glucose (G7). Figure SI-5: UPLC-HR-  
463 MS data for octagallate glucose (G8). Figure SI-6: Macroscopic symptoms induced by gallic  
464 acid infiltration into tobacco leaves.

465  
466 **Funding:** This work was supported by grants from the private company Roullier (Saint-Malo,  
467 France) and the Auvergne Rhone-Alpes region.

468  
469 **Acknowledgments:** P.G and C.R. thank the private company Roullier (Saint-Malo, France)  
470 and Auvergne Rhône-Alpes region for their financial support “Pack Ambition Research” and  
471 the LIT “Laboratoire d’Innovation Territorial”. The authors thank Céline Sac and Amélie  
472 Couston for help with tobacco plant cultures and laboratory assistance and Dominique  
473 Marcon for technical assistance in photographic editing.

474  
475 **Author contributions:** Conceptualization, P.G., C.R., A.T.; Data curation, P.G., C.R.;  
476 Formal analysis, P.G. and C.R.; Investigation, E.P., S.H., A.G., P.G., C.R.; Methodology,  
477 P.G., C.R., M.S.; Supervision, P.G., C.R., M.S.; Writing, Original draft, P.G., C.R., E.P.;  
478 Writing-Review and editing, P.G., C.R, A.K.; Funding acquisition, P.G., C.R., H.E., A.K.; All  
479 authors have read and agreed to the published version of the manuscript.

480

481 **Conflicts of interest:** The authors declare no conflict of interest.

482

483

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