

1 **Title:** Alkyl gallates display elicitor activities in tobacco plants

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3 **Running title:** Alkyl gallates in plant defence

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5 **Authors:** Pascale Goupil^{1,2} *, Razik Benouaret^{1,2}, Claire Richard^{3,4}

6

7 **Affiliations:**

8 ¹Université Clermont Auvergne, UMR 547-UBP/INRA PIAF, Campus universitaire des Cézeaux, 8 avenue
9 Blaise Pascal, 63178 Aubière cedex, France,

10 ²INRA, UMR 547 PIAF, Clermont-Ferrand, France

11 ³Université Clermont Auvergne, CNRS, UMR 6296, Campus universitaire des Cézeaux, Institut de Chimie de
12 Clermont-Ferrand (ICCF), 24 avenue Blaise Pascal, 63178 Aubière cedex, France

13 ⁴Equipe Photochimie CNRS, UMR 6296, ICCF, 63178 Aubière, France

14

15 *Author for correspondence:

16 *Pascale Goupil*

17 *Phone: (33) 04 73 40 79 40*

18 *Fax: (33) 04 73 40 79 42*

19 *E-mail: pascale.goupil@uca.fr*

20

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25 **Authors' e-mails:** *pascale.goupil@uca.fr; rbenouaret@vertal.fr; claire.richard@uca.fr*

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27 **Highlights**

28

29 – Alkyl gallates elicited defence reactions in tobacco

30 – Alkyl gallates induced local biochemical changes in tobacco leaves

31 – Alkyl gallates caused modification of plasma membrane properties

32 – Ethyl gallate led to defence transcript accumulation and dose-dependent cell death associated with
33 hypersensitive response

34 – Alkyl gallates are novel elicitor agents well-suited to crop protection schemes.

35

36 **Abstract**

37

38 Alkyl gallates showed elicitor activities on tobacco in both whole plants and cell suspensions. Methyl gallate
39 (MG), ethyl gallate (EG) and *n*-propyl gallate (PG) infiltration into tobacco leaves induced hypersensitive
40 reaction-like lesions and topical production of autofluorescent compounds revealed under UV light. When
41 sprayed on tobacco plants at 5 mM, EG promoted upregulation of defence-related genes such as the
42 antimicrobial *PR1*, β -1,3-glucanase *PR2*, chitinase *PR3* and osmotin *PR5* target genes. Tobacco BY-2 cells
43 challenged with EG underwent cell death in 48 h, significantly reduced in the presence of the protease inhibitor
44 aprotinin. The three alkyl gallates all caused alkalinisation of the BY-2 extracellular medium, whereas gallic acid
45 did not trigger any pH variation. Using EGTA or LaCl_3 , we showed that Ca^{2+} mobilisation occurred in BY-2
46 cells elicited with EG. Overall, our findings are the first evidence of alkyl gallate elicitor properties with early
47 perception events on plasma membrane, potential hypersensitive reactions and PR-related downstream defence
48 responses in tobacco.

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50

51 **Keywords:** Alkyl gallates, BY-2, Elicitors, Ethyl gallate, Pathogenesis-related protein genes, Plant defence
52 reactions, Tobacco

53 **Introduction**

54 Open-field crops are always vulnerable to pathogens, and so extensive farming requires constant control of
55 pathogen populations. Synthetic pesticides are commonly used for direct pest control, but need repeated
56 spreading for season-long protection. Pesticide action is mostly selective and requires combinations of active
57 compounds in an integrated pest management approach. A promising alternative that emerged in the 1990s is
58 based primarily on improving the endogenous defence capabilities of plants. When plants are attacked by
59 pathogens, they defend themselves with an arsenal of defence mechanisms. These active plant defence responses
60 comprise three successive events: (i) recognition of the pathogen, (ii) signal transduction, and (iii) execution of
61 the defence programmes with activation of defence genes (Fu and Dong, 2013). The recognition events involve
62 pathogen- or plant-derived signalling entities termed elicitors (Choi and Klessig, 2016). Intracellular signal
63 conversion and transduction include the very early elicitor-induced signalling processes such as protein
64 phosphorylation, influx of Ca^{2+} , efflux of H^+ , K^+ and Cl^- leading to extracellular alkalinisation, depolarisation of
65 the plasma membrane, and active oxygen species production, culminating in the hypersensitive response, a
66 localised cell suicide (Yang *et al.*, 2011). Ultimately, the pathogen challenge leads to the production of
67 phytoalexins and pathogenesis-related proteins, which prevent pathogen development. Point inoculation of the
68 pathogen, in addition to triggering the local acquired resistance response (LAR), can mobilise systemic acquired
69 resistance (SAR), which alerts and predisposes the tissues to a more intensive response on subsequent infection
70 (Kachroo and Robin, 2013; Fu and Dong, 2013). Salicylic acid (SA) plays a crucial role in the SAR induction
71 processes. SA is involved in the signal transduction systems by activating enzyme expression catalysing
72 biosynthetic reactions to produce defence compounds (Chen *et al.*, 2009; Boatwright and Pajeroska-Mukhtar,
73 2013) and provide protection for plants against pathogens.

74 As it is pathogenesis that initiates SAR, impact on disease is lower than if the SAR response had been activated
75 before the arrival of the pathogen. The use of plant defence inducers (PDIs) to establish SAR intentionally and
76 preventively in plants are thus currently a promising strategy in phytoprotection. PDIs are organic, inorganic,
77 botanical or synthetic stimulus-triggering factors (elicitors) that mobilise plant defence reactions (Walters *et al.*,
78 2013; Lyon, 2014). By stimulating plant immunity, PDIs provide defence against subsequent infection (Gozzo
79 and Faoro, 2013; Walters *et al.*, 2013). To date, only very few substances with high SAR activity have been
80 described (Gozzo and Faoro, 2013), and they mostly combine PDI and pesticide actions, not necessarily a
81 disadvantage since both properties are inimical to pathogens (Dewen *et al.*, 2017).

82 Agricultural applications of plant activators are far from valued (Gozzo and Faoro, 2013). The synthetic
83 agrochemicals now available are SA-like substances that mimic the action of this phytohormone. Of these, 2,6-
84 dichloro isonicotinic acid (INA) and acibenzolar-*S*-methyl (BTH) and their derivatives are the most thoroughly
85 studied, currently marketed as BOOST®, Bion® and Actigard™. These resistance activators serve as efficient
86 chemical inducers of SAR, with effectiveness against diverse plant diseases (Bektas and Eulgen, 2015; Dufour
87 *et al.*, 2016).

88 The identification of new chemicals able to induce disease resistance is beneficial not only for elucidating the
89 pathways leading to SAR, but also for developing new plant protection agents. The effectiveness of various
90 elicitors depends on plant taxonomy, and there are many examples where an elicitor is more effective on some
91 plant species than on others (Lyon, 2014). We have been engaged in seeking naturally-occurring or synthetic
92 substances able to induce SAR reactions. As part of this research we evaluated the PDI activity of alkyl gallates
93 (3,4,5-trihydroxybenzoates) on tobacco plants. Gallic acid derivatives had already been found to display
94 antimicrobial properties. Octyl, nonyl and lauryl gallates exhibit fungicidal and bacterial activity (Kubo *et al.*,
95 2002; Leal *et al.*, 2009). Silva *et al.* (2013) showed their potential for use in disease management, evaluating the
96 inhibitory activity of a set of gallic acid alkyl esters on the plant pathogenic bacterium *Xanthomonas citri*. Here
97 we demonstrate for the first time that alkyl gallates act at various points in the signalling pathway leading to
98 defence reactions. We report evidence that treatment of tobacco with methyl, ethyl and *n*-propyl gallates induces
99 hypersensitive-like reactions with activation of defence-related genes in the tobacco plant. These gallic acid
100 derivatives trigger rapid extracellular alkalinisation of BY-2 plant cells with Ca²⁺ mobilisation, suggesting that
101 tobacco cells perceive the chemicals by modifying plasma membrane physical properties. Finally, the naturally-
102 occurring and semisynthetic gallic acid derivatives revealed potent PDI actions, revealing a valuable source of
103 compounds that could find use in green agriculture practices.

104

105 **Materials and Methods**

106 *Biological compounds and chemicals*

107 Methyl gallate (MG), ethyl gallate (EG), *n*-propyl gallate (PG), salicylic acid (SA), and gallic acid (GA) were
108 purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). All the compounds were dissolved in ultrapure
109 water (MilliQ). PG needed warm (25 °C) ultrapure water to dissolve completely.

110

111 *Plant material and treatments*

112 The biological activity of alkyl gallates was measured on 3-month old tobacco plants (*Nicotiana tabacum* L.)
113 bearing 20–22 leaves (preflowering stage). Tobacco plants were grown in a greenhouse under controlled
114 conditions (22 ± 5 °C with a photoperiod of 16 h light). The chemical solutions to be tested (50 μ l) were
115 infiltrated into the leaf tissue using a plastic syringe (without a needle), on an area of 1–2 cm². As a positive
116 control, leaves were infiltrated with 2 mM SA. For the negative control, leaves were infiltrated with ultrapure
117 water. The compounds were infiltrated into distinct areas on the same leaf for the examination of macroscopic
118 symptoms under bright field or UV light (at 312 nm). For PR transcript quantification, three mature leaves were
119 sprayed on both their upper (adaxial) and lower (abaxial) surfaces. Foliar spray treatments were administered
120 with a fine atomiser until the solutions dripped (3 mL per leaf on average). The negative control was leaves
121 sprayed with ultrapure water.

122 Tobacco cell suspensions of *Nicotiana tabacum* L. cv. Bright Yellow-2 (BY-2) were grown in MS medium
123 (Duchefa, Netherlands) supplemented with sucrose (30 g.L⁻¹), thiamine (1 mg.L⁻¹), myo-inositol (102 mg.L⁻¹)
124 and 2,4-dichlorophenoxyacetic acid (0.2 mg.L⁻¹) at pH 5.8. Cells were agitated in the dark (140 rpm, 25 °C) and
125 subcultured weekly by dilution at a 1:15 ratio in fresh medium. For determination of extracellular pH changes,
126 cells from culture in exponential-phase growth were collected by filtration, washed with 175 mM mannitol, 0.5
127 mM CaCl₂, 0.25 mM MgCl₂, 1 mM KCl and 1 mM sucrose, and resuspended at 0.1 g fresh weight (FW)/mL in
128 the same medium. The initial pH was about 4.8. For transcript accumulation analysis, cells were collected by
129 filtration, and resuspended at 0.3 g FW/mL in fresh MS medium using open 6-well microplates (Greiner Bio-
130 one, Germany). Cells were first equilibrated for 2 h with continuous stirring before elicitation with alkyl gallates.

131

132 *Transcription levels of defence genes*

133 The patterns of PR gene expression were analysed by real-time quantitative PCR (RT-qPCR) using SYBR-green
134 and the iCycler iQv5 (Bio-Rad). Total RNA was extracted from tobacco leaf tissue (200 mg) in
135 cetyltrimethylammonium bromide extraction buffer, as described by Chang *et al.* (1993). Total RNA was
136 cleaned up with 0.5 U DNase I solution (Euromedex, France) containing 20 U RNase inhibitor (Euromedex,
137 France). RNA integrity was verified on a 1% agarose gel by detecting ribosomal RNA. RNA concentrations

138 were measured spectrophotometrically at 260 nm. First-strand cDNA was synthesised from up to 1 μg of total
139 RNA with Euroscript Reverse Transcriptase (Eurogentec, France) according to the manufacturer's instructions.
140 PCR reactions were prepared using the qPCR kit Mastermix for SYBR green (Eurogentec) according to the
141 manufacturer's protocol. The cDNA concentration used produced a threshold cycle value (C_T) of between 15 and
142 30 cycles. Amplification specificity was checked by melting-curve analysis. The relative quantity (Q_R) of PR
143 gene transcripts using the EF-1 α gene (Peng *et al.*, 2004) as internal standard was calculated with the delta-delta
144 mathematical model (Pfaffl 2001). Values are given as $\log_2 Q_R$. For every PR gene analysed, three independent
145 biological replicates were run, and every run was carried out at least in triplicate. Primers and amplicon sizes are
146 given in Benouaret and Goupil (2015).

147

148 *BY-2 Extracellular alkalinisation*

149 The pH variations of the culture medium were recorded by introducing a glass microelectrode (Hanna
150 Instrument, HI1330B, France) into 6 ml of the equilibrated cell suspension culture. The pH was measured every
151 10 min starting immediately after treatment with elicitors or ultrapure water (negative control). The ΔpH was the
152 pH measured every 10 min relative to the pH measured at t_0 immediately after adding elicitors or ultrapure
153 water. Each experiment was repeated at least three times to check reproducibility. Figures depict the results of
154 typical experiments. Pharmacological treatments were conducted using the chelator EGTA (2 mM) and the Ca^{2+}
155 channel blocker LaCl_3 (2 mM).

156

157 *Cell death measurement*

158 The Evans Blue assay was performed according to Amano *et al.* (2003) using a plastic syringe device. The
159 pigment enters cells with damaged plasmalemma. The assay was first calibrated with a set of viability standards
160 using mixtures of living and dead tobacco BY-2 cells (Benouaret *et al.*, 2014). Tobacco cells (500 μL) were
161 withdrawn from the culture medium immediately (t_0) and then 24 h after elicitation. Pharmacological treatment
162 was conducted with the serine protease inhibitor aprotinin (0.2 μM) 30 min before cell death rate determination.
163 Absorbance at 600 nm was measured to estimate cell death. Each experiment was repeated three times with three
164 independent BY-2 cultures.

165

166 **Results**

167

168 *Macroscopic changes of tobacco tissues challenged with alkyl gallates*

169 Foliar tobacco tissues were infiltrated with the three alkyl gallates (Fig. 1) at 5 mM, and the macroscopic
170 changes were examined under bright and UV light. Fig. 2 shows the kinetics of appearance of macroscopic
171 symptoms on the abaxial face of tobacco leaves exposed to bright light (Fig. 2A-D) and UV light (Fig. 2E-H).
172 UV light examination ($\lambda = 312$ nm) revealed that the methyl gallate (MG)-, ethyl gallate (EG)- and *n*-propyl
173 gallate (PG)-infiltrated zones and the surrounding tissues displayed autofluorescence indicative of the
174 accumulation of phenolic compounds (Fig. 2F-H). At 2 and 4 dpi, tobacco mesophyll infiltrated with MG was
175 unchanged under bright light, but the chemical induced autofluorescence in the surrounding tissues. A restricted
176 chlorotic zone appeared at 8 dpi without production of fluorescent compounds. By contrast, the EG-infiltrated
177 tissues turned chlorotic by 4 dpi and tissue injury was enlarged at 8 dpi. The surrounding zone was loaded with
178 phenolic compounds. The PG-infiltrated tissues showed the most pronounced signs, with restricted brown areas
179 at 2 dpi and a brown extended zone at 4 and 8 dpi. Examination of the leaf tissue by UV light revealed that the
180 necrotic zone was surrounded by a scattered pattern of fluorescence. Overall, tobacco leaves were receptive to
181 the three alkyl gallates tested. Since the alkyl gallates themselves did not emit fluorescence (Fig. 2E), these
182 results evidence that the chemical compounds induced marked metabolic changes over the interveinal areas of
183 the leaves. The naturally-occurring EG was retained for further work, since it proved highly active in inducing
184 accumulation of autofluorescent compounds.

185 A dose-response experiment was undertaken on EG-infiltrated tobacco tissues. Macroscopic symptoms were
186 examined under bright and UV light for EG concentrations ranging from 5 mM to 0.5 mM. Fig. 3 shows the
187 infiltrated areas at 4 dpi. The 5 mM-infiltrated zone appeared slightly chlorotic and displayed a large fluorescent
188 surrounding area. Expansion of autofluorescence was induced dose-dependently. EG infiltration at 2 mM
189 resulted in faint autofluorescence, and at 1 mM had no effect. As expected, SA (2 mM) induced a necrotic area
190 with local accumulation of fluorescent compounds.

191

192 *Responses of cultured cells to alkyl gallates*

193 We used medium alkalinisation as a readily measurable effect on suspension-cultured BY-2 cells to test the
194 potential elicitor activity of alkyl gallates. As shown in Fig. 4A, alkalinisation of the culture medium in response
195 to AG was evidenced for the 1 mM and 5 mM concentrations. Suspended cells responded to AG by a rapid,
196 sustained alkalinisation of the incubation medium. Extracellular alkalinisation was detected immediately after
197 adding the AG. The three alkyl gallates induced a Δ pH reaching 0.8 to 1.1 units at 1 mM and 1.5 to 1.8 units at
198 5 mM after 150 min. Fig. 4B shows that the extent of pH variation was dose-dependent in the concentration
199 range used for EG. The threshold value was not reached in our experimental system; the lowest concentration
200 (0.5 mM) could still cause a pH change of 0.8 units. As expected, no pH shift was detected with the control cells
201 treated with water.

202 The calcium mobilisation in EG-induced pH changes was investigated by adding the chelator agent EGTA
203 (2 mM) and the Ca^{2+} -channel blocker LaCl_3 (2 mM) to the culture medium concomitantly with the EG. Both
204 chemicals radically suppressed the pH shift. Adding the chemicals during the alkalinisation phase, i.e. 30 min
205 after elicitation, resulted in rapid abolition of the pH rise. EDTA and LaCl_3 had an inverse effect on the
206 extracellular pH medium in control cells, with slight medium acidification (Fig. 4C).

207 The eliciting activity of gallic acid (GA) was measured by medium pH determination. Addition of GA at 0.5 mM
208 and 5 mM to the BY-2 cell suspension did not trigger any pH variation in the extracellular medium, suggesting
209 that the compound was not perceived by the tobacco cells, at least as noted above (Fig. 4D).

210 The EG-triggered hypersensitive-like response was investigated in BY-2 cells using the Evans blue test (Fig. 5).
211 Cell death rate was measured in tobacco cells exposed to EG at 0.5 mM, 2 mM and 5 mM for 48 h (Fig. 5A).
212 Cell death rate was 10% on average in control cells (without EG). The EG elicitation induced significantly
213 higher cell death. The exposure of tobacco cells to 2 mM and 5 mM EG increased the magnitude of the cell
214 death rate, which reached 25% and 45% respectively. The action of the plant cell death inhibitor aprotinin was
215 examined on 5 mM EG-elicited cells. As shown in Fig. 5B, the protease inhibitor lowered cell death rate by
216 19%, whereas the inhibitor did not affect cell viability in control tobacco cells.

217

218 *Ethyl gallate-induced PR transcript accumulation*

219 The ability of EG to induce defence-related gene expression was investigated in tobacco plants. Transcript levels
220 of four PR target genes were quantified by quantitative real-time PCR in EG-treated leaves 4 days after spraying.

221 At 1 mM, EG failed to induce significant transcript accumulation of the four PR markers. At 5 mM, EG led to
222 high PR transcript accumulation: 60-fold for PR1, 50-fold for PR2, 35-fold for PR3, and 30-fold for PR5, on
223 average (Fig. 6).

224

225 **Discussion**

226 The present work demonstrates the ability of three alkyl gallates to elicit plant defence reactions. Using tobacco
227 as a plant model, we show the potential of methyl gallate (MG), ethyl gallate (EG) and *n*-propyl gallate (PG) to
228 trigger local injuries, biochemical changes and PR transcript accumulation. Infiltration of tobacco leaves with the
229 chemicals resulted in foliar symptoms and local accumulation of fluorescent compounds. The three compounds
230 induced restricted chlorotic tissues visualised under bright light, with production of phenolics in the surrounding
231 areas, suggesting cell death initiation of the mesophyll cells. The topical reactions induced by the three alkyl
232 gallates were of different severity in terms of tissue injury and production of phytoalexin-like entities. The fact
233 that the intensity of effects varied with the alkyl gallates (MG < EG < PG) suggests that the compounds were
234 active to different degrees at the 5 mM concentration, or that their modes of action differed in the necrotic
235 reaction associated with cell death. EG and PG seemed to be the most potent inducers of local cell death in our
236 experiment. Localised cell death in plant tissue resulting from attack by pathogens can occur either in
237 hypersensitive resistant response or in harmful reactions such as necrosis potentially caused by toxins (Iakimova
238 *et al.*, 2005). We demonstrate here that BY-2 cell death is sensitive to protease inhibitor, suggesting that
239 infiltrated tobacco tissues may evolve towards hypersensitive status. However, phytotoxic reactions caused by
240 alkyl gallate cannot be ruled out.

241 Rapid changes in membrane permeability are involved in plant defence-associated early responses to various
242 biotic elicitors (Garcia-Brugger *et al.*, 2006). Increased influx of protons and calcium mobilisation are both early
243 events in the elicitor signalling network. Perception of alkyl gallates visualised by the pH rise in the incubation
244 medium was evidenced on BY-2 cells. Medium alkalinisation is one of the very early events occurring in
245 elicitor-binding sites in plant cells (Zhao *et al.*, 2005), and results from elicitor-induced depolarisation of the
246 plasma membrane with subsequent K⁺/H⁺ exchanges and Ca²⁺ influx/Cl⁻ efflux. In our experiments, the effect of
247 Ca²⁺ influx inhibitors EGTA or LaCl₃ caused the decreased pH shift of the extracellular medium, suggesting that
248 Ca²⁺ mobilisation occurred in BY-2 cells elicited with alkyl gallates. As a consequence of ion fluxes, a predictive
249 cytoplasmic acidification should take place rapidly in BY-2 cells. Cytoplasmic acidification is regarded as an

250 essential step in signal transduction, leading to early defence gene expression and secondary metabolite
251 biosynthesis (Sakano, 2001).

252 Kinetics of extracellular pH changes vary among elicitors, with two main different behaviours towards cells.
253 According to the nature of the elicitor, the perception can induce a long-sustained pH rise, or the pH variation
254 can be transient, with a return to the resting value. It is noteworthy that the modification of H⁺ flux in BY-2 cells
255 following treatment by alkyl gallates was similar to the defence response elicited by a polyphenolic-enriched
256 grape extract (Benouaret *et al.*, 2014). The induced H⁺ flux modification lasting 150 min presented similar
257 characteristics in the respective time course of the effect. This result suggests that the underlying processes are
258 basically the same. EG is a naturally-occurring gallic acid derivative found in several plant sources and identified
259 in wines from different varieties of *Vitis vinifera* (Monagas *et al.*, 2005). It was not detected in a red winery by-
260 product extract (data not shown), suggesting that this biomolecule is not active in grape marc extract.

261 Alkyl gallates are antioxidants. One of the earliest cellular responses following elicitor recognition is oxidative
262 burst with production of active oxygen species (AOS). Despite their damaging effect, AOS are well-described
263 second messengers producing plant responses. Whether AOS will act as a destructor or as a signalling entity
264 depends on the equilibrium between AOS production and scavenging. Low concentrations of AOS act as
265 signalling entities mediating the plant responses: however, high concentrations cause acute damage to cell
266 components (Sharma *et al.*, 2012). In elicitor-challenged cells, disruption in cellular redox homeostasis appear to
267 switch on a signalling cascade leading to programmed cell death, encountered during hypersensitive response
268 (HR) (Van Breusegem and Dat, 2006). Using pharmacological approaches, our group came near to the
269 hypersensitive status of BY-2 cells that led to cell death on EG treatment. In this BY-2 system, the antioxidant
270 gallates acting as free radical scavengers could subsequently help to reduce physiological AOS equilibrium and
271 induce HR-related cell death. The potential ability of phenolics to induce HR-related cell death has been
272 previously demonstrated with polyphenolic-enriched grape marc extracts (Benouaret *et al.*, 2014). Even though
273 EG was not detected in the grape marc extracts, it can be assumed that the antioxidant properties of
274 polyphenolics could be involved in HR establishment. Investigation of AOS as potential agents with
275 pharmacological approaches will help to characterise the pathways leading to the hypersensitive status of EG-
276 induced cell death in tobacco.

277 Alkyl gallates evidently activate the SAR pathway, since they induced expression of the same set of SAR genes,
278 i.e. *PR1*, *PR2*, *PR3* and *PR5*, that are induced by SA (Cordelier *et al.*, 2003). Numerous synthetic and/or bio-

279 based elicitors can induce plant immune responses. The most marked chemical ones, acibenzolar-*S*-methyl
280 (ASM) and 2,6-dichloroisonicotinic acid (INA), both induce SAR without SA accumulation; probenazole (PBZ)
281 induces SAR through SA biosynthesis (Bektas *et al.*, 2015; Burketova *et al.*, 2015). SA involvement in alkyl
282 gallate-regulated plant defences and the potential of these compounds to protect cultivated crops are being
283 investigated in laboratory studies.

284 In conclusion, the present work offers evidence for plant immunity inducer properties of alkyl gallates. Plants
285 can synthesise a wide range of phenolics as an integral part of their non-host resistance strategy. These
286 secondary metabolites are natural defensive antibiotics, ensuring efficient chemical barriers to pathogens. Their
287 elicitation properties are demonstrated here for the first time in alkyl gallates, which emerge as PDI agents suited
288 to phytoprotection. Being naturally-occurring and ubiquitous, EG is the most promising of these agents. It is
289 potentially non-toxic for the human being, and offers consumer benefits, with antioxidant, anti-inflammatory and
290 anticarcinogenic activities. Given that EG can play a dual role in plant protection, as it also possesses direct
291 antimicrobial activities, this biocontrol agent constitutes a potent product well-suited to environmentally-oriented
292 schemes for high crop protection.

293

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297

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371 **Figure captions**

372 Fig. 2 and Fig. 3 to be in colour on-line only

373

374 **Fig. 1.** Structures of the active ingredients studied

375

376 **Fig. 2.** Macroscopic symptoms induced in tobacco leaves by methyl gallate (MG), ethyl gallate (EG) and *n*-
377 propyl gallate (PG) infiltration observed under bright field (A-D) and UV light (E-H) at 0 dpi (A,E), 2 dpi (B,F),
378 4 dpi (C,G) and 8 dpi (D,H). Alkyl gallates were infiltrated at 5 mM. Bars 2 cm

379

380 **Fig. 3.** Macroscopic symptoms induced in tobacco leaves by EG infiltration observed under bright field (A) and
381 UV light (B) at 4 dpi. Tobacco leaves were infiltrated with different EG concentrations: 5 mM (1), 4 mM (2), 3
382 mM (3), 2 mM (4), 1 mM (5) and 0.5 mM (6), ultrapure water (control), 2 mM SA. Bars 1 cm

383

384 **Fig. 4.** Extracellular alkalinisation in tobacco BY-2 suspension cells over time elicited with methyl gallate (MG),
385 ethyl gallate (EG), and *n*-propyl gallate (PG). A. Alkyl gallates used at 5 and 1 mM, B. EG in the presence of
386 Ca²⁺ inhibitors. EGTA and LaCl₃ at 2 mM were added to the BY-2 medium at the onset of elicitation or at 30
387 min (arrow), C. EG added at 0.5 to 5 mM, D. Gallic acid (GA) and EG used at 5 and 0.5 mM. The control is
388 water. The data are from representative experiments out of a total of five.

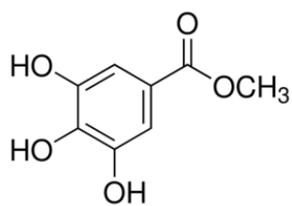
389

390 **Fig. 5.** Cell death rate assessed by Evans Blue staining of tobacco BY-2 cell suspension treated with ethyl gallate
391 (EG) at 0.5, 2 and 5 mM for 48 h. The protease inhibitor aprotinin was added 30 min before cell death
392 measurement in BY-2 cells treated with water (control) or 5 mM EG. Values are expressed relative to control
393 (water treatment) values. The experiments were performed in triplicate with three different BY-2 cultures. Bars
394 represent means ±SEM. Letters indicate significant differences between treated and control samples according to
395 one-way ANOVA followed by LSD test ($p \leq 0.05$). Bars represent the mean values ± SE, $p \leq 0.05$.

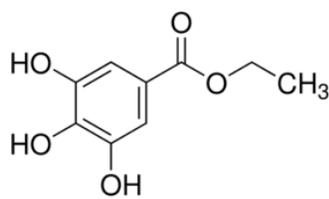
396

397 **Fig. 6.** PR transcript accumulation in tobacco leaves 4 days after 2 mM SA (positive control) or 1 and 5 mM
398 ethyl gallate (EG) spraying. Transcripts were quantified by real-time RT-PCR in the sprayed leaves. Values are
399 expressed relative to control (water treatment) values. Three leaves per plant were treated for each experiment.
400 Each independent experiment was performed in triplicate. Bars represent means \pm SEM. Letters indicate
401 significant differences between treated and control samples according to one-way ANOVA followed by LSD test
402 ($p \leq 0.05$).

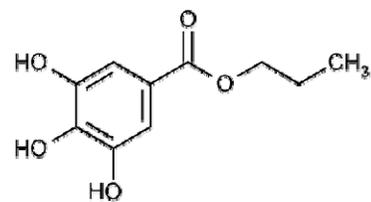
Fig.1



Methyl gallate



Ethyl gallate



Propyl gallate

Fig.2

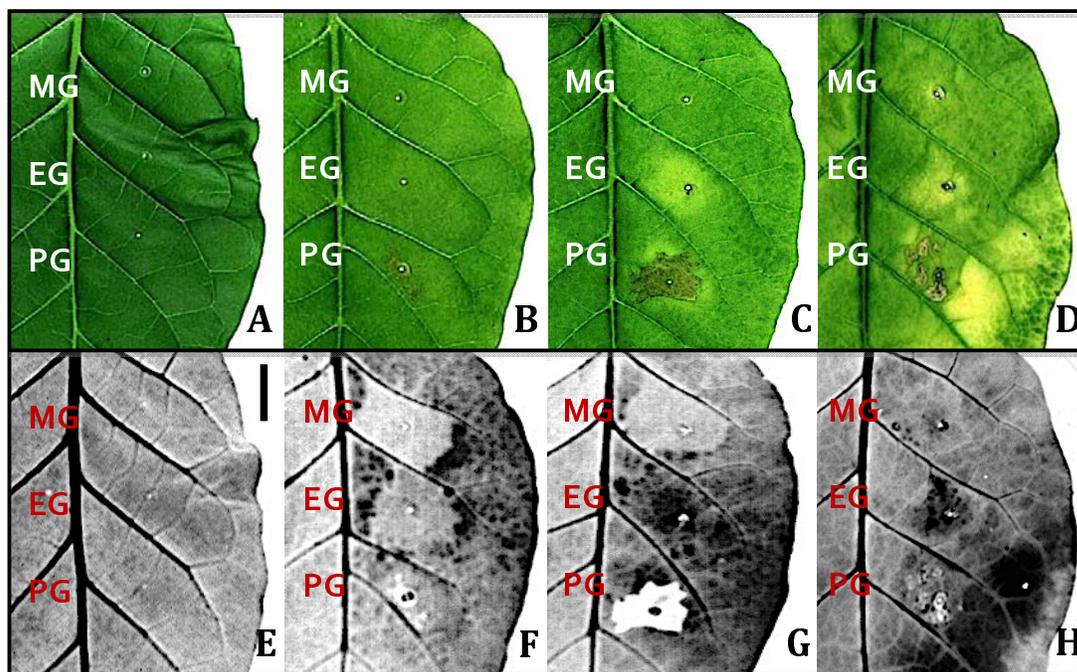


Fig.3

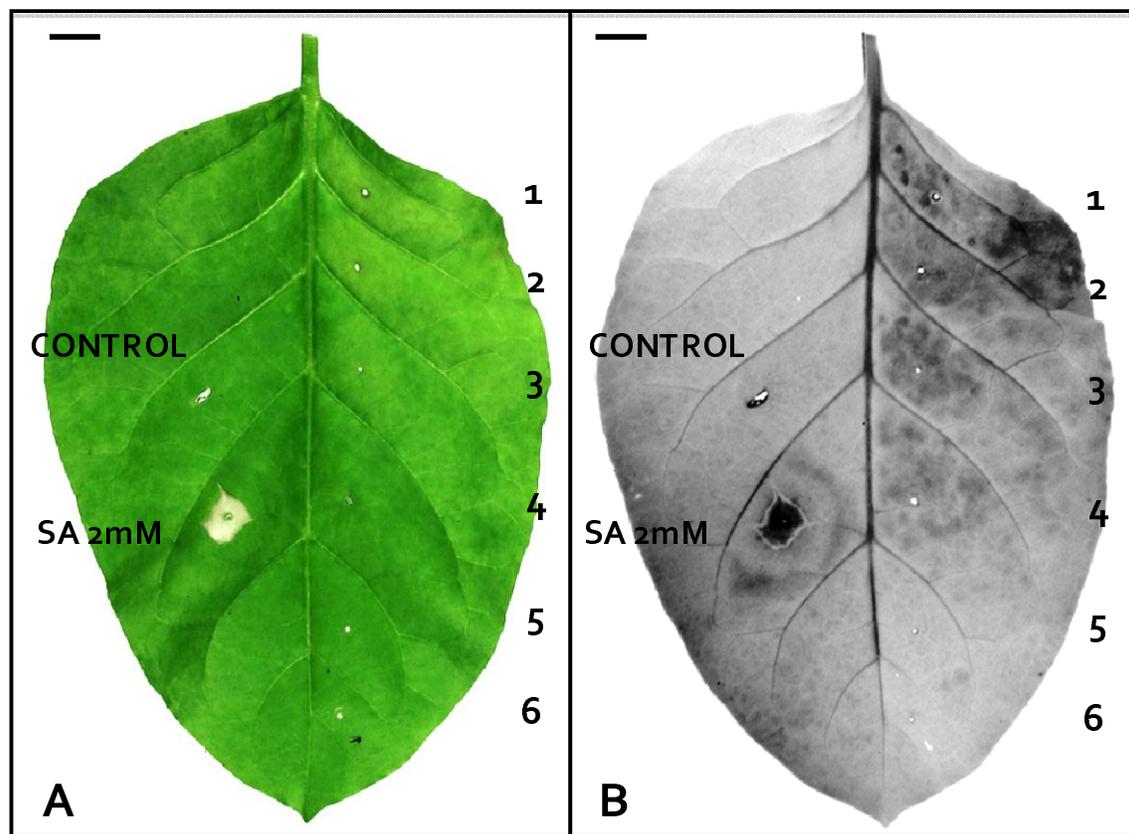


Fig.4

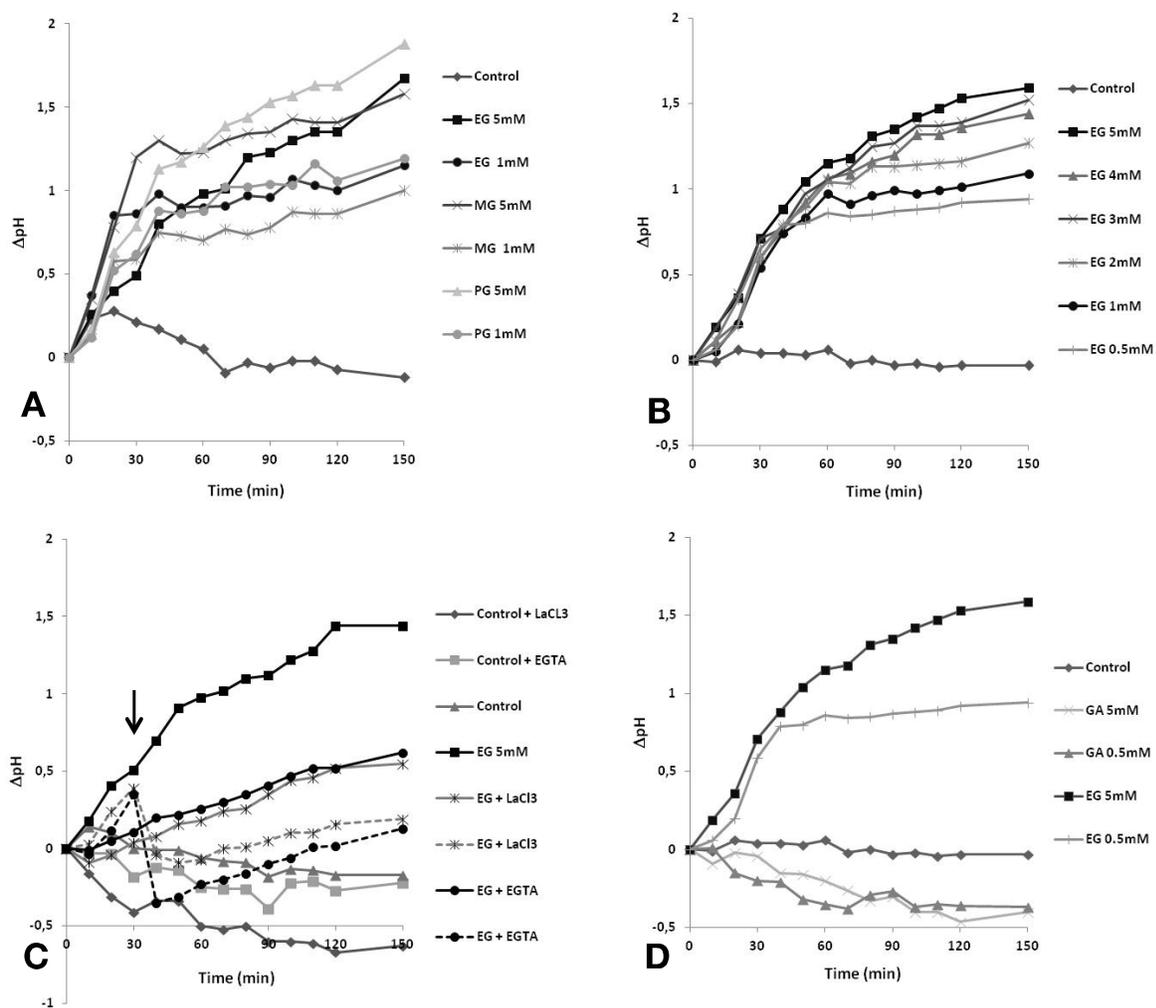


Fig.5

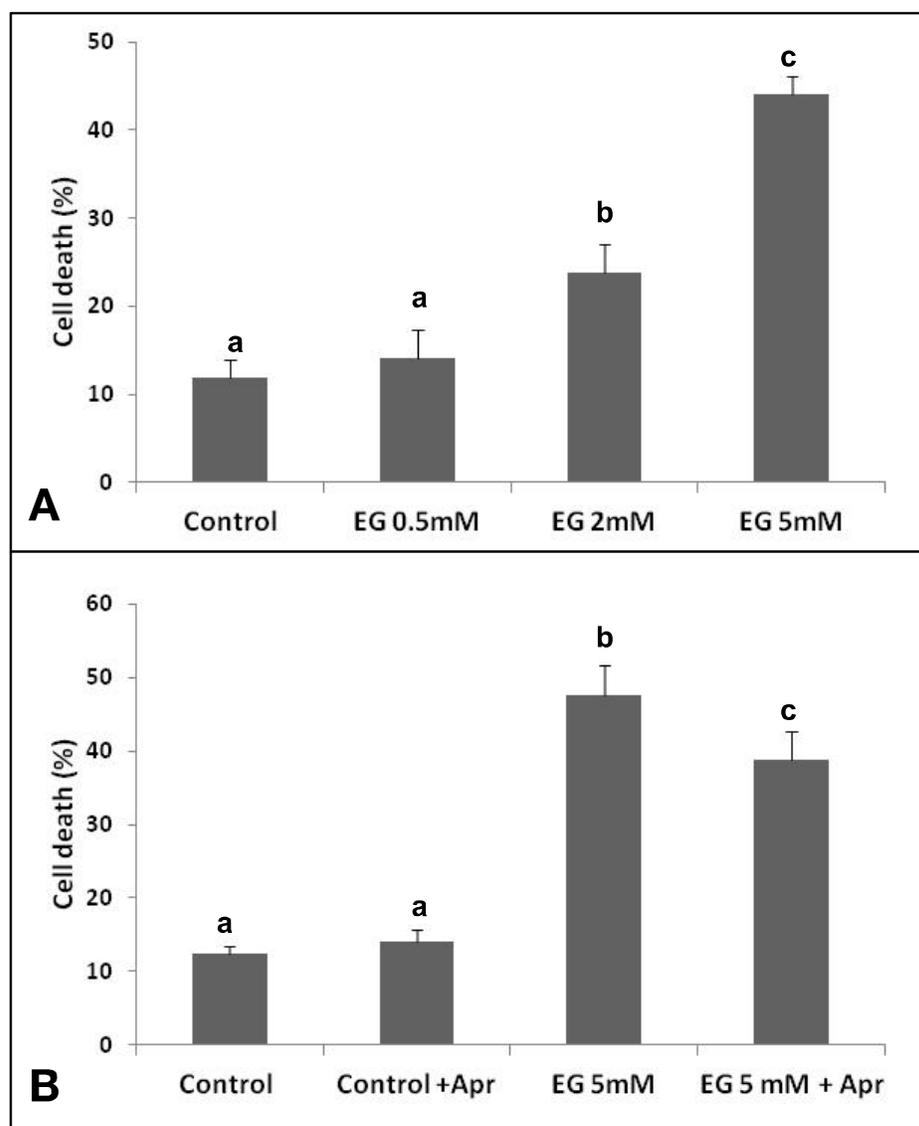


Fig.6

