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]

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7   **Affiliations:**

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

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

11   [3]Université 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   [4]Equipe 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**

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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

Abstract

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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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Introduction

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

As it is pathogenesis that initiates SAR, impact on disease is lower than if the SAR response had been activated before the arrival of the pathogen. The use of plant defence inducers (PDIs) to establish SAR intentionally and preventively in plants are thus currently a promising strategy in phytoprotection. PDIs are organic, inorganic, botanical or synthetic stimulus-triggering factors (elicitors) that mobilise plant defence reactions (Walters *et al*., 2013; Lyon, 2014). By stimulating plant immunity, PDIs provide defence against subsequent infection (Gozzo and Faoro, 2013; Walters *et al*., 2013). To date, only very few substances with high SAR activity have been described (Gozzo and Faoro, 2013), and they mostly combine PDI and pesticide actions, not necessarily a 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 Ca^{2+} 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.

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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 µl) were

115   infiltrated into the leaf tissue using a plastic syringe (without a needle), on an area of 1–2 cm^2. 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 CaCl$_2$, 0.25 mM MgCl$_2$, 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.

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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

6

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-1α 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).

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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).

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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.

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166   **Results**

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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.

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192   *Responses of cultured cells to alkyl gallates*

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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 ΔpH 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.

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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.

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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 Ca^{2+} influx/Cl^- efflux. In our experiments, the effect of

247 Ca^{2+} influx inhibitors EGTA or $LaCl_3$ caused the decreased pH shift of the extracellular medium, suggesting that

248 Ca^{2+} 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

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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-

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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.

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370

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   Ca^{2+} inhibitors. EGTA and $LaCl_3$ 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, $p \leq 0.05$.
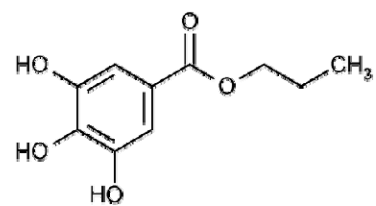
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396

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 ± SEM. Letters indicate

401   significant differences between treated and control samples according to one-way ANOVA followed by LSD test

402   ($p \leq 0.05$).

17

Fig.1
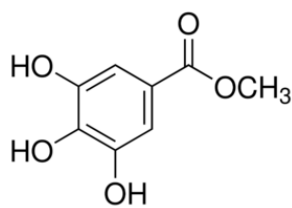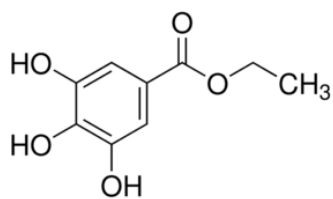
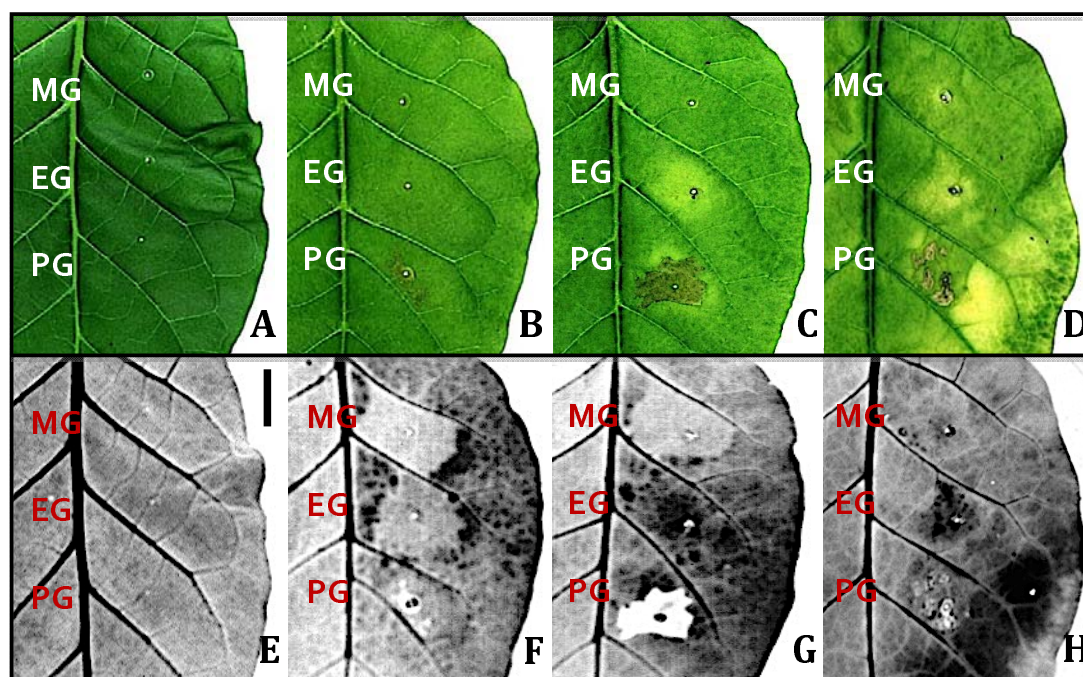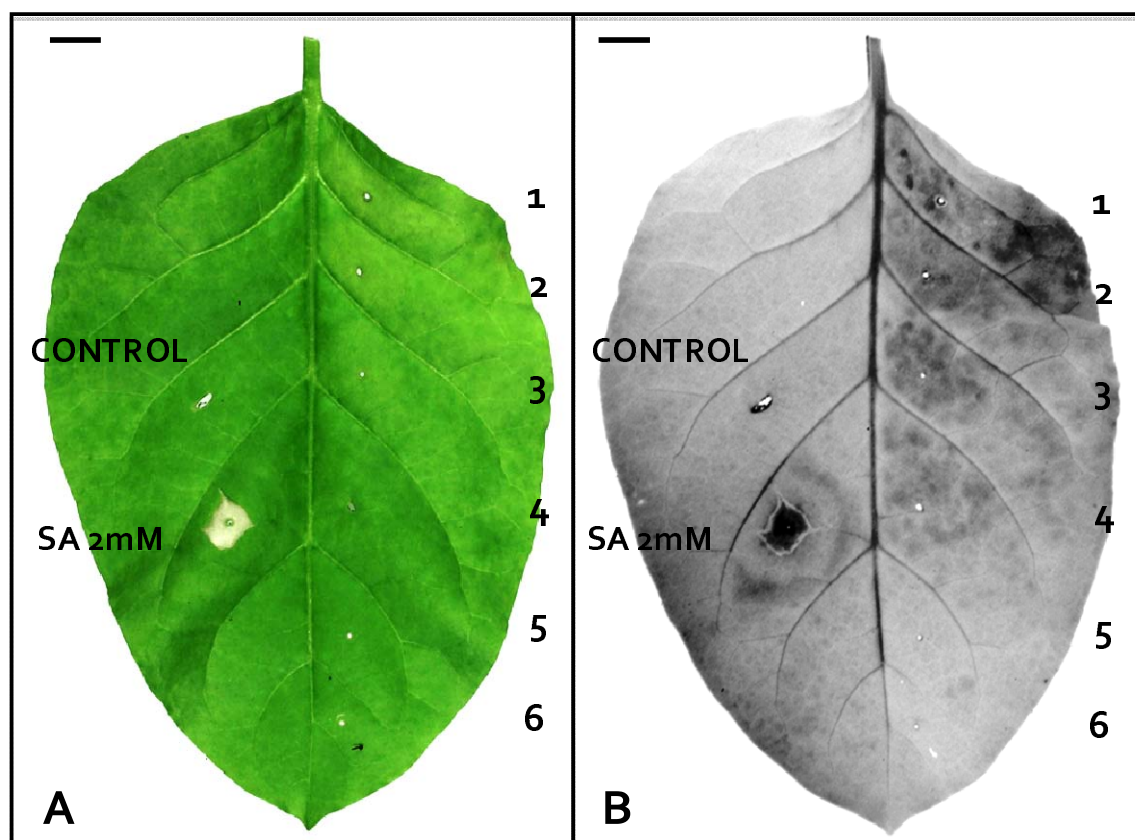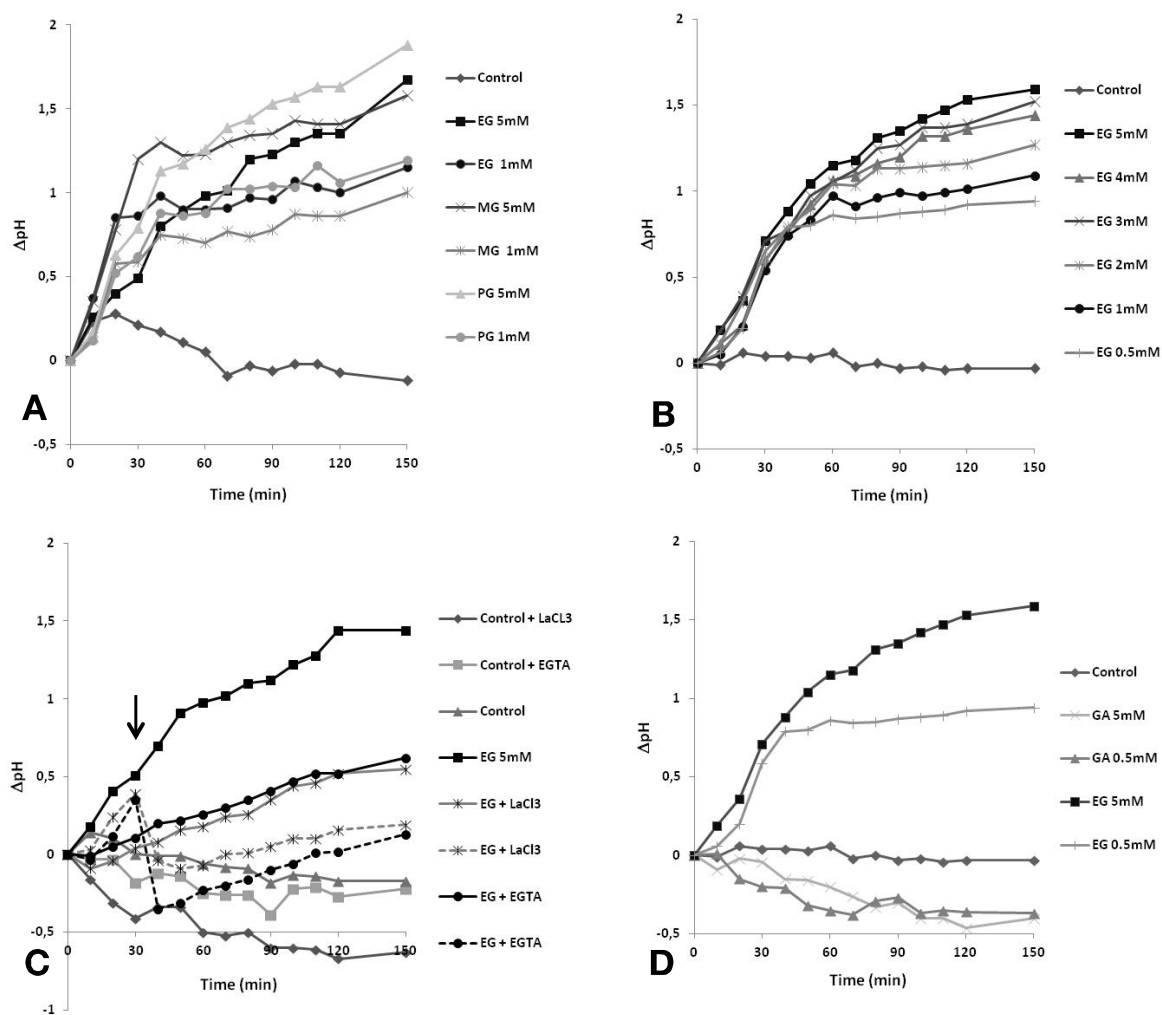Methyl gallate   Ethyl gallate   Propyl gallate

Fig. 2

Fig.3

Fig.4
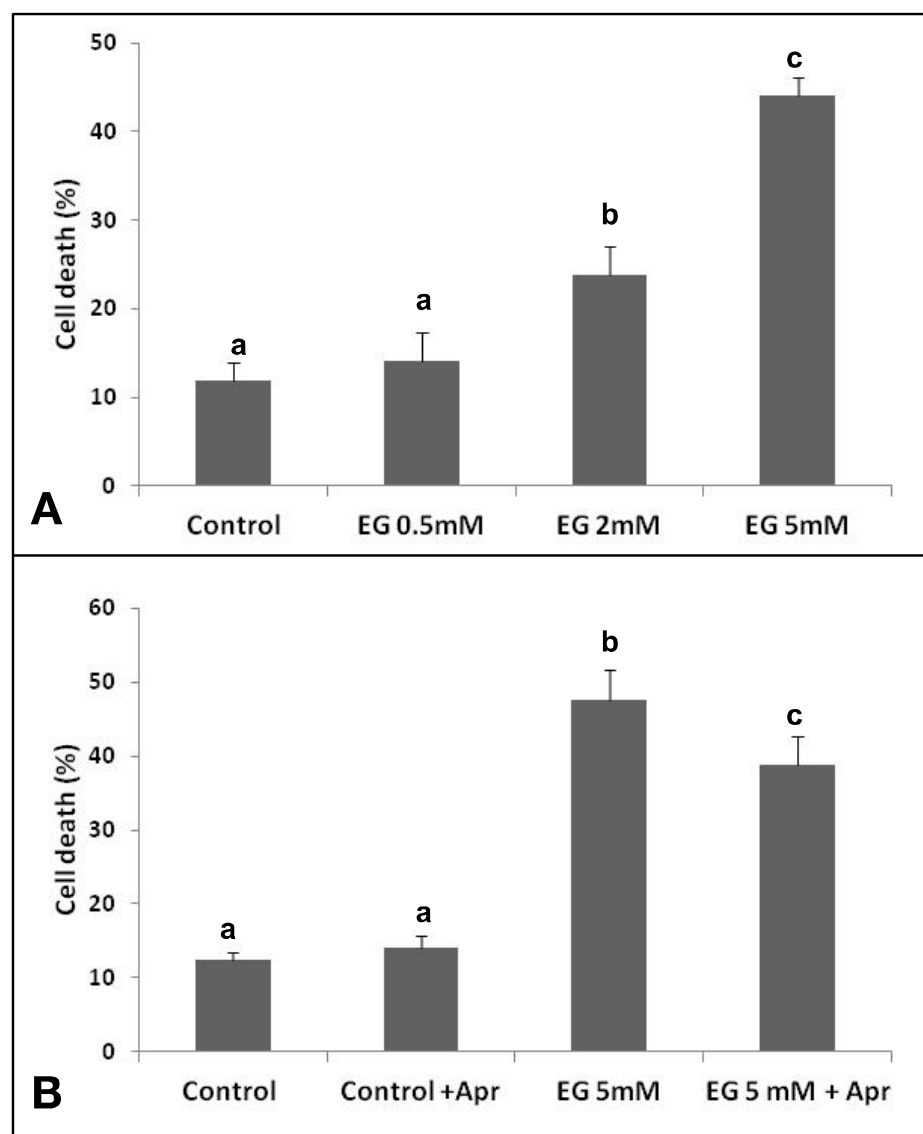
Fig.5

Fig.6