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Grape marc extract causes early perception events, defence reactions and hypersensitive response in cultured tobacco cells

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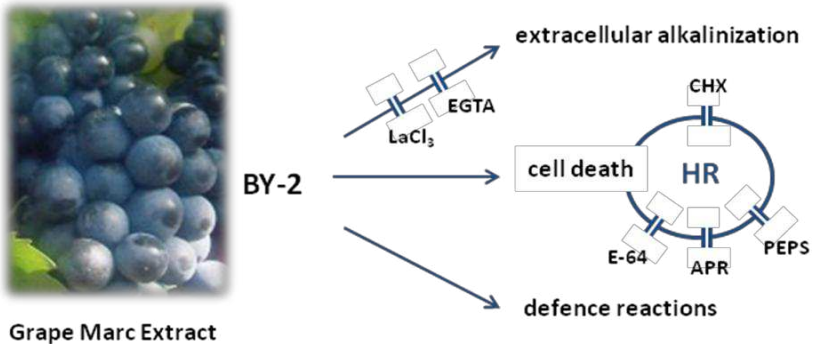
Graphical abstract



Highlights

- A polyphenol-rich extract from grapes induced chemoperception in tobacco suspension cells
- GME induced modification of plasma membrane properties
- GME elicits defence genes in tobacco cells
- GME-induced cell death was mediated by proteases and de novo protein synthesis
- This study evidences the ability of GME to induce hypersensitive response

Abstract: Grape marc extract (GME) showed elicitor activity on suspension-cultured cells of tobacco. The BY-2 cells reacted to GME (0.25% and 0.125%) with a long-sustained pH rise in their growth medium. Using EGTA or $LaCl_3$, we showed that extracellular alkalinization depended on Ca^{2+} mobilization. The tobacco BY-2 cells challenged with GME promoted cell death and the upregulation of defence-related genes such as *PR3*, *PAL* and *CCoAOMT*. Cell death rate was quantified using an experimental calibrated Evans Blue assay. The GME-induced cell death was dose-dependent and occurred in 24 h. Longer exposure increased the extent of tobacco cell death. To investigate a potential hypersensitive reaction, we tested the effect of various inhibitors of protein synthesis (cycloheximide) and proteases (aprotinin, pepstatin and E-64) on GME-induced cell death. All these chemicals reduced GME-induced cell death rate in 30 min. Overall, our findings indicate that GME elicits early perception events, defence reactions and cell death requiring protein synthesis and proteases.

Keywords: Cell death, Grape marc, Hypersensitive reaction, Plant defence, Protease inhibitors, Tobacco BY-2

1. Introduction

In plant cells, pathogen-derived substances elicit a cascade of reactions conferring plant disease resistance. Resistant plants respond rapidly to elicitors at the infection site by the hypersensitive response (HR), forming a localized cell collapse in order to restrict the systemic spread of a virulent pathogen. Hypersensitive cell death, which is distinct from necrosis caused by metabolic toxins or severe trauma, is genetically programmed (programmed cell death, PCD), and requires active host cell metabolism (Van Doorn et al., 2011). In plants, cell death during HR is similar in some features to apoptosis (a specialized form of PCD) in animals (Coll et al., 2011).

During plant-pathogen interactions, the perception of elicitor substances by plant cells, and before the HR reaction, leads to specific physiological perturbations such as ion fluxes across the plasma membrane (Ca^{2+} influx and K^+, Cl^- efflux), pH changes, plasma membrane depolarization, oxidative burst and induction of rapid cell death. The HR is subsequently accompanied by defence gene activation, leading to synthesis of phytoalexins and accumulation of pathogenesis-related (PR) proteins (Garcia-Brugger et al., 2006; Yang et al., 2011). Elicitors include oligosaccharides, glycoproteins, peptides, lipopolysaccharides, and sterols derived from microorganisms or challenged plants (Kasparovsky et al., 2004; Montesano et al., 2003). Highly sophisticated and complex biological processes underlie the interaction between these compounds and the host plants. Early events occurring in the host have largely been deciphered using tobacco cell suspensions (Garcia-Brugger et al., 2006). Modification of membrane properties is not systematically correlated with the activation of downstream defence events. Also, resistance to pathogen invasion is not necessarily associated with cell death (Gilchrist, 1998; Klarzynski et al., 2000). The structural diversity of elicitors can be expected to have a significant influence on plant cell perception, cell death induction and/or defence gene activation.

The prospect of disease control using a plant's own resistance mechanisms has prompted increasing interest in the development of agents that can mimic natural inducers of plant defence systems. These are named plant defence inducers (PDIs), and act at various points in the signalling pathway leading to disease resistance. PDIs can be organic, inorganic, botanical or synthetic (Lyon, 2007; Walters et al., 2005). Although many of these compounds are non-specific and induce resistance in a wide range of crop species against a diverse range of plant pathogens, differences in efficiency are reported (Lyon, 2007). Grape marc extract (GME) is a plant extract

that acts as an efficient PDI. On application to tobacco leaves, this wine by-product elicits a variety of defence reactions such as local injury, biochemical changes and systemic molecular response with upregulation of PR proteins (Goupil et al., 2012). As a potent defence elicitor in tobacco, *Arabidopsis* or tomato (Benouaret et al., 2013), GME might thus prove to be a useful alternative tool for environmentally oriented phytoprotection.

The present report investigates the ability of GME to initiate chemoperception and induce defence mechanisms in tobacco cultured cells. We show that GME triggered rapid extracellular alkalinization and activated defence-related genes, ultimately leading to cell death. Using pharmacological inhibition of proteases, we evidenced a hypersensitive status of tobacco BY-2 cells that led to cell death on GME treatment.

2. Materials and methods

2.1. Biological compounds and chemicals The grape marc extract (GME), a *Vitis vinifera* L. hydroalcoholic extract, was supplied as a red powder by Grap'Sud (Cruviers-Lascours, France) as described by Goupil et al. (2012). Batch #11332 used in our experiments was produced from grapes harvested from several red wine varieties in 2011. According to the company, this grape end-product is 95% dry weight (DW) and contains 66.1% polyphenols including 25.4% anthocyanins. The flavonoid-based compounds are responsible for the red colour of the dry extract, and the acidity (pH 4.2) of the aqueous solution when it is dissolved in water. Control experiments were conducted with ultrapure water acidified with HCl to pH 4.2.

2.2. Plant material Tobacco cell suspensions of *Nicotiana tabacum* L. cv. Bright Yellow-2 (BY-2), were grown at pH 5.8 in MS medium (Duchefa, the Netherlands) supplemented with sucrose (30 $g.L^{-1}$), thiamine (1 $mg.L^{-1}$), myo-inositol (102 $mg.L^{-1}$) and 2,4-dichlorophenoxyacetic acid (0.2 $mg.L^{-1}$). Cells were maintained in the dark on a rotary shaker (140 rpm, 25°C) in 250 mL conical flasks. Subcultures were made weekly by dilution at a 1:15 ratio in fresh medium. All the experiments were performed using cells in exponential growth phase at day 5 after subculture. For alkalinization analysis, cells were collected by filtration, washed with 175 mM mannitol, 0.5 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 the same medium. The initial pH was about 4.8. For transcript accumulation analysis, cells were collected by filtration, and resuspended at 0.3 g FW/L in MS medium. Cells were equilibrated for 2 h in open 6-well microplates (Greiner Bio-one, Germany) with continuous stirring, and then elicited with GME.

2.3. Extracellular alkalinization The pH variations of the culture medium were recorded by introducing a glass microelectrode (Hanna Instrument, HI1330B, France) into 6 ml of the equilibrated cell suspension culture. The pH measurement, performed every 10 min, started immediately after treatment with elicitors or acidified water. The ΔpH was measured at 10 min intervals, relative to the pH measured at t_0 immediately after adding GME or acidified water (control). Each experiment was repeated three to six times to check for reproducibility. Figures describe the results of typical experiments.

2.4. Real-time RT-PCR Tobacco BY-2 cell samples (100 mg) were ground in liquid nitrogen, and total RNAs were isolated using CTAB extraction buffer according to Chang et al. (1993). Total RNAs were cleaned up with 1 U DNase I solution (Euromedex, France) containing 40 U RNase inhibitor (Euromedex, France). RNA integrity was verified on a 1% agarose gel by detecting ribosomal RNAs. First-strand cDNA was synthesized from 1 µg total RNA using oligo $d(T)_{15}$ primers and Euroscript Reverse Transcriptase (Eurogentec, France) according to the manufacturer's instructions.

PCR reactions were prepared using the qPCR kit Mastermix for SYBR green (Eurogentec) according to the manufacturer's protocol. The quantitative assessment of mRNA levels was performed using an iCycler iQ5 (Bio-Rad). The cDNA concentration used produced a threshold cycle value (C_T) of between 15 and 30 cycles. Amplification specificity was checked by melting-curve analysis. The EF-3 was used as an internal control (Goupil et al., 2012). Quantification of expression ratios was performed according to the mathematical model developed by Pfaffl (2001). Primers and amplicon sizes are given in Appendix S1.

Appendix S1 Sequences of gene-specific primers used for quantitative real-time RT-PCR

Gene family	Specific class	Accession number	5′primer	3′primer	*Tm* (°C)	PCR fragment size (pb)
PR1	PR1a, acidic PR1	X12485.1	5′-TGCTAAGGCTGTTGAGATGTGGGTC	5′-ACTGAACCCTAGCACATCCAACACG	58	143
PR3	Endochitinase	X16939.1	5′-GCCATAGGAGTGGACCTGCTAAAC	5′-AAAAGACCTCTGGTTGCCGC	59	335
PAL	Phenylalanine ammonia lyase	AB289452.1	5′-TCCCCTTGTCCTACATTGCTGGTT	5′-TCAATTTGTCCGGGGTGATGCTTC	59	327
CCoAOMT	Caffeoyl-CoA O-methyltransferase	JQ911710	5′-ACACCCTATGGAATGGATCA	5′-CCTTGTTGAGTTCCAATACGA	55	90
EF	Elongation Factor-1α	AF120093	5′-ATTGGTGGAATTGGTACTGTCC	5′-CCGCAACATTCTTGACATTAAA	58	184

PR: pathogenesis-related protein

2.5. Cell death assay The Evans Blue assay was performed according to Amano et al. (2003). Tobacco cells (500 µL) were withdrawn from the culture medium immediately after GME elicitation (t_0) and then every 24 h for 3 days. The cells were spread onto a cotton swab placed at the bottom of a 10 mL plastic syringe. The cells were washed with 10 mL of distilled water and stained with 500 µL of Evans Blue solution (0.25% w/v in distilled water) for 5 min at room temperature. Excess unbound dye was removed by thorough washing with 50 mL of distilled water. The cotton swab containing cells was soaked with 500 µL SDS solution (0.5%, w/v), and the syringe was immediately heated for 3 min in a boiling water bath. The dye bound to dead cells was eluted with 2.5 mL of distilled water, and remaining drops of dye were squeezed out using the syringe plunger. Absorbance at 600 nm was measured to estimate cell death. Each experiment was repeated three times.

2.6. Pharmacological treatments We used EGTA (2 mM and 5 mM), $LaCl_3$ (2 mM) as chelator and Ca^{2+} channel blocker respectively, pepstatin (1 µM), E-64 (1.5 µM), aprotinin (0.2 µM) as protease inhibitors, and cycloheximide (50 µM) as protein synthesis inhibitor. Pepstatin was dissolved in ethanol and the other chemicals in water. All the chemicals were purchased from Sigma-Aldrich (France).

2.7. Statistics All the experiments were performed in biological and technical triplicates. The values were expressed as mean ± standard error of the mean (SE). Letters above bars indicate statistically significant differences between samples, according to one-way ANOVA followed by LSD Significant test ($p \leq 0.05$) using Statistix 9.® software.

3. Results

3.1. Effect of GME on proton flux and Ca^{2+} mobilization GME elicits tobacco BY-2 cells. Suspension cells responded to GME by a rapid, sustained alkalinization of the incubation medium (Fig. 1). Medium alkalinization was detected immediately after addition of GME (0.25%), and the pH shift reached a plateau with a ΔpH of 1.6 units at 80 min. No pH shift was detected with the control cells treated with water. GME added to the cell-free medium did not cause any pH modifications. The intensity of the GME effect was dose-dependent. The pH shift reached 0.9 units with 0.125% GME, and the 0.0625% concentration did not induce any extracellular alkalinization.

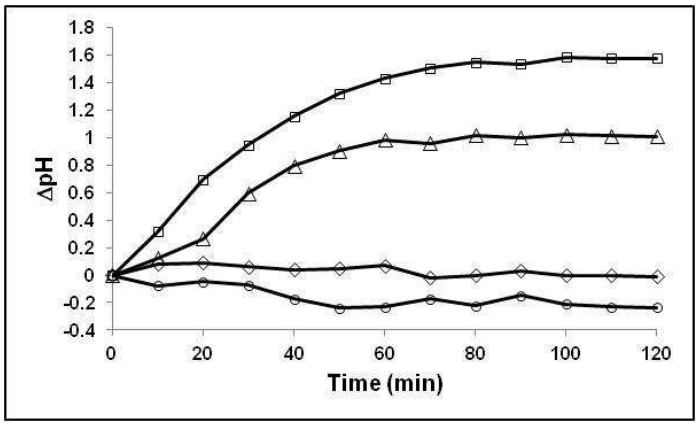


Fig. 1: Extracellular alkalinization in tobacco BY-2 suspension cells on addition of different concentrations of GME. (◊) water control, (o) 0.0625% GME, (Δ) 0.125% GME, (□) 0.25% GME. (x) 0.25% GME without BY-2 cells. The data are from representative experiments out of a total of five.

The involvement of calcium in GME-induced extracellular alkalinization was investigated by adding EGTA (2 mM or 5 mM) or $LaCl_3$ (2 mM) to the culture medium concomitantly with GME. The pH kinetics were measured for 120 min (Fig. 2). Both Ca^{2+}-chelator and the Ca^{2+} surrogate La^{3+} radically suppressed the alkalinization. The extracellular pH rise was reduced to the same extent with both EGTA concentrations, suggesting that the saturating chelation threshold was reached at 2 mM. These compounds dramatically suppressed the pH shift when added subsequently to GME elicitation. They had no effect on the pH medium in control cells.

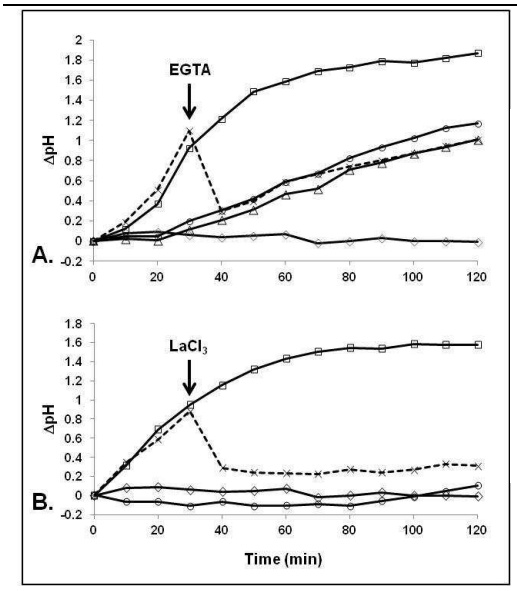


Fig. 2: Extracellular alkalinization in tobacco BY-2 suspension cells of 0.25% GME and Ca^{2+} inhibitors. A: water control (◊), water control with 2 mM EGTA (∗), GME (□), GME with 2 mM EGTA (o), GME with 5 mM EGTA (Δ), GME followed by addition of 2 mM EGTA (-x---x-) at 30 min (arrow). B: water control (◊), water control with 2mM EGTA (∗), GME (□), GME with 2 mM $LaCl_3$ (o), GME followed by addition of 2 mM $LaCl_3$ (-x---x-) at 30 min (arrow). The data are from representative experiments out of a total of five.

3.2. Induction of defence-related gene expression following GME treatment We verified the ability of GME to induce some defence reactions in suspension tobacco cells. Transcript accumulation was measured for two *PR* genes, the antimicrobial *PR1* and the endochitinase *PR3* involved in defence responses. The induction of mRNA accumulation was also assessed for genes involved in the synthesis of phenylpropanoids and lignin encoding phenyl-ammonia-lyase (*PAL*) and caffeoyl CoA 3-*O*-methyltransferase (*CCoAOMT*). Transcript levels were quantified in tobacco BY-2 cells treated with 0.25% GME at different time intervals (from 0 to 24 h). The ratios of transcript levels in treated cells to those in control cells are shown in Fig. 3.

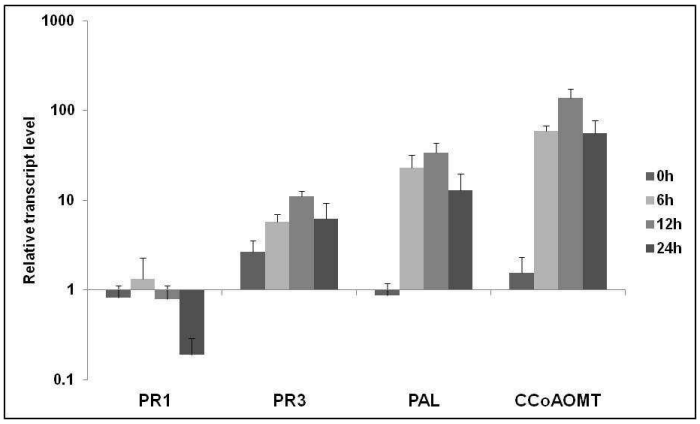
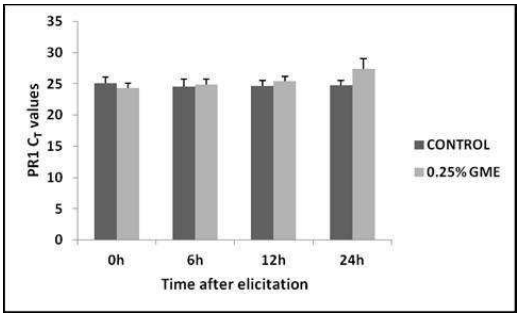


Fig. 3: Effects of GME on transcript accumulation of defence-related genes in tobacco BY-2 cells. Relative transcript levels of genes of *PR1*, *PR3*, *PAL* and *CCoAOMT* in cells challenged for 0, 6, 12 and 24 h with 0.25 % GME. Transcript levels assessed by real-time RT-PCR are expressed as mean ± SE ($n = 3$) of transcript ratios relative to control cells (water treatment).

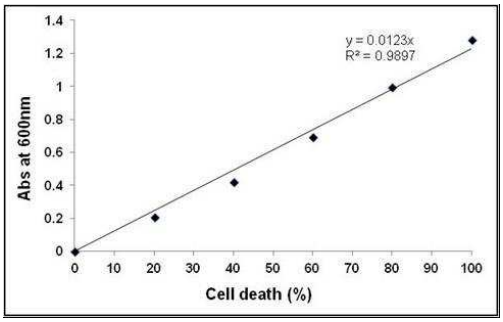
Interestingly, *PAL* and *CCoAOMT* were induced rapidly in GME-treated tobacco cells. The maximum accumulation for both genes was detected at 12 h treatment, and thereafter there was a noticeable reduction. The induction was unexpectedly high, 36-fold for *PAL*, 110-fold for *CCoAOMT* and noticeable for *PR3*. Surprisingly, GME had no effect on the transcriptional induction of the defence marker PR1, even though upregulation had previously been demonstrated on tobacco leaves sprayed or infiltrated with the same GME extracts (Goupil et al., 2012). Since the real-time RT-PCR provided PR1 C_T values similar in control and GME elicited cells (Appendix S2), BY-2 cultured cells should produce high PR1 transcript levels. This could finally reduce the ratio of transcript levels in treated cells to those in control cells making PR1 overaccumulation undetectable in GME-treated cells. GME that might induce different defence responses in tobacco cell cultures and plant cannot be rule out.



Appendix S2 PR1 C_T values provided by real-time RT-PCR performed from tobacco BY-2 cells elicited with GME at different time intervals (from 0 to 24h)

Taken together, modified proton flux, the Ca^{2+} commitment and the defence-related transcript accumulation in GME-treated tobacco cells are evidence that the suspension cellular biological system reacts to GME treatment by inducing characteristic elicitation and defence responses.

3.3. Determination of GME-treated tobacco cell death GME triggers a hypersensitive-like response corresponding to cell death in tobacco plants (Goupil et al., 2012). The ability of GME to cause cell death in tobacco suspension culture was investigated using the Evans Blue assay. The pigment enters cells with damaged plasmalemma (Baker and Mock, 1994). The assay was first calibrated with a set of viability standards using mixtures of living and dead tobacco BY-2 cells (Appendix S3). A near-perfect correlation coefficient (0.989) attests the accuracy of the Evans Blue method for theoretical dead cell content and absorbance at 600 nm.



Appendix S3 Quantitative analysis of cell death by Evans Blue assay: calibration curve for cell death determined by calculating the ratio of total cells to dead cells.

The time course of cell death rate (CDR) was investigated in tobacco cells exposed to 0.25%, 0.125% and 0.0625% GME up to 72 h. When BY-2 cells were cultivated in the absence of GME (control cells), the CDR was below 10%, and this level remained constant for up to 72 h (Fig. 4). The exposure of tobacco cells to 0.25%

GME resulted in an increased CDR, which was 3 to 4 times higher than in the control. The CDR reached 22%, 32% and 39% by 24 h, 48 h and 72 h respectively. The 0.125% GME concentration reduced the magnitude of CDR, which reached 19%, 29% and 30% by 24 h, 48 h and 72 h respectively. A lower concentration of GME (0.0625%) resulted in an even lower CDR, with an average of 21% stabilized over the treatment.

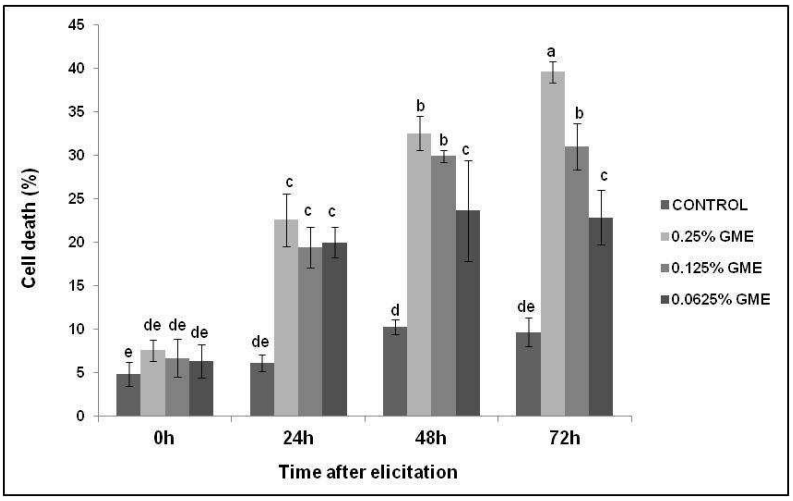


Fig. 4: Time course of cell death rate assayed by Evans Blue staining of tobacco BY-2 suspension cells treated with different concentrations of GME. Bars represent the mean values ± SE, $p \leq 0.05$.

3.4. Pharmacological analysis of GME-induced cell death To determine whether GME-induced cell death was related to hypersensitive reaction (HR), the action of plant cell death inhibitors was investigated on GME-treated cells. Experimentally, BY-2 cells were treated with or without 0.25% GME for 24 h and 48 h, and cell death was quantified in the presence or absence of chemicals added 30 min before CDR determination.

We first examined the effect of cycloheximide, a widely-used inhibitor of protein synthesis. Cycloheximide reduced CDR to 18% and 25% at 24 h and 48 h respectively in GME-treated tobacco cells (Fig. 5), suggesting that the GME-induced cell death process depends on *de novo* protein synthesis. Thereafter, we investigated the action of protease inhibitors on GME-induced cell death. As shown in Fig. 5, aprotinin (0.2 µM), a serine protease inhibitor, suppressed the GME-induced cell death at 24 h or 48 h by 43% and 38% respectively. Pepstatin and E-64, inhibitors of cystein and aspartic proteases, also affected GME-induced cell death. Pepstatin reduced CDR by 36% and 42%, and E-64 inhibitor less efficiently, by 16% and 27% at 24 h and 48 h respectively. Neither inhibitor significantly affected cell viability by itself in control tobacco cells. Taken together, these data evidence the involvement of proteolytic events in GME-induced cell death.

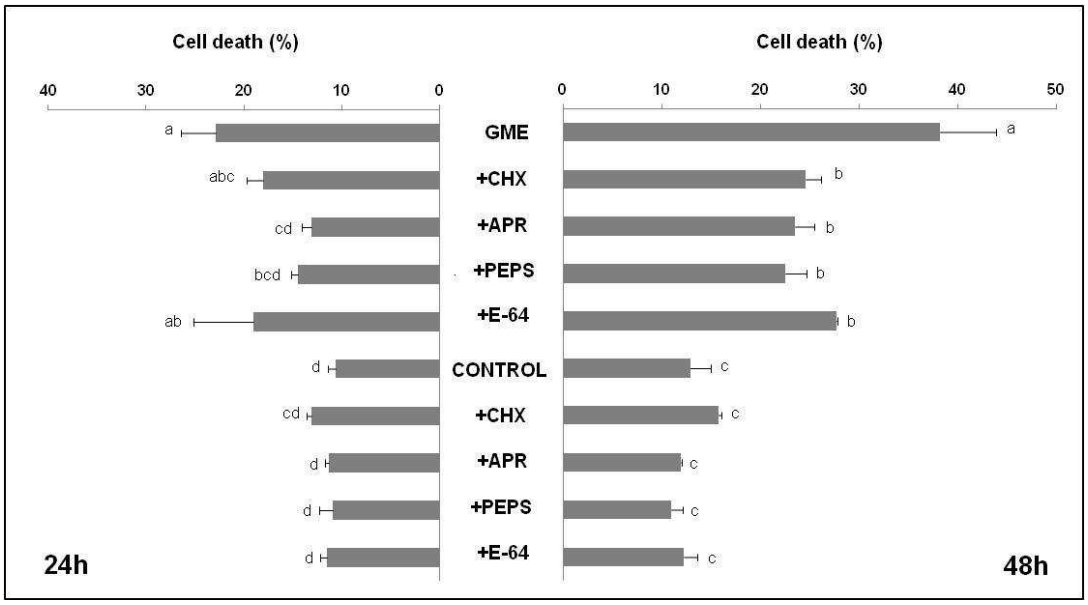


Fig. 5: Effects of cycloheximide and protease inhibitors on GME-induced cell death. Chemicals were added 30 min before cell death measurement in BY-2 cells treated with water (control) or 0.25% GME for 24 h and 48 h. The final concentration of each chemical was 50 µM cycloheximide (+CHX), 0.2 µM aprotinin (+APR), 1 µM pepstatin (+PEPS), and 1.5 µM E-64 (+E-64). Bars

4. Discussion

Medium alkalinization is a common parameter employed to monitor the primary action of elicitors. It has been described as a highly sensitive assay of elicitor perception (Boller, 1995). GME induced extracellular alkalinization of BY-2 cells characterized by long-sustained pH rise. Kinetics of changes in extracellular pH vary among elicitors, and do not predict their type or nature. Tobacco cells reacted to GME with an alkalinization response in a manner resembling the perception of several elicitors such as cyclodextrins, methyljasmonate, oligogalacturonides or cryptogein (Almagro et al., 2012; Binet et al., 1998), whereas the reaction with laminarin or ergosterol was transient, with a return to the resting value (Klarzynski et al., 2000; Vatsa et al., 2011). GME is a polyphenol-enriched extract without measurable jasmonic acid (Goupil et al., 2012) or methyljasmonate (data not shown). The GME early elicitor responses involve rapid modification in proton influx and calcium mobilization, as evidenced by the decreased pH shift in the presence of the Ca^{2+} influx inhibitors EGTA or $LaCl_3$. These data show that tobacco cells perceived GME by modifying plasma membrane biophysical properties. Later on, GME could activate late elicitor responses with *PR3*, *PAL* and *CCoAOMT* gene overexpression in tobacco cells. Since GME induced upregulation of defence-related genes, the GME-bioactive

molecules are presumed to interact with upstream players of a signal transduction pathway leading to defence reactions.

Our group had previously demonstrated that GME was an inducer of dose-dependent cell death in tobacco plants (Goupil et al., 2012). Here, using a calibrated Evans Blue assay, we evidenced GME-induced cell death in cultured tobacco cells. GME reduced BY-2 cell viability by 24 h. The three GME doses (0.25%, 0.125% and 0.0625%) tested were effective. The lowest GME concentration was ineffective on tobacco leaves (Goupil et al., 2012), but sufficient to induce cell death in tobacco cells. Such a difference could be related to tobacco suspension cells and leaf tissues varying in their sensitivities to GME-bioactive molecules.

Localized cell death on plant tissue resulting from attack by pathogens might occur either in hypersensitive resistant response or in susceptible reactions such as necrotic symptom potentially caused by toxins (Iakimova et al., 2005). Plant defence inducers (PDIs) used as stimulus-triggering factors could mobilize plant defence and provoke local necrotic tissue when infiltrated in plant leaves (Kulye et al., 2012; Mercier et al., 2000). Whether the PDI mediates hypersensitive lesions or phytotoxicity should be investigated to provide an exploitable perspective of these biomolecules for phytoprotection. Our previous investigation of GME elicitor activity on tobacco leaves demonstrated local defence reaction with tissue injury on tobacco, *Arabidopsis* and tomato leaves (Benouaret et al., 2013; Goupil et al., 2012). The growth stage of the plant organ could markedly influence the GME-induced resistance reactions. Young growing leaves were much less reactive to GME elicitor molecules than mature ones. The importance of phenology in achieving the GME-defence reaction supports the idea that GME elicits cells that enter a highly regulated cell death process rather than a phytotoxicity process.

In the present study, we investigated the GME-induced HR on BY-2 cells using pharmacological approaches. The inhibitory effect of cycloheximide on GME-induced cell death indicates that GME triggered an active process requiring protein synthesis. Also, proteolytic events may take part in the mechanisms leading to cell death. Serine-, aspartic- and cysteine- protease inhibitors all partially suppressed GME-induced cell death. All these classes of proteases have been shown to be involved in programmed cell death (PCD) in mammalian systems, with the cysteinyl-aspartic-proteases (caspases) as key cell death executioners (Grütter, 2000). Caspase-like activity also executes PCD in plant cells (Coll et al., 2011; Woltering et al., 2002), and the active process operates through constitutively expressed machinery (Elbaz et al., 2002). Their potential involvement in HR has

been reported following the infection of TMV in tobacco (Del Pozo and Lam, 2003). Our data demonstrate that proteolytic events with caspase-like proteases take part in the mechanisms leading to cell death in GME-elicited BY-2 cells, and the regulated process was arrested by chemicals within minutes (experimentally 30 minutes). Since plant cells constitutively express caspase-like machinery to run cell death programs (Elbaz et al., 2002), our data suggest that chemicals could rapidly reverse the induction of PCD and block the morphological changes that tobacco cells undergo under the influence of GME.

GME is typically regarded as a waste by-product in the lucrative winemaking industry (Bustamante et al., 2008). This biomass is a rich source of antioxidative phenolic compounds, typically including anthocyanins, catechins, resveratrol, phenolic acids and procyanidins (Lu and Foo, 1999), with a broad spectrum of pharmacological, medicinal, and therapeutic properties (Vislocky and Fernandez, 2010). The anticancer effects of grape antioxidants have been shown to induce cell cycle arrest and apoptosis in cancer cells with proposed mechanisms involving antioxidant, anti-inflammatory, and antiproliferative activities (Seeram et al., 2005; Zhou and Raffoul, 2012). Grape antioxidants acting as free radical scavengers and chelating agents help to reduce physiological reactive oxygen species (ROS), which are known as important mediators of apoptosis (Matès and Sánchez-Jiménez, 2000). In plants, research on redox-dependent cell death has demonstrated that ROS levels may orchestrate necrosis *vs.* PCD. ROS overaccumulation building up to phytotoxic levels leads to a necrotic phenotype. By contrast, when the accumulation of ROS is insufficient to kill the cell directly, changes in cellular redox homeostasis appear to switch on a signalling cascade, leading to PCD encountered during HR (Van Breusegem and Dat, 2006). In our plant biological system, we demonstrate that the grape antioxidant-rich extract induced cell death closely associated with proteases and dynamic changes in protein synthesis. The GME-induced cell death may be part of a defensive process related to the HR. An investigation of ROS as a potential player in the activation of the HR, along with the identification of GME-containing bioactive ingredients, should help characterize the pathways leading to hypersensitive GME-induced cell death in tobacco.

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Contributions: RB performed the research and statistical analysis, EG set up the cell death procedure, RB and PG drew the figures and analysed the data. PG designed the research and drafted the manuscript. RB and PG revised the manuscript.

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