METHYL JASMONATE/ETHEPHON COTREATMENT SYNERGISTICALLY INDUCES STILBENE PRODUCTION IN *VITIS VINIFERA* CELL SUSPENSIONS BUT FAILS TO TRIGGER RESISTANCE TO *ERYSIPHE NECATOR*

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Abstract

Aim: The aim of the present investigation was to determine whether methyl jasmonate and ethylene could synergistically induce grapevine foliar cuttings (*Vitis vinifera*) defense mechanisms and enhance resistance to *Erysiphe necator*.

Methods and results: We cotreated grapevine foliar cuttings (Cabernet-Sauvignon) with ethylene-releasing ethephon in association with methyl jasmonate. However, this cotreatment did not improve resistance to powdery mildew induced by MeJA or ethephon, alone. Quantitative PCR analysis performed on grape cell suspensions showed that the association ethephon/MeJA triggered an enhancement of phytoalexin biosynthesis by synergistically inducing *PAL* and *STS* genes. This gene expression was correlated with accumulation of stilbenes (antimicrobial compounds), assessed by HPLC analysis. However, ethephon seemed to inhibit MeJA-dependent induction of PR protein gene expression mainly for the first eighteen hours.

Significance and impact of study: Since methyl jasmonate and ethephon can separately enhance grapevine resistance to *Erysiphe necator*, it was interesting to study the effect of the association of the two molecules on it. Although we observed a synergistic effect on phytoalexin production, no improved resistance against the fungus was obtained. These results can be exploited for the development of new pest control strategies in vineyard.

Key words: ethylene, powdery mildew, phytoalexins, methyl jasmonate, grapevine

Résumé

Objectifs : L'objectif de ces travaux a été de déterminer dans quelles mesures l'association du méthyle jasmonate (MeJA) avec l'éthylène exerce une action synergique sur l'induction des mécanismes de défense de la vigne et sur l'augmentation de sa résistance à *Erysiphe necator*, agent responsable de l'oïdium.

Méthodes et résultats : Après traitement de boutures foliaires de vigne (Cabernet-Sauvignon) avec de l'éthéphon, précurseur de l'éthylène, en association avec le méthyle jasmonate, des tests de résistance à l'oïdium sont réalisés sur feuilles détachées. Le traitement par l'éthylène n'augmente pas la résistance de la plante à l'oïdium, induite par le MeJA. Toutefois, des analyses réalisées par PCR quantitative sur des suspensions cellulaires de vigne montrent que l'association MeJA/éthéphon induit un effet synergique sur l'expression des gènes (PAL et STS) impliqués dans le métabolisme des stilbènes, phytoalexines de la vigne. Corrélée à cette surexpression, des dosages par HPLC ont montré une production synergique de stilbènes induite par l'association MeJA/éthéphon. Cependant, l'éthéphon semble inhiber l'induction du MeJA sur l'expression de gènes codant des protéines PR, principalement dans les dix-huit premières heures de traitement.

Signification et impact de l'étude : Des travaux réalisés en laboratoire ont montré que le MeJA et l'éthéphon peuvent, séparément, augmenter la résistance de la vigne à l'oïdium. Il est donc intéressant d'étudier l'impact de l'association des deux molécules sur cette résistance. Bien que nous ayons observé un effet synergique des deux molécules sur la production de stilbènes, nous n'avons pas obtenu une meilleure résistance au champignon. Ces résultats pourront être utilisés pour le développement de nouvelles stratégies de contrôle des agents pathogènes au vignoble.

Mots-clés : éthylène, oïdium, phytoalexines, méthyle jasmonate, vigne

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INTRODUCTION

Grapevine, the major fruit crop worldwide, is affected by many diseases due to bacteria, fungi or viruses. To contain pathogen development, plants possess physical barriers and inducible defense mechanisms like programmed cell death (hypersensitive response), cell-wall reinforcement, production of low molecular weight molecules with high antimicrobial properties, phytoalexins, and production of Pathogenesis-Related proteins (PR proteins), which, like phytoalexins, possess antimicrobial activities (BENHAMOU, 1996; HAMMOND-KOSACK and JONES, 1997). Some of these defense mechanisms seem under the control of two phytohormones, methyl jasmonate and ethylene.

Jasmonates induce the expression of genes encoding PR proteins, such as chitinases and glucanases in tomato (DING *et al*., 2002), maize (BRAVO *et al*., 2003), rice (RAKWAL *et al*., 2004) and lily anthers (WANG *et al*., 1999). They also stimulate proteinase inhibitor gene expression in tobacco (FARMER *et al*., 1992; PLUSKOTA *et al*., 2007). Moreover, jasmonic acid induces the production of a wide range of secondary plant metabolites, like alkaloids, terpenoids, flavonoids, and phytoalexins in various cell cultures (*Taxus cuspidate, Glycine max*) (GRAHAM and GRAHAM, 1996; MIRJALILI and LINDEN, 1996) and in *Arabidopsis thaliana* plants (BRADER *et al*., 2001). Treatments with methyl jasmonate, the methyl ester of jasmonic acid, induce the accumulation of alkaloid compounds in jaborandi leaves (*Pilocarpus microphyllus*) (AVANCINI *et al*., 2003) and phytoalexins in *Cupressus lusitanica* cell cultures (ZHAO *et al*., 2004) or barley (*Hordeum vulgare*) leaf segments (LEE *et al*., 1996). In grapevine, MeJA has been shown to stimulate deposition of callose and accumulation of PR proteins in leaves (HAMIDUZZAMAN *et al*., 2005). Recently, BELHADJ *et al*. (2006) showed that MeJA treatment of grapevine in the vineyard induces the production of stilbene phytoalexins, the accumulation of PR protein-related RNAs, and triggers enhanced resistance to *Erysiphe necator*. Moreover, jasmonic acid pathway was found to contribute to the sulfated laminarin-induced resistance against *Plasmopara viticola* (TROUVELOT *et al*., 2008).

Ethylene is also involved in the activation of plant defense-related processes such as the production of PR proteins (TORNERO *et al*., 1994; van KAN *et al*., 1995), and phytoalexins (YOSHIKAWA *et al*., 1990). DIAZ *et al*. (2002) demonstrated that ethylene pre-treatment of tomato plants resulted in an increased resistance to *Botrytis cinerea*. Conversely, treatment of tomato plants with MCP (1-methylcyclopropene), an ethylene perception inhibitor, considerably reduced resistance to *Borytris cinerea* (DIAZ *et al*., 2002). Recently, BELHADJ *et al*. (2008a) showed that ethephon treatment of grapevine foliar cutting triggers enhanced resistance to *Erysiphe necator* by inducing expression of various PR protein genes and the production of stilbenes.

Genetic experiments based on *Arabidopsis* mutants have provided additional evidence of the involvement of jasmonates and ethylene in the activation of plant defense mechanisms. Thus ethylene perception mutants like *ein2* (*ethylene insensitive2*) showed enhanced sensitivity of different plant species to various pathogens such as fungi, including *Phytium* spp., *Borytris cinerea* and *Plectosphaerella cucumerina*. ISR (Induced Systemic Resistance) is blocked in *etr1* (*ethylene resistant1*) mutants impaired in their response to ethylene (van LOON *et al*., 2006). Conversely, *Arabidopsis* transgenic plants, constitutively expressing Ethylene Response Factor 1 (ERF1), a downstream component of the ethylene signaling pathway, show increased resistance to *Borytris cinerea* and *Plectosphaerella cucumerina*.

Mutations that impair jasmonate perception (*coi*1, *coronatine insensitive*1 and *jar*1, *jasmonate resitant*1) or synthesis (*fad*3-2, *fad*7-2 and *fad*8, *fatty acid desaturase*) result in an increased sensitivity of *Arabidopsis* to various

Table 1 - Gene accession numbers and sequences of gene primers used for real-time quantitative polymerase chain reaction.

Name	Accession number*	Direct primer	Reverse primer
ACT	TC30205	TCAGCACTTTCCAGCAGATG	TAGGGCAGGGCTTTCTTTCT
CHIT4C	AY137377	GGCGACGAATCCATTTATGTTA	CGGAACAAGGGTTTCATAATTC
GLU	AF239617	TACCTCTTTGCCATGTTTGATG	AGTACTTCGGCTGTTTGTTTGG
LOX	AY159556	AAGGCTTTCCTGAAAACAATCA	AAGATGAACCTCATCGGAAGAA
PAL	AB015571	TTAAAATGGCTGGGATCGAG	CCAGCACATTGGTCTCAAAA
PGIP	AF305093	ACGGAACTTGTTCCAGTTTGAT	CGATTGTAACTCACGTTCAGGA
PIN	AY156047	GCAGAAACCATTAAGAGGGAGA	TCTATCCGATGGTAGGGACACT
STS	AF274281	GTGGGGCTCACCTTTCATT	CTGGGTGAGCAATCCAAAAT

* Obtained from NCBI (National center for biotechnology information) or TC TIGR (Tentative consensus number according to the Institute of genome research).

fungi such as *Borytris cinerea*, *Alternaria brassicicola* and to insects (McCONN *et al*., 1997), indicating that jasmonates are essential to induce defense mechanisms during a pathogen attack.

The two hormones cooperate in various processes of plant defense like during plant/fungus and plant/herbivore interactions, wounding or ozone-exposure stress (ZHAO *et al*., 2005). Jasmonates can induce ethylene synthesis in various plant species like tomato, *Arabidopsis*, tobacco and cell suspensions of *Cupressus lusitanica* and *Taxus* spp., whereas ethylene treatment triggers jasmonate synthesis in a restricted number of plants like tomato and *Arabidopsis* (XU *et al*., 1994; MIRJALILI and LINDEN, 1996; ZHAO *et al*., 2004). Ethylene can enhance production of two phytoalexins, taxol and β-thujaplicin, induced by jasmonate treatments of *Taxus* spp. and *Cupressus lusitanica* cell suspensions respectively (MIRJALILI and LINDEN, 1996; ZHAO *et al*., 2004). Ethylene and jasmonate cooperate to induce defense genes synergistically like *PR1b*, osmotin (*PR5*) and *PDF1.2* (XU *et al*., 1994; PENNINCKX *et al*., 1998). In the case of *PDF1.2*, gene induction depended on the simultaneous activation of both signaling pathways when *Arabidopsis* was challenged with *Alternaria brassicicola* (PENNINCKX *et al*., 1996, 1998). Recently, ethylene and jasmonic acid were shown to cooperate in selenium-stress resistance in *Arabidopsis thaliana* (TAMAOKI *et al*., 2008).

The aim of this article was to determine whether grapevine defense mechanisms can be synergistically induced by ethylene/MeJA treatment. Ethephon, a metabolic precursor of ethylene, and methyl jasmonate were used to perform this study. In the light of previous findings (BELHADJ *et al*., 2006; 2008b), we performed experiments on two grapevine models to investigate MeJA/ethephon cotreatment properties: we used foliar cuttings to assess resistance to *Erysiphe necator* and cell suspension cultures to determine stilbene production and expression of defense-related genes, like *STS, PAL* and various genes encoding PR proteins by HPLC and quantitative PCR, respectively.

MATERIALS AND METHODS

1. Plant material

Cell suspension cultures of *Vitis vinifera* L. cv Cabernet-Sauvignon (CS6) (KRISA *et al*., 1999) were maintained under continuous fluorescent light (5000 lux) at 25 °C ± 1 °C on an orbital shaker (110 rpm). Suspension cultures were subcultured weekly in 250 mL Erlenmeyer flasks containing 50 mL of cell suspension in B5 medium supplemented with 20 $g.L^{-1}$ sucrose, 250 $mg.L^{-1}$ casein hydrolysate, 0.5 $mg.L^{-1}$ 1-naphtalene-acetic acid and 0.12 $mg.L^{-1}$ benzylaminopurin, by inoculating the cells at a 1/5 (v/v) ratio into fresh medium. For the experiments, 7-day-old cell suspensions were inoculated (1/7 (v/v) ratio) into the induction medium containing 2-fold more sucrose than in the maintenance medium (LARRONDE *et al*., 1998).

Plants of cultivated grapevine (*Vitis vinifera* L. cv Cabernet-Sauvignon) were propagated from wood cuttings in a greenhouse (INRA, Villenave d'Ornon, France). They were grown under controlled conditions at 25 °C day/night temperature with 75% relative humidity and 16 h photoperiod (350 $\mu mol.m^{2}.s^{-1}$). Two-month-old plants with 10-12 leaves were used for the experiments.

2. Elicitor treatment on cell suspension cultures

MeJA (Sigma-Aldrich, France) was dissolved in EtOH and added at 25 µM final concentration (BELHADJ *et al*., 2008b); ethephon (Sigma-Aldrich, France) was dissolved in water then sterilized by filtration (0.22 µm) before addition at 25 µM final concentration (optimal concentration for stilbene production in CS6 cell suspensions, according to our experiments, data not shown). These compounds were added, alone or together, to 6-day-old cultures. Control cultures received the corresponding vehicle solvent. At 3, 6, 12, 18, 24, 48 and 120 hours after addition, cells were harvested by vacuum filtration, rapidly washed with distilled water, frozen in liquid nitrogen and stored at -80 °C until analysis. At 120 hours, viability of control and treated cells was performed by Trypan blue coloration. In all cases, less than ten percent of cells were dead.

3. Elicitor treatment on foliar cuttings

Cabernet-Sauvignon foliar cuttings of *Vitis vinifera* were sprayed with MeJA dissolved in EtOH (ten-fold MeJA volume) then added to a sterilized aqueous solution at the final concentration of 5 mM. To allow the solution to penetrate into the leaves, we supplied MeJA solution with the wetting agent Triton X-100 at 0.1% (v/v). Ethephon (Sierra®, Bayer) was dissolved in water at the final concentration of 6.94 mM then sterilized by filtration (0.22 µm). Fosetyl-Al (Aliette®, Syngenta) was dissolved in sterile water at the final concentration of 2.82 mM. A control treatment was performed by spraying an aqueous solution containing 0.1% Triton X-100 (v/v) and 1% EtOH. This treatment had no effect on colonization of leaves by *Erysiphe necator*.

4. RNA extraction and quantification of gene expression by real time quantitative RT-PCR

The frozen cells were ground in a mortar and the resulting powder was used for RNA extraction. Total RNA was extracted from cell samples as described by REID *et al*. (2006). Contaminating DNA in RNA samples was removed by using the Nucleospin® RNA plant kit

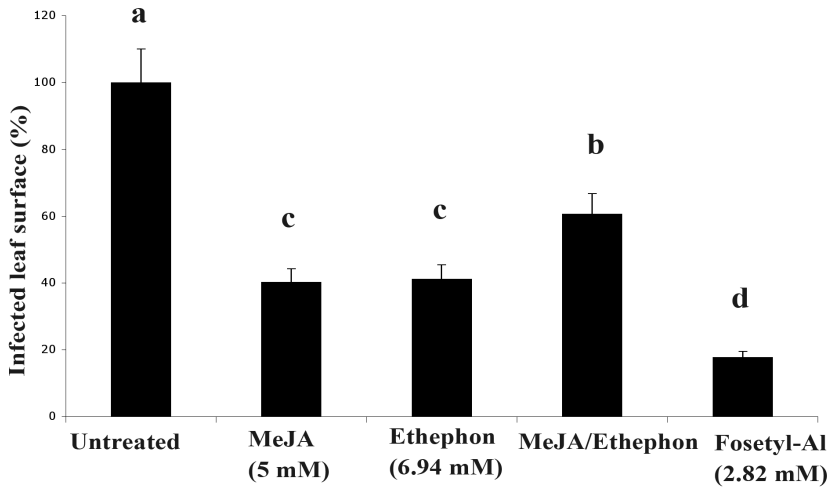

Figure 1 - Protection of grapevine detached leaves of grapevine foliar cuttings in response to ethephon and/or MeJA treatment against *Erysiphe necator*.
Ethephon (6,94 mM) and/or MeJA (5 mM) were sprayed on plants 24 hours before inoculation. Disease was assessed 6 days post-inoculation and expressed as percentage of infected leaf surface. Seven plants were used for each experiment on grapevine foliar cuttings. The experiments were repeated three times with similar results.
Values with different letters are significantly different, $p < 0.05$.

(Macherey-Nagel©). Integrity of total RNA was checked before and after removing DNA by electrophoresis on 1.2% agarose gel stained with ethidium bromide (0.01%).

DNA-free RNA (2 µg) was reverse-transcribed with 3 µM of oligo(dT) using ImProm-II™ (Promega Corp.), Reverse Transcription System (Promega Corp.), according to the manufacturer's instructions. To determine the mRNA copy number of the genes studied, real-time quantitative RT-PCR was performed using the MyiQ detection system (Bio-rad) and iQ SYBR Green Supermix (Bio-rad). PCR reactions were carried out in duplicate in 96-well plates (25 µL per well). Reaction buffer was constituted with 1X iQ SYBR Green Supermix (including Taq polymerase, dNTPs, SYBR Green I dye, 6 mM $MgCl_2$), 400 nM forward and reverse primers and a 1:10 dilution of reverse transcribed RNA. After denaturation at 95 °C for five minutes, amplification was performed in a two-step procedure: 30 s of denaturation at 95 °C and 1 min of annealing and extension at 60 °C, with a total of 45 cycles. The gene-specific primers are indicated in table 1.

PCR reaction specificity was controlled by analyzing melting curves for each data point. Transcript level was calculated as described by ARRIETA-MONTIEL *et al.* (2001). A standard curve was constructed using templates of known copy number for the target sequence. To construct the standard curve, serial dilutions of cloned target sequence in pGEM®-T easy vector (Promega Corp.) were used. The number of copies in each dilution was calculated according to the following formula: (number of moles) (Avogadro constant: 6.023×10^{23}) = number of copies. All standard samples were assayed in triplicate. The copy number of the sample was estimated by plotting the threshold cycle (Ct values) against the logarithm of the starting copy number. The absolute copy number for each sample was calculated from standard curves using the Ct value and normalized against grapevine actin gene as internal control and non-treated cells (t: 0 hours) as reference sample.

Relative gene expression was obtained according to the following formula: fold induction = $2^{-(\Delta\Delta Ct)}$, where $\Delta\Delta Ct$ = (Ct GI (unknown sample) - Ct ACT (unknown sample) - (Ct GI (reference sample) - Ct ACT (reference sample)). The Ct (threshold cycle) value represent the PCR cycle at which the copy number passes the fixed threshold and can be first detected. GI is the gene of interest and ACT is the grapevine actin gene used as internal control. The reference control is the sample chosen to represent 1 x expression of the gene of interest (e.g. non-treated cells).

5. Stilbene extraction and quantification in cells

Stilbenes were extracted from freeze-dried cells (100 mg) overnight with 4 mL of MeOH, at 4 °C. 1 mL of water was added to 1 mL of the methanolic extract and filtered through nylon membrane filters (Titan 2, 0.45 µm, ICS, France). Stilbenes contained in the culture medium were obtained by a triple ethyl acetate extraction using 3 mL of culture medium. The samples were dried-evaporated and extracts were dissolved in 700 µL of H_2O/MeOH (50/50, v/v). Analysis of stilbene content was performed by HPLC on a 250 x 4 mm Prontosil C18 (5 µm) reverse-phase column (Bischoff, Leonberg,

Germany) protected by a guard column of the same material. Separation was performed at a flow rate of 1 mL.min^{-1} with a mobile phase composed of (A) H_2O/TFA 1% (97.5/2.5, v/v) and (B) Acetonitrile/solvent A (80/20, v/v). The run was set as follows: 0 - 1 min, 20% (B), 1 - 8 min, from 20% (B) to 24% (B), 8 - 10 min, from 24% (B) to 25% (B), 10 - 13 min, 25% (B), 13 - 18 min, from 25% (B) to 30% (B), 18 - 35 min, from 30% (B) to 50% (B), 35 - 37 min, from 50% (B) to 100% (B), 37 - 41 min, 100% (B), 41 - 42 min, from 100% (B) to 20% (B), and 20% (B) for 4 min.

The chromatogram was monitored at 286 and 306 nm using an UV detector (Kontron 430, Germany). Stilbene contents were evaluated from calibration curves prepared from standards. *Trans*-resveratrol was purchased from Sigma Chemical Co. (St. Louis, MO). *Cis*- and *trans*-piceids (*cis*- and *trans*-resveratrol 3-O-β glucosides) were purified from *Vitis vinifera* L. cell cultures as previously described (WAFFO-TEGUO *et al*., 1998).

6. Inoculation procedure and disease evaluation

As previous authors noted that older leaves from the bottom of the shoots were more resistant (REUVENI, 1997), inoculation experiments were performed on young leaves from the upper part of the plant. Before inoculation, leaves were washed and dried. *Erysiphe necator* strain belongs to the B genetic group and was isolated from leaves collected in Bordeaux vineyards in 2006 (CORIO-COSTET, 2007).

Detached leaves (one per plant) were cleaned by washing, decontaminated with 5% $Ca(OCl)_2$, rinsed with water and dried. Eight leaves were prepared for each type of treatment. Leaves were deposited lower side down on sterile agar plates and treated with MeJA, ethephon or both under sterile conditions. Twenty-four hours after the treatment, plates were placed at the bottom of a Plexiglas settling tower (DELYE *et al*., 1997). Conidia of *Erysiphe necator* were blown in at the top from sporulating leaves (100 to 200 conidia per cm^2 of leaf). Inoculated leaves were incubated for 6 days at 22 °C under a 16-h photoperiod (25 µE.m^2.s^{-1}).

Disease intensity was estimated by measuring the level of growth and intensity of fungal mycelium and sporulation on leaves. The contamination level was evaluated with a visual scale (0 to 100%) and disk cover was expressed as a percentage according to a scale with steps from 5 to 5 (zero corresponding to the absence of pathogen development). The mycelium and sporulation densities were observed with a microscope.

7. Statistical Analysis

Data were expressed as the means ± standard deviation of 3-5 determinations. Statistical analysis was performed using ANOVA and LSD procedures and $p < 0.05$ was considered to be significant.

RESULTS

1. Effects of ethephon on grapevine resistance to *Erysiphe necator* induced by MeJA

Recently, we have shown that MeJA treatment decreases symptoms severity of *Erysiphe necator* in the vineyard by 75% and also that ethephon can reduce susceptibility to powdery mildew of grapevine foliar cuttings by 70% (BELHADJ *et al*., 2006; 2008a). We used foliar cuttings to determine whether ethephon was able to improve the protection against powdery mildew induced by MeJA treatment.

Leaves excised from grapevine foliar cuttings were sprayed with MeJA, ethephon (Sierra®, Bayer CropScience), or both. Positive control was performed with Fosetyl-Al (Syngenta). 24 hours later, inoculations were performed with the fungus. No phytotoxic effects were observed. Degree of protection against powdery mildew was represented as a percentage of infected leaf surface. Ethephon treatment reduced by 60% colonization of leaves by *Erysiphe necator*. MeJA reduced symptoms severity of the fungus by 60% (figure 1). Cotreatment was less effective than separated hormones since powdery mildew colonization was reduced by only 30%. Fosetyl-Al was efficient against the fungus (85%). We used *Vitis vinifera* cell suspensions, a convenient model for studying defense responses (BELHADJ *et al*., 2008b), to understand why the induction of defense was diminished by ethephon.

2. Effects of ethephon on defense gene expression induced by MeJA

Grapevine cells were cotreated with MeJA and ethephon and the expression pattern of seven selected defense-related genes was analyzed using real-time quantitative polymerase chain reaction (RTq-PCR). Two genes involved in the biosynthesis of phenolic compounds were studied: one phenylalanine ammonia lyase (*PAL1*) gene encoding the first enzyme of the pathway and one stilbene synthase (*STS*) gene encoding the enzyme responsible for the synthesis of resveratrol, the main phytoalexin produced by grapevine in response to biotic and abiotic stresses (LANGCAKE and PRYCE, 1977; ADRIAN *et al*., 1997; COUTOS-THEVENOT *et al*., 2001). Resveratrol derivatives, like piceids (glycosylated resveratrol), viniferins (resveratrol oligomers) or pterostilbene (methylated resveratrol) also play a major role in the resistance of grapevine to fungal attack (BELHADJ *et al*., 2006; 2008a). Moreover, SCHNEE *et al*. (2008) showed that the synthesis of δ - and ε - viniferins at the infection sites seems to be closely linked to the

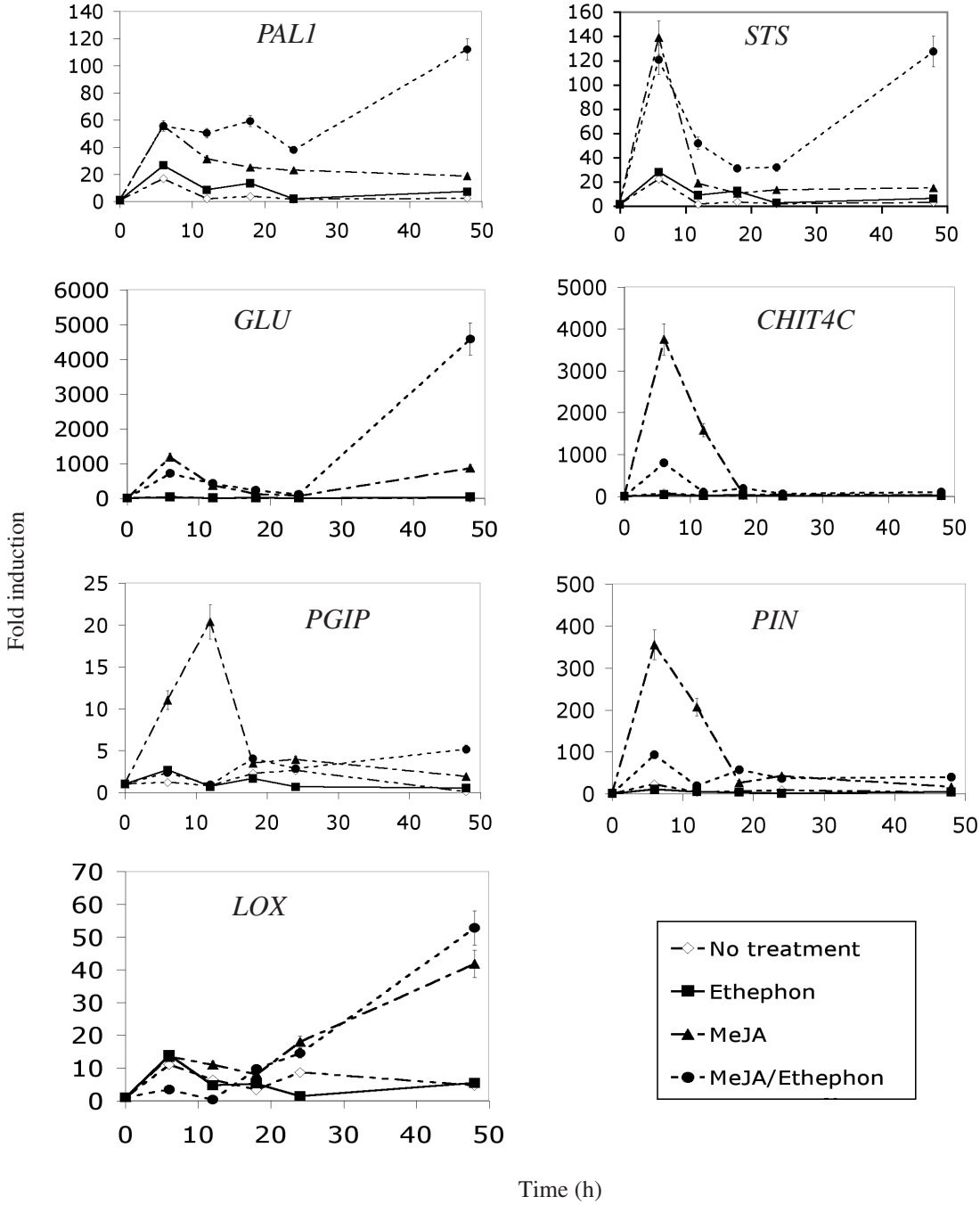

Figure 2 - Time course of defense-related gene transcript accumulation in *Vitis vinifera* cv Cabernet-Sauvignon cell suspensions after treatment with ethephon (25 µM) (■), MeJA (25 µM) (▲), or both (●).

Control cell suspensions were treated with Ethanol/H_2O (◊). Values represent mean ± SD of duplicate assays of one representative experiment out of five. *PAL1, Phenylalanine Ammonia Lyase 1; STS, Stilbene Synthase; GLU, β-1,3 Glucanase; CHIT4C, Chitinase 4C; PGIP, PolyGalacturonase-Inhibiting Protein; PIN, Proteinase Inhibitor; LOX, Lipoxygenase.*

inhibition of grapevine powdery mildew. The expression of four genes encoding PR proteins was also considered: acidic class IV chitinase (*CHIT4C*), serine protease inhibitor (*PIN*), polygalacturonase-inhibiting protein (*PGIP*), and β-1,3-glucanase (*GLU*). Finally, we studied the accumulation of *LOX* transcript gene, encoding one enzyme involved in jasmonate metabolism.

In control cells, the transcript level of the genes remained low during the 48 hours of the experiment (figure 2). In ethephon-treated cells, the expression profile

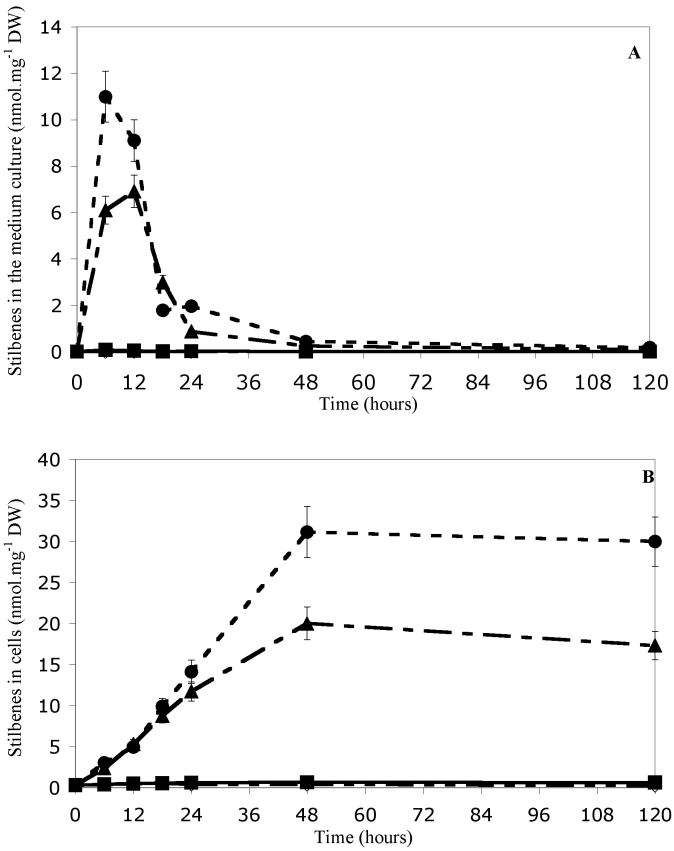

Figure 3 - Time course of stilbene accumulation in *Vitis vinifera* cv Cabernet-Sauvignon cell suspensions after treatment with ethephon (25 µM) (■), MeJA (25 µM) (▲), or both (●).
Control cell suspensions were treated with ethanol/H_2O (◊). A) In cell suspensions medium. B) In cells.
Values represent mean ± SD of duplicate assays of one representative experiment out of five.

of the genes was comparable with that observed in control cells. In MeJA-treated cells, RNAs of genes involved in stilbene metabolism and encoding PR-proteins were rapidly and transiently accumulated after 6 hours of treatment. Maxima observed were respectively 60-fold (*PAL1*), 140-fold (*STS*), 3700-fold (*CHIT4C*), 20-fold (*PGIP*) and 350-fold (*PIN*) (figure 2) higher than in control cells. Transcript level of *LOX* gene was progressively up-regulated and reached a maximum after 48 hours (55-fold higher than in control cells). Transcript level of *GLU* gene was rapidly up-regulated after 6 hours (1,200-fold higher than in control cells), then down-regulated until 24 hours, and reached a new maximum at 48 hours (1,000-fold higher than control cells).

The addition of ethephon in MeJA-treated cells induced a difference in the expression profile of *PAL1*, *STS* and *GLU* genes, mainly by adding a second step of up-regulation. For these three genes, the maximum observed after 6 hours was not modified by ethephon, but it allowed a new accumulation of transcripts from 18 hours post-treatment to 48 hours (120-fold, 125-fold and 4,500-fold induction in comparison with 20-fold, 15-fold and 800-fold in cells treated with MeJA alone, respectively). Concerning the three PR proteins, *CHIT4C*, *PGIP* and *PIN*, ethephon strongly inhibited the gene expression induced by MeJA after 6 hours of treatment (750-fold, 2.5-fold and 100-fold induction in ethephon/MeJA-treated cells instead of 3700-fold, 20-fold and 350-fold induction in MeJA-treated cells, respectively). Similarly, ethephon strongly inhibited the expression of *LOX* gene induced by MeJA treatment at 6 hours (3-fold induction instead of 17.5-fold induction in MeJA treated cells), but the effect of ethephon disappeared after 18 hours of treatment (figure 2).

3. Effects of ethephon on stilbene accumulation induced by methyl jasmonate in cell suspensions

Resveratrol and its derivatives such as piceids, pterostilbene and viniferins are the main forms of phytoalexin in grapevine. They are produced by the plant

Table 2 - Stilbene compostion of *Vitis vinifera* cell suspension cultures (medium and cells) treated with ethephon, MeJA or both, at 6 and 48 hours post-treatment.

In the medium		Values (nmol.mg^{-1} DW)		
		Trans-piceid	Cis-piceid	Trans-resveratrol
6 hours	Untreated	0 (a)	0 (a)	0 (a)
	Ethephon	0 (a)	0 (a)	0.07 ± 0.001 (a)
	MeJA	0.05 ± 0.001 (b)	0.12 ± 0.002 (b)	5.92 ± 0.5 (b)
	MeJA/Ethephon	0.04 ± 0.002 (b)	0.1 ± 0.001 (b)	10.8 ± 0.8 (c)
48 hours	Untreated	0 (a)	0 (a)	0 (a)
	Ethephon	0 (a)	0 (a)	0 (a)
	MeJA	0.01 ± 0.0001 (a)	0.01 ± 0.001 (a)	0.22 ± 0.03 (b)
	MeJA/Ethephon	0.04 ± 0.002 (b)	0.04 ± 0.002 (b)	0.36 ± 0.04 (c)

In cells		Values (nmol.mg^{-1} DW)	
		Trans-piceid	Cis-piceid
6 hours	Untreated	0 (a)	0.42 ± 0.05 (a)
	Ethephon	0 (a)	0.41 ± 0.03 (a)
	MeJA	0.45 ± 0.02 (b)	1.92 ± 0.2 (b)
	MeJA/Ethephon	0.99 ± 0.15 (b)	2.07 ± 0.3 (b)
48 hours	Untreated	0 (a)	0.37 ± 0.04 (a)
	Ethephon	0 (a)	0.67 ±0.05 (b)
	MeJA	8.31 ± 0.5 (b)	11.7 ± 1.1 (b)
	MeJA/Ethephon	18.3 ± 1.5 (c)	12.8 ± 1.1 (b)

Values represent means ± SD of five experiments. No resveratrol was found in the cells.
Values with different letters are significantly different. $p < 0.05$.

in response to pathogen attacks or elicitors. We studied stilbene production in grapevine cell suspensions after MeJA/ethephon treatment in comparison with treatment by MeJA or ethephon alone. *Trans*-resveratrol was found only in the culture medium whereas its glucosides, *trans*- and *cis*-piceids were found both in cells and medium. *Cis*-resveratrol was observed only in the medium at very low levels so we did not quantify it. Other resveratrol derivatives, pterostilbene and viniferins were not detected either in the cells or culture medium, even with a LC-MS detection system.

Cell suspension cultures of Cabernet-Sauvignon (CS6) were treated with MeJA, ethephon or both for 120 hours (figure 3, table 2). During the time course of the experiment, no stilbenes were significantly detected either in the untreated or in ethephon-treated cell culture medium. Moreover, only small quantities of *cis*-piceid were found in the cells (around 0.5 nmol.mg^{-1} DW). In MeJA-treated cell suspensions, the stilbenes studied, mainly *trans*-resveratrol, rapidly and transiently accumulated in the medium. The culture medium of MeJA-treated cells contained 5.92 and 0.17 nmol.mg^{-1} DW of trans-resveratrol and piceids (*cis*- and *trans*- forms), respectively after 6 hours of treatment, whereas only traces of *trans*-resveratrol (0.22 nmol.mg^{-1} DW) and piceids (0.02 nmol.mg-1 DW) were found after 48 hours. Maximum stilbene accumulation in the medium was observed after 12 hours (6.1 nmol.mg^{-1} DW). No *trans*-resveratrol was detected in the cells. MeJA treatment induced linear accumulation of piceids until 48 hours after elicitation (8.31 and 11.7 nmol.mg^{-1} DW of *trans*- and *cis*-piceid, respectively), which slightly diminished until the end of the experiment.

Addition of ethephon to MeJA-treated cell suspensions slightly modified the stilbene accumulation profile (figure 3, table 2). In medium, *trans*-resveratrol production occurred earlier and more strongly than with MeJA treatment, and the maximum was obtained after 6 hours (10.8 nmol.mg^{-1} DW) instead of 12 hours. Nevertheless, no changes in piceid diffusion were noted (around 0.05 nmol.mg^{-1} DW). After 48 hours of MeJA or MeJA in the presence of ethephon treatment, a similar stilbene content was present in the medium (around 0.30 nmol.mg^{-1} DW). In cells, ethephon treatment did not modify the accumulation of stilbenes induced by MeJA before 24 hours, but conferred a more efficient induction of *trans*-piceid accumulation that lasted until 48 hours (18.3 nmol.mg^{-1}). Stilbene content then remained stable until the end of the experiment.

DISCUSSION

When applied on *Vitis vinifera* cell suspensions CS6, MeJA/ethephon cotreatment synergistically up-regulated the expression of *PAL1* and *STS*, genes involved in the phenylpropanoid pathway (figure 2). This induction led to a noteworthy production of stilbenes, mainly *trans*-resveratrol in the medium, and piceids (*cis*- and *trans*-) in cells (figure 3). The activation of this biosynthesis pathway is one of the most important resistance reactions in many plants (DIXON and PAIVA, 1995). Resveratrol and its derivatives are produced by grapevine in response to elicitor and pathogen attacks (ADRIAN *et al*., 1997; PEZET *et al*., 2003). Piceids and resveratrol were

quantitatively the major stilbenes produced. LANGCAKE and PRYCE (1977) showed that resveratrol confers a tolerance to powdery mildew and downy mildew. In response to stress, piceid could constitute a pool of immediately usable resveratrol. Treatment with ethephon did not modify phenylpropanoid pathway gene expressions, *PAL1* and *STS*, but treatment with MeJA led to the up-regulation of phenylpropanoid pathway genes, *PAL1* and *STS*. However, transcript accumulations were lower than observed with MeJA/ethephon cotreatment. Correlated to the poorer induction of *STS* and *PAL1* gene expressions, quantities of stilbenes observed in cell suspensions treated with Ethephon or MeJA were lower than in MeJA/ethephon-treated cells (figure 1 and table 2).

MeJA/ethephon treatment also modified RNA accumulation of various defense-related genes including genes encoding PR proteins and involved in the jasmonate biosynthesis pathway. Grapevine *GLU* gene expression (figure 2) was up-regulated after MeJA treatment, the first maximum induction being observed at 6 hours. When ethephon was added to MeJA-treated cells, *GLU* expression was down-regulated during the first 24 hours. However, after 24 hours, ethephon addition considerably increased the transcript level of the gene induced by MeJA treatment. β-1,3-glucanase is a well-known PR protein that is constitutively expressed at low levels in plants and can be significantly induced when plants are infected by fungal, bacterial or viral pathogens. Several studies have demonstrated that β-1,3-glucanases are able to degrade the structural β-1,3-glucan present in fungal cell walls and inhibit mycelial growth or spore germination of certain pathogenic fungi (LEUBNER-METZGER and MEINS, 1999).

Concerning the acidic chitinase gene, *CHIT4C*, transcript levels increased rapidly after MeJA treatment and peaked at 6 hours (figure 2). Transcript level of this gene was notably modified by addition of ethephon to MeJA-treated cells, reducing the induction observed at 6 hours. Chitinase is one of the most important enzymes involved in pathogenesis-related mechanisms in plants. It degrades chitin which is a major structural component of fungal cell walls (SCHLUMBAUM *et al*., 1986; MAUCH *et al*., 1988). KARASUDA *et al*. (2003) reported that chitinase is able to degrade *Podosphaera aphanis* structure, the strawberry powdery mildew. ROBINSON *et al*. (1997) have shown that the synthesis of chitinase in grapes during ripening coincided with the increased resistance to powdery mildew infection. In our case, we observed that MeJA treatment associated with ethylene was unable to induce *CHIT4C* gene expression, which could be correlated with the loss of protection against the fungus.

Polygalacturonase-inhibiting proteins (*PGIPs*) and proteinase inhibitors (*PINs*) have been shown to be involved in the plant defense response. *PGIPs* interact with fungal endopolygalacturonase and inhibit their activities (de LORENZO *et al*., 1997) and PIN proteins reduce fungal growth (KIM *et al*., 2005). MeJA treatment led to a high accumulation of *PIN* gene transcript and a medium accumulation of *PGIP* gene transcript. When ethephon was added to MeJA-treated cells, accumulations of *PGIP* and *PIN* gene transcripts were notably inhibited (figure 2). Finally, ethephon reduced accumulation of *LOX* gene RNAs induced by MeJA treatment during the first 18 hours. Thereafter, the effect of ethephon disappeared and the transcript level of *LOX* gene was comparable between MeJA and MeJA/ethephon-treated cells.

Ethephon addition to MeJA treatment on *Vitis vinifera* excised leaves was unable to confer a better resistance to *Erysiphe necator*, which was superior to separate treatments with Ethephon or MeJA (60% inhibition of fungal growth for each separate treatment and only 30% inhibition for the cotreatment) (figure 1). The use of mutants in *Arabidopsis*, tobacco (*Nicotiana tabacum*) and soybean (*Glycine max*) demonstrated that both ethylene perception and signaling are required for resistance to some pathogens, but not to others (KNOESTER *et al*., 1998; HOFFMAN *et al*., 1999; THOMMA *et al*., 1999; van LOON *et al*., 2006). Ethylene seems to inhibit symptom development in necrotrophic pathogen infection, as in tomato towards *Botrytis cinerea* (DIAZ *et al*., 2002), but enhances the cell death caused by other types of pathogen infection (WANG *et al*., 2002). However, ethephon treatment was shown to enhance resistance to powdery mildew in grapevine foliar cuttings (BELHADJ *et al*., 2008a). In parallel, the use of mutants in *Arabidopsis*, tobacco (*Nicotiana tabacum*) and soybean (*Glycine max*) showed that both jasmonate perception and signaling are required for resistance to pathogens. Jasmonates have also been reported to induce local and systemic protection against *Phytophthora infestans* in tomato plants and potato (COHEN *et al*., 1993), powdery mildew in barley seedlings (WALTERS *et al*., 2002) and grapevine in the vineyard (BELHADJ *et al*., 2006). The protection triggered by MeJA or ethephon was shown to involve the expression of defense-related genes encoding PR proteins genes or involved in phytoalexin biosynthesis (BELHADJ *et al*., 2006, 2008a).

When compared to MeJA treatment alone, the addition of ethephon to MeJA led to lesser or equal induction of defense-related genes for the first 18 hours and then, in some cases, to up-regulated transcript levels for the rest of the experiment (*PAL1, STS, GLU*). For the other genes (*LOX, CHIT4C, PIN, PGIP*), transcript levels were similar to those obtained with MeJA or ethephon treatments at

the end of the experiment. Ethephon treatment was clearly able to antagonize the effects of MeJA on defense-related gene expression, as MeJA/ethephon treatment led to a systematically reduced induction of *LOX, CHIT4C, PIN* and *PGIP* transcript level for the first 18 hours when compared to MeJA treatment. The first hours of elicitation are known to be essential for the plant to resist a pathogen attack (DIXON *et al*., 1994). The importance of PR proteins for grapevine protection against powdery mildew was shown by GIANNAKIS *et al*. (1998). They reported a correlation between the combined activities of chitinase and glucanase and the observed field resistance to powdery mildew. They also demonstrated that purified chitinase and glucanase have antifungal activity against *Erysiphe necator*. Ethephon can also counteract the jasmonate pathway, particularly the genes induced in wound response (LOX for example) (LORENZO and SOLANO, 2005). Moreover, in some cases, the accumulation of phytoalexins (stilbenes), as observed in MeJA/ethephon treatment, is not sufficient to trigger resistance to pathogens (THOMZIK *et al*., 1997; KOBAYASHI *et al*., 2000). Indeed, transgenic plants (e.g. tomato) expressing the stilbene synthase gene produced *trans*-resveratrol but did not show any improved resistance to *Botrytis cinerea*.

MeJA/ethephon treatment in *Vitis vinifera* cell suspensions induces a more efficient production of phytoalexins than observed with MeJA or ethephon treatment alone. However, the expression of several defense-related genes is lower with it. On foliar cuttings, ethephon treatment was unable to improve the enhanced resistance to *Erysiphe necator*, a pathogenic agent of powdery mildew, conferred by MeJA treatment. This could be due to the antagonistic signaling pathway between MeJA and ethephon, as with the inhibition of *LOX* expression by ethephon via ERF1 (LORENZO and SOLANO, 2005).

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