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GRAPEVINE PROTECTION AGAINST *ERYSIPHE NECATOR* BY METHYL JASMONATE TREATMENT - STUDIES OF DEFENSE RESPONSES -

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RESUME :

PROTECTION DE LA VIGNE CONTRE *ERYSIPHE NECATOR* SUITE A UN TRAITEMENT AU METHYL JASMONATE - ETUDES DES REPONSES DE DEFENSE-

Afin de limiter l'utilisation excessive de produits phytosanitaires au vignoble, des stratégies alternatives basées sur le traitement par des éliciteurs peuvent être développées. L'objectif de ce travail est de déterminer la capacité élicitrice du méthyl jasmonate (MeJA) à stimuler les mécanismes de défense naturelle de la Vigne. Les feuilles traitées au MeJA ont répondu par une augmentation des taux de transcrits codant des protéines PR et codant des enzymes impliquées dans la biosynthèse de stilbènes (composés antimicrobiens). Ces résultats sont corrélés avec une accumulation de ces molécules. L'activité élicitrice du MeJA a été confirmée par l'augmentation de la tolérance à l'oïdium des boutures foliaires et des ceps au vignoble (75% et 73%, respectivement). Le MeJA pourrait donc agir comme un éliciteur efficace dans une stratégie alternative de protection de la Vigne.

Mots-clés : *Vitis vinifera*, éliciteur, stilbènes, protéines PR, oïdium.

SUMMARY :

To limit the excessive use of phytochemicals in the vineyard, alternative strategies based on treatment with elicitors have to be developed. The aim of this work is to check the eliciting capacity of methyl jasmonate (MeJA) to stimulate grapevine natural defenses. MeJA-treated leaves reacted by increasing transcript levels coding pathogenesis-related proteins and coding enzymes involved in stilbene biosynthesis (antimicrobial compounds). This was correlated with the accumulation of these molecules. The eliciting activity of MeJA was confirmed by enhanced tolerance of grapevine foliar cuttings and vineyard against powdery mildew (75% and 73%, respectively). MeJA can therefore act as an efficient elicitor in an alternative strategy of grapevine protection.

Keywords : *Vitis vinifera*, elicitor, stilbenes, PR proteins, powdery mildew.

INTRODUCTION

Grapevine (*Vitis vinifera* L.) is susceptible to many fungi such as *Botrytis cinerea* (grey mould), *Plasmopara viticola* (downy mildew), *Erysiphe necator* (powdery mildew) and *Eutypa lata* (dieback). Fungal infection reduces fruit quality and yield. Disease control is currently achieved by intensive use of fungicides. Although these chemicals are relatively effective when applied as part of a strategic spray program, the cost to the grower and the environmental impact of the residues remain undesirable. Alternative strategies involving genetic manipulation of host defense mechanisms have been shown to increase the resistance of a number of crop species to fungal pathogens (Jach *et al.*, 1995 ; Coutos-Thévenot *et al.*, 2001), but such practices are forbidden in French vineyards (Coutos-Thévenot *et al.*, 2001).

Another approach consists in the induction of natural plant defenses by using elicitors. A variety of molecules can act as elicitors, including oligo- and polysaccharides, peptides, proteins and lipids (Boller, 1995 ; Côté *et al.*, 1998). Elicitor perception triggers various signaling pathways: ion fluxes, oxidative burst and synthesis of signal molecules such as salicylic acid, jasmonic acid and ethylene. Defense-related genes are induced leading to reinforcement of plant cell walls, accumulation of antimicrobial compounds such as phytoalexins, and synthesis of proteins with hydrolytic or inhibitory activity towards microbes (Kombrink and Somssich, 1995).

Owing to their involvement in the signal transduction cascade leading to defense responses, jasmonic acid (JA) and its more active derivative, methyl jasmonate (MeJA), have been used as inducers of defense mechanisms in a number of systems (Creelman and Mullet, 1995).

In grapevine, MeJA has been shown to stimulate deposition of callose and the accumulation of PR-proteins, and to induce production of salicylic acid in leaves and in suspension-cultured cells (Repka *et al.*, 2004).

The present study sought to determine whether exogenous application of methyl jasmonate (MeJA) on vineyard (*Vitis vinifera* L.) is able to induce defense responses and lead to protection towards pathogens. After treatment of plants with MeJA, expression of defense-related genes encoding enzymes involved in the phenylpropanoid pathway (*PAL*, *STS*) and encoding PR proteins (*CHIT4c*, *PIN*, *PGIP* and *GLU*) was monitored by real-time quantitative RT-PCR. We performed quantitative analysis of stilbenes, the major anti-microbial compounds of grapevine, to check for a correlation between the level of expression of the genes involved in the biosynthesis of stilbenes and the accumulation of these products. Grapevine protection with MeJA was evaluated towards downy (*Plasmopara viticola*) and powdery (*Erysiphe necator*) mildew on detached leaves of MeJA-treated plants (Cabernet Sauvignon) and in vineyard (Merlot).

MATERIALS AND METHODS

Plant material. Two months-old plants of cultivated grapevine (*Vitis vinifera* L. cv Cabernet Sauvignon) were used for the experiments. They were grown under controlled conditions at 25/20°C day/night temperature, with 75% relative humidity and a 16h photoperiod (350 µmol/m^2/s).

Experiments in vineyard were conducted in commercial vineyards in Arbis (South-West Region of France) on *Vitis vinifera* L. cv Merlot healthy plants. During the experiments, the average relative humidity was 50-70% and temperature was 24-28 °C.

MeJA treatment on foliar cuttings. MeJA (Sigma, France) was dissolved in 1 % EtOH to the final concentration of 5 mM, and added to an aqueous solution containing the wetting agent Triton X-100 at 0.1% (V/V) (Sigma, France). Ten ml of this solution were sprayed per foliar cuttings. Control plants were sprayed with the Triton solution at 0.1%. Positive control plants were treated with Aliette® (Fosetyl-Al, Fertiligène, France) (3 g/l) while negative ones were untreated.

For all experiments, 15 plants per treatment were entirely sprayed and each experiment was repeated in triplicate. After 72 hours of treatment, leaves were collected for fungal inoculation or stilbene quantification. For molecular analysis, leaves were collected 12, 18, 24 and 72 h after treatment, frozen in liquid nitrogen and stored at - 80°C until analysis.

MeJA treatment in vineyard. MeJA was dissolved in 1 % EtOH to a final concentration of 5 mM (0.67 kg/ha) and 15 mM (2 kg/ha) and added to an aqueous solution containing the wetting agent Triton X-100 (0.1%) (Sigma, France). Control plants were sprayed only with the Triton solution (0.1%). Other control plants were treated with Thiovit® (Syngenta, France) (12.5 kg/ha). Treatments were replicated every 7 to 10 days from May to August (from the bloom to the beginning of veraison) on four adjacent vines arranged in a randomized complete block design.

RNA extraction and quantification of gene expression by real-time quantitative RT-PCR. The leaves were ground in presence of liquid nitrogen and the resulting powder was used for RNA extraction. Total RNA was extracted from leaf samples as described by Chang *et al.* (1993). Gene expression was quantified as described by Aziz *et al.* (2003).

Inoculation procedures and disease evaluation. Inoculations were performed 72 hours after MeJA treatment on leaves. *Plasmopara viticola* and *Erysiphe necator* fungal strains were obtained from INRA, Villenave d'Ornon, France.

Plasmopara viticola (strain Cou100 - Bordeaux) was maintained on grapevine leaf-disk at - 22°C and sub-cultured twice before the assay. Sporangia were collected with a paint brush and suspended in dematerialized water at 4°C to obtain a final density of 5000 sporangia/mm^2. Thoroughly rinsed, cleaned and dried leaves were placed upside down on moist filter paper in Petri dishes. Lower surfaces of the leaves were inoculated with freshly prepared sporangia suspension (fifteen 10µl droplets per leaf, one leaf per plant) and incubated for 16h at 22°C in the dark. The droplets were then gently removed with a pipette connected to an air pump and leaves were reincubated for seven more days at 22°C under a 16h photoperiod (25µE/m^2/s) and 75% relative humidity.

For inoculation with *Erysiphe necator* (strain BE3 – Bordeaux), detached leaves (one per plant) were cleaned, decontaminated with NaOCl 5%, rinsed and dried. Eight leaf disks (1.8 cm diameter) were taken from each leaf (4 disks per side) of each tested plant using a cork borer. Disks were deposited lower side down on sterile agar plates and placed at the bottom of a Plexiglas settling tower (Délye and Corio-Costet, 1998). Conidia were blown in at the top from sporulating leaves (60 to 80 conidia per mm^2 of leaf). Inoculated leaves were incubated for 14 days at 22°C under a 16 h photoperiod (25µE/m^2/s).

Disease intensity was estimated by measuring the level of growth and intensity of fungal mycelium and sporulation on leaves. The contamination level was visually evaluated and expressed as percentage of total leaf area according to a 0 to 100% scale with steps from 5 to 5 (the note zero corresponding to the absence of pathogen development). The mycelium density and the aspect of spores were observed with a microscope.

In vineyard, the parcel in which the experiments were conducted is particularly susceptible to powdery mildew and numerous disease symptoms are detected there each year. Powdery mildew (*Erysiphe necator*) contaminations were natural and promoted by rainfall. Disease intensity was evaluated visually by estimating pathogen attack frequency and percentage of infected leaf surface.

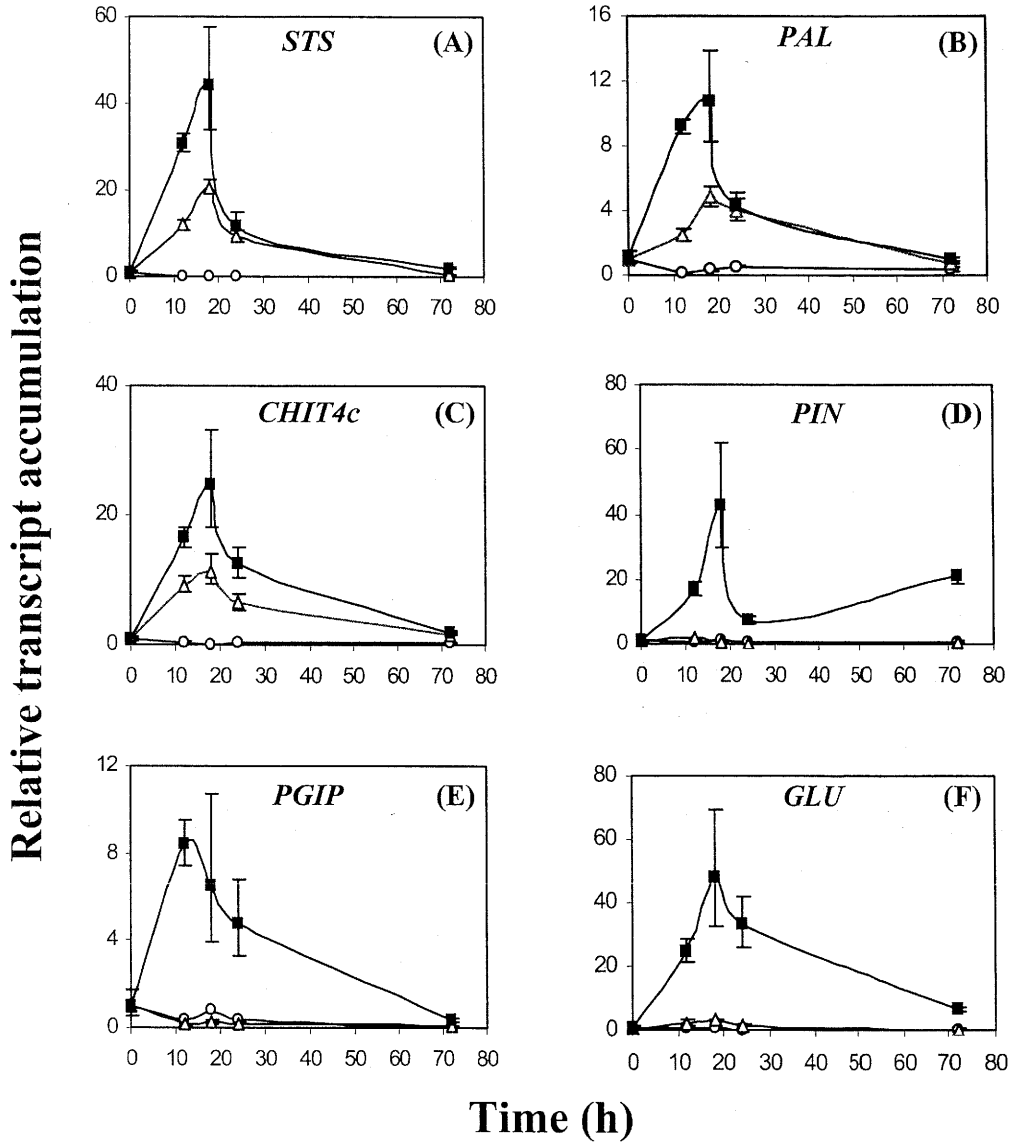
Quantification of stilbenes. Stilbenes were extracted from freeze-dried leaves (100 mg) overnight with MeOH (10 ml) at 4 °C as described by Larronde *et al.* (2003).

RESULTS

Effects of MeJA on defense gene expression in grapevine foliar cuttings. The expression pattern of 6 genes involved in defense was analyzed using real-time quantitative polymerase chain reaction (RTq-PCR). Two genes involved in the biosynthesis of polyphenol compounds were studied: one phenylalanine ammonia lyase (*PAL*) gene encoding the first enzyme of the pathway and one stilbene synthase (*STS*) gene encoding the enzyme

responsible for the synthesis of stilbenes like piceids and resveratrol, the main phytoalexins produced by grapevine in response to biotic and abiotic stresses (Langcake and Pryce, 1977 ; Adrian *et al.*, 1997 ; Coutos-Thévenot *et al.*, 2001). The expression of four genes encoding PR proteins was also considered: an acidic class IV chitinase (*CHIT4c*) gene, a serine protease inhibitor (*PIN*) gene, a polygalacturonase-inhibiting protein (*PGIP*) gene and a β-1,3-glucanase (*GLU*) gene.

Figure 1: Transcript accumulation of defense genes in untreated (open circles), Triton- (open triangles) and MeJA-treated grapevine leaves (closed squares).
Accumulation des transcrits des gènes étudiés dans des feuilles de Vigne non traitées (cercles vides), traitées au Triton (triangles vides) ou au MeJA (carrés pleins).



In untreated leaves, no significant transcript accumulation of the studied genes was detected during the 72 h incubation period (**Figure 1**). In MeJA-treated leaves, the expression of *PAL*, *STS* and *CHIT4c* genes was rapidly and transiently up-regulated (**Figure 1 A, B, C**). Serine protease inhibitor (*PIN*) gene was transiently induced (**Figure 1 D**). The RT-PCR analysis

also showed that the *PGIP* and *GLU* genes were up-regulated in grapevine leaves in response to MeJA (**Figure 1 E, F**).

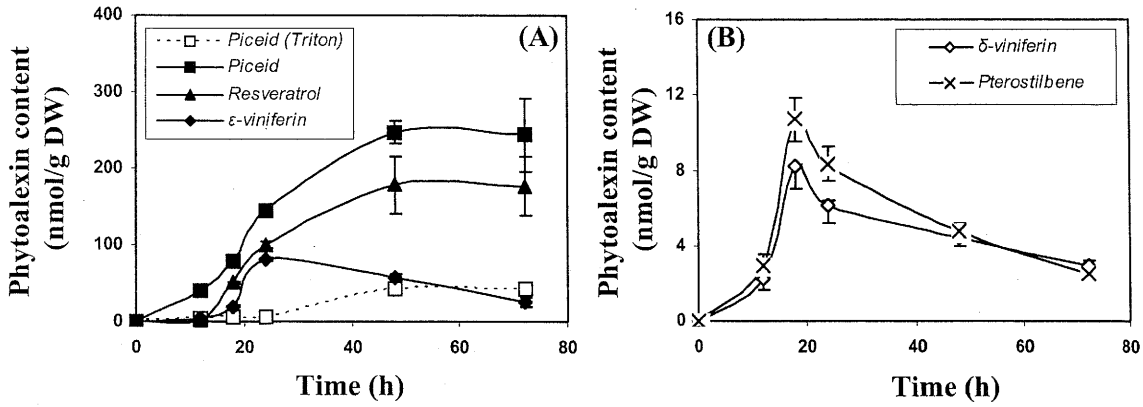
Effects of MeJA on stilbene accumulation. The *trans*-form content (the main form found in leaves) of five major stilbenic phytoalexins - resveratrol, piceid, ε-viniferin, δ-viniferin and pterostilbene – was quantified.

During the 72 h-analysis, *trans*-piceid (**Figure 2 A**) was quantitatively the most prolific stilbene produced (244 nmol/g DW). *Trans*-resveratrol (**Figure 2 A**) accumulated with a similar profile but in slightly less quantity (176 nmol/g DW).

ε-viniferin (**Figure 2 A**) was produced after about 18 h and accumulated quantitatively to about 80 nmol/g DW , then decreased slowly to 25 nmol/g DW at 72h.

Pterostilbene and δ-viniferin (**Figure 2 B**) were also detected in MeJA-treated leaves, but at relatively lower levels compared to the quantity of the other phytoalexins.

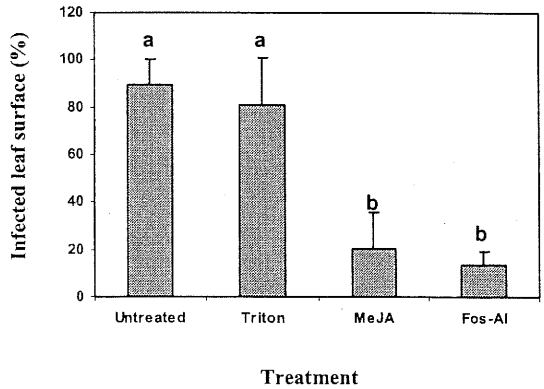
Figure 2: Time course of accumulation of several *trans*-stilbenes in *Vitis vinifera* (L.) cv Cabernet Sauvignon leaves in response to MeJA treatment.
Cinétique d'accumulation des différents *trans*-stilbènes détectés dans les feuilles de Vigne (Cabernet Sauvignon) en réponse au traitement par le MeJA.



Effects of MeJA on grapevine protection.

On excised leaves from foliar cuttings. Degree of protection for powdery mildew was represented as a percentage of infected leaf surface (**Figure 3**).

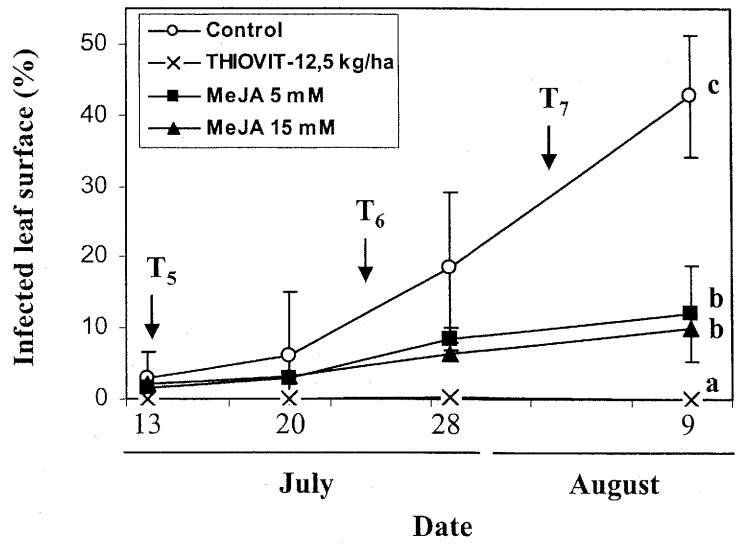
Figure 3: Protection of grapevine detached leaves in response to MeJA treatment against *Erysiphe necator.*
Protection de feuilles détachées de Vigne contre *Erysiphe necator* suite à un traitement au MeJA.



Test validity was checked by using the Aliette® compound (Fosetyl-Al) (3 g/l) as a positive control.
Pre-treatment with MeJA three days before inoculation induced a strong reduction of the infection by *E.* necator (75%) compared to control leaves. Aliette® protection rate was about 83 % for *E.* necator. No significant decrease in the development of *P.viticola* was observed (14%) (data not shown).

In vineyard. Figure 4 shows the evolution of *Erysiphe necator* contamination on vineyard grapevine leaves. Disease intensity was estimated as a percentage of infected leaf surface. Test validity was checked by monitoring the effects of THIOVIT®, a protective and curative commercial extract containing 80% of sulphur (12.5 kg/ha).
In our vineyard assays, a low degree of natural infection was detected on grapevine before July 20 2005, owing to absence of rainfall at the beginning of the experiments. The temperature was high and the average relative humidity low.
From July 20 to August 9, powdery mildew progressed constantly and strongly, infecting from 3 to 43% of control leaf surfaces. In contrast, in MeJA-treated plants, the percentage of leaf surface infection by *E.necator* was only 12.1% with MeJA 15mM and 10.1% with MeJA 5 mM. Owing to its curative effect, THIOVIT® treated plants were completely protected (100 %).

Figure 4: Protection of grapevine plants (Merlot) in response to MeJA against *Erysiphe necator* in vineyard.
Protection de plants de Vigne (cépage Merlot) contre *Erysiphe necator* suite à un traitement au MeJA.



DISCUSSION

On grapevine foliar cuttings (**Figure 3**), a concentration of 5 mM MeJA is sufficient to trigger considerable protection against *Erisyphe necator*: i.e. 75 % of disease reduction. MeJA pre-treatment of plants in vineyard (**Figure 4**) reduced the development of *Erisyphe necator* by approximately 73 % compared to control plants, as shown mainly by a significant reduction in the infected leaf area.
The protection afforded by MeJA could be due to induction of defense-related proteins and enhanced production of antimicrobial compounds as phytoalexins. Indeed to our knowledge,

MeJA have no fungitoxic effect (Prost *et al.*, 2005) and to prevent this possible effect, the foliar cuttings leaves were washed before fungal inoculations.
In our experiment, MeJA treatment on grapevine leaves induces expression of several classes of PR proteins. For example, transcript levels of an acidic chitinase gene, *CHIT4c,* increased rapidly after MeJA treatment and peaked after 18 h (**Figure 1 C**). Grapevine chitinase genes are known to be inducible in stress conditions such as fungal challenge, elicitor treatment or exposure to phytohormones such as ethylene, jasmonic acid and salicylic acid (Graham and Sticklen, 1994 ; Kombrink and Somssich, 1995). Chitinase accumulated in plants to degrade chitin, a major cell wall component of most fungi (Schlumbaum *et al.*, 1986 ; Mauch *et al.*, 1988). However, this induction varies according to the stress applied, the organ studied and the grapevine cultivar.
Grapevine *GLU* gene expression (**Figure 1 F**) was highly up-regulated after MeJA treatment. The class I β-1,3-glucanases are antifungal vacuolar proteins involved in plant defense and their production was enhanced in response to developmental, hormonal and pathogenesis-related conditions (Sela-Buurlage *et al.*, 1993). The antifungal activity of plant β-1,3-glucanases is thought to hydrolyze the structural β-1,3-glucan present in fungal cell walls. Moreover, the combination of chitinase and β-1,3-glucanases is believed to potentiate the antifungal activity and was shown experimentally to inhibit the growth of many pathogenic fungi (Sela-Buurlage *et al.*, 1993). Giannakis *et al.* (1998) reported a correlation between the combined activities of chitinase and β-1,3-glucanase in a range of grapevine cultivars and observed field resistance to powdery mildew. They also demonstrated that chitinase and glucanase proteins purified from the leaves of a resistant cultivar inhibited the growth of powdery mildew in an *in vitro* bioassay.
PIN and *PGIP* mRNA transcripts also accumulated in MeJA-treated leaves. The inhibitors of serine proteases (PIN) belong to the class of antifungal PR-6 proteins, which have a potent activity against plant and animal pathogens (Van Loon and Van Strien, 1999). Farmer and Ryan (1990) showed that airborne MeJA induces the expression of PIN proteins in tomato leaves. Polygalacturonase inhibitor proteins (PGIPs) act to thwart fungal penetration of the plant cell wall as an early induced plant defense to fungal attack (Caprari *et al.*, 1996). PGIPs are ubiquitous cell wall proteins that specifically inhibit the activity of fungal polygalacturonases (PGs), some of which are important fungal pathogenicity factors. PGIP levels have been shown to correlate in several cases with an increased resistance of plants to fungi (De Lorenzo and Ferrari, 2002). Therefore, a putative role of PGIP and PIN could partly explain the reduced infection of grapevine infected leaves by *E. necator.*
In leaves of MeJA-treated plants, the phenylpropanoid pathway was also up-regulated. *PAL* and *STS* gene induction (**Figure 1 A, B**) led to a noteworthy increased production of resveratrol and its derivatives: piceid, viniferins and pterostilbene (**Figure 2 A**). 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 such as viniferins (dimerization), pterostilbene (methylation) and piceid (glycosylation) represent the major forms of phytoalexins in grapevine (Coutos-Thévenot *et al.*, 2001 ; Jeandet *et al.*, 2002 ; Pezet *et al.*, 2004) and are produced by grapevine in response to elicitor and pathogens attacks (Adrian *et al.*, 1997). Piceid and resveratrol were quantitatively the major stilbenes produced, a finding in accordance with our previous study (Larronde *et al.,* 2003). Resveratrol is synthesized by a stilbene synthase after a stress (Jeandet *et al.*, 2002) and has been shown to confer a tolerance to powdery mildew and downy mildew (Dai *et al.*, 1995). Its accumulation in leaves of MeJA-treated plants before inoculation by *E. necator* could also explain the reduction in disease development. Concerning piceid, it could be a form of storage or resveratrol transport in the plant. In response to a stress, the presence of basal piceid levels could constitute a pool of immediately usable resveratrol, which can rapidly be mobilized as a primary defense response. We also noted a transient accumulation of dimers of resveratrol, ε- and δ-viniferins, in elicited leaves. Both compounds are highly fungitoxic and the presence of both viniferins in stressed grapevine leaves has been correlated with enhanced protection against downy mildew (Pezet *et al.*, 2004).

CONCLUSION

Under grapevine field conditions, MeJA triggered a significant protection against *Erysiphe necator*, the causal agent of powdery mildew. This raises the hypothesis that all the defense mechanisms elicited by MeJA treatment on grapevine foliar cuttings also occurred in vineyard, thus accounting for the protection observed against *E. necator*.
To our knowledge, this is the first report which describes the efficiency of MeJA in vineyard. In routine agronomic use, management programs integrating MeJA might reduce the intensive use of fungicides against *Erysiphe necator*.

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