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Ethephon Elicits Protection Against *Erysiphe necator* in Grapevine

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The grapevine (*Vitis vinifera*) is susceptible to many pathogens such as *Botrytis cinerea*, *Plasmopara viticola*, *Erysiphe necator*, and *Eutypa lata*. Phytochemicals are used extensively in vineyards to reduce pathogen infections, but the appearance of pesticide-resistant pathogen strains and the need for environmental protection require the use of alternative strategies. The phytohormone ethylene is assumed to play a role in the development of disease resistance. In the present study, we have treated grapevine foliar cuttings (Cabernet Sauvignon) with ethylene-releasing ethephon. This resulted in an increase in the number of pathogenesis-related protein (*CHIT4c*, *PIN*, *PGIP*, and *GLU*) gene copies and in an enhancement of phytoalexin biosynthesis by inducing the *PAL* and *STS* genes that correlated with the accumulation of stilbenes (antimicrobial compounds). Moreover, ethephon treatment triggered the protection of grapevine detached leaves and grapevine foliar cuttings against *Erysiphe necator*, the causal agent of powdery mildew (64% and 70%, respectively). These studies emphasize the major role of ethylene in grapevine defense.

KEYWORDS: Elicitor; powdery mildew; phytoalexins; PR proteins; *Vitis vinifera*

INTRODUCTION

Grapevine (*Vitis vinifera*) is currently one of the major fruit crops in the world in terms of economic value and cultivated area (7.6 million ha; Food and Agricultural Organization of the United Nations, 2005). Many diseases such as gray mold and powdery and downy mildews affect the development of grapevine. Powdery mildew is a very widespread fungus that has a narrow host range. In grapevine, this disease is caused by the biotrophic fungus *Erysiphe necator*. Many popular wine grape varieties such as Cabernet Franc, Cabernet Sauvignon, Chardonnay, Chasselas, Gamay, Sauvignon blanc, and Syrah are very susceptible to powdery mildew. The disease leads to important yield losses in vineyards if no protective measures are taken. To limit damage, an intensive use of phytochemical compounds is required. 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 their residues are generally considered undesirable. Moreover, pesticide-resistant pathogen strains appear; therefore, efforts are being made to develop alternative protection strategies.

An approach to prevent plant diseases consists of inducing natural plant defenses by using elicitors. A variety of pathogen- or plant-derived molecules can act as elicitors, including polysaccharides, peptides, and lipids (*1, 2*). Elicitor perception triggers the formation of ion fluxes, oxidative burst, protein phosphorylation, and the synthesis of signal molecules such as salicylic acid, jasmonic acid, and ethylene (*3*). The signaling pathways induce defense-related genes leading to the reinforcement of plant cell walls, accumulation of antimicrobial compounds such as phytoalexins, and synthesis of pathogenesis related-proteins (*4*).

Ethylene is a gaseous hormone in higher plants that derives from *S*-adenosyl-L-methionine. It has a profound effect on plant growth and developmental processes, including senescence, abscission, flowering, fruit ripening, and seed germination (*5*). Ethylene production is induced in response to various stresses as biotic ones, for example, pathogens or elicitors (*6*). Ethylene is assumed to play a role in the development of disease resistance, as it has been shown to induce the formation of phytoalexins, lignin, and other phenolic compounds as well as enzymes such as chitinases, β-1,3-glucanases, phenylalanine-ammonia-lyase (PAL), peroxidase, and other defense-related proteins (*7*), and protection against *Botrytis cinerea* in tomato (*8, 9*). However, Wang et al. (*10*) describe a mutual role for ethylene in disease-resistance pathways, depending on the type of pathogen and plant species. Enhanced resistance and increased susceptibility have been reported. Because of its

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Table 1. Gene Accession Numbers and Sequences of Gene Primers Used for the Real-Time Quantitative Polymerase Chain Reaction

names	NCBI[a] accession numbers or TC TIGR[b]	forward primer (5′-3′)	reverse primer (5′-3′)
PAL	X75967	TGCTGACTGGTGAAAAGGTG	CGTTCCAAGCACTGAGACAA
STS	AF274281	GTGGGGCTCACCTTTCATT	CTGGGTGAGCAATCCAAAAT
CHIT4c	AY137377	GGCGACGAATCCATTTATGTTA	CGGAACAAGGGTTTCATAATTC
PGIP	AF305093	ACGGAACTTGTTCCAGTTTGAT	CGATTGTAACTCACGTTCAGGA
PIN	AY156047	GCAGAAACCATTAAGAGGGAGA	TCTATCCGATGGTAGGGACACT
GLU	AF239617	TACCTCTTTGCCATGTTTGATG	AGTACTTCGGCTGTTTGTTTGG
ACT	TC30205	TCAGCACTTTCCAGCAGATG	TAGGGCAGGGCTTTCTTTCT

[a] NCBI, National Center for Biotechnology Information. [b] TC TIGR, Tentative consensus no. according to The Institute of Genome Research. PAL, phenylalanine ammonia lyase gene; STS, stilbene synthase gene; CHIT4c, acidic class IV chitinase gene; PGIP, polygalacturonase-inhibiting protein gene; PIN, serine protease inhibitor gene; GLU, β-1,3-glucanase gene; ACT, actin gene.

gaseous nature, ethylene is very difficult to handle; therefore, an ethylene-releasing substance called ethephon (2-chloroethylphosphonic acid) is regularly used. Ethephon induces, as ethylene does, defense responses (*11–14*) and is also able to trigger plant protection against *Botrytis cinerea* (*8*), for example, reduction of tobacco mosaic virus proliferation in tobacco (*15*). Grimmig et al. (*16*) showed by application of the ethylene inhibitor 1-methylcyclopropene to transgenic tobacco plants that stilbene synthase Vst1 induction is dependent on ethylene. In grapevine, Jacobs et al. (*17*) demonstrated that enzyme activities of two PR proteins, a chitinase and a β-1,3-glucanase, were induced in leaves and preveraison berries by ethephon treatment.

The present study sought to determine whether exogenous application of ethephon on grapevine foliar cuttings (*Vitis vinifera*) is able to induce defense responses and lead to protection toward pathogens. After treatment of plants with ethephon, we monitored the expression of defense-related genes encoding enzymes involved in the phenylpropanoid pathway (*PAL*, *STS*) and encoding PR proteins (*CHIT4c*, *PIN*, *PGIP*, and *GLU*) by real-time quantitative polymerase chain reaction (RTq-PCR). We performed quantitative analysis of stilbenes, the major antimicrobial 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 ethephon against powdery mildew (*Erysiphe necator*) was evaluated on detached leaves of treated plants and on grapevine foliar cuttings of the cv. Cabernet Sauvignon.

MATERIALS AND METHODS

Plant Material. Plants of cultivated grapevine (*Vitis vinifera* cv. Cabernet Sauvignon) were propagated from wood cuttings in a greenhouse (INRA, Villenave d'Ornon, France or Biorizon S.A., Martillac, France). They were grown under controlled conditions at 25/20 °C day/night temperatures, with 75% relative humidity and a 16 h photoperiod (350 μmol/m^2/s). Depending on the subsequent experiment, we used plants at different stages of development: two-month-old plants with 10 to 12 leaves for experiment on foliar disks and on one-month-old plants with 5 to 6 leaves for experiment on whole foliar cuttings.

Ethephon Treatment on Foliar Cuttings. Ethephon (Sierra, Bayer Cropscience, France) was used to a final concentration of 0.5 g/L.

For experiments on foliar disks, 15 plants per treatment were entirely sprayed, and each experiment was repeated in triplicate. Leaves were collected for fungal inoculation after 72 h of treatment and from 0 to 72 h for 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. In the case of experiments on whole foliar cuttings, seven plants per treatment were used. In all cases, control plants were untreated.

RNA Extraction and Quantification of Gene Expression by Real-Time Quantitative RT-PCR. The leaves were ground in the 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. (*18*). To eliminate contaminating DNA, RNA samples were incubated with 20 units of RNase-free DNase I (Promega Corp., Madison, USA) for 30 min, the reaction was stopped, and DNase was removed with a phenol/chloroform/isoamylic alcohol mixture (25/24/1, v/v/v). The integrity of the total RNA was checked by electrophoresis on 1.2% agarose gel stained with ethidium bromide (66 ng/L). DNase-treated RNA (2 μg) was reverse-transcribed with 3 μM of oligo(dT) using the ImProm-II reverse transcription System (Promega Corp., Madison, USA) 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 detection system MyiQ (Biorad, Hercules, USA) and iQ SYBR Green Supermix (Biorad, Hercules, USA). PCR reactions were carried out in triplicate in 96-well plates (25 μL per well) in a reaction buffer containing 1× iQ SYBR Green Supermix (including Taq polymerase, dNTPs, SYBR Green I dye, and 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 5 min, 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 45 cycles.

To check the specificity of the PCR product, melting curves were analyzed for each data point. Transcript levels were calculated as described by Arrieta-Montiel et al. (*19*) using the standard curve method from triplicate data, with actin gene as the internal control and control leaves (at time zero) as the reference sample.

The standard curve was constructed using templates of known copy number for target sequence: serial dilutions of cloned target sequence in pGEM-T easy vector (Promega Corp., Madison, USA) were used. The number of copies in each dilution was calculated with the following formula: (number of moles) (6.02×10^{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 was normalized against grapevine actin gene as the internal control and control leaves as the reference sample.

The gene-specific primers are indicated in **Table 1**. Relative gene expression was obtained with the formula: fold induction = $2^{-[\Delta\Delta Ct]}$, where $\Delta\Delta$Ct = [Ct GI (unknown sample) − Ct VACT (unknown sample)] − [Ct GI (reference sample) − Ct VACT (reference sample)]. GI is the gene of interest, and VACT is the grapevine actin gene used as the internal control. The reference sample is the sample chosen to represent 1 × expression of the gene of interest (e.g., control leaves) (*20*).

Inoculation Procedure and Disease Evaluation. *On Foliar Disks.* Inoculations were performed 72 h after ethephon treatment on leaves. As previous authors had noted that older leaves from the bottom of the shoots are more resistant (*21*), inoculation experiments were therefore performed on young leaves from the upper part of the plant. Before inoculation, leaves were washed and dried.

Erysiphe necator strain was isolated from a Merlot leaf sample collected in the Begadan Bordeaux vineyard in 1999. Detached leaves (one per plant) were cleaned by washing, decontaminated with 5% NaOCl, rinsed with water, 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 the plates placed at the bottom of a Plexiglas settling tower (*22*). Conidia of *E. necator* were blown in at the top from sporulating leaves (60 to 80 conidia per cm^2 of leaf). Inoculated leaves were incubated for 14 days at 22 °C under a 16 h photoperiod (25 $\mu E/m^2/s$).

Plasmopara viticola (strain Cou100, Bordeaux) was maintained on grapevine leaf disk at −22 °C and subcultured 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/mL. 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 10 μL droplets per leaf, one leaf per plant) and incubated for 16 h at 22 °C in the dark. The droplets were then gently removed with a pipet connected to an air pump, and leaves were reincubated for seven more days at 22 °C under a 16 h photoperiod (25 $\mu E/m^2/s$).

Disease intensity was estimated by measuring the level of growth and intensity of fungal mycelium and sporulation on leaves. The contamination level was evaluated with a visual scale (0 to 100%), and the disk covering was expressed as percentage according to a scale with steps from 5 to 5 (with zero corresponding to the absence of pathogen development). At the estimation point, we could observe with a microscope the fungal mycelium as well as conidiophores.

On Grapevine Foliar Cuttings. These experiments were performed by Biorizon S.A. (Martillac, France). Three days after ethephon treatment, plants were placed in a Plexiglas tower and sprayed at 800 to 1000 conidia/cm^2 of *E. necator*. Inoculated plants were incubated at 22 °C under a 14 h photoperiod for 14 days.

Disease intensity was estimated on each leave as described in the previous experiment on excised leaves.

Quantification of Stilbenes. Stilbenes were extracted from freeze-dried leaves (100 mg) overnight with MeOH (10 mL) at 4 °C. After centrifugation (3500*g*, 10 min), the supernatant was concentrated and then prepurified on a Sep-Pak C18 cartridge (Waters, USA) to remove chlorophylls. After elution with methanol 90% (v/v), the extract was evaporated to dryness (at temperature <40 °C). Leaf extracts were then dissolved in 1 mL of methanol 50% (v/v). Samples were then filtered through nylon membrane filters (Titan 2, 0.45 μm, ICS, France). During sample preparation, extracts were protected from light to avoid photochemical isomerization of *trans*-stilbenes to the less fluorescent *cis* forms. Analysis of stilbenes was performed by HPLC on a 250 × 4 mm Prontosil C18 (5 μm) reverse-phase C18 column (Bischoff Chromatography, Leonberg, Germany) protected by a guard column of the same material. Separation was performed at a flow rate of 1 mL/min with a mobile phase composed of (A) H_2O/TFA 1% (97.5/2.5, v/v) and (B) ACN/A (80/20, v/v). The run was set as follows: 0 to 13 min, from 14% B to 18% B; 13 to 15 min, 18% B; 15 to 34 min, from 18% B to 32% B; 34 to 36 min, 32% B; 36 to 40 min, from 32% B to 40% B; 40 to 49 min, from 40% B to 80% B; 49 to 50 min, from 80% B to 100% B; 50 to 56 min, 100% B. Fluorimetric detection was performed at $\lambda ex = 300$ nm and $\lambda em = 390$ nm. Stilbene contents were estimated from calibration curves prepared with pure standards.

trans-Resveratrol was purchased from Sigma Chemicals Co. (St Louis, MO) and *trans*-pterostilbene from Sequoia Research Products (Pangbourne, United Kingdom). *trans*-Piceid (*trans*-resveratrol 3-*O*-β glucoside) was purified from *Vitis vinifera* cell cultures as previously described (*23*). *trans*-δ Viniferin was synthesized by horseradish peroxidase from *trans*-resveratrol (*24*). *trans*-ε-Viniferin was purified from woody material and characterized by NMR and MS as previously described (*25*).

Statistical Analysis. Data were expressed as the means ± standard deviations of 3 to 15 determinations. Statistical analysis was performed using Newman−Keuls or Student's *t*-tests, and $P < 0.05$ was considered to be significant.

RESULTS

Effects of Ethephon on Defense Gene Expression in Grapevine Foliar Cuttings. Grapevine leaves were challenged with ethephon at a concentration of 0.5 g/L, and the expression pattern of six selected defense-related genes 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 such as piceids and resveratrol, the main phytoalexins produced by grapevine in response to biotic and abiotic stresses (*26–28*). The expression of four genes encoding PR proteins (acidic class IV chitinase (CHIT4c), serine protease inhibitor (PIN), polygalacturonase-inhibiting protein (PGIP), and β-1,3-glucanase (GLU)) was also considered.

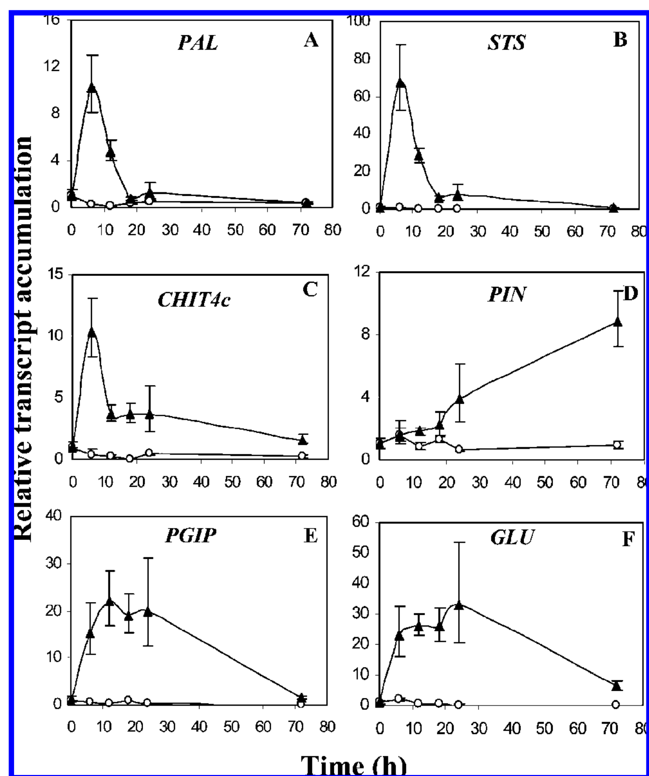

Figure 1. Transcript accumulation of defense genes in untreated (○) and ethephon-treated grapevine leaves (▲). Genes of interest encode the following proteins: (**A**) a phenylalanine ammonia lyase (*PAL*), (**B**) a stilbene synthase (*STS*), (**C**) an acidic class IV chitinase (*CHIT4c*), (**D**) a serine protease inhibitor (*PIN*), (**E**) a polygalacturonase-inhibiting protein (*PGIP*), and (**F**) a β-1,3-glucanase (*GLU*). Analyses were performed by real-time quantitative polymerase chain reaction (RTq-PCR). Transcript levels were calculated by using the standard curve method from triplicate data, with grapevine actin gene as the internal control and nontreated leaves (at time zero) as the reference sample. Results represent the mean fold increase of mRNA level over control leaves, plotted against the 1x expression level. Absolute copy number of mRNA for each gene in the reference sample was 29 (*PAL*), 8.5 (*STS*), 1.3 (*CHIT4c*), 14 (*PIN*), 7.6 (*PGIP*), and 14 (G*LU*) × 10^5 molecules/μg total RNA. Results are the means ± standard deviations of three experiments.

In control leaves, the transcript level of defense genes was very low during the 72 h incubation period (**Figure 1**). In ethephon-treated leaves, the expression of *PAL*, *STS*, and *CHIT4c* genes was rapidly and transiently up-regulated with a peak at 6 h and a maximal induction of 10-, 67-, and 11-fold higher, respectively, than that in control leaves (**Figures 1 A−C**).

RNA of the serine protease inhibitor (*PIN*) gene progressively accumulated from 12 to at least 72 h after treatment (9-fold higher than control leaves; **Figure 1D**).

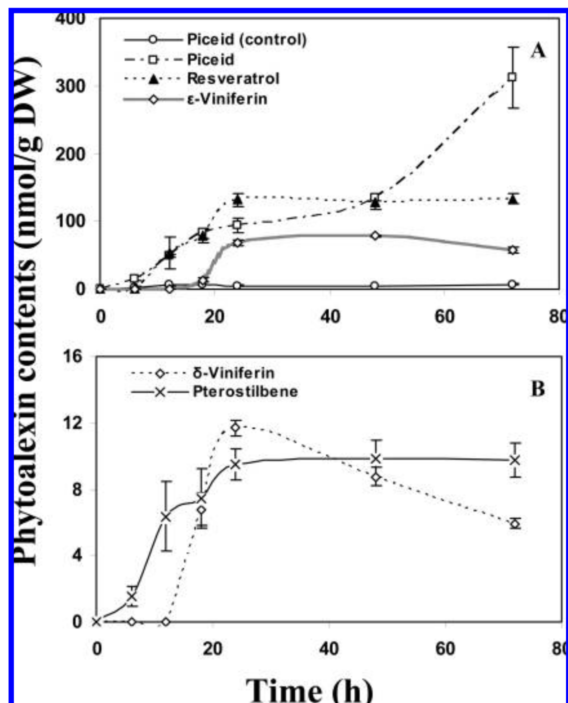

Figure 2. Time course of accumulation of several *trans*-stilbenes in *Vitis vinifera* (L.) cv Cabernet Sauvignon leaves in response to ethephon treatment. (**A**) Piceid, resveratrol, and ε-viniferin, and (**B**) δ-viniferin and pterostilbene. No stilbene except piceid was detected in control leaves (○). Results represent the means ± standard deviations of three experiments.

RNA accumulation of *PGIP* and *GLU* genes began immediately after ethephon treatment. The maximal level of transcripts was reached at 12 and 24 h for *PGIP* and *GLU* genes, respectively. *GLU* gene induction was slightly greater than that of *PGIP* (33- and 22-fold induction, respectively) (**Figure**s **1E** and **F**). Thereafter, the expression of these two genes distinctly decreased from 24 to 72 h after treatment.

Effects of Ethephon 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. In control leaves, the sole phytoalexin slightly detected was the *trans*-piceid (**Figure 2A**), whereas in ethephon-treated grapevine foliar cuttings, all of the phytoalexins under study were produced (**Figure 2**).

During the 72 h analysis, *trans*-piceid was quantitatively the most prolific stilbene produced (**Figure 2A**). Its production started 6 h post-treatment and gradually increased to reach 312 nmol/g DW 72 h post-treatment. *trans*-Resveratrol (**Figure 2A**) similarly accumulated at the beginning, and the production reached a plateau from 24 to 72 h (133 nmol/g DW).

Accumulation of ε-viniferin (**Figure 2A**) started from 18 h, reached the maximal value at 24 h (58 nmol/g DW), and remained constant until 72 h after treatment with ethephon.

Pterostilbene and δ-viniferin (**Figure 2B**) were also detected in ethephon-treated leaves, but at relatively lower levels compared to the quantity of the other phytoalexins. δ-Viniferin accumulated from 12 h, peaked at 24 h (12 nmol/g DW), and subsequently decreased slightly until 72 h post-treatment to reach 6 nmol/g DW, whereas pterostilbene production started increasing at 6 h and reached a plateau from 24 to 72 h (10 nmol/g DW) after ethephon treatment (**Figure 2B**).

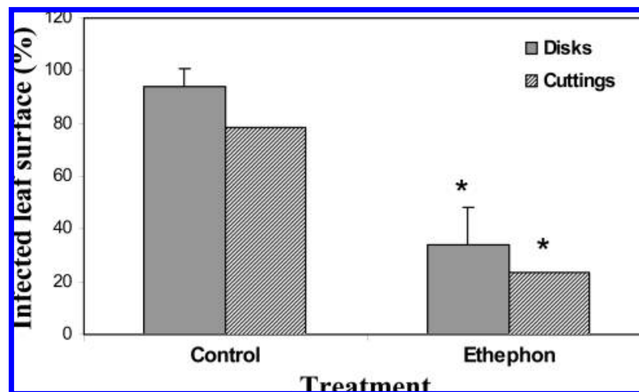

Figure 3. Protection of grapevine detached leaves and grapevine foliar cuttings in response to ethephon treatment against *Erysiphe necator.* Ethephon was sprayed on plants three days before inoculation. Disease was assessed 14 days postinoculation and expressed as the percentage of infected leaf surface. Results represent the means ± standard deviations. * indicates values significantly different from those in control experiments ($P < 0.05$). The experiments were repeated in triplicate with similar results.

Effects of Ethephon on Grapevine Protection. Grapevine leaves were pretreated with ethephon 3 days before inoculation, and any phytotoxic effect was observed. Degree of protection for powdery mildew was represented as a percentage of infected leaf surface (**Figure 3**). Fourteen days postinoculation, on treated and untreated disks or leaves, the fungus gets to the same stage of development (mycelium and conidiophores fully developed) but not with the same final intensity of growth. Ethephon treatment reduced by 64% the colonization of leaf disks by *E. necator*. On grapevine foliar cuttings, ethephon triggered a similar intense decrease in powdery mildew infection of about 70%.

DISCUSSION

Ethephon treatment on grapevine leaves resulted in an increased resistance to *Erysiphe necator*: fungal development was reduced by 64 to 70% compared to that in the untreated control. Ethephon was also able to prevent the infection of *Plasmopara viticola* but to a lesser degree (34 and 19%; data not shown). This positive impact of ethylene treatment against biotrophic fungi is of prime interest. Indeed, the application of exogenous ethylene was found to induce resistance or susceptibility, or to have no effect, depending on the plant pathogen interaction studied (*29*). 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 (*30–32*). Ethylene seems to inhibit symptom development in necrotrophic pathogen infection, as in tomato toward *Botrytis cinerea* (*8*), but enhances the cell death caused by other types of pathogen infection (*10*).

The enhanced protection against powdery mildew was probably due to the accumulation of defense-related proteins and antimicrobial compounds such as phytoalexins. Genes corresponding to PR proteins were up-regulated. Transcript levels of the acidic chitinase gene, *CHIT4c*, increased rapidly after ethephon treatment and peaked after 6 h. This result is in agreement with studies by Jacobs et al. (*17*) who previously demonstrated that grapevine leaves respond to ethephon treatment by the induction of chitinase activities. Chitinase is one of the most important enzymes participating in pathogenesis-related mechanisms in plants. It degrades chitin,

which is a major structural component of fungal cell walls (*33, 34*). Karasuda et al. (*35*) reported that chitinase is able to degrade the powdery mildew infecting strawberry.

Grapevine *GLU* gene expression (**Figure 1F**) was up-regulated after ethephon treatment. Similar experiments (*17*) showed that β-1,3-glucanase activities were induced by ethephon. 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 (*36*). Several studies have demonstrated that β-1,3-glucanases were 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 (*36*).

Moreover, β-1,3-glucanases appear to be coordinately expressed along with chitinases after pathogen infection, wounding, ethylene treatment, and chemical stress in several plants (*37, 38*). Giannakis et al. (*39*) 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.

We also observed *PIN* and *PGIP* mRNA transcript accumulation in ethephon-treated leaves. Protease inhibitors are ubiquitous in tubers and plant seeds (*40*) and are generally thought to act as storage and defense proteins (*41*). They have been shown to participate in the wound-induced defense response of plants against herbivores and pathogens (*42*). For example, the potato PT-1 serine protease inhibitor inhibits the growth of the rice fungal pathogen *R. solani* and *Clavibacter michiganense* subsp. *michiganense*, which is a pathogenic bacterium for a variety of agriculturally important plants such as tomato, potato, and maize (*43*).

Polygalacturonase inhibitor proteins (PGIPs) interact with fungal endopolygalacturonases and inhibit more or less their enzymatic activity (*44*). Moreover, Ferrari et al. (*45*) demonstrated that the overexpression of two differentially regulated PGIP genes in *Arabidopsis* significantly reduced the disease symptoms caused by *B. cinerea*. Furthermore, the partial inhibition of fungal polygalacturonases by PGIPs leads to the production of longer oligomeric degradation products, which are large enough to act as elicitors of plant defense responses (*46*). Therefore, PR gene induction could partly explain the reduced infection of grapevine infected leaves by *E. necator*.

Moreover, the phenylpropanoid pathway was also up-regulated in ethephon-treated grapevine leaves. *PAL* and *STS* gene expression (**Figures 1A** and **B**) is up-regulated, and this induction is correlated to the accumulation of stilbenes as resveratrol and its derivatives, piceid, viniferins, and pterostilbene (**Figure 2**). The activation of this biosynthesis pathway is one of the most important resistance reactions in many plants (*47*). Resveratrol and its derivatives are produced by grapevine in response to elicitor and pathogens attacks (*25, 28*). Piceid and resveratrol were quantitatively the major stilbenes produced. Langcake and Pryce (*27*) showed that resveratrol confers a tolerance to powdery mildew and downy mildew (*48*). Piceid, the glycosylated form of resveratrol, is protected from enzymatic oxidation (*49*). In response to a stress, piceid could constitute a pool of immediately usable resveratrol. In ethephon-treated leaves, we noted a transient accumulation of polymerized oligomers of resveratrol: ε- and δ-viniferins. These compounds were shown to be produced also in response to different biotic and abiotic stresses (*50*). ε- and δ-viniferins are highly fungitoxic, and their presence in stressed grapevine leaves has been correlated with enhanced protection against downy mildew (*24, 51*).

Our data show that pterostilbene is also detected in leaves of ethephon pretreated plants, even if the amounts seemed low compared to that in the other stilbenes analyzed. Pterostilbene is indeed a biologically very active phytoalexin that is usually found in low quantities in grapevine (*52*).

In conclusion, the application of ethephon as a foliar treatment reduces powdery mildew in grapevine foliar cuttings, and this is associated with up-regulation of PR gene expression and induction of the phenylpropanoid pathway. In view of this eliciting activity, ethylene plays a major role in grapevine defense, especially against the biotrophic fungus *E. necator*.

ABBREVIATIONS USED

PR proteins, pathogenesis-related proteins; PAL, phenylalanine ammonia lyase; STS, stilbene synthase; CHIT4c, acidic class IV chitinase; PIN, inhibitors of serine proteases; PGIP, polygalacturonase-inhibiting protein; GLU, β-1,3-glucanase.

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