JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2005,** *53,* 7043−7051 **7043**

Transformation of Eutypa Dieback and Esca Disease Pathogen Toxins by Antagonistic Fungal Strains Reveals a Second Detoxification Pathway Not Present in *Vitis vinifera*

DANILO CHRISTEN,[†] MANUEL THARIN,[‡] SANDRINE PERRIN-CHERIOUX,[‡] ELIANE ABOU-MANSOUR,[‡] RAPHAËL TABACCHI,[‡] AND GENEVIÈVE DÉFAGO*[,†]

Phytopathology Group, Institute of Plant Sciences, Swiss Federal Institute of Technology, 8092 Zürich, Switzerland, and Analytical and Structural Organic Chemistry, University of Neuchâtel, 2007 Neuchâtel, Switzerland

Eutypine, 4-hydroxybenzaldehyde, and 3-phenyllactic acid are some of the phytotoxins produced by the pathogens causing Eutypa dieback and esca disease, two trunk diseases of grapevine (*Vitis vinifera*). Known biocontrol agents such as *Fusarium lateritium* and *Trichoderma* sp. were screened for their ability to consume these toxins. Transformation time courses were performed, and an high-performance liquid chromatography-based method was developed to analyze toxin metabolism and to identify and quantify the converted products. The results show that the aldehyde function of eutypine was reduced to eutypinol, as by *V. vinifera* cv. Merlot, the cultivar tolerant to Eutypa dieback. We revealed a supplementary detoxification pathway, not known in Merlot, where the aldehyde function was oxidized to eutypinic acid. Moreover, some strains tested could further metabolize the transformation products. Every strain tested could transform 4-hydroxybenzaldehyde to the corresponding alcohol and acid, and these intermediates disappeared totally at the end of the time courses. When biological assays on cells of *V. vinifera* cv. Chasselas were carried out, the transformation products exhibited a lower toxicity than the toxins. The possibility of selecting new biocontrol agents against trunk diseases of grapevine based on microbial detoxification is discussed.

KEYWORDS: ***Vitis vinifera*; trunk disease toxins; microbial detoxification; biocontrol agents**

INTRODUCTION

Trunk diseases of grapevine (e.g., Eutypa dieback and esca disease) are becoming increasingly frequent in all vine-producing regions of the world. This is due to expanded mechanization (*1*), new pruning practices (*2*), the worldwide banning of sodium arsenite (*1*, *3*), the absence of alternative direct control, the propagation of the pathogens in the nurseries (*4*, *5*), and the absence of resistant *Vitis* cultivars.

Eutypa dieback and esca disease are characterized by a slow decline that leads to the death of the plants (*6*, *7*). Eutypa dieback is caused by *Eutypa lata*. Esca disease is caused by a complex of pathogens including the ascomycetes *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum* and the basidiomycete *Fomitiporia mediterranea*. These xylem-inhabiting fungal pathogens infect the plants through large wounds and produce cankers or rot in the wood (*3*). Eutypa dieback and esca disease pathogens produce several toxic compounds, the majority of which have an aldehyde function as an active site (**Figure 1**). Some of these toxins were detected in the inflorescences, in the sap, and in the leaves of diseased grapevine plants but were absent in the healthy plants (*8*, *9*). Several in vitro-produced toxins have been chemically characterized (*9*−*14*) and tested for their toxicity on protoplasts or calli of different *Vitis* cultivars (*9*, *15*). Foliar symptoms can be reproduced through the absorption of culture filtrates of *E. lata* (*8*, *15*) or of esca pathogens (*3*) by detached grapevine leaves.

Among these toxins, the mode of action of 4-hydroxy-3-(3-methyl-3-butene-1-ynyl)benzaldehyde, commonly named eutypine and secreted by *E. lata*, is largely documented. Eutypine is a lipophilic weak acid. It enters the grapevine cells through passive diffusion and damages the membrane systems by inserting lipids (*16*, *17*). Eutypine, as a mobile proton transporter (protonophoric activity) due to the dissociable alcohol function, disturbs the respiration (uncoupling effect of the oxidative phosphorylation) and the energy balance of the cells by decreasing the membrane potential (*18*). Another toxic compound, 4-hydroxybenzaldehyde, is believed to be the common precursor of several aromatic toxins (*12*).

The response of various grape plants to trunk disease pathogen attacks, and especially to trunk toxins, reveals large differences. No *Vitis* cultivar is resistant to trunk diseases. In contrast to Cabernet Sauvignon, which is highly susceptible, Merlot shows a high tolerance to Eutypa dieback, probably because of the

* To whom correspondence should be addressed. Tel: +41-1-632-3869. Fax: +41-1-632-1572. E-mail: genevieve.defago@ipw.agrl.ethz.ch.
[†] Swiss Federal Institute of Technology.
[‡] University of Neuchâtel.

10.1021/jf050863h CCC: $30.25 © 2005 American Chemical Society
Published on Web 07/29/2005

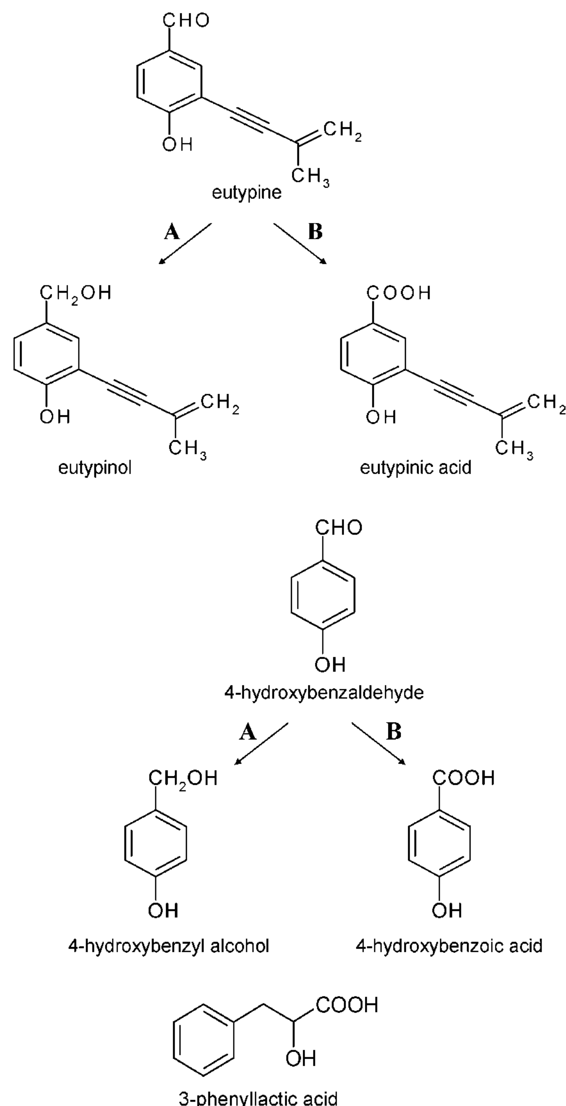

Figure 1. Chemical structures of eutypine and 4-hydroxybenzaldehyde and their derivatives and of 3-phenyllactic acid. (**A**) Reduction of the aldehyde function to the corresponding alcohol; (**B**) oxidation of the aldehyde function to the corresponding acid.

presence of an aldehyde reductase that metabolizes the eutypine to the nontoxic eutypinol (*19*). This NADPH-dependent eutypine reductase enzyme (VR-ERE) was isolated, purified, and cloned from the plant *Vigna radiata*. VR-ERE cDNA was expressed in *Escherichia coli* K38 and in *Vitis vinifera* cv. Gamay cells using *Agrobacterium tumefaciens* C58-mediated transformation (*20*). This transformation was found to confer resistance to eutypine in grapevine cells (*21*).

The absence of direct control of grapevine trunk diseases requires the development of new control strategies to reduce their incidence. In fact, it is not possible to avoid the synthesis and the transportation of the toxins. The only effective approach to control trunk diseases effectively seems to be partial detoxification, the same mechanism as in the tolerant cultivar Merlot. Microorganisms are already known to be efficient in metabolizing biotic toxins (*22*). Tomato plants can be protected from Fusarium wilt by fusaric acid-degrading mutant strains of *Pseudomonas solanacearum* (actual name *Ralstonia solanacearum*) (*23*). Other bacterial strains, such as *Klebsiella oxytoca* (*24*), *Pseudomonas cepacia*, and *Pseudomonas aeruginosa* (*25*) and the fungi *Cladosporium werneckii* (*26*), are also reported to be able to degrade fusaric acid.

The purpose of this study was to screen some potential biocontrol fungal strains for their ability to transform the different toxins tested. To evaluate the most effective strains, we aimed to perform a kinetic analysis of the toxin consumptions, to identify the transformation products and to assess their toxicity. To our knowledge, this study is the first report of microbial transformation of the toxins produced by the pathogens that cause Eutypa dieback and esca disease and of the consumption of the metabolic intermediates.

MATERIALS AND METHODS

Fungal Cultures and Growth Conditions. Four well-known biocontrol fungal species were chosen for this study. *Trichoderma polysporum* strains 503.82 (isolated from *Abies alba*) and 997.87 (from *Picea abies*), *Trichoderma harzianum* strains 753.68 (from *Populus* sp.) and 389.36 (from *Pinus* sp.), and *Fusarium lateritium* strains 633.95 (from *Corylus avellana*), 268.51 (from *Populus* sp.), and 541.88 (from *Pyrus* communis) were obtained from Centraalbureau voor Schimmelcultures (CBS, Utrecht, The Netherlands). *T. polysporum* strain 657 and *F. lateritium* strains 146 and 825 (from *Malus* sp.) were obtained from the culture collection of the Phytopathology group of the Institute of Plant Sciences (Federal Institute of Technology, Zurich, Switzerland). *Trichoderma atroviride* strain P1 (ATCC 74058) was obtained from LGC Promochem (Molsheim, France), and *T. harzianum* strain 616 was obtained from the collection of the Swiss Federal Research Station for Plant Production (Agroscope RAC, Changins, Switzerland). The strains were maintained on malt extract agar (15 g/L malt extract, 12 g/L agar) (Oxoid) or potato dextrose agar [4.8 g/L potato dextrose broth (Difco), 12 g/L agar (Oxoid)] and were grown at 24 °C. Fungal growth was assessed by inoculation of four agar plugs of the fungal strains in flasks containing 30 mL of malt extract (0.7%) liquid cultures and incubated in the dark at 24 °C on a rotary shaker (180 rpm). After 17, 29, 41, 53, 65, and 96 h, the mycelium was filtered and lyophilized, and the dry weight was measured relative to the mycelium dry weight at time 0.

Toxins and Chemicals. Eutypine and the reference transformation products eutypinol and eutipinic acid were synthesized as described previously (*27*, *28*). 3-Phenyllactic acid, 4-hydroxybenzaldehyde, the reference transformation products 4-hydroxybenzyl alcohol and 4-hydroxybenzoic acid, the organic solvents (analytical quality), the salts, the vitamins, and the sucrose, if not specified, were purchased from Fluka Chemie GmbH (Buchs, Switzerland) and used without further purification.

Instrumentation. Qualitative and quantitative analyses were performed by high-performance liquid chromatography (HPLC)-UV-MS using an Agilent 1100 series LC/MSD (Agilent Technologies, Palo Alto, CA) with an electrospray ion source equipped with an autoinjector (Agilent G1313A) and a degasser unit (Agilent G1379A) and coupled with a UV diode array detector (Agilent 1315A). The samples were injected (10 μL) onto a 250 mm × 4.6 mm i.d., 7 μm, Nucleosil-C18 analytical column (Bischoff GmbH, Leonberg, Germany), using a flow rate of 1 mL/min (0.7 mL/min for the analyses of 4-hydroxybenzaldehyde and its metabolites). The mobile phase consisted of solvent A (water/acetic acid, 98:2, v/v) and solvent B (acetonitrile) or, for 4-hydroxybenzaldehyde, solvent C (acetonitrile/tetrahydrofuran, 75:25, v/v). For the analyses of eutypine and its derivatives, an isocratic solvent system was used, consisting of 40% B for 20 min, followed by 4 min at 95% B. For 4-hydroxybenzaldehyde, elution started at 30% of solvent C rising to 70% in 10 min and maintained for 4 min. Finally, for 3-phenyllactic acid, the analyses were carried out under isocratic conditions at 17% B. The toxins and their derivatives were detected at 254 nm (270 nm for 4-hydroxybenzaldehyde) with diode array on-line detection. UV spectra were recorded between 200 and 400 nm.

Toxin Consumption and Extraction of the Metabolites. Four agar plugs of well-known biocontrol fungal isolates were added to flasks containing 30 mL of malt extract (0.7%) liquid cultures with 225 μM eutypine, 550 μM 4-hydroxybenzaldehyde (dissolved in ethanol), and 550 μM 3-phenyllactic acid (dissolved in bidistilled water) and incubated in the dark at 24 °C for 4 days on a rotary shaker (180 rpm).

As control, the same toxin concentration was added to culture medium without fungi. After 17, 29, 41, 65 (53 for 4-hydroxybenzaldehyde), and 96 h, 4 mL of each culture medium was removed. These aliquots were then acidified with 200 μL of 1 N HCl, extracted with 2.5 mL of ethyl acetate by shaking (400 rpm) for 30 min at room temperature, and stored at −20 °C. One milliliter of the organic phase was transferred into HPLC vials (8002-CV−H/V15μ) (Infochroma AG, Zug, Switzerland) and dried by vacuum evaporation (Hetovac; Heto-Holten A/S, Allerød, Denmark). The residues were used for HPLC-UV analysis. All transformation time courses were performed twice with two replicates each. Both experiments provided similar results. The results shown are averages of one experiment.

Calibration and Recovery. To perform calibration curves, 30 mL of malt extract liquid cultures (0.7%) containing 550, 330, 110, 77, 33, and 11 μM 3-phenyllactic acid, 4-hydroxybenzaldehyde, 4-hydroxybenzyl alcohol, or 4-hydroxybenzoic acid were prepared. For eutypine, eutypinol, and eutypinic acid, concentrations of 225, 135, 45, 31.5, 13.5, and 4.5 μM were used. For the calibrations, samples were removed after 17 and 96 h only and used for HPLC-UV analysis. The HPLC peak area was correlated to the compound concentration. Equations and R^2 values were calculated using Systat version 10. To assess the recovery of each compound, the same amount of metabolite, as for the calibration, was diluted in ethanol and directly dried by vacuum evaporation without extraction. For the calibration and recovery experiments, the same protocol as the kinetic analyses of toxin transformation was used.

Analysis of the Metabolites. The residues were suspended in 300 μL of acetonitrile, and 10 μL was injected in HPLC-UV for quantitative analyses. Qualitative analysis was performed by HPLC-MS [electrospray ionization (ESI) mode]. Metabolite concentrations were determined by reference to standard calibration curves, which were linear ($R^2 > 0.99$) for each compound. Biotransformation velocity (reaction kinetics) was estimated between the end of the lag phase and the time of the complete disappearance of the toxin, or after 96 h, when the toxin was not completely consumed. The linear transformation rates are defined as the changes of concentration in time and are expressed in μM/h.

Toxicity Assays on *V. vinifera* cv. Chasselas Cells. Biotests were performed to confirm the toxicity of the toxins and to check the toxicity of the transformation products. *V. vinifera* cv. Chasselas cells, originating from callus, were grown in NOVER-MSMO medium [4.4 g/L Murashige and Skoog Basal Salts with minimal organics (*29*) (Sigma); 30 g of sucrose; 170 mg of KH_2PO_4; 5 mg of 1-naphthyl acetate diluted in ethanol and 2 mg of N^6-benzyladenine diluted in 2 N KOH (*30*); 1 mL of the stock solution of vitamin AT 1.000× (*31*); adjusted to pH 5−5.5 and autoclaved]. Cells were subcultured at 1 week intervals by diluting 15 mL of the suspension in 50 mL of fresh NOVER-MSMO medium and grown on a rotary shaker (150 rpm) at 24 °C with a 16 h light/8 h dark system. After 8 days of incubation, grapevine cells were rinsed three times and resuspended in a minimal isotonic NOVER-MSMO medium (MSMO, sucrose and KH_2PO_4 only) and aliquots of 0.5 mL were distributed in separated wells of a 24 well tissue culture test plate (TPP AG, Trasadingen, Switzerland) containing 1 mL of minimal NOVER-MSMO medium supplemented with the toxins or the transformation products at different concentrations. As control, ethanol (or water for the control test of 3-phenyllactic acid) was added to one well. The toxicity was assessed by testing for cell death and membrane integrity, using the vital dye neutral red. Accumulation of the dye in the vacuoles and plasmolysis was observed after 24 h by light microscopy. A neutral red aqueous stock solution of 5 mg/mL was diluted for staining to a final concentration of 0.05% in minimal NOVER-MSMO medium. Four repetitions were performed, for each metabolite concentration, by examining 60 μL of cell suspension, and the results are reported as an average.

RESULTS

HPLC Analysis. In the present study, the HPLC analytical conditions were determined after investigating the effect of various combinations of solvents, flow rates, and gradient elution conditions on the separation of standard compounds. The use of LC-ESI-MS method provided a sensitive and powerful technique for the identification of the metabolites. All compounds were quantitatively analyzed by a diode array detector and characterized by the UV spectra, the retention time, and the mass spectra of reference compounds. The unconventional solvent system and the reduced flow rate of 0.7 mL/min, used for 4-hydroxybenzaldehyde, allowed a better separation of the metabolites and consequently a precise quantification. Acidification, with HCl, of the liquid medium prior to ethyl acetate extraction permitted a level of recovery to be obtained, which was linear for every metabolite, independently of the concentration in the range of interest.

Kinetic Analyses of Eutypine Consumption and Identification of Its Derivatives. The kinetic analyses of eutypine transformation were monitored for up to 96 h by quantification of the toxin and its metabolites with HPLC-UV, following inoculation in the dark of the different fungi in liquid culture medium containing 225 μM eutypine. No change of eutypine concentration was observed in the control sample without fungi (data not shown). In liquid medium, eutypine is therefore stable for 96 h in the dark. Every fungal strain tested showed at least a partial conversion of eutypine (**Figure 2**). The different strains were grown similarly in the absence of the toxin (data not shown). Therefore, fungal growth and transformation ability were not correlated.

Major differences in reaction kinetics were found at the strain level (**Table 1**). *T. polysporum* strains 657 and 503.82, *T. harzianum* 389.36, and *F. lateritium* 268.51 converted the toxin slowly (2.8−3.5 μM/h), but only *T. polysporum* 503.82 started the transformation with a delay. Rapid reaction kinetics (11.9−13.2 μM/h) was exhibited by *T. atroviride* P1 and by *T. harzianum* 616 and 753.68. The other strains tested showed intermediate reaction kinetics (4.7−7.7 μM/h). *T. polysporum* 997.87 and *F. lateritium* 825 highlighted a two-phase kinetics, beginning with a 40 h slow rate (1 μM/h). *F. lateritium* 825 and *T. polysporum* 503.82 were able to convert eutypine only partially.

Eutypine was reduced to eutypinol by every strain. The oxidation of eutypine producing eutypinic acid was also found when using many strains, except for *T. polysporum* 657 and *T. harzianum* 753.68 (**Table 2** and **Figure 2**). The HPLC-UV analysis provided the following retention times: R_T eutypine = 16.7 min, R_T eutypinol = 8.0 min, and R_T eutypinic acid = 9.6 min (elution profile not shown). The reduction of eutypine to eutypinol occurred, in many kinetic analyses, earlier than the oxidation to eutypinic acid but not in those of *F. lateritium* 146 and *F. lateritium* 541.88. No emergence of other aromatic structures was detected in the medium by HPLC-UV. At the end of the time course, *T. harzianum* 389.36 formed a yellow product, which was not identified. The regression curves of the concentration ranges showed a R^2 of 0.9992, 0.9998, and 0.9999 for eutypine, eutypinol, and eutypinic acid, respectively, allowing an accurate quantification of the three metabolites between 4.5 and 225 μM.

In the presence of *T. harzianum* and *T. atroviride* strains, eutypinol disappeared within 96 h. Eutypinic acid disappeared only in the presence of *T. harzianum* 389.36. The disappearance of eutypinol with *T. harzianum* 753.68 was more rapid than for other strains (**Figure 2**). For both transformation products, the maximal production (μM) and the percentage of consumption after 96 h are shown in **Table 2**. To analyze these supplementary metabolisms, time course transformations by *F. lateritium* 825 and 633.95, by *T. polysporum* 503.82, and by *T.*

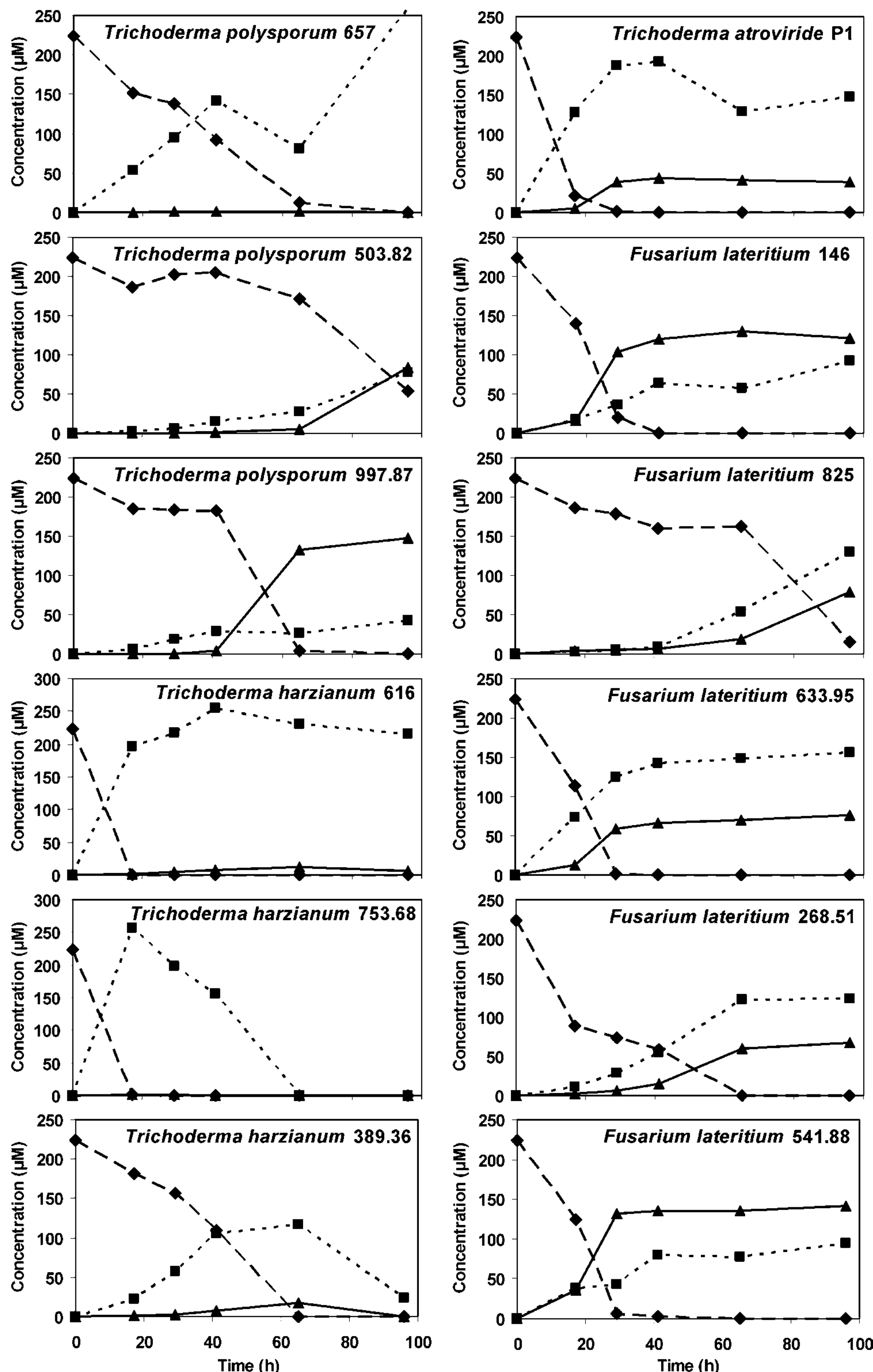

Figure 2. Time course of eutypine (◆) transformation by potential biocontrol agents and production of eutypinol (■) and eutypinic acid (▲) in eutypine-containing liquid medium.

harzianum 389.36 were prolonged to 150 h (data not shown). *Trichoderma* strains were able to convert >98% of both derivatives, whereas *Fusarium* strains only converted eutypinol (>98% consumption by *F. lateritium* 825 and 50% by *F. lateritium* 633.95).

Kinetic Analyses of 4-Hydroxybenzaldehyde Consumption and Identification of Its Derivatives. The protocol described for eutypine modification analysis was used for the kinetic experiment of the 4-hydroxybenzaldehyde transformation. The initial toxin concentration used in this experiment was 550 μM. The control without fungi revealed no change of 4-hydroxybenzaldehyde concentration for 96 h (data not shown), which proves the stability of the toxin in the dark. Every strain tested was able to convert 4-hydroxybenzaldehyde completely (**Figure 3**).

All fungal species exhibited transformation velocity differences among the strains (**Table 1**). The consumption generally occurred in two phases, with a reaction kinetics of 1.2−6.6 μM/h (slow phase) at first, followed by more rapid rates of 18−40 μM/h.

Table 1. Transformation Rates (μM/h) and Percentage Consumption after 96 h of Eutypine, 4-Hydroxybenzaldehyde, and 3-Phenyllactic Acid by Potential Biocontrol Fungal Strains

	eutypine		4-hydroxybenzaldehyde		3-phenyllactic acid	
biocontrol agents	transformation rates (μM/h)	consumption (%)	transformation rates (μM/h)	consumption (%)	transformation rates (μM/h)	consumption (%)
F. lateritium 541.88	7.5	>98	19.7	>98	1.4	24
F. lateritium 146	7.0	>98	35.6	>98	1.6	27
F. lateritium 825	4.7	93	20.3	>98	1.9	32
F. lateritium 633.95	7.7	>98	20.4	>98	3.1	55
F. lateritium 268.51	3.5	>98	19.7	>98	1.9	33
T. polysporum 657	3.3	>98	23.4	>98	1.3	22
T. polysporum 503.82	2.8	76	18.2	>98	3.9	68
T. polysporum 997.87	7.5	>98	36.7	>98	4.8	75
T. atroviride P1	11.9	>98	39.9	>98	8.3	>98
T. harzianum 616	13.2	>98	33.8	>98	4.9	86
T. harzianum 753.68	13.2	>98	32.7	>98	5.3	94
T. harzianum 389.36	3.5	>98	25.3	>98	8.3	>98

Table 2. Analysis of the Derivatives Produced (μM) after 96 h in the Transformation of Eutypine and of 4-Hydroxybenzaldehyde by Different Biocontrol Agents

	eutypine				4-hydroxybenzaldehyde			
	eutypinol		eutypinic acid		4-hydroxybenzyl alcohol		4-hydroxybenzoic acid	
biocontrol agents	yield (μM)	consumption (%)	yield (μM)	consumption (%)	yield (μM)	consumption (%)	yield (μM)	consumption (%)
F. lateritium 541.88	95	0	140	0	20	>98	180	>98
F. lateritium 146	90	0	130	0	60	>98	280	>98
F. lateritium 825	130	0	80	0	20	>98	160	>98
F. lateritium 633.95	150	0	75	0	50	>98	180	>98
F. lateritium 268.51	125	0	70	0	20	>98	250	>98
T. polysporum 657	260	0	0		120	0	120	60
T. polysporum 503.82	80	0	80	0	60	0	50	>98
T. polysporum 997.87	40	0	150	0	10	>98	290	>98
T. atroviride P1	190	20	40	0	50	0	280	>98
T. harzianum 616	250	15	15	0	100	40	80	>98
T. harzianum 753.68	250	>98	0		20	>98	80	>98
T. harzianum 389.36	120	80	20	>98	130	90	50	60

Every strain tested metabolized 4-hydroxybenzaldehyde to both 4-hydroxybenzyl alcohol and 4-hydroxybenzoic acid (**Table 2** and **Figure 3**). The HPLC-UV retention times of the metabolites were as follows: R_T 4-hydroxybenzaldehyde = 8.6 min, R_T 4-hydroxybenzyl alcohol = 5.8 min, and R_T 4-hydroxybenzoic acid = 7.7 min (elution profile not shown). Several analyses revealed a faster production of 4-hydroxybenzoic acid than of 4-hydroxybenzyl alcohol, except for *T. harzianum* 389.36. Unidentified yellow compounds were produced during the reactions by *T. polysporum* 503.82, *T. harzianum* 389.36, and *F. lateritium* 146. The regression curves of the concentration range exhibited a R^2 of 0.9999, 0.9988, and 0.9993 for 4-hydroxybenzaldehyde, 4-hydroxybenzyl alcohol, and 4-hydroxybenzoic acid, respectively, permitting a precise quantification of each compound between 11 and 550 μM.

In the presence of every strain, 4-hydroxybenzoic acid disappeared within 96 h, while for every strain, except by *T. polysporum* 657 and 503.82 and *T. atroviride* P1, 4-hydroxybenzyl alcohol disappeared (**Table 2**). The disappearance of the metabolic intermediates was rapid and occurred simultaneously with the toxin consumption, resulting in a relatively low production of 4-hydroxybenzyl alcohol and 4-hydroxybenzoic acid (**Figure 3**). The maximal production (μM) and the percentage consumption (after 96 h) of both derivatives are reported in **Table 2**. No other aromatic compound was found in the culture medium after total consumption of the derivatives.

Kinetic Analyses of 3-Phenyllactic Acid Consumption. The same protocol as for eutypine was used for the analysis of the utilization of 3-phenyllactic acid. The toxin was diluted in water and used at an initial concentration of 550 μM. No utilization of 3-phenyllactic acid was observed in the control without fungi up to 96 h (data not shown), which proves the stability of the toxin in the dark. Most of the strains tested exhibited only a partial conversion of 3-phenyllactic acid (**Table 1** and **Figure 4**).

In general, the transformation occurred slower than for the other toxins. *F. lateritium* strains and *T. polysporum* 657 showed a limited reaction rate after 96 h (1.3−3.1 μM/h). *T. polysporum* strains 503.82 and 997.87 and *T. harzianum* strains 616 and 753.68 were more efficient and exhibited reaction rates between 3.9 and 5.3 μM/h. *T. harzianum* 389.36 and *T. atroviride* P1 transformed 3-phenyllactic acid completely in 65 h, which represents reaction kinetics of 8.3 μM/h (**Table 1**).

The retention time of 3-phenyllactic acid was 12.9 min with the HPLC system used (elution profile not shown). *T. harzianum* 753.68 and *F. lateritium* 146 produced yellow-colored compounds, which were not identified. The calibration curve of 3-phenyllactic acid was linear in the range of the concentrations tested (R^2 = 0.9998). The toxin detection limit was therefore under the lowest calibration concentration of 11 μM.

Toxicity Assays on *V. vinifera* cv. Chasselas Cells. Biotests were performed on *V. vinifera* cv. Chasselas cells by using neutral red to check for cell viability. The concentration range

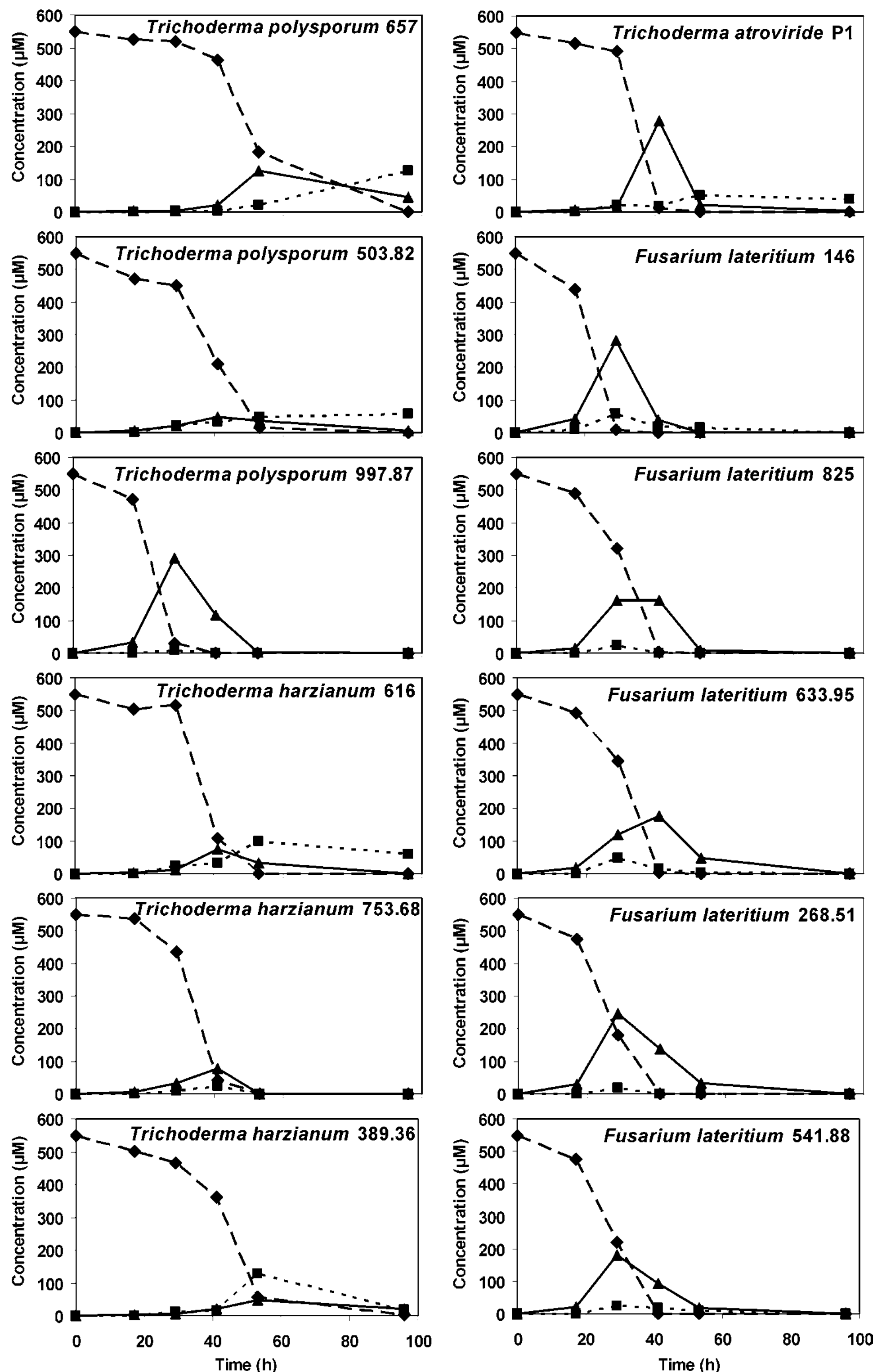

Figure 3. Time course of 4-hydroxybenzaldehyde (◆) transformation by potential biocontrol agents and production of 4-hydroxybenzyl alcohol (■) and 4-hydroxybenzoic acid (▲) in 4-hydroxybenzaldehyde-containing liquid medium.

used for the toxicity test was 550, 275, 137, and 55 μM for 3-phenyllactic acid and for 4-hydroxybenzaldehyde and its derivatives and 225, 112, 56, and 22 μM for eutypine and its derivatives. The results presented are averages of four repetitions and are expressed as remaining living cells in percent of the control (**Figure 5**). The control without toxin exhibited about 10% of dead cells. Cytotoxicity was metabolite dependent as well as concentration dependent. Eutypine showed a high toxicity at a low concentration (90% dead cells at 225 μM), 3-phenyllactic acid exhibited an intermediate toxicity (38% dead cells at 275 μM and 62% dead cells at 550 μM), and 4-hydroxybenzaldehyde was only moderately toxic (22% dead cells at 275 μM and 27% dead cells at 550 μM). The substitution of the aldehyde function of eutypine and 4-hydroxybenzaldehyde with an alcohol or an acid resulted in a prominent diminution of dead cells. This loss of toxicity could even be observed at a low level of toxin (at 56 μM for eutypine and its derivatives and at 55 μM for 4-hydroxybenzaldehyde and its derivatives).

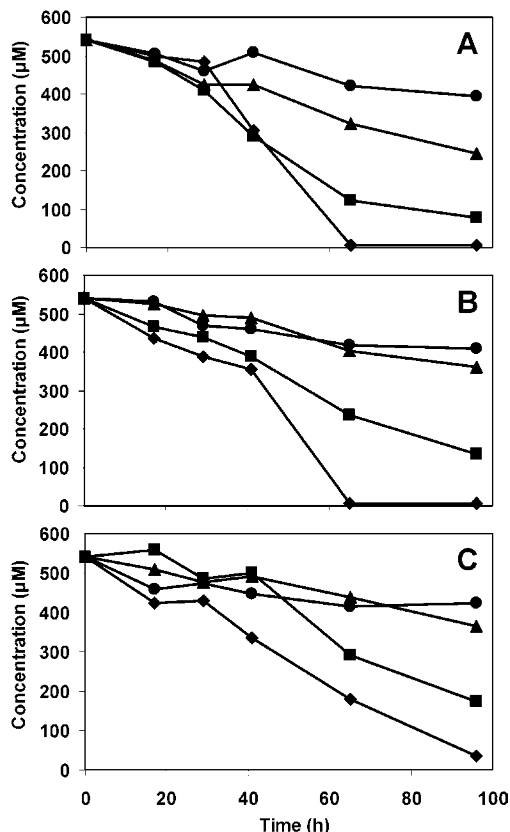

Figure 4. Time course of 3-phenyllactic acid transformation by (**A**) *T. atroviride* P1 (◆), *T. harzianum* 616 (■), *F. lateritium* 633.95 (▲) and *F. lateritium* 146 (●); (**B**) *T. harzianum* 389.36 (◆), *T. polysporum* 997.87 (■), *F. lateritium* 268.51 (▲), and *F. lateritium* 541.88 (●); and (**C**) *T. harzianum* 753.68 (◆), *T. polysporum* 503.82 (■), *F. lateritium* 825 (▲), and *T. polysporum* 657 (●) in liquid medium.

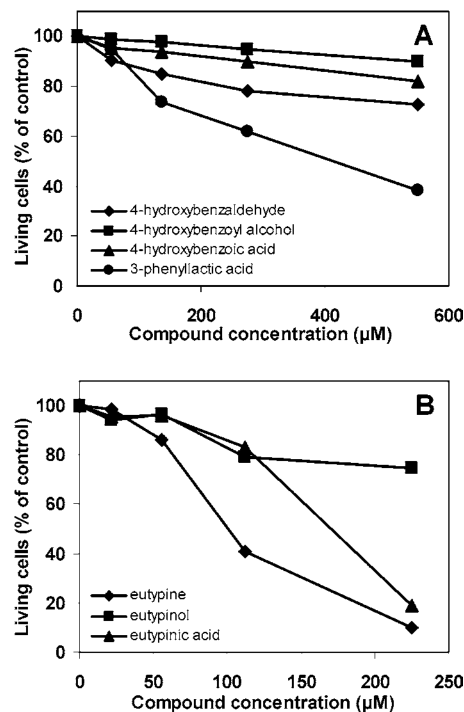

Figure 5. Cytotoxicity of (**A**) 3-phenyllactic acid (●) and of 4-hydroxybenzaldehyde (◆) and its derivatives 4-hydroxybenzyl alcohol (■) and 4-hydroxybenzoic acid (▲) and of (**B**) eutypine (◆) and its derivatives eutypinol (■) and eutypinic acid (▲) expressed as percent reduction of living cells relative to the control.

DISCUSSION

The causal agents of Eutypa dieback and esca disease of grapevine produce numerous toxins (e.g., eutypine, 4-hydroxybenzaldehyde or 3-phenyllactic acid). *V. vinifera* cv. Merlot cells are able to transform eutypine, the main toxin of *E. lata*. This detoxification is thought to confer on Merlot a good tolerance to Eutypa dieback (*19*). On the basis of this detoxification by the cultivar Merlot, we screened potential microbial biocontrol agents for their ability to modify not only eutypine but also other trunk disease toxins.

All strains tested in our study were able to convert totally at least one toxin of the trunk disease pathogens investigated. Detoxification by microorganisms has frequently been documented, especially the fungus *Trichoderma*, which is known to degrade xenobiotics (*32*−*35*), mycotoxins (*36*, *37*), fatty acids (*38*), amines (*39*), or cyanides (*40*). Concerning the other stains used in our study, *F. lateritium* is a cyanide degrader (*41*).

In our experiments, the biotransformation of eutypine and of 4-hydroxybenzaldehyde was rapid for all strains; however, 3-phenyllactic acid was consumed slowly. The lower transformation rates of this toxin can probably be explained by the previously known antifungal activity of 3-phenyllactic acid against *Penicillium* spp., *Aspergillus* spp., and *Fusarium* spp. (*42*). *T. atroviride* P1 exhibited a rapid transformation rate for all three toxins tested, and *F. lateritium* 268.51 was a slow transformer of all three toxins tested. Thus, the reaction kinetics were influenced by the metabolites as well as by the degrader strains.

The reduction of eutypine to eutypinol could be observed in our experiments and confirmed a similar detoxification ability by microorganisms, as is known by plants (*20*). However, surprisingly, we could also identify a second derivative, eutypinic acid, that is the oxidized form of eutypine. The biotransformation of this intermediate has never been described until now. The absence of the oxidative pathway in the tolerant Merlot cultivar (*19*) suggests that fungi are more efficient than plants in metabolizing aldehyde toxins with this alternative pathway. Both reduction and oxidation were also found in the metabolism of the other aldehyde toxin, 4-hydroxybenzaldehyde.

In the kinetic analyses of eutypine transformation by *F. lateritium* and by *T. polysporum* isolates, the metabolic intermediates recovered were not consumed. The quantification of the metabolites was precise, i.e., the molar quantity of eutypine that disappeared was closely correlated with the sum of eutypinol and eutypinic acid produced. This equivalence suggests that no other mechanism beside detoxification (e.g., passive diffusion in the fungal cells) was involved in the consumption of eutypine. On the contrary, in *V. vinifera* cells, eutypine does diffuse passively (*16*).

In the transformation of eutypine by *T. atroviride* and by *T. harzianum* and in every transformation of 4-hydroxybenzaldehyde, the metabolic intermediates were further consumed during the time course of the experiments. Therefore, the stoichiometric equality was not valid. Furthermore, no other aromatic compound could be detected by HPLC in the culture medium at the end of the period. Consequently, the use of the alcohol and acid derivatives in other metabolic pathways or a putative sequestration certainly took place in the fungal cells. To our knowledge, this study is the first report of biological utilization of eutypinol and eutypinic acid. In contrast, the consumption of 4-hydroxybenzyl alcohol and of 4-hydroxybenzoic acid is in accordance with other studies. 4-Hydroxybenzoate is ac-

cumulated against a concentration gradient in *E. coli* (*43*). 4-Hydroxybenzyl alcohol and especially 4-hydroxybenzoic acid are intermediates in several metabolic pathways. The hydroxylation of 4-hydroxybenzoate leads to protocatechuate, one of the important intermediates in the aromatic ring cleavage of the β-ketoadipate pathway (*44*). 4-Hydroxybenzoate also participates as precursor in ubiquinone biosynthesis (*45*) and in the benzoate degradation pathway via CoA ligation (*46*, *47*). Yellow compounds occurred at the end of the transformation by *T. polysporum* 503.82 (4-hydroxybenzaldehyde), by *T. harzianum* 389.36 (eutypine and 4-hydroxybenzaldehyde), by *T. harzianum* 753.68 (3-phenyllactic acid), and by *F. lateritium* 146 (4-hydroxybenzaldehyde and 3-phenyllactic acid). For the biotransformation by the other strains, the formation of yellow compounds was not observed. In the absence of toxin, *F. lateritium* 146 also produced undetermined yellow compounds. Therefore, it is likely that they are produced by some potential biocontrol agents and are not metabolic intermediates in the consumption of the alcohol and acid derivatives.

The structure of eutypine differentiates it from 4-hydroxybenzaldehyde by the substitution of the hydrogen positioned at C_3 of the aromatic ring with an aliphatic chain. Despite this structural distinction, the reaction velocity of eutypine and 4-hydroxybenzaldehyde transformation was relatively close. Nevertheless, this difference could explain the delay of any further conversion of eutypine biotransformation intermediates when compared with the 4-hydroxybenzaldehyde intermediates.

Our toxicity test used cell suspension cultures originated from callus of *V. vinifera* cv. Chasselas. Eutypine was found to be highly toxic and the transformation of its carbonyl group to an acid or to an alcohol resulted in a reduced toxicity. The lower cytotoxicity of eutypinic acid has not been described until now. On the contrary, the decreased toxicity of eutypinol is consistent with other studies presenting biotests performed on cell suspension cultures of *V. vinifera* cv. Gamay, originated from the skin of grape berries and on leaf protoplasts (*19*), on grapevine calli (*48*), or on grapevine leaves (*49*, *50*). The sensitivity of our test was similar to the biotests carried out on cell suspensions from berry skin, on leaf protoplasts, and on calli but was higher than tests on grapevine leaves. Furthermore, our biotest was more rapid and necessitated a lower amount of toxic compounds than assays on calli and was easy-to-handle comparatively to tests on leaf protoplasts and on leaves. We also showed an intermediate toxicity of 3-phenyllactic acid and a low toxicity of 4-hydroxybenzaldehyde. Like eutypine, the metabolism of the aldehyde function of 4-hydroxybenzaldehyde in alcohol or in acid also resulted in a reduced toxicity. These results are comparable with toxicity assays on calli of *V. vinifera* cv. Gamay (*9*).

Microbial detoxification and consumption of the metabolic intermediates were established for the fungi used in our research, which are known to be implicated in different biological control systems (*51*−*54*). Differences were shown at the strain and species level. For eutypine transformation, some strains exhibited one pathway and other strains two pathways. This second new transformation pathway was revealed with 10 of the 12 strains tested. Therefore, the selection of potential new biocontrol agents, especially some endophytic strains, could be based on the ability to effectively consume trunk disease toxins and the choice of biocontrol microbial strains could consequently be enlarged.

ACKNOWLEDGMENT

Special thanks to Isabelle Wagschal for providing cell cultures and to Carmen Saez-Wanzenried for technical assistance.

LITERATURE CITED

(1) Graniti, A.; Surico, G.; Mugnai, L. Considerazioni sul mal dell'esca e sulle venature brune de l'*E. lata* legno della vite. *Informatore Fitopatologico* **1999**, *49*, 6−12.

(2) Bolay, A.; Siegfried, W. Maladies et ravageurs de nos vignobles. *Rev. Suisse Vitic. Arboric. Hortic.* **1993**, *25*, 37−40.

(3) Mugnai, L.; Graniti, A.; Surico, G. Esca (Black measles) and brown wood-streaking: Two old and elusive diseases of grapevines. *Plant Dis.* **1999**, *83*, 404−418.

(4) Bertelli, E.; Mugnai, L.; Surico, G. Presence of *Phaeoacremonium chlamydosporum* in apparently healthy rooted grapevine cuttings. *Phytopathol. Mediterr.* **1998**, *37*, 79−82.

(5) Morton, L. Viticulture and grapevine declines: Lessons of black goo. *Phytopathol. Mediterr.* **2000**, *39*, 59−67.

(6) Moller, W. J.; Kasimatis, A. N.; Kissler, J. J. Dying arm disease of grape in California. *Plant Dis. Rep.* **1974**, *58*, 869−871.

(7) Crous, P. W.; Gams, W.; Wingfield, M. J.; vanWyk, P. S. *Phaeoacremonium* gen. nov. associated with wilt and decline diseases of woody hosts and human infections. *Mycologia* **1996**, *88*, 786−796.

(8) Tey-Rulh, P.; Philippe, I.; Renaud, J. M.; Tsoupras, G.; De Angelis, P.; Fallot, J.; Tabacchi, R. Eutypine, a phytotoxin produced by *Eutypa lata* the causal agent of dying-arm disease of grapevine. *Phytochemistry* **1991**, *30*, 471−473.

(9) Poliart, C. Isolement et caractérisation de métabolites secondaires issus de champignons impliqués dans la maladie de l'esca. Ph.D. Thesis, University of Neuchâtel, 2000.

(10) Nair, M. S. R.; Anchel, M. Frustulosinol, an antibiotic metabolite of *Stereum frustulosum*—Revised structure of frustulosin. *Phytochemistry* **1977**, *16*, 390−392.

(11) Renaud, J. M.; Tsoupras, G.; Tabacchi, R. Biologically active natural acetylenic-compounds from *Eutypa lata* (Pers-F) Tul. *Helv. Chim. Acta* **1989**, *72*, 929−932.

(12) Tabacchi, R.; Fkyerat, A.; Poliart, C.; Dubin, G. M. Phytotoxins from fungi of esca of grapevine. *Phytopathol. Mediterr.* **2000**, *39*, 156−161.

(13) Dubin, G. M.; Fkyerat, A.; Tabacchi, R. Acetylenic aromatic compounds from *Stereum hirsutum*. *Phytochemistry* **2000**, *53*, 571−574.

(14) Molyneux, R. J.; Mahoney, N.; Bayman, P.; Wong, R. Y.; Meyer, K.; Irelan, N. Eutypa dieback in grapevines: Differential production of acetylenic phenol metabolites by strains of *Eutypa lata*. *J. Agric. Food Chem.* **2002**, *50*, 1393−1399.

(15) Deswarte, C.; Rouquier, P.; Roustan, J. P.; Dargent, R.; Fallot, J. Ultrastructural changes produced in plantlet leaves and protoplasts of *Vitis vinifera* cv. Cabernet Sauvignon by eutypine, a toxin from *Eutypa lata*. *Vitis* **1994**, *33*, 185−188.

(16) Deswarte, C.; Canut, H.; Klaebe, A.; Roustan, J. P.; Fallot, J. Transport, cytoplasmic accumulation and mechanism of action of the toxin eutypine in *Vitis vinifera* cells. *J. Plant Physiol.* **1996**, *149*, 336−342.

(17) Amborabe, B. E.; Fleurat-Lessard, P.; Bonmort, J.; Roustan, J. P.; Roblin, G. Effects of eutypine, a toxin from *Eutypa lata*, on plant cell plasma membrane: Possible subsequent implication in disease development. *Plant Physiol. Biochem.* **2001**, *39*, 51−58.

(18) Deswarte, C.; Eychenne, J.; deVirville, J. D.; Roustan, J. P.; Moreau, F.; Fallot, J. Protonophoric activity of eutypine, a toxin from *Eutypa lata*, in plant mitochondria. *Arch. Biochem. Biophys.* **1996**, *334*, 200−205.

(19) Colrat, S.; Deswarte, C.; Latche, A.; Klaebe, A.; Bouzayen, M.; Fallot, J.; Roustan, J. P. Enzymatic detoxification of eutypine, a toxin from *Eutypa lata*, by *Vitis vinifera* cells: Partial purification of an NADPH-dependent aldehyde reductase. *Planta* **1999**, *207*, 544−550.

(20) Guillen, P.; Guis, M.; Martinez-Reina, G.; Colrat, S.; Dalmayrac, S.; Deswarte, C.; Bouzayen, M.; Roustan, J. P.; Fallot, J.; Pech, J. C.; Latche, A. A novel NADPH-dependent aldehyde reductase gene from *Vigna radiata* confers resistance to the grapevine fungal toxin eutypine. *Plant J.* **1998**, *16*, 335−343.
(21) Colrat, S.; Latche, A.; Guis, M.; Pech, J. C.; Bouzayen, M.; Fallot, J.; Roustan, J. P. Purification and characterization of a NADPH-dependent aldehyde reductase from mung bean that detoxifies eutypine, a toxin from *Eutypa lata*. *Plant Physiol.* **1999**, *119*, 621−626.
(22) Karlovsky, P. Biological detoxification of fungal toxins and its use in plant breeding, feed and food production. *Nat. Toxins* **1999**, *7*, 1−23.
(23) Toyoda, H.; Hashimoto, H.; Utsumi, R.; Kobayashi, H.; Ouchi, S. Detoxification of fusaric acid by a fusaric acid-resistant mutant of *Pseudomonas solanacearum* and its application to biological control of Fusarium wilt of tomato. *Phytopathology* **1988**, *78*, 1307−1311.
(24) Toyoda, H.; Katsuragi, K.; Tamai, T.; Ouchi, S. DNA-sequence of genes for detoxification of fusaric acid, a wilt-inducing agent produced by *Fusarium* species. *J. Phytopathol.* **1991**, *133*, 265−277.
(25) Ouchi, S.; Toyoda, H.; Utsumi, R.; Hashimoto, H.; Hamada, T. A promising strategy for the control of fungal diseases by the use of toxin-degrading microbes. In *Phytotoxins and Plant Pathogenesis*; Graniti, A., Durbin, R. D., Ballio, A., Eds.; Springer-Verlag: Berlin, Germany, 1989; pp 301−317.
(26) Utsumi, R.; Hadama, T.; Noda, M.; Toyoda, H.; Hashimoto, H.; Ohuchi, S. Cloning of fusaric acid-detoxifying gene from *Cladosporium werneckii*—A new strategy for the prevention of plant diseases. *J. Biotechnol.* **1988**, *8*, 311−316.
(27) Defranq, E.; Zesiger, T.; Tabacchi, R. The synthesis of natural acetylenic-compounds from *Eutypa lata* (Pers-F) Tul. *Helv. Chim. Acta* **1993**, *76*, 425−430.
(28) Fkyerat, A.; Dubin, G. M.; Tabacchi, R. The synthesis of natural acetylenic compounds from *Stereum hirsutum. Helv. Chim. Acta* **1999**, *82*, 1418−1422.
(29) Linsmaier, E. M.; Skoog, F. Organic growth factor requirements of tobacco tissue cultures. *Physiol. Plant.* **1965**, *18*, 100−127.
(30) Nover, L.; Kranz, E.; Scharf, K. D. Growth cycle of suspension cultures of *Lycopersicon esculentum* and *Lycopersicon peruvianum*. *Biochem. Physiol. Pflanzen* **1982**, *177*, 483−499.
(31) Adams, T. L.; Townsend, J. A. A new procedure for increasing efficiency of protoplast plating and clone selection. *Plant Cell Rep.* **1983**, *2*, 165−168.
(32) Smith, W. H. Forest occurrence of *Trichoderma* species—Emphasis on potential organochlorine (xenobiotic) degradation. *Ecotoxicol. Environ. Saf.* **1995**, *32*, 179−183.
(33) Ravelet, C.; Krivobok, S.; Sage, L.; Steiman, R. Biodegradation of pyrene by sediment fungi. *Chemosphere* **2000**, *40*, 557−563.
(34) Esposito, E.; da Silva, M. Systematics and environmental application of the genus *Trichoderma. Crit. Rev. Microbiol.* **1998**, *24*, 89−98.
(35) Rigot, J.; Matsumura, F. Assessment of the rhizosphere competency and pentachlorophenol-metabolizing activity of a pesticide-degrading strain of *Trichoderma harzianum* introduced into the root zone of corn seedlings. *J. Environ. Sci. Health, Part B* **2002**, *37*, 201−210.
(36) Shantha, T. Fungal degradation of aflatoxin B1. *Nat. Toxins* **1999**, *7*, 175−178.
(37) Nishimura, A.; Yoshizako, F.; Chubachi, M. Purification and characterization of an enzyme that catalyzes ring cleavage of aspergillic acid, from *Trichoderma koningii* ATCC 76666. *Biosci., Biotechnol., Biochem.* **1997**, *61*, 1527−1530.
(38) Kinderlerer, J. L. Fungal strategies for detoxification of medium-chain fatty acids. *Int. Biodeterior. Biodegrad.* **1993**, *32*, 213−224.
(39) Frebort, I.; Matsushita, K.; Adachi, O. Involvement of multiple copper/topa quinone-containing and flavin-containing amine oxidases and NAD(P)(+) aldehyde dehydrogenases in amine degradation by filamentous fungi. *J. Ferment. Bioeng.* **1997**, *84*, 200−212.
(40) Ezzi, M. I.; Lynch, J. M. Cyanide catabolizing enzymes in *Trichoderma* spp. *Enzyme Microb. Technol.* **2002**, *31*, 1042−1047.
(41) Barclay, M.; Day, J. C.; Thompson, I. P.; Knowles, C. J.; Bailey, M. J. Substrate-regulated cyanide hydratase (chy) gene expression in *Fusarium solani*: The potential of a transcription-based assay for monitoring the biotransformation of cyanide complexes. *Environ. Microbiol.* **2002**, *4*, 183−189.
(42) Lavermicocca, P.; Valerio, F.; Visconti, A. Antifungal activity of phenyllactic acid against molds isolated from bakery products. *Appl. Environ. Microbiol.* **2003**, *69*, 634−640.
(43) Nichols, N. N.; Harwood, C. S. PcaK, a high-affinity permease for the aromatic compounds 4-hydroxybenzoate and protocatechuate from *Pseudomonas putida*. *J. Bacteriol.* **1997**, *179*, 5056−5061.
(44) Harwood, C. S.; Parales, R. E. The beta-ketoadipate pathway and the biology of self-identity. *Annu. Rev. Microbiol.* **1996**, *50*, 553−590.
(45) Uchida, N.; Suzuki, K.; Saiki, R.; Kainou, T.; Tanaka, K.; Matsuda, H.; Kawamukai, M. Phenotypes of fission yeast defective in ubiquinone production due to disruption of the gene for *p*-hydroxybenzoate polyprenyl diphosphate transferase. *J. Bacteriol.* **2000**, *182*, 6933−6939.
(46) Egland, P. G.; Harwood, C. S. HbaR, a 4-hydroxybenzoate sensor and FNR-CRP superfamily member, regulates anaerobic 4-hydroxybenzoate degradation by *Rhodopseudomonas palustris*. *J. Bacteriol.* **2000**, *182*, 100−106.
(47) Merkel, S. M.; Eberhard, A. E.; Gibson, J.; Harwood, C. S. Involvement of coenzyme a thioesters in anaerobic metabolism of 4-hydroxybenzoate by *Rhodopseudomonas palustris*. *J. Bacteriol.* **1989**, *171*, 1−7.
(48) Perrin-Cherioux, S.; Abou-Mansour, E.; Tabacchi, R. Synthesis and activity of grape wood phytotoxins and related compounds. *Phytopathol. Mediterr.* **2004**, *43*, 83−86.
(49) Mahoney, N.; Lardner, R.; Molyneux, R. J.; Scott, E. S.; Smith, L. R.; Schoch, T. K. Phenolic and heterocyclic metabolite profiles of the grapevine pathogen *Eutypa lata*. *Phytochemistry* **2003**, *64*, 475−484.
(50) Smith, L. R.; Mahoney, N.; Molyneux, R. J. Synthesis and structure−phytotoxicity relationships of acetylenic phenols and chromene metabolites, and their analogues, from the grapevine pathogen *Eutypa lata*. *J. Nat. Prod.* **2003**, *66*, 169−176.
(51) Di Marco, S.; Osti, F.; Cesari, A. Experiments on the control of esca by *Trichoderma*. *Phytopathol. Mediterr.* **2004**, *43*, 108−115.
(52) John, S.; Scott, E. S.; Wicks, T. J.; Hunt, J. S. Interactions between *Eutypa lata* and *Trichoderma harzianum*. *Phytopathol. Mediterr.* **2004**, *43*, 95−104.
(53) McMahan, G.; Yeh, W.; Marshall, M. N.; Olsen, M.; Sananikone, S.; Wu, J. Y.; Block, D. E.; VanderGheynst, J. S. Characterizing the production of a wild-type and benomyl-resistant *Fusarium lateritium* for biocontrol of *Eutypa lata* on grapevine. *J. Ind. Microbiol. Biotechnol.* **2001**, *26*, 151−155.
(54) Munkvold, G. P.; Marois, J. J. Efficacy of natural epiphytes and colonizers of grapevine pruning wounds for biological control of Eutypa dieback. *Phytopathology* **1993**, *83*, 624−629.

Received for review April 15, 2005. Revised manuscript received June 30, 2005. Accepted June 30, 2005. This project was funded by the National Centre of Competence in Research (NCCR) Plant Survival, research program of the Swiss National Science Foundation.

JF050863H