JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Dying-Arm Disease in Grapevines: Diagnosis of Infection with *Eutypa lata* by Metabolite Analysis

NOREEN MAHONEY,[†] RUSSELL J. MOLYNEUX,*[,†] LEVERETT R. SMITH,[†,‡] THOMAS K. SCHOCH,[†] PHILIPPE E. ROLSHAUSEN,[§] AND W. DOUGLAS GUBLER[§]

Western Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, 800 Buchanan Street, Albany, California 94710, and Department of Plant Pathology, University of California, One Shields Avenue, Davis, California 95616

Dying-arm disease in grapevines, produced by infection with the ascomycete *Eutypa lata*, is responsible for major production losses in vineyards. Dieback of the shoots and cordon is believed to be due to acetylenic phenol metabolites produced by the fungus. To identify specific metabolites that could potentially be used for diagnosis of infection, eight *E. lata* isolates were grown in vitro on hot water extracts from grape varieties with various degrees of tolerance to the foliar symptoms of *E. lata* dieback. HPLC analysis showed that eutypinol was consistently produced in large amounts, together with smaller amounts of methyleutypinol and eulatachromene; eutypine, the putative toxin, was produced solely on Sauvignon Blanc extract and then in only barely detectable amounts. When *E. lata* isolates from Cabernet Sauvignon and Merlot were grown on identical media, the amounts of metabolites produced differed significantly between isolates but the pattern of metabolites was quite similar, with eutypinol again predominating. The consistent production of eutypinol indicated that this was the most suitable metabolite for which to analyze in order to diagnose the presence of *E. lata*. Extraction and analysis of grapevine tissues exhibiting symptoms of dieback failed to show the presence of any metabolites. However, when infected cordon sections were placed in water and cultured for 5 days, eutypinol was readily detected in the aqueous solution; metabolites were not produced from uninfected tissue. This provides a method for detection of infected tissue and indicates that the toxic metabolites react at the point of production, disrupting the vascular structure and inhibiting transport of nutrients, rather than being translocated to tissues that exhibit symptoms.

KEYWORDS: Grapes; *Vitis vinifera*; *Eutypa lata*; dieback; eutyposis; dying-arm disease; eutypinol

INTRODUCTION

Dying-arm disease or *Eutypa* dieback is an important perennial canker disease that affects grapevines (*Vitis vinifera*; Vitaceae) worldwide, including the United States, with particular economic impact in the primary wine-producing areas of California. The causative agent, the ascomycete *Eutypa lata* (*1*), enters the plant principally through pruning wounds, leading to necrosis of woody tissues in the vicinity of the point of infection and may subsequently expand into the trunk of the vine. The disease is cryptic, becoming apparent only after bud break as stunting of new shoots, formation of small, deformed, chlorotic leaves, and development of small fruit clusters. The disease is progressive over many years, and failure to control it leads to severe economic losses, primarily as a consequence of decreased yields, increased vineyard management costs, and reduced longevity of the grapevines. Yield decreases for five vineyards in California growing either Chenin Blanc or French Columbard grapes were estimated to range from 30 to >60%, whereas vineyards over 20 years old had up to 83% yield reduction, relative to their peak production at ∼10−12 years of age (*2*). There are significant differences in tolerance to foliar symptoms of infection, with some of the most valuable cultivars, including Cabernet Sauvignon, being particularly sensitive (*3*). The cost to wine grape production alone in California has been estimated to be in excess of $260 million per annum, or ∼16% of the gross producer revenue of $1.672 billion for 1999 (*4*).

Phytotoxicity has been attributed to one or more of a number of phenolic metabolites (**Figure 1**), bearing an unusual pentenyne side chain ortho to the hydroxyl group, that have been isolated from laboratory cultures of the fungus, together with structurally related compounds in which the aromatic ring has been reduced and epoxidized and the side chain cyclized (*5*, *6*). A recent study using the yeast *Saccharomyces cerevisiae* as a model bioassay system to investigate gene targets of *E. lata* metabolites has shown that eutypinol, **1**, and eulatachromene, **2**, inhibit mitochondrial respiration, suggesting a probable mode

* Corresponding author [telephone (510) 559-5812; fax (510) 559-6129; e-mail molyneux@pw.usda.gov].
[†] Western Regional Research Center.
[‡] Present address: Department of Chemistry, Contra Costa College, 2600 Mission Bell Dr., San Pablo, CA 94806.
[§] University of California, Davis.

10.1021/jf0510236 CCC: $30.25
Published on Web 09/16/2005

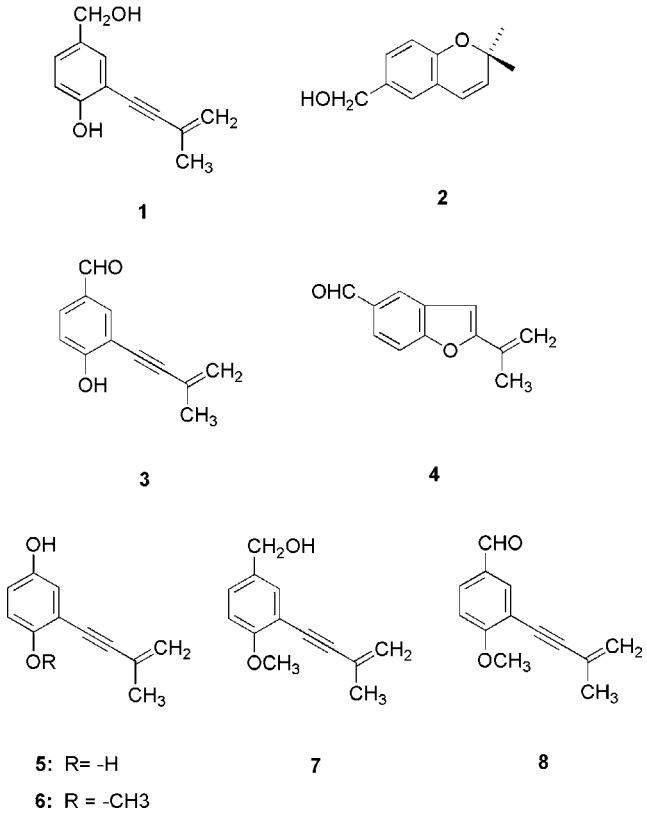

Figure 1. Chemical structures of *E. lata* metabolites: eutypinol, **1**; eulatachromene, **2**; eutypine, **3**; 2-isopropenyl-5-formylbenzofuran, **4**; siccayne, **5**; eulatinol, **6**; methyleutypinol, **7**; methyleutypine, **8**.

of action in grapevine cells (*7*). These results conflict with attribution of the phytotoxicity of *E. lata* to the aldehyde, eutypine, **3** (*8*), with tolerance of some cultivars to the disease being inferred from the ability of a reductase in mung bean (*Vigna radiata)* to reduce eutypine to eutypinol, **1**, which was not found to be phytotoxic to grape callus tissue in vitro (*9*); however, there is no evidence for the presence of a similar reductase in grapevines and this lack of phytotoxicity has not been confirmed in planta (*10*). It is noteworthy that eutypine is particularly sensitive to pH, cyclizing to 2-isopropenyl-5-formylbenzofuran, **4**, even under mildly acidic conditions (*5*), and the relative phytotoxicity of the latter compound has also not been established.

In view of the uncertainty as to the specific metabolites of the fungus responsible for its phytotoxicity we have previously analyzed by high-performance liquid chromatography (HPLC) the metabolite composition of 11 fungal isolates from California, Australia, New Zealand, Italy, and France grown on four artificial media. These profiles were also compared with those of the same isolates grown on Cabernet Sauvignon grapevine wood hot water extracts, with and without sucrose supplementation. Only six compounds were produced in detectable amounts, namely, eutypinol, **1**, and its chromene analogue, eulatachromene, **2** (*11*); eutypine, **3**, and its cyclization product, the benzofuran, **4**, together with the quinol, siccayne (**5**), and its monomethyl analogue, eulatinol (**6**) (**Figure 1**). The two most widely distributed and abundant metabolites were eutypinol and eulatachromene, which were present in eight of the isolates grown on grapewood aqueous extract fortified with sucrose (*11*). Metabolite production on grapevine extract was greatly increased relative to the artificial media, indicating that this native substrate provides enhanced conditions and a more representative profile of the metabolites produced in the natural disease state. These experiments established that eutypine was not consistently produced, and when present, the amounts were generally low, indicating that it is not the sole constituent responsible for phytotoxicity, in contrast to earlier reports (*8*, *9*). This was confirmed in a grapeleaf disk bioassay that showed that eulatachromene, eulatinol, eutypine, and the benzofuran all exhibited necrotic effects with consequent chlorophyll reduction relative to controls (*11*, *12*). Furthermore, eutypine was barely detectable or absent in cultures from a strain of *E. lata* from California (E120) known to be pathogenic to grapes, even though it was present in an Italian strain (E125) (*11*).

Table 1. Identification of *E. lata* Isolates Investigated, Grape Variety from Which Obtained, and Source of Fungal Inoculum

E. lata isolate ID	grape varietal/origin	source
B001	Shiraz/Australia	University of Adelaide
D010	Merlot/Australia	University of Adelaide
E002	Chardonnay/Australia	University of Adelaide
N04	grape/Australia	University of Adelaide
E113	grape/Napa Co., California	E&J Gallo
E190	grape/Mendicino Co., California	E&J Gallo
E203	grape/Sonoma Co., California	E&J Gallo
E206	grape/San Joaquin Co., California	E&J Gallo
8M	Merlot/Napa Co., California	University of California, Davis
21M	Merlot/Napa Co., California	University of California, Davis
7CS	Cabernet Sauvignon/Napa Co., California	University of California, Davis
24CS	Cabernet Sauvignon/Napa Co., California	University of California, Davis

The frequent and abundant occurrence in vitro of eutypinol and eulatachromene from isolates of *E. lata* suggested that either or both of these compounds could be used as diagnostic markers for the presence of the fungus in infected grapevines. A study was therefore undertaken to establish that they were consistently produced in hot water extracts from canes of grapevines other than Cabernet Sauvignon, including varieties known to be susceptible and tolerant, respectively, to dying-arm disease. Grapevine tissues from a vineyard known to be infected with dieback and showing visible symptoms of the disease were then collected to develop both the protocol for sampling and the analytical procedures necessary to use metabolite detection as a surrogate for diagnosis of infection.

MATERIALS AND METHODS

Reagents. Pyridine (Fisher Scientific) was dried by storage over molecular sieves, type 4A, 8−12 mesh (Aldrich Chemical Co.). *N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) in 1 mL ampules was obtained from Pierce (Rockford, IL).

Instrumentation. UV spectra were measured at 190−500 nm in MeOH solution, using a Hewlett-Packard 8452A diode array spectrophotometer, and IR spectra were determined with a Nicolet Magna-IR 550 series II spectrometer. Melting points were taken on a Mel-Temp apparatus and are uncorrected. NMR spectra were obtained at 298 K from samples dissolved in $CDCl_3$ with TMS as an internal standard on a Bruker ARX400 spectrometer at frequencies of 400.13 MHz (^{1}H) and 100.62 MHz (^{13}C). A 90° pulse at a 7−8 s repetition rate was used for ^{1}H experiments, and a 30° pulse at a 2.3 s repetition rate was used for ^{13}C experiments. The number of attached protons for ^{13}C signals was determined from DEPT90 and DEPT135 assays.

Fungal Isolates. The fungal isolates investigated (**Table 1**) were obtained as pure cultures from the E & J Gallo Winery Culture Collection (Modesto, CA), the collection of Dr. Richard Lardner (University of Adelaide, Glen Osmond, South Australia, Australia), and the University of California (Davis, CA). *E. lata* isolates were grown on potato dextrose agar (Difco) from which 5 mm plugs were cut after 4 weeks and stored in water at 4 °C for use as inoculum in all tested media.

Preparation of Grape-Based Media. Tested media consisted of a hot water extract of selected grape cane varieties fortified with 1% sucrose. Dormant canes (Napa Valley, CA) collected during the 2002 season were ground in a Wiley mill with a 1 mm screen. Liquid cane-based medium was prepared by sonication (Branson) of 100 g of ground cane of each variety with 500 mL of hot water (100 °C). This extract was clarified by filtration through the following series of filters: Miracloth (CalBiochem), multigrade GMF 150 (Whatman), GF/F (Whatman), and Supor-200 0.2 μm (Gelman). The cane extract was supplemented with 1% sucrose for the varietal cane media and with 1% sucrose or glucose for the time course Cabernet cane medium.

Inoculation and Incubation of Cultures. Varietal cane media (50 mL of cane hot water extract per 250 mL flask) and Cabernet cane media used for the time course (250 mL per 1 L flask) were autoclaved followed by the addition of three plugs of *E. lata* inoculum. All cultures were incubated at 22 °C. Varietal cane cultures were incubated for 30 days before metabolite extraction. Aliquots (20 mL) were removed from the time course cultures for metabolite extraction after 12, 20, 28, 34, 39, 42, 46, 50, and 57 days of incubation.

Quantitative Analysis of Metabolites in *E. lata* Growth Media. *E. lata* liquid cultures (50 mL) were filtered through Whatman no. 4 paper, and the filtrate was extracted with Et_2O (2 × 50 mL). Time course aliquots (20 mL) were extracted without filtration with Et_2O (2 × 20 mL). The Et_2O extracts were combined and extracted with H_2O (50 mL). The organic phase was collected and the Et_2O removed under reduced pressure. The residue was dissolved in MeOH (1 mL) and filtered through a 0.45 μm, 13 mm, syringe filter (Gelman). Samples (20 μL) were analyzed by HPLC (Agilent 1100) using a 250 mm × 4.6 mm i.d., 5 μm, Microsorb C18 column (Varian) with gradient elution at 1.0 mL/min of 100% H_2O containing 0.5% AcOH changing to 100% CH_3CN over 30 min and held at 100% CH_3CN for 5 min. Detection was either by UV at 254 nm (Agilent 1100 VWD) or photodiode array (Agilent 1100 DAD). Metabolite concentrations were determined by reference to standard curves prepared for each compound, which were linear over the range tested, 0.2−20 μg/20 μL injection.

GC-MS Analysis of TMS Derivatives. TMS derivatives of individual compounds or mixtures were prepared by suspension of the sample (~0.5 mg) in dry pyridine (100 μL) in a 1.0 mL Reacti-Vial (Pierce), to which was added MSTFA (100 μL). The reaction mixture was then heated at ~60 °C for 1 h with periodic shaking to ensure complete dissolution of all reactants. Analyses were performed on a Hewlett-Packard 5890 series II instrument equipped with a 5971 mass-selective detector (MSD) and a 60 m × 0.32 mm i.d., 0.25 μm, SE-30 fused Si capillary column (J&W Scientific, Folsom, CA). The column was held at an initial temperature of 105 °C for 0.2 min, ramped at 30 °C/min for 0.5 min, programmed from 120 to 300 °C at 10 °C/min, and held at the final temperature for 10 min. Helium was used as carrier gas with a head pressure of 60 psi. Derivatized samples (0.1−0.2 μL) were introduced through an SGE model OC1-3 on-column injector held at ambient temperature. The MSD was operated at 70 eV in the EI mode with an ion-source temperature of 180 °C and scanning from *m*/*z* 75 to 600 at a sampling rate of 1.5 scans/s. A postinjection delay of 7.0 min was set to avoid MS data acquisition during elution of the solvent and derivatization reagent.

Synthesis of Methyleutypinol, 7, and Methyleutypine, 8 (Figure 2). *3-Iodo-4-methoxybenzaldehyde.* 3-Iodo-4-hydroxybenzaldehyde (2.14 g, 8.63 mmol) in Et_2O (40 mL) was methylated with diazomethane prepared from Diazald (*N*-methyl-*N*-nitroso-*p*-toluenesulfonamide) (10.0 g, 46.7 mmol) in Et_2O (100 mL) using the manufacturer's (Aldrich, Milwaukee, WI) apparatus and procedure. When the yellow color of the solution disappeared (30 min), the volume was reduced to ~50 mL under N_2 with a warm water bath; rotary evaporation was then used to remove remaining solvent. Subsequent higher vacuum (<1 Torr) gave a white powder (2.26 g; 8.63 mmol; 100%): mp 96−101 °C [lit. 112 °C (*13*)]; IR and ^{1}H NMR spectra consistent with literature values (*13*); ^{13}C NMR ($CDCl_3$) δ 56.8 (−OCH_3), 86.5 (C-3), 110.6 (C-5), 131.5 (C-4), 132.1 (C-6), 141.1 (C-2), 162.8 (C-1), 189.3 (−CHO).

*Methyleutypine, 4-Methoxy-3-(3′-methylbut-3′-en-1′-ynyl)benzaldehyde, **8**.* A mixture of 3-iodo-4-methoxybenzaldehyde (1.05 g, 3.84 mmol), CuI (0.21 g, 1.11 mmol), and $Pd(Ph_3P)_4$ (0.24 g, 0.208 mmol) under an N_2 atmosphere was treated with anhydrous THF (6.0 mL), then triethylamine (10.0 mL), and, finally, after a clear yellow solution was obtained, 2-methyl-1-buten-3-yne (3.0 mL, 31.5 mmol). The mixture rapidly darkened (1−2 min), and stirring was continued at ambient temperature for 25 h in the dark, after which it was diluted with hexane/Et_2O (9:1 v/v, 30 mL). The supernatant was filtered through cotton wool, rinsed through with 20 mL of additional solvent, then washed successively with saturated aqueous NH_4Cl (3 × 20 mL), 5% aqueous $NaHCO_3$ (2 × 20 mL), and saturated aqueous NaCl (2 × 20 mL). The solution was dried over anhydrous Na_2SO_4 and the solvent removed by rotary evaporation to give an amber oil, which was dissolved in Et_2O (30 mL), and then purified by passage through silica gel (5.0 g) using additional Et_2O (100 mL) to complete elution. Reconcentration gave an oil from which **8** was isolated by preparative HPLC. The product solidified, giving a total purified yield of 0.544 g (2.72 mmol; 71%): mp 45.5−48 °C [lit. 49−50 °C (*5*)]; GC-MS (underivatized by treatment with MSTFA) t_R 13.99 min; *m*/*z* 200 [M^+] (100), 185 (13), 159 (37), 128 (76), 115 (15); UV (MeOH) λ_{max} nm (log ϵ) 210 (4.11), 262 (4.56); IR and ^{1}H NMR matched literature values (*13*); ^{13}C NMR ($CDCl_3$) δ 23.4 (−CH_3), 56.3 (−OCH_3), 83.2 (C-1′), 95.8 (C-2′), 110.7 (C-5), 113.7 (C-3), 122.6 (=CH_2), 126.7 (C-3′), 129.7 (C-1), 131.5 (C-6), 135.5 (C-2), 164.4 (C-4), 190.2 (−CHO).

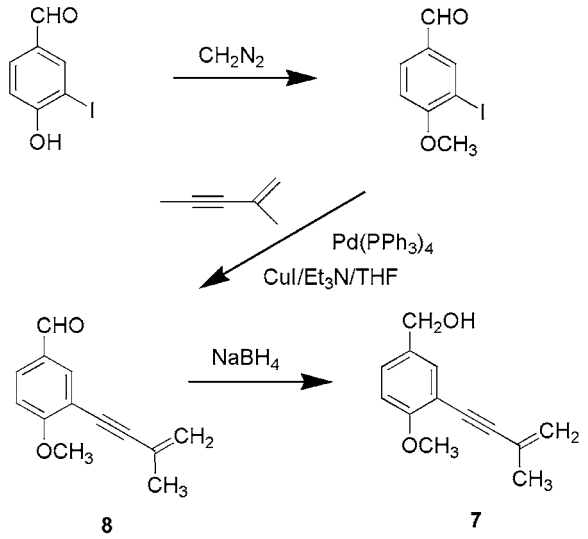

Figure 2. Synthetic route to methyleutypinol, **7**, and methyleutypine, **8**.

*Methyleutypinol, 4-Methoxy-3-(3′-methylbut-3′-en-1′-ynyl)benzyl alcohol, **7**.* Methyleutypine, **8** (0.093 g; 0.465 mmol), was dissolved in MeOH (3.0 mL) and treated with $NaBH_4$ (0.10 g; 2.6 mmol). After 30 min of stirring at ambient temperature, Et_2O (15 mL) was added, followed by 2% aqueous HCl (2.5 mL). The organic phase was washed with saturated aqueous NaCl (2 × 5 mL) and dried over anhydrous Na_2SO_4, and the solvent was removed (rotary evaporator, then <1 Torr) to give 0.091 g (0.45 mmol; 97%) of an almost colorless oil: GC-MS (mono-TMS derivative) t_R 15.38 min; *m*/*z* 274 [M^+] (65), 259 (17), 185 (100), 141 (12), 115 (13); UV (MeOH) λ_{max} nm (log ϵ) 216 (4.33), 276 (4.12), 264 (4.11), 306 (3.91); IR and ^{1}H NMR spectra consistent with literature data (*13*); ^{13}C NMR ($CDCl_3$) δ 23.5 (−CH_3), 56.0 (−OCH_3), 64.6 (−CH_2OH), 84.5 (C-1′), 94.8 (C-2′), 110.9 (C-5), 112.6 (C-3), 121.9 (=CH_2), 127.0 (C-3′), 128.6 (C-6), 132.5 (C-4), 133.0 (C-1), 159.5 (C-4).

Detection and Analysis of Metabolites in Infected Grapevine Tissues. Grapevine tissues (leaves, stems, fruits, canes, spurs, cordons, and trunks) were obtained from a Zinfandel vineyard in the Sacramento Valley and from Merlot and Cabernet Sauvignon vineyards in the Napa Valley, California; we thank the vineyard management and staff for identification of infected and uninfected vines and assistance in collecting the material.

Canes, spurs, cordons, and trunks were ground in a Wiley mill with a 1 mm screen. Leaves, stems, and fruits were separated by hand. Plant samples (50 g) were homogenized with a Polytron (Brinkmann Instruments) in water, water adjusted to pH 4 with HCl, water adjusted

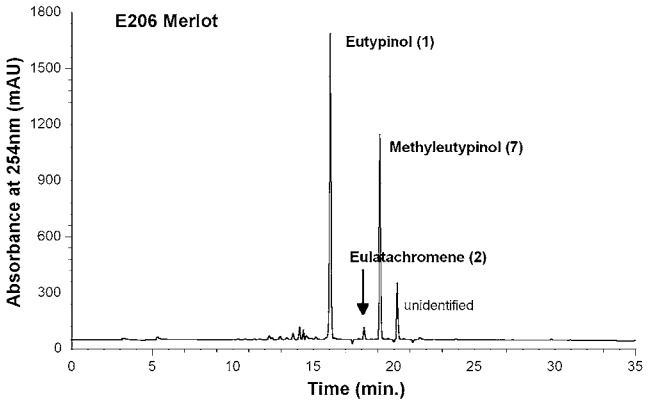

Figure 3. HPLC analysis of metabolites produced by *E. lata* isolate E206 grown on Merlot cane hot water extract supplemented with 1% sucrose.

to pH 10 with NaOH, methanol, acetone, and dioxane (all 500 mL) followed by filtration (Whatman no. 4). The water-based extracts were extracted with Et_2O (500 mL). Solvents were removed from the organic extracts under reduced pressure and the extracts resuspended in water and extracted with Et_2O as above. The organic phases were collected, and the Et_2O was removed under reduced pressure. The residues were dissolved in MeOH (1 mL) and filtered through 13 mm, 0.45 μm, nylon syringe filters (Gelman) followed by HPLC analysis.

Preparation, Culture, and Analysis of Cordon Sections. Highly symptomatic *E. lata*-infected cordons (six samples) and nonsymptomatic cordons (three samples) from Zinfandel grapevines, selected by visible inspection for evidence of dieback, were sectioned into 1 cm slices with a handsaw and debarked with a chisel. Cordon sections were dipped in 70% EtOH, flame sterilized, and placed in 100 mm $\times$ 50 mm crystallizing dishes containing 30 mL of sterile water. The dishes were covered with foil and incubated at 22 °C for 5 days. The water was removed from the crystallizing dish and extracted with 50 mL of Et_2O. The organic phase was collected and the Et_2O removed under reduced pressure. The residue was dissolved in 1 mL of MeOH, filtered through a 0.45 μm syringe filter (Gelman), and analyzed for metabolites by HPLC.

RESULTS AND DISCUSSION

In previous work we established that 11 isolates of *E. lata* obtained from grapevine, apricot, and oak in California, France, Italy, Australia, and New Zealand consistently produced higher levels of metabolites when grown on a hot water extract of Cabernet Sauvignon supplemented with 1% sucrose than on four artificial media (potato dextrose broth, PDB; malt yeast broth, MYB; Pezet's; and Vogel's) (*11*). The sucrose-fortified medium was chosen to represent a nutrient source as similar as possible to that present in planta at the time of the early-season flush of growth in grapevines. Cabernet Sauvignon was selected because it is a variety of great economic value, generally regarded as being particularly susceptible to *Eutypa* dieback (*3*). The ability to produce fungal metabolites on grapevine extract medium provided an opportunity to compare metabolite profiles and yields on extracts from varieties susceptible and tolerant to the disease in order to determine whether there could be constituents in the tolerant varieties that suppressed toxin production. In this work, eight *E. lata* isolates, all isolated from grapevines, were therefore grown on Cabernet Sauvignon, Sauvignon Blanc, and Syrah (susceptible) extracts and on Merlot and Semillon (tolerant) extracts, respectively. The culture filtrates were analyzed by HPLC, and a representative analysis for isolate E206 grown on Merlot extract is shown in **Figure 3**. In all isolates and grapevine extracts, eutypinol was consistently produced in largest amounts, together with smaller quantities of two additional metabolites, one of which was eulatachromene,

Table 2. Metabolite Production by *E. lata* Isolates Grown on Grape Variety Cane Hot Water Extracts with 1% Added Sucrose at 30 Days Growth Stage

medium / metabolite	metabolite yields[a] (μg/mL) for fungal isolate							
	B001	D010	E002	N04	E113	E190	E203	E206
Susceptible Varieties								
Cabernet Sauvignon								
eutypinol, **1**	5.6	16.4	1.7	5.8	4.8	17.6	3.3	40.8
eulatachromene, **2**	0.2	1.1	0.2	0.6	0.5	1.0	0.3	3.1
methyleutypinol, **7**	0.1	7.6	0.1	4.6	0.2	0.2	0.4	4.3
eutypine, **3**	–	–	–	–	–	–	–	–
Sauvignon Blanc								
eutypinol, **1**	7.0	7.8	3.2	0.1	6.2	19.3	23.6	10.5
eulatachromene, **2**	0.3	0.9	0.2	–	0.2	0.8	1.1	1.1
methyleutypinol, **7**	0.2	11.1	0.3	–	1.5	0.4	7.9	3.3
eutypine, **3**	–	tr	–	–	tr	0.1	tr	tr
Syrah								
eutypinol, **1**	6.8	5.6	1.1	0.2	5.2	11.3	11.2	9.0
eulatachromene, **2**	0.4	0.9	tr	0.2	0.2	0.8	1.0	1.5
methyleutypinol, **7**	0.6	2.1	0.2	0.8	3.8	0.4	11.0	4.1
eutypine, **3**	–	–	–	–	–	–	–	–
Tolerant Varieties								
Merlot								
eutypinol, **1**	15.3	17.9	1.8	1.0	0.6	20.6	7.2	21.0
eulatachromene, **2**	0.8	1.3	0.1	0.1	0.6	0.8	0.7	2.0
methyleutypinol, **7**	0.4	11.1	0.2	2.5	0.1	0.2	1.8	9.2
eutypine, **3**	–	–	–	–	–	–	–	–
Semillon								
eutypinol, **1**	2.4	10.7	1.0	0.6	20.6	6.0	9.9	16.5
eulatachromene, **2**	0.2	1.0	0.2	0.2	0.6	0.6	0.5	1.4
methyleutypinol, **7**	0.1	12.8	0.2	1.8	2.3	0.2	2.1	4.8
eutypine, **3**	–	–	–	–	–	–	–	–

[a] –, none detected; tr, above detection limit but <0.1 μg/mL.

whereas the other did not correspond to any metabolites available to us; eutypine, the putative toxin, was produced solely on Sauvignon Blanc and then only in trace amounts. The unidentified metabolite eluted at a retention time ~3 min later than that of eutypinol but had a similar UV spectrum, suggesting that it was a less polar derivative of eutypinol. Methyleutypinol, **7**, has been isolated previously from cultures of *E. lata* (*13*), and this compound therefore seemed to be the most likely candidate for the unidentified metabolite. The synthesis of methyleutypinol via methyleutypine, **8**, another known metabolite, was reported by Defranq et al. (*13*), and both of these compounds were prepared by using the same general approach, but with some slight modifications of the route (**Figure 2**). IR and ^{1}H NMR spectra for methyleutypinol and methyleutypine were consistent with literature data (*13*), and the ^{13}C NMR data, which had not previously been reported, also supported the structures. HPLC comparison of synthetic methyleutypinol with the unknown metabolite showed that these were identical, having the same retention times and UV spectra.

Table 2 shows the levels of production of metabolites for the eight *E. lata* isolates on five different grape variety cane extracts. No obvious correlations could be made for metabolite levels produced on either susceptible or tolerant grape varieties. For example, the most productive isolate, E206, produced 48.2 μg/mL total metabolites on Cabernet Sauvignon extract but only 14.9 and 14.6 μg/mL, respectively, on Sauvignon Blanc and Syrah extracts, all of which are regarded as susceptible grape varieties. In contrast, the metabolite levels were 32.2 and 22.7 μg/mL, respectively, on the tolerant varieties Merlot and Semillon. The only consistent feature was the production of eutypinol as the principal metabolite, with methyleutypinol and eulatachromene in lesser amounts. Eutypinol and methyleutypinol, which are obviously structurally closely related, together

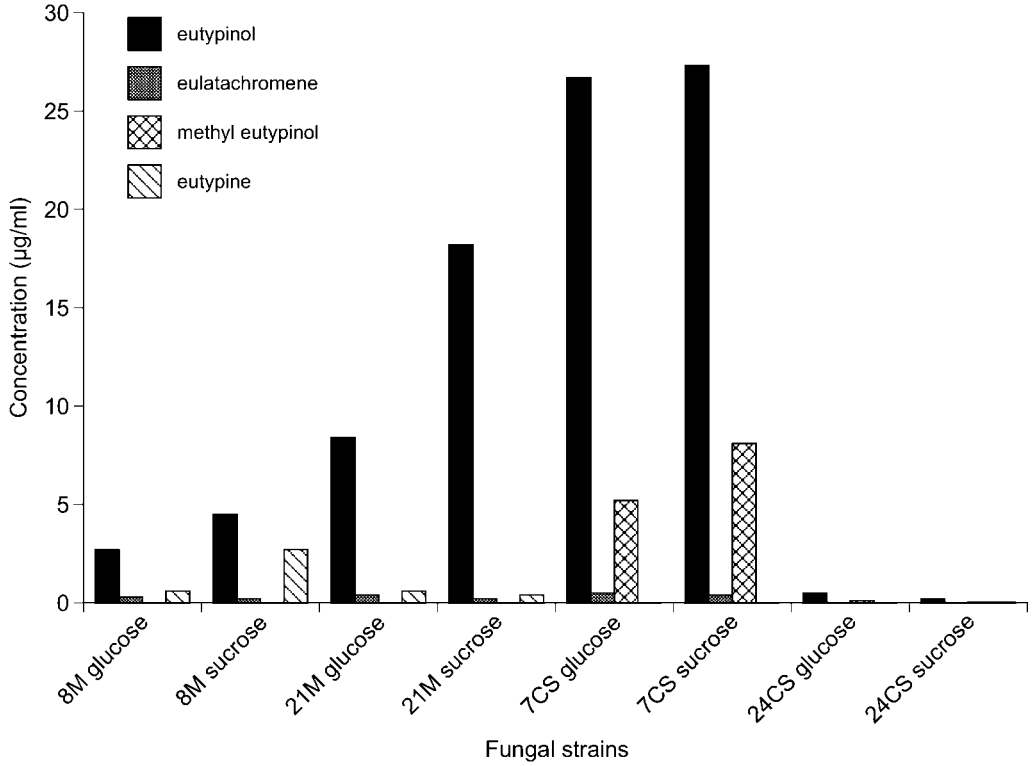

Figure 4. Metabolite production on Cabernet Sauvignon cane hot water extract, supplemented with sucrose or glucose, by *E. lata* isolates isolated from Merlot (8M, 21M) or Cabernet Sauvignon (7CS, 24CS) grapevines.

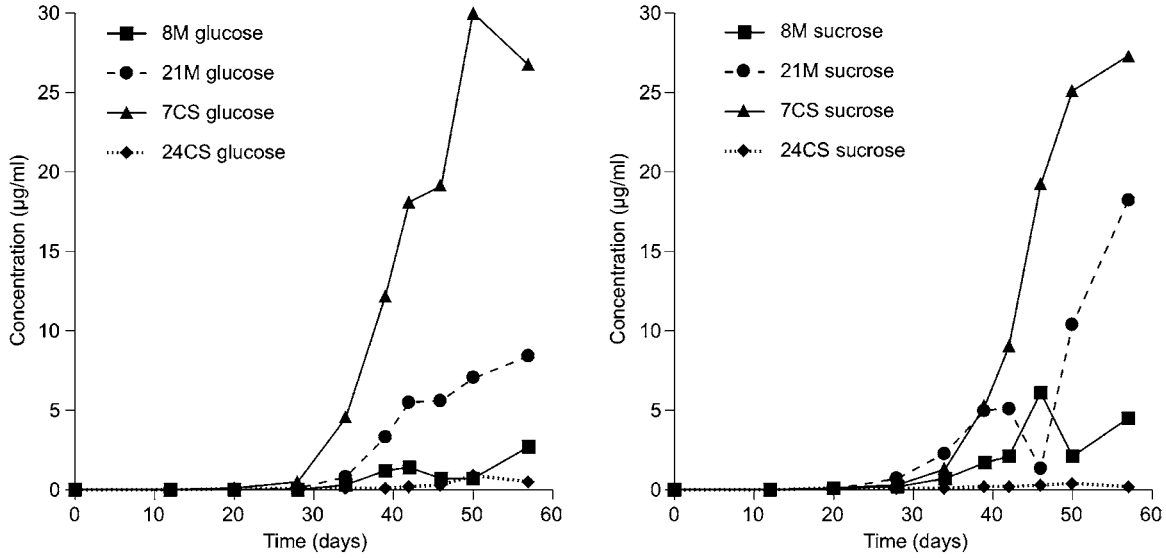

Figure 5. Time course for eutypinol, **1**, production on Cabernet Sauvignon cane hot water extract, supplemented with sucrose or glucose.

comprised 83−100% of the total metabolites, with the exception of isolate E113 grown on Merlot extract, which had a comparable amounts of eutypinol and eulatachromene.

Because differences in metabolite production did not correlate with the variety of grapevine from which cane extracts were derived, the time course and profiles of metabolites produced by *E. lata* isolates isolated from Cabernet Sauvignon and Merlot grown on identical media were examined to determine the effect of fungal source. Cabernet Sauvignon is generally accepted to be the more susceptible cultivar, so it was hypothesized that isolates from the latter would produce a different pattern of metabolites than those from Merlot; this proved not to be the case. Two sets of media were tested, consisting of Cabernet Sauvignon hot water extract supplemented with sucrose or glucose. Although levels of metabolites varied with strain, the general pattern of metabolites was similar, with eutypinol again predominating (**Figure 4**); supplementation with sucrose stimulated metabolite production somewhat more than glucose supplementation. A time course study (**Figure 5**) showed that there was very little eutypinol production during the first 20 days of the culture period but that production increased rapidly thereafter, attaining levels as high as 27 μg/mL for one strain isolated from Cabernet Sauvignon at the end of the experiment on day 57, whereas another strain from the same grape variety produced <1 μg/mL. The isolates from Merlot had intermediate eutypinol production levels, ranging from 2 to 18 μg/mL. The

long time period prior to biosynthesis of significant amounts of the metabolite and subsequent rapid increase suggest that either an unidentified precursor must first attain a particular level before eutypinol production is initiated or that eutypinol biosynthesis is stimulated by nutrient depletion. The latter would appear to be more likely and may have important implications for the situation in grapevines, where cordon wood is likely to be limited in nutrients relative to more actively growing tissues. These results indicate that although eutypinol is the primary metabolite, its level is dependent on the specific isolate of *E. lata*, suggesting that some isolates can be highly pathogenic, whereas others may be relatively innocuous, assuming this compound is a marker of pathogenicity. It is noteworthy that only the isolates from Merlot produced any eutypine, also supporting the hypothesis of variability in pathogenicity of *E. lata* isolates. Nevertheless, correlation between quantitative and/or qualitative production of secondary metabolites and phytotoxicity still has to be demonstrated.

Because the fungus is restricted to the perennial wood of the host plant (*14*), it has been postulated that fruiting spurs and stems are damaged by translocation of toxins (*13*), although no evidence has been advanced to support this. We therefore undertook analysis of grapevine tissues exhibiting clearly visible signs of *Eutypa* dieback for typical metabolites as well as some control samples showing no symptoms of the disease. HPLC analysis of the methanol extract of ground plant material was performed on cordon, spurs, grape canes, young shoots, inflorescences, and fruit bunches from Cabernet Sauvignon, Merlot, and Zindfandel varieties. No *E. lata* metabolites were detected in a total of 39 samples analyzed (results not shown), even though the detection limit (0.01 μg/mL) for these compounds by the HPLC method was very low. To ensure that the absence of metabolites was not due to an inability to extract them from the plant matrix with methanol, other solvents were used. These encompassed a range of polarities and included water, acetone, and dioxane; again, no metabolites were detected. It is possible that the metabolites could be converted into glycosides or other derivatives in planta, compounds which would not be formed by culturing the fungus alone. In the event of such transformations, such derivatives might not be extractable from plant samples with organic solvents, and in order to deal with this eventuality, the plant material was subjected to treatment with either acid or base prior to extraction; once again no metabolites were detectable.

These results indicate that translocation of metabolites from the site of fungal biosynthesis, with direct effects on vegetative tissues, does not occur. In retrospect this is not surprising, because if the compounds are sufficiently reactive to damage plant tissues, then such phytotoxicity should be just as likely in the vicinity of fungal infection. Furthermore, the toxins probably react in such a way that they are bound irreversibly, so that it is unlikely that appreciable amounts of metabolites are present at any particular time. As phenolic compounds, the metabolites would be susceptible to oxidative polymerization by plant phenol oxidases, possibly accounting for the dark, wedge-shaped areas typical of *E. lata* infection. Such a scenario renders the detection of metabolites in planta as a surrogate for identification of *E. lata* infection highly unlikely.

A different approach was therefore adopted, in which cordon tissue was cultured in water to initiate fungal growth and the aqueous solution analyzed for the presence of metabolites. Cross sections ~1 cm thick were cut from cordons that had been classified as infected or uninfected by experienced vineyard managers on the basis of visible symptoms; the bark was removed with a chisel, and the wood samples were briefly surface-sterilized in a flame. The samples were partially immersed in water and kept at 22 °C. After 5 days, fungal growth was visible to a greater or lesser extent on all samples that had shown signs of dying-arm disease, and even earlier on some samples, but not on samples with no symptoms of the disease. After 14 days, the symptomatic cordons showed extensive fungal growth (**Figure 6A**), whereas the nonsymptomatic cordons showed no fungal growth even after 60 days (**Figure 6B**). Extraction of aliquots of the aqueous media with diethyl ether after culturing for 5 days and analysis by HPLC showed the presence of eutypinol, **1**, and methyleutypinol, **7** (**Figure 7**). The presence of these metabolites correlated with visible evidence of pathogenic *E. lata* in the tissues, whereas the control samples showed no eutypinol or any other metabolites of similar structure. In addition, a major peak was also observed at 16.63 min in some samples. Although this constituent had a UV spectrum typical of other *E. lata* compounds, it did not correspond to any of the metabolites produced in culture and may therefore be an artifact produced by the action of plant enzymes. Culturing of larger amounts of cordon tissue will be

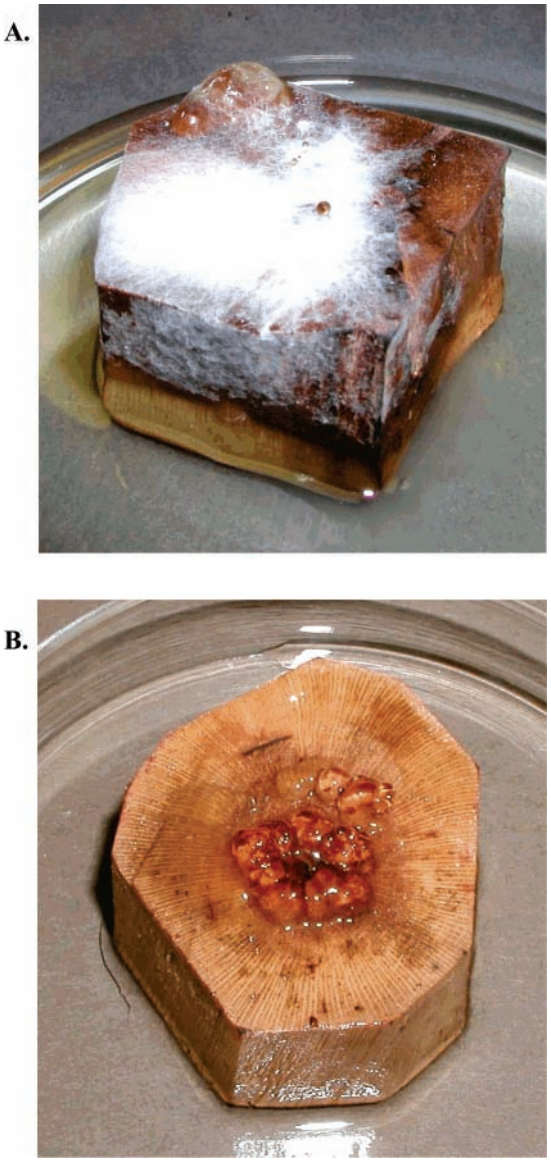

Figure 6. Cordon sections cultured in water at 22 °C: (**A**) highly symptomatic cordon, 14 days; (**B**) nonsymptomatic cordon, 60 days.

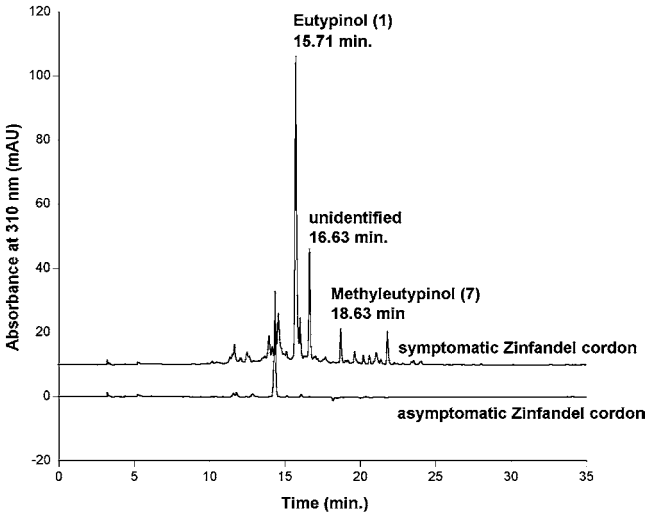

Figure 7. HPLC analysis of highly symptomatic and nonsymptomatic cordon culture medium (water) after 5 days.

required to isolate sufficient quantities of this compound for structural identification.

The analytical techniques developed in the course of this research should be applicable to the detection of specific phytotoxic metabolites in grapevines infected by *E. lata*, possibly before visible symptoms of dying-arm disease develop. Although a PCR assay for the fungus in grapevine wood has been developed (*15*), it does not distinguish between pathogenic and nonpathogenic isolates, nor does it provide a measure of the degree of pathogenicity. The primers designed for ITS sequences used by Lecomte et al. (*15*) have now been shown not to be specific for *E. lata* (*16*), and Lardner et al. (*17*) have recently demonstrated that the SCAR primer pairs were also not specific to Australian populations of *E. lata*. Furthermore, several diatrypaceous species occur on grapevines. A *Diatrypella* species was recently recovered from wood canker of grapevines (unpublished observations), Rohlshausen et al. (*18*) confirmed the presence of a *Diatrype* species on grapevines, and Trouillas et al. (*19*) also found the teleomorphs of *Diatrype* and *Diatrypella* species on dead wood of grapes. Moreover, *Eutypa leptoplaca* (*20*) and *Cryptovalsa ampelina* (*21*) have been reported to be pathogenic on grapes. The presence of several related taxa on grapevine indicates that particular care has to be taken for positive identification of the causal agent responsible for disease. Disease diagnosis is commonly done by isolating the organism from wood canker on PDA medium, but because of the morphological resemblance of this group of fungi in culture, specialized mycological expertise is required and misidentification can occur (*16*). Further research is required to establish species delimitation within the Diatrypaceae to better identify the organisms causing disease, and metabolite analysis of cultured grapewood samples as developed herein should enable the presence of *E. lata* to be distinguished from other diatrypaceous species, because it has been shown that production of such secondary metabolites is a genetically derived character (*18*). This is supported by the fact that 2 of the 11 isolates in our previous study, E178 and SS1#1, which did not produce metabolites (*11*), have subsequently been classified on the basis of PCR amplification of DNA as *Diatrype* and *Cryptovalsa* species, respectively (*17*). It is important to note that in our previous study (*11*) and in the current work the metabolite yields are much higher and profiles quite different when using grape cane extracts from those obtained using artificial media (*10*). Use of grape cane extract for in vitro experiments is therefore likely to provide a much more representative picture of the metabolite composition in planta. A recent publication (*22*) has described similar findings when metabolites produced by *Penicillium* species responsible for storage rot in root vegetables and flower bulbs were compared for artificial media and plant material agars.

The techniques developed in this exploratory study can be performed in any suitably equipped analytical chemistry laboratory and do not require expert knowledge of fungal taxonomy. We plan to investigate the metabolite profiles of other *Eutypa* species by HPLC analysis to determine whether they differ significantly from that of *E. lata*. Moreover, *Botryosphaeria* species produce symptoms remarkably similar in appearance to those of *Eutypa* dieback in grapevines, almonds, pistachios, and walnuts, including the wedge-shaped necrosis of trunks and cordons (*23*, *24*). The metabolites produced by *Botryosphaeria* on grapevine media have not been identified; it will be of interest to determine whether they are structurally related to those of *E. lata*. Such information should permit discrimination of *Eutypa* dieback from other grapevine trunk diseases, without the need to rely upon symptomatic descriptors, and also could establish the structural features essential for phytotoxicity.

The metabolite profiles produced by the limited number of *E. lata* isolates examined in this study, their heterogeneity with growth media and time, and potential for structural transformation within the grape plant matrix suggest that phytotoxicity is probably a consequence of a suite of compounds rather than eutypine alone. In fact, eutypine was a rarely produced metabolite in all of our studies and notable by its absence from most *E. lata* isolates grown on grape cane extracts. Identification of the complete range of metabolites produced by analysis of a larger number of *E. lata* isolates, representative of those parts of the world where the disease is a problem for grape producers, and assessment of the relative phytotoxicity of each using a standardized grape-tissue bioassay should permit decisions to be made as to the economic impact in any particular situation. The cordon culturing procedure and HPLC analysis of the medium provides a proof-of-concept that the technique could be applied to experimental studies of transmission and spread of the disease and to ensure that tissues used for grafting are not infected. It could also be used to ensure that reworked trunks to which tissues are being grafted are free of *E. lata*. Improved cordon sampling methods, such as removal of smaller tissue samples by coring or plug cutting would permit high-throughput testing of numerous samples, without sacrificing the entire cordon. Appropriate management techniques can then be devised to limit the spread of the most virulent isolates and ensure the continued productivity of individual vineyards.

ACKNOWLEDGMENT

We gratefully acknowledge Rosalind Wong, Western Regional Research Center, Albany, CA, for obtaining NMR spectra. We thank Dr. Richard Lardner, University of Adelaide (Waite Campus), Glen Osmond, South Australia, for providing selected *E. lata* isolates. We greatly appeciate the cooperation of Andrew C. Johnson, Beringer Blass Wine Estates, St. Helena, CA, in providing grape cane material for preparation of hot water extract nutrient solutions.

LITERATURE CITED

(1) Carter, M. V.; Bolay, A.; Rappaz, F. An annotated host list and bibliography of *Eutypa armeniacae*. *Rev. Plant Pathol.* **1983**, *62*, 251−258.

(2) Munkvold, G. P.; Duthie, J. A.; Marois, J. J. Reductions in yield and vegetative growth of grapevines due to *Eutypa* dieback. *Phytopathology* **1994**, *84*, 186−192.
(3) Munkvold, G. P.; Marois, J. J. Factors associated with variation in susceptibility of grapevine pruning wounds to infection by *Eutypa lata*. *Phytopathology* **1995**, *85*, 249−256.
(4) Siebert, J. B. Eutypa: the economic toll on vineyards. *Wines Vines* **2001**, 50−56.
(5) 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.
(6) Renaud, J. M.; Tsoupras, G.; Stoecklievans, H.; Tabacchi, R. A novel allenic epoxycyclohexane and related compounds from *Eutypa lata* (Pers: F.) TUL. *Helv. Chim. Acta* **1989**, *72*, 1262−1267.
(7) Kim, J. H.; Mahoney, N.; Chan, K. L.; Molyneux, R. J.; Campbell, B. C. Secondary metabolites of the grapevine pathogen *Eutypa lata* inhibit mitochondrial respiration, based on a model bioassay using the yeast *Saccharomyces cerevisiae*. *Curr. Microbiol*. **2004**, *49*, 282−287.
(8) Tey-Rulh, P.; Philippe, I.; Renaud, J. M.; Tsoupras, G.; Deangelis, 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) 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.
(10) 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.
(11) 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.
(12) Smith, L R.; Mahoney, N.; Molyneux, R J. Synthesis and structure-phytotoxicity relationships of acetylenic phenols and chromene metabolites, and their analogs, from the grapevine pathogen *Eutypa lata. J. Nat. Prod*. **2003**, *66*, 169−176.
(13) Defranq, E.; Zesiger, T.; Tabacchi, R. The synthesis of natural acetylenic compounds from *Eutypa lata* (Pers: T.) Tul. *Helv. Chim. Acta* **1993**, *76*, 425−430.
(14) Munkvold, G. P. Eutypa dieback of grapevine and apricot. *Plant Health Prog*. on-line doi:10.1094/PHP-2001-0219-01-DG.
(15) Lecomte, P.; Péros, J.-P.; Blancard, D.; Bastien, N.; Délye, C. PCR assays that identify the grapevine dieback fungus *Eutypa lata*. *Appl. Environ. Microbiol.* **2000**, *66*, 4475−4480.
(16) Rolshausen, P. E.; Trouillas, F. P.; Gubler, W. D. Identification of *Eutypa lata* by PCR-RFLP. *Plant Dis*. **2004**, *88*, 925−929.
(17) Lardner, R.; Stummer, B. E.; Sosnowski, M. R.; Scott, E. S. Molecular identification of *Eutypa lata* in grapevine. *Mycol. Res.* **2005**, *109*, 1−10.
(18) Rolshausen, P. E.; Mahoney, N.; Molyneux R. J.; Gubler, W. D. A re-assessment of the species concept in *Eutypa lata*, the causal agent of Eutypa dieback of grapevine. *Phytopathology* **2005**, in press.
(19) Trouillas, F. P.; Rolshausen, P. E.; Gubler, W. D. Importance of *Eutypa lata* and occurrence of other Diatrypaceous fungi in northern Californian vineyards. *Phytopathology* **2001**, *91*, S89.
(20) Trouillas, F. P.; Gubler, W. D. Identification and characterization of *Eutypa leptoplaca*, a new pathogen of grapevine in northern California. *Mycol. Res*. **2004**, *10*, 1195−1204.
(21) Mostert, L.; Halleen, F.; Creaser, M. L.; Crous, P. W. *Cryptovalsa ampelina*, a forgotten shoot and cane pathogen of grapevines. *Australas. Plant Pathol*. **2004**, *33*, 295−299.
(22) Overy, D. P.; Blunt, J. W. Corymbiferan lactones from *Penicillium hordei*: stimulation of novel phenolic metabolites using plant tissue material. *J. Nat. Prod.* **2004**, *67*, 1850−1853.
(23) Michailides, T. J.; Morgan, D. P. Panicle and shoot blight of pistachio: A major threat to the California pistachio industry. *APSnet: Plant Pathology Online* 2004; www.apsnet.org/online/feature/pistachio (accessed Feb 3, 2005).
(24) van Niekerk, J. M.; Crous, P. W.; Groenewald, J. Z.; Fourie, P. H.; Halleen, F. DNA phylogeny, morphology and pathogenicity of *Botryosphaeria* species on grapevines. *Mycologia* **2004**, *96*, 781−798.

Received for review May 3, 2005. Revised manuscript received July 28, 2005. Accepted August 11, 2005. This research was conducted under a Cooperative Agreement between USDA-ARS (No. 58-5325-0-158) and the American Vineyard Foundation (Project V200); we thank the AVF for financial support.

JF0510236