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Eutypa Dieback in Grapevines: Differential Production of Acetylenic Phenol Metabolites by Strains of *Eutypa lata*

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The production of acetylenic phenol metabolites in vitro by three strains of the ascomycete *Eutypa lata*, the causative agent of dying-arm disease in grapevines, has been investigated. Metabolite composition and yields differed significantly between strains and with growth medium but usually reached a maximum after 24−30 days of fungal growth. A general method for the analysis and identification of metabolites by gas chromatography/mass spectrometry of their trimethylsilyl ether derivatives was developed and individual compounds were quantitated by analytical high-performance liquid chromatography (HPLC) and separated by preparative HPLC. The phenolic aldehyde, eutypine (**1**), reported to be the grape phytotoxin, occurred in only one of the strains examined whereas the primary metabolite was the corresponding alcohol, eutypinol (**2**), the presumptive detoxification product. A novel metabolite was isolated as a major constituent, together with a minor component, and their structures were established by spectroscopic methods as a methoxyquinol, named eulatinol (**4**), and a chromene analog (**9**) of **2**, respectively. The evidence suggests that **1** is not solely responsible for phytotoxicity in grapevines but that dying-arm disease may result from a suite of compounds elaborated by the fungus, with the composition dependent on fungal strain and nutritional source.

KEYWORDS: Grapes; *Vitis vinifera*; *Eutypa lata*; dieback; eutyposis; vine decline; dying-arm disease; chromene

INTRODUCTION

Eutypa dieback, also known as eutyposis or "dying-arm disease", is a perennial canker disease that affects grapevines (*Vitis vinifera*; Vitaceae) in major grape-producing areas around the world, including but not restricted to, the United States, Europe, Australia, and South Africa (*1*). The causative agent, the ascomycete *Eutypa lata* (initially named *E. armeniacae*), enters the plant primarily through pruning wounds, leading to necrosis of woody tissues in the vicinity of the point of infection and stunting of new shoots. This results in small, deformed, chlorotic leaves and meager development of 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 and reduced longevity of the grapevines. In Australia, yield losses of at least 850 and 740 kg/ha have been reported for Shiraz and Cabernet Sauvignon vines, respectively (*2*). Yield decreases for five vineyards in California growing either Chenin blanc or French Columbard grapes were estimated to range from 30% to greater than 60%, while vineyards over 20 years old had up to 83% yield reduction, relative to their peak production at about 10−12 years of age (*3*). There are significant differences in susceptibility to infection, with some of the most valuable cultivars, including Cabernet Sauvignon, being particularly sensitive (*4*). *E. lata* is also known to infect other important agricultural crops in the Central Valley of California, including almond, apricot, cherry, olive, peach, and walnut (*1, 5*). The fungus is distributed throughout California, and the economic impact is a result of decreased yields, increased vineyard management costs, and shortened life span of the vines. The cost to wine grape production alone in California has been estimated to be in excess of $260 million per annum, or ca. 16% of the gross producer revenue of $1.672 billion for 1999 (personal communication, J. B. Siebert, University of California, Berkeley).

A considerable number of phenolic metabolites bearing an unusual pentenyne side chain ortho to the hydroxyl group have been isolated from a laboratory culture of the fungus, together with structurally related compounds in which the aromatic ring has been reduced and epoxidized and the side chain cyclized (*6*, *7*). The phytotoxicity has been attributed primarily to the aldehyde, eutypine (**1**; **Figure 1**) (*8*), which is believed to act as a protonophoric agent, uncoupling mitochondrial oxidative phosphorylation (*9*−*11*). The tolerance of some cultivars to the disease has been ascribed to their ability to reduce **1** to the

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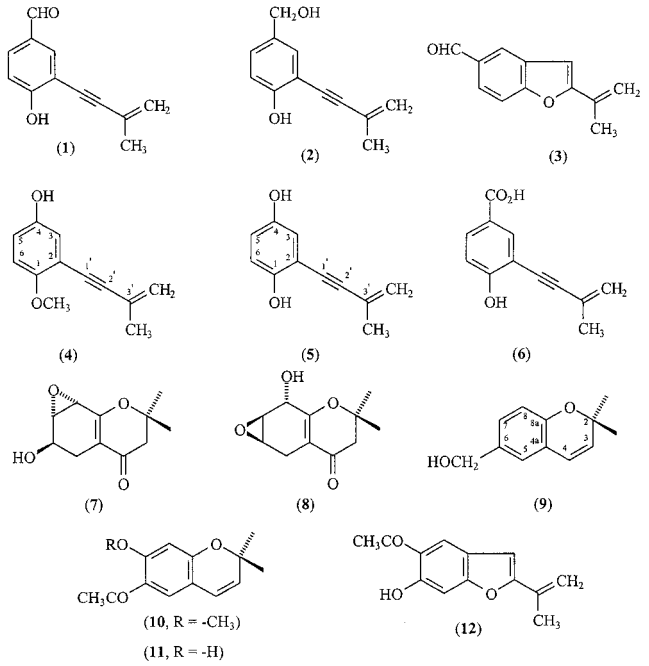

Figure 1. Chemical structures of *Eutypa lata* metabolites: **1**–**5**, eutypine carboxylic acid analogue (**6**), epoxy-tetrahydrochromanone isomers (**7** and **8**), and **9**.

corresponding alcohol, eutypinol (**2**), which was not phytotoxic to grape callus tissue in vitro, but this lack of effect has not been confirmed in planta (*12*). Moreover, **1** is particularly sensitive to pH, cyclizing to 2-*iso*propenyl-5-formylbenzofuran (**3**) even under mildly acidic conditions (*6*), and the relative phytotoxicity of this compound has also not been established.

In preliminary work designed to identify the phytotoxic agents responsible for eutyposis in California grapevines, we were unable to confirm the phytotoxicity of **1** in a grape plantlet bioassay and could not detect the presence of this metabolite in cultures from a strain of *E. lata* from California known to be pathogenic to grapes, even though it was present in an Italian strain. We therefore undertook an investigation of the time−course of metabolite production and the effect of growth media on metabolite composition in order to establish the range of conditions influencing production of individual compounds for future investigations of phytotoxicity. Concurrently, we developed a high-performance liquid chromatography (HPLC) method for the separation and quantitation of metabolites and a gas chromatography (GC)/mass spectrometry (MS) method for their analysis and isolated and identified two novel metabolites.

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. High-resolution mass spectral (HRMS) measurements were determined on a VG Micromass 7070 mass spectrometer. Nuclear magnetic resonance (NMR) spectra were obtained at 298 K from samples dissolved in $CDCl_3$ with tetramethylsilane (TMS) as an internal standard on a Bruker ARX400 spectrometer at frequencies of 400.13 (^{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 experiments. One- and two-dimensional experiments were run for both nucleii. ^{1}H−^{1}H and ^{13}C−^{1}H two-dimensional correlation spectroscopy (COSY) experiments were obtained using the pulse programs COSY90 and HXCOCP, respectively. Long-range ^{13}C−^{1}H COSY experiments, optimized for $^3J_{C,H}$, were obtained using the pulse program HXCOLR.

Fungal Strains. The fungal isolates used were obtained from the E. & J. Gallo Winery Culture Collection (Modesto, CA) and were selected on the basis of host species and geographical origin. Strains E120 and E178 were isolated from grape and oak, respectively, in Sonoma County, California, and strain E125 was isolated from grape in Italy (*13*).

Preparation of Media and Fungal Cultures. *Eutypa* isolates were grown on potato dextrose agar (Difco) for use as inoculum in either potato dextrose broth (PDB, pH 4.9) or malt extract/yeast extract broth (MYB, pH 5.9) liquid media. Liquid broths (50 mL per 250 mL flask) were prepared in triplicate from dehydrated culture media (Difco) for both PDB (24 g/L) and MYB (20 g/L malt extract, 10 g/L yeast extract) to which several plugs (6.5 mm diameter) of *Eutypa* inoculum were added and incubated at 20 °C.

Preparative HPLC. Crude extracts from *Eutypa* mycelial mats and liquid cultures were dissolved in ethanol and filtered through 0.2 μm Acrodisc CR 25 mm syringe filters (Gelman). *Eutypa* metabolites were isolated by preparative chromatography using a C18 Dynamax 8 μm column, 250 mm × 21.4 mm i.d., and a 50 mm guard column (Rainin), with isocratic elution by CH_3CN:H_2O (1:1) pumped (Beckman 110B) at a flow rate of 10 mL/min and UV detection at 254 nm (Gilson 115). *Eutypa* metabolites had the following retention times: eutypinol (**2**), 9.8 min; siccayne (**5**), 10.7 min; eulatachromene (**9**), 14.2 min; eutypine (**1**), 18.5 min; and, eulatinol (**4**), 19.4 min.

GC/MS Analysis of TMS Derivatives. TMS derivatives of individual compounds or mixtures were prepared by suspension of the sample (ca. 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 ca. 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. SE-30 (0.25 μm) fused Si capillary column (J&W Scientific). 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 the 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 scanning from 75 to 600 amu at a sampling rate of 1.5 scans/sec. A postinjection delay of 7.0 min was set in order to avoid MS data acquisition during elution of the solvent and derivatization reagent.

Isolation of Metabolites from *Eutypa* Liquid Culture and Mycelia. *Eulatinol (4) and Siccayne (5).* *Eutypa* strain E120 mycelial mats (140 total) were removed from MYB medium, and the mats were extracted with acetone (2 L) using a Polytron disintegrator (Brinkmann Instruments) and filtered through Whatman 50 paper. The acetone was removed under reduced pressure, and the remaining H_2O (1 L) was extracted with diethyl ether (2 × 1 L). The ether fractions were combined, and the solvent was removed under reduced pressure. Compounds **4** and **5** were isolated from the remaining residue (270 mg) by preparative HPLC.

Eulatinol (4). Compound **4** was obtained as a colorless oil. HREIMS: *m/z* 188.0834 (calcd for $C_{12}H_{12}O_2$, 188.0837). GC/MS (mono-TMS derivative): R_T 12.95 min, *m/z* 260 [M^+] (100), 245 (45), 215 (4), 187 (4), 115 (5). Analytical HPLC: R_T 8.94 min. ^{1}H and ^{13}C NMR, see **Table 1**; UV, see **Figure 5**.

Siccayne (5). Compound **5** was obtained as a colorless oil. GC/MS (di-TMS derivative): R_T 14.03 min, *m/z* 318 [M^+] (100), 303 (46), 287 (5), 263 (6). Analytical HPLC: R_T 4.90 min. ^{1}H and ^{13}C NMR, see **Table 1**; UV, see **Figure 5**.

Eutypine (1), Eutypinol (2), and Eulatachromene (9). *Eutypa* strain E125 mycelial mats were removed from the PDB culture medium (10 L). The liquid medium was filtered through Whatman no. 4 paper and extracted with diethyl ether (10 L). The ether extract was reduced to 1 L under reduced pressure and extracted with an equal volume of H_2O. The organic phase was collected, and the ether was removed under

Table 1. ^1H and ^{13}C NMR Assignments for **4**, **5**, and **9** in $CDCl_3$

	4			**5**			**9**	
position	^1H	^{13}C	^{13}C–^1H COSY ($^3J_{CH}$)	^1H	^{13}C	position	^1H	^{13}C
1		154.40	H(3), H(5), –OCH_3		150.87	8a		152.63
2		113.31	H(6)		109.90	4a		121.33
3	6.89 (dd, J = 2.4, 0.8)	120.00	H(5)	6.81 (d, J = 3.2)	117.37	5	6.99 (d, J = 2)	125.46
4		149.15	H(6)		148.69	6		133.20
5	6.77 (dd, J = 8.8, 2.4)	116.52	H(3)	6.74 (dd, J = 8.8, 3.2)	117.98	7	7.09 (dd, J = 8.4, 2)	128.16
6	6.74 (dd, J = 8.8, 0.8)	112.39	none obsvd	6.82 (dd, J = 8.8, 0.4)	115.49	8	6.75 (d, J = 8.4)	116.37
1′		84.29	H(3)		81.91	4	6.31 (d, J = 10)	122.18
2′		94.87	=CH_2, –CH_3		97.63	3	5.62 (d, J = 10)	131.10
3′		126.92			126.09	2		76.32
–OCH_3	3.83	56.58						
=CH_2	5.29; 5.41 (m)	122.06	–CH_3	5.36; 5.43 (m)	123.05			
–CH_3	1.94 (dd, J = 1.6, 1.2)	23.48	=CH_2	2.01 (dd, J = 1.6, 1.2)	23.43	–CH_3	1.43 (×2)	28.00
						–CH_2OH	4.57	65.15
–OH				4.46, 5.38		–OH	5.29	

reduced pressure. Compounds **1**, **2**, and **9** were isolated from the remaining residue (1.3 g) by preparative HPLC.

Eutypine (1). Compound **1** was obtained as a colorless oil. GC/MS (mono-TMS derivative): R_T 13.77 min, m/z 258 [M^+] (100), 243 (83), 227 (8), 199 (41), 185 (26), 141 (11), 128 (12), 115 (13). Analytical HPLC: R_T 7.92 min. ^1H and ^{13}C NMR, identical to literature values (*6*, *8*); UV, see **Figure 5**.

Eutypinol (2). Compound **2** was obtained as a colorless oil. GC/MS (di-TMS derivative): R_T 14.92 min, m/z 332 [M^+] (100), 317 (50), 243 (76), 227 (11), 147 (11). Analytical HPLC: R_T 4.37 min. ^1H and ^{13}C NMR, identical to literature values (*6*); UV, see **Figure 5**.

Eulatachromene (9). Compound **9** was obtained as a colorless oil. HREIMS: m/z 190.0993 (calcd for $C_{12}H_{14}O_2$, 190.0994). GC/MS (mono-TMS derivative): R_T 12.08 min, m/z 262 [M^+] (8), 247 (100), 173 (14), 158 (5). Analytical HPLC: R_T 7.02 min. ^1H and ^{13}C NMR, see **Table 1**; UV (MeOH) λ_{max} (log ϵ): 224 (4.54), 266 (3.59), 312 (3.47).

Quantitative Analysis of Metabolites in *Eutypa* Growth Media. *Eutypa* MYB and PDB liquid cultures (50 mL) were filtered through Whatman no. 4 paper, and the filtrate was extracted with diethyl ether (2 × 50 mL). The ether extracts were combined and partitioned with H_2O (50 mL). The organic phase was collected, and the ether was removed under reduced pressure. The residue was dissolved in methanol (1 mL) and filtered through a 0.45 μm 13 mm syringe filter (Gelman). Samples (20 μL) were analyzed by HPLC (Hewlett-Packard 1050) using a C18 Dynamax 8 μm, 250 mm × 4.6 mm i.d. and 50 mm guard column (Rainin), with isocratic elution by CH_3CN:H_2O (1:1) at a flow rate of 1.0 mL/min with UV detection at 254 nm. Metabolite concentrations were determined by reference to the standard curves prepared for each compound, which were linear over the range 0−100 μg/mL.

RESULTS AND DISCUSSION

To investigate the variability of metabolite production in vitro with respect to a specific isolate, growth medium, and time−course, three representative strains of *E. lata* were selected. Two of these were isolated from grape, one from California (E120) and the other from Italy (E125), and despite the difference in geographical origin were found to be closely affiliated within a large monophyletic group of 104 strains, based on amplified fragment-length polymorphism and internal transcribed spacer rDNA sequence analyses (*13*). A third isolate from oak (E178) was selected as a representative of a group of seven isolates from native host species in California. The latter small group was inferred as monophyletic, sister to the major clade containing the E120 and E125 grape strains (*13*). The E178 oak strain was therefore expected to serve as an outlier sample in which the metabolite composition should theoretically be as distinctly different as possible from the grape isolates.

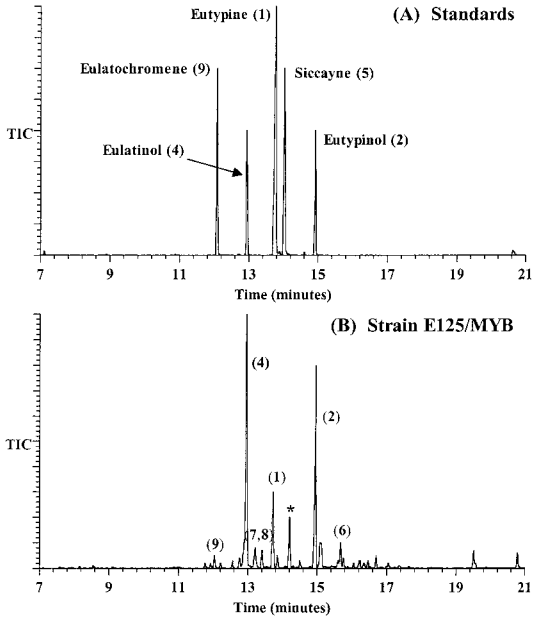

Figure 2. (**A**) GC/MS analysis of TMS derivatives of standards of the *E. lata* metabolites **1**, **2**, **4**, **5**, and **9**. (**B**) GC/MS analysis of TMS derivative of the ether extract of *E. lata* strain E125 grown on MYB; the asterisk denotes methyl ether of **2**.

It was also necessary to develop analytical methods suitable for determining the composition of metabolites and following the time−course of their production. Because the phytotoxic effects in grapevines are observed primarily on new growth removed from the initial point of infection, analysis was concentrated on metabolites excreted into the filtrate; mycelial mats were not analyzed but were extracted to obtain additional material for structural elucidation. Compounds **1** and **2** were found to be resolved on capillary GC, but there was some tailing of the peaks. Because the reported metabolites from *E. lata* were phenolic in nature (*6*, *7*), it appeared that these compounds would be more amenable to analysis as their trimethylsilyl derivatives. The derivatives were therefore prepared by treating samples with MSTFA, a reagent that generates only volatile byproducts and thus is suitable for on-column injection. This method proved to be generally applicable to all of the metabolites subsequently isolated, with the trimethylsilyl derivatives well-resolved (**Figure 2A**). In combination with a mass-selective detector (GC/MS), structural information could be deduced with regard to the number of derivatizable hydroxy groups, including phenolic, alcoholic, and carboxylic acid

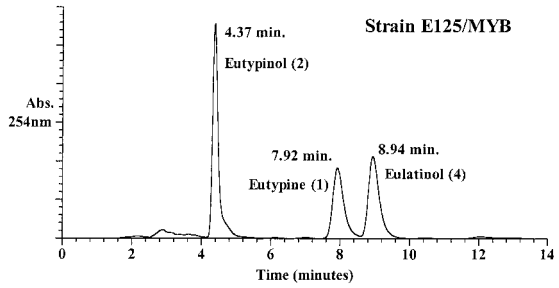

Figure 3. HPLC analysis of the ether extract of *E. lata* strain E125 grown on MYB.

moieties. The purity of isolated compounds was established by using this technique and also by thin-layer chromatography.

Analytical HPLC on a reversed-phase C18 column eluted with aqueous acetonitrile was also used for the detection of metabolites, with UV detection at 215 and 254 nm. The higher wavelength was expected to be optimal for the detection of all phenolic components, and the lower wavelength was monitored in order to ensure that any significant amounts of compounds lacking an aromatic ring would also be observed. The HPLC was amenable to preparative scale separations and was subsequently modified accordingly to enable the isolation of individual constituents from the fungal culture filtrate and also mycelium when necessary to obtain additional material.

The three *Eutypa* strains were inoculated on MYB medium, and the filtrate was extracted and analyzed for the presence of acetylenic phenols by HPLC monitored at 254 nm. Both E120 and E125 showed the presence of such metabolites, but none were detectable in strain E178, indicating that the latter oak-derived strain was significantly different from the two grape strains, in accord with previous genetic analysis (*13*). The effect of other growth media on metabolite composition was therefore not examined further with strain E178, but both E120 and E125 were also grown on PDB and analyzed under the same conditions as for the MYB samples. It was observed that globules of clear exudate appeared on the fungal mat and that these darkened with the age of the culture, eventually becoming dark brown or black. Samples of this exudate were collected by aspiration with a syringe for analysis by HPLC. It was also noted that with certain cultures the pH of the filtrate changed significantly over the time−course of the experiment and that with others the filtrate darkened considerably as growth progressed. The filtrate pH and transmittance at 400 nm were therefore measured at 6 day intervals in order to determine whether these observations could be correlated with the production of specific metabolites.

HPLC analysis (**Figure 3**) of the filtrate from strain E125 grown on MYB revealed the presence of **1** and **2**, metabolites previously reported to be produced by an unspecified strain of *E. lata* (*6*, *7*). However, some peaks observed by both HPLC and GC/MS (**Figure 2B**) could not be correlated with known compounds, and these needed to be isolated and identified in order to compare metabolite production between the E120 and the E125 strains. When grown on MYB, both strains produced the same unidentified compound as the primary metabolite, eluting at 8.94 min on analytical HPLC, following **1**, which eluted at 7.92 min (**Figure 3**). Preparative HPLC gave this compound as a colorless oil for which the HRMS established a molecular formula of $C_{12}H_{12}O_2$. Trimethylsilylation and GC/MS analysis indicated a mono-TMS derivative and displayed a molecular ion and base peak at *m*/*z* 260 and a major ion at *m*/*z* 245 corresponding to loss of a $-CH_3$ moiety from a TMS group, as typically observed for such derivatives. This suggested that the compound was a monophenol, lacking the aldehyde substituent present in **1**; we therefore named this new metabolite eulatinol.

The structure of eulatinol was established as the quinol monomethyl ether (**4**) by ^{1}H and ^{13}C NMR spectroscopy (**Table 1**). The presence of the pentenyne side chain and substitution pattern of the aromatic ring identical to that of **1** was immediately apparent. The presence of an aromatic methoxyl group was also established by signals at δ 3.83 and at δ 56.6 in the ^{1}H and ^{13}C spectra, respectively, leaving only the question of which of the two phenolic hydroxyl groups was methylated. This was unambiguously established by a long-range ^{1}H−^{13}C COSY experiment optimized for $^3J_{C,H}$. Specifically, the methoxyl protons showed three-bond coupling to the quaternary aromatic carbon at δ154.4. This carbon in turn showed three-bond couplings to the *o*,*m*- and *m*-coupled aromatic protons at δ 6.77 and δ 6.89, respectively. The location of the methoxyl group at the alternative (para-) aromatic carbon would have resulted in three-bond coupling of the aromatic ring carbon atom to only a single *o*-coupled proton. Similar arguments enabled the assignments of all proton and carbon resonances to be established unequivocally, as shown in **Table 1**.

In addition to **4**, both E120 and E125 strains grown on MYB produced moderate amounts of a metabolite that preceded **1** on analytical HPLC, eluting at 4.9 min. GC/MS analysis after trimethylsilylation of this compound showed a molecular ion at *m*/*z* 318, which was therefore indicative of a di-TMS derivative. Examination of the ^{1}H and ^{13}C NMR spectra established the structure as desmethyleulatinol. This is identical to a metabolite previously named ciccayne (**5**), first isolated from *Helminthosporium siccans* (*14*) and subsequently reported to occur in the marine basidiomycete *Halocyphina villosa* (*15*). The ^{1}H NMR spectrum was consistent with that reported for synthetic **5** (*16*, *17*); the complete assignments, including ^{13}C chemical shifts, are given in **Table 1**.

GC/MS analysis of the trimethylsilylated extract of the fungal filtrates indicated the presence of further constituents in trace amounts (**Figure 2B**). On the basis of their mass spectra, these were inferred to be the acid (**6**) (*6*), corresponding to **1**, the two epoxidized chromanones (**7** and **8**) previously reported (*7*), and the methyl ether derivative of **2**, with a retention time of 14.36 min. In addition, a minor compound was also observed as a small shoulder present on the major eutypine peak on analytical HPLC, and this was isolated by repeated preparative HPLC. The compound was obtained as an oil, with the molecular formula $C_{12}H_{14}O_2$, and a molecular ion at *m*/*z* 262 for the TMS derivative established the presence of one hydroxyl group and a second nonderivatizable oxygen atom. The ^{1}H NMR spectrum revealed signals for a pair of geminal methyl groups (δ 1.43), a hydroxymethyl singlet (δ 4.57), two olefinic protons (δ 5.62 and δ 6.31), and three aromatic protons, and the ^{13}C NMR spectrum was consistent with the presence of these moieties. ^{1}H−^{1}H and ^{1}H−^{13}C shift COSY experiments permitted assignments of the resonances and corroborated the structure as 2,2-dimethyl-6-hydroxymethylchromene (**9**). This compound has been reported to occur in desert plants of the genus *Encelia* (Asteraceae), but the ^{1}H NMR reported (*18*) showed significantly different shifts from those that we observed, and no other physical data, including ^{13}C NMR, were described. Whereas the values for protons in the chromene ring and the hydroxymethyl group were virtually identical, those observed for the two aromatic ring protons adjacent to the hydroxymethyl group were approximately 0.5 ppm upfield (δ 6.99 and δ 7.09) relative to the literature values (δ 7.52 and δ 7.65), respectively. It thus

Table 2. Metabolite Yields (μg/mL) in Filtrate of *E. lata* Strains Grown in MYB and PDB at 30 Days Growth Stage[a]

strain/media	1	2	4	5
E120/MYB	nd	nd	6.9	1.1
E120/PDB	nd	0.1	nd	nd
E125/MYB	7.8	16	22	nd (9.7)*
E125/PDB	9.0	78	nd	nd
E178/MYB	nd	nd	nd	nd

[a] Detection limit, 0.01 μg/mL; the asterisk denotes day 24.

appears that the structure originally reported (*18*) for the *Encelia* chromene may be incorrect, and the isomeric 7-hydroxymethyl compound is more probable. Our structure (**9**) is supported by the disposition of the hydroxymethyl group para to the chromene ring oxygen functionality, which is consistent with the structures of all other fungal metabolites occurring in *E. lata*, and it can be considered to be a transformation product of **2** in which the pentenyne chain is reduced and cyclized onto the adjacent phenol. This chromene proved to be surprisingly labile, rapidly darkening in color and giving rise to several as yet uncharacterized products, as evidenced by GC/MS. We were therefore unable to obtain additional long-range $^{13}C-^{1}H$ NMR data, which would have provided additional support for the proposed structure **9**. Further investigation of the chemical and biological properties of this compound and its transformation products, together with unequivocal structural identification, will require the isolation or synthesis of larger amounts of material. The synthesis of the chromene (**9**) as an intermediate in the preparation of biologically active chroman derivatives has been reported (*19*), but the physical properties of the compound were not described, and no spectroscopic data was provided in support of this structure.

Comparison of the metabolites of strains E120 and E125 grown on the two types of media showed significant differences. Standard response curves were determined for **1**, **2**, **4**, and **5** at 254 nm, and the separations were monitored by HPLC in order to quantitate each component. In general, the metabolic profile of E125 was considerably more complex than that of E120, especially when grown on MYB (**Table 2**). Four metabolites were produced in a significant quantity when E125 was grown on MYB, namely, **1**, **2**, **4**, and **5**, whereas E120 produced only **5** and **4**. In contrast, PDB was a much less productive growth medium in terms of metabolite diversity, with E125 producing only **2** and **1**, and E120 producing **2** as the sole metabolite. However, the highest level of any metabolite was **2** produced by E125 growing on PDB, which attained a maximum level of 78 μg/mL, whereas E120 produced only 0.2 μg/mL of the same compound. When grown on MYB, **4** was also produced in significant quantities, amounting to 22 and 6.9 μg/mL from E125 and E120, respectively. In contrast, **1** was not a major metabolite relative to **2** and **4**; it was produced only by E125 in concentrations of 7.8 and 9.0 μg/mL when grown on MYB and PDB, respectively.

The time−course for the production of **1**, **2**, **4**, and **5** was studied by growing the *Eutypa* strains on both media and analyzing the filtrate by HPLC, with monitoring at 254 nm, at 6 day intervals until day 30 and then at 8 day intervals until day 48 (**Figure 4**). All four of these compounds exhibit absorption maxima close to this wavelength, with **2**, **5**, and **4** having very similar UV spectra (**Figure 5**). The UV spectrum of **1** showed a significantly higher absorbance in this region, as expected, due to the increased conjugation of the aldehyde substituent with the phenolic ring. Most of the fungal filtrates showed maximum metabolite accumulation around day 30, with

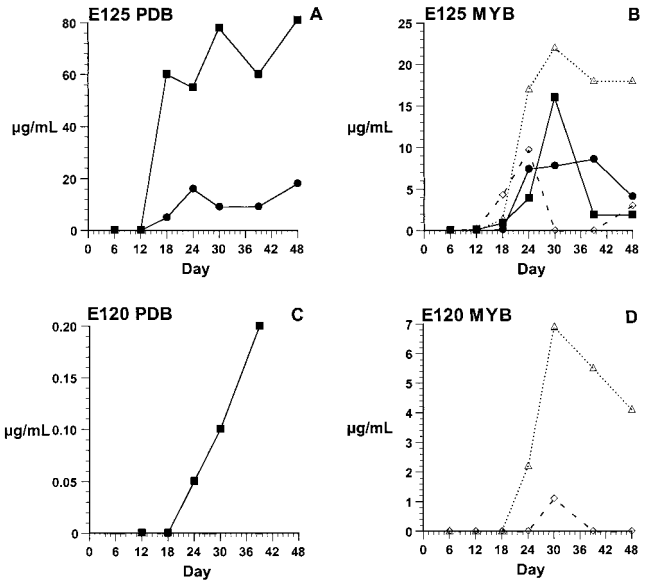

Figure 4. Time−course for metabolite production from *E. lata* strains grown on two different media over 48 days. (**A**) Strain E125 on PDB; (**B**) strain E125 on MYB; (**C**) strain E120 on PDB; and (**D**) strain E120 on MYB. Symbols: ●, **1**; ■, **2**; △, **4**; ◇, **5**.

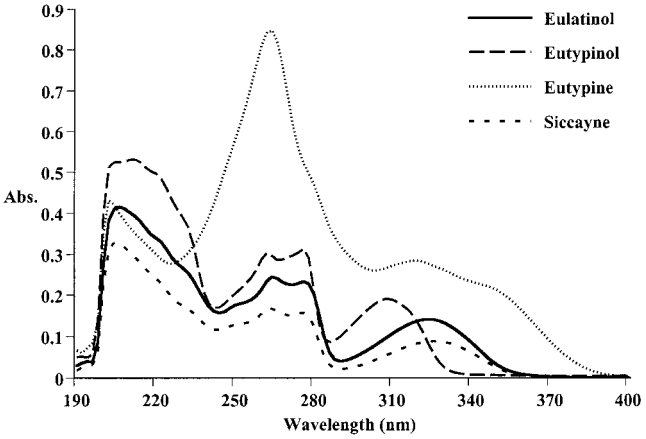

Figure 5. UV spectra of **1** (2.58×10^{-5} M), **2** (2.13×10^{-5} M), **4** (2.13×10^{-5} M), and **5** (2.30×10^{-5} M) in MeOH solution.

some decline thereafter. It was not immediately apparent whether this was due to the instability of metabolites, catabolism, or transformation into other compounds further along the biosynthetic pathway. However, for E125 growing on MYB, **5** attained a maximum concentration of 9.7 μg/mL at day 24 and declined to negligible amounts by day 30, whereas **4** increased to a maximum of 22 μg/mL at day 30 (**Figure 4**). This supports the reasonable hypothesis that **4** may be formed biosynthetically by the monomethylation of **5**. In contrast to this, there was no obvious correlation between the production of **2** and **1**, both of which appeared to increase in concert until about day 30, although these two metabolites could be visualized as being biosynthetically interrelated, through either oxidation of the former or reduction of the latter. It is apparent from these experiments that to culture *E. lata* in order to obtain significant quantities of specific metabolites for bioassay experiments, the particular strain and culture medium must be selected appropriately. Thus, **2**, and to a lesser extent **1**, can be most effectively obtained from E125 grown on PDB, whereas **4** can be procured most easily from either E120 or E125 grown on MYB. The isolation of **5** from E125 grown on MYB necessitates separation from the other three primary metabolites by prepara-

tive HPLC. In all cases, optimal yields from the filtrate would be obtained from cultures grown for a 24−30 day time period.

In addition to metabolite production, other factors associated with fungal growth in culture over a 48 day time period were investigated. The fungal mat dry weight was found to attain a maximum at 24−30 days and subsequently decline slightly under all growth conditions, except for E178 on MYB, which continued to increase up to day 48, the limit of the experiment. Fungal mat production was approximately twice as great for the other two strains when grown on MYB as on PDB, but this did not appear to correlate with metabolite yield since the highest levels were produced by E125 on PDB. It was also hypothesized that the observation that some culture filtrates darkened significantly during growth might correlate with the presence of the quinol **5** and its consequent oxidation to a highly colored quinone. This was investigated by measuring the percentage transmittance at 400 nm. The three strains grown on MYB showed very little visible darkening or measurable decline in transmittance throughout growth. However, the two strains grown on PDB darkened significantly, with the final transmittance approximately 50% of that at the start of the experiment. Because **5** is produced only in cultures on MYB and not on PDB, the darkening phenomenon does not appear to be associated with this compound, as initially suspected. However, the production of phenolic metabolites does seem to have some relationship with filtrate darkening, since E120 and E125 cultures all showed reduced transmittance with growth, whereas E178 filtrate actually increased in transmittance with age, although correlation with the presence of specific metabolites is not obvious.

Appreciable changes in pH were observed with the different strains and growth media. Cultures of E120 and E125 grown on PDB started at pH 5 and remained essentially unchanged throughout the 48 day growth period. All of the strains grown on MYB were initially at pH 6, but whereas the E178 culture rose only to pH 7, E120 and E125 increased rapidly after day 18 to pH 7.5 at day 24 and then more slowly to pH 8−8.5 at day 48. These changes may have a considerable significance in relation to the comparative hydrophilicity and lipophilicity of the primary metabolites of *E. lata*. All of these compounds are phenolic weak acids and as such are likely to remain unionized in acidic solutions and therefore more soluble in lipid materials. In contrast, at pH 8−8.5, the phenolic hydroxyl group would be ionized, and the metabolites would therefore become much more water-soluble. Deswarte et al. (*10*) have established the pK_a of **1** as 6.21, and **1** would therefore be 50% ionized at pH 6.2 and 90% ionized at pH 7.2, thus becoming readily soluble in water. These properties have been confirmed by the determination of the octanol/water partition coefficients for the ionized and unionized forms of **1** as 14 at pH 8.4 and 86 at pH 5.7, respectively (*10*). It is probable that the solubility properties of all of the other phenolic metabolites produced by *E. lata* are similarly pH-dependent. In contrast, the solubility properties of **9**, with no free phenolic group, should be pH-independent, a situation that also applies to **3**, the cyclization product readily formed from **1** under acidic conditions.

With respect to the potential phytotoxicity of **9** and the benzofuran (**3**), it is interesting to note that two acetylchromenes, encecalin (**10**) and demethylencecalin (**11**), isolated from the roots of *Helianthella quinquenervis*, have been shown to inhibit radicle growth of *Amaranthus hypochondriacus* (Prince's feather) and *Echinochloa crusgalli* (barnyard grass) (*20*); structural similarities to **9** suggest that the latter might also exhibit phytotoxicity. These chromenes and euparin (**12**) (*20*), a benzofuran structurally analogous to the cyclization product (**3**) of **1**, variously inhibited photophosphorylation and electron transport in isolated spinach chloroplasts (*21*), demonstrating that such oxygen heterocyclic compounds are capable of interfering with fundamental metabolic processes in plants.

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 polymerase chain reaction assay for the fungus in grapevine wood has been developed (*22*), it does not distinguish between pathogenic and nonpathogenic strains, nor does it provide a measure of the degree of virulence. The diversity of metabolites produced by the limited number of *Eutypa* strains examined in this study, their heterogeneity with growth media and time, the potential for structural transformation within the grape plant matrix, and our inability to confirm the phytotoxicity of **1** in a grape plantlet bioassay suggest that phytotoxicity may be a consequence of a suite of compounds rather than **1** alone. Identification of the complete range of metabolites produced by the analysis of a larger number of *E. lata* strains, 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. Appropriate management techniques can then be devised to limit the spread of the most virulent strains and ensure the continued productivity of individual vineyards.

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Supporting Information Available: Change in fungal mat dry weight, pH of medium, and UV transmittance at 400 nm of medium with *Eutypa* strain and growth stage. This material is available free of charge via the Internet at http://pubs.acs.org

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