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Phenolic and heterocyclic metabolite profiles of the grapevine pathogen *Eutypa lata*

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Dedicated to memory of Professor Jeffrey B. Harborne

Abstract

The ascomycete *Eutypa lata* is the causative agent of eutypa dieback in grapevines, a serious economic problem in major wine grape producing areas. In order to develop a predictive, non-destructive assay for early detection of fungal infection, the phenolic metabolite profiles of 11 strains of *E. lata* grown on four different artificial growth media were analyzed by HPLC and their variability compared with growth on Cabernet Sauvignon grapevine wood and wood extracts. Six compounds were generally produced in significant amounts, namely eutypinol, eulatachromene, and eutypine and its benzofuran cyclization product, together with siccayne and eulatinol. The two most widely distributed and abundant metabolites were eutypinol and eulatachromene, which were present in 8 of the strains grown on grapewood aqueous extract fortified with sucrose. Metabolite production on grapevine extract was greatly enhanced relative to the artificial media, indicating that this native substrate provides optimal conditions and a more representative profile of the metabolites produced in the natural disease state. The primary metabolites were tested in a grapeleaf disc bioassay to establish their relative toxicity. Neither eutypinol nor siccayne were phytotoxic; eulatachromene, eulatinol, eutypine, and the benzofuran exhibited necrotic effects in the bioassay. The results indicate that eutypa dieback may be caused by several *E. lata* metabolites rather than a single compound.
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Keywords: *Vitis vinifera*; Vitaceae; Grapes; *Eutypa lata*; Dying-arm disease; Dieback; Eutyposis; Vine decline; Bioassay

1. Introduction

Many major grape-producing areas around the world, including California and Australia, suffer serious economic losses from a fungal infection affecting grapevines (*Vitis vinifera*; Vitaceae) known as eutypa dieback or "dying arm disease. The problem is also known as "eutyposis" after the ascomycete *Eutypa lata* (formerly *E. armeniacae*), which was identified as the causative agent by Moller and Kasimatis (1978). Symptoms of the disease include stunting of new shoots, with small, cupped, chlorotic and tattered leaves, meager development of fruit clusters, and characteristic dark, wedge-shaped necrosis of the trunk and cordons (Fig. 1A and B). Progression of the disease over a period of years results in decreased yields and reduced longevity of the grapevines. Grape cultivars show significant differences in susceptibility to infection, with some of the most valuable, including Cabernet Sauvignon, being particularly sensitive (Munkvold and Marois, 1995). Yield decreases in California for infected Chenin blanc and French Columbard grapes were estimated to range from 30 to 60%, with vineyards over 20 years old having up to 83% reduction, relative to their peak productivity (Munkvold et al., 1994) while in Australia, yield losses

Abbreviations: PCR, polymerase chain reaction; RAPD, Random amplification of polymorphic deoxyribonucleic acid; RFLP, Restriction-fragment length polymorphism.

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for Shiraz and Cabernet Sauvignon vines have been reported as 850 and 740 kg/ha, respectively (Wicks and Davis, 1999). The cost to wine grape production in California has been estimated to be in excess of $260 million per annum, due to decreased yields, increased vineyard management costs and shortened life span of the vines (Siebert, 2000).

E. lata has been isolated from numerous woody species, including grapevines, almond, apricot, cherry, olive, peach and walnut (Carter et al., 1983; Munkvold and Marois, 1994), and from a wide range of non-cultivated hosts (Carter, 1991). The fungus enters the grapevine through pruning wounds, and colonizes the woody tissues. The foliar symptoms may therefore be due to toxins produced by the fungus in the wood and translocated to the shoots. Since foliar symptoms do not become visible until several years after infection, non-destructive procedures for early prediction and removal of infected vines are urgently needed. A PCR-based assay for detecting the fungus in grapevine wood has been developed (Lecomte et al., 2000), but this does not distinguish between pathogenic and non-pathogenic strains, nor provide a measure of the degree of virulence. Methodology based upon the detection in grapevine tissues of the fungal metabolites responsible for phytotoxicity therefore appears to be the best approach for rapid, early diagnosis of the disease.

(a)

(b)

Fig. 1. (A) Symptoms of eutypa dieback in new shoots of grapevine; (B) Necrosis of grapevine trunk wood caused by *Eutypa lata*.

Examination of a laboratory culture of an unspecified strain of the fungus by Renaud et al. (1989a,b) led to the identification of several phenolic compounds bearing a pentenyne side chain *ortho*- to the hydroxyl group, including the benzylic alcohol, eutypinol (**1**), the corresponding aldehyde, eutypine (**3**) and its cyclization product, 2-*iso*propenyl-5-formylbenzofuran (**4**) (Fig. 2), together with highly modified compounds in which the aromatic ring is reduced and the side chain cyclized. Eutypine **3** was postulated as the constituent phytotoxic to grapevines on the basis of in vitro experiments (Tey-Rulh et al., 1991) and was shown to be a protonophoric agent, uncoupling mitochondrial oxidative phosphorylation (Deswarte et al.,1996a,b; Fallot et al., 1997), and acting on chloroplasts (Deswarte et al., 1994) and the plasma membrane (Amborabé et al., 2001). The ability to reduce eutypine **3** to eutypinol **1**, which was not

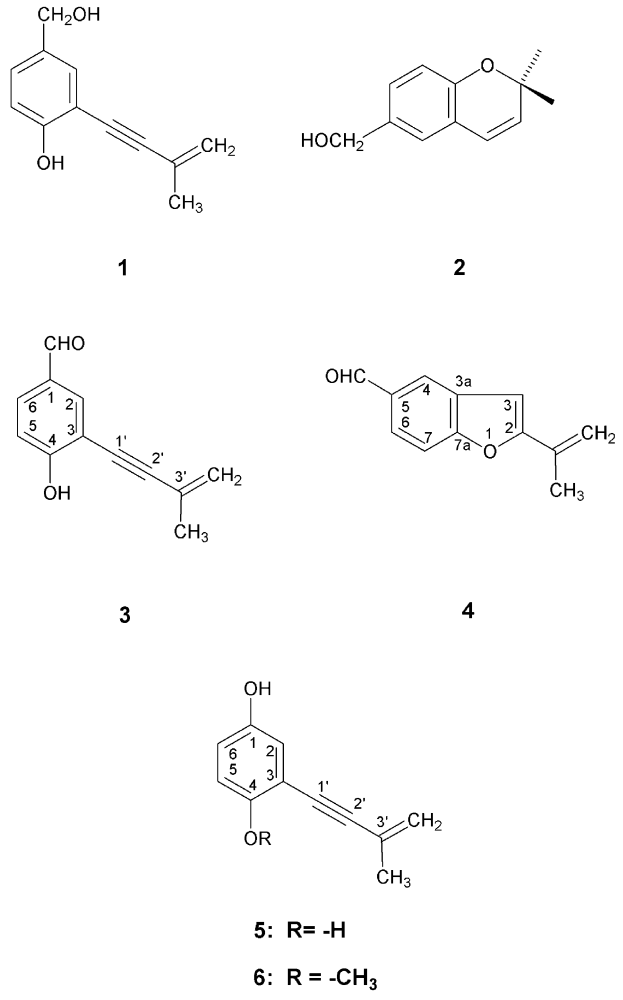

Fig. 2. Chemical structures of *Eutypa lata* metabolites: eutypinol (**1**); eulatachromene (**2**); eutypine (**3**); 2-*iso*propenyl-5-formylbenzofuran (**4**); siccayne (**5**); eulatinol (**6**).

phytotoxic to grape callus tissue in vitro, has been proposed as an explanation for the tolerance of some cultivars to the disease (Guillen et al., 1998). More recently, a comparison of metabolites produced by Californian and Italian strains on malt yeast broth (MYB) and potato dextrose broth (PDB) has identified eulatachromene (**2**), a cyclic analog of eutypinol, and siccayne (**5**) together with its *O*-methyl analog, eulatinol (**6**), as new constituents of *E. lata* (Molyneux et al., 2002); furthermore, eutypine **3** was not produced by the Californian strain even though it has been implicated as being pathogenic to grapevines.

A preliminary structure–activity study (Smith et al., 2003) of synthetic *E. lata* metabolites and analogues, using an in vitro bioassay based on necrosis of grape leaf disks, showed that some phenolic metabolites (**1** and **5**) had no phytotoxic activity, and that eutypine (**3**) had reduced phytotoxic activity compared with the cyclic compounds eulatachromene (**2**) and 2-*iso*propenyl-5-formylbenzofuran (**4**). The variety of metabolites biosynthesized by the fungus, and conflicting information as to those responsible for dieback required that more fundamental information be acquired prior to the use of metabolite detection in grapevines as a diagnostic tool for the disease. This investigation was therefore designed to establish the fungal growth conditions best suited to metabolite production using a number of different strains, especially when grown on grapevine wood and wood extracts, and to identify those metabolites most representative as indicators of fungal infection.

2. Results and discussion

Eleven strains of *E. lata* were chosen for examination, eight of which were originally isolated from grapevines in Australia, California, France, Italy and New Zealand (Table 1). Two strains were from apricot (*Prunus armeniaca*) in Australia and one from Valley oak (*Quercus lobata*) in California. Four of the isolates, E1, E120, E125, and E178, the first from apricot in Australia, the second and third from grape in California and Italy, respectively, and the fourth from oak, have been subjected to genetic analysis using amplified fragment-length polymorphism (AFLP) and the ribosomal DNA (rDNA) internal transcribed spacer (ITS) region (DeScenzo et al., 1999). Although the strains were widely separated geographically, the first three were closely affiliated within a large monophyletic group of 104 strains. In contrast, the oak isolate (E178) was a member of a small, genetically distinct group consisting of four isolates from Valley oak, three isolates from madrone (*Arbutus menziesii*), and only one from grape. This isolate was included as representative of an isolate from a native plant species, with the expectation that its metabolic profile might differ significantly from strains present on the major cultivated plant hosts, grape and apricot, and thus serve as an outlier. The remaining isolates examined in this study had not been genetically screened at the commencement of our experiments on metabolite production.

In an attempt to establish the most productive growth medium for metabolites, all of the *E. lata* strains were initially grown in stationary culture on four artificial liquid media, the defined Vogel's medium (Vogel, 1964; Davis and de Serres, 1970) and that of Pezet (1983), and the complex potato dextrose broth (PDB) and malt/yeast extract broth (MYB). Culture filtrates were extracted after 3–4 week incubation periods, previously shown to be optimal for metabolite production (Molyneux et al., 2002) and the range and amounts of metabolites analyzed by HPLC on a reversed phase column. The standards **1–6** showed baseline resolution with retention times from 16.4–24.3 min (Fig. 3A). Calibration curves were constructed for these major metabolites, either isolated from fungal filtrate (Molyneux et al., 2002) or prepared synthetically (Smith et al., 2003) and characterized by spectroscopic properties and GC–MS, and were linear over a range of 0.2–2.0 μg/20 μl injection. Identity of the peaks was confirmed by retention times and comparison of the UV spectra obtained by photodiode array detection; these spectra provided characteristic distinction between the alcohol, aldehyde, quinol and chromene structural types.

The metabolites identified and their yields are shown in Table 1. It is apparent that the various media supported production of significantly different levels of metabolites and also different profiles, even for the same isolate. In general, the greatest diversity of metabolic products was produced on MYB and Pezet's medium, although the specific compounds varied greatly with fungal strain. In contrast, only three metabolites were produced on PDB (**1**, **5**, and **6**), and Vogel's medium (**1**, **3**, and **6**), respectively, with occasional occurrence of the chromene, **2**. Two of the metabolites from the PDB filtrate, siccayne, **5** and eulatinol, **6**, are obviously structurally related quinols, but this correlation did not extend to the Vogel's medium, which contained no **5**, even though **6** was a common constituent. In fact, siccayne **5** was consistently produced only on PDB, whereas eulatinol **6** was produced by most isolates on all four artificial media. This is somewhat surprising, since **6** would be expected to be produced biosynthetically by methylation of **5**.

The highest amount of metabolites was produced by strain M279, isolated from apricot in Australia, grown on Pezet's medium, which produced 14.7 μg/ml eutypinol (**1**), 1.3 μg/ml eulatachromene (**2**), 0.5 μg/ml eutypine (**3**) and 5.6 μg/ml eulatinol (**6**), together with unidentified peaks that may correspond to *O*-methyl derivatives of eutypinol **1** or eutypine **3**, based on their

Table 1
Metabolite profiles in filtrate of *Eutypa lata* strains grown in four artificial media

Media/metabolite	Metabolite yields (μg/ml) for fungal isolate/source										
	E1 apricot Australia	E120 grape California	E125 grape Italy	E178 oak California	M266 grape France	M279 apricot Australia	M280 grape Australia	M355 grape Australia	NO4 grape Australia	SS1#1 grape New Zealand	SS6 grape Australia
Malt yeast broth (MYB)											
Eutypinol **1**	0.3	0.1	–	–	0.3	0.1	0.1	–	0.1	–	–
Chromene **2**	0.2	–	0.2	0.1	–	–	0.1	–	–	0.1	–
Eutypine **3**	2.5	tr	0.1	tr	3.9	tr	tr	tr	tr	tr	–
Benzofuran **4**	0.1	–	tr	–	0.2	–	–	–	–	–	–
Siccayne **5**	–	0.1	–	–	–	–	0.1	–	–	–	–
Eulatinol **6**	1.1	1.7	2.4	0.2	0.1	–	1.6	0.1	0.1	tr	0.2
Potato dextrose broth (PDB)											
Eutypinol **1**	0.2	–	0.9	–	0.2	0.1	0.1	0.1	0.2	tr	0.1
Chromene **2**	–	–	0.2	–	0.1	–	–	–	–	–	–
Eutypine **3**	–	–	–	–	–	–	–	–	–	–	–
Benzofuran **4**	–	–	–	–	–	–	–	–	–	–	–
Siccayne **5**	0.1	0.2	2.0	tr	0.3	tr	0.3	–	0.1	–	tr
Eulatinol **6**	0.1	0.1	0.1	tr	–	0.1	tr	–	0.1	–	tr
Pezet's											
Eutypinol **1**	0.8	0.1	0.2	–	0.7	14.7	0.4	0.2	1.2	0.2	1.3
Chromene **2**	–	–	0.1	–	–	1.3	0.7	–	0.1	0.1	0.4
Eutypine **3**	tr	–	0.1	–	tr	0.5	tr	–	0.1	tr	0.1
Benzofuran **4**	–	–	–	–	–	–	–	–	–	–	–
Siccayne **5**	–	–	–	–	–	0.3	–	–	0.1	tr	tr
Eulatinol **6**	–	–	–	–	tr	5.6	0.1	0.1	0.8	0.1	0.1
Vogel's											
Eutypinol **1**	0.8	0.1	0.2	tr	2.2	0.1	0.1	tr	0.1	tr	tr
Chromene **2**	0.1	–	–	–	0.6	0.1	–	–	–	–	–
Eutypine **3**	0.3	tr	0.1	–	0.3	–	tr	–	tr	–	–
Benzofuran **4**	–	–	–	–	–	–	–	–	–	–	–
Siccayne **5**	–	–	–	–	–	–	–	–	–	–	–
Eulatinol **6**	0.4	1.7	0.2	–	0.1	0.1	2.0	–	tr	–	0.1

– = None detected. tr = < 0.05-detection limit. Detection limits (μg/ml) at 254 nm: **1**, 0.03; **2**, 0.05; **3**, 0.01; **4**, 0.005; **5**, 0.01; **6**, 0.01.

similarities in UV spectra to the latter compounds (Fig. 3B). Surprisingly, all other strains produced less than 0.1 μg/ml of **3** on Pezet's medium, even though this was the medium used for the initial isolation of the compound, although neither its yield or strain of *E. lata* used was specified (Renaud et al., 1989a). Eutypine **3** was produced in significant amounts, 2.5 and 3.9 μg/ml, respectively, only by strains E1 and M266 grown on MYB, accompanied by small amounts of its cyclization product **4**, and was not produced by any of the 11 strains grown on PDB. The benzofuran **4** was not observed for any of the strains grown on PDB, Pezet's or Vogel's media. However, samples that initially contained eutypine **3** showed extensive conversion into the benzofuran **4** on re-analysis, even though the sample had been evaporated to dryness and stored at 0 °C. This confirms our previous observation that eutypine **3** is quite labile and undergoes facile cyclization even when not in solution (Smith et al., 2003). Eutypine **3** was originally proposed as the phytotoxic metabolite of *E. lata* but the low levels produced by most of the strains examined, even on different media, indicate that other metabolites may be involved. Furthermore, we have shown by measurement of chlorophyll reduction in a grapeleaf disk bioassay, that eutypine **3** is approximately 50% less active than the chromene **2** and the benzofuran **4** when tested at a concentration of 50 μg/ml (Smith et al., 2003), suggesting that the latter are more likely to be responsible for the activity.

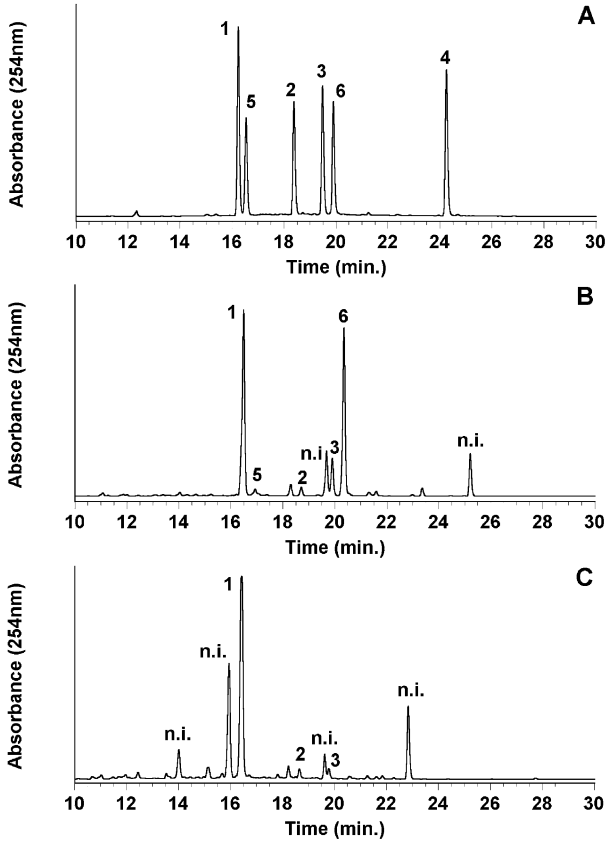

Fig. 3. HPLC analysis of *E. lata* metabolites. (A) Standards **1**–**6**; (B) culture filtrate of isolate M279 grown on Pezet's medium; (C) culture filtrate of isolate E125 grown on grapevine cane aqueous extract + 1% sucrose. n.i. = unidentified metabolites.

The oak isolate, E178, showed little or no metabolite production on any of the four artificial media examined and two grape isolates, M335 from Australia and SS1#1 from New Zealand, showed a similar lack of significant amounts of metabolites. It is therefore probable that not all strains of *E. lata* are capable of inducing foliar symptoms in grapevines and detection of the presence of the fungus is not sufficient evidence to indicate potential for expression of the disease.

In view of the great variability in metabolite production with both media and isolate, it was decided to investigate the effect of growing the fungi on media which more closely approximated the natural situation. The experiments were therefore repeated with inoculation of either finely ground Cabernet Sauvignon cane material or a hot water extract of the cane. Because symptoms of eutypa dieback correlate closely with the Spring flush of growth in the grapevines when sap is freely flowing, the inoculations were also conducted with the same media amended with 1% sucrose. The results of HPLC analysis are shown in Table 2.

The metabolite profiles produced during growth of the fungus on these "natural" media were in marked contrast to those on artificial media, with much more consistent production between strains for each type of medium. The ground cane and cane hot water extracts without added sucrose were relatively non-productive, eutypinol (**1**) being the only metabolite produced at levels above 1 μg/ml, by grape isolates M266 and NO4. Siccayne (**5**) and eulatinol (**6**) were either not produced at all or only at extremely low levels. This was markedly different from the artificial media, on which certain of the strains produced significant amounts, especially of **6**, suggesting that the constituents of grape cane do not favor biosynthesis of the quinol metabolites or else suppress their formation. Previous isolations of siccayne (**5**) from the fungus *Helminthosporum siccans* (Ishibashi et al., 1968) and the marine basidiomycete *Halocyphina villosa* (Kupka et al., 1981) did not report the co-occurrence of acetylenic monophenol metabolites of the eutypinol or eutypine structural type, indicating that the phenol and quinol classes arise from a branching of the biosynthetic pathway.

In contrast, the two sucrose-amended, cane-derived media were especially high in yields of metabolites but these were confined exclusively to eutypinol (**1**), eulatachromene (**2**) and eutypine (**3**); the other three known metabolites were not formed, including the benzofuran (**4**), even when the level of eutypine (**3**) was reasonably high. Unidentified metabolites were also produced, which appeared to be methyl ether derivatives of eutypinol **1** or

Table 2
Metabolite profiles in filtrate of *Eutypa lata* strains grown in "natural" grapevine media with and without sucrose

Media/metabolite	Metabolite yields (μg/ml) for fungal isolate/source										
	E1 apricot Australia	E120 grape California	E125 grape Italy	E178 oak California	M266 grape France	M279 apricot Australia	M280 grape Australia	M355 grape Australia	NO4 grape Australia	SS1#1 grape New Zealand	SS6 grape Australia
Cane hot water extract + 1% sucrose											
Eutypinol **1**	24.7	22.3	29.2	–	30.2	23.1	30.2	0.2	27.5	–	4.7
Chromene **2**	1.5	2.0	2.4	–	0.7	2.8	0.7	–	3.7	–	0.3
Eutypine **3**	0.2	0.1	0.2	–	0.3	5.0	0.8	–	0.1	–	0.2
Benzofuran **4**	–	–	–	–	–	tr	–	–	–	–	–
Siccayne **5**	–	–	–	–	–	–	–	–	–	–	–
Eulatinol **6**	–	–	–	–	–	–	–	–	–	–	–
Cane hot water extract/no sucrose											
Eutypinol **1**	0.9	–	0.2	–	0.1	0.2	0.2	–	1.1	–	–
Chromene **2**	–	–	–	–	–	–	–	–	0.5	–	–
Eutypine **3**	tr	–	–	–	–	–	–	–	tr	–	–
Benzofuran **4**	–	–	–	–	–	–	–	–	–	–	–
Siccayne **5**	0.1	0.1	0.1	–	tr	tr	0.1	–	0.1	–	tr
Eulatinol **6**	0.1	–	–	–	–	–	–	–	–	–	–
Ground cane + 1% sucrose											
Eutypinol **1**	1.1	5.1	12.8	–	21.1	0.1	16.3	–	3.0	–	–
Chromene **2**	0.2	0.1	0.5	–	–	–	0.3	–	0.1	–	–
Eutypine **3**	0.1	0.1	0.1	–	0.1	–	0.1	–	tr	–	–
Benzofuran **4**	–	–	–	–	–	–	–	–	–	–	–
Siccayne **5**	–	–	–	–	–	–	–	–	–	–	–
Eulatinol **6**	–	–	–	–	–	–	–	–	–	–	–
Ground cane/no sucrose											
Eutypinol **1**	0.2	0.1	1.2	–	2.8	–	0.4	–	0.5	–	–
Chromene **2**	–	–	0.1	–	–	–	0.1	–	–	–	–
Eutypine **3**	tr	tr	0.1	–	tr	–	tr	–	tr	–	–
Benzofuran **4**	–	–	–	–	–	–	–	–	–	–	–
Siccayne **5**	–	–	–	–	–	–	–	–	–	–	–
Eulatinol **6**	–	–	–	–	–	–	–	–	–	–	–

– = none detected. tr = < 0.05-detection limit. Detection limits (μg/ml) at 254 nm: **1**, 0.03; **2**, 0.05; **3**, 0.01; **4**, 0.005; **5**, 0.01; **6**, 0.01.

siccayne **5** from the similarities in their UV spectra to the latter compounds; attempts will be made to isolate and elucidate the structures of these constituents from large scale cultures on cane extract medium. The levels of **1** were much higher than those of **2** and **3**, amounting to ca. 30 μg/ml for the grape isolates E125 (Fig. 3C), M266 and M280, from Italy, France and Australia, respectively. As with the artificial media, oak isolate E178 and grape isolates M355 and SS1#1 produced essentially no metabolites. All of the other apricot and grape isolates, except for SS6 from Australia, produced **1** in excess of 20 μg/ml. The second most abundant metabolite was eulatachromene (**2**) with levels generally on the order of 1–4 μg/ml, far in excess of eutypine (**3**) with levels from 0.1 to 0.8 μg/ml. This trend is illustrated in Fig. 4, the only major exception being apricot isolate M279 with 5.0 μg/ml of **3** compared to 2.8 μg/ml of **2**. Previous results have shown that **2** is more phytotoxic than **3**, based on measurement of chlorophyll loss in a grape leaf disk bioassay (Smith et al., 2003). The relative activity at 50 μg/ml for the primary metabolites **1**–**6** in the current study are illustrated in Fig. 5. It is noteworthy that the most active metabolites are eulatachromene (**2**) and the benzofuran (**4**), rather than the acetylenic phenols. Castañeda et al. (1996, 1998) have shown that the acetylchromenes, encecalin and demethylencecalin, and the benzofuran, euparin, isolated from the roots of *Helianthella quinquenervis,* suppress radicle growth of Prince's feather (*Amaranthus hypochondriacus*) and barnyard grass (*Echinochloa crusgalli*) and inhibit photophosphorylation and electron transport in isolated spinach chloroplasts. In addition, the benzofuran (**4**) is the dehydroderivative of fommanoxin [(±)-5-formyl-2-isopropenyl-2,3-dihydrobenzofuran], a metabolite of the basidiomycete *Heterobasidion annosum*, a common wood rot fungus affecting conifers. Fommanoxin has been shown to inhibit protein biosynthesis in *Picea abies* and *Nicotiana tabacum* protoplasts (Sonnenbichler et al., 1989) and cause growth retardation in *Pinus sylvestris* (Hirotani et al., 1977). The compound has been isolated from the stained stem heartwood zone of affected Sitka spruce trees and is toxic to germinating seeds and young seedlings (Heslin et al., 1983). The structural analogies of these compounds with the cyclic *E. lata* metabolites **2** and **4**, suggests that the latter may have a similar mode of action.

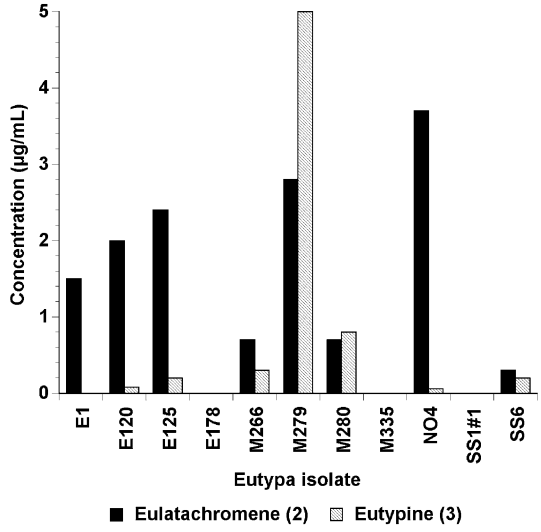

Fig. 4. Comparison of eulatachromene (**2**) and eutypine (**3**) yields for 11 *Eutypa lata* isolates grown on grapevine cane aqueous extract + 1% sucrose.

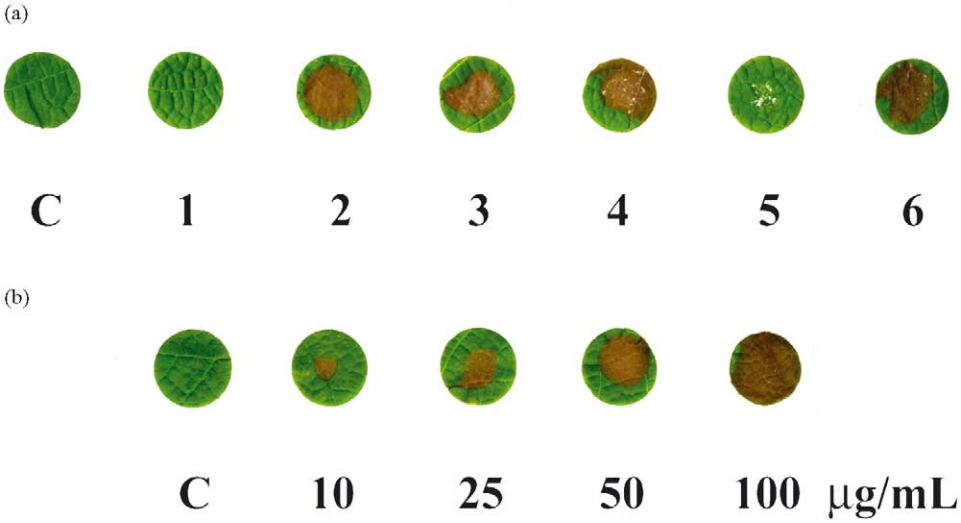

Fig. 5. (A) Comparison of phytotoxicity at 24 h at 50 μg/ml for methanol control (**C**) and the primary metabolites **1–6** in grapeleaf bioassay; (B) comparison of phytotoxicity at 24 h for methanol control (**C**) and eulatachromene (**2**) at 10, 25, 50 and 100 μg/ml.

3. Conclusions

The great variability in metabolite profiles and yields indicates that isolates cultured on artificial media are not useful for establishing the likelihood of any specific *E. lata* isolate producing phytotoxic metabolites in a field situation. However, grape cane hot water extract produced consistent profiles and high levels of metabolites in a medium that best approximates under laboratory conditions the environment available to the fungus during expression of eutypa dieback in planta. We therefore intend to concentrate on use of this medium in the future for examination of additional isolates and to evaluate comparative resistance of different grape cultivars to the disease.

The consistent pattern of metabolites observed for *E. lata* isolates from different plant hosts and locations indicates that these isolates are substantially the same and confirms that eutyposis, as defined on the basis of symptoms, is not a different disease in different geographical areas of the world. Nevertheless, the essential absence of metabolites from isolates E178, M355 and SS1#1 demonstrates that not all *E. lata* strains are likely to be capable of producing characteristic foliar symptoms. This is supported by preliminary results from genetic analysis of the 11 isolates, showing that 9 of them had similar profiles when screened with 10 RAPD primers but that E178 and SS1#1 were consistently different from the rest and from each other; similar results were obtained with RFLP analysis (R. Lardner, unpublished results). Examination of asci produced by SS1#1 has confirmed that it is not *E. lata* (P. Long, personal communication), whereas E178 was previously shown to differ from the majority of *E. lata* strains examined, using AFLP and ITS sequence analysis (DeScenzo et al., 1999). As a consequence, mere detection of the fungus in a grapevine is not sufficient diagnostic evidence for the disease and chemical methods for detection of metabolites are likely to be more suitable. The higher levels produced in grapevine medium, and greater activity of eulatachromene (**2**) than eutypine (**3**) in our bioassay, suggests that the former should be the better diagnostic constituent. However, the much higher levels of eutypinol (**1**) than any other metabolites for all of the producing isolates examined in this study indicates that this compound would be most readily detected in infected tissue, even though bioassay results show that it lacks phytotoxicity. It must be recognized that inactive **1** could serve as a precursor for transformation into active **2** in plant tissue through a simple reduction of the acetylenic bond of the side chain and subsequent cyclization. Future experiments will focus on detection of specific metabolites present in emerging shoots at a time when signs of infection and disease expression are noticeable, and pathogenicity testing of isolates of *E. lata*.

4. Experimental

4.1. Reagents

Pyridine (Fisher Scientific) was dried by storage over molecular sieves, Type 4A, 8-12 mesh (Aldrich Chemical Co.). *N*-Methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) in 1 ml ampules was obtained from Pierce (Rockford, IL).

4.2. General

UV spectra were measured at 190–500 nm in MeOH solution, using a Hewlett-Packard 8452A diode array spectrophotometer. GC–MS analyses were performed on a Hewlett-Packard 5890 Series II instrument equipped with a 5971 mass-selective detector (MSD). HPLC analyses were run on an Agilent 1100 system with detection either by UV at 254 nm (Agilent 1100 variable wavelength detector) or photodioarray at 190–400 nm (Agilent 1100 diode array detector).

4.3. Microorganisms and culture conditions

The fungal isolates were obtained from the E. & J. Gallo Winery Culture Collection (Modesto, CA) (E1, E120, E125, E178); from Drs. Maurice Carter (SS6), Mary Cole (M280, M335, M266, M279) and Peter Long (SS1#1) (Australia); and a collection by R. Lardner (N04). *E. lata* isolates were grown on potato dextrose agar (Difco) and cut into plugs for use as inoculum in all tested media.

4.3.1. Preparation of artificial media

Tested media comprised 2 complex and 2 defined media. Complex media were potato dextrose broth (PDB, Difco) and malt extract/yeast extract (MYB;10 and 20 g/l, respectively; Difco). Defined media were Vogel's Media N (VMN) and Pezet's. VMN consisted of the following nutrients per liter: glucose, 10 g; sodium citrate.$5H_2O$, 3 g; KH_2PO_4, 5 g; NH_4NO_3, 2 g; $MgSO_4.7H_2O$, 0.2 g; $CaCl_2.2H_2O$, 0.1 g; biotin, 5 ml of a 5% solution in 50% ethanol; and 5 ml of a trace element solution containing (per 100 ml) citric acid.$1H_2O$, 5 g; $ZnSO_4.7H_2O$, 5 g; $Fe(NH_4)_2(SO_4)_2.6H_2O$, 1 g, $CuSO_4.5H_2O$, 250 mg; $MnSO_4.1H_2O$, 50 mg; H_3BO_3, 50 mg; and $Na_2MoO_4.2H_2O$, 50 mg. Pezet's medium consisted of the following nutrients per liter: glucose, 10 g; sucrose, 5 g; KNO_3, 5 g; KH_2PO_4, 1 g; NaCl, 0.5 g; $CaCl_2.2H_2O$, 0.1 g; $FeCl_3$, 0.05 g; and 1 ml of a trace element solution containing (per 100 ml) $CuSO_4$, 10 mg; $MnSO_4$, 2 mg; H_3BO_3, 1 mg; $ZnSO_4$, 2 mg; $(NH_4)_2MoO_4$, 2 mg; and pH adjusted between 5.5 and 6.0 with 1 N NaOH or 1 N HCl before sterilization.

4.3.2. Preparation of grape-based media

Tested media comprised grape cane and the hot water extract of grape cane. Dormant Cabernet Sauvignon canes (Napa Valley, CA; 2000) were ground in a Wiley mill with a 1 mm screen. The Cabernet cane based medium consisted of 10 g ground cane with 50 ml H_2O; cane medium supplemented with sugar also contained 1 g sucrose per 10 g cane. Liquid cane-based media was prepared by sonifying (Branson) 100 g ground Cabernet cane with 500 ml hot water (100 °C). This extract was clarified by filtration though the following series of filters: Miracloth (CalBiochem), multigrade GMF 150 (Whatman), GF/F (Whatman), and Supor-200 0.2 μm (Gelman). Cane extract supplemented with sugar also contained 1 g sucrose per 50 ml extract.

4.3.3. Inoculation and incubation of cultures

Liquid media (50 ml per 250 ml flask) and cane media (10 g cane per 250 ml flask) were autoclaved followed by the addition of several plugs (6.5 mm diameter) of *E. lata* from margins of the inoculum. All cultures were incubated at 25 °C in the dark for 30 days except for those isolates grown in Vogel's media, which were incubated for 24 days.

4.4. Quantitative analysis of metabolites in Eutypa growth media

Liquid cultures (50 ml) of *E. lata* were filtered through Whatman #4 paper and the filtrate 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 removed under reduced pressure. The residue was dissolved in methanol (1 ml) and filtered through a 0.45 μm 13 mm syringe filter (Gelman). Cane-based media was extracted by sonification with $CHCl_3$ (50 ml) and filtered though Whatman No. 4 paper. The extract was partitioned with H_2O (50 ml) and the organic phase collected with the $CHCl_3$ removed under reduced pressure. The residue was diluted and filtered as above. Samples (20 μl) were analyzed by HPLC using a Microsorb C_{18} 5 μm column, 250×4.6 mm i.d. (Varian) with a gradient elution at 1.0 ml/min of 100% H_2O with 0.5% HOAc to 100% CH_3CN over 30 min and held at 100% CH_3CN for 5 min. Detection was either by UV at 254 nm or photodioarray. Metabolite concentrations were determined by reference to the standard curves prepared for each compound, which were linear over the range tested, 0.2–20 μg/20 μl injection.

4.5. GC/MS analysis of metabolites

The GC/MS method for analysis of fungal extracts and metabolites as their trimethylsilyl ether (TMS) derivatives has been reported previously (Molyneux et al., 2002). In brief, the sample in dry pyridine was treated with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) and the resulting TMS derivatives separated on a temperature programmed, 60 m×0.32 mm i.d. SE-30 fused Si capillary column, with on-column injector. The MS was operated at 70 eV in the EI mode with scanning from 75 to 600 a.m.u at a sampling rate of 1.5 scans/s.

4.6. Phytotoxicity to grape leaf disks

Leaf disks (1 cm diameter) were cut from greenhouse grown Cabernet Sauvignon grape leaves (4–5 cm at the widest point) with a cork borer with the major veins being avoided. Ten leaves were used for each concentration such that each of the tested compounds and its associated control had one leaf disk from each of the same ten leaves. The ten leaf disks for each sample were arranged around the perimeter of filter paper (7.0 cm Whatman No. 3) saturated with H_2O (2 ml) and placed in a Petri dish (100×15 mm). Compounds to be tested were dissolved in MeOH and a volume of 5 μl applied as a single spot at the center of the surface of each leaf disk; the control disks were similarly treated with 5 μl of MeOH. The Petri dishes were covered with lids and the test samples exposed at room temperature to ambient lighting on a laboratory bench; this consisted of approximately 12 h of mixed natural and artificial light and 12 h of darkness. An additional volume of H_2O (1.5 ml) was added to each Petri dish after 24 h.

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