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Secondary Metabolites of the Grapevine Pathogen *Eutypa lata* Inhibit Mitochondrial Respiration, Based on a Model Bioassay Using the Yeast *Saccharomyces cerevisiae*

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Abstract. Acetylenic phenols and a chromene isolated from the grapevine fungal pathogen *Eutypa lata* were examined for mode of toxicity. The compounds included eutypine (4-hydroxy-3-[3-methyl-3-butene-1-ynyl] benzyl aldehyde), eutypinol (4-hydroxy-3-[3-methyl-3-butene-1-ynyl] benzyl alcohol), eulatachromene, 2-*iso*prenyl-5-formyl-benzofuran, siccayne, and eulatinol. A bioassay using the yeast *Saccharomyces cerevisiae* showed that all compounds were either lethal or inhibited growth. A respiratory assay using 2,3,5-triphenyltetrazolium (TTC) indicated that eutypinol and eulatachromene inhibited mitochondrial respiration in wild-type yeast. Bioassays also showed that 2-*iso*prenyl-5-formyl-benzofuran and siccayne inhibited mitochondrial respiration in the *S. cerevisiae* deletion mutant *vph2*Δ, lacking a vacuolar type H (+) ATPase (V-ATPase) assembly protein. Cell growth of *tsa1*Δ, a deletion mutant of *S. cerevisiae* lacking a thioredoxin peroxidase (cTPx I), was greatly reduced when grown on media containing eutypinol or eulatachromene and exposed to hydrogen peroxide (H_2O_2) as an oxidative stress. This reduction in growth establishes the toxic mode of action of these compounds through inhibition of mitochondrial respiration.

Eutyposis, or "dying-arm disease", is a fungal disease of grapevines (*Vitis vinifera*; Vitaceae). It is caused by the ascomycete *Eutypa lata* (Pers.) Tul. & C. Tul. This perennial canker disease is progressive over several years and affects vineyards worldwide, including California and Australia [3]. Infected vines have a shortened lifespan and produce fewer and smaller grapes than uninfected vines. Failure to control the disease results in severe economic losses to the viticulture industry [18], up to approximately US$260 million per annum in California alone [20].

E. lata produces a number of structurally related secondary metabolites, mainly acetylenic phenols and heterocyclic analogs [14, 17] (Fig. 1). Eutypine has been implicated as the principal phytotoxin from a European strain causing the symptoms of dying-arm disease [23]. However, strains of *E. lata* isolated in California, Australia, and New Zealand produce dying-arm disease but do not synthesize eutypine. These strains produce high quantities of eutypinol or eulatinol [14]. Thus, phytotoxicity of *E. lata* probably results from a suite of structurally related compounds. Each of these compounds may have a different level of toxicity and possibly different molecular targets within the plant cell [17, 21].

To date, thorough investigation of the relative phytotoxicities of these secondary metabolites has not been performed. Such investigations have been hampered by an insufficient supply of the compounds and lack of an appropriately sensitive bioassay system. One assay using grapeleaf discs enables evaluation of phytotoxicity of individual metabolites by measuring chlorophyll decrease [14, 21]. However, this method is limited because individual metabolites do not have equal propensity to penetrate the leaf cuticle, a major physical barrier to entry of compounds into plant cells.

In this study, a bioassay employing selected strains of the yeast *Saccharomyces cerevisiae* was used to study the toxicity and mode of action of *E. lata* secondary metabolites. Molecular targets of *E. lata* metabolites have been investigated with mutant strains of *S. cerevi-*

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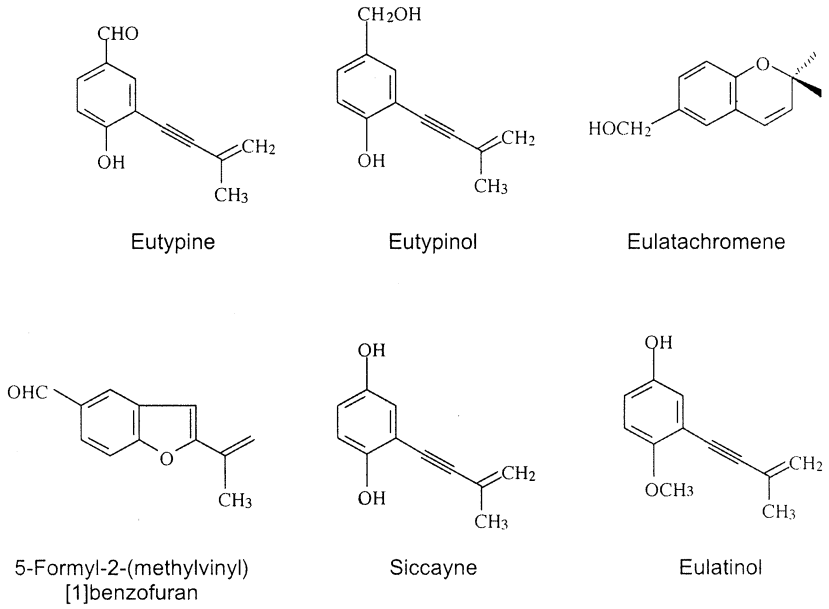

Fig. 1. Chemical structures of secondary metabolites of *Eutypa lata* examined in this study.

Table 1. Deletion mutant strains of *Saccharomyces cerevisiae* used in bioassays to elucidate the mode of action of toxicity of secondary metabolites of *Eutypa lata*

Yeast strain (deleted gene)	Function of deleted gene	Deletion mutant phenotype	Rationale	References
4969 (*vph2*Δ)	V-ATPase assembly protein	Defects in mitochondrial respiration, vacuolar acidification	Phenotypically analogous to dying-arm disease	[1]
3840 (*crd1*Δ)	Mitochondrial CL synthase	Defects in mitochondrial respiration and maintaining mitochondrial membrane potential	Phenotypically analogous to dying-arm disease	[10, 12]
5933 (*yor1*Δ)	ABC transporter (efflux)	Hypersensitivity to xenobiotics, e.g., fumonisin B1	Increased sensitivity to *E. lata* metabolites	[15]
3323 (*ypc1*Δ)	Alkaline ceramidase w/ reverse activity	Defects in signal transduction and stress response	Increased sensitivity to *E. lata* metabolites	[9, 15]
545 (*tsa1*Δ)	Thioredoxin peroxidase (cTPXI)	Hypersensitive to oxidative stress under mitochondrial dysfunction	Confirm toxic effects of eutypinol and eulatachromene on mitochondrial respiration	[4]

siae having singular deletions of individual genes. Using this model system, we have examined inhibition of mitochondrial respiration as the mode of action of eutypinol and eulatachromene phytotoxicity.

Materials and Methods

Microorganisms, cultures, and chemicals. Strains of deletion mutants of *S. cerevisiae* (Invitrogen, Carlsbad, CA), function of the deleted genes, and the rationale for selecting the particular strain for this study are listed in Table 1. *S. cerevisiae* BY4741 (*mat a his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0*) was used as the wild-type strain. Each of the deletion mutants was derived from BY4741. Yeasts were cultured on either rich medium (YPD: Bacto yeast extract 1%, Bacto peptone 2%, glucose 2%) or minimal medium (SG: yeast nitrogen base w/o amino acids 0.67%, glucose 2% with appropriate supplements i.e., uracil 0.02 mg/mL, amino acids 0.03 mg/mL) at 30°C without light. Methods for growth of *E. lata* and isolation and purification of eutypine, eutypinol, eulatachromene, 2-*iso*prenyl-5-formyl-benzofuran, siccayne, and eulatinol have been previously described [17, 21]. *E. lata* secondary metabolites were dissolved in dimethyl sulfoxide (DMSO) before use. 2,3,5-Triphenyltetrazolium (TTC), hydrogen peroxide (H_2O_2), and antimycin A were obtained from Sigma.

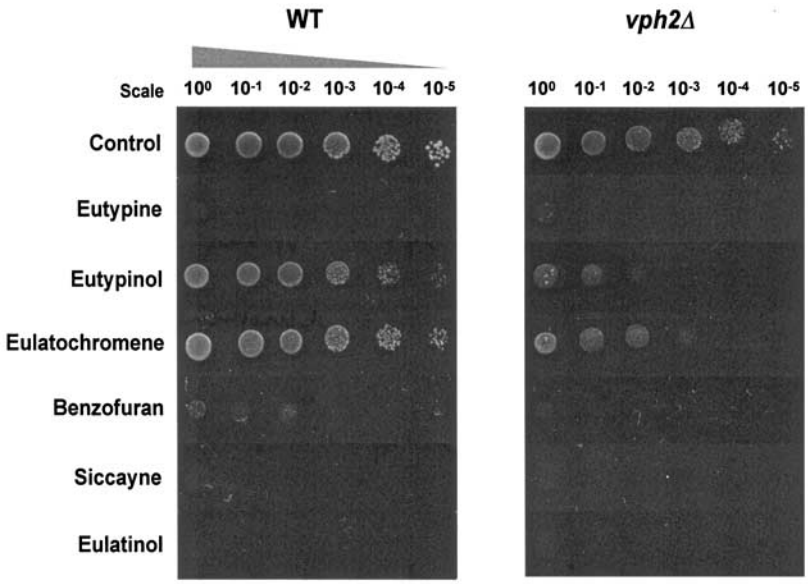

Fig. 2. Representative bioassays showing sensitivity of *S. cerevisiae* wild-type (WT) and the *vph2*Δ mutant strain to *E. lata* metabolites. Control cohorts were grown on media treated with DMSO without any test compound. "Scale" shows the level of dilution of approximately 10^6 cells spotted on plates prior to a 7 day incubation at 30°C.

2,3,5-Triphenyltetrazolium (TTC) overlay assay. This assay was used to test whether secondary metabolites of *E. lata* inhibited mitochondrial respiration. The TTC overlay assay was performed as described by Barclay et al. [2]. Briefly, *S. cerevisiae* cells (5×10^6 cells/5 mL YPD soft agar) were overlaid onto YPD hard agar medium, and 70 μL (1.4 mg) of individual *E. lata* metabolites was added into a small well in the center of the plate. Yeast cells were grown overnight, allowing diffusion of metabolites. The following day, plates were overlaid with 5 mL TTC (1.5% agar, 2% glucose, and 0.1% TTC). Yeast growth and mitochondrial respiratory activity were monitored in an incubator (30°C) for 2 days.

Yeast dilution bioassays. Yeast cells of the different test strains were cultured in YPD liquid medium overnight. Approximately 1×10^6 cells were serially diluted 10-fold five times using SG liquid medium. This provided six samples of yeast cells from ~10^6 to ~10 cells. Cells from each dilution were adjacently spotted on SG agar medium containing nutrient supplements and 0.5 mg/mL of individual *E. lata* secondary metabolites. Cell growth was monitored after incubation at 30°C for 7 days. *S. cerevisiae tsa1*Δ strain, a deletion mutant lacking thioredoxin peroxidase (cTPx I), was used to determine whether eutypinol and eulatachromene inhibited mitochondrial respiration. Cells of *tsa1*Δ (1×10^6) were serially diluted and dispensed, as described above, onto YPD plates containing 0.35 mM H_2O_2 and 0.5 mg/mL of eutypinol or eulatachromene. Cell growth was monitored after incubation at 30°C for 2 days.

Results and Discussion

Effects of *E. lata* secondary metabolites on growth of yeast strains. Representative yeast dilution bioassays are presented in Fig. 2, showing cell growth of both wild-type and 4969 (*vph2*Δ) yeast strains were greatly inhibited by eutypine, 2-*iso*prenyl-5-formyl-benzofuran, siccayne, and eulatinol. Furthermore, *S. cerevisiae* 4969 was shown to be somewhat more sensitive to eutypinol and eulatachromene than the wild-type strain. The dilution bioassays were visually assessed and cell growth of the yeast strains scored as tolerant, slightly sensitive or hypersensitive to the *E. lata* metabolites. *S. cerevisiae* strains 3840 (*crd1*Δ), 3323 (*ypc1*Δ), and 5933 (*tsa1*Δ) showed essentially the same levels of sensitivity to *E. lata* metabolites as the wild-type strain (Table 2). *S. cerevisiae* 4969 was clearly the most sensitive of the strains examined to the *Eutypa* metabolites. This strain has a defect in mitochondrial respiration as a result of intracellular fluctuation of pH [1].

Inhibition of mitochondrial respiration by *E. lata* secondary metabolites (TTC assay). Based on the yeast dilution bioassays eutypinol or eulatachromene putatively exacerbate mitochondrial dysfunction in strain 4969. In experiments with grapevine cells in vitro, it has been shown that eutypine increases proton leakage via a cyclic protonophore mechanism [5]. This leakage results in uncoupling of mitochondrial oxidative phosphorylation and a decrease in the ADP/O ratio, ultimately contributing to mitochondrial dysfunction. We therefore tested the effects of eutypine and the other *E. lata* metabolites on mitochondrial function, especially mitochondrial respiration, in yeast cells of the wild-type and 4969 strains using a TTC assay.

In principle, cells having normal respiratory activity develop a reddish color when overlaid with TTC while cells having inhibited respiration remain white or pink

Table 2. Visual bioassay responses (see Fig. 2) of wild-type (BY4741) and various deletion mutant strains of *Saccharomyces cerevisiae* to secondary metabolites of *Eutypa lata*

Yeast strain	Eutypine	Eutypinol	Eulatachromene	2-*Iso*prenyl-5-formyl-benzofuran	Siccayne	Eulatinol
BY4741	−	+	+	+/−	−	−
4969	−	+/−	+/−	−	−	−
3840	−	+	+	+/−	−	−
5933	−	+	+	+/−	−	−
3323	−	+	+	+/−	−	−

Level of sensitivity is represented as tolerant (+), slightly sensitive (+/−), or hypersensitive (−).

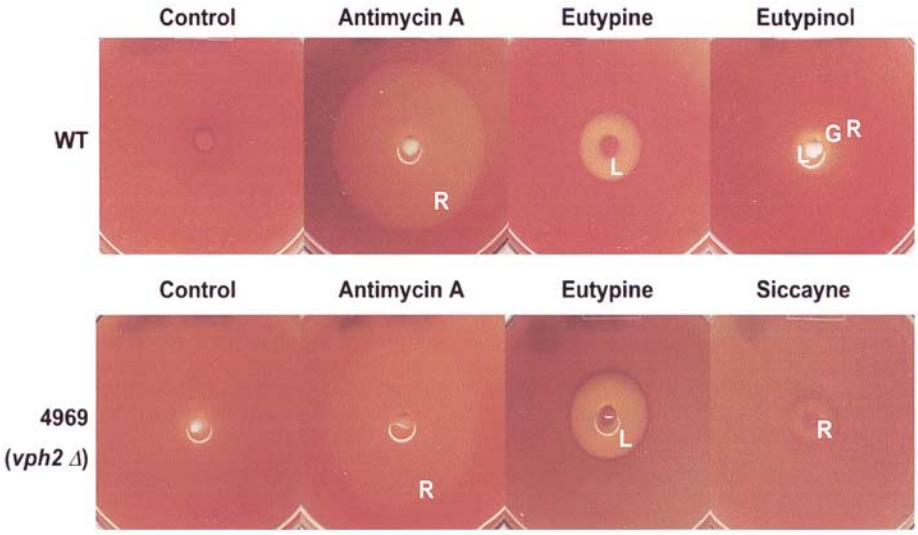

Fig. 3. Examples of TTC overlay bioassays showing comparative levels of cell growth and respiration (color) between wild-type (WT) and 4969 (*vph2*Δ) yeast strains as affected by no treatment (control), antimycin A, or various metabolites of *Eutypa lata*. Responses to the treatments were scored as lethal (L), inhibitory to growth (G), or inhibitory to respiration (R).

Table 3. Tabulation of results from TTC overlay assays (see Fig. 3) to determine the effects of *Eutypa lata* metabolites on cell growth and respiration of wild-type and 4969 yeast strains

Eutypa lata metabolite	BY4741 (wild-type)	4969 (*vph2*Δ)
Eutypine	L	L
Eutypinol	L, G, R	L
Eulatachromene	L, R	L
2-*Iso*prenyl-5-formyl-benzofuran	L	L, R
Siccayne	No effect	R
Eulatinol	L	L

Responses to treatments are scored as lethal (L), inhibitory to growth (G), or inhibitory to respiration (R).

(Fig. 3). Depending upon the color change in the TTC assay, toxicity levels for the compounds were defined as lethal (L), cell growth inhibiting (G) and/or respiration inhibiting (R). Results of the TTC assays are summarized in Table 3. Both strains grew and respired normally under control conditions. Treatment with antimycin A resulted in inhibition of respiration of both strains. Antimycin A is a fungicide that inhibits mitochondrial electron transfer [24]. The responses to antimycin A showed the assay effectively visualized inhibition of respiration. Eutypinol and eulatachromene caused mitochondrial respiratory inhibition in wild-type yeast cells. Additional assays showed that 2-*iso*prenyl-5-formyl-benzofuran and siccayne triggered similar respiratory inhibition in the 4969 (*vph2*Δ) strain. Siccayne had no effect on the wild-type strain. Thus, the mitochondrial dysfunction inherent to the 4969 mutant renders it more sensitive to *E. lata* metabolites. Eutypine and eulatinol were lethal to both yeast strains under the test conditions.

Differential growth inhibition of *S. cerevisiae tsa1*Δ under exposure to oxidative stress and *E. lata* metabolites. The growth of wild-type and the *tsa1*Δ deletion mutant, lacking thioredoxin peroxidase (cTPx I), was compared under exposure to eutypinol or eulatachro-

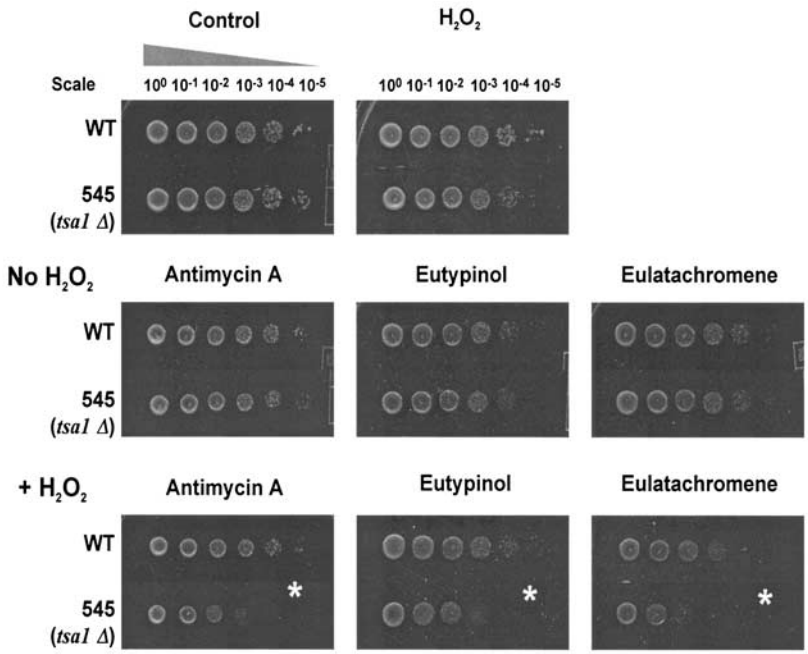

Fig. 4. Yeast dilution bioassays showing relative sensitivities of yeast cells of wild-type (WT) and 545 (*tsa1*Δ) strains under oxidative stress conditions and treatment with antimycin A or *Eutypa lata* metabolites. Asterisk (*) indicates assays in which growth of the 545 strain was significantly less (approximately 100-fold) than that of the wild-type strain.

mene with additional exposure to oxidative stress. Since cTPx I is essential for the antioxidant defense of yeast having dysfunctional mitochondria [4], deletion of the cTPx I gene renders cells more sensitive to oxidative stress under conditions causing mitochondrial respiratory inhibition. Both strains grew normally under exposure to minor amounts of H_2O_2 (0.35 mM) without treatment with antimycin A, eutypinol, or eulatachromene (Fig. 4). Also, both strains showed equivalent levels of sensitivity to antimycin A, eutypinol, and eulatachromene when not exposed to oxidative stress. However, when exposed to both H_2O_2 and any of the compounds, growth of the *tsa1*Δ deletion mutant was approximately 100-fold lower than that of the wild-type strain (Fig. 4). These results establish the mode of action at the molecular level of the toxicity of eutypinol and eulatachromene as inhibition of mitochondrial respiration.

In fungi and plants, vacuolar compartmentalization of toxic substances (e.g., xenobiotics using vacuolar transporters and ATPase) is a well-known mechanism for detoxification [6, 8, 13]. Acidification mediated by V-ATPases is also necessary for the accumulation of ions and metabolites, such as Ca^{2+} and amino acids in the vacuoles [19, 22]. Since the 4969 mutant lacks the V-ATPase assembly protein, its cells are likely to be dysfunctional in transporting toxic *E. lata* metabolites into vacuoles, explaining the hypersensitivity of this strain to these metabolites. In contrast, *S. cerevisiae* 5933, a strain representing a nonfunctional ATP-binding cassette (ABC) efflux transporter, showed a similar level of sensitivity to *E. lata* metabolites as the wild-type strain. It is our current view that either vacuolar sequestration or enzymatic conversion/degradation are the mechanisms of detoxification of *E. lata* metabolites in yeast cells. Future experiments involving other mutant strains deficient in functional efflux pumps are required to confirm this view.

The *S. cerevisiae* 3840 mutant, lacking mitochondrial cardiolipin (CL) synthase and hence unable to maintain normal mitochondrial function, was not as sensitive to eutypinol as the 4969 strain. These mutant strains may have different degrees of cellular sensitivity to the secondary metabolites or culturing conditions. For example, strain 4969 is very sensitive to higher temperatures, when cultured in a nonfermentable carbon-source, and pH than strain 3840 (unpublished).

The model bioassay system using *S. cerevisiae* mutants has future applications for controlling dying-arm disease. It is noteworthy that *S. cerevisiae* has long been a model system used to study mycotoxins affecting human health, such as aflatoxin [7, 11], fumonisin [15], citrinin [1] and zearalenone [16]. Use of mutant strains of *S. cerevisiae* as screening tools for identifying detoxifying genes may be particularly useful for control of plant pathogens. With this bioassay we found that *E. lata* metabolites have different levels of toxicity depending

on the mutant yeast strain tested. The most significant observation was that eutypinol and eulatachromene inhibited mitochondrial respiration in wild-type yeast, and 2-*iso*prenyl-5-formyl-benzofuran and siccayne inhibited respiration in the *vph2*Δ strain. The effects of eutypinol and eulatachromene on promoting mitochondrial dysfunction are confirmed using the *tsa1*Δ strain. It is likely that mitochondrial dysfunction is the phytotoxic mode of action of these compounds on grapevine cells leading to cell death and the symptoms of dying-arm disease.

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