FINAL PROJECT REPORT **WTFRC Project Number:** CP-07-701

Project Title: Augmenting fungal control in apples with natural compounds

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Total Project Funding:

Budget History:

Item	Year 1: 2007	Year 2: 2008	Year 3: 2009
Salaries	$20,276	$21,880	$23,924
Benefits			
Wages			
Benefits			
Equipment	$7,146	$4,491	$4,449
Supplies	$3,857	$5,987	$6,357
Travel	$1,142	$1,197	
Miscellaneous			
Total	$32,421	$33,555	$34,730

RECAP ORIGINAL OBJECTIVES

The goal of this research is to find new, safe natural compounds that effectively improve activity of conventional fungicides for pre/ post-harvest treatment of apple. We are trying to identify molecular targets of these compounds using genomic tools and determine effective methods for delivery of discovered compounds. This research will greatly improve the ability to suppress decay of apples, a priority identified by the WTFRC. Our specific objectives are:

1. Determine an effective method for delivery of newly discovered natural compounds, leading to a target-specific strategy for a safe and economic approach to fungal pathogen control in the field or during processing and storage.
2. Identify the most efficient molecular targets [*e.g.*, mitochondrial superoxide dismutase (Mn-SOD), cellular antioxidation system, *etc*.] for newly discovered compounds using functional genomics approaches.
3. Augment antifungal activity of natural compounds, through synthesis of structural derivatives, based on structure-activity relationships of analogs of identified antifungal natural compounds.

SIGNIFICANT FINDINGS (Last 3 years):

[A] Identify the most efficient molecular targets:

- Fungal response (tolerance) to 2,3-dihydroxybenzaldehyde or 2,3-dihydroxybenzoic acid was found to rely upon mitochondrial superoxide dismutase (*SOD2*) or glutathione reductase (*GLR1*), genes regulated by the *HOG1* signaling pathway, respectively. Thus, certain benzo analogs can be effective at targeting cellular oxidative stress response systems.
- The *SLT2* and *BCK1* genes, encoding mitogen-activated protein kinase (MAPK) and MAPK kinase kinase (MAPKKK) in cell wall integrity pathway, respectively, were essential for fungal tolerance to thymol, *o*-coumaric acid, 2,3-dihydroxybenzaldehyde and berberine hemisulfate.

[B] Determine an effective method for delivery of newly discovered natural compounds:

- Activity of conventional antifungal agents, fludioxonil, strobilurin and antimycinA, which target the oxidative and osmotic stress response systems, was elevated by co-application of certain benzo analogs (aldehydes and acids).
- The activity of conventional antifungal compounds interfering with cell wall integrity, *i.e.*, Congo red or calcofluor white, was elevated by coapplication with thymol or 2,3-dihydroxybenzaldehyde, demonstrating the chemosensitizing capacity of the identified compounds on fungal growth.

[C] Overcome the tolerance of *Penicillium expansum* to fludioxonil and strobilurin through chemosensitization by using natural compounds:

- We investigated the mechanism of two fludioxonil resistant strains of *Penicillium expansum, i.e.*, FR2 and FR3, provided by Dr. Xiao. Our study indicates that fludioxonil resistance of the FR2 and FR3 strains is a result of UV-induced mutation of cellular redox homeostasis.
- Increased sensitivity of FR3 to oxidizing agents, compared to its parental strain (W2), indicated the oxidative stress response system of FR3, such as the mitogen-activated protein kinase (MAPK) pathway, had become defective as a result of UV-treatment.
- Alternatively, the other resistant strain, FR2, showed higher tolerance to oxidizing agents compared to its parental strain (W1), suggesting fludioxonil resistance of FR2 was based upon gain-of-function, possibly increased antioxidation activity.
- To overcome the resistance in both strains, redox-active natural phenolics were successfully used as chemosensitizing agents that targeted various elements of the oxidative stress-response pathway. Co-application of certain natural phenolic compounds with fludioxonil overcame fludioxonil-resistance in two mutant strains, FR2 and FR3, of *P. expansum*.

- Natural phenolics were also found that served as chemosensitizing agents for overcoming resistance to strobilurin.

Methods:

Chemicals. Test chemosensitizing agents: thymol [5-methyl-2-(isopropyl)phenol], 2,3-dihydroxybenzaldehyde, gallic acid and ester analogs (methyl-, ethyl- and octyl- gallates); antifungal agents: fludioxonil, kresoxim-methyl, antimycin A; and oxidizing agents: menadione, hydrogen peroxide (H_2O_2), diamide; other compounds such as alkaloid, cell wall interfering agents (Congo red, calcofluor white) were purchased from Sigma Co. (St. Louis, MO). Each compound was dissolved in dimethylsulfoxide (DMSO; absolute DMSO amount: < 2% in media) except H_2O_2 and diamide, which were dissolved in water, for incorporation into media.

Microorganisms and culture condition. *Penicillium expansum* FR2 and FR3, fludioxonil resistant mutants (Li and Xiao, 2008) and their parental strains, W1 and W2, respectively, were grown at 28 °C (82.4 °F) on potato dextrose agar (PDA). *Aspergillus fumigatus* AF293, wild type, and *A. fumigatus* MAPK deletion mutants *sakA*Δ and *mpkC*Δ (Xue *et al*. 2004; Reyes *et al*. 2006) were grown at 37 °C (98.6 °F) on PDA (5 to 7 days). *P. expansum* NRRL974 and *A. flavus* NRRL3357 (obtained from National Center for Agricultural Utilization and Research, USDA, Peoria, IL) were cultured at 28 °C (82.4 °F) on PDA (5 to 7 days).

Saccharomyces cerevisiae wild type BY4741 (*Mat* a *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0*) and selected deletion mutants lacking genes in (a) antioxidative stress response/multidrug resistance systems or (b) cell wall construction/integrity system were obtained from Invitrogen (Carlsbad, CA) and Open Biosystems (Huntsville, AL), as follows (See also Kim *et al*., 2005, 2007, 2008b): Gene regulation mutants: *yap1*Δ, *msn2*Δ, *msn4*Δ, *hot1*Δ, *sko1*Δ, *rim101*Δ; Transporter/assembly protein mutants: *flr1*Δ, *yor1*Δ, *pdr5*Δ, *vph2*Δ, *tfp1*Δ/*vma1*Δ; Signal transduction mutants: *sho1*Δ, *sln1*Δ, *ste50*Δ, *ste20*Δ, *ypd1*Δ, *ssk1*Δ, *ptp2*Δ, *ptp3*Δ, *hog1*Δ, *hog4*Δ, *ssk22*Δ, *ssk2*Δ, *ste11*Δ; Antioxidation mutants: *ctt1*Δ, *cta1*Δ, *osr1*Δ, *trr1*Δ, *trr2*Δ, *tsa1*Δ, *grx1*Δ, *grx2*Δ, *trx1*Δ, *trx2*Δ, *glr1*Δ, *gsh1*Δ, *gsh2*Δ, *sod1*Δ, *sod2*Δ, *ahp1*Δ; DNA damage control/energy metabolism mutants: *rad54*Δ, *sgs1*Δ, *acc1*Δ, *gpd1*Δ, *hor2*Δ;Osmoregulation mutant: *hog1*Δ (MAPK), *hog4*Δ (MAPK kinase; MAPKK; Scaffold activity), *ssk22*Δ (MAPKK kinase; MAPKKK), *ssk2*Δ (MAPKKK), **ste11*Δ (MAPKKK); Cell wall construction/integrity mutant: *slt2*Δ (MAPK), *mkk1*Δ (MAPKK), *mkk2*Δ (MAPKK), *bck1*Δ (MAPKKK), *wsc1*Δ (Sensor-transducer), *mid2*Δ (Sensor for cell wall integrity signaling), **kss1*Δ (MAPK), **ste7*Δ (MAPKK), **ste11*Δ (MAPKKK); Morphological switch mutant: **kss1*Δ (MAPK), **ste7*Δ (MAPKK), **ste11*Δ (MAPKKK); Mating response mutant: *fus3*Δ (MAPK), **ste7*Δ (MAPKK), **ste11*Δ (MAPKKK), *ste5*Δ□ (Scaffold protein), *ste2*Δ (Receptor for "alpha" factor pheromone), *ste3*Δ (Receptor for "a" factor pheromone); Sporulation mutant: *smk1*Δ (MAPK); PKC-signaling pathway mutant: *rom2*Δ (GDP/GTP exchange protein), *fks1*Δ (β-1,3-D-glucan synthase), *fks2*Δ (β-1,3-D-glucan synthase), *rlm1*Δ (Mcm1p-Agamous-Deficiens-Serum Response Factor <MADS>-box transcription factor), *swi4*Δ (Transcription factor), *pkc1*Δ (Protein serine/threonine kinase; dipoid), where the asterisk (*) indicates overlapping biological roles of the marked gene in more than one pathway (Reference for the description of each deletion mutant: www.yeastgenome.org). Yeast strains were grown on YPD (1% Bacto yeast extract, 2% Bacto peptone, 2% glucose) or SG (0.67% Yeast nitrogen base w/o amino acids, 2% glucose with appropriate supplements: 180 μM uracil, 200 μM amino acids) medium at 30 °C (86 °F; 5 to 7 days).

Antifungal bioassays. Yeast cell dilution bioassays were performed as described (See Kim *et al*., 2005, 2007). Sensitivities of filamentous fungi to the compounds were based on percent radial growth of treated (T) compared to control (C), receiving only DMSO, colonies and/or based on the Vincent equation [% inhibition of growth = 100 (C-T)/C, C: diameter of fungi on control plate; T: diameter of fungi on the test plate] (Vincent 1947), if necessary. Fungi (5 x 10^3 spores) were diluted in phosphate buffered saline and spotted on the center of PDA plates (triplicates) with or without antifungal compounds. Growth was observed for 3 to 7 days. Effectiveness of chemosensitization by thymol (0.2

to 0.6 mM), octylgallate (0.05 to 0.2 mM) or 2,3-dihydroxybenzaldehyde (2,3-D; 0.05 to 0.3 mM) was assessed by incorporating each compound into growth media with fludioxonil or strobilurin (kresoxim-methyl; 0.02, 0.04, 0.06 mM). Radial growth was recorded as described above. Oxidizing agents, menadione (0.001 to 0.512 mM), hydrogen peroxide (0.5 to 5 mM) or diamide (0.5 to 5 mM) were incorporated into media at respective levels and fungal sensitivities were measured by fungal radial growth, as described above. Minimum inhibitory concentration (MIC) was defined as the lowest concentration of a compound where no fungal growth was observed.

RESULTS & DISCUSSION:

Fungal tolerance to benzo analogs depends on cellular Mn-SOD. Based on yeast cell dilution bioassays, 2,3-dihydroxybenzaldehyde had the highest antifungal activity, *i.e.*, no visible growth of wild type *S. cerevisiae* at $\geq$ 80 μM, among eight benzo analogs tested. Highest to lowest antimicrobial activity was, as follows: 2,3-dihydroxybenzaldehyde > 2,5-dihydroxybenzaldehyde > 2,4-dihydroxybenzaldehyde > 3-hydroxybenzaldehyde > vanillin, 4-hydroxybenzaldehyde, veratraldehyde > benzaldehyde. An almost identical relationship in the relative antifungal activities of the analogs was observed among the various fungi, *i.e., Penicillium* and aspergilli tested.

Among forty-five mutants of *S. cerevisiae* examined, where genes in oxidative stress response/multidrug resistance systems were individually deleted, the *sod2*Δ [mitochondrial superoxide dismutase (Mn-SOD) deletion] mutant showed hypersensitivity to 2,3-dihydroxybenzaldehyde (at 10 μM) compared to the wild type strain. This greater sensitivity strongly indicated Mn-SOD activity is crucial for fungal response/tolerance against toxicity of benzaldehyde derivatives. It appears this gene is a promising candidate as a potential target for fungal control.

The *sakA*Δ mutant of *A. fumigatus* is hypersensitive to benzaldehyde derivtives. *Aspergillus* can be used as a model filamentous fungal pathogen to validate the target or mode of action of natural compounds identified through yeast screening in view that the entire genome of several species of *Aspergillus* (*i.e.*, *A. nidulans*, *A. fumigatus*, *A. flavus*) have been sequenced and annotated to a great extent, and manipulation of its genome has been well established, allowing the studies of functional genomic responses to treatments. Responses of *sakA*Δ and *mpkC*Δ mutants derived from *A. fumigatus* AF293, to the benzo analogs was also examined. The MAPKs SakA and MpkC are orthologous proteins to Hog1p of *S. cerevisiae* (Xue *et al*., 2004; Reyes *et al*., 2006). Growth of AF293 and *mpkC*Δ was inhibited by 32 to 72% with 0.2 to 0.25 mM 2,3-dihydroxybenzaldehyde, respectively. The *sakA*Δ strain was more sensitive to these treatments, showing 56 to 100% reduction, respectively, in radial growth. Like Hog1p in *S. cerevisiae*, SakA may play a role in regulating Mn-SOD activity and, thus, tolerance to 2,3-dihydroxybenzaldehyde. The same trends were observed with 2,5-dihydroxybenzaldehyde, but higher concentrations (0.8 to 1 mM) were needed to achieve similar levels of growth inhibition.

2,3-Dihydroxybenzoic acid inhibits fungal growth by disrupting cellular glutathione homeostasis. The acid derivative of 2,3-dihydroxybenzaldehyde, 2,3-dihydroxybenzoic acid, was also examined in order to investigate structure-activity relationships with regard to acid or aldehyde moieties. The 2,3-dihydroxybenzoic acid inhibited growth of *S. cerevisiae* (MIC in wild type $\geq$ 7 mM). Also, growth of a number of *S. cerevisiae* deletion mutants was inhibited by 2,3-dihydroxybenzoic acid at 4 mM, including *glr1*Δ, *gsh1*Δ, *gsh2*Δ, *vph2*Δ (vacuolar ATPase assembly protein deletion), *vma1*Δ (vacuolar ATPase deletion). Also, like in treatments with 2,5-dihydroxybenzoic acid in our prior study (Kim *et al*., 2007), exogenously supplemented GSH resulted in a strong recovery of growth of these *S. cerevisiae* strains. These findings suggest the mechanism of antifungal activity of 2,3-dihydroxybenzoic acid is, as with the 2,5- analog, disruption of cellular GSH homeostasis. Thus, the GSH reductase gene (*GLR1*), another gene relatively downstream within the *HOG1* signaling pathway, may play an important role for fungal tolerance to this, or related,

compounds. The sensitivities of the *vph2*Δ and *vma1*Δ mutants may result from disruption of the normal ability for transportation, sequestration and detoxification of toxic compounds in vacuoles.

The concordance of these results demonstrates there is a structure-activity relationship between the acid and aldehyde moieties in that they affect different target genes in the *HOG1*-signaling pathway. The 2,3- and 2,5- dihydroxybenzaldehydes targeted *SOD2*. Whereas, 2,3- and 2,5-dihydroxybenzoic acids targeted *GLR1*, disrupting glutathione homeostasis.

Chemosensitization to conventional fungicides by 2,3-dihydroxy benzaldehyde and benzoic acid derivatives: targeting cellular signal transduction/oxidative stress response systems. Some fungi having mutations in certain MAPK genes, involved in signal transduction of oxidative stress responses, can escape toxicity of phenylpyrrole fungicides, such as fludioxonil (Kojima *et al*., 2004). In this regard, we found MAPK mutants, *sakA*Δ and *mpkC*Δ, of *A. fumigatus* were tolerant to fludioxonil toxicity. However, co-application of 2,3-dihydroxybenzaldehyde (at 0.2 mM) or 2,3-dihydroxybenzoic acid (at 11 mM) with fludioxonil effectively prevented these mutants from developing this tolerance to fludioxonil. This prevention of tolerance by co-application of either of these compounds may result from the disruption of genes downstream in this MAPK pathway. In particular, based on the results with the deletion mutants of *S. cerevisiae* it is likely that these aldehyde and acid analogs target the antioxidative gene *sod2* and the glutathione homeostasis genes.

The potential chemosensitizing effect of 2,3-dihydroxybenzaldehyde was also tested on the activity of kresoxim-methyl, a strobilurin fungicide, and antimycin A. Co-application of 2,3-dihydroxybenzaldehyde enhanced the antifungal activity of both fungicides against the filamentous fungi examined, *A. fumigatus*, *A. flavus* and *P. expansum*. Co-application of 100 or 200 µM 2,3-dihydroxybenzaldehyde to kresoxim-methyl (25 µM; **Figure 1a**) or antimycin A (5 µg mL^{-1}; **Figure 1b**) resulted in complete (100%) inhibition of fungal growth, except *A. flavus* (70% inhibition). Whereas, if any of these compounds are applied alone at these rates fungal growth is only slightly inhibited.

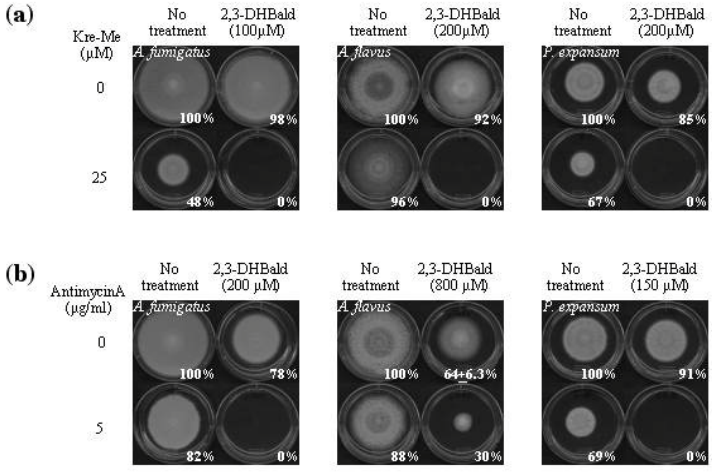

Figure 1. Targeting the mitochondrial antioxidative stress system with 2,3-dihydroxybenzaldehyde in combination with (**a**) strobilurin (kresoxim-methyl) or (**b**) antimycin A had an enhanced antifungal effect against the filamentous fungi, *A. fumigatus* AF293, *A. flavus* NRRL 3357 or *P. expansum* NRRL 974. Standard deviation: <5%, except where noted.

Natural compounds to which *slt2*Δ/*bck1*Δ mutants of *S. cerevisiae* showed sensitivity: chemosensitization of cell wall-interfering agents. We then tested the antifungal effects of natural

compounds by using *slt2*Δ, *bck1*Δ, *wsc1*Δ and *swi4*Δ mutants (See Methods) of *S. cerevisiae*. We found that *slt2*Δ and *bck1*Δ mutants showed the highest sensitivity (10^3 to 10^4 times) to thymol, 2,3-dihydroxybenzaldehyde, *o*-coumaric acid and berberine comparing to the wild type or *wsc1*Δ/*swi4*Δ strains.

In the bioassay using *S. cerevisiae*, thymol showed chemosensitizing effect to Congo red, where co-application of thymol (0.5 mM) and Congo red (100 and 500 μg/ml) resulted in ~10 to 10^4 times higher sensitivity of yeast cells (wild type, *slt2*Δ and *bck1*Δ mutants) comparing to the independent treatment of each compound. Results also showed that co-application of Congo red (0.4 to 1.0 mg/ml) with thymol (1.0 mM) or 2,3-dihydroxybenzaldehyde (0.2 mM) enhanced its antifungal activity against *A. fumigatus* (*i.e.*, ~95% to 100% growth inhibition; **Figure 2A**), while no chemo-sensitizing effect was observed with *o*-coumaric acid or berberine (data not shown), indicating thymol or 2,3-dihydroxybenzaldehyde may affect common cellular target of cell wall-interfering agents. We found that 100% of growth inhibition can be achieved in *A. fumigatus* AF293, *A. flavus* 3357 and *P. expansum* 974 (**Figure 2B**) by co-application of thymol and 2,3-dihydroxybenzaldehyde, strongly indicating these two compounds affect common cellular target.

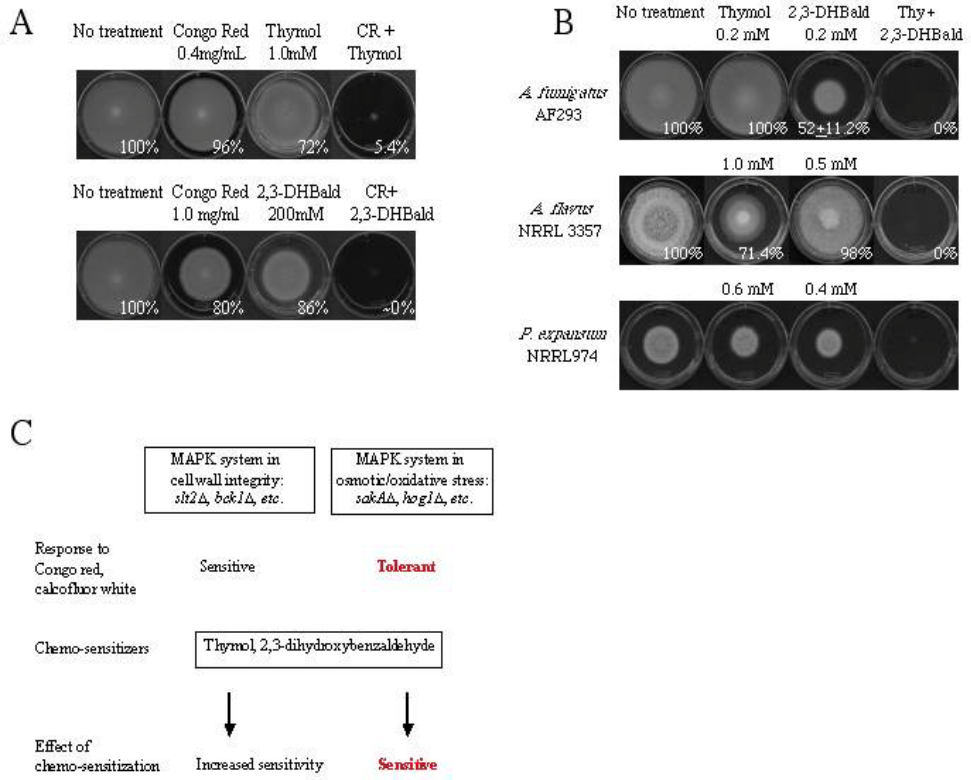

Figure 2. (A) Co-application of Congo red (0.4 to 1.0 mg/ml) with thymol (1.0 mM) or 2,3-dihydroxybenzaldehyde (0.2 mM) enhanced its antifungal activity against *A. fumigatus* (*i.e.*, ~95% to 100% growth inhibition), (B) Co-application of thymol and 2,3-dihydroxybenzaldehyde achieved 100% growth inhibition in *A. fumigatus* AF293, *A. flavus* 3357 and *Penicillium expansum* 974, indicating these two compounds affect common cellular target. (C) Diagram summarizing the chemosensitizing effect of thymol and 2,3-dihydroxybenzaldehyde on fungi.

Overcoming fungal tolerance to antifungal agents by using natural compounds: *characteristics of* P. expansum *fludioxonil resistant mutants.* UV-induced generation of *P. expansum* FR2 and FR3, fludioxonil-resistant mutants, was previously reported elsewhere (Li and Xiao, 2008). Further characterization of these mutants performed in this study is summarized as follows:

(A) Reduced growth rate of FR2 and FR3 on normal medium: FR2 and FR3 mutants showed reduced radial growth on PDA (w/o fludioxonil) compared to their respective parental strains, *i.e*., W1 and W2, respectively (FR2: 11-17% reduction; FR3: 25-30% reduction). Results indicated that FR2 and FR3 possessed inherently lower hyphal-growth activity.

(B) Fludioxonil resistance of FR3: When grown on fludioxonil-containing medium, FR3 showed higher resistance to fludioxonil than W2. FR3 showed only a 3-14% growth reduction as compared to a 17-100% growth reduction of W2 on media containing 0.02-1.0 mM fludioxonil compared to W2 controls. Interestingly, the growth rate of FR3 exposed to fludioxonil always exceeded that of FR3 controls [*i.e.*, 19-35% higher radial growth with fludioxonil (0.02-1 mM) than without fludioxonil].

(C) Fludioxonil resistance of FR2: FR2 also showed higher fludioxonil resistance than W1, with FR2 showing a 21-38% growth reduction compared to a 66-100% reduction of W1 under fludioxonil treatments (0.02-1 mM) compared to growth of W1 controls. However, unlike FR3, the growth rate of FR2 exposed to fludioxonil was always lower than that of untreated FR2 [4-25% lower growth exposed to fludioxonil (0.02-1 mM) compared to control FR2]. Sporulation of fludioxonil treated FR2 was very poor, whereas that of fludioxonil treated FR3 was normal. Hence, though both strains exhibited resistance to fludioxonil, different mechanisms were probably involved between the strains.

Differential responses of* P. expansum *mutants to oxidizing agents. We hypothesized that the mechanism(s) of fludioxonil resistance in *P. expansum* FR2/FR3 mutants resulted from a mutation in the HK-MAPK signaling system. Some fungi having mutations in certain MAPK genes can escape toxicity of fludioxonil. We observed in FR2/FR3 the characteristics of fungi having a mutation in the HK signaling system, showing reduced hyphal growth while resistant to fludioxonil (Hagiwara *et al*. 2007). Since the HK-MAPK pathway is the key signaling system for fungal defense against environmental stresses, such as oxidative stress, we reasoned that the FR2/FR3 mutants should be hypersensitive to exogenously applied oxidizing agents.

Menadione, a redox cycling quinone, is a source of toxic superoxide radicals (Castro *et al*. 2007; Fernandes *et al*. 2007). FR3 was approximately twice as sensitive to menadione than the parental W2 strain (MICs for menadione: 0.256 mM < W2 < 0.512 mM *vs.* 0.128 mM < FR3 < 0.256 mM; **Fig. 3**). FR3 was also more sensitive to other oxidizing agents, H_2O_2 (3 mM) or diamide (3.5 mM; thiol-oxidizing agent) than W2 (**Fig. 3**).

Interestingly, the response of the FR2 mutant to the oxidizing agents was directly opposite to that of the FR3 mutant. Unlike FR3, FR2 was almost twice as tolerant to menadione than its parental strain, W1 (MICs for menadione: 0.128 mM < W1 < 0.256 mM *vs.* 0.256 mM < FR2 < 0.512 mM; **Fig. 3**) and was also relatively tolerant to H_2O_2 (at 4 mM), where W1 showed no growth (**Fig. 3**), or diamide (at 2.5 mM) (FR2: 29% less growth than FR2 w/o diamide; W1: 42% less growth than W1 w/o diamide; **Fig. 3**).

The hypersensitivity of the FR3 mutant to oxidizing agents, in addition to characteristic reduced hyphal-growth, indicates the HK-MAPK oxidative stress response pathway was defective in this strain. Since fungicidal activity of fludioxonil is exerted through a normal/functional MAPK system, mutation of the HK-MAPK pathway may explain why the FR3 strain was able to escape the mode of action of fludioxonil (Kojima *et al*. 2004).

On the other hand, FR2 appeared to represent a gain-of-function phenotype. This strain showed increased tolerance to the oxidizing agents. This increased tolerance suggests an increased antioxidative capacity that possibly could ameliorate cellular redox fluctuations under fludioxonil treatment. However, the energy expenditure for heightened antioxidative activity, under conditions without stress, may explain the lower hyphal growth of FR2. We previously observed that overexpression of the antioxidation gene *sodA*, encoding mitochondrial superoxide dismutase (Mn-

SOD) of *A. flavus*, in the *S. cerevisiae* wild type strain resulted in reduced growth on normal growth medium (*i.e*., w/o oxidative stress) (Kim *et al*. 2006). Collectively, our results indicate that disruption of normal cellular redox homeostasis, either through up- or down-regulation of antioxidation activity, can be at least one mechanism of fludioxonil resistance in *P. expansum*.

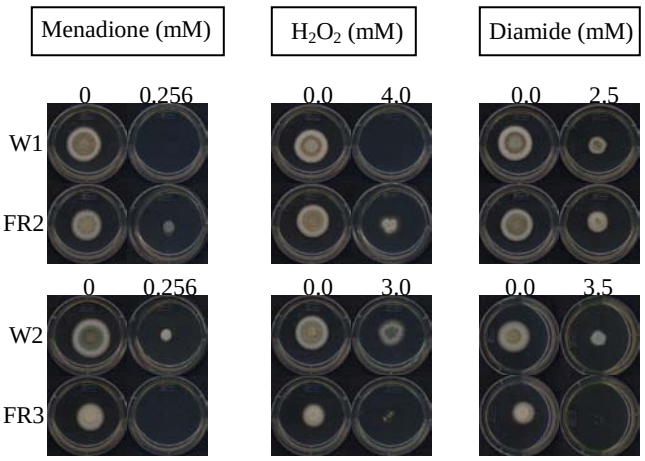

Figure 3. Differential responses of *Penicillium expansum* parental strains, W1 and W2, and respective fludioxonil-resistant mutants, FR2 and FR3, to oxidizing agents, menadione, hydrogen peroxide (H_2O_2), or diamide.

Chemosensitization of fludioxonil resistant strains using redox-active natural compounds. Chemosensitization using redox-active natural compounds, such as thymol or 2,3-dihydroxybenzaldehyde (2,3-D), was found to be effective in overcoming fungal resistance to conventional antifungal drugs (Kim *et al*. 2008a,b). We examined the chemosensitizing activity of octylgallate, thymol and 2,3-D in co-applications with fludioxonil. Growth of FR2, FR3 and their parental strains was almost completely inhibited when fludioxonil (0.02 or 0.06 mM) was co-applied with 0.15 mM of octylgallate (**Fig. 4**). Thymol or 2,3-D also exhibited some chemosensitizing activity to fludioxonil in the *P. expansum* strains. However, this activity for 2,3-D was negligible in the mutant strains (**Table 1;** See below).

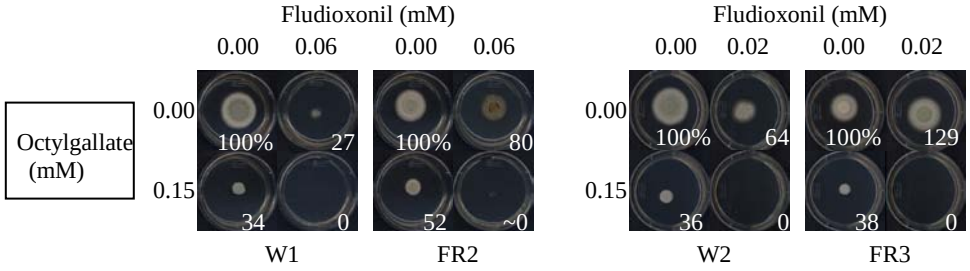

Figure 4. Representative bioassay showing growth of FR2, FR3 (fludioxonil-resistant mutants of *P. expansum*) and their respective parental strains, W1 and W2, in co-applications of fludioxonil (0.02 or 0.06 mM) and octylgallate (0.15 mM). (See also Table 1). Note: Radial growth rates of each strain w/o any treatment was considered as 100% growth (control), and the relative growth rate in each treatment was determined accordingly (SD < 5%).

Chemosensitization of* P. expansum *to a mitochondrial respiration inhibitor. The responses of FR2 and FR3 to the oxidizing agents (See **Fig. 3**) showed the redox homeostasis systems of these mutants were abnormal. We decided to further investigate the responses of these mutants to another fungicide, strobilurin (kresoxim-methyl). The responses of these mutants to strobilurin treatments were not substantially different from those of the respective parental strains (**Table 1**). Hence, there was no indication the mutant strains were resistant to this fungicide. The mode of action of strobilurin is different from that of fludioxonil. Strobilurin inhibits complex III of the mitochondrial respiratory chain, resulting in a disruption of energy production. Coinciding with this disruption is an abnormal

release of electrons that additionally damages cellular components by oxidative stress. Mn-SOD plays an important role in protecting cells from such oxidative damage. We were interested to see if co-applying redox reactive chemosensitizing agents, targeting Mn-SOD, could augment the fungicidal effects of strobilurin. As shown in **Table 1**, octylgallate (as low as 0.05 mM) in combination with strobilurin (0.02 mM), resulted in complete growth inhibition of both parental and mutant strains of *P. expansum*. This chemosensitization also occurred with co-application of 2,3-D or thymol (**Table 1**).

Table 1. Percent growth of strains of *Penicillium expansum*, fludioxonil-resistant mutants (FR2 and FR3) and respective parental strains (W1 and W2), to fungicides fludioxonil (Flud) and strobilurin (kresoxim-methyl) (Kre-Me) and redox-active natural phenolics, alone and in combination (co-applied). The phenolics include octylgallate (OcGal), 2,3-dihydroxybenzaldehyde (2,3-D) and thymol (Thy). Numbers in parentheses are concentrations (mM) of each compound used, and percent numbers (%) are relative growth rate (radial growth) of fungi compared to "no treatment" controls of each strain (=100%) (SD < 5%). *P. expansum* strains were grown at 28°C (82.4°F) on potato dextrose agar. Growth was observed for 3 to 7 days.

	Fungicide alone	Chemosensi-tizer alone	Co-applied		Fungicide alone	Chemosensi-tizer alone	Co-applied
W1	Flud (0.06) 27%	OcGal (0.15) 34%	0%	**W2**	Flud (0.02) 64%	OcGal (0.15) 36%	0%
	Flud (0.06) 14%	2,3-D (0.20) 71%	~0% (few colonies)		Flud (0.04) 78%	2,3-D (0.30) 75%	0%
	Flud (0.04) 44%	Thy (0.60) 50%	0%		Flud (0.04) 26%	Thy (0.20) 58%	0%
	Kre-Me (0.02) 54%	OcGal (0.05) 69%	0%		Kre-Me (0.02) 52%	OcGal (0.05) 73%	0%
	Kre-Me (0.02) 50%	2,3-D (0.15) 81%	0%		Kre-Me (0.02) 54%	2,3-D (0.20) 79%	0%
	Kre-Me (0.02) 44%	Thy (0.60) 40%	0%		Kre-Me (0.02) 47%	Thy (0.40) 50%	~0% (few colonies)
FR2	Flud (0.06) 80%	OcGal (0.15) 52%	~0% (few colonies)	**FR3**	Flud (0.02) 129%	OcGal (0.15) 38%	0%
	Flud (0.06) 78%	2,3-D (0.20) 70%	48%		Flud (0.04) 119%	2,3-D (0.30) 85%	96%
	Flud (0.06) 86%	Thy (0.60) 36%	~0% (few colonies)		Flud (0.04) 135%	Thy (0.20) 83%	~0% (few colonies)
	Kre-Me (0.02) 59%	OcGal (0.05) 77%	0%		Kre-Me (0.02) 67%	OcGal (0.05) 79%	0%
	Kre-Me (0.02) 59%	2,3-D (0.15) 77%	0%		Kre-Me (0.02) 65%	2,3-D (0.20) 82%	0%

	Kre-Me (0.06) 41%	Thy (0.40) 41%	~0% (few colonies)	Kre-Me (0.02) 61%	Thy (0.20) 83%	~0% (few colonies)

Chemosensitization of* P. expansum *with newly identified cinnamaldehyde. We recently identified cinnamaldehyde as a potent antifungal agent. In combination with thymol, additive antifungal effect was identified in *P. expansum* NRRL974.

Table 2 . Ranges of Minimum Inhibitory Concentrations (MICs) of thymol and cinnamaldehyde (mM), tested alone or in combination against *Penicillium expansum*. Compound interactions were determined as Fractional Inhibitory Concentrations (FIC)[a].

Compounds	MIC: alone	MIC: combined	FIC
	P. expansum NRRL974		
Cinnamaldehyde	$0.4 < n_1 < 0.8$ $0.4 < n_2 < 0.8$	$0.2 < n_1 < 0.4$ $0.0 < n_2 < 0.1$	0.625 additive

[a]Compound interactions were determined as described by Isenberg as follows: FIC (Fractional Inhibitory Concentration) = (MIC of compound A in combination with compound B / MIC of compound A alone) + (MIC of compound B in combination with compound A / MIC of compound B alone). Compound interactions: synergistic (FIC index ≤ 0.5), additive ($0.5 <$ FIC index ≤ 1), neutral ($1 <$ FIC index ≤ 2) or antagonistic ($2 <$ FIC index). n_1, MIC of cinnamaldehyde alone. n_2, MIC of thymol alone. n_3, MIC of cinnamaldehyde in combination with thymol. n_4, MIC of thymol in combination with cinnamaldehyde.

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EXECUTIVE SUMMARY:

During last three years, we identified a potentially effective approach to fungal control using newly discovered natural compounds that have a target-specific basis of activity, as follows:

(1) Identify the most efficient molecular targets: Antioxidative stress response and cell wall integrity systems of fungi can be an efficient molecular target of phenolics for pathogen control.

(2) Determine an effective method for delivery of newly discovered natural compounds: Results also show certain natural compounds are effective synergists to commercial fungicides and can be used for improving control of fungal pathogens. We proved positive interaction between phenolics and conventional fungicides significantly augment the fungicidal effects of commercial fungicides by reducing the costs of application or contamination of the environment.

(3) Overcome the tolerance of *Penicillium expansum* to fludioxonil and strobilurin through chemosensitization by using natural compounds: Certain safe natural phenolic compounds have the potential to serve as potent chemosensitizing agents to enhance activity of conventional antifungal drugs or commercial fungicides. We demonstrated how a number of phenolic compounds greatly improved effectiveness of fludioxonil and activated a process for overcoming fludioxonil-resistance (**Fig. 5**). These compounds also greatly enhanced the activity of strobilurin. Our results indicate this enhanced activity is from disruption of cellular redox homeostasis by targeting the antioxidative stress response systems (*e.g*., Mn-SOD) with redox-active natural compounds. Chemosensitization by safe, natural compounds can lower effective dosages of conventional antifungal drugs and fungicides.

In conclusion, our data proved the effectiveness of targeting cellular stress response system such as oxidative stress response or cell wall construction/integrity pathway for control of fungi. Results also show certain natural compounds are effective synergists to commercial fungicides and can be used for improving control of food-contaminating pathogens. Use of such compounds for fungal control reduces environmental and health risks associated with commercial fungicides, and lowers cost for control and the probability for development of resistance to these fungicides.

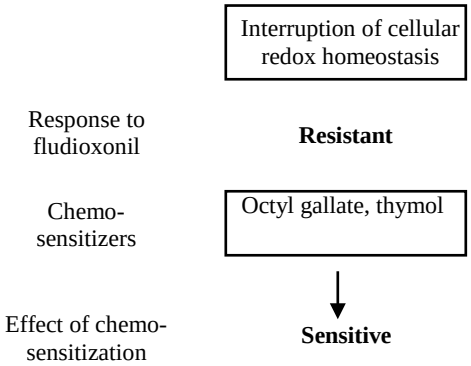

Figure 5. Diagram showing chemosensitizing effects of safe, natural compounds, which enhance antifungal activities of and/or overcome fungal resistance to conventional fungicides such as fludioxonil.