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Controlling food-contaminating fungi by targeting their antioxidative stress-response system with natural phenolic compounds

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Abstract The antioxidative stress-response system is essential to fungi for tolerating exposure to phenolic compounds. We show how this system can be targeted to improve fungal control by using compounds that inhibit the fungal mitochondrial respiratory chain. Targeting mitochondrial superoxide dismutase with selected phenolic acid derivatives (e.g., vanillyl acetone) resulted in a 100- to 1,000-fold greater sensitivity to strobilurin or carboxin fungicides. This synergism is significantly greater with strobilurin than with carboxin, suggesting that complex III of the mitochondrial respiratory chain is a better target than complex II for fungal control, using phenolics. These results show certain natural compounds are effective synergists to commercial fungicides and can be used for improving control of food-contaminating pathogens. These results suggest that the use of such compounds for fungal control can reduce environmental and health risks associated with commercial fungicides, lower cost for control, and the probability for development of resistance.

Introduction

Natural compounds have the potential to serve as alternatives to many conventional antimicrobial agents. Recent studies demonstrated the effectiveness of certain natural compounds such as derivatives of benzoic or cinnamic acid as antifungal or antimycotoxigenic agents (Tawata et al.

1996; Florianowicz 1998; Beekrum et al. 2003; Kim et al. 2004; Mahoney and Molyneux 2004). Moreover, many fungi-toxic phenolics are actually produced or released by the host plant during fungal infection. The fact that fungi must detoxify these compounds to be infective demonstrates how such natural compounds can serve as potential sources of antimicrobial agents.

The yeast *Saccharomyces cerevisiae* Meyen ex. E.C. Hansen can serve as a model system for examining the functional basis of antifungal compounds. This usefulness is mainly because the entire genome of *S. cerevisiae* has been sequenced and well annotated (viz., ~6, 000 open reading frames). Deletion mutants of *S. cerevisiae*, wherein individual open reading frames are deleted (Winzeler et al. 1999), can assist in ascertaining target genes for control of fungi (Tucker and Fields 2004; Parsons et al. 2004) in the view that many genes in yeast are orthologs of genes of fungal pathogens. Using yeast deletion mutants, we recently confirmed structural homology of several signal transduction and antioxidative stress-response genes between *S. cerevisiae* and the aflatoxigenic filamentous fungus *Aspergillus flavus* Link (Kim et al. 2005). Signal transduction and stress-response genes of fungal pathogens are known to play important roles for virulence and pathogenesis (Hamilton and Holdom 1999; Clemons et al. 2002; Lopez-Malo et al. 2002; Garrido et al. 2004; Yamauchi et al. 2004). Hence, the pathway(s) in which these genes play a role can potentially serve as a promising molecular target for control of fungal pathogens.

In this investigation, we examine the potential to target these genes in a number of food-contaminating fungal pathogens using *S. cerevisiae* as a model. Results support development of a target-specific strategy (e.g., the antioxidative response system) for an effective, safe, and economic approach to fungal pathogen control. Moreover, our data show that antifungal activity of known commercial fungicides can be greatly enhanced if used in combination with certain natural compounds.

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Materials and methods

Microorganisms

Strains of *S. cerevisiae*, i.e., wild-type (BY4741) and 43 deletion mutants having defects in antioxidative stress-response genes, used in this study were described previously (Kim et al. 2005). *Aspergillus niger* NRRL 326, *A. flavus* NRRL 3357, and *Penicillium expansum* NRRL 974 were obtained from the National Center for Agricultural Utilization and Research, USDA-ARS, Peoria, IL. Field isolates of other pathogenic filamentous fungi were a mixture of those contaminating walnut kernels, mainly species of *Aspergillus* and *Penicillium*. Contaminated walnuts were provided by the Walnut Marketing Board, Sacramento, CA.

Table 1a Effects of varying concentrations (mM) of the five most active phenolics selected from those tested (see text) on growth of *Aspergillus flavus*, *Aspergillus niger*, and *Penicillium expansum*^a

	Fungal species		
	<i>A. niger</i> NRRL 326	<i>A. flavus</i> NRRL 3357	<i>P. expansum</i> NRRL 974
Phenolic (mM)			
No treatment			
0.0	100	100	100
Salicylic acid			
0.5	100	100	99
1.0	100	100	96
5.0	99	88±5	64
≥10	0	0	0
Thymol			
0.5	85	90±7	79
1.0	32±12	50±6	49±3
≥5.0	0	0	0
Vanillyl acetone			
0.5	100	100	99
1.0	100	100	96
5.0	98	99	88
10	55±2	70	66
15	15	39	32
≥25	0	0	0
Vanillin			
0.5	100	100	99
1.0	100	100	93
5.0	95	36	66
≥10	0	0	0
Cinnamic acid			
0.5	100	100	100
1.0	100	100	91
5.0	65±2	8±14	0
≥10	0	0	0

^aGrowth of fungi is expressed as a percentage of radial growth of the fungal culture of treated compared to controls (no treatment). Values are means of three replicates; standard deviations of all measurements were less than 2% except where noted

In vitro susceptibility bioassays

For yeast bioassays, wild-type or mutant yeast cells ($\sim 1 \times 10^6$) cultured in yeast extract, peptone, and dextrose (YPD) (1% Bacto yeast extract, 2% Bacto peptone, and 2% glucose) liquid medium were serially diluted, by ten-fold increments, five times to yield aliquots of $\sim 10^6 \sim 10$ cells in SG (0.67% yeast nitrogen base without amino acids, 2% glucose with appropriate supplements: 0.02 mg/ml uracil and 0.03 mg/ml amino acids) liquid medium. The cells from each serial dilution were spotted adjacently on SG agar medium incorporated with each compound to be tested (i.e., fungicides and/or phenolic agents). The appearance of colonies from each of the dilutions was assessed after incubating plates in the dark at 30°C for 7 days.

For assays of filamentous fungi, *A. niger* NRRL 326, *A. flavus* NRRL 3357, and *P. expansum* NRRL 974 (~ 200 spores) were diluted in phosphate-buffered saline (PBS) and spotted on the center of potato dextrose agar (PDA) plates containing test phenolic agents and/or carboxin/strobilurin fungicides. Phenolics tested (italicized numbers correspond to position on aromatic ring) were as follows: vanillin (*1*: -CHO; 3: -OCH₃; and 4: -OH) and cinnamic acid (*1*: -CH=CH-COOH) and their structural derivatives, i.e., vanillic acid (*1*: -COOH; 3: -OCH₃; and 4: -OH), vanillyl acetone (*1*: -CH₂-CH₂-CO-CH₃; 3: -OCH₃; and 4: -OH), *o*-coumaric acid (*1*: -CH=CH-COOH and 2: -OH), *m*-coumaric acid (*1*: -CH=CH-COOH and 3: -OH), *p*-coumaric acid (*1*: -CH=CH-COOH and 4: -OH), caffeic acid (*1*: -CH=CH-COOH; 3: -OH; and 4: -OH), ferulic acid (4-hydroxy-3-methoxycinnamic acid; *1*: -CH=CH-COOH; 3: -OCH₃; and 4: -OH), gallic acid (3,4,5-trihydroxybenzoic acid; *1*: -COOH; 3: -OH; 4: -OH; and 5: -OH), methyl and decyl gallic acid, salicylic acid (2-hydroxybenzoic acid; *1*: -COOH; and 2: -OH), thymol (2-isopropyl-5-methylphenol; 2: -CCH₃CH₃; 3: -OH; and 5: -CH₃). A more detailed examination of the synergistic inhibition of fungal growth was performed with carboxin or strobilurin incorporated into the medium with vanillyl acetone (See Table 1a and 1b for concentrations used). Compounds were obtained from Sigma (St. Louis, MO) and dissolved in dimethylsulfoxide (DMSO) before use. Colony size was measured after growing 7–10 days at 28°C.

Results and discussion

Antifungal activities of phenolic agents against food-contaminating pathogens

In preliminary assays, salicylic acid, thymol, vanillyl acetone, vanillin, and cinnamic acid completely inhibited the germination of the guild of fungi contaminating walnut kernels. The remaining compounds among the 14 tested showed there were moderate to low levels of antifungal activity. Observed structure–activity relationships showed that cinnamic acid and vanillyl acetone/vanillin had higher

Table 1b Effects of different concentrations of fungicides (μM), carboxin, and strobilurin combined with vanillyl acetone (mM) on growth of the fungal species *P. expansum* (*Pe*), *A. niger* (*An*), and *A. flavus* (*Af*)^a

Fungicide (μM)	Vanillyl acetone (mM)														
	0			5			10			15			20		
	<i>Pe</i>	<i>An</i>	<i>Af</i>	<i>Pe</i>	<i>An</i>	<i>Af</i>	<i>Pe</i>	<i>An</i>	<i>Af</i>	<i>Pe</i>	<i>An</i>	<i>Af</i>	<i>Pe</i>	<i>An</i>	<i>Af</i>
None															
0	100	100	100	80	100	100	64	60	91	40	19	58	24	0	29
Carboxin															
50	75	100	98	60	89	91	29	32	64	~0	17	29	0	0	0
100	71	100	96	52	83	85	19	24	47	0	0	17	0	0	0
150	52	100	96	~0	70	70	0	20	32	0	0	0	0	0	0
Strobilurin															
50	71	100	100	24	28 ^b	62	0	0	18	0	0	0	0	0	0
100	69	100	98	0	0	55	0	0	0	0	0	0	0	0	0
150	63	100	96		ND ^c										

~0 Almost no growth was observed

^aResponses for growth of fungi as a percentage of radial growth of the fungal culture of treated compared to control (no inhibitors of mitochondrial respiratory chain). Values are means of three replicates; standard deviations of all measurements were less than 2% except where noted

^bSD=17%

^cNot detected due to precipitation

activities in comparison to their structural derivatives *o*-, *m*-, and *p*-coumaric acid or vanillic acid (data not shown).

Because of the above preliminary results, we focused on the five compounds (salicylic acid, thymol, vanillyl acetone, vanillin, and cinnamic acid) most active against *A. niger*, *A. flavus*, and *P. expansum*. All five compounds showed somewhat similar activity in inhibiting the growth of *A. niger*, *A. flavus*, and *P. expansum* at the concentrations tested, with thymol showing the highest activity (i.e., complete inhibition of growth at 5 mM) (Table 1a). *A. niger* was slightly more tolerant to cinnamic acid compared with the other two fungi. In a prior study, it was shown that salicylic acid attenuated virulence of the bacterial pathogen *Staphylococcus aureus* in producing endovascular infections. This loss of virulence was a result of salicylic acid activating the stress-response gene *sigB*, thereby targeting the global regulatory pathways of this bacterium (Kupferwasser et al. 2003). In our study, salicylic acid and thymol completely inhibited growth of the wild-type and the 43 deletion mutants of yeast at 2.5 mM (data not shown).

Responses of fungal pathogens and *S. cerevisiae* mutants to inhibitors of mitochondrial respiration

Because we were targeting the fungal antioxidative stress-response system with phenolic compounds, we selected two fungicides, carboxin and strobilurin, that are known to disrupt mitochondrial respiration. Our hypothesis was that treatment with phenolics would synergize the activity of these fungicides. Carboxin inhibits complex II (succinate dehydrogenase; EC 1.3.99.1) of the mitochondrial respiratory chain, resulting in succinate accumulation/inhibition

of succinate oxidation in cells, since succinate cannot be further metabolized. It is estimated that there is only moderate potential for fungi to develop resistance to carboxin (<http://www.frac.info>; target site of mutation for specific fungi is H257L). The molecular target for strobilurin-related fungicides such as azoxystrobin or kresoxim-methyl is the mitochondrial respiratory *bc1* complex (complex III; ubiquinol-cytochrome *c* oxidoreductase, EC 1.10.2.2). These fungicides specifically bind to the Q_p (Q_o) center of cytochrome *b* (Hnatova et al. 2003; Zheng et al. 2000) and inhibit the functioning of the respiratory chain. This inhibition eventually leads to cellular oxidative stress caused by an abnormal release of electrons from the respiratory chain. It is estimated that there is a high potential for fungi to develop resistance to strobilurin (<http://www.frac.info>; target sites for mutations in various fungi are G143A, F129L, and additional mechanisms).

Our in vitro susceptibility bioassay of yeast mutants showed that *sod1* Δ , *sod2* Δ , and *vph2* Δ were especially hypersensitive to carboxin being more than 100 times more sensitive compared to the wild-type (data not shown). All three of these genes are involved in the antioxidative stress-responses of yeasts. Cu, Zn-SOD (Sod1), which resides mainly in the cytosol, plays a key role in antioxidative stress responses in cells (Zelko et al. 2002). Mn-SOD (commonly named Sod2) is located in the mitochondrial matrix and detoxifies superoxide radicals generated by the mitochondrial respiratory chain. The *vph2* Δ yeast mutant lacks the V-ATPase assembly protein and is dysfunctional in transporting toxic compounds into vacuoles. Acidification, mediated by V-ATPases, is necessary for accumulation of ions and metabolites, such as Ca⁺⁺, amino acids, etc., in vacuoles (Nelson 1992). Vacuolar compartmentalization of toxic substances (e.g., xenobiotics using vacuolar

transporters and ATPase) is a well-known detoxification mechanism in fungi (Dietz et al. 2001; Hamilton et al. 2002), and mitochondrial functioning/respiration is debilitated in the absence of the *vph2* gene (Ammar et al. 2000).

No discernable differences between wild-type and gene deletion mutants of *S. cerevisiae* were seen when treated with strobilurin alone. However, there was clear synergism of antifungal activity of this fungicide when applied in combination with certain phenolic compounds (e.g., vanillin, veraldehyde, cinnamic acid, etc; unpublished observation). Based on our previous complementation bioassay, we reasoned that this synergism was a result of the effect of these phenolics on Mn-SOD activity (see also Kim et al. 2004).

Identification of an effective antifungal target on the mitochondrial respiratory chain

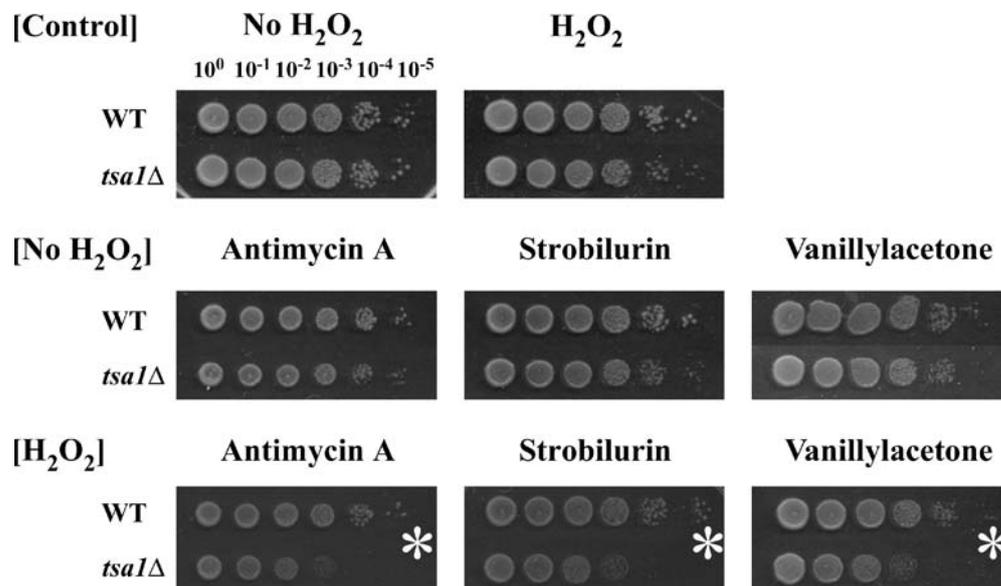
Bioassays using the *tsa1* Δ mutant of *S. cerevisiae* showed vanillyl acetone affects the normal function of mitochondria of yeast (Fig. 1; see Demasi et al. 2001 for methods). Tsa1p (thioredoxin peroxidase; cTPxI) is essential in the antioxidant defense of yeast containing dysfunctional mitochondria. Thus, deletion of the cTPxI gene renders cells more sensitive to oxidative stress under conditions where mitochondrial function is inhibited (Fig. 1). Vanillyl acetone inhibits growth of the *tsa1* Δ mutant (lacking the cTPxI gene) in the presence of hydrogen peroxide compared to the wild-type strain. This growth reduction of the *tsa1* Δ mutant indicates that vanillyl acetone inhibits normal mitochondrial function. Antimycin A and strobilurin also are known inhibitors of mitochondrial function, especially the respiratory chain, by inhibiting electron transfer at complex III. The activity of these fungicides paralleled that of vanillyl acetone. However, vanillyl acetone presumably disrupted Mn-SOD activity (Kim et al. 2004).

To target mitochondrial respiration and the antioxidative response system (e.g., Mn-SOD) for fungal pathogen control, we applied vanillyl acetone to synergize the effects of the fungicides carboxin or strobilurin. Application of vanillyl acetone enhanced the level of growth inhibition by these fungicides (Table 1b). There was greater synergism in the activity of strobilurin than that of carboxin by vanillyl acetone. This result suggests that complex III of the respiratory chain is an efficient target for fungal control. Thus, using vanillyl acetone as a synergist to these fungicides may significantly reduce potential for development of resistance to these types of fungicides that inhibit mitochondrial respiration, a frequent problem with conventional fungicides.

According to prior complementation assays using the *sodA* of *A. flavus* in a mutant of *S. cerevisiae* having the orthologous gene deleted (Kim et al. 2004), oxidative stress, triggered by vanillyl acetone, appears to be the major cause of toxicity to fungi. Vanillyl acetone is significantly more toxic to the *sod2* Δ deletion mutant of yeast than to the wild-type. Expression of *A. flavus sodA* in the *sod2* Δ yeast mutant recovered tolerance to vanillyl acetone equivalent to that of the wild-type. This recovery indicates that vanillyl acetone disrupts normal mitochondrial function resulting from enhanced oxidative stress caused by reduced Mn-SOD activity. The combined treatment of strobilurin and vanillyl acetone may overwhelm Mn-SOD activity. Mn-SOD would be required to detoxify both abnormally released electrons from the respiratory chain (strobilurin) and induced oxidative stress (vanillyl acetone).

We conclude that natural compounds such as phenolic agents that do not have any significant medical or environmental shortcomings can be useful in control programs involving conventional antifungal or antimycotoxic agents. Moreover, they can significantly augment the utility of commercial fungicides by reducing costs of applica-

Fig. 1 Yeast serial dilution bioassays of wild-type and the *tsa1* Δ strain of yeast. The assays show vanillyl acetone inhibits mitochondrial function. WT indicates wild-type; H_2O_2 , 0.35 mM; antimycin A, 50 μ g/ml; strobilurin, 50 μ M; and vanillyl acetone, 10 mM; *indicates growth inhibition



tion, potential for development of resistance, and concerns over environmental safety.

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