Prevention of Preharvest and Postharvest Fungal Infection in Almonds by Application of Natural Compounds as Chemosensitizers

Project No.: **09-PATH8-Campbell**

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Objectives:

1. Identify natural compounds highly effective as antifungal or anti-mycotoxigenic.
2. Identify the most efficient molecular targets for newly discovered compounds using functional genomic approaches.
3. Determine an effective method for delivery of newly discovered natural compounds, leading to a target-specific strategy for fungal pathogen control in the field or during processing and storage.
4. Test and compare the efficacy of treatments to control *Alternaria* leaf spot in areas with historically high levels of fungal infection

Interpretive Summary:

We have identified a number of natural antifungal compounds that effectively control fungal pathogens that infect almonds. In so doing we have discovered a number of ways to chemically enhance the activities of natural compounds that play a role in almond antifungal defenses. Using comparative genomics we have been able to identify molecular targets in these fungal pathogens so that we can use these natural compounds to promote synergistic activity when combined with commercial fungicides. For example, salicylaldehyde (SA), a volatile natural compound, exhibited potent antifungal and antimycotoxigenic activities to *Aspergillus flavus* and *A. parasiticus*. Co-application of SA with strobilurin resulted in complete growth inhibition of *Aspergillus* at much lower doses than treatment of either agent, alone. Therefore, natural compounds such as SA can enhance antifungal activity of commercial antifungal agents required to achieve effective fungal control. We are currently trying to determine an effective method for delivery of these newly discovered natural compounds so

that they can be used for almond fungal pathogen control, especially under stockpile conditions.

Materials and Methods:

1. Antifungal bioassays.

For fungal (*Aspergillus* spp., *etc.*) assays, 5 x 10^3 spores were diluted in phosphate-buffered saline (PBS) and spotted at the center of potato dextrose agar (PDA; triplicates) containing natural compounds and/or inhibitors of mitochondrial respiration, *etc*. Cell growth was monitored after 3 to 7 days at 28 ^oC. Sensitivities of fungi to the compounds was 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.

Antifungal MICs (minimum inhibitory concentrations; defined as concentrations with no visible fungal growth) and chemosensitization resulting from interactions between antifungal compounds were also determined by the broth microdilution method in liquid medium (5 x 10^3 conidia/ml inoculum) following the modified methodology published by the Clinical Laboratory Standards Institute (CLSI) M38-A (See References).

Sensitivities of *A. flavus* and *A. parasiticus* to the volatile reagent, salicylaldehyde (SA), were measured based on percent radial growth of fungal colonies as described above. SA was dissolved in DMSO (9.5 to 95 mM; Final volume: 1 ml in DMSO) and placed next to the PDA plates inoculated with fungal spores. Fungal plates and SA were placed in the same sealed plastic container (739 ml volume; see Fig. 3a), allowing SA vapors to directly contact fungal spores in a given space (vapor-agar contact method; Sekiyama *et al*. 1994), and incubated at 28 °C. The antifungal treatments, therefore, consist of: (1) Control plates: *A. flavus* or *A. parasiticus* inoculum without SA vapor (DMSO only) and (2) Treated plates: *A. flavus* or *A. parasiticus* inoculum with SA vapor. Results were means of three replicates. Growth was observed for 5 to 7 days.

To determine the chemosensitizing activity of SA, SA (12.0 or 13.5 mM) was placed next to the *Aspergillus*-inoculated PDA plates incorporated with strobilurin (Kresoxim-methyl; 25 μM) or antimycin A (10 μg/ml), which are inhibitors of mitochondrial respiration. Fungal radial growth was recorded as described above.

2. *In vitro* susceptibility bioassays using the model yeast.

The model yeast *Saccharomyces cerevisiae* was used to identify target genes and/or mode of action of natural compounds tested. Wild type or mutant yeast cells (~1 x 10^6), cultured in YPD (1% Bacto yeast extract, 2% Bacto peptone, 2% glucose) medium, was serially diluted from 10-fold to 10^5-fold in SG (0.67% Yeast nitrogen base w/o amino acids, 2% glucose with appropriate supplements: 0.02 mg/ml uracil, 0.03 mg/ml amino acids) liquid medium. The cells from each serial dilution were spotted adjacently on SG agar medium incorporated with each phenolic reagent to be tested. Numerical scoring for the sensitivity was as follows: 6-colonies are visible in all dilutions, 0- no colonies are visible in any dilution, 1- only the undiluted colony is visible, 2- the undiluted and 10-fold diluted colonies are visible, *etc.* Cells were grown at 30 ^oC for 3 to 5 days.

Sensitivities of gene deletion mutants of *S. cerevisiae* to SA (Concentrations: 0, 0.5, 0.6, 0.7, 0.8, 0.9 mM) were assessed by a yeast-cell dilution bioassay on SG agar as described above. Unlike the antifungal test for *Aspergillus* (see above), SA was directly incorporated into the agar plate (absolute DMSO amount: < 2% in media), and colony growth was monitored at 30 ^{o}C for 3 to 5 days.

S. cerevisiae wild type BY4741 (*Mat* a *his3*Δ1 *leu2*Δ*0 met15*Δ*0 ura3*Δ*0*) and selected gene deletion mutants were procured from Invitrogen (Carlsbad, CA) and Open Biosystems (Huntsville, AL), as follows: 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 (Reference for the description of each deletion mutant: www.yeastgenome.org, Accessed on July 13, 2010)

3. Analysis of aflatoxin.

Spore suspensions (200 spores) were inoculated onto the center of membrane filters (Poretics polycarbonate membrane filters, 0.2 µm pore size, 50 mm diameter; GE Osmonics, Minnetonka, MN) placed on top of the PDA. Cells were grown at 28 °C for 5 days with or without SA (9.5 mM; Control: DMSO only). SA was provided by vapor-agar contact method as described above.

Aflatoxin extracts were prepared by crushing fungal mats or agar media in 50 ml MeOH with a pestle, followed by filtration of a 1.0 ml aliquot through a 0.45 µm nylon syringe filter (Pall Co., Ann Arbor, MI). Extracts were analyzed for aflatoxins B_1, B_2, G_1, and G_2 (AFB_1, AFB_2, AFG_1, AFG_2) using a HPLC system consisting of a degasser, autosampler, quaternary pump, and fluorescence detector (Agilent 1100, Santa Clara, CA) with injection volumes of 20 µl on a 4.6 x 250 mm Inertsil 5 µm ODS-3 column (GL Sciences, Torrance, CA) and a mobile phase consisting of H_2O : CH_3CN : MeOH (45 : 25 : 30) at a flow rate of 1.0 ml/min. Fluorescence detection at 365 nm excitation and 455 nm emmission was enhanced with “PHRED” post column photochemical derivatization (Aura Industries, New York, NY). Aflatoxin standard solutions were prepared as described in AOAC 971.22 (Official Methods: See references). Quantification was linear in the range of 0.1 – 8.0 ng for AFB_1 and AFG_1 and 0.1 – 4.0 ng for AFB_2 and AFG_2, with retention times of 7.8 min for AFG_2, 8.7 min for AFG_1, 9.4 min for AFB_2, and 10.6 min for AFB_1, respectively.

4. Field trials to examine efficacy of treatments (To be conducted in the future).

Plot design will be determined based upon lab findings. Plot size will range from single trees from 1 up to 20 acres, depending on the compounds selected, rates used and their availability. Treatments will include untreated controls and 1 or 2 standard treatments of fungicide, alone. Additional treatments will then include chemosensitizing agents in combination with 2 fungicide rates, with at least 4-5 replications.

Results and Discussion:

1. Identification of new natural compounds effective for controlling almond fungal pathogens: use of a high throughput model yeast system, *Saccharomyces cerevisiae*

The model yeast *S. cerevisiae* was used in a high throughput bioassay to identify new natural compounds for control of almond fungal pathogens. *S. cerevisiae* is a useful tool for examining antifungal compounds and identifying gene targets in view that the entire genome of *S. cerevisiae* has been sequenced and well annotated. Many genes in yeast are orthologs of genes of fungal plant pathogens. Forty-four mutant strains of *S. cerevisiae* are currently chosen for analyzing sensitivity to natural compounds. These mutant strains can be categorized into five groups lacking particular functional genes in stress tolerance systems. These groups are those lacking genes for 1) signal transduction, 2) gene regulation, 3) antioxidation, 4) DNA damage control, and 5) enzymes for energy metabolism (See Materials and Methods). Use of such mutants to screen biological activity of natural compounds will also provide us insights as to mode of action of the compounds.

2. Fungal tolerance to benzo analogs depends on cellular mitochondrial superoxide dismutase (Mn-SOD) or glutathione reductase.

2-1. 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 ≥ 80 μM (microM), 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.*, aspergilli tested.

Among forty-four 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 microM) 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. Mn-SOD gene (*SOD2*) is downstream in the yeast oxidative stress response (HOG1-MAPK signaling) pathway **(Figure 1)**. It appears this gene is a promising candidate as a potential target for fungal control.

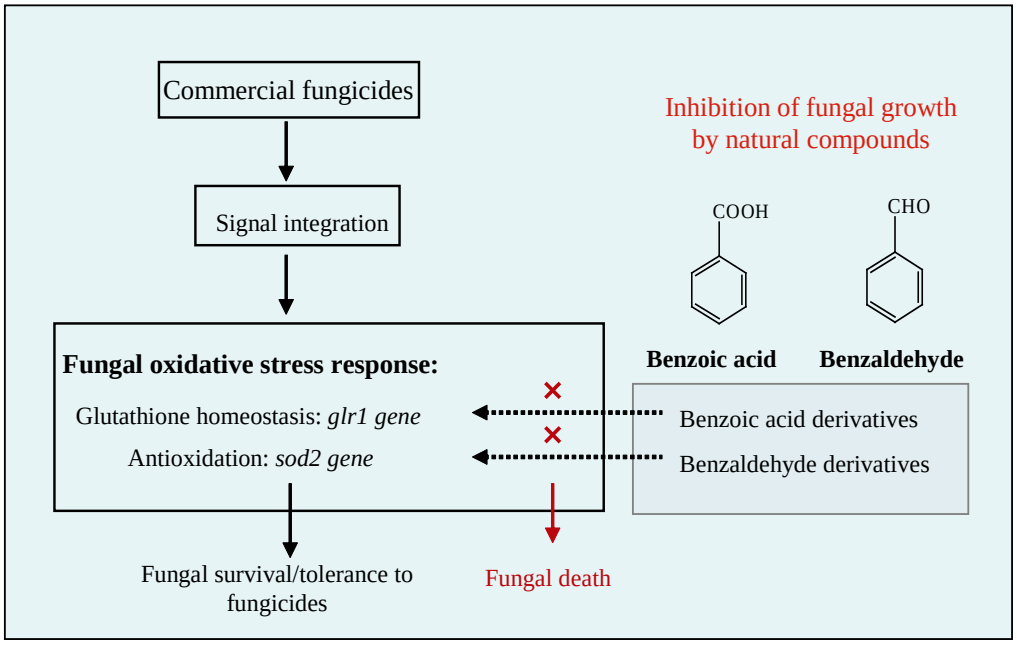

Figure 1. Strategies for targeting oxidative stress response systems of fungi using benzo analogs.

2-2. 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 ≥ 7 mM). Also, growth of *S. cerevisiae glr1*Δ (glutathione reductase deletion) mutant was inhibited by 2,3-dihydroxybenzoic acid at 4 mM. These findings suggest the mechanism of antifungal activity of 2,3-dihydroxybenzoic acid is, as with the 2,5- analog, disruption of cellular glutathione (GSH) homeostasis. Thus, the GSH reductase gene (*GLR1*), a gene also relatively downstream within the oxidative stress response (HOG1-MAPK signaling) pathway, may play an important role for fungal tolerance to this, or related, compounds (Figure 1).

2-3. The concordance of our results demonstrates there is a structure-activity relationship between the acid and aldehyde moieties in that they affect different target genes in the oxidative stress response (HOG1-MAPK signaling) pathway. The 2,3-dihydroxybenzaldehyde targeted *SOD2*. Whereas, 2,3- and 2,5- dihydroxybenzoic acids targeted *GLR1*, disrupting glutathione homeostasis (**Figure 1**).

3. Chemosensitization to conventional fungicides by 2,3-dihydroxybenzaldehyde and benzoic acid derivatives: overcoming fungal tolerance to antifungal agents by using natural compounds

Chemosensitization involves enhancing the effectiveness of antifungal agents by co-applying a second compound. The second compound does not necessarily have much antifungal potency

alone, but debilitates the ability of the fungus to launch a protective response to the antifungal agent **(**See **Figure 2).**

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. In this regard, we found MAPK mutants of *Aspergillus* 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.

4. The potential chemosensitizing effect of 2,3-dihydroxybenzaldehyde was also tested on the activity of strobilurin fungicide

Co-application of 2,3-dihydroxybenzaldehyde enhanced the antifungal activity of strobilurin against the filamentous fungi examined. Co-application of 100 or 200 microM 2,3-dihydroxybenzaldehyde to strobilurin (25 microM) 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.

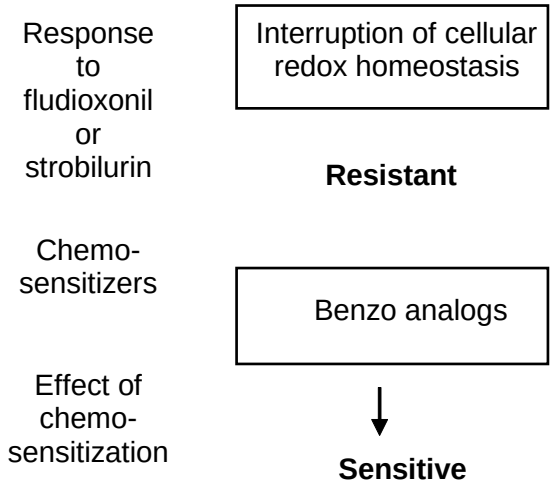

Figure 2. Diagram showing chemosensitizing effects of safe, natural compounds, which enhance antifungal activities of and/or overcome fungal resistance to conventional fungicides such as fludioxonil or strobilurin.

5. Antifungal activity of salicylaldehyde (SA)

Antifungal activity of SA was examined in *A. flavus* and *A. parasiticus* by a vapor-agar contact method described by Sekiyama *et al.* (See references) (**Figure 3a; see Figure 3b** for SA

structure). The volatile molecules of SA were self-evaporated and distributed inside the sealed plastic container (739 ml volume), resulting in direct contact to fungal spores spotted on the PDA plates (triplicate). At 9.5 mM ≤ SA ≤ 16.0 mM, the radial growth of *A. parasiticus* was gradually decreased (*i.e.*, Vincent equation: 10% - 75%; **Table 1**) as the concentrations of SA increased. Similar trends were also observed with *A. flavus* at 9.5 mM ≤ SA ≤ 13.5 mM (*i.e.,* Vincent equation: 35% - 87%; **Table 1**). Coinciding with this growth inhibition was the decrease in the formation of green pigments in fungal spores, leading to the development of pale colonies (**Figure 3c**; at 9.5 mM SA). This result indicates that SA also has an inhibitory effect on the secondary metabolism for fungal pigment formation. At 16.0 mM ≤ SA or 19.0 mM ≤ SA, the growths of *A. flavus* or *A. parasiticus*, respectively, were completely inhibited, resulting in no colony formation on PDA plates (**Table 1**). Therefore, results proved that SA could act as an effective antifungal agent, where the level of antifungal activity was commensurate with SA concentrations applied.

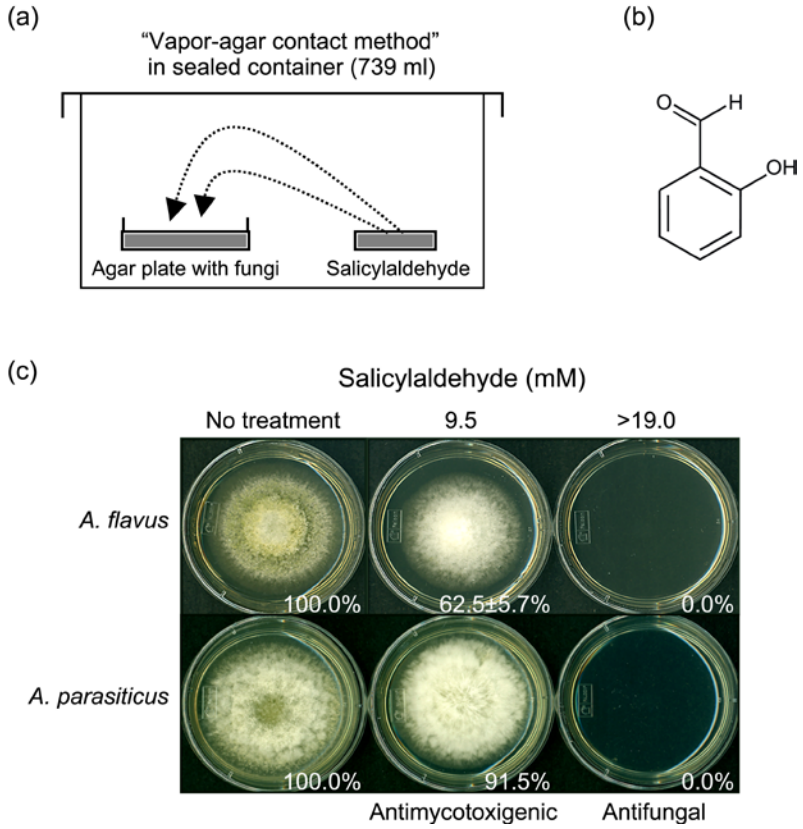

Figure 3. Antifungal and anti-aflatoxigenic activity of SA against *Aspergillus flavus* and *A. parasiticus*. **a** Vapor-agar contact method showing how fungal cells were exposed to volatile SA. **b** Chemical structure of SA. **c** Effect of different concentrations of SA on fungal growth (see also **Table 1**). Note that 9.5 mM of SA, where spores lacked pigmentation, was used for anti-aflatoxigenic assays in this study.

Table 1. Antifungal activity of salicylaldehyde (SA) volatilized in a sealed plastic container (739 ml volume).[1]

SA Conc. (mM)	0.0	9.5	10.5	12.0	13.5	16.0	19.0-95.0
Aspergillus							
A. flavus	100.0	65.2±5.7	60.9±6.5	43.5±5.7	13.0	0.0	0.0
A. parasiticus	100.0	91.5	76.6	72.3	51.1	25.5±7.4	0.0

[1]Numbers are in %, which is based on the Vincent equation (See Materials and Methods). SD < 5% except where noted.

6. SA inhibits aflatoxin production in *A. flavus* and *A. parasiticus*

We then tested the antimycotoxigenic activity of SA in *A. flavus* and *A. parasiticus*. As mentioned above, volatilized SA could inhibit the development of green pigments in fungal spores. Considering that fungal pigments are synthesized by secondary metabolism in cells, we reasoned that SA could also inhibit the secondary metabolic pathway for aflatoxin production in *Aspergillus*.

As shown in **Table 2**, the aflatoxin production in SA-exposed fungi was reduced by ~13% to 45% compared with the untreated control, depending on the types of fungi and/or types of aflatoxins synthesized. *A. flavus* produces AFB_1/B_2 (aflatoxin B_1 and B_2) only, while *A. parasiticus* produces AFG_1/G_2 (aflatoxin G_1 and G_2) as well as AFB_1/B_2. As expected, we could not detect AFG_1/G_2 in the culture of *A. flavus*, whereas the production of AFG_1/G_2 in *A. parasiticus* was reduced by 23% to 32% with the treatment of SA. Altogether, our results demonstrated that volatilized SA possesses both antifungal and anti-mycotoxigenic activities in *A. flavus* and *A. parasiticus*.

Table 2. Anti-aflatoxigenic activity of salicylaldehyde (SA; 9.5 mM)[1]

Strains	SA (mM)	Partition	AFB_1	AFB_2	AFG_1	AFG_2
A. flavus 3357	0	Fungal mat (Retained)	171.6±16.6	3.7±0.5	ND[2]	ND
		Media (Secreted)	326.5±42.5	11.8±1.8		
		Total:	498.1±56.3 (100%)	15.5±2.2 (100%)		
	9.5	Fungal mat (Retained)	148.3±8.5	3.1±0.2	ND	ND
		Media (Secreted)	283.2±25.2	10.0±1.0		
		Total:	431.5±32.4 (86.6%)[3]	13.1±1.1 (84.5%)		
A. para-siticus 5862	0	Fungal mat (Retained)	93.3±5.5	1.4+0.2	42.5±4.8	1.2±0.2
		Media (Secreted)	148.9±2.8	4.0±0.2	123.1±4.8	2.8±0.1
		Total:	242.2±3.2 (100%)	5.4±0.3 (100%)	165.6±9.3 (100%)	4.0±0.2 (100%)
	9.5	Fungal mat (Retained)	60.9±16.1	0.8±0.3	29.8±5.9	0.8±0.3
		Media (Secreted)	112.8±29.4	2.5±0.9	98.0±21.1	1.9±0.4
		Total:	173.7±45.4 (71.7%)	3.3±1.2 (54.8%)	127.8±26.8 (77.2%)	2.7±0.7 (67.5%)

[1]Unit of aflatoxins produced: μg aflatoxin/100 mg dried fungal mat. [2]ND= Not detectable. [3]Treated percentages in parentheses are relative to control percentages.

7. Target identification: Sensitive responses of *S. cerevisiae* mutants lacking genes in oxidative stress response system and vacuolar function

Using gene deletion mutants of *S. cerevisiae*, we performed yeast dilution bioassays on SG agar containing SA (see Materials and Methods). We observed the sensitive responses of the downstream antioxidation gene mutants, *i.e.*, *sod1*Δ [cytosolic superoxide dismutase (SOD) gene deletion], *sod2*Δ [mitochondrial SOD (Mn-SOD) gene deletion] and *glr1*Δ (glutathione reductase gene deletion) to SA, while none of the mutants for the upstream signal transduction pathway, *e.g.*, *yap1*Δ, controlling downstream antioxidation system, *etc.*, were sensitive to the same treatment (Fig. 4a). Sod2p and Sod1p detoxify mitochondrial and cytosolic superoxide radicals, respectively. *GLR1* encodes glutathione reductase, which converts GSSG (oxidized glutathione) to GSH (reduced glutathione: antioxidant) (See above). The *glr1*Δ mutant cannot reduce GSSG to GSH efficiently, resulting in higher sensitivity to SA. Collectively, we

hypothesized that the mechanism of antifungal action of SA is by targeting the downstream functional/structural genes in the antioxidation system (see also **Figure 4b**).

In addition, the *vph2*Δ mutant was also sensitive to SA (**Figure 4a, b**). The gene *VPH2* encodes a vacuolar ATPase (V-ATPase) assembly protein, and the *vph2*Δ mutant has dysfunctional vacuolar and/or mitochondrial respiration resulting from intracellular fluctuations of pH (Ammar *et al*. 2000). Since the *vph2*Δ mutant lacks the V-ATPase assembly protein, its cells are likely to be dysfunctional in transporting toxic compounds, such as SA, into vacuoles.

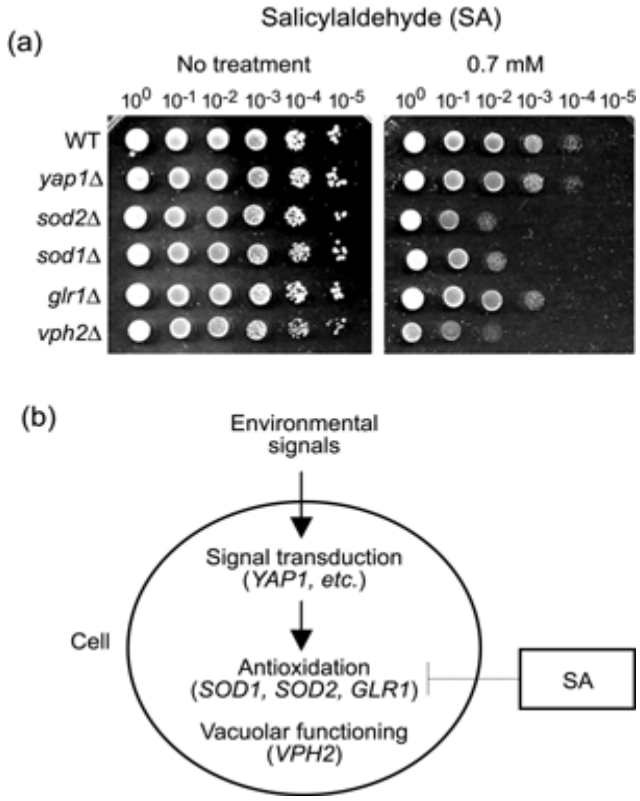

Figure 4. a Yeast dilution bioassays showing sensitive responses of *Saccharomyces cerevisiae* gene deletion mutants (*sod2*□,*sod1*□,*glr1*□,*vph2*□) to SA (representative result shown from 0.7 mM treatment). Note that *yap1*□, lacking the *YAP1* gene controlling downstream antioxidation system, was not sensitive to the same treatment (see also text). **b** Diagrammatic representation of gene targets tested in this study showing the downstream antioxidation system was affected by SA.

8. Chemosensitization by SA of strobilurin or antimycin A

Chemosensitization refers to the ability to synergistically enhance effectiveness of antimicrobial agents by co-applying a second compound. With regard to fungi, the second compound (chemosensitizing agent) debilitates the ability of the fungus to exert a protective/tolerant response to commercial, or other, antifungal agents. Such chemosensitizing agents can affect common cellular targets, such as the antioxidation system of fungi, which

results in synergistic inhibition of fungal growth. In this study, the chemosensitizing effect of SA to antimycin A or strobilurin, both inhibitors of complex III of the mitochondrial respiratory chain which disrupts energy production (Wood and Hollomon 2003), was tested. Coinciding with this disruption is an abnormal release of electrons that additionally damages cellular components by oxidative stress (Takimoto *et al*. 1999). Therefore, antioxidation enzymes such as Mn-SOD play important roles in protecting cells from such oxidative damage.

As shown in **Figure 5a**, co-application of antimycin A (10 μg/ml) or strobilurin (Kresoxim-methyl, 25 μM) with volatilized SA (as low as 13.5 mM) completely inhibited the colony formation of *A. flavus* or *A. parasiticus*, while independent treatment of each reagent at the given concentrations still allowed the growth of *Aspergillus*.

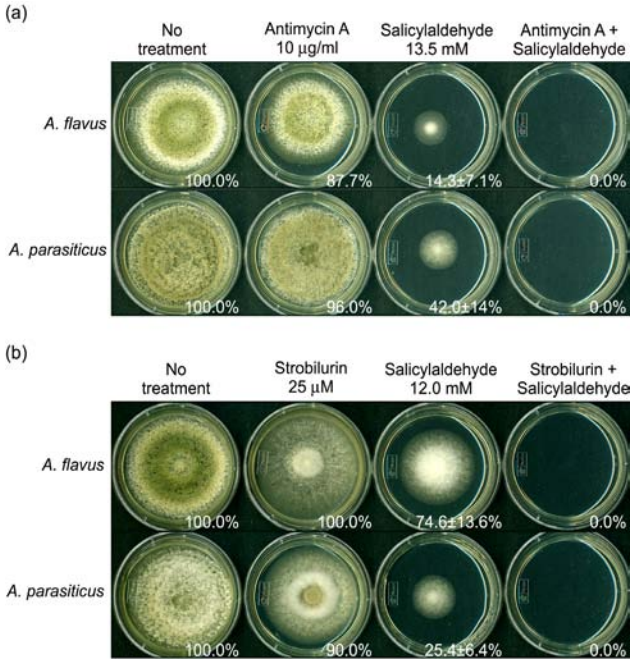

Figure 5. Chemosensitizing activity of SA to inhibitors of mitochondrial respiration complex III [*i.e.*, **a** Antimycin A (upper panel) or **b** Strobilurin (lower panel)], resulting in complete inhibition of fungal growth by co-application.

Therefore, results demonstrated the efficacy of chemosensitization strategy by using volatile SA for enhancing activities of antimycin A or strobilurin. Considering the serious mammalian cytotoxicity caused by antimycin A (through the intra-cellular production of reactive oxygen species; Takimoto *et al*. 1999), and also the increased development of fungal resistance to strobilurin (http://www.frac.info; target sites for mutations in various fungi are G143A, F129L, and additional mechanisms), our chemosensitization approach will, thus, advance effective control of fungal pathogens having either medical or agricultural importance.

Summary:

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

9-1. Identify the most efficient molecular targets: Antioxidative stress response can be an efficient molecular target of natural compounds for pathogen control.

9-2. Determine an effective method for delivery of newly discovered natural compounds: Certain natural compounds are effective synergists to commercial fungicides and can be used for improving control of fungal pathogens. Positive interaction between natural compounds and conventional fungicides significantly augment the fungicidal effects of commercial fungicides.

9-3. Overcome the tolerance of fungi to fludioxonil and strobilurin through chemosensitization by using natural compounds: A number of natural compounds greatly improved effectiveness of strobilurin and fludioxonil, and activated a process for overcoming fungicide-resistance. We also presented the potential for using SA as antifungal and/or anti-mycotoxigenic agent, and especially as a chemosensitizing agent. As shown in this study, SA can enhance activity, lower resistance, and alleviate health and environmental risks by reducing amounts of commercial antifungal agents required to achieve effective control. Further *in vivo* studies are necessary to determine if the *in vitro* activities demonstrated herein can translate to *in vivo* treatment efficacy and safety. The use of SA has promising implications for agriculture, as a safe, natural fumigant to inhibit fungal growth and/or mycotoxin production in crops, or crop products, under storage, such as stockpiled almonds.

Research Effort Recent Publications:

Campbell, B., Kim, J., Faria, N., Yu, J., Mahoney, N., Chan, K., Molyneux, R., Bhatnagar, D., Martins, M.L., Cleveland, T., Nierman, W. 2009. Use of natural compounds as anti-mycoxigenic and chemosensitizing agents. International Society for Mycotoxicology Conference 2009. September 9-11, Tulln, Austria.

Kim, J.H., Campbell, B.C., Mahoney, N., Chan, K.L., Molyneux, R.J. and Xiao C.L. 2010. Use of chemosensitization to overcome fludioxonil-resistance in *Penicillium expansum*. Lett. Appl. Microbiol. 51:177-183.

Kim, J. H., Campbell, B. C., Mahoney, N., Chan, K. L. and Molyneux, R. J. 2010. Chemosensitization of *Aspergillus* to antimycin A and strobilurin using salicylaldehyde, a volatile natural compound targeting cellular antioxidation system. Mycopathologia (Submitted).

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