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

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

Project Leader: Bruce Campbell
PMR Unit
USDA-ARS, Western Regional Research Center
800 Buchanan Street
Albany, CA 94710
(510) 559-5846
fax: (510) 559-5737
bruce.campbell@ars.usda.gov

Project Cooperators and Personnel:
Jong Kim, CO-PI; PMR Unit, Western Regional Research Center, USDA-ARS
Brad Higbee, Paramount Farming Company

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 safe, natural antifungal compounds that effectively control a wide variety of 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. 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.

Results and Discussion:

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-three 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 for stress tolerance system. 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. Use of such mutants to screen biological activity of natural compounds will also provide us insights as to mode of action of the compounds.

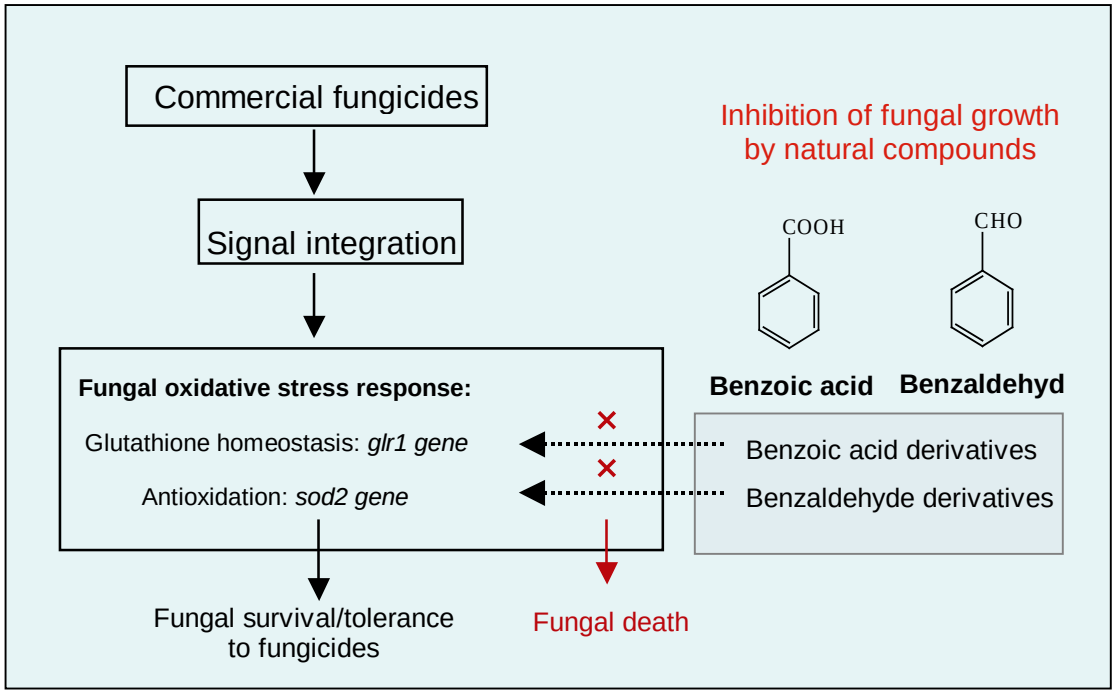

Figure 1. Strategies for targeting oxidative stress response systems of fungi using benzo analogs.

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

(a) 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 M (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-three mutants of *S. cerevisiae* examined, where genes in oxidative stress response/multidrug resistance systems were individually deleted, the *sod2*□ [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 is downstream in the yeast oxidative stress response (HOG1-MAPK signaling) pathway. It appears this gene is a promising candidate as a potential target for fungal control.

(b) 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*□ (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.

(c) 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.

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.

<u>The potential chemosensitizing effect of 2,3-dihydroxybenzaldehyde was also tested on the activity of strobilurin fungicide</u>

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.

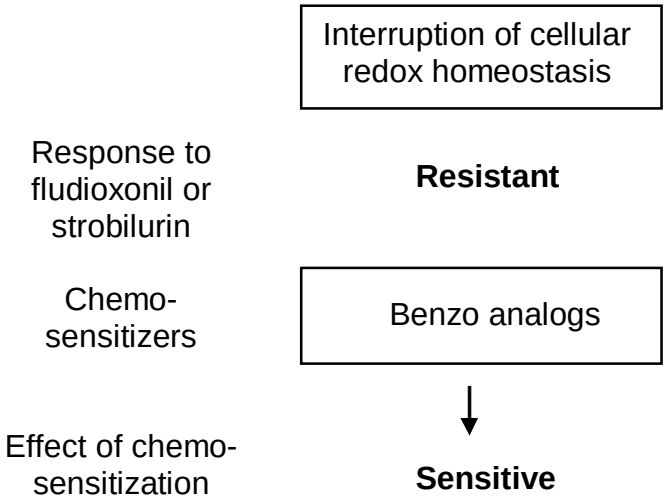

Figure 2. Diagram showing chemosensitizing effects of safe, natural compounds, which enhance antifungal activities of and/or overcome fungal resistance to conventional fungicides such as fludioxonil or strobilurin.

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:

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

(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.

(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 fludioxonil and strobilurin, and activated a process for overcoming fungicide-resistance.