AES/CE MAR 84 Workgroup/Department: Plant Mycotoxin Research Unit, WRRC, USDA-ARS

University of California
Division of Agricultural Sciences

PROJECT PLAN/RESEARCH GRANT PROPOSAL

Project Year FY2007-2008 Anticipated Duration of Project 3 to 4 Years

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Project Title Prevention of Preharvest and Postharvest Fungal Infection in Walnut Based on Antimicrobial Natural Compounds and Target Gene-Specific Control

Keywords Walnut, Fungal diseases, Pathogen control, Aflatoxin, Natural compounds, Molecular targets

Commodity(s) Walnut Relevant AES/CE Project No.

Problem and its Significance:

Infection of walnut trees by a variety of pathogenic and saprophytic fungi can result in considerably reduced viability of trees and quality of harvested tree nuts. Moreover, non-visible preharvest

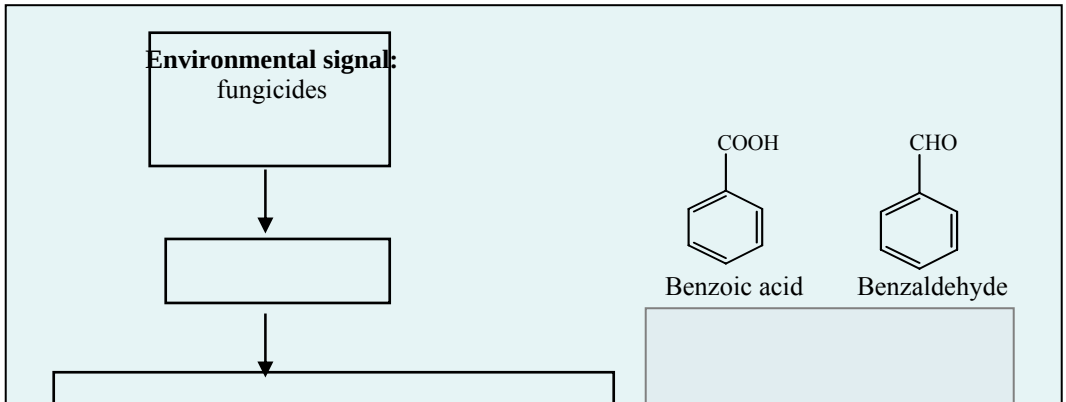
infection can result in post-harvest fungal breakouts on nuts in storage or during shipping. Significant amounts of walnuts can be made unsuitable for sale and consumption as a result of infection by a wide number of different fungi. As such, these infectious fungi have a perniciously negative effect on economic value from year to year. Moreover, infection of tree nuts by certain fungi capable of producing mycotoxins could result in economic losses and reduced exports to foreign markets due to food-safety issues.

Natural compounds can serve as promising alternatives to conventional fungicides or chemosensitizers to enhance the antifungal activities of commercial fungicides, and can be used to prevent mycotoxin biosynthesis. Although synthetic chemicals have been used to keep the quality of stored products, such antifungal reagents will be phased out eventually due to their adverse effects on human health and the environment. We recently identified a set of safe natural compounds with great promise for controlling fungal pathogens, which includes derivatives of benzaldehyde, a key component of the scent of tree nuts. We have found antioxidative stress response systems are essential for fungal tolerance to the natural compounds we tested. Caffeic acid also almost completely shuts down the expression of key genes triggering aflatoxin biosynthesis. This is a vivid demonstration of how natural compounds from plants can be useful sources of antimicrobial activities, and therefore, shows great promise for control of fungal pathogens such as aflatoxin-producing *Aspergillus* (Kim *et al*. 2006).

The potential use of such antimicrobials, which would be both environmentally and consumer safe, could occur at all stages of developing walnuts as a commodity. Natural antifungals could be applied pre-harvest, in the orchard, to prevent early infection of nut kernels. They could also be effective during processing as a means of preventing any cross-contamination between infected and uninfected walnuts. Lastly, they would have utility in preventing fungal infections under storage or shipping conditions as a back-up to maintaining appropriate temperature and humidity. The types of compounds we have discovered thus far that are effective could be applied as a mist simultaneously with applications of other commercial compounds (*i.e*., chemosensitizing activity).

Objectives:

1. Identify natural compounds highly effective as antifungal or antimycotoxigenic in walnut orchards.
2. Identify the most efficient molecular targets [*e.g*., mitochondrial superoxide dismutase (Mn-SOD), glutathione reductase, *etc*.] for newly discovered compounds using functional genomics approaches.
3. Determine an effective method for delivery of newly discovered natural compounds, leading to a target-specific strategy for an easy, safe and economic approach to fungal pathogen control in the field or during processing and storage.



Signal integration

Fungal oxidative stress response:

Glutathione homeostasis: *glr1gene*

Antioxidation: *sod2 gene*

Inhibition of fungal growth by natural compounds:

Benzoic acid derivatives

Benzaldehyde derivatives

Growth inhibition

Figure 1. Strategies for targeting antioxidative stress response systems (*i.e.*, glutathione homeostasis and mitochondrial superoxide dismutase) of fungi using benzoinc acid / benzaldehyde derivatives.

Plans and Procedures:

Methods:

All research will be conducted within the Plant Mycotoxin Research (PMR) unit at the USDA-ARS Western Regional Research Center, Albany, CA. The PMR labs are fully equipped to conduct the analytical natural product chemistry (NMR, HPLC, GC-MS, *etc*.) and molecular biological procedures (PCR, RT-PCR, nucleotide sequencing, computer algorithms for database searches, *etc*.) required to carry out the planned research. Dr. Campbell and those in his lab (*e.g.,* Dr. Kim) are approved by the USDA-ARS Institutional Bio-safety Committee for the Pacific West Area to perform procedures concerning functional and chemo-genomics on fungi, license # 7-04-4. All appropriate laboratory hazard and safety procedures have been reviewed and approved by the on-site Environmental Hazard Safety Officer.

(1) Antifungal bioassays. For fungal (*Aspergillus flavus*, *A. niger*, *Penicillium* spp., *etc.*) assays ~200 spores will be diluted in phosphate-buffered saline (PBS) and spotted in the center of potato dextrose agar (PDA) plates containing phenolic reagents and/or inhibitors of mitochondrial respiration, *etc*., and cell growth will be monitored after 5 to 7 days at 28 ^{o}C. Colony growth will be measured based on percent radial growth compared to control colonies [**Vincent equation:** % inhibition = 100 (C-T)/C, C: diameter of fungi on control plate; T: diameter of fungi on the test plate; Vincent, 1947]. Result will be means of three replicates.

For the volatile compound, antifungal activity will be monitored by the vapor-agar contact method (Sekiyama, Y. *et al.*, 1996). Fungal spores (~200) will be inoculated at the center of PDA medium, and will be incubated at 28 ^{o}C for 3 to 5 days. The antifungal treatments will consist of:

1. Control plate- *A. flavus* inoculation without treatment of volatile compound (water only)
2. Treated plate- *A. flavus* inoculation with the treatment of volatile compound. Result will be means of three replicates.

(2) *In vitro* susceptibility bioassays using the model yeast. The model yeast *Saccharomyces cerevisiae* will be used to identify the 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, will be 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 will be spotted adjacently on SG agar medium incorporated with each phenolic reagent to be tested. Numerical scoring for the sensitivity will be 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 will be grown at 30 ^oC for 7 days.

[Natural compounds tested so far in our laboratory: Veratraldehyde (1: -CHO; 3: -OCH_3; 4: :-OCH_3) and cinnamic acid (1: -CH = CH-COOH) and their phenolic compound derivatives, *i.e.*, vanillin (1: -CHO; 3: -OCH_3; 4: -OH), vanillic acid (1: -COOH; 3: -OCH_3; 4: -OH), vanillyl acetone (1: -CH_2-CH_2-CO-CH_3; 3: -OCH_3; 4: -OH), *o*-coumaric acid (1: -CH = CH-COOH; 2: -OH), *m*-coumaric acid (1: -CH = CH-COOH; 3: -OH), *p*-coumaric acid (1: -CH = CH-COOH; 4: -OH), caffeic acid (1: -CH = CH-COOH; 3: -OH; 4: -OH), benzaldehyde (1: -CHO), benzoic acid (1: -COOH), 4-hydroxybenzoic acid (1: -COOH; 4: -OH), 3-chlorobenzoic acid (1: -COOH; 3: -Cl), 3,4,5-trimethoxybenzoic acid (1: -COOH; 3:-OCH_3; 4: -OCH_3; 5:-OCH_3), ferulic acid (4-Hydroxy-3-methoxycinnamic acid; 1: -CH = CH-COOH; 3: -OCH_3; 4: -OH), *etc.*, where numbers in the parenthesis represent positions on the benzene ring; 5, 10, 15, 25mM].

(3) Testing chemosensitizing effect of natural compounds. For testing chemosensitizing/synergistic effects of the compounds, natural compounds will be added to the growth medium together with known fungicides. Colony growth will be measured based on percent radial growth compared to control colonies grown on PDA plates receiving only DMSO (Dimethylsulfoxide).

[Natural compounds tested for chemosensitization in our laboratory: cinnamic acid, 3-hydroxycinnamic acid, vanillic acid, vanillyl acetone, vanillin, or veratraldehyde, *etc.*; Fungicides tested in our laboratory: antimycin A, strobilurin (kresoxim-methyl), fludioxonil, *etc.*].

(4) Determination of inhibition of mitochondrial respiration by phenolic reagents. To confirm whether the phenolic reagents used inhibit mitochondrial respiration of fungi, we will use the *tsa1*Δ strain, a deletion mutant lacking the thioredoxin peroxidase (cTPx I) gene, of the yeast *S. cerevisiae*. Wild type yeast cells (~1 x 10^6) will be serially diluted and dispensed, as described above, onto YPD plates containing 0.35 mM H_2O_2 and phenolic reagents at given concentrations. If the growth of *tsa1*Δ mutant is inhibited, the phenolic compound will be considered to inhibit mitochondrial respiration in the model-yeast system.

Procedures & Preliminary Results:

A. Identification of new natural compounds effective for the control of walnut fungal pathogens using a high throughput model yeast system, *Saccharomyces cerevisiae*

Many natural phenolic compounds are promising antifungal agents through their ability to disrupt oxidative stress-response systems. When applied above antioxidant levels, phenolics are potent redox-cyclers that inhibit cell growth by interfering with cellular redox homeostasis (Guillen & Evans, 1994; Shvedova *et al*., 2000).

Stress-activated signal transduction pathways serve as targets for fungal control (Smits & Brul, 2005). Fludioxonil, a phenylpyrrole fungicide, blocks the protein kinase involved in glycerol biosynthesis (Rosslenbroich & Steubler, 2000). Fungal mutants having defects in certain mitogen-activated protein kinase (MAPK) genes are tolerant to fludioxonil (Kojima *et al*., 2004). The emergence of such resistance in fungi, especially by natural mutation in the target gene, is a major contamination issue in orchard. Therefore, approaches are needed to curtail development of such tolerance or resistance to this and other antifungal agents.

S. cerevisiae will be used in a high throughput bioassay to identify new natural compounds for control of walnut 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* were 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 provide us insights as to mode of action of the compounds. Several of the deletion mutants showed different levels of sensitivity to the tested compounds compared to the wild type strain of *S. cerevisiae*. Heightened sensitivity of certain genetic mutants to natural products indicates functional defects that can, therefore, show genetic and molecular targets for pathogen control. As mentioned above, many of the genes in *S. cerevisiae* are directly relatable to genes of other fungal pathogens. We have already confirmed structural homology of signal transduction and antioxidative stress genes between *S. cerevisiae* and aflatoxigeneic *A. flavus* using a recently available *A. flavus* Expressed Sequence Tag (EST) database (Kim *et al*. 2005). Functional analyses of these genes are underway to find molecular targets.

So far, we tested more than fifteen phenolic reagents (*i.e.* phenolic acids and their structural derivatives; Table 1); many of which are natural compounds found ubiquitously in a wide variety of plants, including tree nuts. Veratraldehyde, cinnamic acid, and respective benzoic acid derivatives vanillin, vanillic acid, vanillyl acetone, benzaldehyde, benzoic acid, 3-chlorobenzoic acid, 3,4,5-trimethoxybenzoic acid, and cinnamic acid derivatives *o*-, *m*- and *p*-coumaric acids, and ferulic acid, *etc*., showed significant antifungal activities (highest to lowest: cinnamic acid, vanillin, benzoic acid, 3-chlorobenzoic acid > veratraldehyde, ferulic acid > *o*-, *m*-, *p*-coumaric acids, vanillic acid, vanillyl acetone, benzaldehyde, 3,4,5-trimethoxybenzoic acid) in the yeast system with caffeic acid and 4-hydroxybenzoic acid having little to no effect. Certain of the compounds identified act as potent antifungal agents when applied individually. For example, a benzaldehyde derivative at micromolar concentrations completely inhibits the growth of fungi such as *A. flavus*. We are currently working on the identification of effective method(s) to deliver this compound to the target sites.

In our test on a fungal pathogen, antifungal activity levels against mycotoxin-producing *A. flavus* were similar to that in the model yeast. This similarity in antifungal activity demonstrated the usefulness of the *S. cerevisiae* bioassay for screening antifungal compounds against a variety of fungal pathogens (See below). Assays using deletion mutants of yeast identified signal transduction and antioxidative stress response genes important to fungal tolerance. Different structural derivatives

of natural compounds exhibit different levels of toxicity to fungal cells. Currently we are focusing on identifying additional antifungal natural compounds by using model yeast. Inclusion of additional types of mutant strains will be necessary to identify specific genes whose functions are affected by the screened compounds. Additional factors requiring consideration are: a) Optimum concentration of natural product for pathogen control, b) Potential combination of antimicrobial natural compounds to give best results (See below), c) Selection of additional target microorganisms, and d) Mode of action of compound and safety to humans, or other non-target species. Origin of the natural compounds is also an important factor in considering an effective supply (*e.g*., whether the source is a readily available animal, plant, microbe, *etc*.).

Table 1. Level of antifungal activity of phenolic reagents tested[a]

Level of antifungal activity	Criteria	Benzoic acid derivatives	Cinnamic acid derivatives
High	Completely inhibits the growth of both fungi at>5-10mM of the compound.	Vanillin (4-hydroxy-3-methoxybenzaldehyde), Benzoic acid, 3-Chlorobenzoic acid	Cinnamic acid (*trans*-3-phenylacrylic acid)
High to moderate	Completely inhibits the growth of at least one fungus at>5-10mM of the compound, and inhibit the growth of the other fungi as the concentration of compound increased.	Veratraldehyde (3,4-Dimethoxybenzaldehyde)	Ferulic acid (4-hydroxy-3-methoxycinnamic acid)
Moderate to low	Inhibits the growth of both fungi as the concentration of compound increased.	Vanillyl acetone, Vanillic acid (4-hydroxy-3-methoxybenzoic acid), Benzaldehyde, 3,4,5-Trimethoxybenzoic acid	*o*-Coumaric acid, *m*-Coumaric acid, *p*-Coumaric acid
No	Doesn't inhibit the growth of fungi at recognizable level.	4-Hydroxybenzoic acid	Caffeic acid

[a]Responses for yeast growth were represented as a logarithmic number and for growth of *A. flavus* as a percentage of radial growth of the fungal mat of treated (5, 10, 15, 25mM) compared to control (non-treated). Level was determined based on means of three replicates.

B. Effect of antifungal phenolic reagents on the growth of walnut fungal pathogens

It is very important that the identified antifungal natural compounds exhibit antimicrobial activities on a wide range of walnut fungal pathogens. To confirm the effects of our antifungal phenolic reagents on the growth of walnut fungal pathogens, fungal strains were directly isolated from heavily contaminated-walnut seed coats. Cell survivability of each fungus was tested in the presence of each natural compound. At least three compounds completely inhibit the growth of any walnut fungal pathogen (Figure 2). Other compounds showed a medium-level inhibitory effect with some interpretable basis for a structure-activity relationship. To confirm the "fungus-compound" specificity, antifungal activity of each compound needs to be tested with pure isolation of fungal species (*e.g., Penicillium, A. niger, A. flavus/A. parasiticus, Armillaria* spp.*, Phytophthora* spp.*, Alternaria* spp.*, Cladosporium, Rhizopus, etc.*) from the contaminated walnuts.

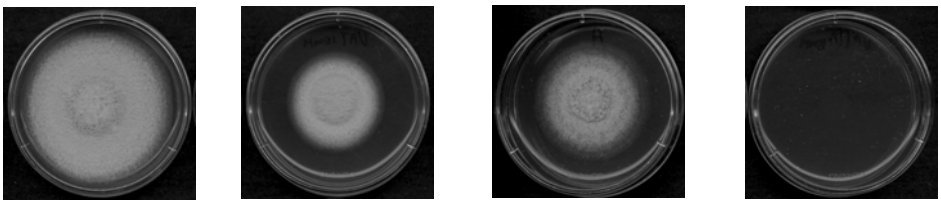
(i) (ii)

Figure 2. Bioassay showing inhibition of fungal growth by a natural compound. Fungal colonies (seen in ii) were a mixture of those contaminating walnut kernels. The total fungal mixture was inoculated onto Potato Dextrose Agar (PDA) plates containing individual natural compounds to be tested for antifungal activity. Colonies were grown for 7 days at 28°C. (i) With antifungal compound, (ii) Control- no natural compound.

C. Chemosensitization of fungal pathogens to conventional fungicides using natural compounds: enhancement of antifungal activities by targeting fungal stress response system

The primary goal of this study is to provide antifungals, which are environmentally safe and to reduce use of toxic fungicides to a minimal level. Therefore, studies on synergistic inhibition of fungal targets with combined application of natural compounds and known fungicides are currently underway in our laboratory. Selected phenolic agents showed enhanced antifungal activity of conventional fungicides when applied together against fungal pathogens (*i.e.*, synergistic inhibition/chemosensitization).

(1) Inhibition of cell growth using redox-potent phenolic reagents and inhibitors of the mitochondrial respiratory chain (*e.g.*, strobilurin fungicides) was tested for control of fungi. The *A. flavus* mitochondrial superoxide dismutase (Mn-SOD) gene (*sodA*) was chosen for a target-gene bioassay in the model yeast *S. cerevisiae*. Intervention of mitochondrial respiration and the antioxidative stress system by synergistic application of antimycin A or strobilurin-antifungals with phenolic reagents, such as vanillyl acetone, vanillic acid, vanillin, cinnamic acid, veratraldehyde, or *m*-coumaric acid, proved to be more effective than individual treatment of these compounds for inhibiting fungal growth (Figure 3). Treatment with vanillyl acetone increased sensitivity of the fungi to strobilurin fungicides by almost 100-fold (in the yeast test). This result clearly demonstrated that the amount of fungicides for field application could be reduced by synergistic application of natural compounds. Our study also showed that vanillyl acetone inhibited mitochondrial respiration, while other compounds tested seemed to affect the antioxidative system in this organelle.



(i) (ii) (iii) (iv)

Figure 3. Synergistic effects of vanillyl acetone and Strobilurin on the growth of *A. flavus*. Fungal spores (~200) were incubated for 7 days at 28 °C. (i) Control-no natural compound, (ii) Vanillyl acetone, (iii) Strobilurin, (iv) Vanillyl acetone + Strobilurin.

(2) Chemosensitization of fungal pathogens to conventional antimicrobial agents was also achieved by using benzaldehyde analogs. This approach increases sensitivity of the fungus to strobilurins (Figure 4).

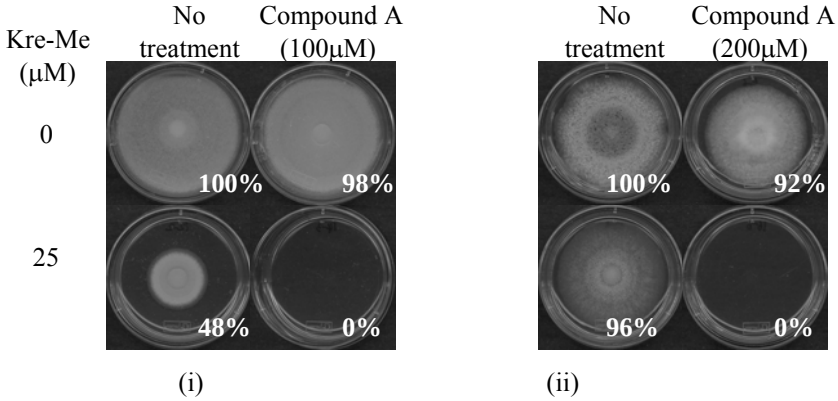


Figure 4. Targeting the mitochondrial antioxidative stress response system with benzaldehyde analog in combination with strobilurins (Kresoxim-methyl). (i) *A. fumigatus* AF293, (ii) *A. flavus* 3357.

(3) Enhancement of fludioxonil fungicidal activity by disrupting cellular glutathione homeostasis with 2,5-dihydroxybenzoic acid: Activity of fludioxonil, a phenylpyrrole fungicide, is elevated by co-application of the aspirin/salicylic acid metabolite, 2,5-dihydroxybenzoic acid (2,5-DHBA). Fludioxonil activity is potentiated through a mitogen-activated protein kinase (MAPK) pathway that regulates osmotic/oxidative stress-responses. 2,5-DHBA disrupts cellular GSH (reduced glutathione)/GSSG (oxidized glutathione) homeostasis, further stressing the oxidative stress-response system, enhancing fludioxonil activity. 2,5-DHBA treatment also prevents tolerance of MAPK mutants resistant to fludioxonil (Figure 5).

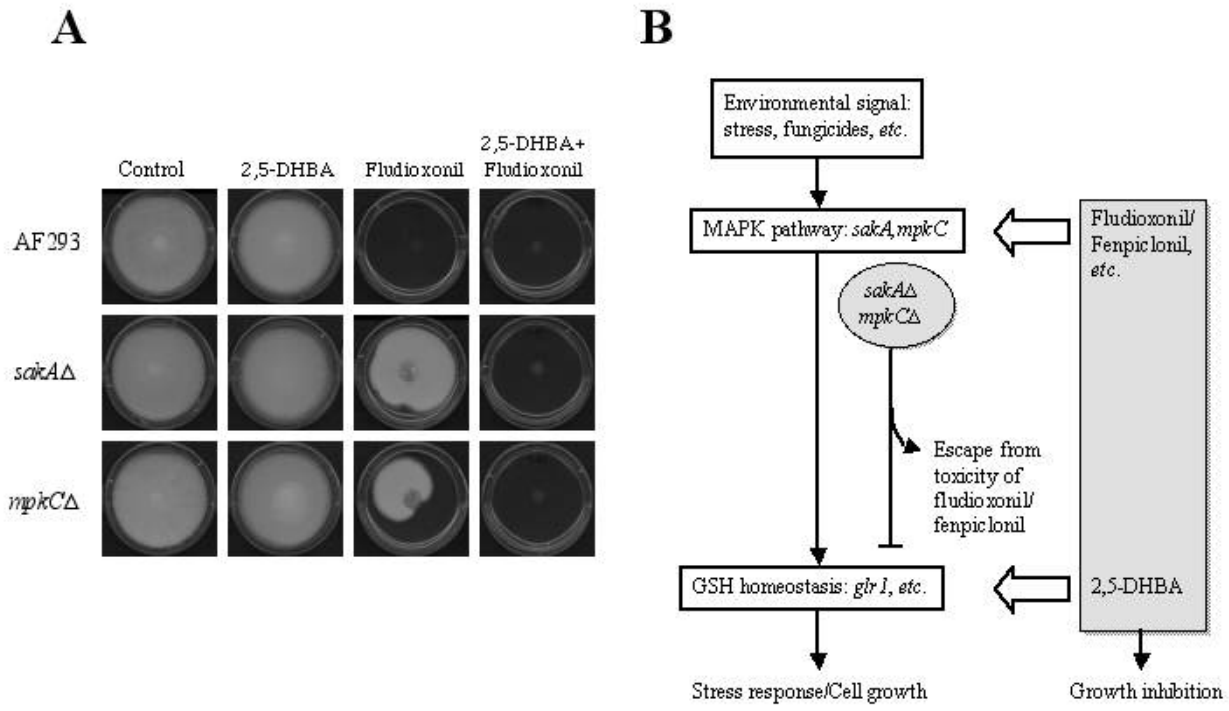


Figure 5. Inhibiting tolerance (escape) of *sakA*Δ and *mpkC*Δ MAPK mutants of *A. fumigatus* to phenylpyrrole fungicides by co-application of 2,5-DHBA. (**a**) Representative bioassays of wild type (AF293) and MAPK mutants with no treatment (Control), 2,5-DHBA (12 mM), fludioxonil (50 µM) and fludioxonil (50 µM) + 2,5-DHBA (12 mM). Note that co-treatment of 2,5-DHBA prevents *sakA*Δ and *mpkC*Δ MAPK mutants from escaping fludioxonil toxicity. (**b**) Scheme showing where phenylpyrrole fungicides (*e.g.*, fludioxonil) target MAPK signaling pathway genes. MAPK mutants escape toxicity by missing the signal stimulated by phenylpyrrole fungicides and, thus, avoiding the induced osmotic/oxidative stress response. Application of 2,5-DHBA disrupts cellular GSH homeostasis, which enhances the toxicity in the wild type cells or helps prevent escape of MAPK mutants from antifungal effects.

Currently, we are testing additional fungicides to examine the chemosensitizing effects of selected natural compounds on walnut fungal pathogens and the model yeast. Use of safe, natural compounds to augment effectiveness of commercial fungicides or antifungal drugs lowers dosages of commercial fungicides required for effective control. Consequently, this lower dosage reduces environmental impact and risks to human health by lowering exposure to fungicides.

D. Reduction of costs, labors and time by using volatile natural compounds

Our recent finding also showed that one compound identified (another benzaldehyde analog) has volatile characteristic, and therefore, can be easily disseminated to the target sites for fungal control. This will significantly reduce the costs, labors and time to apply/deliver the identified compound to trees in orchards (Figure 6).

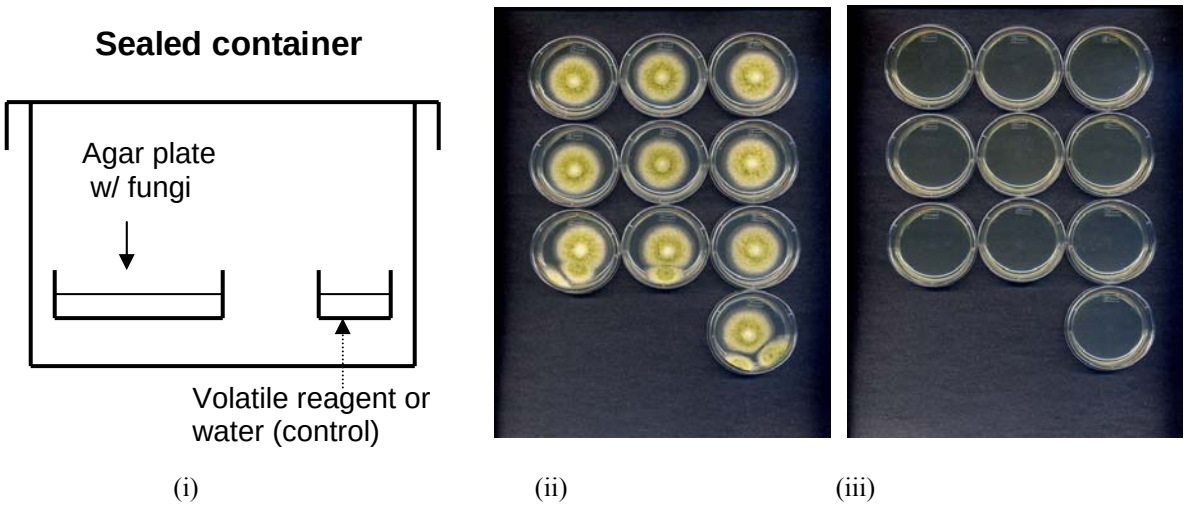


Figure 6. Bioassay (vapor-agar contact method) showing inhibition of fungal growth by volatile natural compound. *A. flavus* was inoculated onto potato dextrose agar (PDA) plates and incubated in sealed containers containing water (control) or volatile natural compound to be tested for antifungal activity. Colonies were grown for 3 days at 28°C. (i) System showing how fungal cells were exposed to the antifungal volatile compound, (ii) Control- no natural compound (water only), (ii) With antifungal compound.

E. Effect of antifungal phenolic reagents on the growth of bacterial pathogens

The antimicrobial activities of phenolic reagents identified in our laboratory were also examined in a crown gall-inducing, *Agrobacterium tumefaciens,* and a model enteric, *Escherichia coli,* bacteria. Most of the compounds showed strong antibacterial activities (complete inhibition of bacterial growth), while vanillyl acetone and gallic acid showed less activity. Currently, we are planning to see the antibacterial effects of these compounds by including more bacterial pathogens. The mechanism of antibacterial activity as well as synergistic effects with known bactericides will also be confirmed to provide background for the practical use of the natural compounds in the future.

Summary

This proposal outlines research that will: (1) identify safe, natural antifungal compounds that effectively control the wide variety of fungal pathogens that infect walnuts, (2) enhance activities of natural compounds that play a role in walnut antifungal defenses, (3) determine an effective method for delivery of newly discovered natural compounds, and (4) use comparative genomics to identify molecular targets in these fungal pathogens that promote synergistic activity when combined with commercial fungicides. Thus far, we have already demonstrated how a model bioassay system using *S. cerevisiae* is a valid and effective tool for high throughput screening for discovery of antimicrobial natural compounds. In addition, our ability to use available yeast deletion mutants provides a potent genetic basis to identify the mode of action of any discovered compound(s). We have already identified a few promising compounds, that may be effective against fungal pathogens of walnut, using these approaches. We have

a promising safe, antifungal natural compound that could be used as a fumigant against aflatoxin-producing fungi; especially during storage of walnuts in piles under plastic tarps, prior to processing. Funding would greatly enhance our ability to widen our research by increasing technical support to conduct laboratory assays and eventual field and post-harvest testing.

References:

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BUDGET REQUEST

Budget Year FY2007-2008

Funding Source Walnut Marketing Board

Salaries and Benefits			
Postdocs/RA's			
SRA's		(GS-3 8 mos)	$15,873
Lab/Field Assistance			
Subtotal	Sub 2		$15,873
Employee benefits	Sub 6		$1,587
	SUBTOTAL		$17,460
Supplies and Expenses	Sub 3		$2,000
Equipment	Sub 4		
Travel	Sub 5		
	TOTAL		$19,460

Department account number 401-5325-420 (35)

Date

Originator's Signature

COOPERATIVE EXTENSION County Director Date

Program Director Date 11/5/07

AGRICULTURAL EXPERIMENT STATION Department Chair Date 11/5/07

LIAISON OFFICER Date

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