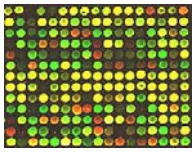

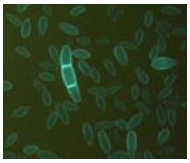

Proceedings of the 4th Annual Fungal Genomics 5th Annual Multi-Crop Fumonisin 17th Annual Multi-Crop Aflatoxin Elimination Workshops

October 25 – 28, 2004
Sacramento, California USA

Please join us in thanking the following tree nut industry organizations for their generous support which made this workshop possible:

DFA of California

California Pistachio Commission

California Fig Board

Almond Board of California

California Walnut Marketing Board

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INTRODUCTION: FUNGAL GENOMICS AND FUMONISIN ELIMINATION AND AFLATOXIN ELIMINATION WORKSHOP

SACRAMENTO, CA
OCTOBER 25-28, 2004

Introduction: Aflatoxin and Fumonisin Elimination and Fungal Genomics Workshop – 2004 Sacramento, CA

Despite tremendous advances in both applied and basic research, mycotoxin contamination of food and feed continues to be a global safety problem. The sobering reality of the impact of mycotoxins on the daily lives of individuals living in developing countries was demonstrated by the report of the aflatoxin poisoning outbreak in Kenya in 2004 where 125 people died and more than 300 became sick. Quick intervention by the Center for Disease Control and World Health Organization staff with urgent replacement of the aflatoxin contaminated maize proved to be critical to disease control. However, aflatoxin poisoning will likely continue to be a public health problem until culturally appropriate dry maize storage methods are implemented by the local population.

Fungal Genomics

The USDA sponsored *Aspergillus flavus* and *Fusarium verticilloides* EST projects are essentially complete and have been published. Microarray chips have also been developed based on these ESTs. These chips will allow for the simultaneous expression analysis of the genes involved in mycotoxin biosynthesis, regulation and signal transduction, pathogenesis, fungal development, stress responses and other cellular processes under diverse environmental conditions. Data from these studies can be used to develop strategies to identify targets to regulate and control toxin formation and fungal development and will help in gene annotation of both fungi. Analysis of the *FUM* gene ESTs led to the discovery of a new *FUM* gene (*FUM*20), the presence of alternative splice form transcripts which appear to be differentially regulated and potential regulatory genes. Comparison of EST data sets from two closely related species, *A. flavus* and *A. oryzae* revealed that domestication of *A. oryzae* resulted in the loss of metabolic genes and metabolic diversity relative to *A. flavus*. The research proposal to sequence and compare the genomes of *F. verticilloides* and *Fusarium oxysporum* was funded and will be conducted by the Broad Institute. Syngenta Inc. contributed a 4X sequence coverage of the *F. verticillioides* genome to the project which were assembled at the Broad Institute. The overall goal of this project is to develop a comprehensive gene catalog for the genus *Fusarium* by comparing the sequences of these two species with the already available *F. graminearum* genome.

Fumonisins

Fumonisins continue to receive considerable attention in both scientific and general literature due to their health effects especially in relation to neural tube defects, craniofacial anomalies and other birth defects due to their apparent interference with folate utilization in neural crest cells. The molecular mechanisms involved in *F. verticilloides* colonization of maize and regulation of fumonisin biosynthesis is complex and highly regulated. Recent research looking at the influence of kernel tissue type, stage of development and genotype showed that FB1 production is greater in endosperm of dent corn at the dent stage of development. Fungal metabolism of maize kernel components plays a major role in FB1 biosynthesis regulation. Sweetcorn ears from produce/grocery stores were shown to be as sensitive as those grown under greenhouse and field conditions and can serve as experimental material for analyzing plant-fungal interactions. Maize germplasm evaluation and QTL analysis identified four inbreds (GE440, TBA76125, CG1 and CQ201) with resistance to *F. verticilloides* ear rot and reduced fumonisin levels which could be readily transferred to a susceptible commercially-used inbred (FR1064). This research showed that levels of resistance necessary to minimize fumonisin in grain under most environments can be incorporated into commercial germplasm through marker-assisted breeding. Under laboratory conditions, fumonisin B1 is taken up by maize seedling roots and affects root and seedling growth in a dose-dependent manner by causing an elevation in sphingoid base and their respective 1-phosphates which may play a role in the *F. verticilloides* damping-off disease in sweetcorn. In sweetcorn, FB1 may serve as a pathogenicity factor resulting in necrotic leaf lesions and abnormal leaf development. Endophytic infection is not necessary to cause disease development and FB1 may function as a translocated phytotoxin functioning in a germplasm specific manner. Heterologous expression showed that *FUM3* is a 2-ketoglutarate dependent dioxygenase required for the C-5 hydroxylation of fumonisin and *FUM13* is an NADPH dependent ketoreductase for the C-3 ketoreduction. A bioassay was developed using a *F. verticilloides* strain containing both an antibiotic resistance and visual reporter gene was used monitor ear colonization. A new sensitive, reproducible and reliable analytical method to separate fumonisins from corn silage is now available with recoveries for FB1, FB2 and FB3 of 50, 25 and 25 ng/g of dried silage with similar recovery levels for wet silage.

Aflatoxins

Since many agricultural commodities are vulnerable to attack by aflatoxin producing fungi research efforts are divided into common themes.

Natural Products for Prevention of Fungal Invasion and/or Aflatoxin Biosynthesis

Saccharomyces cerevisiae was used as a model system for the development of a high throughput bioassay to screen natural products for anti-aflatoxigenic and antifungal activities. *S. cerevisiae* was selected because its genome is sequenced and numerous

strains are available with single gene deletions. Comparison of the yeast genome with the *A. flavus* EST library revealed that many yeast genes are orthologous to genes in *A. flavus*; therefore, once activity is identified in the yeast-based bioassay the compounds can be screened against *A. flavus*. Hydrolyzable tannins from tree nuts, including walnut and pistachio, both inhibit growth of *Aspergillus flavus* and aflatoxin production. When evaluated individually, several organic acids released from hydrolysable tannins, including gallic acid, ellagic acid and quinic acid actively suppress aflatoxin production but had no effect on fungal growth while tree nut hydrolysable tannins both inhibit aflatoxin biosynthesis and prevent fungal growth more than any of the individual tannin components. Gallic acid appears to function by interfering with transcription of aflatoxin biosynthesis genes. Radial growth and sporulation of a tannase knockout mutant of *A. flavus* was not affected by low concentrations of gallic acid (≤ 0.05%); however, radial growth and sporulation decreased significantly when challenged by 0.25% gallic acid. A 20-37kDa protein fraction retained by cation exchange resin from mature Tex6 corn seeds, called "ABI1" inhibited aflatoxin biosynthesis but not the growth of *A. flavus*. An anion exchange resin retained fraction with a mass of less than 10kDa called "ABI2" also had aflatoxin inhibition activity. Both proteins are thought to act as transcriptional repressors of the aflatoxin biosynthetic pathway. Efforts are now underway to purify the active proteins and use the sequences to reverse clone the respective genes and develop DNA probes for resistance breeding efforts. Seven prenylated stilbenes, including Mucilagin A, were isolated from peanut root tip mucilage and postulated to serve a role in the interactions between roots and soil-borne plant pathogens, including *A. flavus*.

Crop Resistance: Genetic Engineering

Glyoxalase I (GLX-I)is one of several proteins found at elevated levels in mature maize seeds resistant to *A. flavus* and is thought to function by decreasing methyglyoxal, an aflatoxin inducer. Gene silencing studies using RNAi technology are underway to confirm the regulatory role of GLX-I. Identification of a selectable marker with greater efficiency than hygromycin for peanut transformation is still ongoing at the University of Georgia. Two mercury-resistance genes, *merA* and *merB*, failed to confer to somatic embryos the ability to grow on media containing mercuric chloride or phenylmercuric acetate, respectively. The antifungal gene, *cpo-c*, a non-heme chlorperoxidase from *Pseudomonas pyrrocinia* was introduced into peanut and transmitted to progeny with field trials planned for 2006. The gene encoding Bcl-XL, a stress-related anti-apoptotic protein from humans was also introduced into peanut and gene product activity was monitored using paraquat tolerance. Progress continues in the identification of naturally occurring antifungal compounds from cotton. Class I cotton chitinase with neutral pI's are active against *A. flavus in vitro*. A 20 kDa cleavage fragment from vicilins, one of the major cotton seed storage proteins, is being evaluated for antifungal activity. New generations of lytic peptides with increased antifungal activity and decreased phytotoxicity are also being evaluated. Optimization of other antifungal proteins continues, including purothionin, hodohionin, synthetic cpo-c and MOD1, a synthetic gene encoding the

active form of RIP. In order to protect transgenic antifungal peptides from cytoplasmic proteolysis and to prevent transgene escape via pollen, chloroplast transformation was evaluated for cpo-p and D4E1which were confirmed by Western analysis. 9-lipoxygenases and their metabolites are thought to function as susceptibility factors in maize to increase *Aspergillus* sporulation and aflatoxin production while 13-lipoxygenases and 12-oxo-phytodienoate reductases (OPR) are thought to increase resistance because their products, C6 volatile aldehydes and jasmonates, inhibit aflatoxin biosynthesis. LOX and OPR mutants created by insertion of the *Mu* transposable element are being evaluated for changes in ear rot resistance.

Crop Resistance: Conventional Breeding

The most desirable method for fungal resistance and mycotoxin control is through host plant resistance; however, despite extensive selection in a wide variety of environments, no commercial varieties are available containing both traits. Recent data showing that visibly sound seed may well contain high levels of mycotoxins dictates that breeders select for both traits (toxin sensitivity and resistance to rot). Development of molecular markers associated with reduced mycotoxin levels and resistance to rot is receiving a lot of attention and offers the possibility of improving plant health attributes while maintaining high yields. Nine corn lines originating from aflatoxin-resistant germplasm from the International Institute of Tropical Agriculture and containing varying levels of aflatoxin-resistant US germplasm had significantly less aflatoxin contamination than their respective US resistant parents in the laboratory-based kernel screening assay. Proteome analysis of these lines showed that stress-related proteins were more abundant in the embryos of the resistant lines possibly making them able to more readily defend against pathogens while under stress conditions. Previous Quality-trait locus screening projects identified probes for chromosomal regions linked to reduced toxin formation and lower disease development. These probes were used in both yellow and white corn marker-assisted breeding programs to introgress regions associated with reduced aflatoxin contamination into germplasm commonly used in commercial US breeding programs. Regions on Chromosomes 2, 5, 8 and 10 from Tex6 were transferred to FR4341 and chromosome 4 regions from Mp313E were transferred to FR1064. Crosses between the two aflatoxin resistant FR lines had significantly lower aflatoxin contamination than the original lines. In Texas, the white corn inbred, CML176, and Tx experimental lines derived from crosses among CML269, Tx110, CML78 and CML270 provided resistance in hybrid evaluations and had good agronomic traits when crossed onto LH195 and LH210 tester lines. The movement of GFP-tagged *A. flavus* is suppressed in the rachis and rachilla of resistant maize lines. Progress continues towards the development of a "southern hybrid-type" maize germplasm with resistance to ear-feeding insects, drought tolerance and resistance to *Aspergillus* infection or aflatoxin formation and "acceptable" yield. Under a diverse set of environmental conditions in Texas, Argentine maize was less susceptible than U.S. material with some association between grain texture and aflatoxin accumulation. Using one isolate of *A. flavus* is an effective approach to screen for resistance in

maize in Texas. Generation mean analysis is an effective way to distinguish the type of inheritance for aflatoxin in parental maize inbreds and their generations. An algorithm was developed based on various agronomic characteristics and environmental variables as a way to more to accurately predict the influence of environmental factors on aflatoxin production in maize. High throughput analysis of the proteome of maize ears grown under different cultural conditions is being evaluated for processes involved in ear rot and mycotoxin resistance. Expression of the 13-Zmlox gene in developing and mature maize embryos is associated with resistance to *A. flavus* and may serve as a potential molecular marker for maize breeding programs. Gene expression profiles of GT-A1-1 maize line grown under various stress conditions were monitored using the microarray chips-Master Unigene from the Maize Gene Discovery Project. Individual gene function will be verified using RT-PCR. A new field screening technique was used to identify eleven "core" peanut accessions with a 70 percent reduction in preharvest aflatoxin contamination (PAC) as well as accessions with both drought tolerance and PAC. Two polymorphic markers associated with resistance to peanut seed infections were identified. Activities of the PR protein ß-1,3-glucanase were similar in un-infected seeds of both resistant and susceptible peanut genotypes but increased significantly in resistant genotypes following infection by *A. flavus* and has a high degree of homology to the seed storage protein, conglutin whereas both a nonspecific lipid transfer protein and the allergen vicilin were constitutive expressed at higher levels in three resistant peanut genotypes. Almonds with a very thin but high-seal endocarp (*web*-trait) and a non-Gallic acid based resistance mechanisms showed good promise in both lab and field trials. The Calimyrna-type fig selection 6-38W has the agronomic attributes of Calimyrna figs but has less fungal decay.

Microbial Ecology

A model system was developed using soil inoculation of wounded, viable peanut seeds to monitor the interactions between fungi species and the influence of environmental factors on aflatoxin formation. In May 2004, the US Environmental Protection Agency granted a section 3 registration for the use of Afla-guard® as a biopesticide on peanuts with the incidence of nontoxigenic *A. flavus* averaging greater than 98 percent of the *A. flavus* population in treated fields. In Louisiana, 30 percent of corn kernels were colonized by a proprietary atoxigenic *A. flavus* isolate belonging to VCG24 when fixed to barley seed and distributed on the soil surface between corn rows after planting. Under field conditions, the yeast, *Pichia anomala* strain WRL-076 reduced the spore production of toxigenic and atoxigenic *A. flavus* isolates in pistachio flowers, nut fruits and in almond and pistachio leaves and in wounded pistachio nuts. Bacterial isolates from almond flowers, hulls, immature and mature kernels grown under orchard conditions and collected through out the growing season showed varying levels of antagonism to *A. flavus*. Examination of *A. flavus* strains from several Southern and Southeastern states that are unable to produce aflatoxin or cyclopiazonic acid showed that they lost either part or the entire aflatoxin biosynthetic gene cluster. Use of the atoxigenic strain, AF36 continues to be improved and expanded to crops besides cotton. During 2004, the AF36

manufacturing process was modified which resulted in improved product quality and allowed for the treatment of approximately 30,000 acres of cotton in AZ and 5,000 acres in South TX. When applied on colonized wheat seed to a fig orchard, the strain AF36 colonized 91 percent of the noncarprified figs and was the only isolate observed under the drip irrigation lines in the treated orchard area three months after application. Additionally AF36 was shown to occur naturally in the major pistachio-growing area in California. Because *A. flavus* is a strong colonizer of maize cobs, aflatoxin management strategies for South TX include prompt harvest with techniques to minimize corn cob debris on the ground. An increase in the aflatoxigenic populations of *A. flavus* and *Aspergillus parasiticus* was observed in a five year period in a California orchard. In 1998, 33 percent of the isolates were toxigenic which increased to 66 percent by 2003 while 88 percent of the isolates collected from wounded pistachio nut-fruits collected in 2003 produced AF1. Variation in the D1/D2 domain of the 26S ribosomal subunit was used to identify yeast and filamentous fungi, including *A. flavus*, isolated from almond and pistachio orchards. Culture medium and nitrogen source strongly influence stromata and ascocarp formation in *Petromyeces alliaceus* section *FLAVI*. Currently it is unclear what contributions the fertile and non-fertile stromata play in the fungal disease cycle. A DNA probe, pAF28, was described which can be used to estimate genotypic diversity of *P. alliaceus*.

Crop Management and Handling, Insect Control and Fungal Relationships

An *Arabidopsis* chitinase-like gene with possible insecticidal activity was cloned into maize resulting in insect mortality levels that were positively correlated with enzyme activity. Constitutive expression of a regulatory gene that regulates multiple defense mechanisms, significantly enhanced resistance in transgenic *Arabidopsis* plants to fall armyworm, however seed production was drastically reduced. Substantial progress continues in the development of a neural network to detect fungal infected corn kernels and classify the kernels by pathogen with two spectral bands, 715 nm and 965 nm distinguishing 98.1 percent of the controls and 96.6 percent of the severely infected kernels. Root galling by root knot nematodes (*Meloidogyne arenaria*) predisposes peanut plants to drought stress and may increase aflatoxin contamination in peanuts. The pear odor, ester, ethyl (2E,4Z)-2,4-decadienoate (PE) discovered by USDA-ARS and co-patented and developed with Trece Incorporated significantly influences the behavior of adult male and females and larvae of codling moth (CM). Novel formulations containing PE have been tested and commercialized as monitoring systems for orchards treated conventionally and/or with mating disruptants. A micro-encapsulated, tank sprayable formulation of the PE kairomone had good residual field release for more than a month resulting in reduced walnut injury and the number of multiple-matings by female CMs. A rapid, easy and low cost method to screen aflatoxin in maize and peanuts using weakly polar organic polar solvents and activated neutral alumina was statistically similar to results using commercially available immunoaffinity methods. Application of a commercial diatomaceous earth product (Protect-It™) to groundnuts during storage minimized damage by *Tribolium confusum* and colonization by *A. flavus*.

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AGENDA

4th Annual Fungal Genomics
5th Annual Multi-Crop Fumonisin
17th Annual Multi-Crop Aflatoxin
Elimination Workshops

October 25-28, 2004

Holiday Inn – Capitol Plaza Hotel, Sacramento, CA

SUNDAY, OCTOBER 24, 2004

4:00 – 6:00 REGISTRATION / POSTER ASSIGNMENTS

MONDAY, OCTOBER 25, 2004

8:00 – 6:00 FIELD/FARM TOUR

8:00	Bus Departs Hotel Brief UCD Campus Tour Enroute
8:45	Visit to UCD Transformation and Genomics Facility Abhaya Dandekar, Tom Gradzeil, and Gale McGranahan
9:45	UCD Orchards – Tour Nut Crop Breeding Programs (Germplasm, Transgenic, Seedling, Selection Blocks, etc.)
11:00	Visit to Commercial Almond and Water Orchards near Dixon (Adjacent Blocks – one stop) Bruce Lampinen, CE Specialist Wilbur Reil, Yolo County Farm Advisor (Wilbur on bus for enroute narration of Yolo County Ag Scene)
12:15	Lunch – Jack Mariani Residence, Winters
1:30	Mariani Nut Company Plant Tour, Winters
3:00	Visit to Harry Dewey, Pistachio Orchard near Yolo Wilbur Reil and Themis Michailides
4:30	Blue Diamond Plant Tour, Sacramento Gary Gray
6:00	Return to Hotel

4:00 – 6:00 REGISTRATION / POSTER ASSIGNMENTS

TUESDAY, OCTOBER 26, 2004

7:00 – 8:00 CONTINENTAL BREAKFAST

7:00 – 4:30 REGISTRATION / POSTER ASSIGNMENTS AND SET UP

8:00 WELCOME
Mike Hurley
DFA of California

8:05 INTRODUCTORY REMARKS
Jane F. Robens, National Program Leader
Food Safety and Health, USDA, ARS, Beltsville, MD

4TH ANNUAL FUNGAL GENOMICS AND FUMONISIN WORKSHOP

Moderator: Scott Azerhoff, Grower, Waxahachie, TX

8:10 **Profiling of Genes Involved in Aflatoxin Formation by *Aspergillus flavus* EST and Microarray Analysis for Solving Aflatoxin Contamination of Crops.**
Jiujiang Yu,[1] Jeffery Wilkinson,[1] Stanley H. Kim,[2] William C. Nierman,[2] Deepak Bhatnagar,[1] and Thomas E. Cleveland.[1] [1]USDA/ARS, Southern Regional Research Center, New Orleans, LA; [2]The Institute for Genomic Research, Rockville, MD.

8:30 **Analysis of Expressed Sequence Tags (ESTs) from *Fusarium verticillioides*; Towards the Elimination of Fumonisin from Corn.**
Daren W. Brown,[1] Robert H. Proctor,[1] Robert A.E. Butchko,[1] Ronald D. Plattner,[1] Foo Cheung,[2] Christopher D. Town,[2] and David F. Kendra.[1] [1]USDA/ARS, National Center for Agricultural Utilization Research, Peoria, IL; [2]The Institute for Genomic Research, Rockville, MD.

8:50 **An Early Peak into the *Aspergillus flavus* Genome: Status of the Project and Access to Sequence Data.**
Gary A. Payne,[1] Jiujiang Yu,[2] William C. Nierman,[3] Bethan Pritchard,[1] Deepak Bhatnagar,[2] Thomas E. Cleveland,[2] and Ralph Dean.[1] [1]North Carolina State University, Raleigh, NC; [2]USDA/ARS, Southern Regional Research Center, New Orleans, LA; [3]The Institute for Genomic Research, Rockville, MD.

9:10 **Utilization of *Fusarium verticillioides* EST's to Identify Potential Regulators of the FUM Gene Cluster.**
Robert A.E. Butchko,[1] Daren W. Brown,[1] and Robert H. Proctor.[1] [1]USDA/ARS, National Center for Agricultural Utilization Research, Peoria, IL.

9:30 **An Overview of the Relatedness of EST Sequences from *Aspergillus flavus* and *Aspergillus oryzae* and Their Genome Sequences.**
William C. Nierman,[1] Catherine Ronning,[1] Masayuki Machida,[2] Jiujiang Yu,[3] Gary A. Payne,[4] Deepak Bhatnagar,[2] and Thomas E. Cleveland.[3] [1]The Institute for Genomic Research, Rockville, MD; [2]National Institute of Advanced Industrial Science and Technology, Ibaraki, Japan; [3]USDA/ARS Southern Regional Research Center, New Orleans, LA; [4]North Carolina State University, Raleigh, NC.

9:50 – 10:05 BREAK AND POSTER VIEWING

10:05 **Genetic Makers Track Movement of *Fusarium verticillioides* in Corn Ears.**
Ida E. Yates[1] and D. Sparks.[1] [1]USDA/ARS, Richard B. Russell Research Center, Athens, GA.

10:20 **Biochemical Analyses of FUM Genes for the Biosynthesis of Fumonisins in *Fusarium verticillioides*.**
Yousong Ding,[1] Ravi S. Bojja,[1] Kathia Zaleta-Rivera,[1] Han Yi,[1] Chunping Xu,[1] Patrick H. Dussault,[1] and Liangcheng Du.[1] [1]University of Nebraska-Lincoln, Lincoln, NE.

10:40 **Inheritance of and Markers Associated with Resistance to *Fusarium* Ear Rot and Fumonisin Production in Corn.**
Don White,[1] Craig Kleinschmidt,[1] and Michael Clements.[2] [1]University of Illinois, Urbana, IL; [2]USDA/ARS, Mississippi State University, Mississippi State, MS.

11:00 **Accumulation of Sphingoid Bases and Sphingoid Base 1-Phosphates: A Possible Mechanism for *Fusarium verticillioides* Corn-Seedling Disease.**
Lonnie D. Williams,[1] Anthony E. Glenn,[1] and Ronald T. Riley.[1] [1]USDA/ARS, Russell Research Center, Athens, GA.

11:20 **Fumonisin Biosynthesis by *Fusarium verticillioides* in the Maize Kernel Environment.**
Charles P. Woloshuk,[1] Robert A.E. Butchko,[2] and Won-Bo Shim.[3] [1]Purdue University, West Lafayette, IN; [2]USDA/ARS, National Center for Agricultural Utilization Research, Peoria, IL; [3]Texas A&M, College Station, TX.

11:40 – 12:00 PANEL DISCUSSION
Panel Chair: Gary A. Payne, North Carolina State University, Raleigh, NC

12:00 – 12:30 BREAK AND POSTER VIEWING

12:30 – 1:30 LUNCH

17TH ANNUAL AFLATOXIN ELIMINATION WORKSHOP

Session 1: Potential Use of Natural Products for Prevention of Fungal Invasion and/or Aflatoxin Biosynthesis in Crops

Moderator: Merle Jacobs, Almond Board of California

1:30 **Inhibition of Aflatoxin by Tree Nut Hydrolyzable Tannins.**
Bruce C. Campbell,[1] Russell J. Molyneux,[1] Noreen E. Mahoney,[1] and Jong H. Kim.[1] [1]USDA/ARS, Western Regional Research Center, Albany, CA.

1:50 **Identifying Antiaflatoxigenic and Antifungal Natural Compounds and Their Modes of Action Using High-Throughput, Target-Gene Based Bioassays.**
John H. Kim,[1] Kathleen L. Chan,[1] and Bruce C. Campbell.[1] [1]USDA/ARS, Western Regional Research Center, Albany, CA.

2:10 **Inhibition of Aflatoxin Production by Corn Seed Proteins.**
Gary A. Payne,[1] Robert A. Holmes,[2] and Rebecca S. Boston.[2] [1]Dept. of Plant Pathology, North Carolina State University, Raleigh, NC; [2]Dept. of Botany, North Carolina State University, Raleigh, NC.

2:10 – 3:00 PANEL DISCUSSION
Panel Chair: Bruce Campbell, USDA/ARS, Western Regional Research Center, Albany, CA

3:00 – 3:30 BREAK AND POSTER VIEWING

Session 2: Crop Resistance - Genetic Engineering

Moderator: Bob Klein, California Pistachio Commission

3:30 **Investigating the Role(s) of Corn Glyoxalase I Protein in Host Resistance to *Aspergillus flavus* Infection/Aflatoxin Production using RNAi Technology.**
Zhi-Yuan Chen,[1] Robert L. Brown,[2] Bronwyn R. Frame,[3] Kan Wang,[3] Thomas E. Cleveland,[2] and Kennith E. Damann.[1] [1]Louisiana State University Agricultural Center, Baton Rouge, LA; [2]USDA/ARS, Southern Regional Research Center, New Orleans, LA; [3]Iowa State University, Ames, IA.

3:50 **Introduction of Antifungal Genes into Peanut.**
Chen Niu,[1] Xiang-Yang Deng,[1] Sulekha Hazra,[1] Ye Chu,[1] and Peggy Ozias-Akins.[1] [1]University of Georgia Tifton Campus, Tifton, GA.

4:10 **Cotton Antifungal Compounds.**
C. A. Chlan,[1] V. C. Chigarapati,[1] J. W. Cary,[2] K. Rajasekaran,[2] and T. E. Cleveland.[2] [1]University of Louisiana at Lafayette, Lafayette, LA; [2]USDA/ARS, Southern Regional Research Center, New Orleans, LA.

4:30 **Function of Oxylipin Biosynthetic Enzymes in the Reduction of Aflatoxin and Fumonisins in Maize: Functional Genomics Approach.**
Mike Kolomiets,[1] Jinglan Zhang,[1] Andriy Nemchenko,[1] Carl Simmons,[2] Xiquan Gao,[1] and Nasser Yalpani.[2] [1]Texas A&M University, College Station, TX; [2]Pioneer Hi-Bred International, Johnston, IA.

4:50 **Control of Preharvest Aflatoxin Contamination in Cotton--Update on New Antifungal Peptides and Chloroplast Expression.**
Kanniah Rajasekaran,[1] Jeffrey W. Cary,[1] Tracey A. Ruhlman,[2] and Thomas E. Cleveland.[1] [1]USDA/ARS, Southern Regional Research Center, New Orleans, LA; [2]University of New Orleans, New Orleans, LA.

5:10 **Transgenic Peanut Expressing Maize RIP I Inhibits *Aspergillus* Growth *In vivo*.**
Arthur Weissinger,[1] Minsheng Wu,[1] Xingfen Wang,[1] K. Rajasekaran,[2] and Thomas E. Cleveland.[2] [1]North Carolina State University, Raleigh, NC; [2]USDA/ARS, Southern Regional Research Center, New Orleans, LA.

5:30 **Biochemical and Genetic Analysis of Gallic Acid in Walnuts in Relation to Aflatoxin Accumulation.**
R. M. Muir,[1] G. McGranahan,[1] C. Leslie,[1] and A. M. Dandekar.[1] [1]Department of Pomology, University of California at Davis, Davis, CA.

5:30 – 6:10 PANEL DISCUSSION
Panel Chair: Robert Brown, USDA/ARS, Southern Regional Research Center, New Orleans, LA

7:00 – 10:30 RECEPTION

WEDNESDAY, OCTOBER 27, 2004

7:00 – 8:00 CONTINENTAL BREAKFAST

7:00 – 4:00 REGISTRATION / POSTER ASSIGNMENTS

Session 3: Crop Resistance - Conventional Breeding

Moderator: Phil Wakelyn, National Cotton Council

8:00 **Identification of Aflatoxin-Resistance and Potential Markers in Maize Breeding Materials Developed in West Africa.**
Robert L. Brown,[1] Zhi-Yuan Chen,[2] Abebe Menkir,[3] and Thomas E. Cleveland.[1] [1]USDA/ARS, Southern Regional Research Center, New Orleans, LA; [2]Louisana State University, Baton Rouge, LA; [3]International Institute of Tropical Agriculture, Ibadan, Nigeria.

8:20 **Development of Field Based Techniques for Assessing Variability among Cotton Cultivars in Susceptibility to Aflatoxin Contamination During the Second Phase of Contamination.**
M. W. Olsen,[1] P. J. Cotty,[1] and S. Husman.[1] [1]The University of Arizona, Tucson, AZ.

8:40 **Use of Molecular Markers to Create Commercially Acceptable Corn Hybrids with Resistance to Aflatoxin Production in Grain.**
Don White[1] and Torbert Rocheford.[1] [1]University of Illinois, Urbana, IL.

9:00 **Screening Corn Accessions for Resistance to Aflatoxin.**
S. H. Moore,[1] H. K. Abbas,[2] and M. J. Millard.[3] [1]Louisiana State University, Baton Rouge, LA; [2]USDA/ARS, MSA, Stoneville, MS; [3]NCRPIS.

9:20 **Taking the Cob Out: Genetic and Proteomic Approaches for Investigating Induced and Constitutive Resistance to *Aspergillus flavus* during Maize (*Zea mays* L.) Ear.**
D. S. Luthe,[1] O. Pechanova,[1] B. Peethambaran,[1] G-Y. Liu,[1] Z. V. Magbanua,[1] T. Pechan,[3] S. Bridges,[4] L. K. Hawkins,[2] G. L. Windham,[2] and W. P. Williams.[2] [1]Dept. of Biochemistry and Molecular Biology, Mississippi State University, Mississippi State, MS; [2]USDA/ARS, Corn Host Plant Resistance Laboratory, Mississippi State University, Mississippi State, MS; [3]Life Sciences and Biotechnology Institute, Mississippi State University, Mississippi State, MS; [4]Department of Computer Science, Mississippi State University, Mississippi State, MS.

9:40 **Breeding Corn Germplasm to Reduce Aflatoxin Contamination.**
Javier Betran,[1] Tom Isakeit,[1] Gary Odvody,[1] and Kerry Mayfield.[1] [1]Texas A&M University, College Station, TX.

10:00 **Tools for Breeding Peanut with Resistance to Preharvest Aflatoxin Contamination.**
C. C. Holbrook,[1] B. Z. Guo,[1] D. M. Wilson,[2] X. Liang,[3] M. Luo,[2] P. Timper,[1] H. Q. Xue,[4] and T. Isleib.[4] [1]USDA/ARS, Tifton, GA; [2]University of Georgia,

Tifton, GA; [3]Guangdong Academy of Agricultural Science, Guangzhou, China; [4]North Carolina State University, Raleigh, NC.

10:00 – 10:30 BREAK AND POSTER VIEWING

10:30 **Field Evaluation and Gene Expression Analysis of Corn Genotypes for Resistance to Preharvest Aflatoxin Contamination and Drought Stress.**
B. Z. Guo,[1] M. Luo,[2] A. E. Coy,[2] and R. D. Lee.[2] [1]USDA/ARS, Crop Protection and Management Research Unit, Tifton, GA; [2]University of Georgia, Tifton, GA.

10:50 **Progress in Peanut Functional Genomics, a Strategy to Mitigate Aflatoxin Contamination and Improve other Important Traits.**
B. Z. Guo,[1] M. Luo,[2] X. Liang,[1,3] M. L. Wang,[4] and C. C. Holbrook.[5] [1]USDA/ARS, Crop Protection and Management Research Unit, Tifton, GA; [2]University of Georgia, Tifton, GA; [3]Guangdong Academy of Agricultural Sciences, Guangzhou, China; [4]USDA/ARS, Plant Genetic Resources Conservation Unit, Tifton, GA; [5]USDA/ARS, Crop Genetics and Breeding Research Unit, Tifton, GA.

11:10 **Breeding Stress-Tolerant and Low-Aflatoxin Corn Hybrids for the Southern States.**
Wenwei Xu,[1] Gary Odvody,[2] and W. Paul Williams.[3] [1]Texas A&M University, Lubbock, TX; [2]Texas A&M University, Corpus Christi, TX; [3]USDA/ARS, Corn Host Plant Resistance Research Unit, Mississippi State, MS.

11:30 **Computational Support for Research in Maize Proteomics and Marker Assisted Selection.**
Susan M. Bridges,[1] Julia E. Hodges,[1] Yong Wang,[1] Hui Xian,[1] Dawn S. Luthe,[2] and W. Paul Williams.[3] [1]Dept. of Computer Science and Engineering, Mississippi State University, Mississippi State, MS; [2]Dept. of Biochemistry and Molecular Biology, Mississippi State University, Mississippi State, MS; [3]USDA/ARS, Corn Host Plant Resistance Research Unit, Mississippi State University, Mississippi State, MS.

11:50 **Asymmetris in Fungal Development on Almond may indicate Differential Intra-Tissue Vulnerabilities to Aflatoxin Contamination.**
T. M. Gradziel[1] and A. M. Dandekar.[1] [1]Department of Pomology, University of California, Davis, CA.

11:50 – 12:30 PANEL DISCUSSION
Panel Chair: Paul Williams, USDA/ARS, Corn Host Plant Resistance Research Unit, Mississippi State, MS

12:30 – 1:30 LUNCH

Session 4: Microbial Ecology

Moderator: David Ramos, California Walnut Commission

1:30 **Colonization of Peanut Seeds by Aspergillus section *Flavi* in Soil: Selective Effects of Water Activity and Temperature.**
Bruce W. Horn.[1] [1]USDA/ARS, National Peanut Research Laboratory, Dawson, GA.

1:50 **Commercial Production and Application of Afla-Guard®, a Biopesticide for Aflatoxin Control in Peanuts.**
Joe W. Dorner.[1] [1]USDA/ARS, National Peanut Research Laboratory, Dawson, GA.

2:10 **Biological Control of *Aspergillus flavus* by *Pichia anomala*.**
S.-S. T. Hua.[1] [1]USDA/ARS, Western Regional Research Center, Albany, CA.

2:30 **Field Test of a Biocontrol Yeast in Pistachio Orchard.**
D. E. Parfitt,[1] S. B. Ly,[2] A. A. Almehdi,[1] H. Chan,[1] and S.-S. T. Hua.[2] [1]University of California, Davis, CA; [2]USDA/ARS, Western Regional Research Center, Albany, CA.

2:50 **Isolation and Initial Characterization of Bacterial Antagonists of *Aspergillus flavus*.**
Jeffrey D. Palumbo[1] and James L. Baker.[1] [1]USDA/ARS, Western Regional Research Center, Albany, CA.

3:10 **Deletions in the Aflatoxin Biosynthetic Gene Cluster of Nonaflatoxigenic and Cyclopiazonic Acid-Nonproducing *Aspergillus flavus* Isolates.**
Perng-Kuang Chang,[1] Bruce W. Horn,[2] and Joe W. Dorner.[2] [1]USDA/ARS, Southern Regional Research Center, New Orleans, LA; [2]USDA/ARS, National Peanut Research Laboratory, Dawson, GA.

3:10 – 3:30 BREAK AND POSTER VIEWING

3:30 **Aflatoxin Control in Figs: Biocontrol and New Resistant Cultivars.**
Mark Doster[1] and Themis Michailides.[1] [1]University of California, Davis/Kearney Agricultural Center, Davis, CA.

3:50 **Aflatoxin Control in Pistachios: Biocontrol Using Atoxigenic Strains.**
Themis Michailides[1] and Mark Doster.[1] [1]University of California, Davis/Kearney Agricultural Center, Davis, CA.

4:10 **Refinements in Atoxigenic Strain Production, Distribution, and Application for Suppression of Aflatoxin Producing Fungi in Arizona Cotton.**
Larry Antila[1] and Peter Cotty.[2] [1]Arizona Cotton Research and Protection Council, Phoenix, AZ; [2]USDA/ARS, Food and Feed Safety Research, Tucson, AZ.

4:30 **Progress in Aflatoxin Management for South Texas.**
Peter Cotty[1] and R. Jaime-Garcia.[1] [1]USDA/ARS, Food and Feed Safety Research, Tucson, AZ.

4:50 – 5:30 PANEL DISCUSSION
Panel Chair: Mark Doster, University of California, Davis/Kearney Agricultural Center, Davis, CA.

6:00 – 7:30 COCKTAILS

7:30 – 10:30 BANQUET

THURSDAY, OCTOBER 28, 2004

7:00 – 8:00 CONTINENTAL BREAKFAST

7:00 – 10:00 REGISTRATION / POSTER ASSIGNMENTS

Session 5: Crop Management and Handling, Insect Control, and Fungal Relationships

Moderator: Gary Gray, Blue Diamond Growers

8:00 **New Plant-Derived Genes and Gene Products with Activity Against Corn Ear Pests and Strategies for Deployment.**
P. F. Dowd[1] and E. T. Johnson.[1] [1]USDA/ARS, National Center for Agricultural Utilization Research, Peoria, IL.

8:20 **Neural Network Classification of Single Maize Seeds Infested with Kernel Rotting and Mycotoxin-Producing Fungi.**
T. C. Pearson[1] and D. T. Wicklow.[2] [1]USDA/ARS, Grain Marketing Research and Production Research Center, Manhattan, KS; [2]USDA/ARS, National Center for Agricultural Utilization Research, Peoria, IL.

8:40 **Development of Field Based Techniques for Assessing Variability among Cotton Cultivars in Susceptibility to Aflatoxin Contamination during the Second Phase of Contamination.**
M. E. Olsen,[1] P. J. Cotty,[2] and S. Husman.[2] [1]The University of Arizona, Tucson, AZ; [2]USDA/ARS, Food and Feed Safety Research, Tucson, AZ.

9:00 **The Influence of Pod and Root Galling by Root-Knot Nematodes on Preharvest Aflatoxin Contamination of Peanut.**
P. Timper,[1] D. M. Wilson,[2] and C. C. Holbrook.[1] [1]USDA/ARS, Crop Protection and Management, Tifton, GA; [2]University of Georgia, Tifton, GA.

9:20 **Advances in Mating Disruption Control of the Codling Moth and *Aspergillus* in California Walnuts.**
D. M. Light,[1] K. M. Reynolds,[1] P. Bouyssouncouse,[1] and B. C. Campbell.[1] [1]USDA/ARS, Western Regional Research Center, Albany, CA.

9:40 – 10:00 PANEL DISCUSSION
Panel Chair: Pat Dowd, USDA/ARS, National Center for Agricultural Utilization Research, Peoria, IL

10:00 – 10:30 BREAK

10:30 – 11:45 COMMODITY BREAKOUT SESSIONS

11:45 – 12:00 CLOSING REMARKS
Jane F. Robens, National Program Leader
Food Safety and Health, USDA, ARS, Beltsville, MD

FUNGAL GENOMICS/FUMONISIN/AFLATOXIN ELIMINATION WORKSHOP

4th ANNUAL FUNGAL GENOMICS AND FUMONISIN WORKSHOP

Moderator: *Scott Azerhoff, Grower, Waxahachie, TX*

Profiling of Genes Involved in Aflatoxin Formation by *Aspergillus flavus* EST and Microarray Analysis for Solving Aflatoxin Contamination of Crops

Jiujiang Yu,[1] Jeffery Wilkinson,[1] Stanley H. Kim,[2] William C. Nierman,[2] Deepak Bhatnagar,[1] and Thomas E. Cleveland.[1]
[1]USDA/ARS, Southern Regional Research Center, New Orleans, LA; [2]The Institute for Genomic Research, Rockville, MD.

Aflatoxins are extremely toxic and carcinogenic secondary metabolites produced primarily by the fungi *Aspergillus flavus* and *A. parasiticus*. Molecular studies on the genetics of aflatoxin biosynthesis have established a well organized aflatoxin pathway gene cluster consisting of 25 genes within a 70 kb DNA region. These genes have been systematically renamed from *aflA* to *aflY* according to the gene naming convention in *Aspergillus*. Many factors (such as media or environmental conditions) are found to affect aflatoxin formation. In order to better understand the molecular mechanisms regulating aflatoxin biosynthesis, such as the signal transduction pathway(s) in turning on aflatoxin production, gene profiling through *A. flavus* expressed sequence tags (EST) and microarray have been carried out. A total of 7,218 unique EST sequences have been obtained from a normalized *A. flavus* cDNA library. Genes that are putatively involved in aflatoxin biosynthesis, regulation and signal transduction, fungal virulence or pathogenicity, and fungal development have been identified from the ESTs. Microarray containing over 5,000 unique *A. flavus* gene amplicons has been constructed. Comparisons and annotation results from gene profiling under aflatoxin supportive and non-supportive medium conditions have showed that hundreds of genes were found to be expressed or up-regulated under aflatoxin-producing conditions. Further investigations on the functions of up-regulated genes are underway by gene knockout experiments. The results of this research are expected to provide information for developing new strategies for control of aflatoxin contamination of agricultural commodities.

Analysis of Expressed Sequence Tags (ESTs) from *Fusarium verticillioides*; Towards the Elimination of Fumonisin from Corn

Daren W. Brown,[1] Robert H. Proctor,[1] Robert A.E. Butchko,[1] Ronald D. Plattner,[1] Foo Cheung,[2] Christopher D. Town,[2] and David F. Kendra.[1]
[1]USDA/ARS, National Center for Agricultural Utilization Research, Peoria, IL; [2]The Institute for Genomic Research, Rockville, MD.

Fusarium verticillioides is a pathogen of maize worldwide and produces fumonisins, a family of mycotoxins that cause several animal diseases and is epidemiologically associated with human esophageal cancer and birth defects in some regions of the world. This fungus is generally an endophyte of corn but, under some conditions, it can cause seedling blight and ear, root and stalk rots. Fumonisins are often found in diseased tissue and sometimes in corn kernels with little-to-no disease symptoms. One of the primary goals of our labs is to eliminate fumonisins contamination of corn and corn products, and thus prevent them from entering animal and human food supplies. We believe that understanding how and why these toxins are produced as well as the biology of *F. verticillioides*-maize *(should be consistent corn vs. maize)* interactions will allow us to develop novel strategies to limit fumonisin contamination and corn diseases caused by the fungus.

We, in collaboration with The Institute for Genomic Research (TIGR), have sequenced the 5'ends (and in some cases the 3'ends) of *F. verticillioides* cDNAs derived from eight different fungal growth conditions. Three cDNA libraries were constructed from RNA after growth for different lengths of time (e.g. 24, 48/72, and 96 hours) on a synthetic medium that supports fumonisin synthesis between 72 and 96 hours. We reasoned that a comparison of these ESTs would identify differentially expressed genes important in fumonisin biosynthesis. Four libraries were constructed from RNA after growth on maize-derived media. We hoped to identify genes that were differentially expressed and involved in fungal response to maize as well as required for growth on maize as a sole substrate. The final library was a suppressive subtracted library that was prepared from fungal culture with or without 2-benzoxazolinone (BOA), an antimicrobial compound produced by maize. In this case, we sought to identify specific and general fungal genes that provide protection from plant defense molecules.

A total of over 87,000 expressed sequence tags (ESTs) were generated which corresponded to 11,119 unique sequences. A comparative analysis between the eight different library sequences found thousands of differentially expressed genes including all 15 genes in the fumonisin gene cluster. We identified numerous candidate fumonisin regulatory genes and a number of genes that may play a role in *F. verticillioides*-plant disease process. Analysis of over 700 *FUM* gene ESTs led to the discovery of a new *FUM* gene designated *FUM20* and to the presence of alternative splice form transcripts (ASFs). ASFs are transcripts with unspliced introns or spliced introns with different 3' splice sites. Most ASFs would yield truncated proteins because the retained sequence introduces stop codons and/or frameshifts. ASFs appear to be differentially expressed as more were present in libraries derived from older cultures. This trend was not observed for ASFs of genes located outside the cluster. This, coupled with the high frequency of occurrence of some ASFs suggests they serve a biological role.

An Early Peak into the *Aspergillus flavus* Genome: Status of the Project and Access to Sequence Data

Gary A. Payne,[1] Jiujiang Yu,[2] William C. Nierman,[3] Bethan Pritchard,[1] Deepak Bhatnagar,[2] Thomas E. Cleveland,[2] and Ralph Dean.[1]
[1]North Carolina State University, Raleigh, NC; [2]USDA/ARS, Southern Regional Research Center, New Orleans, LA; [3]The Institute for Genomic Research, Rockville, MD.

Aspergillus flavus remains an agriculturally important pathogen because it produces aflatoxin, one of the most toxic and carcinogenic naturally occurring compounds. . A whole genome sequencing project funded by the USDA/NRI and USDA/ARS and conducted at TIGR is nearing completion. A 5X coverage without annotation is being released by TIGR. The sequence can be assembled into 17 scaffolds representing 2995 contigs. Gene finding using SNAP software predicts that the genome contains approximately 13,800 genes. Thus the *A. flavus* genome appears to be larger than either *A. nidulans* or *A. fumigatus*, but similar in size to *A. oryzae*. Among the predicted genes are 24 putative polyketide synthases and 25 putative non-ribosomal peptide synthases. Manual annotation and analysis of the genome is being coordinated through North Carolina State University and will be made available at www.Aspergillusflavus.org.

Utilization of *Fusarium verticillioides* EST's to Identify Potential Regulators of the FUM Gene Cluster

Robert A.E. Butchko,[1] Daren W. Brown,[1] and Robert H. Proctor.[1]
[1]*USDA/ARS, National Center for Agricultural Utilization Research, Peoria, IL.*

Fusarium verticillioides is the causal agent of stalk and ear rot of maize. *F. verticillioides* also produces the mycotoxins fumonisins which have been shown to cause diseases in horses and swine as well as cancer in laboratory animals. In the effort to reduce or eliminate fumonisin contamination of maize, we are interested in understanding the genetic regulation of fumonisin production. A fumonisin (*FUM*) biosynthetic gene cluster, consisting of 15 co-regulated genes, has been described in *F. verticillioides* and predicted amino acid sequences of these genes suggest that most are involved in fumonisin biosynthesis. Through gene deletion analysis, we have shown that many of the genes in the cluster are required for fumonisin production. Similarly, other genes have been shown to be required for certain steps in the biosynthetic pathway and accumulate fumonisin-like intermediates. However, absent from the *FUM* gene cluster is a transcription regulatory element.

Expressed Sequence Tag (EST) libraries are useful tools for identifying genes expressed under different conditions. EST libraries have been constructed from *Fusarium verticillioides* mRNA's isolated from the fungus grown under various conditions (including synthetic fumonisin inducing liquid media and maize seedlings). Comparison of libraries from different growth conditions allows the identification of genes that are expressed under those conditions. Comparison of libraries from conditions where the *FUM* gene cluster is not transcribed and conditions where the *FUM* gene cluster is transcribed has allowed the identification of a number of regulatory type genes which may have a role in the transcriptional regulation of the *FUM* gene cluster.

We have created disruption constructs using information from the EST libraries in order to study the role, if any, of candidate genes on the activity of the *FUM* gene cluster. Our disruption constructs contain a portion of the EST nucleotide sequence as well as an antibiotic resistance gene. Incorporation of these constructs into the genomic region of the genes of interest results in the disruption of the gene with the antibiotic resistance gene. The recipient strain for disruption analysis contains a *FUM1::GUS* fusion construct which allows us to assess *FUM* gene expression by the activity of the *GUS* gene product (β-glucoronidase) under the control of the *FUM1* promoter. β-glucoronidase catalyzes the conversion of the colorless substrate X-Gluc to a blue compound. This system allows us to use a blue/white screen for activity of the *FUM1* promoter. We have disrupted a number of these candidates and are characterizing the affects of the mutation on toxin production and *FUM* gene cluster activity.

An Overview of the Relatedness of EST Sequences from *Aspergillus flavus* and *Aspergillus oryzae* and Their Genome Sequences

William C. Nierman,[1] Catherine Ronning,[1] Masayuki Machida,[2] Jiujiang Yu,[3] Gary A. Payne,[4] Deepak Bhatnagar,[2] and Thomas E. Cleveland.[3]
[1]The Institute for Genomic Research, Rockville, MD; [2]National Institute of Advanced Industrial Science and Technology, Ibaraki, Japan; [3]USDA/ARS Southern Regional Research Center, New Orleans, LA; [4]North Carolina State University, Raleigh, NC.

A comparison of EST data sets from *Aspergillus flavus* and *Aspergillus oryzae* was conducted relative to the genomic sequences to obtain an early view of the unique gene content of each genome relative to the other. The *A. flavus* EST set containing 7,218 unique sequences was obtained from a library prepared as a normalized mix of RNAs obtained from the utilization of 8 media. The *A. oryzae* set contained 6,539 unique sequences obtained from 10 non-normalized libraries prepared after growth on different media. The *A. oryzae* unique set is composed of 38 sequences; 9 of identified genes, 1 similar to a hypothetical protein, and 28 with no homology to the 4X *A. flavus* genome sequence. In contrast, 477 *A. flavus* EST sequences are unique relative to the *A. oryzae* genome. Of these, 185 have homology to known genes, 17 are similar to hypothetical proteins, and 292 have no homology to the *A. oryzae* genome. Our tentative conclusion from this analysis is that *A. oryzae* has lost metabolic genes and metabolic versatility relative to *A. flavus* as a consequence of centuries of selection through domestication as a biotechnology production organism.

Genetic Makers Track Movement of *Fusarium verticillioides* in Corn Ears

Ida E. Yates[1] and D. Sparks.[1]
[1]*USDA/ARS, Richard B. Russell Research Center, Athens, GA.*

Corn kernels have been identified as the key to solving the problem of mycotoxin synthesis during corn colonization by the fungus *Fusarium verticillioides (1)*. However, experimentation with the reproductive phase of the corn plant is limited under field conditions to once each year in temperate climates due to the seasonality of the organisms, and under greenhouse conditions by the number of plants that can be grown. The objective of this study was to examine grocer ears from retail stores as an alternative source for experimental material to utilize in bioassays to study this important food safety problem. *Fusarium verticillioides* migration to kernels distant from the inoculation site was compared in sweet corn ears from a local grocery store and from greenhouse and field grown plants. Ears were inoculated with a *F. verticillioides* transformant tagged with a selection gene encoding resistance to hygromycin, a fungicidal antibiotic, and a reporter gene encoding for ß-glucuronidase, an enzyme detectable by histochemical staining. Kernels screened for both genes ensure unequivocal identification of the source of subsequent mycelia. *Fusarium verticillioides* colonized sweet corn ears towards the ear apex and base from the inoculation site regardless of ear source, incubation protocol, and attachment of the ear to the plant or the shuck to the ear. Thus, ears from retail grocers can serve as experimental material for analyzing sweet corn and *F. verticillioides* interactions throughout the year.

1. Woloshuk, C.P. and W-B. Shim. 2000. Regulation of fumonisin biosynthesis: the corn kernel holds the key. Aflatoxin/Fumonisin Wkshp. 2000:48.

Biochemical Analyses of *FUM* Genes for the Biosynthesis of Fumonisins in *Fusarium verticillioides*

Yousong Ding,[1] Ravi S. Bojja,[1] Kathia Zaleta-Rivera,[1] Han Yi,[1] Chunping Xu,[1] Patrick H. Dussault,[1] and Liangcheng Du.[1]
[1]*University of Nebraska-Lincoln, Lincoln, NE.*

Fumonisins are mycotoxins produced by filamentous fungus *Fusarium verticillioides*, which is a widespread pathogen of corn. We have expressed several *FUM* genes in heterologous hosts to study the mechanism for fumoinisin biosynthesis. The produced proteins from five *FUM* genes have been purified and used for enzymatic assays. The data showed that *FUM3* is a 2-ketoglutarate dependent dioxygenase required for the C-5 hydroxylation of fumonisins and that *FUM13* an NADPH dependent ketoreductase for the C-3 ketoreduction. Three other genes, *FUM10, FUM7,* and *FUM14*, were proposed to function in the addition of tricarballylic esters of fumonisins using a mechanism similar to nonribosomal peptide synthetases. These three proteins were tested for their roles in this addition by using chemically synthesized substrates. Preliminary results suggested that Fum14p may be responsible for the transfer of the acyl thioester, presumably activated by Fum10p, to hydrolyzed fumonisin. Experiments are underway to obtain further information for the nature and activity of these enzymes. The results will provide directly evidence for the role of the *FUM* genes in the biosynthesis of fumonisins.

Inheritance of and Markers Associated with Resistance to *Fusarium* Ear Rot and Fumonisin Production in Corn

Don White,[1] Craig Kleinschmidt,[1] and Michael Clements.[2]
[1]University of Illinois, Urbana, IL; [2]USDA/ARS, Mississippi State University, Mississippi State, MS.

Commercial corn hybrids grown in an environments that favors Fusarium ear rot and fumonisin production often produce grain with unacceptably high concentrations of fumonisin. Therefore resistance must be identified from novel genetic sources and incorporated into commercial germplasm. In 2000, we evaluated more than 1500 inbreds as F1 hybrids with the susceptible, commercially-used inbred, FR1064. The F1 hybrids were evaluated in inoculated trials in Illinois and in two naturally infected trials in North Carolina. Several of the F1 hybrids were highly resistant to fumonisin accumulation in grain at all trials. The resistant F1 hybrids were developed with inbreds representing a variety of endosperm types and maturity. In the summer of 2001 replicated trials evaluated "progeny groups" comprised of FR1064, the other inbred used to make the resistant F1, the F1, the F2, and the backcross to FR1064. Thirty five "progeny groups" were evaluated in Urbana, Illinois and 29 in Haubstadt, Indiana in cooperation with Scott Walker of Monsanto. From that study we identified seven inbreds with low fumonisin concentration in grain of the F1 and the parent crossed with FR1064 to make the resistant F1. From further evaluation we have identified a total of four inbreds (GE440, TBA76125, CG1, and CQ201) where the inbred per se is resistant, the F1 is resistant, and F2 and backcross to FR1064 also are relatively resistant. For the inbred GE440, 215 backcross families were evaluated in North Carolina and Illinois in 2002 and in 2003 in North Carolina in cooperation with Leilani Robertson, Jim Holland and Gary Payne. Their research indicates resistance is associated with two QTL on chromosome 5. With resistant inbred TBA76125, 225 backcross to the susceptible self families were grown in Illinois and Indiana in 2002 and in 2003 in Illinois. Genotyping of the backcross susceptible families has been done by AgReliant Genetics. Genotyping indicates one or more QTL associated with resistance on chromosome 6 that account for 315% of the variation. Therefore it would appear that the chromosome regions from TBA76125 that account for resistance are different from those that have been identified from GE440. With resistant inbred CG1, 346 backcross susceptible self families were evaluated in 2003 in Illinois and Indiana and reevaluated at both locations in 2004. At this time we do not have a commercial partner who will provide genotyping. For resistant inbred CQ201, in 2003 we evaluated 270 backcross susceptible self families in Illinois and evaluated the same families in 2004 in both Illinois and Indiana. Here again we do not have a commercial partner to provide genotyping. By the end of 2004 we will have phenotypic data on backcross to the susceptible self families for four sources of resistance. We will have determined inheritance of resistance from the four sources and have genotyping information from two sources.

This research will provide sources of resistance with known inheritance of resistance and QTL's associated with resistance that can be used by us and by commercial corn breeders to improve currently used corn hybrids. Our research strongly suggests that levels of resistance necessary to minimize fumonisin accumulation in grain in most environments can be incorporated into commercial germplasm through marker-assisted selection.

Accumulation of Sphingoid Bases and Sphingoid Base 1-Phosphates: A Possible Mechanism for *Fusarium verticillioides* Corn-Seedling Disease

Lonnie D. Williams,[1,2] Anthony E. Glenn,[2] C. W. Bacon,[2] M. A. Smith,[1] and Ronald T. Riley.[2,1]
[1]*College of Agriculture and Environmental Sciences, University of Georgia, Athens, GA;*
[2]*USDA/ARS, Russell Research Center, Athens, GA.*

Sphingolipids are important structural components of membranes involved in signaling pathways that regulate cell growth and death. Fumonisins (FB) are water soluble mycotoxins produced by *F. verticillioides*, which is parasitic to corn. FBs are inhibitors of ceramide synthase (CS), a key enzyme in sphingolipid biosynthesis. Inhibition of CS causes an increase in sphinganine and sphingosine (phytosphingosine in plants), as well as their respective 1-phosphates, which is used as a biomarker for FB activity. While FB is not known to cause plant disease in the field, it is found in the ear, roots, and stalks of corn. The objectives of this study were to determine the dose- and time-dependent effects of FB1 in soils on i) root development and ii) disruption of sphingolipid metabolism in roots. Sterilized corn seeds were germinated and planted in sterile potting soil (10/pot) and watered with solutions of FB_1 (0, 1, 5, 10, and 20 μg/ml). The seedlings were dosed with FB_1 on days 2, 4, and 6 after planting, followed by ddH2O as needed until harvest. The experiment was split into two time groups (3 reps per dose), the first group was harvested on day 8 and the second group was harvested on day 21. There was a dose dependent reduction in root mass in the 21 day time group at 5, 10, and 20 μg/ml FB_1, while there was a slight increase in root growth at 1 μg/ml FB_1. FB_1 was detected in the soils as well as the roots, and the levels were closely correlated with the FB_1 dosage. There was a dose dependent elevation in sphingoid bases and their 1-phosphates in the root tissues from both time groups. These results show that under laboratory conditions FB_1 in soils can i) affect root growth and alter seedling performance, ii) FB_1 in soil can be taken up by roots, iii) FB_1 in soils can cause marked dose-dependent elevation in sphingoid bases and their 1-phosphates that precede reduced root growth.

Fumonisin Biosynthesis by *Fusarium verticillioides* in the Maize Kernel Environment

Charles P. Woloshuk,[1] Robert A.E. Butchko,[2] and Won-Bo Shim.[3]
[1]Purdue University, West Lafayette, IN; [2]USDA/ARS, National Center for Agricultural Utilization Research, Peoria, IL; [3]Texas A&M, College Station, TX.

The molecular mechanisms that regulate fumonisin B_1 (FB_1) biosynthesis in *Fusarium verticillioides* during colonization of maize kernels appear complex. Deletion of the regulatory genes *FCC1* (C-type cyclin), *FCK1* (cyclin dependant kinase), and *ZFR1* (transcription factor) in *F. verticillioides* results in reduced FB1 biosynthesis in corn kernels. With the long-term goal to identify strategies for eliminating fumonisin contamination, our collaborative study has focused on the perception of cues within the kernel environment that initiate toxin production by *F. verticillioides*. Results thus far indicate that kernel composition as related to tissue type, genotype, and development greatly influences the initiation fumonisin biosynthesis. FB1 production during colonization is greater in the endosperm tissue than in germ tissue, greater in dent hybrids than in lines homozygous for the *shrunken-2* allele, and greater in the dent stage of development than in the blister, milk and dough stages. By microarray analysis, we also identified 13 genes with expression profiles similar to *F. verticillioides* genes. Our findings suggest that fungal metabolism of maize kernel components, especially amino acids and starch, plays an important role in the regulation of FB1 biosynthesis. Our current research objectives are to identify factors in developing maize kernels that influence FB_1 biosynthesis, determine if Zfr1 is a cis-acting regulatory protein of FB_1 biosynthesis, and generate disruption mutants in *F. verticillioides* for the 13 genes that displayed expression patterns similar to the FB_1 biosynthetic genes.

Panel Discussion: Fungal Genomics and Fumonisin Workshop

Panel Chair: Gary A. Payne
Panel Members:

Presentations in this section focused on progress in the genomic analysis of *Aspergillus flavus* and *Fusarium verticillioides*. Jiujiang Yu reported on the EST sequencing project directed the USDA/ARS/SRRC in New Orleans. Over 7,000 unique ESTs were sequenced, and they represent genes involved in aflatoxin biosynthesis, regulation and signal transduction, fungal virulence or pathogenicity, stress response or antioxidation, and fungal development. TIGR has the subcontract to prepare DNA microarrays and is spotting 5000 unique elements derived from these ESTs on glass slides. These genetic resources will greatly augment studies on gene expression analysis and will help in gene annotation in *Aspergillus flavus*.

Gary Payne reported on the status of the *Aspergillus flavus* sequencing project.
Preliminary studies of the 36.2 Mb draft sequence, which consists of 29 scaffolds representing 1407 contigs, indicate that the genome contains in the region of 11,760 genes. Thus the *A. flavus* genome appears to be larger than either *A. nidulans* or *A. fumigatus*. An initial scan of the *A. flavus* genome for proteins involved in the production of secondary metabolites revealed the presence of 29 putative polyketide synthases and 24 putative non-ribosomal peptide synthases. Manual annotation and analysis of the genome is being coordinated through North Carolina State University and will be made available at www.Aspergillusflavus.org.

William Nierman, from TIGR, reported on the relatedness of the EST sequences from *A. flavus* and *A. oryzae*. He reported that 38 *A. oryzae* ESTs are unique compared to the *A. flavus* genome. Ten of these have homology to other genes while 28 have no homology to sequences in the database. Interestingly, he found that 477 *A. flavus* genes are unique compared to the *A. oryzae* genome. Of these, 292 have no homology to sequences in the database. Genes unique to *A. flavus* include an ABC transporter, choline kinase, a DNA ligase III, NADH oxidoreductase, and a zinc finger protein. A whole genome analysis of these two fungi, which will be possible soon, will greatly aid our understanding about the biology of these two fungi.

Daren Brown reported on the characterization of ESTs in *Fusarium verticillioides*. He reported the generation of over 87,000 ESTs that represent 11,119 genes. Interestingly, within these ESTs he found that 78 of the 700 ESTs that match portions of the 15 fumonisin genes were alternative splice forms. Further studies are underway to determine biological significance of alternate splicing in fungi. A better understanding of the role of alternate splicing in fungi may reveal new strategies for the control of fumonisin contamination.

Robert Butchko reported on expression analysis of the *F. verticillioides* ESTs. He compared gene expression in a library prepared at 24 hr with one prepared at 96 hr. He found many genes more highly expressed at 96 hr. ESTs from the 96 hr library had similarities to transcription factors and activators, DNA binding proteins, and zinc finger proteins. These genes are targets for gene replacement experiments to determine their role in fumonisin biosynthesis. Certainly, understanding the regulation of fumonisin biosynthesis will provide information that can be used to control fumonisin contamination of maize.

Fumonisin B_1 as a Phytotoxin of Maize: Examining Mechanisms of Pathogenicity, Cellular Toxicity, and Resistance in Maize and Possible Translocation of Fumonisin

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Fungi produce a vast array of secondary metabolites, often of unknown function but generally believed to enhance fitness and ecological interactions. The fungus *Fusarium verticillioides* infects and endophytically colonizes corn, forming an association of significant concern due to production of various metabolites, most notably fumonisin B1 (FB1). FB1 is a water soluble mycotoxin that causes species-specific animal diseases, including cancer in experimental animals. We have examined an apparent seedling pathogenicity factor produced by the fungus that results in necrotic leaf lesions and abnormal leaf development. Genetic analyses indicated a single locus segregated for ability to cause disease. Seedlings inoculated with non-pathogenic strains were identical with uninoculated control plants. Endophytic infection was not necessary or sufficient to cause disease symptoms, suggesting the pathogenicity factor may be a translocated phytotoxin. FB1 production was assessed among the parental and progeny strains and also segregated as a single locus. Linkage between pathogenicity and FB1 production was supported because only the pathogenic strains produced FB1. Free sphingoid bases were elevated significantly in diseased seedlings inoculated with FB1-producing strains, which is concordant with the disruption of sphingolipid biosynthesis by FB1 due to inhibition of ceramide synthase. Seedlings watered with a 1 ppm solution of FB1 showed a stimulatory effect on growth, yet seedlings watered with 10 ppm and higher concentrations showed dose-dependent toxicity, stunting, and elevated sphingoid bases and their 1-phosphates. Thus, we propose that FB1 is the phytotoxin and pathogenicity factor causing the disease symptoms. Disease development was dependent upon corn hybrid, indicating that resistance to FB1 toxicity exists. Efforts are underway to examine FB1 toxicity and resistance mechanisms (i.e., *LAG1* homologs) in differing hybrids, including evaluation of FB1 translocation and accumulation in corn tissues.

Liquid Chromatographic Determination of Fumonisins B_1, B_2 and B_3 in Corn Silage

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Corn silage is a popular feed source for dairy and beef cattle. Fumonisins, toxic substances produced by molds, are prevalent in field corn and potentially could remain intact in corn silage. Diseases in livestock have been linked to the presence of fumonisins in feed.

Although a number of analytical methods for the determination of fumonisins in corn and other cereals have been developed, silage is still a notoriously difficult material to test for fumonisins because of its composition. To date there has been no chemical methods for fumonisins developed such as liquid chromatographic (LC).

In view of this situation, we developed sensitive, reproducible, and reliable analytical method to separate and quantify fumonisins from corn silage samples. The method was applied to corn silage samples collected from the midwestern area of the United States during 2001-2002. Of 89 corn silage samples, FB_1, FB_2 and FB_3 were found in 86 (97%), 64 (72%), and 51 (57%) of samples. The mean positive levels of FB_1, FB_2 and FB_3 were 615 ng/g, 93 ng/g, and 51 ng/g, respectively in dried silage.

This suggests fumonisins may be frequent low-level contaminants in corn silage. Because no systematic survey of corn silage for fumonisins has yet been undertaken, the results also provide useful information about the exposure of livestock to fumonisins from silage.

Intervention of Cellular Redox Homeostasis for Control of Fungal Pathogens: Mitochondrial Antioxidative Stress System as a Molecular Target

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To establish target-gene specific control of aspergilli, intervention of cellular redox homeostasis by synergistic application of phenolic compounds (or derivatives) and inhibitors of mitochondrial respiratory chain was studied. *Aspergillus flavus* mitochondrial superoxide dismutase (Mn-SOD) gene (*sodA*) was used for exemplary target-gene bioassay in the model yeast *Saccharomyces cerevisiae*. The hydrophobic to basic amino acid ratio in the signal sequence of this enzyme, which is roughly 1:1, indicates SodA is a manganese SOD. Expression of *sodA* in a cDNA clone alleviated the sensitivity of *S. cerevisiae sod2Δ* mutant to oxidative stress while that from genomic clone could not. This difference suggests SodA is a functional ortholog of *S. cerevisiae* Sod2p but the mechanism/machinery for RNA splicing of *A. flavus* genes is different from that in the model yeast. Overexpression of Mn-SOD has been known to lengthen the chronological life span of yeast cells optimized for G_0 maintenance but leads to dramatic Sir2p-independent shortening of the replicative life span, where mitochondrial defects, *i.e.*, failure in segregation of the mitochondrion from the mother to daughter cells, are the proposed mechanism. In our study, growth inhibition was also observed when *A. flavus sodA* was overexpressed in the *S. cerevisiae* wild-type strain, indicating tight control of Mn-SOD activity, and hence maintaining cellular redox homeostasis, is necessary for normal cell growth. Therefore, this antioxidative enzyme and/or a system linked to this enzymatic activity can serve as a good molecular target for the control of fungal pathogens.

We proved synergistic inhibition of Mn-SOD by combined application of natural compounds, such as vanillyl acetone, vanillic acid, vanillin, cinnamic acid, 3,4-dimethoxybenzaldehyde or *m*-coumaric acid, with mitochondrial respiration-inhibitory reagents (*e.g.* antimycin A/strobilurin-fungicides) effectively suppresses the growth of fungal pathogens. Combined application of antimycin A or strobilurin with these natural compounds will intensify the antifungal effects by depleting Mn-SOD activity in fungal cells.

Since differences in the respiratory chains between host and fungal pathogens can be a pharmacological target for the control of pathogens, understanding the structure of the respiratory system in aspergilli is very important. In the case of *A. fumigatus*, a human pathogen causing aspergillosis, this fungus contains a functional respiratory chain (complex I to V) as well as an alternative NADH-ubiquinone oxireductase, an alternative oxidase and an uncoupling protein. Searches of the *A. fumigatus* genomic database using orthologs from *A. flavus* showed these two species share significant structural homology in their antioxidative stress pathways. Application of yeast high throughput bioassays, identifying specific gene-targets in aspergilli using comparative functional genomics, and achieving fungal control using new antimicrobials targeting specific genes will be discussed.

Analysis of Expressed Sequence Tags (ESTs) from *Fusarium verticillioides*; Towards the Elimination of Fumonisin from Corn

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Fusarium verticillioides is a pathogen of maize worldwide and produces fumonisins, a family of mycotoxins that cause several animal diseases and is epidemiologically associated with human esophageal cancer and birth defects in some regions of the world. This fungus is generally an endophyte of corn but, under some conditions, it can cause seedling blight and ear, root and stalk rots. Fumonisins are often found in diseased tissue and sometimes in corn kernels with little-to-no disease symptoms. One of the primary goals of our labs is to eliminate fumonisins contamination of corn and corn products, and thus prevent them from entering animal and human food supplies. We believe that understanding how and why these toxins are produced as well as the biology of *F. verticillioides*-maize *(should be consistent corn vs. maize)* interactions will allow us to develop novel strategies to limit fumonisin contamination and corn diseases caused by the fungus.

We, in collaboration with The Institute for Genomic Research (TIGR), have sequenced the 5'ends (and in some cases the 3'ends) of *F. verticillioides* cDNAs derived from eight different fungal growth conditions. Three cDNA libraries were constructed from RNA after growth for different lengths of time (e.g. 24, 48/72, and 96 hours) on a synthetic medium that supports fumonisin synthesis between 72 and 96 hours. We reasoned that a comparison of these ESTs would identify differentially expressed genes important in fumonisin biosynthesis. Four libraries were constructed from RNA after growth on maize-derived media. We hoped to identify genes that were differentially expressed and involved in fungal response to maize as well as required for growth on maize as a sole substrate. The final library was a suppressive subtracted library that was prepared from fungal culture with or without 2-benzoxazolinone (BOA), an antimicrobial compound produced by maize. In this case, we sought to identify specific and general fungal genes that provide protection from plant defense molecules.

A total of over 87,000 expressed sequence tags (ESTs) were generated which corresponded to 11,119 unique sequences. A comparative analysis between the eight different library sequences found thousands of differentially expressed genes including all 15 genes in the fumonisin gene cluster. We identified numerous candidate fumonisin regulatory genes and a number of genes that may play a role in *F. verticillioides*-plant disease process. Analysis of over 700 *FUM* gene ESTs led to the discovery of a new *FUM* gene designated *FUM20* and to the presence of alternative splice form transcripts (ASFs). ASFs are transcripts with unspliced introns or spliced introns with different 3' splice sites. Most ASFs would yield truncated proteins because the retained sequence introduces stop codons and/or frameshifts. ASFs appear to be differentially expressed as more were present in libraries derived from older cultures. This trend was not observed for ASFs of genes located outside the cluster. This, coupled with the high frequency of occurrence of some ASFs suggests they serve a biological role.

FUNGAL GENOMICS/FUMONISIN/AFLATOXIN ELIMINATION WORKSHOP

17TH ANNUAL AFLATOXIN ELIMINATION WORKSHOP

Session 1: Potential Use of Natural Products for Prevention of Fungal Invasion and/or Aflatoxin Biosynthesis in Crops

Moderator: *Merle Jacobs, Almond Board of California*

Inhibition of Aflatoxin by Tree Nut Hydrolyzable Tannins

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Pistachio and walnut fruit tissues contain hydrolyzable tannins that inhibit the growth and aflatoxin production of *Aspergillus flavus*. Walnut hydrolyzable tannins, located in the kernel seed coat, are composed of glucose esterified with gallic acid and hexahydroxydiphenic acid, which spontaneously lactonizes into ellagic acid upon hydrolysis. Pistachio hydrolyzable tannins, located in the hull, are composed of quinic acid esterified with gallic acid. *A. flavus* produces an extracellular tannase which hydrolyzes tannins into their polyol and phenolic components. The hydrolyzed components of tree nut tannins are potent inhibitors of aflatoxin; ellagic acid, quinic acid, and gallic acid reduced aflatoxin by 70%, 84%, and 88%, respectively, when incorporated at 0.2% (w/v) in nut kernel media. These compounds did not affect *A. flavus* radial growth. Despite the fungal tannase activity, the tree nut hydrolyzable tannins are more powerful inhibitors of aflatoxin biosynthesis and fungal growth than any of the individual tannin components. When incorporated at 0.2% (w/v) in walnut kernel agar, walnut tannin reduced aflatoxin production by 99.9% and significantly reduced *A. flavus* growth. Notable is the ability of pistachio tannin to completely inhibit the growth of *A. flavus* at 0.04% (w/v) in pistachio kernel agar.

Identifying Antiaflatoxigenic and Antifungal Natural Compounds and Their Modes of Action Using High-Throughput, Target-Gene Based Bioassays

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We have developed a high throughput bioassay to screen natural products for antiaflatoxigenic or antifungal activity against *Aspergillus flavus*. The assay uses the yeast, *Saccharomyces cerevisiae*, as the initial target organism. This yeast serves as a good model system because its genome is entirely sequenced and is well annotated. Moreover, strains of *S. cerevisiae* are commercially available that have deletion mutations of individual genes. These deletion mutants can be used to ascertain genes (or their products) being affected by any active compounds identified in the bioassays. Based on the new Expressed Sequence Tag (EST) library of *A. flavus* developed by the Food and Feed Safety Research Unit, USDA-ARS, Southern Regional Research Center, New Orleans, LA, we have found many of the yeast genes are orthologous to genes in *A. flavus.* Hence, once activity is discovered using the yeast-based bioassay, promising compounds can then be screened against target fungi, such as *A. flavus.*

Gallic, tannic and caffeic acids and methyl gallate are potent inhibitors of aflatoxin biosynthesis in *A. flavus.* To determine if this antiaflatoxigenic activity was associated with alleviation of oxidative stress, 22 strains of singular gene deletion mutants of *S. cerevisiae* were treated with hydrogen peroxide (H_2O_2) and the antiaflatoxigenic compounds. Results clearly indicated these compounds alleviated oxidative stress. Analysis of the *A. flavus* EST database identified 43 orthologs of *S. cerevisiae* genes involved in gene regulation, signal transduction (*e.g., SHO1, HOG1, etc.*) and oxidative stress response (*e.g., CTT1, CTA1, etc.*). An exemplary functional complementation of an antioxidative stress gene from *A. flavus*, mitochondrial superoxide dismutase (*sodA*), was succesful in a *sod2*Δyeast mutant, indicating *S. cerevisiae* deletion mutants may serve as a model system to study *A. flavus*.

The yeast high throughput bioassay was also used to identify a number of phenolic agents for control of *A. flavus*. Veratraldehyde, 1, and cinnamic acid, 5, and respective benzoic acid derivatives vanillin, 2, vanillic acid, 3, and vanillyl acetone, 4, and cinnamic acid derivatives *o*-coumaric acid, 6, *m*-coumaric acid, 7, and *p*-coumaric acid, 8, showed significant antifungal activities (highest to lowest: 2, 5 > 1 > 6, 7 > 4 > 3, 8) in the yeast system with caffeic acid, **9**, having little to no effect. Results of the compounds against *A. flavus* were parallel to those of the initial yeast bioassays, demonstrating the usefulness of the *S. cerevisiae* bioassay for screening antifungal compounds. Assays using deletion mutants of yeast identified signal transduction and antioxidative stress responses genes important to fungal tolerance. Targeting the antioxidative stress response system with certain compounds (*e.g.*, vanillyl acetone) in combination with strobilurin-fungicides had a synergistic antifungal effect against both fungi.

In summary, the high throughput yeast bioassay shows great promise in identifying compounds that can reduce aflatoxin production and/or fungal development. In addition, the assay provides some insights on the mode of action of any active compounds identified. This knowledge can then be used to develop methods of fungal control, such as the synergistic effect demonstrated in combining strobilurin fungicides with certain natural products that target gene activities.

Inhibition of Aflatoxin Production by Corn Seed Proteins

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Research has focused on characterizing a compound (ABI) from Tex6 corn seeds that inhibits aflatoxin production but not growth of Aspergillus flavus. This protein, which is present in mature corn seeds, interacts with cation exchange resin and is approximately 20-37 kDa, based on electrophoretic mobility. Purification has yielded preparations of ABI1 that inhibit aflatoxin biosynthesis but have little effect on fungal growth. ABI1 activity has been purified to a few candidates, which will be submitted for mass spectrometry. Once the protein is identified the encoding gene sequence will be used to develop DNA primers specific for the gene. These primers will allow amplification and cloning of the gene for subsequent use in a marker assisted breeding program. We have also begun purification of a second aflatoxin inhibitory activity, ABI2. Unlike ABI1, ABI2 interacts with an anion exchange resin and is not retained by a 10kDa MWCO centrifugation filter, suggesting that it is smaller than 10 kDa. We have also performed preliminary measurements of fungal gene transcription in response to inhibitory fractions containing ABI1 and ABI2 activity. These experiments suggest that the inhibitory activity of these is a result of transcriptional repression of the aflatoxin biosynthetic pathway. Once the protein identity is determined we intend to clone the respective genes in order to develop DNA specific probes that can be used by researchers breeding for resistance.

Panel Discussion: Potential Use of Natural Products for Prevention of Fungal Invasion and/or Aflatoxin Biosynthesis in Crops

Panel Chair: Bruce Campbell
Panel Members:

Dr. Bruce Campbell led the discussion of this session. He began by presenting a brief history of the numbers and types of presentations that had appeared in this session over the previous six years of Aflatoxin Elimination Workshops. The purpose was to provide an overview of what types of natural products had been examinied, to date, with regard to their effects on curtailing either aflatoxin biosynthesis or antifungal. Most of the studies had historically involved laboratory-based bioassays. For example, at the Memphis meeting in 1997, there were six presentations in this session. Compounds described included naphthaquinones, triglycerides and sugar metabolites, but also included enzymatic studies on lipoxygenase and initial molecular biological studies on gene expression and regulation. The following year, 1998 in St. Louis, there were also six presentations. Specific natural products discussed included carotenoids and terpenoids, but, again, this session included discussions on lipids, enzyme activities, such as amylase, and also gene regulation. The next year, 1999 in Atlanta, there were five papers presented in this session. Discussions continued on lipids and lipid metabolism and their role in gene regulation of aflatoxin biosynthesis. The role of sugars and amylase activity also continued and a number of compounds that affected insect behavior were also presented. In 2000, in Yosemite, CA, there were five presentations. Some of the molecular biological studies presented in this session were now being presented in a separate workshop on genomics. Again, the effects of certain metabolites of sugars and triglycerides were shown to have an antifungal effect. A number of natural spice compounds, such as curcumin, were shown to have an inhibitory effect on the transformation of aflatoxin to its carinogenic epoxide. Also, lipid metabolism and development of aspergilli continued to be discussed. However, in this session, a new lure of codling moth, didecenoate, was revealed. It was in this session that first revelations of a walnut constituent inhibited aflatoxin biosynthesis. The following year, 2001 in Phoenix, this session had seven presentations that again, included an admixture of natural products chemistry and molecular biological subjects. It was in this session that the walnut antiaflatoxigenic compound appeared to be most active in the walnut variety 'Tulare'. Also, it was at this time the strategies for development of an Expressed Sequence Tag (EST) library for *Aspergillus flavus* were revealed. In ending the overview, it was pointed at that the previous year's workshop, 2003 in Savannah, had five presentations, one of them showing that gallic acid was an antiaflatoxigenic factor in walnuts. This session also included some initial genetic analyses for biosynthesis of gallic acid in walnuts. Seed proteins of corn inhibitory to fungal growth were also discussed. But it was also at this meeting that many of the presentations with regard to molecular biology and gene regulation were presented in an adjoining genomics workshop. Thus, in the 2004 Aflatoxin Elimination Workshop in Sacramento, there were only three presentation in the "natural products" session. These presentations included more examinations of the antiaflatoxigenic and antifungal activites of hydrolysable tannins and corn seed proteins, but also included a new dimension of how to perform a rapid, high throughput analysis of the functional genomic basis for antiaflatoxigenic activity.

Thus, although there were few presentations in this year's session, in actuality an historical perspective of this session revealed tangible progress in developing and implementing ways to inhibit aflatoxin biosynthesis. In the "Fungal Genomics and Fumonisin Workshop" that preceded this session, one audience member had questioned the investment over the past few years in molecular biological and genetic efforts in coming up with applicable methods to curtail mycotoxin contamination. However, it was pointed out in the "Natural Products" session that all the approaches to the problem work somewhat hand-in-hand. With the discovery of a natural product that actually

prevented aflatoxin biosynthesis, in addition to the genetic tools of an EST library and a soon to be available genomic library of *A. flavus*, major advances in understanding what triggers aflatoxin biosynthesis and the genes involved in producing these signals would soon be determined. Thus, natural products are actually the "metabolomics" end of the research cycle into solving the aflatoxin problem. It was pointed out by one of the panelists that this "metabolomic" knowledge combined with "genomic" tools will probably result in an applicable method to prevent aflatoxin biosynthesis within the first decade of this 21st century.

Walnut and Pistachio Hydrolyzable Tannins: Comparison of Chemical Structures and Aflatoxin Inhibiting Properties

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In order to identify natural sources of resistance to aflatoxin accumulation, tree nuts were screened for their ability to support aflatoxin production by *Aspergillus flavus* using agar-based media composed of kernels of almond, pistachio, or walnut cultivars. *A. flavus* grown on walnut cultivars produced significantly less aflatoxin than on pistachio or almond cultivars, and the English walnut cultivar 'Tulare' completely inhibited the production of aflatoxin. The inhibitory factor in 'Tulare' kernels was found to reside in the seed coat and bioassay-directed chemical fractionation of the seed coat resulted in the identification of hydrolyzable tannins with potent aflatoxin inhibiting properties. In order to elucidate the mode of action it was first confirmed that *A. flavus* isolate NRRL 25347 (isolated from pistachios) produced a previously reported extracellular tannase. This tannase produced gallic acid, ellagic acid, an unidentified ellagic acid analog, and glucose upon hydrolysis of walnut tannin. When incorporated into an agar-based medium consisting of 'Tulare' kernels without seed coat, walnut tannin, gallic acid, and ellagic acid all strongly inhibited aflatoxin production. At 0.2% (w/v), gallic acid and ellagic acid inhibited aflatoxin by 86% and 70%, respectively. However, walnut tannin was an especially potent inhibitor of aflatoxin, with a 99.9% reduction in aflatoxin production at 0.2% (w/v). Walnut tannin also significantly reduced *A. flavus* growth, while neither gallic acid nor ellagic acid had any visible effect on fungal growth.

The hulls of 'Kerman' pistachios also contain high levels of hydrolyzable tannins (20% dry wt.). The aflatoxin inhibitory effect of these hull tannins were demonstrated by the inoculation of fresh, wounded pistachio kernels with and without the hull. Aflatoxin was measured in 50 individual kernels after 10 days and the effect of a single nut in an otherwise aflatoxin-free 10kg test lot was calculated. No aflatoxin was detected in any of the individually assayed hulls. Without the hull, 46 out of 50 kernels would cause a test lot to fail the EU standard of 2ppb aflatoxin B1, and 27 kernels would cause the lot to fail the US standard of 20ppb B1. None of the kernels inoculated in-hull would cause a test lot to fail the EU limit of 2ppb aflatoxin B1. Chemical analysis showed that pistachio hydrolyzable tannins consist of quinic acid esterified with gallic acid, both of which would be released upon activity by *A. flavus* tannase. Quinic acid and gallic acid were strong inhibitors of aflatoxin in an agar-based 'Kerman' pistachio medium. At 0.2% (w/v), gallic acid and quinic acid reduced aflatoxin by 88% and 84%, respectively. It was difficult to fully evaluate the aflatoxin inhibiting properties of pistachio tannin since it was such a powerful inhibitor of fungal growth. Pistachio tannin completely inhibited *A. flavus* growth at only 0.04% in the pistachio medium.

Novel Prenylated Stilbenes from Peanut Root Mucilage

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A mucilaginous external layer (mucilage) is often observed on plant root tips. Mucilage consists of sloughed organic matter produced by roots, and is reported to mechanically lubricate the roots, promote formation of mycorrhizae and nitrogen-fixing nodules, enhance nutrient and water absorption, promote soil aggregation, and reduce root desiccation during drought. Excretion of antimicrobial compounds by roots, particularly in response to elicitors such as pathogenic fungi, has been documented in many species.

Several potent antimicrobials, including resveratrol, isopentenyl and isopentadienyl resveratrol analogs, have been isolated from peanut leaves, stems, pegs, roots, and kernels when challenged by pathogens. These compounds likely play a defensive role when water activity level in plant tissues is sufficiently high. Indirect evidence for this defensive role is that aflatoxin contamination, a result of *Aspergillus* spp. infection, increases as peanut kernels lose their capacity for phytoalexin synthesis as a result of dehydration under drought conditions.

Peanut mucilage may play a role in the interactions between roots and soil-borne plant pathogens; however, the occurrence of phytoalexins or preformed antimicrobial compounds in peanut mucilage has not been reported.

The HPLC-PDA-APCI-MS^n investigation revealed 7 prenylated stilbenes in the extract of peanut (*Arachis hypogaea* L.) root tip mucilage. To our knowledge, this is the first report of detection of secondary metabolites in peanut root mucilage. The compound termed mucilagin A, 4-(3-methyl-but-1-enyl)-3,5-dimethoxy-4′-hydroxyl-*trans*-stilbene, was the principal constituent. Mucilagin A structural assignment was strongly supported by our data. Structures of the other constituents are tentative; however, the data are sufficient to conclude that the stilbenoids have not been reported from peanuts. Preliminary research demonstrated that the peanut root tip (with the mucilage removed) did not contain any traces of the new stilbenoids. This suggests that the new compounds are restricted to the mucilage. All but one of the substances detected had a 4′-OH, which has been shown to be strongly associated with stilbenoid biological activity. Mucilagin A concentration in the mucilage was 250 $\mu g\ g^{-1}$. The concentration of the other stilbenes totaled 130 $\mu g\ g^{-1}$.

Inhibition of Aflatoxin Biosynthesis by Tannic Acid

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Tree nut research has demonstrated that aflatoxin (AF) biosynthesis appears to be inhibited by gallic acid (GA). It is hypothesized that the release of GA from hyrolyzable tannins present in the plant seed coat or hull tissue by fungal tannase enzyme is responsible for the observed inhibition of AF production. Studies have shown that GA content of the seed coat or hull correlated inversely with the ability of the fungus to produce AF. We have shown that inhibition of AF production appears to be at the level of transcription of AF biosynthetic genes as GA at 0.25% was shown to almost completely inhibit expression of *nor1*, *ver1* and *omtA* genes while aflR expression was only slightly reduced. In order to test the theory that GA is the major inhibitory compound in the seed coat/hull tissues we have cloned the tannase gene from *A. flavus* and constructed a disrupted version of it by insertion of the *A. parasiticus* nitrate reductase (niaD) gene. We hypothesized that inhibition of toxin production should not be as great in the tannase knockout mutant due to its inability to release GA from tannic acid (TA). The disruption construct was transformed into an *A. flavus* 70 niaD mutant and two isolates were identified, 13B and 20A, that exhibited loss of tannase activity.

Both the wild-type *A. flavus* 70 and the tannase knockout mutant 13B were grown for 3 days on minimal salts/1% sucrose plates supplemented with 0, 0.05, 0.125, or 0.25% GA or TA. Essentially no difference in radial growth was observed when comparing the wild-type to 13B on media supplemented with GA. No significant differences were observed between the two fungi for growth on TA either. However significant differences were observed when comparing fungal growth on GA vs. TA. Radial growth of both isolates was the same at 0 and 0.05% GA or TA but reduced by 13.6% and 37.5% on 0.125 and 0.25% GA respectively, compared to TA. In addition, there was a reduction in sporulation of about 50% observed on media containing 0.125 and 0.25% GA vs. TA. Analyses of growth, aflatoxin production and transcription of aflatoxin pathway and developmental genes during growth of the wild-type and 13B in shake culture in minimal salts/1% sucrose supplemented with TA will also be presented.

Influence of Messenger on Corn and Mycotoxins in Mississippi

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Messenger is a bacterial protein called "Harpin" which can be isolated from certain plant pathogenic bacteria such as *Erwinia amylovora* and *Pseudomonas syringae.* Messenger has been reported to enhance yield and quality of many plants including corn (*Zea may* L.), rice (*Oryza sativa* L.), cotton (*Gossypium hirsutum* L.), tomato (*Lycopersicon esculentum* Mill.), and strawberry (*Fragaria virginiana* Duchesne). Because Messenger is also reported to increase resistance to plant diseases, we tested corn treated with the protein to determine if it might protect against Aspergillus and Fusarium ear rot diseases and decrease the accumulation of aflatoxins and fumonisins. This study was conducted two years in the field at two different locations near the Jimie Whitten Delta States Research Center at Stoneville, Mississippi. Pioneer Hi-Bred brand 32R25, a locally-adapted corn hybrid, was planted in April 2002 and 2003 at both locations. The experimental design was a RCB with 4 replications. The corn was inoculated with *Aspergillus flavus* strain F3W4, which produces large amounts of aflatoxins. The fungus was formulated on autoclaved wheat kernels and applied to the soil surface at 20 lbs/A between the 4 center rows of 6-row plots at growth stage V5 – V6. *Fusarium verticillioides* infected the corn naturally. Messenger was sprayed on top corn leaves at rate of 2.25 oz/A at V1 – V2 and V5 – V6 leaf stages. Six treatments were applied using a CO_2 pressurized backpack sprayer delivering 10 gal/ A (20 psi). The treatments were: 1) MES 2.25 oz/A, at V1 – V2 growth stage; 2) MES 2.25 oz/A, at V1 – V2 growth stage plus fungal inoculum (20 lbs/A); 3) MES 2.25 oz/A, at V5 – V6 growth stage; 4) MES 2.25 oz/A, at V1 – V2 growth stage plus fungal inoculum (20 lbs/A); 5) Untreated control; and 6) Treated control with fungal inoculum (20 lbs/A). Statistical analysis showed no significant differences in yield. Messenger had no effect on the level of aflatoxins or fumonisins or their respective fungi. In conclusion, the 2002 and 2003 trails in two locations showed that Messenger did not increase corn yields or corn quality. The variation noticed within the treatments is normal for yield and grain quality in a small plot test area.

Profiles of Headspace Volatiles from Leaves of Ten Commercial Pistachio Varieties

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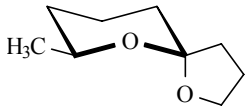
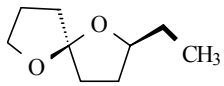
To test this hypothesis we wounded almond hulls mechanically in April, May and June of 2004, allowed them to remain on the trees, then removed them and some undamaged controls two weeks later and analyzed their headspace volatiles by GCMS. The number of volatile components collected from the control almonds increased considerably during the three-month period of the experiment, a reflection of the increasing maturity of the fruit. The April control sample had four prominent volatiles, the May sample had six and the June sample had many more components including several terpenes, sesquiterpenes, aldehydes, and numerous ethyl esters. In addition to the sorts of compounds seen in the controls, the volatiles from the wounded almonds revealed the presence of three spiroketals (**1**, **2** and **3**), natural substances not frequently encountered but that have been found previously in some insects as well as in the bark of several trees.



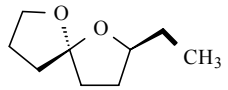
1

(5*S*,7*S*) 7-methyl-1,6-dioxaspiro[4.5]decane
(2*S*,5*R*) isomer



2

(2*S*,5*S*) 2-ethyl-1,6-dioxaspiro[4.4]decane



3

Although **1** was the most abundant of the three spiroketals and appeared in the samples from all three months, its concentration was greatest in April, decreased in May and by June only a small amount was detected. Compounds **2** and **3** were detected in the April sample only. The attraction and egg deposition by adult female navel orangeworm on wounded almonds followed a parallel time course. The greatest numbers of eggs were observed in April, fewer in May and very few in June. Spiroketals **1**, **2** and **3** may be produced by the wounded almonds and attract adult female navel orangeworms, but the possibility that they originate from the female moths and are absorbed by the wound exudates cannot yet be ruled out.

Effects of Four Isoflavones and One Flavone on *Aspergillus flavus* Growth

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Aflatoxin, produced by *Aspergillus flavus* and *A. paraciticus*, is the most potent natural carcinogen. Contaminated grain exceeding aflatoxin levels of 20 parts per billion (ppb) is restricted from interstate trade by the U. S. Food and Drug Administration (FDA). *A. flavus* affects a wide range of agricultural crops such maize, rice, peanuts, figs, pistachios, almonds, cotton, and many other crops (Raper et al, 1965). It has low host specificity, infecting a wide range of plants, insects, and animals (St. Leger et al, 2000). In Kenya as of July 2004, 317 cases of aflatoxicosis had been reported with 125 of those being deaths over a seven month period (CDC, 2004). It has been hypothesized that some flavonoids significantly inhibit the growth of *A. flavus*. Previous studies on the role of flavonoids in decreasing *A. flavus* growth were conducted by Norton (1999) using suspended disk cultures. Flavonoids have been recognized to possess anti-inflammatory, antioxidant, antiallergic, hepatoprotective, antithrombotic, antiviral, and anticarcinogenic actions. They are phenolic compounds that act as potent metal chelators and free radical scavengers (Middleton et al, 2000). Daidzin, daidzein, genistein, and genistin (all isoflavones) are present in soybean and diosmin (a flavone) is present in citrus but all compounds are absent in current commercial maize varieties (Figure 1). We report here the results of testing four flavonoids and one isoflavone compound for ability to reduce *A. flavus* growth in culture. Compounds that significantly reduce *A. flavus* growth may serve a dual purpose *in vivo* of also providing other health benefits such as lowering cancer risks.

Identification of Maize Kernel Proteins Acting as Inhibitors of Aflatoxin Biosynthesis

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The development of molecular markers for resistance to aflatoxin contamination by *Aspergillus flavus* will accelerate the transfer of resistance traits from resistant maize lines to more agronomically superior lines. Here we show the characterization of aflatoxin inhibitory activities present in kernels of the resistant inbred Tex6 (Hamblin and White, Phytopathology 90: 292-296). Using selective (NH4)2SO4 precipitation, gel filtration chromatography and ion exchange chromatography we have identified at least two inhibitory activities that primarily affect aflatoxin biosynthesis, rather than growth. In addition to these two activities, bioassays using recombinant maize ribosome-inactivating protein 1 (an abundant endosperm protein) show that this protein also has an inhibitory effect on aflatoxin biosynthesis. Preliminary real-time PCR data suggest that treatment with either inhibitory activity from Tex6 kernels results in transcriptional repression of the pathway-specific regulator aflR. Further purification steps are in progress to identify the proteins responsible for the inhibitory activities. The genes encoding these proteins will make excellent candidates for stacked resistance. Also, we plan to identify the effect of these inhibitors on regulation of aflatoxin biosynthesis using microarray analysis of *A. flavus*.

Maize Seeds Contain Multiple Proteins with Aflatoxin Inhibitory Activity

Robert A. Holmes[1], Gary A. Payne[2], Rebecca S. Boston[1]
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The development of molecular markers for resistance to aflatoxin contamination by *Aspergillus flavus* will accelerate the transfer of resistance traits from resistant maize lines to agronomically superior lines. Here we show the characterization of aflatoxin inhibitory activities present in kernels of the resistant inbred Tex6 (Hamblin and White, Phytopathology 90: 292-296). Using selective (NH4)2SO4 precipitation, gel filtration chromatography and ion exchange chromatography we have identified at least two inhibitory activities that primarily affect aflatoxin biosynthesis, rather than growth. In addition to these two activities, bioassays using recombinant maize ribosome-inactivating protein 1 (an abundant endosperm protein) show that this protein also has an inhibitory effect on aflatoxin biosynthesis. Preliminary real-time PCR data suggest that treatment with either inhibitory activity from Tex6 kernels results in transcriptional repression of the pathway-specific regulator *aflR*. Further purification steps are in progress to identify the proteins responsible for the inhibitory activities. The genes encoding these proteins will make excellent candidates for stacked resistance. Also, we plan to identify the effect of these inhibitors on regulation of aflatoxin biosynthesis using microarray analysis of *A. flavus*.

FUNGAL GENOMICS/FUMONISIN/AFLATOXIN ELIMINATION WORKSHOP

17TH ANNUAL AFLATOXIN ELIMINATION WORKSHOP

Session 2: Crop Resistance - Genetic Engineering

Moderator: *Bob Klein, California Pistachio Commission*

Investigating the Role(s) of Corn Glyoxalase I Protein in Host Resistance to *Aspergillus flavus* Infection/Aflatoxin Production using RNAi Technology

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Aflatoxins are carcinogens produced mainly by *Aspergillus flavus* and *A. parasiticus* during infection of susceptible crops such as corn (*Zea mays* L.). Though resistant corn genotypes have been identified, the incorporation of resistance into commercial lines has been slow due to the lack of selectable markers. Recently, using a proteomics approach a dozen proteins were found to express at higher levels in resistant corn lines compared to susceptible ones. Over a dozen of these spots have been sequenced. One such protein, glyoxalase I (GLX-I, EC 4.4.1.5), has been further characterized and data suggest that glyoxalase I may play an important role in kernel resistance. GLX-I may contribute to decreased methyglyoxal (an aflatoxin inducer) level inside kernels. However, direct evidence for the involvement of this protein in kernel resistance would be difficult to acquire without the recent discovery of the RNA interference (RNAi) phenomenon. This posttranscriptional gene silencing (PTGS) is a sequence-specific RNA degradation process. It is triggered by a double stranded (ds) RNA, leading to the degradation of homologous RNA encoded by endogenous genes, and transgenes. In the present study, a binary vector containing all the key elements needed to generate a dsRNA structure was constructed using Gateway technology. A 0.6 kb coding region of *glx-I* was integrated into the vector through site-specific recombination. This construct was then transformed into immature corn embryos using Agrobacterium. Callus clones representing independent transformation events are currently being recovered from *in vitro* selection for regeneration to plants. The expression of *glx-I* in the transgenic tissues will be compared using realtime RT-PCR with non-transformed controls to identify the efficacy of gene silencing before poting the transgenic plants for seed production. These RNAi studies may highlight the role glyoxalase I plays in kernel resistance.

Introduction of Antifungal Genes into Peanut

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Aflatoxin reduction through genetic engineering of peanut should be possible through the introduction of anti-fungal genes. Progress is slow, however, because of the relative inefficiency of peanut transformation and untested response of *Aspergillus* to the expression of anti-fungals in peanut tissues. In order to potentially improve transformation efficiency and reduce the dependence of transformation on antibiotic resistance genes, we have tested other selection methods for peanut transformation. Unfortunately, none of the more recently tested selectable markers is more efficient than hygromycin resistance for the recovery of transgenic peanut. Quite the opposite is the observation that neither of two mercury-resistance genes, mercuric ion reductase (*merA*) or organomercurial lyase (*merB*), confer to somatic embryos the ability to grow on mercuric chloride or phenylmercuric acetate (PMA)-containing media, respectively. A third marker, green fluorescent protein, has been used for visual selection and as a reporter in co-bombardments with *merB* to assess the effectiveness of PMA selection. Within ~7 mo we determined that *merB* was not useful for selection of transgenic embryogenic tissues because from 40 lines growing on PMA, 3 were essentially selected visually and 2 of these contained the merB gene as demonstrated by PCR and Southern blot analyses. None of the remaining lines that continued to grow on PMA showed any molecular evidence of being transformed.

A putative antifungal gene that we have introduced into peanut is *cpo-p,* a non-heme chloroperoxidase from *Pseudomonas pyrrocinia*. Rajasekaran et al. (2000; Plant Cell Rep 19:333) showed that this gene conferred antifungal properties to transgenic tobacco. We recently have focused our analysis on progeny from one line that showed ~50% reduction in *A. flavus* hyphal growth using an in vitro assay with tissue extract. Progeny containing cpo-p retain the antifungal properties of the parental line. We have documented gene expression in these plants using northern and western blots to detect RNA and protein, respectively. A seed increase is planned for summer, 2005 so that replicated field trials for aflatoxin reduction can be conducted in 2006.

Another particularly interesting putative antifungal gene encodes for an anti-apoptotic protein from humans, Bcl-XL. Bcl-XL has been transformed into tobacco and was shown to confer broad-spectrum pathogen resistance (Dickman et al. 2001; PNAS 98:6957). We have recovered at least 31 independent lines from 14 bombardment experiments and have selected PCR-positive lines for plant regeneration. Several lines have been tested by western blotting for protein expression during regeneration and 4/5 tested thus far show expression. Since it is speculated that Bcl-XL may be a stress-related protein, we will test the tolerance of transformed plants to paraquat as a quick assay for protein activity. It probably will be necessary to conduct in vivo rather than in vitro fungal assays for this gene product.

A new goal of the project is to produce a TILLING population that can be screened for lipoxygenase mutants or any other gene suspected to enhance aflatoxin production. TILLING stands for Targeting Induced Local Lesions IN Genomes (McCallum et al. 2000; Plant Physiol. 123:439), is a mutation strategy, and was first tested in *Arabidopsis*. TILLING can identify mutants based on screening with gene sequence rather than for phenotype. Presently, ~2000 M1 plants are in the field and a subset will be screened for mutation frequency by AFLP. The TILLING populations would be useful for recovering mutants from any gene once the gene of interest has been identified and sequenced.

Cotton Antifungal Compounds

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Our goal is to engineer cotton with genes that will result in cotton that has lower levels of aflatoxin. We have developed efficient cotton transformation and regeneration protocols that can be used either to express novel structural genes or modify expression of existing cotton genes. This may enable us to enhance resistance to *A. flavus* infection. In the past we have focused on a variety of structural gene candidates that include a small lytic peptide gene, a chloroperoxidase gene, and a neutral cotton chitinase gene. While our studies of transgenic plants expressing some of these structural genes are still ongoing, we have also continued to more completely characterize the anti-flavus activity of the neutral cotton chitinase, and expanded our studies to include other cotton antimicrobial proteins.

Our previous chitinase purification scheme includes a chitin affinity column. Consequently, with this protocol, we obtain Class I cotton chitinases (they contain a chitin binding domain). Our chitin affinity column fraction migrates as a single band on a 1-D gel, however it is composed of three isoelectric isomers. To determine the activity of each isomer singly and in combination, as well as to study the potential activity of other cotton chitinases against *A. flavus*, we have developed an alternate purification scheme. The new purification is based on fractionation of protein extracts by pI and by size. A preliminary study of fractions purified in this fashion shows that fractions with pI's in the neutral range are active against *A. flavus in vitro*. Our ongoing studies will include activity assays of single fractions and combinations of fractions to determine their efficacy against *A. flavus*. This purification scheme may also enable us to purify additional cotton chitinase fractions to determine their efficacy.

We are also interested in studying other cotton proteins that may have antifungal activity. Vicilins are one of the major seed storage protein groups. The cotton vicilin is first synthesized as a precursor protein that is processed (signal peptide cleaved) and then cleaved and degraded during germination. Although all of the proteolytic sites are not definitively identified in the cotton vicilin, there are several obvious candidate sites for cleavage (1). The cotton vicilin precursor is cleaved to generate an approximate 20kD cysteine rich peptidethat has 3 repeats of a 4 cysteine unit. A homolog of this cys rich peptide from *Macadamia integrifolia* has antimicrobial activity (2). Other studies have shown that the cotton cys rich peptide is a component of more complex fractions that have antimicrobial activity (3). We are in the process of purifying the 20 kD cotton fragment and then testing fractions containing this fragment for antifungal activity.

Our overall goal is to identify natural antimicrobial compounds found in cotton. Once these compounds are identified and characterized, they may become targets for genetic engineering. In addition, tracking the expression of these compounds in different cotton cultivars may provide a marker to facilitate identification of cultivars with enhanced natural resistance to *A. flavus.*

1. *Chlan, C., Pyle, J., Legocki, A.B., and Dure, L.S. (1986) Plant Molecular Biology 7:475-489.*
2. *Marcus, J., Green, J.L., Goulter, K.C. and Manners, J.M. (1999) Plant Journal 19:699-710.*
3. *Chung, R., Neumann, G., and Polya, G.M. (1997) Plant Science 127: 1-16*

Function of Oxylipin Biosynthetic Enzymes in the Reduction of Aflatoxin and Fumonisins in Maize: Functional Genomics Approach

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Oxylipins are oxygenated fatty acids, the majority of which are derivatives of linolenic (18:3) and linoleic (18:2) acids, the two most abundant polyunsaturated fatty acids found in both plants hosts and fungi. *Aspergillus flavus* and *A. nidulans* produce their own oxylipins. The best characterized *Aspergillus* oxylipins, termed psi factors, are potent transcriptional regulators of sporogenesis and mycotoxin biosynthesis. In plants, oxylipins are implicated as molecular signals in such diverse processes as growth and development, and defense against pathogens and pests. Recent biochemical and genetic evidence suggests that there is a sophisticated oxylipin-mediated signaling crosstalk between the plant host and *Aspergillus* spp. It is plausible that the outcome of this cross-talk dictates whether the host is resistant or susceptible to pathogen invasion and accumulation of mycotoxins. The key enzymes of the plant oxylipin biosynthetic pathways are lipoxygenases (LOXs) and 12-oxo-phytodienoate reductases (OPRs). LOXs catalyze the hydroperoxidation of linoleic and linolenic acids either at position 9 or 13 of their carbon chains. The resulting fatty acid hydroperoxides are then converted into an array of oxylipins. The function of the 9-LOX derived oxylipins is unclear. The 13-LOX pathway produces C6 volatile aldehydes and jasmonates such as 12-oxo-phytodienoic acid (12-OPDA) and its derivative jasmonic acid (JA). OPRs reduce 12-OPDA to form JA that was shown to inhibit aflatoxin biosynthesis.

Recent biochemical and gene expression analyses of resistant versus susceptible corn lines strongly suggest that 9-lipoxygenases (9-LOXs) and their metabolites may be the *Aspergillus* and aflatoxin susceptibility factors in plants that act to increase *Aspergillus* sporulation and aflatoxin production in maize. In contrast, 13-LOXs and OPRs are hypothesized to increase resistance to aflatoxin contamination because its products, C6 volatiles and JA, act to inhibit aflatoxin biosynthesis. Our RNA profiling data obtained by using massively parallel signature sequencing technique (MPSS) and RNA blot analysis of LOX and OPR gene expression not only further supported these hypotheses but also extended them to include other maize-fungal pathogen systems. The results of our studies showed that some 9-LOXs are induced only or to much higher levels during susceptible but not resistant interactions with two other maize fungal pathogens, *Cochliobolus carbonum* and *Colletotrichum graminicola*. Unlike the 9-LOXs, the transcription level of certain 13-LOXs was only induced in plants resistant to these pathogens. To test these hypotheses that some 9-LOXs are susceptibility factors that cause maize plants to accumulate higher levels of mycotoxins and certain 13-LOXs and OPRs contribute to reducing levels of susceptibility to mycotoxin contamination, we are disrupting all the LOX and some OPR genes in corn lines contrasting in their levels of mycotoxin contamination. We have identified maize mutants in which the function of 9 of the 12 maize LOXs, and 1 of the 8 OPR genes is interrupted by insertions of *Mu* transposable elements in their coding sequences. Currently we are generating near isogenic wild type and mutant LOX and OPR lines in the genetic backgrounds that are either resistant or susceptible to aflatoxin and fumonisin accumulation. We will test them for any changes in resistance to ear rots caused by *Aspergillus flavus* and *Fusarium verticillioides* and accumulation of aflatoxins/fumonisins. If these hypotheses are correct, then (1) knocking out 9-LOX genes in susceptible inbred lines will result in increased resistance to aflatoxin accumulation and (2) inactivating 13-LOX and OPR2 genes will decrease aflatoxin accumulation in resistant lines.

Control of Preharvest Aflatoxin Contamination in Cotton—Update on New Antifungal Peptides and Chloroplast Expression

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In order to combat the problem of preharvest aflatoxin contamination we have made many advances using transgenic approaches in our lab. Most notably, we have demonstrated the antifungal activities of transgenic tobacco or cotton plants expressing the antifungal peptide D4E1 or the chloroperoxidase (cpo-p) that tolerate or resist invasion by many microbial pathogens including *Aspergillus flavus*. We are currently multiplying seed of selected cotton transformants for field testing against soil-borne vascular pathogens and *A. flavus*. Meanwhile, we are also continually evaluating several new gene constructs for efficacy against *A. flavus*. Some of the approaches are listed below:

1. Following the promising results on *A. flavus* control that we demonstrated earlier with transgenic tobacco and cotton plants expressing the synthetic lytic peptide D4E1, we are currently addressing some of the problems inherent in the synthetic peptide technology – a) by making modified synthetic peptides that are more potent against *A. flavus*, yet not harmful to the plant; b) by making peptides that are large enough to be identified *in planta* and analyzed by immunoassays or western blots; c) targeting the expression of these peptides to plastids or peroxisomes to shield them from proteolytic degradation.

2. Antifungal activities of purothionin and hordothionin (provided by Dr. R. Skadsen, ARS, Madison, Wisconsin) were examined in our lab. These proteins, when assayed *in vitro* against germinating conidia of *A. flavus*, showed an inhibitory level of 10 μM. Cotton transformation experiments have been carried out with the horodthionin (Hth 1) construct driven by the cottonseed storage protein promoter (provided by Dr. Caryl Chlan, University of Louisiana, Lafayette. LA). Analysis of these plants are yet to be carried out for expression and phenotype in cottonseeds.

3. We have also engineered a synthetic cpo-p gene in an effort to optimize the codon usage of this bacterial gene for expression in plants. Analysis of crude extracts from transgenic tobacco leaves (nuclear expressing) indicate ample enzyme activity compared to the non-optimized version. We are in the process of making proper gene constructs with the optimized version for chloroplast transformation as well.

4. We have made new constructs with the antifungal gene, MOD 1, a synthetic gene that encodes the active form of RIP 1 from corn. Results from Dr. Weissinger's lab indicate that RIP 1 is not phytotoxic and is effective against *A. flavus* in transgenic peanuts. The efficacy of this gene will be tested in both tobacco and cotton in our lab.

5. In order to improve the efficiency of antifungal gene expression, we have been evaluating expression of transgenes in tobacco chloroplast transformants. Results are being presented in a separate poster (Ruhlman et al).

Transgenic Peanut Expressing Maize RIP I Inhibits *Aspergillus* Growth *In vivo*

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RIP 1 is a ribosome inactivating protein from maize which has been shown to inhibit hyphal growth of *Aspergillus flavus in vitro* when spores were exposed to an activated form of the protein. RIP 1 is expressed in maize as an inactive pro-RIP that must be activated by proteolytic cleavage. Mod 1 is a synthetic gene that encodes an active RIP 1 identical to the proteolysis-activated form of the protein found in maize. The protein encoded by Mod 1 has also been found to retard feeding by certain insects when expressed in transgenic plants. We have transferred this gene into peanut in an attempt to control aflatoxin contamination by retarding fungal growth.

We have developed an improved peanut transformation procedure in which DNA is transferred to imbibed peanut embryos from mature seeds to reduce the time in culture, improve fertility of transgenic plants, and reduce labor needed to produce transgenic peanuts. Transgenic plants typically flower 290 days after initial imbibition of the mature embryos.

Two plasmids were delivered simultaneously by microprojectile bombardment into peanut embryos of cv. 'NCV 11' and 'Georgia Green'. One plasmid carried the Mod 1 gene driven by the ubiquitin 3 promoter from potato (*ubi 3*), flanked with Rb 7 MARs from tobacco. The other plasmid carried a similar construct, but with a gene encoding hygromycin phosphotransferase, conferring resistance against hygromycin, in place of Mod 1. Transgenic plants were recovered after rigorous selection on hygromycin.

From 467 treated embryos of cv. 'Georgia Green', 43 events were recovered that were positive for Mod 1 by PCR. 10 of these events were shown by western blot to produce the Mod 1 protein. From 1024 treated embryos of peanut cv. 'NCV 11', 49 PCR positive events were recovered, of which 18 express the Mod 1 protein. These plants are fully fertile when grown to maturity.

Preliminary efficacy tests of Mod 1 transgenic peanut have been conducted. In these tests, the cotyledons from mature seed (R_1) taken from expressing primary transformants were dissected away from the embryo, which was retained and subsequently grown to maturity in a culture system. The cotyledons were then cut in half and imbibed on water agar. The cotyledons were then inoculated with a suspension of *A. flavus* conidia containing approximately 10^5 conidia from the toxigenic strain "32-8". The cotyledons were then incubated in the dark at 28°C. The experiments were controlled by inoculating cotyledons from untransformed peanut cv. 'Georgia Green' or 'NCV 11' as appropriate. Control and transgenics were photographed daily to record growth of fungal mycelium.

Growth of *A. flavus* mycelium was significantly inhibited on all expressing transgenics. While untransformed controls were completely overgrown by 3 days following inoculation, transgenics showed little mycleium growth even after 8 days. Inhibition varied among transgenic events, and inhibition was positively correlated with level of Mod 1 observed in the R_0 parent plant. This is an especially important observation, because it is consistent with the hypothesis that the Mod 1 protein inhibits fungal growth.

Expression of Mod 1 in R_1 plants was mapped, and the antifungal protein was observed in roots, stems, leaves, flowers, developing pods and immature seeds. Mod 1 also accumulates in mature seeds, but may be expressed at higher levels in cotyledonary epidermis than in internal storage cells.

All lines have now been advanced to R_2 generation, and are currently being tested to identify homozygotes suitable for use in large-scale field trials.

Biochemical and Genetic Analysis of Gallic Acid in Walnuts in Relation to Aflatoxin Accumulation

R. M. Muir,[1] G. McGranahan,[1] C. Leslie,[1] and A. M. Dandekar.[1]
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California nut crops represent a 1.5 billion dollar industry, a significant amount of which is threatened by regulations regarding aflatoxin contamination. Our strategy for reducing aflatoxin contamination has two major goals; 1) develop insect resistance to reduce insect damage and 2) identify genes that reduce toxin production.

For developing insect resistant walnuts we have focused on the *cry*IAc gene from *Bacillus thuringiensis*. We are currently analyzing field grown transgenic plant material and are comparing the expression of *cry*IAc using two promoters; CaMV35S and Ubi3 (from potato). Several of the transformed lines have flowered and the nuts harvested. Analysis of vegetative tissues and nut tissues has revealed that both constructs expressed by CaMV35S and Ubi3, gave significant results compared to control untransformed walnut tissues.

Since walnuts have less of a problem with aflatoxin contamination as compared to other nuts, we have begun examining nut tissue (i.e. seed coat) as potential sources of resistance genes. Studies conducted at the USDA facility at Albany (Mahoney et al., 2003) have shown that seed coat extracts from several walnut varieties, and especially from the cultivar Tulare, contain a 'factor' that strongly inhibits aflatoxin production by *A.flavus*. Our results have suggested that this 'factor' is gallic acid (GA), a key component of hydrolysable tannins (HTs). Estimation of free GA by staining with rhodanine and precipitation with boric acid has indicated that GA is the component of HTs responsible for the observed suppression of aflatoxin synthesis in Tulare pellicles. Genetic and molecular analysis of HT metabolism is underway to identify candidate genes responsible for the accumulation of free GA in Tulare.

We have also been investigating the dehydrogenase directly responsible for gallic acid synthesis. We developed an enzyme activity assay that enables us to visulalize gallic acid production on a native protein gel. The activity levels of the gallic acid synthesizing enzyme increase during the early stages of seed coat development and declines towards the end of the growing season (i.e. August through September). Importantly, these levels correlate with the levels of gallic acid accumulation detected in the Tulare seed coat.

The gallic acid synthesizing dehydrogenase was visualized on a native protein gel and sequenced using mass spectrometry. The hypothetical identity of the protein was determined based on its' sequence similarity with homologous proteins in GenBank. The gallic acid synthesizing dehydrognease was determined to be part of the shikimate pathway and exhibited relatively high sequence homology with shikimate dehydrogenase (SDH) from other plant and bacterial species. We subsequently cloned SDH from Tulare seed coats and purified the protein on a nickel column. Purified SDH synthesized GA *in vitro*, conclusively demonstrating that we had successfully identified the protein responsible for GA biosynthesis. Over-expression analysis of SDH in walnut somatic embryos is currently underway to determine the effects of the protein on GA accumulation *in planta* as well as aflatoxin production by *A. flavus*.

Panel Discussion: Crop Resistance – Genetic Engineering

Panel Chair: Robert Brown
Panel Members: Zhi-Yuan Chen, Peggy Ozias-Akins, Carol Chlan, Mike Kolomiets, Kanniah Rajasekaran, Arthur Weissinger, and Ryan Muir

Highlights**:**

1) Art Weissinger was questioned about why he altered the native wersion of RIP (ribosome inactivating protein) prior to plant transformation. Why didn't he use native RIP? He had no particular reason as to why an alternative form was used. It was then pointed out that native RIP is also bioactive against *Aspergillus flavus.*

2) Chen was asked by Georgia Davis "what was his control for RNAi silenced callus or plant? His answer: "Due to the fact that the immature embryos used for transformation were from hybrid kernels, therefore, each of the non-transformed callus tissues from different embryos may be genetically different. For this reason, callus tissues from over half a dozen individual embryos will be examined for basal level expression of gene of interest, and possible variations between controls."

3) Caryl Chlan was questioned about the purpose of her work with antifungals (chitinases) to cotton resistance. She explained that their objective was to identify a chitinase with superior activity to serve as a candidate gene for plant transformation.

Expressing Antifungal Genes in the Chloropast Genome

T. A. Ruhlman,[1] K. Rajasekaran,[2] and J. W. Cary.[2]
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Phytopathogens and mycotoxin producing saprophytes can significantly impact the value of commodity crops and cause concern for food and feed safety. Introduction of microbial resistance may be achieved through genetic manipulation of susceptible crop species. Genetic transformation of plastid genomes via particle bombardment offers many benefits over nuclear transformation. For example, proteins can accumulate to high levels (up to 50% of total soluble protein) due to increased expression; proteins of interest are retained within the chloroplast envelope protecting them from degradation by host cytoplasmic proteases; maternal inheritance of organellar genomes allows for control of transgene escape through pollen and single copy insertion through homologous recombination eliminates complications such as transgene silencing. Our research aims to confer resistance to microbial pathogens and mycotoxin producers by stable integration and expression of novel antifungal genes such as chloroperoxidase (cpo-p) or D4E1 in the plastid genome. Tobacco transformants were identified by repeated rounds of selection on regeneration media with toxic levels of spectinomycin, resistance to which was conferred by the aminoglycoside-3'-adenyltransferase (*aadA*) gene in the chloroplast transformation cassette. PCR analysis with primers specific for our transgene has confirmed integration in the plastid genome. Western analysis confirms the presence of the gene product in higher abundance in chloroplast transformants over nuclear. Preliminary gene expression analyses have indicated that higher levels of antimicrobial peptides/proteins are produced in chloroplast transformants due to organelle proliferation and protection of proteins within the chloroplast envelope. Following cell and organelle lysis that occurs as a result of microbial invasion, antifungal proteins will be released at high concentration at the site of infection, thus inhibiting pathogen advance and spread of disease and/or production of mycotoxins.

FUNGAL GENOMICS/FUMONISIN/AFLATOXIN ELIMINATION WORKSHOP

17TH ANNUAL AFLATOXIN ELIMINATION WORKSHOP

Session 3: Crop Resistance – Conventional Breeding

Moderator: *Phil Wakelyn, National Cotton Council*

Identification of Aflatoxin-Resistance and Potential Markers in Maize Breeding Materials Developed in West Africa

Robert L. Brown,[1] Zhi-Yuan Chen,[2] Abebe Menkir,[3] and Thomas E. Cleveland.[1]
[1]USDA/ARS, Southern Regional Research Center, New Orleans, LA; [2]Louisana State University, Baton Rouge, LA; [3]International Institute of Tropical Agriculture, Ibadan, Nigeria.

The breeding program at the International Institute of Tropical Agriculture (IITA) had originally developed maize populations from crosses of US aflatoxin-resistant inbreds with IITA resistant inbreds. Populations formed had 75%, 50%, or 25% US germplasm. A total of 65 S_5 (~98% towards inbred development) lines extracted from 75% US x 25% IITA germplasm and 63 S_5 lines derived from 50% US x 50% IITA germplasm with resistance to lowland leaf rust, leaf blight and *Curvularia* leaf spot, were advanced to S_6. Seed samples of the first 65 lines were sent to the USDA-ARS laboratory in New Orleans (SRRC) and were screened for resistance to aflatoxin contamination using the laboratory-based kernel screening assay (KSA). Based on the results of KSA screening, nine inbred lines with significantly lower levels of aflatoxin production compared to their respective US resistant parents and with good agronomic features were selected. Seed samples of these inbred lines were increased in Nigeria in the 2004 dry season (December-April) and sent to SRRC to confirm their resistance to aflatoxin production. Following confirmation of the levels of resistance of these lines, they will be candidates for release to the public as sources of resistance genes to aflatoxin production. These lines are also being tested in the field in Nigeria under artificial inoculation with *Aspergillus flavus* to assess the consistency of their resistance across the different strains of *A. flavus*. The remaining 63 S_6 lines have been sent to SRRC for KSA screening. Several closely-related pairs of lines, differing significantly in aflatoxin accumulation, were identified at SRRC among IITA temperate (75% US) breeding materials. When kernel embryo proteins of these pairs were subjected to differential proteomics, several resistance-associated proteins (RAPs), categorized as stress-related, were found to be more abundantly produced in resistant lines. Also, a putative regulatory protein protein was identified. When endosperm proteins of closely related pairs were compared, stress proteins, antifungals and putative regulatory proteins were identified. Among the antifungals were a 14 kDa trypsin inhibitor and zeamatin, both previously highlighted in our lab as important to kernel resistance. The discovery of abundant stress-related proteins, constitutively expressed in resistant lines, supports the findings of an earlier investigation we conducted. This may be an important find, since drought and high nighttime temperatures are known to enhance aflatoxin accumulation in maize. Possession of unique or higher levels of the identified stress-proteins, may put resistant lines in an advantageous position over susceptible ones in the ability to defend against pathogens while under stress. Future studies include cloning corresponding genes, performing characterization experiments and studies, such as RNAi gene silencing experiments, to investigate the role of RAPs in resistance. These investigations will aid in selecting the best molecular markers for use in breeding strategies and transformation protocols involving IITA-bred germplasm.

Development of Field Based Techniques for Assessing Variability among Cotton Cultivars in Susceptibility to Aflatoxin Contamination During the Second Phase of Contamination

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All cotton producing regions of the U.S. may experience some aflatoxin contamination in some years. In most of the Cotton Belt, occurrence of aflatoxin levels unacceptable for dairy use is infrequent, but in Arizona, southern Texas and the Imperial Valley of California, aflatoxins are a perennial concern. Aflatoxin contamination of cottonseed occurs in two phases. The crop is first contaminated when *A. flavus* infects the developing bolls through wounds or cracks. The second phase of contamination occurs when mature seed is exposed to both conducive temperature (above 27° C) and either high relative humidity (above 85%) or rewetting at or after boll opening. Rank cotton, dense canopies, dew, and late irrigations increase the severity of the second phase. No assays have been developed to compare susceptibility of cotton cultivars to contamination during the second phase of contamination under field conditions. Development of such an assay is the first objective of this project.

Another objective is to determine if seed hardness and seed coat fragility relate to susceptibility to aflatoxin contamination during the second phase of contamination. Rain on open bolls has been shown to be the factor most closely related to aflatoxin content of commercial cottonseed and is the most important factor inciting the second phase of contamination. Therefore, deliberate wetting of open bolls is being used to simulate moisture effects. Field plots have been established at The University of Arizona Maricopa Agricultural Center (MAC). A transgenic Bt cultivar commonly planted in Arizona was utilized in order to reduce variation in aflatoxin content caused by pink bollworm damage. A misting system capable of increasing humidity in the crop canopy and of precisely wetting open bolls has been designed and installed at (MAC). Water is pumped from the irrigation ditch through two inch pipe throughout the field to each of sixty four 20 x 20 ft^2 plots. The misting system consists of 21 one-gal/hr brass foggers spaced about three feet apart in a 10 x 10 ft^2 grid of ½ inch PVC pipe.

In 2004, treatments included 9 hour misting periods initiated at various dates either with or without *A. flavus* inoculation. Plots were replicated eight times, and bolls were harvested two weeks after misting. The harvested cotton was dried in a forced air oven at 45° C and stored dry at room temperature until processed. Harvested seed will be tested for aflatoxin, and the data used to design a field based screening technique cultivars. Subsequent variations in treatments will include frequency of wetting and duration of wetting. Humidity and temperature of treated plots will be monitored to quantify impact of wetting regimen on canopy environment. Ultimately a model will be developed relating wetting period and temperature to aflatoxin content. This will contribute both to development of the screening technique and to a better understanding of the second phase of contamination. Such a model will be useful to the industry in assessing crop aflatoxin risk prior to harvest and to researchers striving to assess efficacy of other aflatoxin management technologies.

Use of Molecular Markers to Create Commercially Acceptable Corn Hybrids with Resistance to Aflatoxin Production in Grain

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The goal of this research is to produce commercially usable corn inbreds that in hybrid combinations have significant levels of resistance to aflatoxin production. For the last five years, research at the University of Illinois has successfully used molecular markers in conjunction with field evaluation to confirm resistance and to move resistance for Aspergillus ear rot and aflatoxin production from the agronomically poor but resistant inbreds MP313E or Tex6 into the commercially used inbred FR1064. In the summer of 2004 we evaluated versions of inbred FR1064 that had chromosome regions from the resistant inbred MP313E associated with resistance on chromosome four as testcross hybrids with the commercially used inbred FR4341 in Mississippi and south Texas with inoculation. The resistant versions of FR1064 had been backcrossed three times to FR1064 and are approximately 94% similar to the susceptible FR1064 except they have chromosome 4 regions from MP313E. The plots in south Texas were provided by BH Genetics and plots in Mississippi provided by Michael Clements, USDA-ARS. In those two trials FR1064 x FR4341 had an average of 866 ppb aflatoxin in grain. The resistant versions of FR1064 x FR4341 ranged from 205 to 434 ppb aflatoxin in grain. Results from 2005 are in the process of being completed. In one experiment, done by Javier Betran in Texas, FR1064 x FR4341 had an average of 2705 ppb aflatoxin and the two resistant versions of FR1064 x FR4341 had 223 and 235 ppb aflatoxin. In another trial done by Steve Moore in Louisiana, FR1064 x FR4341 had 1409 ppb aflatoxin and the two resistant versions had 595 and 667 ppb aflatoxin. Yield of the resistant hybrids was good in both trials. We also are developing commercially usable lines using resistance from the inbred Tex6 which is an unreleased line developed at the University of Illinois. With Tex6 we are selecting chromosome segments for lower aflatoxin associated with QTL on chromosomes 2, 5, 8 and 10. Backcross derived lines with various combinations of these QTL have good levels of resistance in testcrosses with FR4341 in trials in Texas, Mississipi, Louisiana, and Illinois. Therefore inclusion of chromosome regions from MP313E on chromosome four and on chromosome regions from Tex6 reduces the amount of aflatoxin. These trials were done with inoculation in very favorable environments which suggests that under natural conditions the resistance will likely allow grain to be consistently produced with less than 200 ppm aflatoxin.

This past summer we crossed resistant versions of FR1064 with a number of commercially used inbreds that are used as male parents of hybrids grown throughout the Southern United States. It is our expectation, with appropriate male parents, that we can develop a number of hybrids that will be more appropriately grown in the south and have very good agronomic traits and low aflatoxin in grain.

A resistant version of FR1064 will be marketed by IFSI (Illinois Foundation Seeds Inc.). IFSI's major business is the supply of foundation seeds, which makes the seed accessible to any company in the U.S. Also in the winter of 2004-05 IFS will begin crossing Monsanto transgene (Bt, Roundup Ready, CRW) versions of FR1064 as well as a Dow transgene (Herculex I) versions of Fr1064 with resistant versions of FR1064 to begin the process of pyramiding resistance to aflatoxin production with various transgene traits.

We will continue backcrossing and should be commercially testing significant numbers of resistant hybrids in the next few years for agronomic traits and aflatoxin concentration in grain. We expect to

test a backcross 2 resistant line that uses MP313E as a source of resistance in yield trials in south Texas this next summer.

Screening Corn Accessions for Resistance to Aflatoxin

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Genetic improvement for host resistance of peanut to fungal colonization and toxin production is an important approach for the integrated management of aflatoxin contamination. Breeding progress has been limited due to the lack of a cost-effective method for identifying resistant individuals in segregating populations. The objective of this research was to attempt to develop a molecular marker that could be used to select for resistance to seed infection by *Aspergillus flavus*. A resistant (J11) and susceptible (Zhonghua No. 5) parent were selected and used to produce an F_2 population. The DNAs of the two parents were extracted and tested with AFLP protocol. From the 256 primer pairs tested, twenty-four pairs showed polymorphism between the two parental lines. The parents and their 108 F_2 progeny were tested for reaction to *A. flavus* infection by inoculation under laboratory conditions. The DNAs of 12 F_2 segregating lines extremely resistant and susceptible to seed infection were pooled and analyzed along with individual plants. Two polymorphic markers associated with resistance to seed infection were identified.

Taking the Cob Out: Genetic and Proteomic Approaches for Investigating Induced and Constitutive Resistance to *Aspergillus flavus* during Maize (*Zea mays* L.) Ear

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Maize (*Zea mays* L.), one of the world's most important food crops, is susceptible to infection by *Aspergillus* species that produce the very potent mycotoxin, aflatoxin. Aflatoxin contamination of maize kernels is a serious human and animal health threat worldwide and knowledge gained in maize can be applied to crops with less tractable genetics. In maize, resistance against *Aspergillus flavus* infection and aflatoxin accumulation is a complex trait that is influenced by genotype and environmental conditions. We are using multiple approaches, including proteomics, to understand how maize inbred lines with varying levels of resistance and susceptibility to *Apergillus flavus* respond to fungal infection. Because most aflatoxin accumulation occurs in the field during ear development, we are examining the proteomes of cob tissues from inoculated and control ears from several different genotypes. Genetic analysis suggests that the maternally derived tissues contribute to resistance, therefore, we are examining the proteomes of the rachis, silks and pericarp. Because studies with GFP-tagged *A. flavus* indicated that fungal movement is retarded in the rachis and rachilla of resistant lines, the proteome of rachis tissue collected 21 days after silk emergence has been investigated and approximately 75% of 480 proteins have been identified. Differential-in-gel-electrophoresis (DIGE) is being used to analyze differences among rachis proteins from resistant and susceptible inbreds and inoculated and control ears. Differences in the proteomes of silks from resistant and susceptible inbreds have been examined and the induction of a suite of resistance proteins in response to inoculation has been demonstrated. Protein extracts from resistant silks retard the growth of *A. flavus* on filter paper disks. Because drought stress is associated with aflatoxin accumulation in maize, we also are examining the proteome of ears from resistant and susceptible genotypes in response to water stress and inoculation with *A. flavus*. By combining quantitative trait loci mapping, microarray analyses, proteomic studies, bioinformatics and molecular biology, we hope to develop a comprehensive understanding of this complex plant-fungal interaction.

Breeding Corn Germplasm to Reduce Aflatoxin Contamination

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Our program has evaluated and selected corn germplasm to reduce aflatoxin in Texas. Our goal is to identify and develop corn inbreds with resistant factors that can reduce the risk of aflatoxin and have a good agronomic performance in hybrids. We have used three locations in South Central Texas and inoculation with *Aspergillus flavus* (isolate NRRL3357) using the nonwounding silk channel or colonized corn kernels on the soil surface. At harvest, infected ears were husked, rated for kernel integrity and visible fungi colonization, shelled, ground with a mill, and evaluated for aflatoxin. Quantification of aflatoxin was conducted with monoclonal antibody affinity columns and fluorescence determination (Vicam Aflatest™). These experimental screening techniques and inoculation have facilitated the display of genetic differences and increased heritability in aflatoxin evaluations. The response of inbreds to aflatoxin accumulation varies, depending upon the environment and the genetic background. Therefore, their evaluation in several hybrid combinations and multiple environments under inoculation has been necessary to identify the most consistent resistant germplasm. Low aflatoxin accumulation was associated with good husk coverage, flinty endosperm texture, good kernel integrity, high grain yield, and late maturities. Selection for these associated traits having high heritabilities and strong correlation with low aflatoxin, in addition to low aflatoxin accumulation in inbreds and hybrids, has helped in reducing the risk of aflatoxin contamination. The less susceptible white inbreds in hybrids across evaluations in Texas have been CML176, and Tx experimental lines derived from crosses among CML269, Tx110, CML78, and CML270. The less susceptible yellow inbreds have been Tx experimental lines derived from crosses among CML288, Tx772, CML161, and NC300, and several lines from Tx69Q and LAMA breeding populations. Most of these lines have shown good agronomic performance and less aflatoxin than commercial hybrids in southern areas in testcrosses with testers LH195 and LH210, which are representative of heterotic groups commonly used in the U.S. The combined evaluations for aflatoxin and agronomic performance has facilitated the selection for adaptation, yield potential, stability, and reduced aflatoxin risk. Ultimately, we aim to incorporate aflatoxin resistant factors into elite genetic backgrounds suitable to produce commercial hybrids.

Tools for Breeding Peanut with Resistance to Preharvest Aflatoxin Contamination

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Peanuts become contaminated with aflatoxin when subjected to prolonged periods of heat and drought stress. The resulting aflatoxin contamination costs the peanut industry over $20 million annually. The development of peanut cultivars with resistance to preharvest aflatoxin contamination (PAC) would reduce these costs. Two requirements are needed to breed a cultivar with resistance to PAC. First we must have screening techniques that can reliably differentiate genetic resistance from susceptibility. During the course of this project we have developed field screening techniques that can measure genetic differences in aflatoxin contamination. The second requirement is genetic variation for resistance. During the course of this project we have identified 11 core accessions that have shown at least a 70 % reduction in PAC in multiple environments. Recently, some of these accessions have also been shown to have relatively low fungal colonization and aflatoxin contamination when tested under conditions to simulate post harvest contamination. We have also identified significant reduction in PAC in peanut genotypes with drought tolerance. These sources of resistance to PAC have been entered into a hybridization program. They have been crossed with cultivars and breeding lines that have high yield, acceptable grade, and resistance to tomato spotted wilt virus (TSWV). Due to the large environmental variation in PAC, it is not feasible to examine these breeding populations until late generations when there is less heterozygosity and adequate seed are available for field testing using multiple replications. We have identified families and individual breeding lines that have relatively low PAC, relatively high yield, and acceptable levels of resistance to TSWV. However, much faster breeding progress could be achieved through the development and use of indirect selection techniques. We are exploring this with studies on mechanisms of resistance to PAC and attempting to develop molecular markers for resistance.

Field Evaluation and Gene Expression Analysis of Corn Genotypes for Resistance to Preharvest Aflatoxin Contamination and Drought Stress

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The development of "southern hybrid-type" corn with resistance to ear-feeding insects, tolerance to drought, and resistance to *Aspergillus* infection or aflatoxin formation, and with acceptable yield will help southern corn growers produce a quality, profitable crop. The objectives of this program are to evaluate germplasm, hybrid combination of the selected inbreds for aflatoxin contamination and yield performance, and study gene expression in response to different factors using microarray gene expression analysis.

We have conducted the field and laboratory screening of inbreds for resistance and susceptibility to *A. flavus* and aflatoxin production. Few lines, P56, P2, GT-A1-1, GT-A638, have similar aflatoxin levels as Tex6 and MP714, averaged from 44 ppb (GT-A638) to 19 ppb (P56). MP714 averaged 20 ppb of total aflatoxin. Selected single-cross hybrids were tested in the Georgia State Performance Test and SERAT (southeastern region aflatoxin test) in 2004. In comparison with the commercial hybrids, field tests in Georgia and Texas have shown that several crosses have good yield potential for the Southeastern region in comparison with local commercial hybrids in the irrigation and non-irrigation condition.

We also used microarray analysis of gene expression to study the effects of drought stress, *A. flavus* infection, SA induction on gene expression in developing corn ear/kernels in 2003 and 2004. Three corn inbred lines, B73, GT-A1-1, and A638, have been used in the gene expression study. The laboratory bioassay of the seeds harvested from the rain-out shelters has showed that the pre-formed biochemical compounds were accumulated by the treatments used for gene expression study. The lower aflatoxin concentration from the lab assay will be correlated with the gene expression profiles induced by different treatment. Genomic research of gene expression analyses in the developing ears/kernels will help identify and understand the function of genes that control significant biological processes and agronomic performance under stress of drought and fungal infection, which are crucial in the development of genetic approaches for control of preharvest aflatoxin contamination.

Progress in Peanut Functional Genomics, a Strategy to Mitigate Aflatoxin Contamination and Improve other Important Traits

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The peanut genome (2,800 Mb) is large in comparison to the current plant models, *Arabidopsis* (128 Mb), rice (420 Mb), and *Medicago* (500 Mb). *A. thaliana* was estimated to have 27,000 genes; rice is predicted to have 30,000 to 50,000 genes; and soybean is about 60,000 genes. While the peanut genome probably has a similar number of genes as the small-genome species, the large genome size makes it unrealistic to completely sequence the peanut genome in the near future. By partially sequencing large numbers of expressed genes, it is possible to obtain enough information to access genome of peanut and thus ensure continued advance in the biology of this important crop species. This project focuses solely on the expressed component of the peanut genome. The National Center for Biotechnology Information's (GenBank) dbEST database contains (June 25, 2004) 22,165,266 ESTs. There are 5,643,076 human ESTs, and 4,191,008 ESTs for mouse and rat. Among plants, *Triticum aestivum* (wheat) has the most ESTs deposited with 552,245 in the NCBI, and *Zea mays* (maize) is second with 397,515. *Glycine max* (soybean) has 334,668, *Oryza sativa* (rice) has 284,006, *Arabidopsis thaliana* has 258,825, and *Medicago truncatula* has 187,763. Peanut (*Arachis hypogaea*) is dramatically low on the list for such an important crop, with only 1,366 ESTs.

We have generated about 2000 ESTs from two cDNA libraries constructed using mRNA prepared from leaves of peanut line C34-24 (resistant to leaf spots and tomato spotted wilt virus) and immature pods of peanut line A13 (tolerant to drought stress and preharvest aflatoxin contamination), and 1345 ESTs have been released to GenBank (CD037499 to CD038843). Four hundred unigenes have been selected from these ESTs and arrayed on glass slides for gene expression analysis, and 44 EST-derived SSR markers have been characterized for cultivated peanut, in which over 20% of the SSRs produced polymorphic markers among 24 cultivated peanut genotypes. The microarray data have been validated using real-time PCR. In the laboratory screening of the germplasm from China and India, the differences of peanut kernel infection by *A. flavus* were significant. Several Chinese and Indian lines had lower *A. flavus* colonization than Georgia Green, and this resistance has been tested in the field in 2003 and 2004. The 2003 field test supported the resistance detected in the laboratory. The protein profiles (2-D) have revealed the differences in protein contents, which may be related to the resistance observed in the laboratory and the field.

Breeding Stress-Tolerant and Low-Aflatoxin Corn Hybrids for the Southern States

Wenwei Xu,[1] Gary Odvody,[2] and W. Paul Williams.[3]
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[3]*USDA/ARS, Corn Host Plant Resistance Research Unit, Mississippi State, MS.*

Aflatoxin contamination of corn by *A. flavus* is a chronic problem in the southern United States. The objective of this study was to determine if genetic improvement of abiotic stress tolerance and corn earworm resistance can reduce the aflatoxin risk in this region.

Experimental hybrids developed by the corn-breeding program of the Texas Agricultural Experiment Station (TAES) in Lubbock, TX and commercial checks were grown in Lubbock, Halfway, Corpus Christi, and Beeville in Texas and Mississipi State, MS in 2004. In Lubbock and Halfway, plants were inoculated one week after silking by injecting *A. flavus* conidia into silk channels. In Corpus Christi, Beeville, and Mississippi State, corn kernels colonized by *A. flavus* were distributed between all rows when the first hybrid was at the mid-silking stage to provide the increased and uniform aerial dissemination of conidia. In all cases, the inocolum was from a high aflatoxin-producing *A. flavus* strain (NRRL3357). A limited late planting date was used in Corpus Christi, Beeville and Mississippi State to encourage severe drought stress at later stages of maturity. The tests used a randomized complete block design with nine replications. Ears from each plot were hand-harvested. All ears were threshed and agronomic data were recorded including grain yield. All grain from each composite replicate was initially ground in a Romer mill at the coarse grind setting. After thoroughly mixing the ground kernels, a 200 g sub-sample was ground again at the finest grind setting on the mill. Aflatoxin B1 assay was done on 50 g sub-samples of the finely ground material for each composite replication using the Vicam immunoassay/ fluorometer system. Standard ANOVA was performed to test the differences among hybrids. Natural log transformation of aflatoxin data was made before data analysis.

Results showed that hybrids S1W x CML343, S2B73 x NC300 and Tx202 x CML343 had significantly lower aflatoxin than control hybrid. The aflatoxin levels in S1W x CML343, S2B73 x NC300, Tx202 x CML343, and P31B13 (CK) was respectively 5.3, 61.3, 16.7, and 70.0 ppb at Corpus Christi, 10.1, 9.4, 5.8, and 33.3 ppb at Mississippi State, which was in general consistent with the results in 2003. The experimental hybrids were and they yield well in comparison to the checks. For example, the average yield of S1W x CML343 in Halfway and Lubbock in 2003 and 2004 was yielded 140212 kg/ha in while 31B13 produced 13,003 kg/ha in the same environments. Hybrid B110 x SGP3 had high yielding and high aflatoxin in both years in most environments. The TAES experimental hybrids and their parental lines have at least 25% tropical germplasm and were selected for drought and heat tolerance, CEW resistance and overall agronomic performance. They have tight husk, good ear tip coverage, significantly lower grain mold and less ear injuries by corn earworm. Results indicate that breeding for drought tolerance and earworm resistance is a promising approach to reduce aflatoxin contamination in corn grown in Southern environments.

Computational Support for Research in Maize Proteomics and Marker Assisted Selection

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The Corn Host Plant Resistance Research Unit, USDA-ARS, Mississippi State University is conducting a number of different types of studies to determine the effects of biotic and abiotic factors on *Aspergillus flavus* infection and aflatoxin accumulation in maize with the goal of developing resistant maize cell lines. Computational support for this research has provided scientists with improved methods for data access and retrieval, new methods to analyze the effects of environmental factors on aflatoxin accumulation in different cell lines, and tools to improve protein identification rates in proteomics studies and to support efficient and informative annotation of the proteome.

An integrated database system with data management, data mining, and data modeling capabilities has been developed that provides a comprehensive view of the maize genetics research at MSU. The database archives raw data, derived data and metadata collected or generated by the biologists. The database system has of five partitions for germplasm data, field data, quantitative trait loci (QTL) analysis data, proteomics data, and weather data. A web-based interface provides fast, flexible access to the database system for investigators.

The effects of environmental variables on aflatoxin levels are complex and difficult to predict. A genetic algorithm approach has been developed to extract features describing environmental factors that are correlated to aflatoxin levels. Data available for this study include aflatoxin levels at maturity, middle silk (flowering) date, and environmental data from 1998 to 2003. Functions for computing values of environmental variables are represented as a population of randomly initialized artificial chromosomes. Each chromosome represents the environmental variable to be measured, the period of time over which it should be measured, and how the values should be combined over the interval. The correlation of the index specified by each chromosome and measured aflatoxin levels is used to evaluate the "fitness" of each chromosome. The highest scoring environmental variables derived using this method were found to have R^2 values of greater 0.90.

Characterization of the maize proteome of the developing ear under different conditions has the potential to reveal the fundamental processes that confer resistance in some cell lines. Advances in proteomics have been made possible by high-throughput methods for gel electrophoresis and new technologies for mass spectrometry such as LC/MS/MS However, these technologies can only be used to their full potential for protein identification if they are supported by the availability of high quality, annotated protein data sets. A method for generating such a data set from EST clusters was developed that is based on homology-based search. The effectiveness of this method for generating a high quality annotated set of translated ESTs for identification of proteins was tested by comparing the protein identification rates in proteins from cob using a data set (called the PIE Maize set) generated from assembled ESTs available from TIGR and with those generated using the NCBI protein database for corn, rice, and Arabidopsis. The PIE data set resulted in identification of 87.5% of the spots, while only 56% of the spots were identified with the NCBI database. Additional tools have been developed to streamline the protein identification process and to provide the Gene Ontology annotation of the identified proteins.

Asymmetris in Fungal Development on Almond may indicate Differential Intra-Tissue Vulnerabilities to Aflatoxin Contamination

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With a value at $1.3 billion in 2004, almond has become the largest horticultural export for the U.S. and the largest agricultural export for California. Approximately 70% of the crop is exported to European and Asian countries with very low to zero import aflatoxin tolerance levels. Stress during seed cotyledon development is correlated with greater fungal proliferation/sporulation and possibly aflatoxin formation. Within both naturally and artificially infected kernels, greater mold development/sporulation were found in the cotyledon showing greater developmental stress as determined by reduced growth rate and final size. This knowledge is leading to a better understanding of aflatoxin induction *in-situ*, and consequently, more efficient resistance selection. Genetic (inheritance) and genomic (molecular) characterization of resistance also shows promise for the development of efficient markers to facilitate the timely incorporation of aflatoxin resistance to more widely adapted California almond varieties. . Findings concerning genetic sources and mechanisms of resistance are being incorporated into an industry supported breeding program. Second generation selections, combining resistance and improved commercial quality are now in regional field-testing. Resistance options demonstrating particular promise based on field and lab performance and good heritability include the *web*-endocarp and *pers*-kernel traits. The *web*-trait is expressed as a very thin but very high-seal endocarp. The *pers*-trait confers non-Gallic acid based reduction in toxin levels following kernel inoculation by *A. flavus*. Aflatoxin production in field-infected nuts is erratic, apparently due to differences in tissue stress.

Large-scale field-testing from 2000 through 2004 in northern, central and southern California growing regions has shown effective aflatoxin control, with selection *UCD36-52* showing particularly good promise as a resistant and commercially desirable variety.

Panel Discussion: Crop Resistance – Conventional Breeding

Panel Chair: Paul Williams
Panel Members: Javier Betrán, Susan Bridges, Robert Brown, Tom Gradziel, Baozhu Guo, Corley Holbrook, Dawn Luthe, Steve Moore, M. W. Olsen, Don White, and Wenwei Xu

Summary of Presentations: Phil Wakelyn of the National Cotton Council served as moderator of the session. Presentations included not only reports on conventional breeding for resistance to aflatoxin contamination, but also other approaches toward identifying and using molecular markers to enhance aflatoxin resistance. The majority of the presentations were related to aflatoxin resistance in corn. Such topics as field screening for resistance, association between drought stress and aflatoxin accumulation, gene expression, and molecular markers were included. Proteomic approaches toward investigating aflatoxin resistance at two locations were discussed. Efforts to develop computational support for proteomics are currently underway as well. New tools are being developed to increase aflatoxin resistance in peanut, cotton, and almond.

Summary of Panel Discussion: Because of lack of time after the presentation were completed, the panel discussion was abbreviated. Dave Wilson, who has been engaged in research on aflatoxin for many years, urged those in the audience who are now conducting research on aflatoxin to familiarize themselves with research that was done in the past. He strongly encouraged young scientists in the audience to avoid spending their time and resources repeating research done in the past.

Distribution of Aflatoxin in B73

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Aflatoxin contamination in corn is well known to be highly variable, a characteristic that has added to the difficulty of selecting for resistance. No literature has been found that documents the normal distribution of aflatoxin concentration in natural or inoculated corn populations. A 2-year field study was conducted in central Louisiana using conventional wide-row spacing on a Norwood silt loam soil. The test included inbred 'B73' (susceptible) both years and four treatments; 1=Open-pollinated non-inoculated ears, 2=Open pollinated inoculated (*A. flavus*) ears, 3=Self-pollinated non-inoculated ears, and 4=Self-pollinated inoculated (*A. flavus*) ears. A split-plot design was used with inbred as main plot and treatment as sub-plot. Each inbred-treatment combination was replicated 20 times. Inoculation was made after anthesis using a pin bar cushion dipped in a container containing spores in liquid suspension (90 million spores/ml). Ears were harvested after physiological maturity, shelled, and ground to a fine meal. Samples were then sent to the USDA-ARS facility at Stoneville, MS and analyzed for aflatoxin using commercially available assay kits distributed by Neogen Corporation of Lansing, MI. Several preliminary findings resulted from the first year of this study. Inoculating with *Aspergillus flavus* greatly increased fungal growth, BGYF, and aflatoxin. Fungal growth and aflatoxin were greater in the susceptible line. Aflatoxin increased when plants were self-pollinated. There were no significant interactions between inbreds and inoculation or pollination methods, indicating that the ranking of inbreds did not change due to these treatments. The correlation coefficients of aflatoxin with BGYF and fungal growth were 0.93 and 0.87, respectively. Interactions between inbreds, pollination, and inoculation were not the same in 2003, using only data from open-pollinated B73. Average aflatoxin contamination in naturally infected plots was 317 ppb in 2002 and 112 ppb in 2003. Aflatoxin contamination in inoculated plots was 3066 ppb in 2002 and 7536 ppb in 2003. The standard deviation was higher in naturally infected plots in 2002 (859) than in 2003 (330). The standard deviation was lower in inoculated plots in 2002 (2156) than in 2003 (19403). Distribution of aflatoxin was heavily skewed in most populations. Aflatoxin appeared more broadly distributed in naturally-infected populations. Inoculating with *Aspergillus flavus* spores not only increased aflatoxin contamination but seemed to narrow the distribution.

Response to Aflatoxin Contamination of Argentine Maize Hybrids in Texas Environments

B. Ochs, C. McKee, T. Isakeit, G. Odvody, K. Mayfield, and J. Betran.
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Argentine maize, which has hard endosperm, orange grain color, good husk cover, and temperate adaptation, can contribute to improve grain quality of U.S. hybrids and reduce aflatoxin accumulation. Our objective was to determine relative performance and adaptation of Argentine hybrids across diverse Texas environments ranging from subtropical to temperate, and compare their response to aflatoxin contamination with commercial hybrids under inoculation with *Aspergillus flavus*. Argentine commercial hybrids from seed companies Nidera, Syngenta, Monsanto, and Agricom (A933, AX877, AX878, AX882, AX884IT, AX888IT, AX889, AX934, AX956, AX882MG, AX890MG, DK682, CONDOR, NK900TDMAX, AGRI124) together with popular Southern U.S. commercial hybrids (DKC66-80, DKC69-70, P31B13, P32R25, LH195 x LH210) were evaluated for grain yield, agronomic performance and response to aflatoxin across Texas locations. Plots in Weslaco and College Station, TX were inoculated with *A. flavus* spores through silk channel inoculation, and plots in Corpus Christi, TX were inoculated by scattering *A. flavus* colonized kernels between rows. Aflatoxin concentrations were quantified with Vicam Aflatest® antibody columns. Aflatoxin concentrations were transformed using the base 10 logarithm, and analyzed with SAS procedures. Five ear samples were taken from each replication and location to determine grain traits such as test weight (kg hl^{-1}) and 1000 kernel weights (g). Combine or hand harvested grain yield (Mg ha^{-1}) data was recorded on plot basis and adjusted to 15.5% grain moisture. Average aflatoxin concentration was 121.6 ng g^{-1} (range: 19.1 to 537.0 ng g^{-1}) for College Station, 297.9 ng g^{-1} (range: 16.5 to 1597.3 ng g^{-1}) for Weslaco, 70.7 ng g^{-1} (range: 5.1 to 344.1 ng g^{-1}) for Corpus Christi, and 104.6 ng g^{-1} (range 17.9 to 342.22 ng g^{-1}) across all locations. Average test weight was 76.2 kg hl^{-1} in College Station, 75.3 kg hl^{-1} in Weslaco, 75.7 kg hl^{-1} in Corpus Christi, and 75.7 kg hl^{-1} across all environments. Average grain yield was 9.3 Mg ha^{-1} in College Station, 8.7 Mg ha^{-1} in Weslaco, 7.3 Mg ha^{-1} in Corpus Christi, and 8.4 Mg ha^{-1} over all environments. Kernel texture rating averages varied little across locations with a 1.96 rating in College Station, a 2.26 in Weslaco, a 2.25 in Corpus Christi, and a 2.16 rating across all environments. Although there were a few Argentine hybrids that had softer kernels, the majority were flintier. The averages for 1000 kernel weight was 279.9 g in College Station, 287.8 g in Weslaco, 253.5 g in Corpus Christi, and 274.2 g across all environments. In general, U.S. hybrids were more susceptible to aflatoxin than Argentine hybrids, and some association was found between texture rating and aflatoxin accumulation. Preliminary data show Argentine hybrids AX889, CONDOR, AX888IT, and NKTD900MAX have competitive yields and aflatoxin levels below 50 ng g^{-1}. Argentine hybrids as a group showed higher test weights than the U.S. hybrids but less grain yield and lower 1000 kernel weight. Although Argentine hybrids appeared to be adapted to South and Central Texas, future performance of these hybrids in different environments will determine their range of adaptation and potential contribution to reduce aflatoxin.

Aflatoxin Accumulation in Testcrosses Between Exotic Lines and an Elite Temperate Tester

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Incorporation of exotic subtropical and tropical germplasm can contribute alleles for increased productivity, grain quality, and resistance to biotic stress, to temperate maize. We have characterized maize lines (Tx-LAMA) developed in Texas from exotic breeding populations in testcrosses with LH195, an elite Stiff Stalk temperate tester, for both agronomic traits and response to aflatoxin. Selections during line development were based on maturity, grain color, endosperm texture, lodging, and plant characteristics. The testcrosses were evaluated for grain yield and agronomic performance (lodging, grain moisture, test weight, maturity) in summer 2004 across subtropical, transitional, and temperate environments in Texas locations. At three locations, College Station, Corpus Christi, and Weslaco, TX, the testcrosses were evaluated for response to aflatoxin under inoculation with *Aspergillus flavus* using silk channel (College Station, Weslaco) or colonized kernel (Corpus Christi) inoculations. Alpha lattices design with 3 replicates were used in all locations. Commercial hybrids from Dekalb (DKC66-80, DKC69-70, and DKC69-72), Pioneer Hi-Bred (P31B13 and P32R25), and Holdens (LH195 x LH210) were used as checks. Vicam Aflatest® antibody columns were used for aflatoxin quantification. Statistical analysis at each location and across locations was conducted with Proc Mixed in SAS. Significant differences were observed for aflatoxin and grain yield at most of the locations and across locations. In general, the Tx-LAMA testcrosses showed less aflatoxin accumulation than the commercial checks both across environments and at each individual location. The mean aflatoxin concentration for the Tx-LAMA testcrosses across environments was 67.5 ng g^{-1} (range: 9.3 to 407.4 ng g^{-1}), and for the commercial checks was 128.2 ng g^{-1} (range: 24.9 to 292.3 ng g^{-1}). Aflatoxin concentrations at the Corpus Christi location were lower than the other environments with a mean level of 24.3 ng g^{-1} (range: 4.4 to 63.1 ng g^{-1}), compared to 146.6 ng g^{-1} at College Station (range: 1.2 to 1174.9 ng g^{-1}), and 198.6 ng g^{-1} at Weslaco (5.2 to 831.8 ng g^{-1}). Corpus Christi results showed no significant differences between testcrosses, this probably occurred because the colonized kernel inoculation method was not able to produce levels of aflatoxin high enough to separate testcrosses in a season with more rain than usual at flowering time. Grain yield did not appear significantly different between the Tx-LAMA testcrosses and the commercial check hybrids as groups, but was significantly different for individual testcrosses both between and within environments. Overall, Tx-LAMA testcrosses showed lower levels of aflatoxin accumulation than the commercial checks at individual locations and across all environments. Tx-LAMA testcrosses 1, 15, 17, 37, 45, 49, and 51 showed aflatoxin levels below 50 ng g-1, and grain yields above the mean grain yield across all environments. These testcrosses seem to be competitive with commercial hybrids and reduce the risk of aflatoxin.

Interaction between *A. flavus* Strains and Host Plant Genotypes

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Pre-harvest aflatoxin (AF) contamination is a major limitation to maize production in Texas and the southern United States, causing major economic losses and severe health problems. Screening for resistance to AF accumulation is commonly conducted through inoculation with a highly concentrated solution of *Aspergillus flavus* spores. Only one isolate of *A. flavus* has been used for inoculation in our studies, NRRL3357 (AF3357), although isolates of this species are known to exhibit a range of toxigenity. Our objective is to determine if there is interaction between genetically-different isolates of *A. flavus* and several genotypes of maize. Two experiments were conducted, one with hybrids and the other with inbreds. The hybrid trial included eight hybrids and the inbred trial five inbreds. Inbreds and hybrids were selected for maturity and previous contrasting response to AF. Hybrids were planted at three Texas locations: College Station (CS), Weslaco (WE) and Corpus Christi (CC), while inbreds were planted at two locations, CS and WE. An alpha-lattice field experimental design was used in the hybrid trial, and a randomized complete block design in the inbred trial, both with four replications. Hybrids and inbreds were inoculated using the silk channel inoculation method using isolates L1, F1, I5 (all isolated from soil in a maize field in San Patricio County, TX) and AF3357. Each isolate was diluted to a concentration of 10^7 spores ml^{-1}. Isolates were inoculated within the same row of maize and kept separate by marking individual plants with different colored tape. Inoculation occurred 10-12 d after plants reached mid-silk. Plots were hand harvested, shelled and ground prior to quantification of AF. Aflatoxin was quantified with monoclonal antibody affinity columns using fluorescent determination (Vicam AflaTest®). Data analysis was conducted using SAS. Hybrid data from individual locations CS, WE and CC showed significant differences among genotypes and isolates and showed no genotype by isolate interaction. Across location analysis showed significant genotype*environment and isolate*environment interactions, and no significant genotype*isolate and environment*genotype*isolate interactions. Isolate I5 produced the most aflatoxin in CS and CC (196.4 ng g^{-1} and 552.7 ng g^{-1}, respectively) and F1 produced the most aflatoxin in WE (227.2 ng g-1). SR470 had the most aflatoxin in hybrids in CS (345.2 ng g^{-1}) and WE (314.7 ng g^{-1}), and CML172/CML161 had the most AF in CC (1118.8 ng g^{-1}). Inbred data from CS and WE showed significant differences among genotypes and isolates as well, and non significant genotype*isolate interaction. Across location analysis showed significant genotype*environment and no significant isolate*environment or environment*genotype*isolate interactions. Isolate AF3357 produced the most AF in CS (1024.3 ng g^{-1}) and I5 produced the most AF in WE (340.7 ng g^{-1}). Inbred CML269 had the most AF at CS (919.9 ng g^{-1}) and Tx804 had the most AF at WE (1269.8 ng g^{-1}). There are differences in the levels of aflatoxin produced by the different isolates. There are differences in the levels of aflatoxin accumulated in different hybrids and inbreds, and also across different environments. However, no significant interaction was detected between genotypes and isolates in either inbreds or hybrids. Therefore, based on these results, it appears that screening for resistance using one isolate of *A. flavus* can be effective but inoculation with multiple isolates needs further consideration and evaluation.

Generation Mean Analysis of Aflatoxin Accumulation

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In generation mean analysis (GMA), means of different generations (e.g., P1, P2, F1, F2, BC1P1, BC1P2) are used to estimate genetic effects in a cross between two inbreds. Some of these generations are genetically homogeneous (P1, P2, F1), which helps to determine environmental and error variation, and the remainder are segregating (F2, BC1P1, BC1P2). We have used GMA to characterize and validate the presence of resistant factors in inbreds such as CML176, CML161 and CML269. A total of six crosses among maize inbreds with contrasting response to aflatoxin accumulation were study following GMA. Inbreds CML176, CML161 and CML269 have shown less susceptibility to aflatoxin than Tx811, Tx804 and Tx114 in previous evaluations. Six generations (P1, P2, F1, F2, BC1P1, and BC1P2) from six crosses were evaluated in replicated trials at two locations in Texas, Weslaco (WE) and College Station (CS) in 2003. Twice the number of observations was recorded in the segregating generations (F2, BC1P1, and BC1P2) as compared with the non-segregating generations (P1, P2, and F1). Inoculation was with a conidial suspension of *A. flavus* injected 6-10 days after midsilk by using the nonwounding silk channel inoculation technique. At harvest, inoculated ears per generation were husked, dried, shelled, and bulked. The whole kernel sample was ground with a mill and evaluated for aflatoxin. Quantification of aflatoxin was conducted with monoclonal antibody affinity columns and fluorescence determination (Vicam Aflatest^{TM}). Means were obtained using REMLtool^{TM} software and used to estimate: a = additive effects, d = dominance effects, aa = additive by additive epistatic effects, ad = additive by dominant epistatic effects, and dd = dominant by dominant epistatic effects using SAS procedures. The average aflatoxin concentrations across locations was 158 ng g^{-1} for CML176, 597 ng g^{-1} for Tx114, 38 ng g^{-1} for the F1 hybrid, 213 ng g^{-1} for the F2 generation, 76 ng g^{-1} for the BC to CML176, and 252 ng g^{-1} for the BC to Tx114. The average aflatoxin concentration was 354 ng g^{-1} for CML269, 847 ng g^{-1} for Tx114, 140 ng g^{-1} for the F1 hybrid, 300 ng g^{-1} for the F2 generation, 252 ng g^{-1} for the BC to CML269, and 519 ng g^{-1} for the BC to Tx114. The average aflatoxin concentration was 340 ng g^{-1} for CML161, 2273 ng g^{-1} for Tx804, 123 ng g^{-1} for the F1 hybrid, 523 ng g^{-1} for the F2 generation, 280 ng g^{-1} for the BC to CML161, and 588 ng g^{-1} for the BC to Tx804. Inbred CML176 and its generations (F1s and BC1s) showed reduced levels of aflatoxin, suggesting that it has resistant factors that are heritable. Furthermore, CML176 had less susceptibility than CML269, another line that has been associated with less aflatoxin. Inbreds Tx114 and Tx804, and its generations were susceptible to aflatoxin. Dominant effects and dominant by dominant epistatic effects were prevalent in these group of GMA crosses except for the cross CML269 x CML176 that had more additive effects. In most of the cases, backcrosses to the most resistant parent were less susceptible than backcrosses to the susceptible parent, which is further evidence of the presence of resistant genetic factors in these lines. GMA was an effective approach to characterize the type of inheritance for aflatoxin response in these parental inbreds and their generations.

Aflatoxin Accumulation in a Mapping Population of RILs derived from the CML176 x Tx811 Cross

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Recombinant inbred line (RIL) populations, which are developed through continuous selfing of the cross between two contrasting parental inbreds, can be used to estimate heritabilities and to map quantitative trait loci (QTL) of relevant traits in maize. Inbred lines CML176 and Tx811, which differ significantly for many agronomic characteristics were crossed and then selfed for at least 6 generations to produce a mapping population. This population of 162 S6 RILs, was evaluated in replicated trials in two Texas locations, Weslaco (W) and College Station (CS) under inoculation with *Aspergillus flavus*. This population was created to map quantitative trait loci (QTLs) associated with the response to aflatoxin accumulation. The objectives of this study were: (1) to analyze the phenotypic characteristics of days to silking, % root lodging, grain texture, aflatoxin accumulation, and grain yield of the (CML176 x Tx811) RIL mapping population, including parental lines; and (2) to estimate heritability for each trait and phenotypic and genotypic correlations of aflatoxin accumulation with secondary traits. CML176 and Tx811 are two quality protein maize (QPM) inbreds. CML176 has been less susceptible to aflatoxin contamination than Tx811 in previous experiments. The mapping population, including both parents, was grown in three replications at two locations using an alpha lattice design. Parents were replicated twelve times. The population was severely affected by root lodging at both locations as a consequence of heavy rains and winds around flowering. Percentage of root lodging was measured. Flowering time was also measured as number of days from planting to 50 % silking. Inoculation was with a conidial suspension of *A. flavus* injected 6-10 days after midsilk by using the nonwounding silk channel inoculation technique. The isolate was NRRL3357. At harvest, infected ears were husked, dried, shelled, and bulked. The grain was rated for texture (1 indicating flinty endosperm, 5 indicating floury endosperm) and kernel integrity (1 showing intact kernels, 5 indicating fully damaged kernels). Thousand kernel weight and test weight were measured in grams, and grain yield was measured in grams per ear. The whole kernel sample was ground with a mill and evaluated for aflatoxin. Quantification of aflatoxin was conducted with monoclonal antibody affinity columns and fluorescence determination (Vicam Aflatest™). Data was analyzed using SAS procedures and means were obtained using REMLtool™ software. There were significant differences for all traits except kernel texture. The mapping population showed significant differences and broad ranges for all agronomic traits studied, with the offspring showing transgressive segregation for each of the traits. Adjusted averages were 90 days to silking for flowering, 13 % for root lodging, 46 g per ear for grain yield, 558 ng g^{-1} for aflatoxin, 2.27 for the logarithm transformation of aflatoxin, 2.66 for kernel integrity rating, 2.37 for kernel texture rating, and 156 g for thousand kernel weight. The populations showed transgressive segregation, with the range of the RILs significantly different from the parental inbreds. Heritability estimates on genotypic mean basis at Weslaco were high for all the traits: 0.85 for days to silking, 0.76 for % root lodging, 0.73 for kernel integrity, 0.84 for 1000 kernel weight, 0.65 for grain yield, and 0.72 for aflatoxin. Aflatoxin was significantly correlated with kernel integrity (0.51, 0.71 for phenotypic and genotypic correlations, respectively). This trait is highly heritable and also easy to select for in the field. This provides possibilities for future selection indices that may expedite selection for aflatoxin resistance and provide a more inexpensive initial selection criterion.

Southern East Regional Aflatoxin Test (SERAT)

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Aflatoxin, a potent toxin and carcinogen produced by the fungus *Aspergillus flavus*, limits corn marketability, causes enormous economic losses, and poses a risk to animal and human health. Aflatoxin contamination of corn grain is a chronic problem for growers in the southeast United States. For several years, research groups at Louisiana, Mississippi, Georgia, Illinois and Texas have been screening corn germplasm for response to aflatoxin contamination at specific locations. Although several sources of resistance have been identified and released, at present, there are no elite inbred lines resistant to aflatoxin that can be used directly in commercial hybrids. Aflatoxin accumulation is severely affected by the environment. Genotype by environment interaction is normally significant with genotypes showing different relative response across environments. A testing network of environments across major growing areas affected by aflatoxin has been established to identify the most consistent stable sources of resistance. SERAT is a multilocation and multistate regional test of the most promising germplasm from each breeding program. Participants provide seed of a few hybrids and a testing location. Evaluations are conducted under inoculation with *A. flavus* following the protocols commonly used by each research group. In addition to aflatoxin, grain yield and other agronomic traits such as maturity, lodging, grain moisture, test weights, etc. are recorded. Each research group conducts the analysis for its location and single location data are compiled and analyzed across environments. In 2004, SERAT tests were conducted at six locations: Alexandria, LA; Tifton, GA; Starkville, MS; Urbana, IL; Halfway, TX; and Weslaco, TX. The silk channel inoculation method was used at all locations except Urbana, where inoculation with a pinboard was used, and Starkville, where inoculum was injected through husk leaves into the side of the ear. Currently we have aflatoxin accumulation data for Weslaco and Alexandria. Aflatoxin concentrations in Weslaco ranged from 31 ng g^{-1} to 3709 ng g^{-1} with an average of 652 ng g^{-1}. Average aflatoxin concentration at Alexandria was 730 ng g^{-1} with a range from 56 to 1759 ng g^{-1}. In general, some experimental hybrids had less aflatoxin but lower yield than commercial checks. Response of materials from different programs was variable, in that hybrids showed desirable expression for different traits such as aflatoxin, grain yield and standability. This suggests possibilities of combining positive traits by crossing germplasm from different programs. With this collaborative regional testing, we expect to identify the most stable sources of aflatoxin resistance, assess their consistency across different environments and treatments, characterize their agronomic performance, increase the collaboration among research groups in different states, and to assess the magnitude and nature of genotype x environment interaction for aflatoxin.

Development of a Molecular Marker for Resistance to Seed Infection by *Aspergillus flavus* in Peanut

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Genetic improvement for host resistance of peanut to fungal colonization and toxin production is an important approach for the integrated management of aflatoxin contamination. Breeding progress has been limited due to the lack of a cost-effective method for identifying resistant individuals in segregating populations. The objective of this research was to attempt to develop a molecular marker that could be used to select for resistance to seed infection by *Aspergillus flavus*. A resistant (J11) and susceptible (Zhonghua No. 5) parent were selected and used to produce an F_2 population. The DNAs of the two parents were extracted and tested with AFLP protocol. From the 256 primer pairs tested, twenty-four pairs showed polymorphism between the two parental lines. The parents and their 108 F_2 progeny were tested for reaction to *A. flavus* infection by inoculation under laboratory conditions. The DNAs of 12 F_2 segregating lines extremely resistant and susceptible to seed infection were pooled and analyzed along with individual plants. Two polymorphic markers associated with resistance to seed infection were identified.

Gene Differential Expression of Corn Lines at the Late Development Stage under Drought Stress and *Aspergillus flavus* Infection

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We have been using maize microarray chips-Master Unigene from Maize Gene Discovery Project, which has over 20,000 unique ESTs (expressed sequence tags), to analyze gene expression profiles and to identify the genes associated with the stress responses. Research experiments have been conducted in the field rain-out shelters with 3 corn genotypes, GT-A1-1, GT-A638, and B73. Treatments include regular water, water stress after pollination, salicilic acid (SA) spray, and inoculation with *Aspergillus flavus* 20 DAP. The water contents in leaves and soil have been measured since 15 DAP every two days. The seeds have been harvested for laboratory bio-assay. Samples of developing ears have been harvested for gene expression profiling analysis at 20, 25, and 30 DAP. The results presented here are gene expression profiles of only GT-A1-1 samples from drought stress, *A. flavus* challenge under well water or stressed, and SA spray under well water or stressed condition at 20, 25, and 30 DAP. The water content in the drought-stressed soil was below 60% of the well-watered soil at 24 DAP. The responses to different challenges have been shown on the gene expression profiles as indication of the up- or down-expression of genes. The expression profiles will be compared among the 3 genotypes, and individual gene function will be verified by real-time PCR.

Microarray-Based Screening of Differentially Expressed Genes in Peanut in Response to Aspergillus parasiticus infection and Drought Stress

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Aflatoxin contamination caused by Aspergillus fungi is a great concern in peanut production worldwide. Pre-harvest A. parasiticus infection and aflatoxin contamination are usually severe in peanuts that are grown under drought stressed conditions; however, drought tolerant peanut lines have less aflatoxin contamination. The objective of this study was to identify resistance genes in response to A. parasiticus infection under drought stress using miccroarray and real-time PCR. To identify transcripts involved in the resistance, we studied the gene expression profiles in peanut genotype A13 which is drought tolerant and resistant to preharvest aflatoxin contamination, using cDNA microarray containing 384 unigenes selected from two EST (expressed sequenced tag) cDNA libraries challenged by abiotic and biotic stresses. A total of 83 up-regulated spots (Log2 ratio>1) representing 42 genes in several functional categories were detected under both A. parasiticus infection and drought stress. A total of 104 up-regulated spots representing 52 genes were detected in response to drought stress alone. There were forty-nine up-regulated spots (25 genes) commonly expressed in both treatments. The top 20 genes were selected for validation of their expression levels using real-time PCR. A13 was also used to study the functional analysis of these genes and a possible link of these genes to the resistance trait. Microarray technology and real-time PCR were used for comparison of gene expression. The selected genes identified by microarray analysis were validated by real-time PCR. Further investigations are needed to characterize each of these genes. Gene probes could then be developed for application in breeding selection.

Peanut Storage Protein, Conglutin-like Protein, has β-1,3-glucanase Activity and is Induced by Colonization with *Aspergillus flavus*

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Infection of peanut (*Arachis hypogaea* L.) seeds by *Aspergillus flavus* and *A. parasiticus* is a serious problem of contamination of the seeds with aflatoxins. Breeding the resistant cultivars would be an effective approach to eliminate aflatoxin accumulation. The objective of this study was to investigate the expression of the PR protein *β*-1,3-glucanase and the isoform patterns in peanut seeds inoculated with *A. flavus*. Peanut genotypes, GT-YY9 and GT-YY20 (resistant to *Aspergillus flavus* infection), and Georgia Green and A100 (susceptible to *A. flavus* infection), were used in this study. The activities of *β*-1,3-glucanase were similar in the un-infected seeds of all genotypes, but increased significantly in the resistant genotypes after inoculation in comparison with the susceptible genotypes. An in-gel (native PAGE) enzymatic activity assay of *β*-1,3-glucanase revealed that there were more protein bands corresponding to *β*-1,3-glucanase isoforms in the infected seeds of resistant genotypes than in the infected seeds of susceptible genotypes. Both acidic and basic *β*-1,3-glucanase isoforms were detected in the IEF gel. Thin layer chromatography (TLC) analysis of the hydrolytic products from the reaction mixtures of total protein extract and individual bands of native PAGE revealed the presence of the enzymatic hydrolytic oligomer products. Two bands were revealed on the SDS-PAGE with molecular weight of 10-kDa and 13-kDa, respectively, which are of the individual bands corresponding to the bands of *β*-1,3-glucanase isoforms from native PAGE. The 13-kDa protein was the majority. The sequences of the 13-kDa protein showed a high degree of homology to conglutin, a storage protein in peanut seeds. Conglutin is reported as a peanut allergen, Ara h2, and has trypsin inhibitor function. Our results suggest this 13-kDa conglutin-like protein has *β*-1,3-glucanase activity and may be associated with resistance to *A. flavus* conolization in peanut seeds.

A Nonspecific Lipid Transfer Protein and an Allergan *Ara* h1-like Protein are Associated with the Resistance to *Aspergillus sp* in Peanut

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Peanut genotypes, resistant or susceptible to *Aspergillus sp.*, were extracted with acid buffer, pH 3.0. Although, the antifungal *in vitro* bioassays showed that protein extracts from all genotypes inhibited *A. flavus* spore germination and hypha growth, the extracts from all resistant genotypes had stronger antifungal activities than the susceptible genotypes. The protein extract SDS-PAGE profiles revealed that two proteins with molecular weight of 9 kDa and 14 kDa, respectively, were present constitutively in higher concentrations in the seeds of three resistant genotypes. These two proteins were excised from the gels and sequenced using ESI-MS/MS and Edman degradation after trypsin digestion. The sequence suggested that the 9 kDa protein is homologous to the nonspecific lipid transfer proteins 1 (nsLTPs 1). The 14 kDa protein has homology with peanut allergen *Ara* h1 (vicilin), but the molecular weight is much smaller than peanut *Ara* h1 protein. It has been reported that LTPs have antifungal activities. These two small proteins may be novel antifungal proteins and contribute to the resistance against *Aspergillus flavus* in peanut. Further studies of these proteins will be needed in gene expression and regulation.

Laboratory and Field Screening of Peanut Germplasm for Sources of Resistance to Preharvest Aflatoxin Contamination

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Peanut germplasm and breeding lines from China, India (International Crop Research Institute for the Semi-Arid Tropics), and Tifton/Georgia were evaluated in the laboratory and in the field rain-out shelters for sources of resistance to *Aspergillus flavus* infection and preharvest aflatoxin contamination. Total protein extracts were also analyzed by 1-D and 2-D polyacrylamide gel electrophoresis. In the laboratory bio-assay, marked differences in fungal colonization were detected among the tested genotypes at 7 days after inoculation. In the field evaluation, the experiment was randomized completely block design with 9 replications in 2003 and 2004. Only the 2003 data are presented. The aflatoxin levels were ranged from 4 ppb to163 ppb. GT-PE2 had the lowest aflatoxin concentration and A100 had the highest. The breeding lines GT-YY20, a Spanish market-type, had 16 ppb and C34-24, a runner market-type, had 70 ppb. The differences in protein profiles were detected among the genotypes. Unique proteins have been excised from the gels for sequencing. The resistance observed in the laboratory was demonstrated in the field test in 2003. Introgression of the resistance to U.S. breeding lines has been initiated, and the resistant mechanisms are under investigation.

Further Investigation of Resistance Mechanisms Responsible for Reduced Levels of Aflatoxin Accumulation in Maize: QTL Studies of Two Resistant Inbreds

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This study was designed to discover additional sources of genetic resistance to aflatoxin accumulation in maize. Previous studies have been performed that located quantitative trait loci(QTL)in the maize inbred Mp313E. Two separate mapping studies involving different susceptible parents and multiple environments resulted in the identification of two primary and many secondary loci which influence aflatoxin accumulation. Many of these QTL are shared between the two studies while some appear to be unique raising questions about the effect of genetic background on their performance. Currently these QTL are being backcrossed into susceptible inbreds and evaluated for their effect. Data from these tests are also being combined to examine genetic background effects on QTL performance.

Further investigation into sources of resistance continue using different resistant and susceptible lines. Mp92:673 is a resistant inbred developed in the CHPRRU breeding program that is derived from a different genetic background than Mp313E. It is slightly less resistant than Mp313E while being less tropical in growth habit and earlier to flowering. NC300 was chosen as the susceptible inbred. It is a southern adapted inbred with relatively good combining ability. 280 F_2 families derived from a cross of these parents were evaluated in an initial trial in 2003. Variation was observed to be sufficient for QTL analysis. In choosing these parents to develop a mapping population a new source of resistance will be studied, a susceptible inbred adapted to southern growing conditions will be used, and a larger population will be phenotyped. In addition, field data will be collected in Mississippi and Georgia locations so that genotype x environment interactions can be better studied.

One year of field evaluation has been completed and aflatoxin levels of replicated plots are being determined. Genotyping of the population is under way. One more year of field data will be collected in Mississippi and Georgia. QTL analysis will be undertaken to determine relevant genetic regions affecting aflatoxin accumulation and their interactions with each other and the environment. Examination of these collective genetic studies should prove useful in characterizing the complex interactions of resistance loci with the environment, different genetic backgrounds, and other resistance genes.

Assessing Germplasm Enhancement of Maize (GEM) Project Germplasm as a Source of Resistance to Aflatoxin Accumulation in Grain

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Although publicly available corn (*Zea mays* L) inbreds with superior resistance to aflatoxin accumulation have been identified, agronomic performance of these sources as inbred lines *per se* and in hybrid combination is generally poor. Breeding populations and inbred lines *per se* developed through the USDA Germplasm Enhancement of Maize (GEM) project are derived from crosses of temperate and tropical or subtropical germplasm with the goal of widening the germplasm base of commercial hybrid corn in the U.S. Lines available to GEM cooperators and/ or released to the public through GEM have been selected for agronomic performance; however, they have typically not been evaluated for resistance to aflatoxin accumulation in grain. The objective of this study is to assess GEM breeding crosses and GEM lines for resistance to aflatoxin accumulation in grain, and for agronomics that are suitable for hybrid corn production in the southern U.S.

All experiments were planted in replicated, inoculated trials at Mississippi State University in 2003 and 2004. Aflatoxin concentration in grain among 87 GEM lines and experimental checks ranged from 9 to 2147 ng g^{-1} in 2003. Aflatoxin concentration was lowest from the resistant check, inbred Mp313E. Aflatoxin concentration in grain from four GEM lines (02GEM00281, 02GEM00276, 02GEM00277, and 02GEM00279) did not differ significantly ($P>0.05$) from Mp313E. All four of these lines had moderate maturity for the southern U.S., and severity of Aspergillus ear rot that did not differ significantly ($P>0.05$) from Mp313E in 2003 and 2004. Aflatoxin concentration in grain among 19 GEM breeding crosses and experimental checks ranged from 38 to 1087 ng g^{-1} in 2003. Aflatoxin concentration was lowest from the resistant check, hybrid Mp313E x Mo18W. Aflatoxin concentration in grain from five breeding crosses (01GEM80064, 01GEM80058, 98GEM80076, 01GEM80062, and 95GEM80061) did not differ significantly ($P>0.05$) from Mp313E x Mo18W. All five of these breeding crosses had severity of Aspergillus ear rot that did not differ significantly ($P>0.05$) from Mp313E x Mo18W in 2003 and 2004. All five of these breeding crosses have moderate maturity for the Southern U.S.

GEM lines in this study will be evaluated for agronomic characteristics and resistance to aflatoxin accumulation in grain as testcrosses developed with commercial inbreds Holden's LH195 and LH210 in 2005. All GEM lines also are being evaluated for resistance to leaf feeding by southwestern corn borer (*Diatraea grandiosella* Dyar). Ear to row and recurrent selection programs are currently underway with several of the most promising lines and breeding crosses.

Express of Different Lipoxygenase Genes in Relation to Resistance in Corn Developing and Mature Embryos to Aflatoxin Contamination by *Aspergillus flavus*

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The accumulation of aflatoxin, a mycotoxin produced by the fungus *Aspergillus flavus* (*A. flavus*) Link: Fr., during maize grain fill continues to be a problem. The impact of aflatoxins on the agricultural economy and human and animal health is well established. Because most aflatoxin problems develop in the field, the best strategy for eliminating mycotoxin production is to develop preharvest host resistance to aflatoxin contamination. Developing natural genetic resistance should be the most effective control for aflatoxin production in maize and is currently used for the development of resistant hybrids in conventional plant-breeding programs. USDA-ARS scientists at Mississippi State University (MSU) have contributed to this intense field research by releasing several corn inbreds as a source of resistance to kernel infection by *A. flavus*. However, incorporating resistance from these sources into commercial hybrids requires identification and characterization of factors shown to be associated with resistance. The ability to identify resistant corn genotypes has been enhanced by the combination of genomic analysis and molecular biology tools. Molecular markers associated with resistance would help to advance the breeding program and provide clues about the mechanisms of resistance. Therefore, working in this direction we have found that a lipoxygenase could be a molecular marker consistently associated with corn resistance. Lipoxygenases are enzymes responsible to initiate the octadecanoid pathway in response to insect and fungi attack. We compared lox expression levels of two different genes (13-lox and 9-lox) of immature embryos from different resistant and susceptible inbreds and of mature embryos from six crosses among the same resistant and susceptible genotypes. We sampled at different time points after inoculation with *A. flavus* spores. We found that 13- Zmlox gene expression in developing and mature embryos, appears to be associated with the corn resistance response to *A. flavus* attack. This gene codes a 13-LOX isoform that acts on linoleic acid producing 13(S)-hydroperoxides and its derivatives could also function as inhibitors of sporulating factors and aflatoxin gene synthesis in *A. flavus*. 13-lox expression analysis showed higher expression in resistant genotypes than in susceptible genotypes, whereas inoculated embryos showed higher expression than the non-inoculated controls. Similar results were obtained using mature embryos from the hybrids between resistant and susceptible inbreds. We did not detect any 13-lox expression in hybrids obtained from the cross between susceptible inbred lines. Futhermore, the level of aflatoxin content measured in infected kernels from resistant and susceptible genotypes showed good correlation with 13-lox expression levels. Although resistance to contamination by aflatoxin is greatly influenced by environmental effects on the plants, the differences in lox expression between hybrid genotypes could be related to the inherited plant resistant mechanisms. Therefore we propose the use of this corn embryo 13-lox gene as a potential molecular marker that could contribute to get commercially available and agronomically acceptable corn lines.

FUNGAL GENOMICS/FUMONISIN/AFLATOXIN ELIMINATION WORKSHOP

17TH ANNUAL AFLATOXIN ELIMINATION WORKSHOP

Session 4: Microbial Ecology

Moderator: *David Ramos, California Walnut Commission*

Colonization of Peanut Seeds by *Aspergillus* section *Flavi* in Soil: Selective Effects of Water Activity and Temperature

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Insect-damaged peanut seeds are highly susceptible to contamination by carcinogenic aflatoxins produced by *A. flavus* and *A. parasiticus*, fungi belonging to *Aspergillus* section *Flavi*. A laboratory procedure was developed in which viable peanut seeds were wounded and inoculated with field soil containing natural populations of fungi, then incubated under different conditions of seed water activity and temperature. Densities of *Aspergillus* section *Flavi* in soil used for inoculating seeds were low relative to the total numbers of filamentous fungi (< 1%). *Aspergillus* species from section *Flavi* present in soil included *A. flavus* L and S strains, *A. parasiticus*, *A. caelatus*, *A. tamarii* and *A. alliaceus*. Peanut seeds were colonized by section *Flavi* species as well as *A. niger* over broad ranges of water activity (0.82-0.98) and temperature (15-37 C) and the highest incidences of seed colonization occurred at water activities of 0.92-0.96 at 22-37 C. Optimal temperatures for seed colonization were lower for *A. parasiticus* and *A. caelatus* than for *A. flavus*. *A. parasiticus* was the only species from section *Flavi* to colonize peanut seeds at 15 C after extended incubation (35 d). Cool soil temperatures relative to temperatures of aerial crop fruits may explain why *A. parasiticus* is more frequent in peanuts than in corn and cottonseed. Other fungal genera (*Penicillium*, *Fusarium* and *Clonostachys*) colonized seeds primarily at water activities and temperatures suboptimal for section *Flavi* species and *A. niger*. The inoculation of wounded viable peanut seeds with soil provides a model system for studying the infection process, the interactions between fungi, and those factors important in aflatoxin formation.

Commercial Production and Application of Afla-Guard®, a Biopesticide for Aflatoxin Control in Peanuts

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We have worked for many years on the development of technology for the biological control of aflatoxin contamination in peanuts. The strategy behind this technology is to apply a high level of a nontoxigenic strain of *Aspergillus flavus* to soil in which peanuts are growing so that the strain can competitively exclude toxigenic strains when peanuts are subject to invasion and growth during periods of late-season drought. Numerous studies have shown that this strategy can effectively reduce aflatoxin contamination of peanuts in the range of 70-90%. In June, 2002, Circle One Global, Inc., licensed ARS patents related to this technology for the purpose of commercialization. The biocontrol product, which consists of hulled barley coated with conidia of the nontoxigenic *A. flavus*, was given the trade name, afla-guard®. In May, 2004, the US Environmental Protection Agency granted a section 3 registration for use of afla-guard® as a biopesticide on peanuts.

Circle One used seed coating equipment supplied by Gustafson, Inc., to produce afla-guard® in a continuous flow operation capable of rapid production of large, commercial quantities of the biopesticide. In the continuous flow process hulled barley is coated with conidia suspended in soybean oil followed by addition of diatomaceous earth to absorb the oil and make the product free-flowing. Finished product is weighed into 50 pound bags for shipment to peanut buying points for distribution to growers. The process has the capacity to produce 5-6 tons of afla-guard® per hour. Based on orders received from the peanut industry, Circle One produced four 25,000 pound lots of afla-guard® for use on the 2004 peanut crop. Quality control analyses of those four lots showed excellent conidial coverage of the barley as well as low sample to sample variability. The mean colony forming units of nontoxigenic *A. flavus* per gram of barley ranged from 6.4×10^5 to 1.0×10^6, all well above the minimum desired level of 1.0×10^5.

Growers applied afla-guard® at a rate of 20 lb/acre to approximately 4000 acres of peanuts in southeastern Alabama and southwestern Georgia during the summer of 2004. Various analyses are being conducted during and after the growing season to monitor efficacy of the treatments. Incomplete analyses of soil in fields that were treated with afla-guard® showed that the nontoxigenic strain was well established relative to toxigenic strains already present. The incidence of nontoxigenic *A. flavus* averaged 98.8% of the total *A. flavus* population in fields that were treated compared with an incidence of only 14.7% in untreated soils. As the crop is harvested, peanuts from both treated and untreated fields will be analyzed for aflatoxin as well as for colonization by *A. flavus*. Further, treated and untreated peanuts will be stored and shelled separately to determine the final effect on aflatoxin contamination of peanuts destined for edible markets.

Biological Control of *Aspergillus flavus* by *Pichia anomala*

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Demand for biological control products arises from problems in recent decades with the use of chemical pesticides, including resistance, pest resurgence, environmental pollution and risks to human health. There is a growing interest in the food industry, including retail supermarkets, growers cooperatives and large food companies, to adopt BBT (biological based technologies) as a means of minimizing risk and as a marketing advantage to attract environmentally and health-conscious comsumers.

The fungus, *Aspergillus flavus* produces aflatoxin which is the most potent carcinogen known and is very hazardous to the health of both human and animal. National economic losses are in the billions of dollars per year due to aflatoxin contamination of agricultural commodities. Aflatoxin levels of 2-4 ppb have been declared mandatory by importing European Countries (Commission of the European Community, 1998). Even very low levels of infection of the nuts, corn, peanuts and cotton seeds by *A. flavus* can result in aflatoxin levels above these mandatory standards. Managing pre-harvest aflatoxin contamination via biological control is a promising and environmentally-friendly approach.

Aflatoxin contamination is well documented to be associated with wounding in corn, peaunts, cotton seeds and tree-nuts before harvest. A bioassay has been developed to screen for effective yeast inhibiting both the growth of the *Aspergillus flavus* and aflatoxin production (Hua et al., 1999, Appl. Environ. Microbiol. 65: 2738-2740). One particular yeast, *Pichia anomala* strain WRL-076 was tested further for its antagonistic activities to reduce spore production of both toxigenic and aflatoxigenic isolates of *A. flavus* in pistachio flowers and nut-fruits as well as in almond and pistachio leaves. Assessment of the efficacy of *P. anomala* has been achieved by artificially wounding almond and pistachio nut-fruits on the trees and sprayed with the biocontrol yeast in the orchard. The results clearly demonstrate that populations of *A. flavus* and other fungi were significantly reduced in wounded nut-fruits by this yeast

Several properties *Pichia anomala* make the species suitable as a biological control agent. The yeast does not produce allergenic spores or mycotoxins and is capable of growing at low water activity (aw). In particular *P. anomala* WRL-076 grows poorly at 37^0C and does not grow above 38^0C so it will not be an opportunistic pathogen to human and animal. Further field trials to demonstrate biocontrol efficacy are warranted. EPA registration and commercialization of the biocontrol yeast, *P. anomala WRL-076* should benefit the grower, food industry and consumer.

Field Tests of a Biocontrol Yeast in Pistachio Orchards

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Prior research by Dr. Hua has demonstrated that *Aspergillus flavus* is inhibited by several selections of a yeast, *Pichia anomala.* One strain was selected for further studies based on it's durability and ability to inhibit *A. flavus* growth. The present experiments were undertaken to determine if a) the biocontrol yeast can survive in an outdoor environment (eg. Pistachio orchards), b) the yeast has no phytotoxic effects on the pistachio trees or nuts, and c) the yeast can be demonstrated to control *A. flavus* in the field. Studies in 2003 were conducted using a replicated experiment with three yeast concentrations (10^5, 10^6, and 10^7 cells/ml) and a water control. Single spray treatments, applied later in the season, were found to be most effective. Highest yeast concentrations were observed just prior to harvest from both earlier and later spray treatments. In 2004, spray treatments of 10^7, 5 x 10^7, and 10^8 cells/ml, and a water control were applied to evaluate possible phytotoxic effects on pistachio. No differences in leaf or nut appearance were observed for any of the treatments when compared to the water control. No differences in nut percent splits or dry weight were observed between any of the treatments. Artificial wounding experiments were also conducted in 2003 and 2004 to simulate the occurrence of early split nuts, the primary repository for *A. flavus* contamination. Yeast growth was about 10^3 fold higher on wounded nuts when compared to unwounded controls. A 5x reduction in *A. flavus* colonization was observed on treated wounded nuts vs. untreated wounded nuts. A 5x reduction in *A. flavus* sporulation was also observed on treated wounded nuts vs. untreated wounded nuts. While we have seen nothing to suggest that biocontrol yeast would impact yield, large scale tests in commercial orchards with yield evaluations as well as evaluation of aflatoxin using standard commercial measurement systems will be necessary.

Isolation and Initial Characterization of Bacterial Antagonists of *Aspergillus flavus*

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We have initiated studies to identify bacterial isolates with antagonistic activity against mycotoxigenic fungi for their potential development as biological control agents. Candidate bacteria were isolated from almond orchard soils using enrichment cultures selective for bacteria that could utilize *Aspergillus* mycelium as a sole carbon source, as well as from direct plating of almond kernel washes. Phenotypic screens were performed on solid and liquid media to assess antifungal activity against the *nor* mutant *A. flavus* strain Papa827. This strain accumulates norsolorinic acid, a red-orange compound, under conditions conducive to aflatoxin biosynthesis, and facilitates identification of bacteria with aflatoxin-inhibitory activity. From approximately 500 bacterial isolates tested for inhibition of fungal growth and aflatoxin biosynthesis, 35 isolates were selected for further study. These isolates showed a range of activity relative to growth inhibition and/or inhibition of aflatoxin production in *A. flavus* Papa827, as well as production of chitinolytic and yeast cell wall hydrolytic extracellular enzyme activities. Using 16s ribosomal RNA gene sequence analysis, several bacterial genera were identified, including *Bacillus*, *Chryseobacterium*, *Rahnella*, *Streptomyces*, and *Stenotrophomonas* species. Further analysis of these isolates to determine mechanisms of antifungal and aflatoxin-inhibitory activities is ongoing. In a related study, bacterial populations recovered from almond orchard samples at different times during the growing season were assayed for antifungal activity against *A. flavus* Papa827. Antagonistic bacterial populations were recovered from almond flowers, as well as from the hulls and kernels of immature and mature almond fruits throughout the orchards sampled. Further analysis of these populations to characterize the antifungal activities of individual bacterial isolates will be used to identify candidate organisms for development of biological control agents.

Deletions in the Aflatoxin Biosynthetic Gene Cluster of Nonaflatoxigenic and Cyclopiazonic Acid-Nonproducing *Aspergillus flavus* Isolates

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Aflatoxins (AF) and cyclopiazonic acid (CPA) commonly contaminate agricultural commodities. Subpopulations of *Aspergillus flavus* that produce either, neither, or both toxins are present in agricultural soils of several Southern and Southeastern States. We examined the aflatoxin biosynthetic gene cluster of 24 AF-negative and CPA-negative *A. flavus* isolates, which belong to various vegetative compatibility groups. Preliminary results show that five of those *A. flavus* strains have a partial deletion in the aflatoxin gene cluster, and 18 have completely lost the aflatoxin gene cluster. Using PCR we detected two different breakpoints, at one end of the aflatoxin gene cluster, responsible for the partial deletions. One is between *norA* and *ver1* and the other between *avfA* and *omtB*. A breakpoint responsible for the complete loss of the aflatoxin gene cluster was located between *hexA* and *glcA* of the sugar utilization gene cluster, which resides downstream of the aflatoxin gene cluster. For these 23 *A. flavus* strains the breakpoint at the other end lies beyond 10 kb of the aflatoxin gene cluster. In contrast to AF-negative and CPA-positive *A. flavus* isolates, deletion or loss of the aflatoxin gene cluster appears to be more prevalent among AF-negative and CPA-negative *A. flavus* isolates.

Aflatoxin Control in Figs: Biocontrol and New Resistant Cultivars

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For several years, we have investigated the use of atoxigenic strains (strains not able to produce aflatoxins) of *Aspergillus flavus* as biocontrol agents to reduce aflatoxin contamination of figs in California. This approach has been very successful in commercial cotton fields in Arizona where the atoxigenic strain AF36 has substantially reduced aflatoxin contamination of cottonseed. In 2003 we performed an experiment in a research Calimyrna fig orchard that had been converted to drip irrigation. On 9 July, wheat seeds infected with AF36 (the same strain used in cotton fields in Arizona) were applied at the rate of 41.2 g wheat/tree (equivalent to 10 lbs/acre). On 21 August, we collected noncaprified figs from the orchard floor. Almost all *A. flavus* isolates (91%) obtained from the noncaprified figs belonged to the applied atoxigenic strain AF36. On 10 October, leaf and additional soil samples were collected. The soil had a higher density of *A. flavus*/*A. parasiticus* in the areas under the drip lines where infected wheat had been placed (72.6 and 113.2 cfu/g soil for areas treated with AF36 for two years and one year, respectively) than in the middles (5.5 to 11.9 cfu/g) or under the drip lines in the untreated areas (0.9 cfu/g). Three months after applying the wheat, all of the *A. flavus* isolates obtained from under the drip lines in the treated areas belonged to AF36 compared to only 18.2% in the untreated areas. In contrast, in the middles the incidence of AF36 was approximately the same in the treated areas (24.1 and 35.7%) and in the untreated areas (25.0%). The density of *A. flavus* on leaves did not differ significantly between treatments, but the strain AF36 was detected on the leaves at a substantially higher incidence in the areas treated with AF36 (51.7 and 38.8%) than in the untreated areas (13.7%).

In 2004 we repeated the biocontrol experiment done in 2003 in the drip-irrigated research orchard. On 6 July, the atoxigenic strain AF36 was applied to the area beneath the drip emitters at the same rate as in 2003. Soil collected just prior to application of the wheat had a very low density of *A. flavus* in the middles (ranging from 1.3 to 8.5 cfu/g, depending on treatment) and in the areas not treated with AF36 in 2003 (0.6 cfu/g), but a relatively high density in the areas treated with AF36 in 2003 (111.5 and 279.7 cfu/g, depending on treatment). On 26 August, we collected noncaprified figs from the orchard floor and observed colonies of *A. flavus* on them (0.0 to 1.3%, depending on treatment). On 15 September, leaf, fig, and additional soil samples were taken and are currently being evaluated.

New fig selections have been developed by a breeding program that is attempting to produce a Calimyrna-type fig that has the advantages of Calimyrna figs but not the disadvantages of having high levels of decay and aflatoxin contamination. In 2003 the most promising selection, 6-38W, had substantially smaller eye diameter of dried fruit (0.3 mm) than the commercial cultivars Adams (1.7 mm) and Calimyrna (2.2 mm) but the same as Conadria (0.3 mm). Furthermore, the incidence of decay by *Aspergillus* sect. *Flavi* of the dried figs of 6-38W (0.0%) was the same or slightly lower than figs of Adams (0.1%) or Conadria (0.0%) but substantially lower than that of Calimyrna (2.7%). In addition, dried figs of 6-38W and each of the commercial fig cultivars (Calimyrna, Conadria, and Adams) have been collected from commercial orchards in 2004 and are being evaluated for fruit decay. The figs of the new fig selection 6-38W have consistently had less fungal decay than Calimyrna figs.

Aflatoxin Control in Pistachios: Biocontrol Using Atoxigenic Strains

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For the past several years, we have investigated the use of atoxigenic strains (strains not able to produce aflatoxins) of *Aspergillus flavus* as biocontrol agents to reduce aflatoxin contamination of pistachios in California. This approach has been very successful in commercial cotton fields in Arizona where the atoxigenic strain AF36 has substantially reduced aflatoxin contamination of cottonseed. In 2001 and 2002, the three promising atoxigenic strains A564, A815, and AF36 (this strain, the same as used in Arizona cotton fields, was only applied in 2002) were applied in a flood-irrigated research pistachio orchard. In early summer for both years, wheat seeds infected with these strains were applied to the orchard floor at the rate equivalent to 10 lbs/acre. In 2003 no atoxigenic strains were applied and soil samples were collected on 19 August. Soil in the nontreated areas had substantially lower density of *A. flavus*/*A. parasiticus* (68 cfu/g) than in the treated areas (201 to 545 cfu/g, depending on strain). Almost all of the *A. flavus* isolates from the soil in the treated areas belonged to the atoxigenic strain applied there (94.9 to 98.2% of the isolates, depending on strain). Furthermore, the applied strains were detected in the untreated areas (10.3 to 27.6% of the isolates, depending on strain), demonstrating movement of the atoxigenic strains to areas not treated. In 2004 additional soil samples were collected on 30 August and are currently being evaluated.

Starting in 2003, additional biocontrol experiments were performed in a different research pistachio orchard that was irrigated by microsprinklers. The only atoxigenic strain applied in this orchard was the strain AF36. In 2003 wheat seeds infected with AF36 were applied after collecting soil samples on 1 July. On 23 September, leaf and additional soil samples were collected. The density of *A. flavus*/*A. parasiticus* in the soil only increased slightly from before the infected wheat was applied (40.7 cfu/g) to 3 months later (53.4 cfu/g). Also, the density in soil was not significantly different between treated areas and untreated areas for both dates. In the treated areas, the incidence of AF36 among *A. flavus* isolates increased from 1.7% before applying the infected wheat to 31.3% 3 months later. The incidence of AF36 was substantially higher on leaves collected from areas treated with AF36 (53.3%) than from untreated areas (17.8%). Also, early split nuts were collected and found to have very low incidences of decay by *A. flavus*. Only one early split nut had been colonized by *A. flavus* in the treated areas (0.5%), and that isolate did not belong to the strain AF36. In 2004 wheat infected with AF36 was applied on 6 July. The density of *A. flavus*/*A. parasiticus* in the soil collected before applying the infected wheat did not differ significantly between treatments, indicating that there was not an increase due to applying AF36 in 2003. Leaf, nut, and additional soil samples were collected in 2004 and are currently being evaluated.

The incidence of atoxigenic strains among *A flavus* isolates occurring naturally in commercial pistachio orchards in California was determined. Isolates of *A. flavus* from commercial pistachio orchards were evaluated, and all three atoxigenic strains AF36 (5.4% of the 780 isolates evaluated), A564 (1.7%), and A815 (1.0%) were detected. The atoxigenic strain AF36 was detected in all the major pistachio-growing counties (representing 80% of the total acreage of pistachios in California), suggesting that the atoxigenic strain AF36 occurs naturally throughout the pistachio-growing area of California.

Refinements in Atoxigenic Strain Production, Distribution, and Application for Suppression of Aflatoxin Producing Fungi in Arizona Cotton

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The inability of various strains of the common fungus *Aspergillus flavus* to produce aflatoxin has since the late 1980's been investigated as a potential mechanism for biological control. Experimental use of the atoxigenic strain AF36 over multiple years in full field tests in Yuma County, Arizona verified that its ability to competitively displace toxin-producing strains could be effectively employed as a means to limit or prevent aflatoxin contamination of cottonseed. This led to an industry plan for area wide control of aflatoxin and the development of a grower owned facility designed to produce commercial scale quantities of AF36. Based on a partnership between USDA ARS and the Arizona Cotton Research and Protection Council and with the collaborative effort of numerous supportive organizations, limited production of AF36 began in 1999. Progressive improvements in production, distribution and application of AF36 have been made since that time. To date this program has treated approximately 82,961 acres with AF36 with clear beneficial displacement of aflatoxin producing fungi. During 2004 approximately 30,000 acres were treated with AF36 including 5,000 acres in South Texas. All the material for these treatments was produced at the grower run facility in Phoenix

A section 3 registration of AF36 on cotton in Arizona and Texas was granted to the ACRPC by EPA in mid-summer, 2003. This was a watershed event in moving atoxigenic strain technology from the experimental use mode to full commercialization. During 2004, modifications to the AF36 manufacturing process resulted in significant improvements in product quality. A computer-automated system was installed to maintain precise drying temperature control in the final phase of production. The hot-oil heat source and accompanying computer program allow product drying at temperatures up to 20°F less that the original steam heating system. The program also incorporates automated cool down and individual termination of drying vessels. These changes have resulted in measurable improvements in final product quality. Mapping of the distribution of product moisture after drying is also providing insights that will lead to further refinement in manufacture of this important biological control agent.

The distribution of AF36 from manufacturing facility to the field also poses a potential for negative effects on product efficacy. Current packaging and delivery containers include 2000-pound bulk boxes (black) and bulk bags (white). Product is also supplied in fifty-pound polyethylene bags depending on grower choice. Influences of the Arizona environment on product quality when transported in the various containers is being studied and is resulting in improved handling techniques and practical in-field benefits.

AF36 field application strategies have been a subject of investigation since the initiation of the program in Arizona in 1999. Recommended guidelines have been to apply AF36 after termination of field cultivation (known as layby) and just prior to irrigation. Depending on soil type and/or the agronomic practices of individual growers, historically many fields have been treated at very early stages, well ahead of canopy closure. Some evidence from 2003 treatment activities suggested that, where soils were light (reduced moisture carrying capacity) and the crop lacked adequate closure (therefore reduced humidity levels), the ability of AF36 to displace toxigenic strains was diminished. Specific studies designed to address this issue were conducted in 2004 and are currently being analyzed.

During the 2003 cotton growing season AF36 treatments were applied to a total of 19,946 acres in Arizona. This figure equates to 824 individual fields representing ten ginning communities in nine separate geographical regions across five counties in the state. Results of the AF36 program in 2003 were varied. From the standpoint of toxin levels, 2003 was arguably the worst year on record in Arizona. Commercial labs were the source of this information with some cottonseed samples exceeding 16,000 ppb. Based on this, very little clean cottonseed (below 20 ppb) was produced in 2003 including program treatment areas. Despite this fact, significant progress was made in areas where AF36 was utilized. Reflecting a composite sample size of 502 fields tested for AF36 on the crop (seed) after treatment and ginning data summaries resulted in an area wide increase in average AF36 on the crop of 62.9%, 70.4% and 78.7% for the years 2001, 2002 and 2003 respectively. Correspondingly, levels of the principal toxigenic strain (s) were low, 1.9%, 1.8% and 3.6% for the same time sequence. This is significant in view of the fact that of the 1825 fields treated cumulatively over the past five years, more than 77% have only received one treatment of AF36. Efforts continue to address the issues of program efficacy and expansion to fully achieve area wide status in Arizona and beyond.

Progress in Aflatoxin Management for South Texas

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In South Texas aflatoxin contamination is a serious problem for both the corn and cottonseed industries and these two crops are frequently rotated with each other and with sorghum. Thus, aflatoxin contamination cycles in this region involve rotations with three susceptible and frequently affected crops. In this area, reduced tillage results in long-term residence of corncobs on soil surfaces. *Aspergillus flavus*, the causal agent of aflatoxin contamination, can grow and survive in corncobs. We studied the potential of corncobs as sources of *A. flavus* in cotton and corn crops in South Texas from 2001 to 2003 in order to gain insights into potential methods to prevent contamination. The results indicate that corncobs are important sources of crop exposure to *A. flavus*. Corncobs from the previous season contained, on average, around 200 times more *A. flavus* propagules than soil from the same field, and 2-year old corncobs still retained 45 times more propagules than soil. The results suggest that aflatoxin management should include prompt harvest with techniques to reduce the period that corncobs remain in the field, such as incorporation. Delayed harvest of corn resulted in increased quantities of *A. flavus* on the crop.

Strains of *Aspergillus flavus* that do not produce aflatoxins (atoxigenic strains) are being developed as biological control agents for the management of aflatoxin contamination of several crops. *Aspergillus flavus* AF36 was the first atoxigenic strain to receive a Section 3 registration from EPA in 2002. Use of AF36 seeks to reduce the incidence of aflatoxin producers in the soil, on the crop, and throughout the environment without increasing the quantity of fungus in the environment. This strategy seeks both to displace aflatoxin producers during crop development, and thereby reduce aflatoxin contamination, and to cause long-term changes in fungal communities so that incidences of atoxigenic strains remain elevated after over-wintering. Over-wintering allows for both cumulative and area-wide influences of management programs utilizing atoxigenic strains. During 2003, 2000 acres of cotton were treated with atoxigenic strain AF36 in an attempt to assess the efficacy of atoxigenic strain methodology for biological control in limiting aflatoxin contamination in cotton-corn-sorghum rotations in South Texas. Four areas were treated in the Coastal Bend and Upper Coast regions of South Texas extending from Chapman Ranch in the south to the Port Lavaca area in the north. Displacement of aflatoxin producers by the atoxigenic strain was excellent in both non-irrigated areas and areas that received supplemental irrigation. The incidence of AF36 in soils prior to treatment in the four areas was determined by vegetative compatibility analyses on 1700 *A. flavus* isolates from 116 soil samples. AF36 incidence ranged from 0.2% in Port Lavaca to 7.1% in Gregory, and the highly toxigenic S strain ranged from 16% to 36%. On cottonseed from treated fields, incidences of the S strain ranged from 0 to 8% and of the atoxigenic strain AF36 from 88% to 97%. Preliminary analysis of 2004 soil communities from two treatment areas indicate significant long-term influences of applications on fungal community structure with the atoxigenic strain composing between 55% and 75% of the *A. flavus* community one year after application and significant reductions in incidences of the S strain. The results indicate that applications of atoxigenic strains in South Texas have great potential for reducing the average aflatoxin producing ability of fungal communities and aflatoxin contamination of crops in cotton-corn-sorghum rotations.

Panel Discussion: Microbial Ecology

Panel Chair: Mark Doster
Panel Members: L. Antilla, P.-K. Chang, P. Cotty, J.W. Dorner, B.W. Horn, S.-S.T. Hua, J.D. Palumbo, D.E. Parfitt.

Question: How long does afla-guard last?

Dr. Dorner answered: It depends on the temperature at which it is stored. Higher temperatures reduce stability because as the oil, which is part of the formulation, breaks down, volatiles given off are toxic to the spores. Under typical ambient conditions the product is stable for at least six months, and this can be extended to greater than a year if the product is stored under refrigeration. We are evaluating more stable oils for the formulation that could extend the shelf life of the product. However, Circle One's plans are to manufacture only enough product for a given year and not try to carry over product from one year to the next.

Question: How long does the atoxigenic strain product last?

Dr. Cotty answered: 28 month stability has been documented (Biocontrol Science and Technology 9:529-543) with laboratory produced material at room temperature. However, stability is dependent upon storage conditions. At the commercial scale manufacturing facility run by the Arizona Cotton Research and Protection Council, 10 month stability is required so that material can be made in advance for the following season.

Question: Why do Dr. Cotty and his collaborators monitor the S strain of A. flavus when assessing displacement of aflatoxin producers by atoxigenic strains?

Dr. Cotty answered: S strain isolates are of particular interest because S strain isolates make more aflatoxins on average than other *A. flavus* isolates and S strain isolates have been shown to be important causative agents of aflatoxin contamination. It is also practical to separate out the S strain isolates because these can rapidly be differentiated from the atoxigenic strains and other L strain isolates by colony morphology.

Question: How long do the atoxigenic strains persist in the soil?

Dr. Cotty answered: We always see influences of atoxigenic strain applications not only on the crop at harvest, but also in the soil one year after application. This suggests that applications should have both long-term effects and that multiple year applications should provide additive benefits. However, both the extent of carryover and the length of carryover are dependent on location, crop rotation and the environment. It is not yet clear whether relatively stable, long-term changes to the *A. flavus* community resident in entire areas can be achieved.

Question: What level of displacement of aflatoxin-producers was required to have acceptable control levels?

Dr. Cotty answered: The level of control required is dependent on the severity of contamination and is highly variable between regions and seasons. In some situations, when crops are primarily contaminated with between 20 and 50 ppb, an 80% level of displacement will generally result in acceptable aflatoxin levels. However, if contamination is typically over 2,000 ppb, even 99%

displacement will not result in a crop below 20 ppb. In such cases, control is aimed at getting the crop into less profitable animal feed markets that legally can accept up to 300 ppb.

Question: What are the economics of the biocontrol?

Dr. Dorner answered: Dr. Marshall Lamb, an economist at our lab, did an economic analysis based on three years of data from field studies using afla-guard. In one year there was no aflatoxin contamination in treated or untreated peanuts, so there was no economic return from using afla-guard. The second year produced low levels of contamination in peanuts, and the return to shellers averaged about $28.00 per ton of peanuts. In the third year, peanuts were exposed to significant late-season drought and were highly contaminated with aflatoxin, particularly in untreated peanuts. Use of afla-guard produced a return to shellers of about $160.00 per ton. The average return for the three different types of years was about $62.00 per ton of shelled peanuts. These savings are the result of reduced costs associated with re-milling and/or blanching that is done to "clean up" contaminated peanuts so they can enter the edible trade. This is the reason that peanut shelling companies are so interested in having afla-guard used to reduce aflatoxin levels.

Question: What is the situation with fungi growing on noncaprified figs on the orchard floor in fig orchards?

Dr. Doster answered: For certain fig cultivars such as Calimyrna, if the developing fig does not get pollinated by the fig wasp, then the fig falls to the ground and is referred to as a "noncaprified fig". On the ground, these noncaprified figs are colonized by various fungi (similar to fungi that colonize other organic matter in fig orchards), including various *Aspergillus* species. These fungi usually grow where the noncaprified fig touches the soil. Conidia are produced by fungal colonies growing on the noncaprified figs at the time that figs are ripe on the tree and susceptible to fungal decay. Our results suggest that the atoxigenic *A. flavus* strains applied in early summer in the fig orchard can colonize noncaprified figs, thereby increasing the number of conidia of the atoxigenic strains in the orchard.

Question: Are the fig wasps involved in the colonization of figs by aflatoxin-producing fungi?

Dr. Doster answered: The figs are pollinated by the fig wasp in early summer while the fig is still relatively small and not susceptible to decay by *Aspergillus* species. When we have looked at the fungi present in figs in early summer, only rarely do we find figs colonized by *Aspergillus* species. The fig wasp is probably not involved with spreading aflatoxin-producing fungi to figs, although the fig wasp does spread *Fusarium* species.

Question: Where is the location in figs of infections by A. flavus and A. parasiticus?

Dr. Doster answered: Most of the infections occur in the internal cavity of the fig. Normally, very few infections occur on the external surface of the figs, probably due to the dry climate.

Question: Is there any knowledge of dioxin contamination of the diatomaceous earth that is part of the formulation?

Dr. Dorner answered: I have no knowledge of dioxin contamination of the diatomaceous earth that is used in formulating afla-guard. We will check with the supplier of the diatomaceous earth to ensure that it is not contaminated.

Question: Do the biological effects of AF36 produce demonstrable economic benefits as expressed by the reduction in aflatoxin levels below 20 ppb?

Dr. Antilla answered: In many cases this was definitely the case, but where background toxin levels are high, even significant reductions of toxin producers may not result in clean cottonseed at least in the first year of AF36 application. The goal of the program is to progressively alter the makeup of the fungal community in agricultural settings to produce long term areawide aflatoxin reduction and concomitant economic benefits.

Characteristics of a Retrotransposon from *Aspergillus flavus*

S.-S. T. Hua,[1] A. S. Tarun,[1] S. N. Pandey[2] and P. K. Chang.[3]
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Retrotransposons are retrovirus-like DNA elements found in eukaryotic genomes that replicate to new chromosomal locations via RNA intermediates. The *Ty3/gypsy* group of LTR (long-terminal repeat) retrotransposons have been found in a wide range of filamentous fungi. Functional LTR retrotransposons contain the *gag* and *pol* genes flanked by direct LTRs at each end. The *gag* gene encodes protein (Gag) involved in virus-like particle formation while the *pol* gene encodes a polyprotein (Pol) with domains for the following enzyme activities: protease (PR), reverse transcriptase (RT), RNase H (RH) and Integrase (IN). Retrotransposons are a major driving force in genomic rearrangements and mutation induction in fungal genomes. Transposable elements may be important in the evolution of new variants of mycotoxin-producing fungi, such as *A. flavus*, as they can cause insertional inactivation of genes or provide sites for recombination.

A 7784 bp DNA sequence containing the *AFLAV* (*A. flavus* retrotransposon) complete sequence has been submitted to GenBank (accession number AY485785). Two ORFs in *AFLAV* that were flanked by the nearly identical 5' and 3' LTR. The 5' and 3' LTRs of *AFLAV* both begin with TGTTA and terminate with TGTCA. Two possible motifs for promoter initiation are found in the 5' LTR region; a TATA box sequence (TATAA) at position 288-292 and two CAAT boxes (CAAT) at positions 213-216 and 331-334. The first ORF starts 250 bases downstream of the 5' LTR and encodes a predicted protein of 240 amino acids. The second ORF encodes a putative protein of 1726 amino acids. Sequence analysis indicates that various regions of this protein have homology to the CA domain of Gag and to the various domains of the Pol polyprotein: PR, RT, RH and IN found in that order confirming that *AFLAV* belongs to the *Ty3/Gypsy* family of retrotransposons. Other sequence feature found in *AFLAV* is the primer-binding site (PBS) for reverse transcription. Reverse transcription is mediated by a reverse transcriptase (RT) and a primer for minus-strand strong stop cDNA synthesis. This primer is usually a tRNA that binds to the PBS. However, retrotransposons in the *Tf1/sushi* subgroup, appear to form a secondary structure that enables self-priming for reverse transcription. This feature also found in *AFLAV*.

Comparison of Two Populations of *Aspergillus flavus* and *Aspergillus parasiticus* Isolated Five Years Apart in a California Orchard

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Both *Aspergillus flavus* Link and *A. parasiticus* Speare cause aflatoxin contamination in crops such as corn, cottonseed, peanuts and tree-nuts. These two fungal species are saprophytes which infect plants through woundings. *A. flavus* is typically the dominant aflatoxigenic species. *A. flavus* produces aflatoxin B_1 and B_2 and the species has been divided into S and L morphotypes base on sclerotial sizes. Many isolates of *A. flvus* do not produce aflatoxin and are named atoxigenic strains. *A. parasiticus* strains produce aflatoxins B_1, B_2, G_1 and G_2 and atoxigenic isolates has rarely been described. Of the four aflatoxins, B_1 is the most potent carcinogen known. Fungal cultures collected in a California orchard in spring 1998, spring 2003 and summer 2003 were analyzed for aflatoxins production, morphoty and pectinase activities. The data indicate that there is an increase in afltoxigenic population from 1998 to 2003. The percent of toxigencic isolates of both *A. flavus* and *A. parasiticus* is 33% and 66% in spring of 1998 and 2003 respectively. The isolates from wounded pistachio nut-fruits in the summer of 2003 consist 55% of *A. flavus* and 45% of *A. parasiticu*s. The percent of isolates producing AFB_1 is reaching 88% in wounded pistachio nut-fruits collected in the summer of 2003.

Identification of Fungal and Yeast Isolates by Analysis of Nuclear Large Subunit (26S) Ribosomal DNA Sequences

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There are approximately 70,000 to 80,000 species of fungi. While filamentous fungi are identified using mainly morphological characteristics, yeast are identified using biochemical characteristics, such as their ability to utilize carbon and nitrogen compounds. However these methods of identification are often problematic as there can be different morpho/biotypes or variants within a single species. The procedures are also time-consuming. Kurtzman and Robnett published the D1 /D2 sequences of all Ascomycetous yeasts known at that time in 1998 (*Antonie von Leeuvenhoek*, 73: 331-371) and new sequences are continuously being made available by various authors describing new species. This has allowed the formulation of certain generalization on the significance of sequence divergence in species delineation, namely, that con-specific strains seldom differ by more than three bases in their D1 /D2 region. Strains differing by five or six bases or more are likely to be a different species. The D1 /D2 region is also practical for identifying filamentous fungi because the database is available. We report here the identification of yeast and fungal populations by analysis of ribosomal DNA sequences.

Yeast and fungal strains were isolated from pistachio and almond orchards as single colonies. Yeast cells and fungal mycelia were harvested from cultures medium by centrifugation and DNA were extracted for PCR reaction. A pair of universal primers, NL1 and NL4 were used to generate the fragment of D1/D2 region in the 26S ribosomal subunit. The nucleotide sequences of each fragment were determined using the ABI Prism BigDye™ Terminator Cycle Sequencing Ready Reaction Sequencer Kit and ABI Prism 3100 Genetic Analyzer. The sequences were Blast searched using GenBank database and DNA data libraries (DDBJ/EMBL).

Yeast species identified are *Pichia anomala, Rhodotorula mucilaginosa, Pichia fermantans*, *Candida zeylanoides, Issachenkia orientalis, Pichia guillermondi.* Fungal species identified are *Aspergillus flavus, Aspergillus carbonarius, Aspergillus phoenicis, Cladosporium cladosproriodes, Alternaria alternata.* New species of yeast and fungus in orchard may be discovered through the DNA sequence analysis.

Prevention of Fungal Colonization on Wounds of Almonds and Pistachios by a Biocontrol Yeast in Orchard

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The fungus, *Aspergillus flavus* produces aflatoxin. This toxin is the most potent carcinogen known and is very hazardous to the health of human, animal, fish and poultry. National economic losses are in the billions of dollars per year due to aflatoxin contamination of agricultural commodities. Aflatoxin contamination is known to be associated with wounding in corn, peaunt, cotton seed, almomd, pistachio and walnut.

A particular strain of *Pichia anomala* WRL-076 has been demonstrated to be effective in reducing the population of *A. flavus* and aflatoxin biosynthesis in laboratory studies. Field evaluation of the efficacy of *P. anomala* has been carried in 2003 and 2004 in pistachio and almond orchards located in Winters and Arbuckle, CA. Nut-fruits of pistachio or almond were individually wounded with a dental needle and sprayed with an aqueous suspension of yeasts at $3x10^7$ cells/ml. The wounded nut-fruits without yeast-spray were used as controls. Four to five weeks after the yeast spraying wounded pistachio nut-fruit was hand picked from the tree and immediately placed on salt agar medium and incubated at 28^0C for eight days. In order to see the variation among the nuts, every single pistachio nut-fruit collected was analysed for colonization of *A. flavus.* The viable fungal and yeast counts on individual nut were enumerated by standard microbiological techniques. Similar protocol was applied to almond except the collected nut-fruits were not incubated on the salt medium. Randomly selected almond nut-fruits from five trees were wounded with a dental needle and sprayed with yeast cells. For control almond nut-fruits from five additional trees were wounded and sprayed with water. Four weeks later nut-fruits were collected for evaluation. Each analysis consisting 15 wounded almond nut-fruits and four replicates were performed for each tree.

Colonization of *A. flavus* on wounded nut-fruits was 27.1% for the control and 5.1% for the yeast treated nut-fruits respectively. The colony forming unit (CFU) of *A. flavus* from each infected nut-fruit was enumerated. Average spore production of *A. flavus* from infected nuts was $5.6x10^6$ and $1.3x10^6$ respectively for the control and yeast sprayed. The total number of *A. flavus* spores from all infected nut-fruits of the control group was $1.2x10^8$. In contrast the total number of *A. flavus* spores from the yeast sprayed nut-fruits was only $6.2x10^6$. The results clearly demonstrated that *P. anomala* prevented colonization of *A. flavus* on wounds and modulate spore production on infected wounds. A reduction of fungal colonization was observed for the almond nut-fruits sprayed with yeast cells. One can anticipate that field spraying of this effective yeast to tree-nuts may decrease the population of *A. flavus* in orchard environments. Thus, *P. anomala* is likely to provide an economical means of managing aflatoxin contamination.

Effects of Spacing and Timing on the Biocompetitiveness of Atoxigenic *Aspergillus flavus* Against Toxigenic Strains

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Aspergillus flavus, is a complex species composed of many distinct vegetative compability groups (VCGs), produces widely different quantities of aflatoxin. Certain isolates produce no aflatoxin are called atoxigenic strains. Some of these atoxigenic strains can competitatively exclude aflatoxigenic strain during crop infection and thereby reduce aflatoxin comtamination. The parameters influencing the biocompetitiveness of the atoxigenic strain to toxigenic strain as well as toxigenic strain to toxigenic strain were investigated. *A. flavus,* Papa 827 (AF827), a *nor* mutant defective in norsolorinic acid reductase, accumulates norsolorinic acid (a bright red-orange pigmented compound) resulting a red orange colored fungal colony on potato dextrose agar (PDA). The spore color of the mutant is white while the spore color of wild type *A. flavus* is green. Thus AF 827 is an excellent strain for monitoring the interactions with either atoxigenic strain or aflotoxigenic strain because the red orange color can be used as a visual score for aflatoxin production and fungal colony growth of the two interacting *A. flavus* strains can be visually traced by the color of the spores.

AFP8, CA 26 and CA35 are atoxigenic isolates, CA 28 is a S type aflatoxigenic isloate. Fungal spore suspensions are prepared in Tween 80 solution and adjusted to $1x10^5$ spore/ml. Selected testing strains were paired and mixed at 1:1 ratio and the mixtures of spore suspension were further diluted by 10 fold or 100 fold. The undiluted and diluted spore mixtures were spiral plated on PDA plates. A second set of experiments were conducted by spotting the mixed spore suspensions in the center of PDA plates. In the third set of experiments, spore suspension of individual isolate was spotted on PDA at a distance at least 1mm apart from each other. The inoculated plates were incubated at 28^0C for 7 to 14 day. A Sony digital camera was used to record the growth of fungal colonies.

The results indicate that the competing atoxigenic strains do not inhibit either the growth or aflatoxin production of AF827 when the spores are as close as 0.1mm apart (spiral plating) or spotted at least 1 mm apart. It seems that two competing strains in the spore mixtures grow independently and do not interfere the growth or red orange color formation of AF827. AFP8 and CA28 appear to stimulate aflatoxin production of AF827, which can be visualized by the intensity of red-orange color of the fungal colony. When the paired spore mixtures were spotted in the center of PDA plates, sector growth of AF827, AFP8, CA26, CA28 and CA 35 were observed.

In the fourth set of experiments, spore suspension of CA28 was spotted in the center of PDA plates first, then AFP8 was inoculated on the top of CA28 at time 0, 6, 24, 30, 48 and 54 h. After the addition of AFP8, the plates were incubated at 28^0C for additional 7 days. All the plates were evaluated for aflatoxin production by HPLC analysis. The results indicate that inhibitory effect of AFP8 on afatoxin production of CA28 significantly reduced once the toxigenic strain has started to grow on PDA plates.

Culture Media and Sources of Nitrogen which Promote the Formation of Stromata and Ascocarps in *Petromyces alliaceus* section *FLAVI*

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Petromyces alliaceus is the only known sexually reproducing fungus classified in *Aspergillus* section *Flavi.* The fungus was recently associated with ochratoxin A contamination that is occasionally observed in California fig orchards. When grown on solid culture media *P. alliaceus* produces numerous grey-black, sclerenchymatous stromata, some of which may slowly ripen to form ascocarps. Ascospores (meiospores) represent the principal source of genetic variation among naturally occurring clonal populations of *P. alliaceus.* The goal of this research was to identify culture media and sources of nitrogen that best support the formation of stromata with ascocarps. Three cultures of *Petromyces alliaceus* (NRRL 31813, 31814, 31816) isolated from crop field soils were grown on selected agar media in Petri dishes (7 mos. dark incubation, 30C). The largest numbers of stromata were recorded for cultures grown on Czapek's agar (CZA) and a mixed cereal agar (MCA) while the percentage of stromata containing ascocarps was greatest ($p = <0.05$) for cultures grown on MCA (25-28%). When *P. alliaceus* was grown on standard CZA containing 0.3% $NaNO_3$, only 5% of the stromata contained ascocarps. A greater percentage of the stromata (15-22%) formed ascocarps when the NaNO3 in CZA was replaced with an equivalent amount of available nitrogen supplied by ammonium tartrate, glutamic acid, or serine. The information should prove useful in supporting genetic investigations on the significance of recombinant diversity in ochratoxin A production, and in interpreting the contributions of fertile and non-fertile stromata in the fungal disease cycle which, like other species in *Aspergillus* section *Flavi,* may include insects as potential hosts.

DNA fingerprinting analysis of *Petromyces alliaceus* section *FLAVI*

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The objective of this study was to evaluate the *Aspergillus flavus* pAF28 DNA probe's ability to produce DNA fingerprints for distinguishing among genotypes of *Petromyces alliaceus* section *Flavi,* a fungus considered responsible for the ochratoxin A contamination that is occasionally observed in California fig orchards. *Petromyces alliaceus* (14 isolates), *Petromyces albertensis* (1 isolate) and seven species of *Aspergillus* section *Circumdati* (14 isolates) were analyzed by DNA fingerprinting using a repetitive sequence DNA probe pAF28 derived from *A. flavus*. The presence of hybridization bands with the DNA probe and the *P. alliaceus or P. albertensis* genomic DNA indicates a close relationship between *A. flavus* and *Petromyces alliaceus* section *Flavi.* Twelve distinct DNA fingerprint groups or genotypes were identified among the 15 isolates of *Petromyces.* Species belonging to *Aspergillus* section *Circumdati* hybridized only slightly at the 7.0 kb region with the repetitive DNA probe, unlike the highly polymorphic hybridization patterns obtained from *P. alliaceus* and *A. flavus*, suggesting very little homology of the probe to *Aspergillus* section *Circumdati* genomic DNA. The pAF28 DNA probe offers a tool for typing and monitoring specific *P. alliaceus* clonal populations and for estimating the genotypic diversity of *P. alliaceus* in orchards, vinyards or crop fields.

Evaluation of Dietary Corn Contaminated with Aflatoxin and Fumonisin on *Ictalurus punctatus* (Channel Catfish) Productivity and Mycotoxin Residues in Tissues

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Exposure to semi-purified basal diets showed that typically encountered aflatoxin B_1 (AFB_1) and fumonisin B_1 (FB_1) levels are below the NOAEL (No Observed Adverse Effect Level) in channel catfish. Practical diets formulated with aflatoxin-contaminated corn were administered to channel catfish with physiological and behavioral patterns being evaluated. The sphinganine to sphingosine (SA:SO) ratio and catfish growth were evaluated after administration of a semi-purified diet containing FB_1 from *Fusarium verticillioides.* Resistant hybrid and commercial strains of *Zea mays* (corn) were planted in May 2004. Mature corn ears were inoculated via pin-bar, side-needle injection, spray applications and wheat inoculum methods. Corn samples were analyzed for aflatoxin (AF) concentrations using ELISA (Enzyme-Linked Immunosorbent Assay), HPLC (High Performance Liquid Chromatography and VICAM (Immuno-affinity Column Assay). Further research will be conducted to investigate aflatoxin and fumonisin (F) levels in tissues including liver, epidermis, kidney, brain, stomach and muscle (fillet) in channel catfish. Toxicological studies on channel catfish fingerlings will reveal any adverse effects on the intake of mycotoxin-contaminated corn. Effluent water samples will be evaluated for the existence of AF and F in an environmentally-controlled habitat. Determination of the sphingolipid ratio (SA:SO) in channel catfish serum and fecal matter will be assessed.

Frequency of Colonization of Corn Kernels by Atoxigenic *Aspergillus flavus* Applied as a Biocontrol Agent

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Aflatoxin contamination of corn is a chronic problem among Southern states. Others have demonstrated the efficacy of using atoxigenic isolates of this fungus to lessen aflatoxin contamination of cotton by "competitive exclusion" of toxigenic strains from cotton field soils. This decreases the inoculum potential of toxigenic strains, presumably allowing the atoxigenic strains to out compete the toxigenic strains at the infection court. To successfully use this approach with corn, it is necessary to demonstrate that the biocontrol isolate can colonize corn kernels. A proprietary *A. flavus* isolate (Circle One Global, Inc., Shellman, GA) fixed to barley seed, was distributed atop soil between 16 corn rows at the rate of 20lbs/A on June 4, 2003. The plot was combine-harvested on September 4, and kernels from each row were bagged separately. Randomly collected sub samples (200 kernels/row) were plated on AFPA selective medium. *A. flavus* was recovered from approximately 50% of the sampled kernels. Single NIT mutants of the isolates were selected and paired with *cnx* or *nirA* mutants of the biocontrol fungus which is VCG 24, one not previously reported in Louisiana. Approximately 60% of the kernels colonized by *A. flavus*, or 30% of the kernels sampled, were identified as VCG 24 and presumed to be the biocontrol isolated originally applied between the rows.

FUNGAL GENOMICS/FUMONISIN/AFLATOXIN ELIMINATION WORKSHOP

17TH ANNUAL AFLATOXIN ELIMINATION WORKSHOP

Session 5: Crop Management and Handling, Insect Control, and Fungal Relationships

Moderator: *Gary Gray, Blue Diamond Growers*

New Plant-Derived Genes and Gene Products with Activity Against Corn Ear Pests and Strategies for Deployment

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A chitinase-like gene from *Arabidopsis*, potentially producing a protein homologous to one previously identified with activity against insects, was introduced into maize. Insect bioassays and histochemical assays for enzyme activity of regenerated maize tissue indicated mortality levels were significantly positively correlated with histochemical ratings for enzyme activity for two series of plants tested. Further confirmatory assays and introduction of related gene forms are planned. Petunia flower assays (as a model system) coupled with individual assays of purified chemicals, suggested type and structural positioning of anthocyanin sidechain moieties influences activity against insects. Gene knock out assays with petunia, and silk specific transformation of maize are planned to better explore the potential of modifying anthocyanin to increase insect resistance. A peroxidase gene cloned from maize was identified by stable tissue expression assays as one that produces an isozyme previously identified with several types of disease resistance, and may potentially contribute to insect resistance. A transformed line of *Arabidopsis* that continuously expresses a regulatory gene that turns on multiple defensive pathways, including anthocyanin biosynthesis, had significantly enhanced resistance to fall armyworms, but seed production was significantly decreased compared to wild type plants. This information suggests targeting fewer more potent resistance genes may be preferable to continuous induction of multiple defensive genes.

Neural Network Classification of Single Maize Seeds Infested with Kernel Rotting and Mycotoxin-Producing Fungi

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Reflectance spectra (550 - 1700 nm), visible color reflectance images, x-ray images, multi-spectral transmittance images, and physical properties (mass, thickness, cross-sectional area) were analyzed to determine if they could be used to detect fungal infected corn kernels. Corn ears were inoculated with one of several different common fungi several weeks before harvest, then collected at harvest time. It was found that two spectral bands centered at 715 nm and 965 nm can correctly identify 98.1% of controls and 96.6% of the kernels severely infected with *Aspergillus flavus*, *Aspergillus niger*, *Diplodia maydis*, *Fusarium graminearum*, *Fusarium verticillioides*, or *Trichoderma viride*. Histogram features from three transmittance images (blue and red components of visible images and another at 960 nm) can distinguish 91.9% of kernels with severe fungal infection from 96.2% of un-infested kernels. A neural network was trained to classify kernels by species as well as into one of three damage categories (control, minor damage, or severe damage) with good accuracy using principle components of the reflectance spectra. Spectra of single corn kernels can be measured automatically and kernels sorted into different mold species categories using commercial instruments.

Development of Field Based Techniques for Assessing Variability among Cotton Cultivars in Susceptibility to Aflatoxin Contamination during the Second Phase of Contamination

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All cotton producing regions of the U.S. may experience some aflatoxin contamination in some years. In most of the Cotton Belt, occurrence of aflatoxin levels unacceptable for dairy use is infrequent, but in Arizona, southern Texas and the Imperial Valley of California, aflatoxins are a perennial concern. Aflatoxin contamination of cottonseed occurs in two phases. The crop is first contaminated when *A. flavus* infects the developing bolls through wounds or cracks. The second phase of contamination occurs when mature seed is exposed to both conducive temperature (above 27° C) and either high relative humidity (above 85%) or rewetting at or after boll opening. Rank cotton, dense canopies, dew, and late irrigations increase the severity of the second phase. No assays have been developed to compare susceptibility of cotton cultivars to contamination during the second phase of contamination under field conditions. Development of such an assay is the first objective of this project.

Another objective is to determine if seed hardness and seed coat fragility relate to susceptibility to aflatoxin contamination during the second phase of contamination. Rain on open bolls has been shown to be the factor most closely related to aflatoxin content of commercial cottonseed and is the most important factor inciting the second phase of contamination. Therefore, deliberate wetting of open bolls is being used to simulate moisture effects. Field plots have been established at The University of Arizona Maricopa Agricultural Center (MAC). A transgenic Bt cultivar commonly planted in Arizona was utilized in order to reduce variation in aflatoxin content caused by pink bollworm damage. A misting system capable of increasing humidity in the crop canopy and of precisely wetting open bolls has been designed and installed at (MAC). Water is pumped from the irrigation ditch through two inch pipe throughout the field to each of sixty four 20 x 20 ft^2 plots. The misting system consists of 21 one-gal/hr brass foggers spaced about three feet apart in a 10 x 10 ft^2 grid of ½ inch PVC pipe.

In 2004, treatments included 9 hour misting periods initiated at various dates either with or without *A. flavus* inoculation. Plots were replicated eight times, and bolls were harvested two weeks after misting. The harvested cotton was dried in a forced air oven at 45° C and stored dry at room temperature until processed. Harvested seed will be tested for aflatoxin, and the data used to design a field based screening technique cultivars. Subsequent variations in treatments will include frequency of wetting and duration of wetting. Humidity and temperature of treated plots will be monitored to quantify impact of wetting regimen on canopy environment. Ultimately a model will be developed relating wetting period and temperature to aflatoxin content. This will contribute both to development of the screening technique and to a better understanding of the second phase of contamination. Such a model will be useful to the industry in assessing crop aflatoxin risk prior to harvest and to researchers striving to assess efficacy of other aflatoxin management technologies.

The Influence of Pod and Root Galling by Root-Knot Nematodes on Preharvest Aflatoxin Contamination of Peanut

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Infection of peanut by root-knot nematodes (*Meloidogyne arenaria*) can lead to an increase in aflatoxin contamination of kernels when the plants are subjected to drought stress during pod maturation. It is not clear whether the increased aflatoxin contamination is primarily due to greater invasion of the galled pods by toxigenic *Aspergillus* spp. or whether root galling is also involved. Nematode damage to roots may cause greater drought stress or cause physiological changes in the plant which may increase its susceptibility to fungal invasion. Our objective was to determine the contribution of root and pod galling caused by root-knot nematodes to the increase in aflatoxin contamination of peanut.

A greenhouse experiment was conducted in which pods and roots were physically separated. Pod set was restricted to soil-filled pans (41 cm dia. x 10 cm depth), while the roots grew underneath the pan into a pot. Root-knot nematodes (RKN) were applied to the root zone of half the plants; the other plants did not receive nematodes in the root zone. Nematodes were added to the root zone 20 days after planting. Flowers were clipped to allow plants to grow larger before pod formation. Plants received four pod treatments: pods +RKN 20 DAF (days after flower), pods +RKN 40 DAF, pods +RKN 20 & 40 DAF, and pods without RKN. The treatments were arranged in a completely randomized design with 12 replicates/treatment. Conidia of *Aspergillus flavus* (1×10^5) and *A. parasiticus* (1×10^5) were added to each pan 25 DAF. Plants were subjected to drought stress 60 DAF and harvested 100 DAF.

In pots where nematodes were added to the pod zone, 60% of the pods had at least one gall on them regardless of when the nematodes were applied. In pots where nematodes were added to the root zone, 70 to 80% of the root system was galled. Mean aflatoxin concentrations for the nematode treatments ranged from 39 to 184 ppb. Application of root-knot nematodes to the pod zone did not affected aflatoxin concentrations in the kernel. However, in all four pod zone treatments, aflatoxin concentrations were consistently higher ($P = 0.04$) in kernels from plants where the roots were infected with nematodes than in roots without nematodes (128 ppb +RKN vs 51 –RKN). This trend suggests that root galling may play a role in increasing aflatoxin contamination of peanut. Additional experiments are being conducted to support or refute these preliminary results.

Advances in Mating Disruption Control of the Codling Moth and *Aspergillus* in California Walnuts

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Aspergillus invasion of tree nuts is primarily through insect damage by moth larvae. Our goal is to diminish insect-caused nut damage through the use of novel, species-specific control systems based on host-plant kairomones. Because adult female moths lay eggs that hatch into damaging larvae, controlling female codling moth adults would create greater control efficacy. We have identified a single compound isolated from pears, ethyl (2*E*, 4*Z*)-2,4-decadienoate, that is a powerful kairomone, attracting and capturing both male and female codling moth (CM) adults in baited sticky traps. Through a cooperative research and development agreement, and an approved patent and license, between USDA/ARS and Trécé, Inc., a global research program has been underway for five years to demonstrate possible control uses for the kairomone compound. Trécé, Inc. petitioned and attained both Experimental Use Permits and Research Authorizations from the EPA and the California Depart. Food & Agriculture for the experimental application of the kairomone in walnut orchards.

Various control tactics are being investigated using the pear-ester kairomone to directly manage female and male codling moths in walnut orchards. These control tactics include: mass-trapping of male and female moths (as reported last year), mating disruption of male moths, and "attract & kill" "bait-sprays" targeting larvae. Due to the implementation of the Food Quality Protection Act of 1996 the most effective and inexpensive insecticides for codling moth control, the organophosphate insecticides, will in the very near future be highly restricted or completely banned from use. Also, the current alternative control materials, both insecticides and pheromone mating disruptants, and their required application rates are much higher or prohibitive in cost. Thus, both insecticidal and pheromone mating disruption alternative strategies must be made more effective, affordable, and acceptable for control use. Our goal and hypothesis is that the pear-ester kairomone will act to improve the control efficacy and diminish the amount of insecticide and pheromone disruptant required to control damaging populations of CM in walnut orchards. This is based on our prior reported research that male codling moths are more attracted to combination lures that are a blend of kairomone and pheromone than to pheromone-alone baited traps, thus will mating disruption be more effective using the combination of pheromone and kairomone over the current pheromone-alone tactic. Also, newly-hatched neonate CM larvae are highly attracted to the kairomone, thus low-volume bait-sprays of kairomone + insecticide might attract - kill target larvae more effectively.

Trécé, Inc. developed a micro-encapsulated (MEC) sprayable formulation of the kairomone that had good residual field-release activity for over a month. The sprayable MEC-kairomone was tank-mixed with either sprayable MEC-pheromone and applied by standard orchard fan-sprayers. Mating disruption trials were conducted in: 1) 21 replicated walnut orchards, with treatment and standard blocks being 10 -15 acres, and six replicated apple orchards, with five acre blocks. Occurrence and degree of nut damage was evaluated by nut drop, canopy infestation, and will be evaluated by current harvest sampling. In 2003 trials, the addition of kairomone-MEC to sprayable pheromone reduced both walnut injury and the degree of multiple-matings by codling moth females. Results for 2004 are also very encouraging, but await the critical harvest damage assessment of treatments.

These studies show promise that the kairomone can attract and eliminate CM and disrupt male mating orientation, and thereby improve damage control. Next season, these mating disruption and bait-spray studies will expand in block size and number of replicated orchards, to further resolve the efficacy of these kairomone-augmented tactics and diminish CM immigration from orchards.

Panel Discussion: Crop Management and Handling

Panel Chair: Pat Dowd
Panel Members: M.E. Olsen, T.C. Pearson; P. Timper and D.M. Light

Presentations involved new genes for insect resistance, new monitoring methods for insects, nematode involvement in aflatoxin in peanut, and corn kernel sorting for mycotoxin reduction. Questions and responses reflected these themes.

Dr. Wicklow pointed out that to achieve accurate high speed dual-wavelength sorting of white corn, as was done with yellow corn, it will again be necessary to collect and analyze single kernel reflectance spectra (500-1200nm) to select the optimal pair of optical filters for detecting mycotoxin-contaminated white corn kernels. He further noted that high volume optical sorters are limited to scanning at only two absorbance bands and cannot discriminate among symptoms of kernel-discoloration produced by different fungal species. The author's neural network classification of kernels infested with different fungal species was developed for research applications using "bench top" laboratory instruments.

Dr. Timper indicated that nematodes probably would not act as reservoirs for *A. flavus* because their stylet orifices are too small for conidia to pass through. Nematodes typically just vector viruses. Dr. Timper also indicated that she did classify damage by degree, but has not yet analyzed that data. Dr. Light indicated that the microencapsulation methods used for volatiles could encapsulate different types of volatiles, depending on the formulation. Formulations tended to be more compatible with esters, but some satisfactory for alcohols also existed. Dr. Light also indicated that the size range was 5-20 um, and that the size was a trade off between large enough to contain a suitable volume, and small enough to pass through sprayer nozzles. Dr. Light indicated that the esters could potentially autooxidize since they had two double bonds, but were stable up to two months in gray septa. The microcapsules can additionally be stabilized by adding carbon to the formulation. Dr. Dowd indicated that silk-specific promoters were to be used to express anthocyanin producing genes for insect resistance in corn, and initial tests indicated the promoter was highly tissue specific.

Screen Method for Determination of Aflatoxins in Peanut and Corn

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Although different analytical methods have been recommended to determine aflatoxins in food and feeds in developed countries, most of these methods are costly and complex. Therefore, a rapid, easy and inexpensive method to screen aflatoxins in peanut and corn was developed. The method involved several steps including sample preparation and extraction with a relatively weak organic polar solvent (acetone-water 80:20, v/v), cleanup with anhydrous sodium sulfate and activated neutral alumina (800mg) packed in a polyethylene tube, elution with a strong organic polar solvent such as methanol (methanol, HPLC Grade), derivatization using a bromine solution, and quantification with a calibrated Fluorometer. Statistical analysis with SAS/STAT software showed high correlations between the developed method and the inimunoaffinity method (Afla Test P, manufactured by VICAM) in peanut and corn samples with adjusted R values of 0.96 and 0.98 respectively. The initial assessment of dollar cost for each column packed with activated alumina neutral is less than two dollars allowing a substantial cost saving.

Preharvest *Aspergillus* Invasion and Aflatoxin Contamination of Groundnuts and Control of *Tribolium confusum* by Diatomaceous Earth in Stored Groundnuts

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Infection of groundnut, *Arachis hypogaea* L., kernels by aflatoxigenic fungi and subsequent aflatoxin contamination can be an extremely serious problem under conditions of drought stress and elevated soil temperatures. However, discovery of sources of resistance to preharvest aflatoxin contamination of groundnuts and breeding for preharvest aflatoxin contamination can minimize aflatoxin contamination in groundnuts. In addition, a reduction in insect pests during the storage of groundnuts can lead to reduced aflatoxin contamination. In this study, 10 groundnut varieties selected to represent the entire germplasm collection for groundnuts (core collection), 7 developed resistant varieties (breeding lines) and 2 indigenous varieties to Botswana, all grown at Notwane farm, Sebele (Botswana College of Agriculture) were tested for *Aspergillus* and aflatoxin contamination. Major insect pests of stored groundnuts in Botswana and the efficacy of diatomaceous earth (Protect-It™) to control the *Tribolium confusum* were also determined. All the 19 (100%) groundnut varieties were contaminated with *Aspergillus flavus* and *A. niger* while 11(58%), 7(37%) and 3(16%) of the samples contained *A. terreus, A. parasiticus* and *A. niveus* respectively. Only one variety (4%) contained *A. nomius*. When the samples were tested for aflatoxins, 100% of the samples were contaminated with aflatoxin B1 while aflatoxin B2, G1 and G2 were detected in 89%, 89% and 68% of the samples respectively. Eighty percent of the core collection samples had total aflatoxin concentration greater than 100 µg/kg groundnut compared to 71% of the breeding lines. The highest total concentration was 2883 µg/kg groundnut and was found in core collection samples. None of the local varieties exhibited total aflatoxin amounts greater than 100 µg/kg groundnut. All core collection varieties had total aflatoxins above 20 µg/kg groundnut the maximum set by WHO. When stored groundnuts were tested for insect pests *Tribolium confusum* was found to be the most predominant insect pest in both shelled and unshelled stored groundnuts and was found in 79% of the groundnut samples. Other insect pests found were: *Lasioderma serricorne* (59%), *Oryzaephilus surinamensis* (28%) and *Plodia interpunctella* (13%). When losses due to *Tribolium confusum* were assessed over a period of 60 days, it was found that an increase in *T. confusum* population resulted in weight loss of 0.6 g per 400 g of stored groundnuts over a period of 60 days. When stored groundnuts were treated with different dosages of diatomaceous earth, 2.5 g diatomaceous earth/kg groundnut dosage was the most effective against *T. confusum* adults (only 5 insects survived the treatment compared to the control where 38 insects survived). In addition, larval *T. confusum* emergence declined with increased diatomaceous earth dosage. The movement of the insects in the stored product and hence the transfer of *A. flavus* spores by insects was also reduced by diatomaceous earth application. The mean *A. flavus* spore concentration recovered from sterile groundnuts as a result of the migration of insects was 45 CFU/g for 2.5 g diatomaceous earth/kg groundnut treatment compared to 1.08 x 10^3 CFU/g recovered from treatments without diatomaceous earth. This study shows that there is variation in the degree of resistance to preharvest aflatoxin contamination in the different varieties tested. Local varieties appeared to be the least susceptible to aflatoxin contamination in the field when compared to the core collection and breeding lines. *Tribolium confusum* was found to be the most predominant insect attacking stored groundnuts in Botswana. Losses due to *T. confusum* and the transfer of *A. flavus* spores in groundnuts during storage can be minimized by the application of diatomaceous earth (Protect-It™).

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