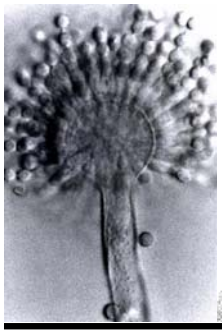

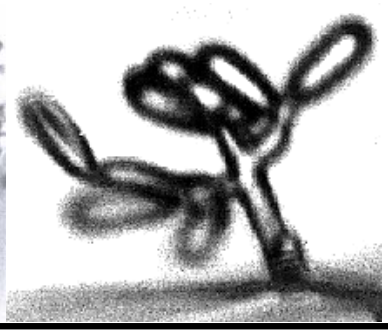

Aflatoxin/Fumonisin Workshop 2000

October 25 - 27, 2000

Yosemite, California

Cover painting: Looking up the Yosemite Valley, Albert Bierstadt, 1865

We would like to thank the following organizations representing the tree nut and dried fruit industries of California for sponsoring this workshop:

Almond Board of California

California Fig Advisory Board

California Pistachio Commission

DFA of California

Walnut Marketing Board

Contents

POSTER PRESENTATIONS

PLATFORM PRESENTATIONS

Moderator: David Ramos, Walnut Marketing Board

Poster Presentation

Session 3: Potential Use of Natural Products for Prevention of Fungal Invasion and/or Aflatoxin Biosynthesis in Crops 73

Platform Presentations

Moderator: Bob Klein, California Pistachio Commission

Poster Presentations

POSTER PRESENTATIONS

SESSION 5: MICROBIAL ECOLOGY 125

PLATFORM PRESENTATIONS

Moderator: Phil Wakelyn, National Cotton Council

POSTER PRESENTATIONS

POSTER PRESENTATIONS

AFLATOXIN/ FUMONISIN WORKSHOP 2000

FISH CAMP (NEAR YOSEMITE), CALIFORNIA
OCTOBER 25-27, 2000

Throughout the world, contamination of food commodities by certain mycotoxins is considered a serious food safety problem. In the past, this workshop focused on chronic problems of aflatoxin contamination of agricultural commodities produced by the United States. These commodities included cottonseed, corn, peanuts, and tree nuts (almonds, pistachios and walnuts). Recent lowering of action threshold levels for aflatoxin by major importing nations has added additional concerns to the issue of aflatoxin contamination.

For over a decade, the 'Aflatoxin Elimination Workshops' brought together the Agriculture Research Service (ARS, USDA), university scientists, and representatives of the cottonseed, corn, peanut, and tree nut industries in a unique cooperative effort to develop aflatoxin control strategies. The ultimate goal of this effort was to implement technologies for eliminating problems of aflatoxin contamination in the U.S. marketplace. Most of this research is currently conducted by ARS. However, an important addition to this ARS effort is a competitive award program, provided by Congressional appropriations, bringing funds to university scientists for collaborative efforts with ARS scientists.

Scientists realized over a decade ago the aflatoxin problem could not be solved using routine technologies for control of "typical" plant pathogens. Aflatoxin contamination results from infection of host plants by spores of a unique class of saprophytic and opportunistic fungi adapted to subsist on organic debris in the field. Classical plant disease prevention methods developed to control fastidious plant pathogens have been generally unsuccessful in excluding aflatoxin-producing fungi from their relatively broad ecological niche. The realization of the unique nature of the aflatoxin problem and need for novel technologies required for its control became a focal point of discussion during the first Aflatoxin Elimination Workshop in 1988. Two areas of research and development were suggested: (1) novel genetic engineering and/or marker-based breeding methods to enhance general resistance to fungi in crops, and (2) use of special strains of the *Aspergillus flavus*-group for biocontrol. These strains do not produce aflatoxin, occupy the same ecological niche and out compete harmful toxin-producing fungi.

This year the scope of the 'Aflatoxin Elimination Workshop' was expanded to include a session on fumonisin contamination of corn. Fumonisins, a family of toxic metabolites produced by the fungus *Fusarium verticillioides* (previously called *F. moniliforme*), are ubiquitous in corn and are unavoidable contaminants in food and feeds. The FDA issued a draft guidance for industry on maximum fumonisin levels the FDA considers adequate to protect human and animal health. The problem of fumonisins in corn is addressed in this year's workshop resulting in a new workshop title, 'Aflatoxin/Fumonisin Workshop 2000'.

Biological Control

Thirteen years after the first aflatoxin workshop, significant progress has been made toward achieving the goal of ARS and Congress to eliminate the aflatoxin contamination problem. This is exemplified by development of biological control technologies for reduction of

aflatoxin contamination. The USDA and the Arizona Cotton Research and Protection Council (ACRPC) received an EPA permit for an area-wide management program in Arizona cotton using the atoxigenic *A. flavus* strain. Thus far, treated areas are demonstrating biological control by atoxigenic-strain technology indicating it is real and sustainable. Acquisition of improved processing equipment has eliminated previous problems with product integrity of the atoxigenic strain. The ACRPC expects to have a full-scale production facility operational by crop year 2001. In South Texas, grain and cotton industry members are enthusiastic at the prospect of testing atoxigenicstrain technology in their area. Similar technology is being developed by ARS and the peanut industry for control of aflatoxin contamination in peanut. This research has demonstrated significant reductions in aflatoxin contamination and steps to obtain EPA registration for this biocontrol product are underway. Additionally, strains of atoxigenic *A. flavus* and a number of newly discovered saprophytic yeasts are being tested as biocontrol agents in pistachio and fig orchards, as a collaborative effort by ARS and university scientists with support from the tree nut and fig industries.

Genetic Modification and Use of Natural Products

Natural products continue to be identified that inhibit fungal growth and/or aflatoxin production. Where feasible, genes encoding these products have been transferred into plants via plant transformation and regeneration technology. ARS scientists at Albany, California in conjunction with researchers at the University of California, Davis discovered the seedcoat surrounding the 'Tulare' walnut almost completely inhibits aflatoxin production. The ARS scientists are currently identifying the structure and mode of action of this seedcoat factor. Genetic crosses of 'Tulare' with susceptible walnut cultivars will be used to study inheritance of increased resistance and aid in cloning of gene(s) encoding the resistance factor for possible introduction into other susceptible crops. Research on both transgenic walnut and peanut plants expressing the *Bacillus thuringiensis* CryIAc gene has continued to show promise as a means of reducing levels of insect damage. Insect-feeding wounds are a major avenue for infection by aflatoxin-producing fungi and *Bt*-transgenic crops offer a promising potential of reducing aflatoxin contamination. Using an optimized microprojectile bombardment protocol scientists at the University of Georgia at Tifton generated a number of transgenic peanut lines showing stable expression of Cry1Ac and resistance to lesser cornstalk borer. Studies in transgenic walnut using the Cry1Ac gene showed excellent mortality levels for codling moth larvae, the major insect pest.

In addition, Albany ARS scientists discovered a novel host-plant volatile (HPV) from pear having pheromone-like potency, but with the added advantage over pheromone of being able to attract female, egglaying moths. This HPV is currently used for monitoring moth flights to time pesticidal applications and is being incorporated into a pesticide-laced bait to attract and kill only codling moths. This work is a cooperative effort by ARS, the Walnut Marketing Board and industrial partner, Trécé, Inc.

Efficacy of antifungal genes such as anionic peroxidase, lipoxygenase, haloperoxidase, and membraneinteracting peptides, D5C and D4E1, has also been analyzed in transgenic crops. Inhibition of fungal growth has been observed with immature cottonseed expressing the haloperoxidase or antifungal peptide gene D4E1 using a green fluorescent protein (GFP)-based assay. Additionally, ARS scientists in New Orleans in conjunction with Dow AgroSciences have initiated the construction of binary vectors in which the D4E1 and Cry1Ac genes will be co-expressed along with an herbicide resistance gene.

Breeding Resistant Lines

A number of corn genotypes have been identified exhibiting superior resistance to infection by *A. flavus*. Significant strides have been made by ARS and university labs on molecular and genetic analyses of these corn genotypes with the goal of using molecular markers to perform marker-assisted selection of desirable germplasm. Quantitative trait loci (QTLs) for insect and fungal resistance, as well as aflatoxin production in select corn inbred lines, have imparted some degree of resistance to commercially desirable lines including QTLs for maysin production and husk tightness. Proteins of 28 kDa and 100kDa in the seed of resistant inbred Tex6 have been identified as resistance sources as well as a 14kDa trypsin inhibitor. Drought tolerant/insect resistant germplasm obtained from Spain and CIMMYT are being field-tested to determine which germplasm will be the best for subsequent crosses with identified aflatoxin resistant corn lines. These corn lines will be used to develop hybrid corn for the Southern U.S. with good husk coverage, insect and aflatoxin resistance and drought tolerance, in addition to providing an acceptable yield. Results of these types of studies indicate additive gene action is important for aflatoxin resistance and resistance genes will need to be 'pyramided' into agronomically desirable inbred lines.

ARS scientists have also scored a core collection of peanut accessions for resistance to preharvest aflatoxin contamination (PAC) and identified a number of lines exhibiting superior resistance. However these lines have less than acceptable agronomic characteristics so they have been entered into a hybridization program to combine resistance to PAC with acceptable agronomic traits such as high yield and drought tolerance. Traditional breeding programs in almond and fig have identified varieties that possess resistance phenotypes such as high shell-seal and reduced ostiole size, respectively. ARS and University of California, Davis scientists identified a number of almond lines with significantly higher resistance to aflatoxin contamination than currently used commercial varieties.

Crop Management and Postharvest Processing

An integrated approach will most likely be required to control aflatoxin contamination of crops. Research on crop management and handling practices, insect control and fungal-plant relationships in the field are providing us with additional strategies for control. Reducing early 'hull-split' in pistachio and improving irrigation techniques in both pistachio and fig orchards results in lower aflatoxin contamination. Development of computer-based "expert system" databases for integrated peanut, cotton, and corn management facilitate transfer of information on crop management practices to the farmer. Improvements in post-harvest sorting has been achieved in almonds and pistachios. ARS scientists, in cooperation with tree nut industrial partners, worked on developing commercially practical sorters that remove aflatoxin contaminated nuts from the processing stream.

Fumonisin in Corn

Fusarium verticillioides, like *A. flavus*, is not a "typical" plant pathogen but rather a biotropic mutualist or an endophyte of corn capable of establishing a long-term association with the crop. As an endophyte, preharvest control of this fungus in corn is difficult. Therefore, as with *A. flavus* and control of aflatoxin, a multifaceted strategy utilizing a number of novel control technologies are likely required for elimination of fumonisins from corn.

ARS scientists are examining use of an endophytic bacterium, *Bacillus mojavensis*, as a biological control agent of *F. verticillioides*. There is commercial interest in developing this control technology for application to seed corn. These scientists also disclosed higher levels of certain hydroxamic acids, DIMBOA and DIBOA. in corn did not provide effective resistance to infection by *F. verticillioides*. Studies using an iodine-based product, Plantpro 45™, indicated this approach merits further investigation into its commercial application to seed. A simple, rapid immunoassay for detection of fumonisins in corn is under development capable of detecting levels in corn extracts over a range of 0.5 to 20 ppm. This technology will be needed by industry to comply with FDA guidelines on acceptable fumonisin levels. ARS and university scientists have made substantial progress in understanding the biosynthesis and regulation of fumonisin production. In addition to characterizing several genes believed to comprise a portion of the fumonisin biosynthetic pathway, other genetic loci have been identified associated with its ability to synthesize fumonisin. A gene from *Gibberella fujikuori* (the perfect state of *F. verticillioides*) was identified that inhibited fumonisin production. This gene is required for conidiation and production of FB1 and may play a role as a regulator of genes involved in fungal development and secondary metabolism. Additional data shows fumonisin production is not required for a fungus to infect the plant or produce ear rot. Thus, biological control of fumonisin in corn by competitive exclusion with non-fumonisin producing strains of *F. verticillioides* might be possible.

The 2000 Workshop

The research summarized in this proceedings provides a technological foundation to understanding multiple strategies for eliminating problems of aflatoxin and fumonisin contamination in the marketplace. Through this research, fundamental strategies are being developed that may prove useful in eliminating future problems associated with other mycotoxins. These workshops have provided a forum for sharing over a decade's worth of research-knowledge and have resulted in a synergism of ARS, university and industry scientists' efforts to curtail aflatoxin contamination. We anticipate presentations and discussions on fumonisins will result in a similar synergism to develop innovative strategies to reduce fumonisin contamination of corn.

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DR. BRUCE C. CAMPBELL
Research Leader
Plant Protection Research
Western Regional Research Center
Agricultural Research Service, USDA
Albany, CA

AGENDA

AFLATOXIN/ FUMONISIN WORKSHOP 2000

OCTOBER 25-27, 2000

TENAYA LODGE, FISH CAMP, CA

WEDNESDAY, OCTOBER 25, 2000

Noon-8:00 PM **REGISTRATION/ POSTER ASSIGNMENTS**

1:45 PM **WELCOME:** J. Michael Hurley, Lab Director, DFA of California, Fresno, CA

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

SESSION 1: ***FUMONISIN CONTAMINATION OF CORN***
Moderator: Kyd Brenner, Corn Refiners Association

2:00 PM **Biological control of *Fusarium moniliform* in corn by competitive exclusion using *Bacillus mojavensis*.** Charles W. Bacon* and Dorothy M. Hinton. USDA, ARS, Toxicology and Mycotoxin Research Unit, Russell Research Center, Athens GA.

2:20 PM **The use of an iodine-based product as a biocompatible control for *Fusarium moniliforme*.** Ida Yates[1], J. Arnold[1], D.M. Hinton[1], W. Basinger[2] and R. Walcott[3]. [1]USDA, ARS, Toxicology and Mycotoxin Research Unit, Russell Research Center, Athens GA; [2]Ajay North America, LLC, Powder Springs, GA; [3]Dept. of Plant Pathology, University of Georgia, Athens, GA.

2:40 PM **Detoxification of corn antimicrobial compounds and the significance to *Fusarium* species.** Anthony E. Glenn and Charles W. Bacon. USDA, ARS, Toxicology and Mycotoxin Research Unit, Russell Research Center, Athens GA.

3:00 PM **Rapid detection of fumonisins in maize using fluorescence polarization.** Chris Maragos. USDA, ARS, National Center for Agricultural Utilization Research, Peoria, IL.

3:20 PM **Progress in understanding the biosynthesis and regulation of fumonisins in *Fusarium verticillioides*.** Ron Plattner, Robert H. Proctor, Daren W. Brown and Anne E. Desjardins. USDA, ARS, National Center for Agricultural Utilization Research, Peoria, IL.

and

Possible minimization of fumonisin levels in corn using fumonisin non-producing strains of *Fusarium verticillioides*. Ron Plattner, Robert H. Proctor and Anne E. Desjardins. USDA, ARS, National Center for Agricultural Utilization Research, Peoria, IL.

4:00 PM **Regulation of fumonisin biosynthesis: the corn kernel holds the key.** Charles P. Woloshuk and Won-Bo Shim. Botany and Plant Pathology, Purdue University, West Lafayette, IN.

4:20- 5:00 PM **PANEL DISCUSSION**
Panel Chair: Ron Plattner, USDA, ARS, National Center for Agricultural Utilization Research, Peoria, IL

5:00-6:00 PM **POSTER VIEWING**

6:00-8:00 PM **MIXER**

THURSDAY, OCTOBER 26, 2000

7:00 AM **REGISTRATION/ POSTER ASSIGNMENTS**

8:00 AM **ANNOUNCEMENTS**

SESSION 2: ***CROP MANAGEMENT AND HANDLING, INSECT CONTROL AND FUNGAL RELATIONSHIPS***
Moderator: David Ramos, Walnut Marketing Board

8:15 AM **Insect Management for Reduction of Mycotoxins in Midwest Corn - FY 2000 Results.** Patrick F. Dowd[1], Jason Barnett[2], Robert J. Bartelt[1], Jim Beck[2], Mark A. Berhow[1], Rebecca S. Boston[3], Jonathan P. Duvick[4], L. Mark Lagrimini[2], Roger A. Larson[5], Gary Molid[6], Michael J. Muhitch[7], and Donald G. White[8]. [1]USDA,ARS,NCAUR,BAR, Peoria, IL; [2]Novartis, Research Triangle Park, NC; [3]Dept. Botany, North Carolina State University, Raleigh, NC; [4]Pioneer Hi-Bred, Johnston, IA; [5]University of Illinois Extension, Havana, IL; [6]Del Monte Foods, Manito, IL; [7]USDA,ARS,NCAUR,MTX, Peoria, IL; [8]Dept. Crop Sciences, University of Illinois, Urbana, IL.

8:30 AM **Aflatoxin control in pistachios: Agronomic practices and biocontrol.** Mark Doster[1], Themis Michailides[1], Robert Beede[2], Brent Holtz[3], and Walter Bentley[1], [1]University of California/Kearney Agricultural Center, Parlier, CA; [2]University of California Cooperative Extension, Kings County, CA; [3]University of California Cooperative Extension, Madera County, CA.

8:45 AM **Aflatoxin control in figs: Agronomic practices, ecological relationships, and biocontrol.** Mark Doster[1], Themis Michailides[1], David Goldhamer[2], James Doyle[3], David Morgan1, and Daniel Felts[1], [1]Dept. of Plant Pathology; [2]Dept. of Land, Air & Water Resources; [3]Dept. of Pomology, Univ. of California, Davis/Kearney Agricultural Center, Parlier, CA.

9:00 AM **Economic input for an 'Expert System' to control preharvest aflatoxin of maize.** Neil Widstrom[1], Marshall Lamb[2] and Ron Williams. [1]USDA, ARS, Crop Genetics and Breeding Research Unit, Tifton, GA; [2]USDA, ARS, National Peanut Research Lab, Dawson, GA.

9:15 AM **Detection of whole corn kernels contaminated with aflatoxin by near infrared spectroscopy**. Tom C. Pearson[1], Floyd E. Dowell[2], Don T. Wicklow[3], Feng Xie[4], and Elizabeth B. Maghirang[2]. [1]USDA, ARS, Western Regional Research Center, Albany, CA; [2]USDA, ARS, Engineering Research Unit, Manhattan, KS; [3]USDA, ARS, National Center for Agricultural Utilization Research, Peoria, IL; [4]Kansas State University, Manhattan, KS.

9:30 AM **Subsampling of pistachios.** Thomas F. Schatzki and Natsuko Toyofuku. USDA, ARS, Western Regional Research Center, Albany, CA

9:45-10:15 AM **PANEL DISCUSSION**
Panel Chair: Patrick F. Dowd, USDA, ARS, National Center for Agricultural Utilization Research, Peoria, IL.

10:15-10:45 AM **BREAK**

SESSION 3: ***POTENTIAL USE OF NATURAL PRODUCTS FOR PREVENTION OF FUNGAL INVASION AND/OR AFLATOXIN BIOSYNTHESIS IN CROPS***
Moderator: Bob Klein, California Pistachio Commission

10:45 AM **Effect of alpha-carotene volatile degradation products, D-talose and glycerol on aflatoxin B1 production by *A. flavus*.** Robert A. Norton, USDA, ARS, National Center for Agricultural Utilization Research, Peoria, IL.

11:00 AM **Anti-aflatoxigenic activity and constituents of walnuts.** Russell J. Molyneux[1], Noreen Mahoney[1], Bruce C. Campbell[1], Gale McGranahan[2] and Jim McKenna[2]. [1]USDA, ARS, Western Regional Research Center, Albany, CA; [2]Department of Pomology, University of California, Davis, CA.

11:15 AM ***In vitro* effects on aflatoxin B1 biotransformation by naturally occurring compounds.** Sung-Eun Lee[1], Bruce C. Campbell[1], Russell Molyneux[1], Shin Hasegawa[1] and Hoi-Seon Lee[2]. [1]USDA, ARS, Western Regional Research Center, Albany, CA; [2]Division of Biotechnology, ChonBuk National University, Chonju, Republic of Korea.

11:30 AM **Development of semiochemical-based control of the codling moth, a key pest in the infection of walnut by *Aspergillus* spp.** Douglas M. Light[1],

Katherine M. Reynolds[1], Ronald G. Buttery[1], Gloria Merrill[1], James Roitman[1], Bruce C. Campbell[1], Alan L. Knight[2], Clive A. Henrick[3], Dayananda Rajapaska[3], Scott Lingren[3], and Bill Lingren[3]. [1]USDA, ARS, Western Regional Research Center, Albany, CA; [2]USDA,ARS, Yakima Agricultural Research Laboratory, Wapato, WA; [3]Trécé, Inc., Salinas, CA

11:45 AM **Role of lipid metabolism in *Aspergillus* development.** Nancy P. Keller and Richard A. Wilson. Department of Plant Pathology and Microbiology, Texas A&M University, College Station, TX.

Noon-12:30 PM **PANEL DISCUSSION**
Panel Chair: Deepak Bhatnagar, USDA, ARS, Southern Regional Research Center, New Orleans, LA

12:30-1:30 PM **LUNCH**

1:30-2:00 PM ***KEYNOTE ADDRESS:***
Food Safety and International Challenges
Julie G. Adams, Almond Board of California, Modesto, CA.

SESSION 4: ***CROP RESISTANCE- GENETIC ENGINEERING***
Moderator: Lynn Jones, National Cottonseed Products Association

2:00 PM **Novel inhibitor for controlling *Aspergillus flavus* growth and aflatoxin production in corn.** Charles P. Woloshuk and Ahmad Fakhoury. Department of Botany and Plant Pathology, Purdue University, West Lafayette, IN.

2:15 PM **Transgenic cottons to combat preharvest aflatoxin contamination: An update.** Kanniah Rajasekaran[1], Jeffrey W. Cary[1], Tom J. Jacks[1], Caryl A. Chlan[2] and Thomas Ed Cleveland[1]. [1]USDA, ARS, Southern Regional Research Center, New Orleans, LA. [2]University of Louisiana, Lafayette, LA.

2:30 PM **Genetic engineering of peanut for reduction of aflatoxin contamination**. Peggy Ozias-Akins[1], Hongyu Yang[1], Evelyn Roberson[1], Yoko Akasaka[1], Robert Lynch[2]. [1]Department of Horticulture and [2]USDA-ARS, University of Georgia Coastal Plain Experiment Station, Tifton, GA.

2:45 PM **Genetic engineering and breeding of walnuts for control of aflatoxin.** Abhaya M. Dandekar[1], Gale McGranahan[1], Patrick Vail[2], Russell Molyneux[3], Noreen Mahoney[3], Charles Leslie[1], Sandie Uratsu[1] and Steven Tebbets[2] [1]Dept. of Pomology, Univ. of California, Davis CA; [2]Horticultural Crops Research Lab., Fresno CA; [3]USDA, ARS Western Regional Research Center, Albany, CA

.

3:00-3:30 PM **BREAK**

3:30 PM **Progress towards the development of cotton with enhanced resistance to *A. flavus*.** Caryl Chlan, Jeffrey Cary, Kanniah Rajasekaran, and Thomas E. Cleveland. USDA, ARS, Southern Regional Research Center, New Orleans, LA.

3:45 PM **Transformation with genes encoding Peptidyl MIM®, as a means of reducing aflatoxin contamination in peanut.** Arthur Weissinger[1], Kimberly Sampson[2], Lori Urban[3], Keith Ingram[4], Gary Payne[1], Sean Scanlon[1], Yan-Sheng Liu[1] and T.E. Cleveland[5]. [1]North Carolina State University, Raleigh, NC; [2]National Institute of Environmental Health Sciences, Research Triangle Park, NC; [3]Demegen, Inc., Pittsburgh, PA; [4]University of Georgia, Griffin, GA; [5]USDA, ARS, Southern Regional Research Center, New Orleans, LA.

4:00-4:30 PM **PANEL DISCUSSION**
Panel Chair: Jeff Cary, USDA, ARS, Southern Regional Research Center, New Orleans, LA

4:30- 5:00 PM **POSTER VIEWING**

5:00 PM **COMMODITY BREAKOUT SESSIONS**

7:00 PM **BANQUET**

FRIDAY, OCTOBER 27, 2000

8:00 AM **ANNOUNCEMENTS**

SESSION 5: ***MICROBIAL ECOLOGY***
Moderator: Phil Wakelyn, National Cotton Council

8:15 AM **Variation in *Aspergillus ochraceus*.** Paul Bayman, James L. Baker and Noreen E. Mahoney. USDA, ARS, Western Regional Research Center, Albany, CA.

8:30 AM **Effect of rainfall on the movement of biocontrol conidia in peanut fields.** Bruce Horn, Ronald Greene, Ronald Sorensen, Paul Blankenship and Joe Dorner. USDA, ARS, National Peanut Research Laboratory, Dawson, GA.

8:45 AM **A simple technique for producing aflatoxin biocontrol formulations**. Joe W. Dorner and Richard J. Cole, USDA, ARS, National Peanut Research Laboratory, Dawson, GA.

9:00 AM **Utilizing atoxigenic strains of *A. flavus* to manage aflatoxins in commercial cotton: Part I. Commercial scale manufacture of inoculum**. Peter J. Cotty[1], Larry Antilla[2], Joe Ploski[3], Kerri Kobbeman[4], and Clive H. Bock[1]. [1]Southern Regional Research Center, ARS, USDA, New Orleans, LA; [2]Arizona

Cotton Research & Protection Council, Phoenix, AZ; [3]Pink Boll Worm Rearing Facility, APHIS, USDA, Phoenix, AZ; [4]Dept. Plant Pathology, Univ. Arizona, Tucson, AZ.

9:15 AM **Utilizing atoxigenic strains of *A. flavus* to manage aflatoxins in commercial cotton: Part II. Field aspects.** Larry Antilla[1] and Peter J. Cotty[2]. [1]Arizona Cotton Research and Protection Council, Phoenix, AZ; [2]USDA, ARS, Southern Regional Research Center, New Orleans, LA.

9:30-10:00 AM **BREAK**

10:00 AM **Biological control of aflatoxin contamination in almond and pistachio.** Sui-Sheng T. Hua. USDA, ARS, Western Regional Research Center, Albany, CA.

10:15 AM **Two new aflatoxin producing species in *Aspergillus* section *Flavi*.** Stephen W. Peterson. USDA, ARS, National Center for Agricultural Utilization Research, Peoria, IL.

10:30-11:00 AM **PANEL DISCUSSION**
Panel Chair: Paul Bayman, USDA, ARS, Western Regional Research Center, Albany, CA

SESSION 6: ***CROP RESISTANCE- CONVENTIONAL BREEDING***
Moderator: Bob Sacher, Hunt Wesson, Inc.

11:00 AM **Characterization of the structural, biochemical and molecular basis for endocarp, seed and and seed-coat based resistance to preharvest aflatoxin contamination in almond.** Thomas M. Gradziel and Abhaya M. Dandekar. Department of Pomology, University of California, Davis, CA.

11:15 AM **Corn varietal response for numbers of aflatoxin contaminated BGYF kernels.** Don Wicklow[1] and Lori C. Marshall[2]. [1]USDA, ARS, National Center for Agricultural Utilization Research, Peoria, IL. [2]Holden's Seeds (Monsanto), Williamsburg, IA.

11:30 AM **Identification and characterization of new corn kernel traits associated with resistance to *Aspergillus flavus* infection/aflatoxin production.** Robert L. Brown[1], Zhi-Yuan Chen[2], Shirley V. Gembeh[1], You-Keng Goh[2], Kenneth E. Damann[2], Casey Grimm[1], Jiu-Jiang Yu[1], Thomas E. Cleveland[1], and Deepak Bhatnagar[1]. [1]USDA-ARS-Southern Regional Center, New Orleans, LA; [2]Department of Plant Pathology and Crop Physiology, Louisiana State University, Baton Rouge, LA.

11:45 AM **Maize resistance to aflatoxin in Texas**. Javier Betran[1], Tom Isakeit[2] and Gary Odvody[2]. [1] Soil & Crop Sciences Department; [2]Pathology Department, Texas A&M University, College Station, TX.

Noon-1:00 PM **LUNCH**

1:00 PM **Identification and pyramiding of genes/markers associated with corn ear resistance to insects and *Aspergillus flavus* for control of preharvest aflatoxin contamination in the South: a research progress report.** Baozhu Z. Guo[1], Ana Butron[1], Neil W. Widstrom[2], David M. Wilson[3], Maurice E. Snook[3], R. D. Lee[3], Thomas E. Cleveland[4], and Robert E. Lynch[1]. [1]USDA, ARS, Crop Protection and Management Research Unit, [2]Crop Genetics and Breeding Research Unit and [3]The University of Georgia, Coastal Plain Experiment Station, Tifton, GA; [4]USDA, ARS, Southern Regional Research Center, New Orleans, LA.

1:15 PM **Identification and selection of molecular markers associated with resistance to aflatoxin production in maize, and value of inbred *CI2* as a resistance source.** Torbert R. Rocheford and Donald G. White. Department of Crop Sciences, University of Illinois, Urbana, IL.

1:30 PM **Preharvest aflatoxin contamination in drought tolerant and drought intolerant peanut genotypes.** C. Corley Holbrook[1], Craig K. Kvien[2], Huiqin Xue[2], David M. Wilson[2], Michael E. Matheron[3], and R. Donald Wauchope[1]. [1]USDA, ARS, CPES, Tifton, GA, [2]Department of Plant Patholology, The University of Georgia, Tifton, GA [3]University of Arizona, Yuma, AZ.

2:00 PM ***Aspergillus* infection of peanut in relation to pod development stage and water deficit, and screening for mechanisms of resistance to *Aspergillus*.** Keith T. Ingram[1], C. Corley Holbrook[2], Arthur K. Weissinger[3], George Pateña[1], Md. Murshidul Hoque[1] and David Wilson[4]. [1]Department of Crop and Soil Sciences, The University of Georgia, Griffin, GA; [2]USDA, ARS, Tifton, GA; [3]Department of Crop Science, North Carolina State University, Raleigh, NC; [4]Department of Plant Pathology, The University of Georgia, Tifton, GA.

2:15-3:00 PM **PANEL DISCUSSION**
Panel Chair: Tom Gradziel, Department of Pomology, University of California, Davis, CA

3:00-3:15 PM **CLOSING REMARKS**: Jane Robens, National Program Leader, Food Safety and Health, USDA. ARS.

POSTER PRESENTATIONS

AFLATOXIN/ FUMONISIN WORKSHOP 2000

OCTOBER 25-27, 2000

TENAYA LODGE, FISH CAMP, CA

A. MICROBIAL ECOLOGY

A-1 **Molecular Characterization of Aflatoxin Biosynthesis Genes in *Aspergillus* Species Other than *A. flavus/ parasiticus*.** Jeff Cary, Maren Klich, Shannon Brennan, and Cecily Bennett. USDA, ARS, Southern Regional Research Center, New Orleans, LA.

A-2 **Monitoring *Aspergillus flavus* AF 36 and S Strain Incidence in the Desert Southwest.** Donna M. Bigelow[1], Thomas V. Orum[1], Peter J. Cotty[2], and Merritt R. Nelson[1]. [1]Department of Plant Pathology, University of Arizona, Tucson, AZ; [2]USDA, ARS, Southern Regional Research Center, New Orleans, LA.

A-3 **Ecology of *Aspergillus* in Mississippi Delta Soils under Corn Production: Aflatoxin Inoculum Production.** Hamed K. Abbas, Robert M. Zablotowicz, and Martin A. Locke. USDA, ARS, Jamie Whitten Delta States Research Center, Stoneville, MS.

A-4 **Desert Legumes: Aflatoxin Contamination of Natural *A. flavus* Reservoirs in the Sonoran Desert.** Maria L. Boyd and Peter J. Cotty. USDA, ARS, Southern Regional Research Center, New Orleans, LA.

A-5 **Molecular Evidence for a Retrotransposon in *Aspergillus flavus*.** Sui-Sheng T. Hua[1], Cesaria E. McAlpin[2], Brian Tibbot[1] and Patricia Okubara[1]. [1]USDA, ARS, Western Regional Research Center, Albany, CA; [2]USDA, ARS, National Center for Agricultural Utilization Research, Peoria, IL.

A-6 **Interactions Among *Fusarium verticillioides*, Insect Pests, and *Aspergillus flavus* in Transgenic and Conventional Corn Hybrids.** Gary P. Munkvold[1], Richard L. Hellmich[2], and Cassandra M. Biggerstaff[1]. [1]Department of Plant Pathology, Iowa State University, Ames, IA; [2]USDA, ARS, Corn Insects and Crop Genetics Research Unit, Ames, IA.

B. POTENTIAL USE OF NATURAL PRODUCTS FOR PREVENTION OF FUNGAL INVASION AND/OR AFLATOXIN BIOSYNTHESIS IN CROPS

B-1 **Proteomics Analysis of Kernel Embryo and Endosperm Proteins of Corn Genotypes Resistant or Susceptible to *Aspergillus flavus* Infection**. Zhi-Yuan Chen[1], Robert L. Brown[2], Kenneth E. Damann[1], Thomas E. Cleveland[2]. [1]Dept. Plant Pathology and Crop Physiology, Louisiana State University Ag. Center, Baton Rouge, LA; [2]Southern Regional Research Center, USDA-ARS, New Orleans, LA.

B-2 **Aflatoxin Reduction by *Liberty* Herbicide Treatment of *Liberty Link* Corn.** Ken E. Damann and Kayimbi M. Tubajika. Department of Plant Pathology and Crop Physiology, Louisiana State University Agricultural Center, Baton Rouge, LA.

B-3 **Inhibitory Effects of Lactones on Aflatoxin-Producing *Aspergillus flavus*.** Sui-Sheng T. Hua, Henry Shu and Nancy Han. USDA, ARS, Western Regional Research Center, Albany, CA.

B-4 **Gas Chromatography/Mass Spectroscopy Analysis of Thin-Layer Chromatography Bands from Corn Kernel Pericarp Wax Associated with Resistance to *Aspergillus flavus* Infection/Aflatoxin Production.** Shirley V. Gembeh, Robert L. Brown, Thomas E. Cleveland, and Casey Grimm. USDA, ARS, Southern Regional Research Center, New Orleans, LA.

B-5 **Epicuticular Wax Composition and Aflatoxin Levels of Developing Corn Kernels of Susceptible and Resistant Genotypes.** You-Keng Goh[1], Kenneth E. Damann[1], Casey Grimm[2], Robert L. Brown[2], Thomas E. Cleveland[2], and John S. Russin[3]. [1]Lousiana State University Agricultural Center, Baton Rouge, LA.; [2] USDA, ARS, Southern Regional Research Center, New Orleans, LA; [3]SIU, Carbondale, IL.

B-6 **Biotransformation of Aflatoxin: Reducing Its Hepatocarcinogenicity.** Sung-Eun Lee, Kathleen L. Chan and Bruce Campbell. USDA, ARS, Western Regional Research Center, Albany, CA.

C. CROP RESISTANCE-CONVENTIONAL BREEDING

C-1 **Multilocation Evaluation of Single Cross Maize Hybrids for Aflatoxin Contamination**. Paul Williams[1], Gary L. Windham[1], Martha C. Willcox[1], Hamed K. Abbas[2], Javier Betran[3], Don G. White[4], Steve H. Moore[5], Rick Mascagni[6], Ken E. Damann[7] and Neil. W. Widstrom[8]. [1]USDA, ARS, Mississippi State, MS; [2]USDA, ARS, Stoneville, MS; [3]Texas A&M University, College Station, TX; [4]University of

Illinois, Urbana, IL; [5]Louisiana State University, Alexandria, LA; [6]Louisiana State University, St. Joseph, LA; [7]Louisiana State University, Baton Rouge, LA; [8]USDA, ARS, Tifton, GA.

C-2 **Aflatoxin and Fumonisin Contamination of Artificially Inoculated Maize Hybrids and Inbreds in Mississippi.** Gary L. Windham and W. Paul Williams. USDA, ARS, Mississippi State, MS.

C-3 **Markers Associated with Silk Antibiotic Compounds, Husk Coverage, and Aflatoxin Concentrations in Two Mapping Populations.** Ana Butrón[1], Baozhu Z. Guo[1], Neil W. Widstrom[2], Maurice E. Snook[3], David M. Wilson[3], and Robert E. Lynch[1]. [1]USDA, ARS, Crop Protection and Management Research Unit and [2]Crop Genetics and Breeding Research Unit, Tifton, GA; [3]The University of Georgia, Coastal Plain Experiment Station, Tifton, GA.

C-4 **Evaluation of Corn Germplasm Tolerance to Drought Stress and Effects on Aflatoxin Production**. Hong Li[1], Ana Butrón[2], Tingbo Jiang[1], Baozhu Guo[2], Anton E. Coy[1], R. Dewey Lee[1], Neil W. Widstom[3], and Robert E. Lynch[2]. [1]The University of Georgia, Coastal Plain Experiment Station, Tifton, GA; [2]USDA, ARS, Crop Protection and Management Research Unit and [3]Crop Genetics and Breeding Research Unit, Tifton, GA.

C-5 **Marker Assisted Backcrossing and Identification of Chromosome Segments Associated with Resistance to Aflatoxin Production in Maize**. Chandra Paul, Torbert R. Rocheford, and Donald G. White. Department of Crop Sciences, University of Illinois, Urbana, IL.

C-6 **Sources of Resistance to Aflatoxin Production in Maize**. Kayimbi M. Tubajika and Kenneth E. Damann. Department of Plant Pathology & Crop Physiology, Louisiana State University Agricultural Center, Baton Rouge, LA.

D. CROP RESISTANCE-GENETIC ENGINEERING

D-1 **Use of A Green Fluorescent Protein-Expressing *Aspergillus flavus* Strain to Study Fungal Invasion of Cotton.** Jeff Cary, Kanniah Rajasekaran, and Peter Cotty. USDA, ARS, Southern Regional Research Center, New Orleans, LA.

D-2 **Does Lipoxygenase Activity Explain Low Aflatoxin Accumulation in Soybeans?** Jay E. Mellon and Peter J. Cotty. USDA, ARS, Southern Regional Research Center, New Orleans, LA.

D-3 **Confirmation of the QTL Regions for Aflatoxin Resistance by Evaluating Tails of the Va35 x Mp313E Mapping Population in Multiple Environments.** Martha Willcox[1], Georgia Davis[2], Gary Windham[1], Paul Williams[1], Hamed Abbas[3], and Javier Betran[4]. [1]USDA, ARS, Corn Host Plant Resistance Research Unit, Mississippi State, MS; [2]Department of Agronomy, University of Missouri, Colum-

bia, MO; [3]USDA, ARS, Physiology and Genetics Research Unit, Stoneville, MS; [4]Soil and Crop Sciences Department, Texas A&M University, College Station TX.

D-4 Aflatoxin and Insect Response of Near-Isogenic *Bt* and Non-*Bt* Commercial Corn Hybrids in South Texas. Gary N. Odvody, Charles F. Chilcutt, Roy D. Parker, and John H. Benedict. Texas A&M Research & Extension Center, Corpus Christi, TX.

D-5 Cloning of Genes Encoding Peptides with Antifungal Activity against *Aspergillus flavus*. Anne-laure Moyne[1], Thomas E. Cleveland[2] and Sadik Tuzun[1]. [1]Entomology and Plant Pathology Department, Auburn University, Auburn, AL; [2]USDA, ARS, Southern Regional Center, New Orleans, LA.

D-6 A Corn *lox* Gene, *cssap 92*, Is Differentially Expressed in Different Corn Lines during *A. flavus* Colonization. Richard A. Wilson[1], Harold W. Gardner[2] and Nancy P. Keller[1]. [1]Department of Plant Pathology and Microbiology, Texas A&M University, College Station, TX and [2]USDA, ARS, National Center for Agriculture Utilization Research, Peoria, IL.

E. CROP MANAGEMENT AND HANDLING, INSECT CONTROL AND FUNGAL RELATIONSHIPS

E-1 Response of Channel Catfish to Diets Containing Aflatoxin Contaminated Moldy Corn. Bruce Manning, Meng H. Li, and Edwin H. Robinson. National Warmwater Aquaculture Center, Mississippi State University, Stoneville, MS.

F. REGULATION OF AFLATOXIN BIOSYNTHESIS

F-1 Variability in pH and Nitrogen Regulation of Aflatoxin Production in *Aspergillus flavus* strains. Kenneth C. Ehrlich and Peter J. Cotty. USDA, ARS, Southern Regional Research Center, New Orleans, LA.

F-2 Inhibition of *Aspergillus* Growth by a Maize Ribosome Inhibiting Protein. Kirsten Nielsen, Rebecca S. Boston, and Gary A. Payne. Departments of Botany and Plant Pathology, North Carolina State University, Raleigh, NC.

F-3 Cloning and Functional Analysis of *avfA* and *omtB* Genes in Aflatoxin Biosynthesis in *Aspergillus parasiticus*, *A. flavus* and *A. sojae*. Jiujiang Yu[1], Charles P. Woloshuk[2], Deepak Bhatnagar[1], and Thomas E. Cleveland[1]. [1]USDA, ARS, Southern Regional Research Center, New Orleans, LA; [2]Department of Botany and Plant Pathology, Purdue University, West Lafayette, IN.

F-4 ***adhA* in *Aspergillus parasiticus* is Involved in Conversion of 5'-Hydroxyaverantin to Averufin.** Perng-Kuang Chang, Jiujiang Yu, Kenneth C. Ehrlich, Stephen M. Boue, Beverly G. Montalbano, Deepak Bhatnagar, and Thomas E. Cleveland. USDA, ARS, Southern Regional Research Center, New Orleans, LA.

G. DETECTION, ANALYSIS, AND EXTRACTION OF AFLATOXINS

G-1 **Simple Cleanup Method for Determination of Aflatoxins in Peanuts.** Victor S. Sobolev and Joe W. Dorner. USDA, ARS, National Peanut Research Laboratory, Dawson, GA.

G-2 **Detection of Ochratoxin by LC/MS/MS: an Alternative Detector for the AOAC Official Method?** Ronald D. Plattner. USDA, ARS, National Center for Agricultural Utilization Research, Peoria, IL.

H. FUMONISIN CONTAMINATION OF CORN

H-1 **Tracking *Fusarium verticillioides* Infection and Fumonisin Accumulation in North Carolina Corn.** Brian J. Bush, Marty L. Carson, Marc A. Cubeta, Winston M. Hagler, and Gary A. Payne. Departments of Plant Pathology and Poultry Science, North Carolina State University, Raleigh, NC.

H-2 **Evaluation of Inoculation Methods for *Fusarium* Ear Rot of Corn.** Michael J. Clements[1], Donald G. White[1] and Chris M. Maragos[2]. [1]Department of Crop Sciences, University of Illinois, Urbana, IL; [2]USDA, ARS, National Center for Agricultural Utilization Research, Peoria, IL.

POSTER PRESENTATIONS
AUTHOR INDEX

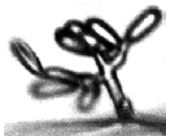
AFLATOXIN/ FUMONISIN WORKSHOP 2000

OCTOBER 25-27, 2000

TENAYA LODGE, FISH CAMP, CA

SESSION 1: FUMONISIN CONTAMINATION OF CORN

***Moderator*: Kyd Brenner**
Corn Refiners Association



BIOLOGICAL CONTROL OF *FUSARIUM MONILIFORME* IN CORN BY COMPETITIVE EXCLUSION

Charles W. Bacon and Dorothy M. Hinton. Toxicology and Mycotoxin Research Unit, ARS, USDA, Russell Research Center, Athens, GA.

Large numbers of fungal symbionts associated with plants are biotrophic mutualists and several of these form endophytic associations Microbial endophytes actively colonize host tissues and establish long-term associations, actually lifelong natural associations, without doing substantive harm to the host. These associations are to be distinguished from the transient visitors, usually dormant or latent infections, which form associations as happenstances that will not survive long. The endophytic fungi are further distinguished by producing a variety of secondary metabolites in planta that impart toxicity to herbivores. Thus, fungal endophytes are capable of producing mycotoxins such as the fumonisins, beauvericin, fusaproliferin, fusaric acid, moniliformin, the ergot alkaloids, tremorgenic toxins, and many other compounds that are biologically active. Endophytic fungi include not only the obligate species of *Neotyphodium*, *Balansia*, *Epichloe*, and *Myriogenospora*, but also the facultative species such as the *Fusarium*. These endophytic fungi are associated with thousands of plant hosts. Some are very restricted and are associated only with grasses, while others are symbiotic with a wide variety of plant species, including monocots and dicots.

Fusarium moniliforme (synonym=*Fusarium verticillioides*; teleomorph = *Gibberella moniliformis*) is one such facultative endophyte that has been isolated from at least 1100 hosts. The fungus is vertically and horizontally transmitted to the next generation of plants via clonal infection of seeds and plant debris. Horizontal infection is the manner by which this fungus is contagiously spread and through which infection takes place from the outside that can be reduced by application of certain fungicides and insecticides during kernel set. The endophytic phase is vertically transmitted and this type infection is important as it is not controlled by seed applications of fungicides, and it remains the reservoir from which infection and toxin biosynthesis in each generation of plants can take place. Thus, vertical transmission of this fungus is equally important as horizontal transmission. This fungus produces five toxins and while it is not known if all five are produced during the endophytic colonizing stage, the fumonisins have been shown to be produced by endophytic hyphae early during maize seedling development.

The endophytic habit of *F. moniliforme* makes it difficult to control with fungicides. However, biocontrols might offer some avenues of hope for endophytic fungi, and there are other nontoxic endophytic microorganisms that offer control for the endophytic hyphae of *F. moniliforme*, such as endophytic bacteria. The biocontrol strategy utilizing an endophytic bacterium is expected to operate under the general mechanism of competitive exclusion, since bacterial growth within the intercellular spaces would preclude or reduce the growth by other microorganisms such as the intercellular hyphae of *F. moniliforme*.

Bacillus subtilis (Ehrenberg) Cohn is the oldest species, and the nomenclatural type for the Bacillaceae and the genus *Bacillus*. This gram positive, spore-forming bacterium, has proven safe over many years as a nonpathogenic species and is consumed in ton quantities in several human food preparations. The bacterium is widely distributed in nature and has been isolated from several botanical environments, primarily the soil, where it has been

shown to have antibiotic properties and biocontrol potential. A strain of *B. subtilis* was discovered that is intercellular, nonpathogenic, enhances plant growth, and offers a protective role for plants against fungi. Root, shoot, and germination are enhanced in the presence of the bacterium. This bacterium readily infects maize seedlings from the topical application to kernels, and is distributed within the roots and above ground foliage of the plants. The plant is internally colonized during the entire growing season of maize. In addition to maize, the bacterium is also an endophyte of wheat, sorghum, barley, oats, and beans. Data indicate that within a range of soil moistures (-0.2 and -0.4 Mpa), the biocontrol bacterium reduced the fumonisin content by 75% over non-bacterial infected plants.

The biocontrol strain was patented (Patent Number 5,994,117) as control for diseases of maize caused by fungi. In addition to *F. moniliforme*, the biocontrol isolate RRC101 is antagonistic to *Alternaria alternata*, *Cladosporium herbarum*, *Colletotrichum graminicola*, *Diplodia zeae*, *Helminthosporijum carborum*, *Penicillium chrysogeum*, *Phythium* sp., and *Rhizoctonia solani*, *Aspergillus flavus*, and *A. parasiticus*. This isolate was subsequently found to belong to the closely related *B. subtilis*-like phenotypes that were recently described as *Bacillus mojavensis* Roberts, Nakamura, & Cohan, which can be distinguished only by differences in whole cell fatty acid composition, divergence in DNA sequences, and resistance to genetic transformation between taxa within the *B. subtilis* group. The type and other strains of this species were isolated from soil samples from the Mojave Desert in California, and other major deserts of the world, respectively.

The ease of infection of maize seedlings and the endophytic colonization by the patented strain of *B. mojavensis*, as well as the mutualistic and intransient nature of the association suggest that endophytism in this strain is a biological requirement for survival in nature. Further, it suggests that this endophytic colonizing ability might be characteristic of this newly erected species. Thus, a comparative study of the biocontrol patented strain with 13 other strains of *B. mojavensis*, including five other species within this subgroup of *B. subtilis*-related species, was undertaken. The five species included *Bacillus amyloliquefaciens*, *B. atrophaeus*, *B. licheniformis*, *Brevibacterium halotolerans*, *Paenibacillus lentimorbus*, and *P. popilliae*.

The major objective of this research was to screen other isolates of *B. mojavensis*, *B. subtilis*, and the other closely related *Bacillus* species for endophytic colonizing ability. Another objective was to screen these bacteria for *in vitro* antagonism to *F. moniliforme* in an effort to determine the distribution of this highly desirable biocontrol quality within the Bacillaceae. Antagonism was determined on nutrient agar and endophytic colonization was established with maize plants following recovery of rifampin-resistant mutants generated from all strains used in the study.

With the exception of one, all eight strains of *B. subtilis*, including the type, were non-inhibitory to the fungus. These strains included several that were used as biocontrol agents. The one strain of *B. subtilis* that was scored with moderately strong inhibition to *F. moniliforme* was ATCC55614, a patented strain for inhibition to microorganisms. The other strain that was negative for *in vitro* inhibition to *F. moniliforme* was *B. licheniformis*. The other species within the Bacillaceae varied in their *in vitro* inhibitory reaction to the fungus. The type strain for *B. amyloliquefaciens* showed the strongest inhibition even though the form of inhibition was contact, and in this regard similar to that produced by some strains of *B. mojavensis*.

The endophytic ability among the other strains of bacteria in maize varied. The one strain

of *B. licheniformis*, and both species of *Paenibacillus* were endophytic in maize but are further characterized as weakly inhibitory to non-inhibitory to *F. moniliforme*, respectively. The strains of *B. subtilis*, *B. amyloliqufaciens*, and *B. atrophaeus* were isolated from roots but not from the above ground portions of plants, indicating that these were not endophytic. Their presence in root tissues probably reflected a very transient association, and while we have no indication, were distributed very randomly within the damaged portions of roots formed during growth in culture.

The study established that all 13 strains of *B. mojavensis*, isolated from major deserts of the world, were capable of the endophytic colonization of maize, and were antagonists to *F. moniliforme*. The ability to colonize maize endophytically by *B. subtilis* and the other four species within this subgroup of the Bacillaceae varied, as well as the ability to antagonize *F. moniliforme*. Thus, this study established that endophytic colonization is another characteristic of the species *B. mojavensis*. This characteristic and antagonism to the test fungus, suggest that isolates of this species might prove to be important biocontrol organisms where the endophytic habit is desired. This study identifies a group of bacteria that can be used as powerful biocontrols for plants as well as conferring other beneficial traits. Strains of *B. mojavensis* should make excellent agents for biological control of diseases, as suggested here, or insect pests. The endophytic location, the variations in secondary metabolites (*i.e.*, fungal inhibitors), and the natural harsh desert environment reported for most isolates of this endophytic species should make it more persistent and reliable than common biocontrol agents that function in the harsh environment on the plant (usually root) exterior. Because of their intimate association with plants, *B. mojavensis* endophytes might also improve general plant health by producing beneficial compounds. Indeed, the biocontrol isolate reported here improves the growth and greening of seedlings early in their life, and reduces the accumulation of the fumonisins.

THE USE OF AN IODINE-BASED PRODUCT AS A BIO-COMPATIBLE CONTROL OF *FUSARIUM MONILIFORME*

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Control of *Fusarium moniliforme* growth was analyzed using a bio-compatible agent, Plantpro 45™ . The iodine-based active ingredient in Plantpro has been used as a disinfectant in human and animal health care products, but applications to control plant pathogens is not common. Plantpro was tested for efficacy in controlling *F. moniliforme* by assaying conidia on agar and *F. moniliforme*-infected corn kernels incubated both on agar and in perlite. *F. moniliforme* growth from conidia was inhibited with 5 ppm active ingredient at a concentration of 5.0×10^4 conidia/ml, but growth from infected corn kernels required a much higher concentration. An active ingredient concentration of 250 ppm reduced *F. moniliforme* growth by 50% from infected kernels under the treatment conditions used. Thus, laboratory studies indicate Plantpro merits investigation into commercial techniques of seed application for use as a bio-compatible control of *F. moniliforme* growth and ensuing production of the mycotoxin FB_1. Plantpro may prove to have commercial applications to control growth of *F. moniliforme* during pre-harvest to prevent seed rot in unfavorable growing seasons and during post-harvest when kernels are inadequately stored.

Fusarium moniliforme infections of corn are of agricultural concern because the fungus produces mycotoxins harmful to animal and human health. Despite a century of research on the fungus, methods do not exist to control corn infections by this fungus. Iodine is one of the naturally occurring basic elements of which all matter is composed. Iodine is classified as a halogen, which means salt-former, along with fluorine, chlorine, bromine and astatine, in the periodic chart of elements. This element is essential to human growth and metabolism and required in small amounts in the animal diet for proper functioning of the thyroid glands. Iodine-based compounds have been used as microbial control agents in human and animal health for centuries. Consequently, obtaining approval for the commercial use of a compound containing this bio-compatible element as a control agent for *F. moniliforme* might be less complex, time-consuming, and costly than other potential means. However, iodine-based compounds have not been used for fungicides.

Control of *Fusarium moniliforme* growth was analyzed for Plantpro 45™ which has an iodine-based active ingredient. Five methods were used to determine the sensitivity of *F. moniliforme* to Plantpro under conditions conducive to *F. moniliforme* growth. One method was to assay isolated *F. moniliforme* conidia and all others involved *F. moniliforme*-infected corn seed and non-infected corn seed as the control. Corn seed were immersed in Plantpro and then one group of seed incubated on agar at 25°C and another group in perlite at 15°C for the second and third methods. Other corn seed were sprayed in a commercial type apparatus and then incubated in perlite at either 25°C or 15°C for the fouth and fifth treatments, respectively.

Plantpro 45™, an agent with an iodine-based active ingredient, was tested for control of *Fusarium moniliforme* growth. Isolated conidia at a biological load of 5.0×10^4 conidia/ml failed to develop colonies following treatment with less than 5-ppm Plantpro active ingredient. However, growth from infected corn kernels required a much higher

concentration. An active ingredient concentration of 250 ppm reduced *F. moniliforme* growth by close to 75% from infected kernels.

Growth of isolated *F. moniliforme* conidia was inhibited at up to 5.0 x 10^4 conidia/ml of 5 ppm active iodine concentration. Suppression of *F. moniliforme* growth from infected corn seeds required a much higher concentration of active iodine, regardless of treatment or incubation method. A 20 min. immersion of infected seed in Plantpro at 250 ppm active iodine reduced *F. moniliforme* growth from infected seeds by 50% without causing detrimental effects on plant development. The optimum concentration for inhibition of fungal growth and optimum plant survival and development was 1000 ppm Plantpro active ingredient when treating seed in a commercial type apparatus by spraying. Thus, laboratory studies indicate Plantpro merits investigation in field applications for use as a bio-compatible control agent for *F. moniliforme* growth and ensuing production of the fumonisin mycotoxins. Plantpro may prove commercially applicable to control growth from air dispersed conidia during pre-harvest growth of the corn plant, as well as an agent to protect seeds from FB_1 accumulation during inadequate storage and/or to prevent seed rot during unfavorable growing seasons. Plantpro 45™ would be a novel, biocompatible material for agricultural use because an iodine-based compound has not been used as a means of fungal control in agrochemicals in spite of the centuries of use as a disinfectant in human and animal health care products.

Thus, laboratory studies indicate Plantpro merits investigation into commercial techniques of application for use as a biologically compatible control agent of *F. moniliforme* growth and ensuing production of the mycotoxins, fumonisins. Plantpro may prove to have commercial applications as a pre-harvest control for seed rot in unfavorable growing seasons and as a post-harvest control to prevent *F. moniliforme* growth and mycotoxin production when kernels are inadequately stored.

DETOXIFICATION OF CORN ANTIMICROBIAL COMPOUNDS AND THE SIGNIFICANCE TO *FUSARIUM* SPECIES

Anthony E. Glenn[1] and Charles W. Bacon[2]. [1]Department of Plant Pathology, University of Georgia, Athens, GA; [2]USDA, ARS, Russell Research Center, Toxicology and Mycotoxin Research Unit, Athens, GA.

Fusarium verticillioides (synonym = *F. moniliforme*; teleomorph = *Gibberella moniliformis*; mating population A of the *G. fujikuroi* species complex) is a cosmopolitan ascomyceteous fungus consistently found in association with maize throughout the world. The fungus is capable of systemic, endophytic infections, resulting in its transmission from the germinating seed to the developing plant to the newly formed seed. Marasas (2) provides a historical outline of animal toxicoses associated with *F. verticillioides*. Because of the potential effects on human and animal health, the U.S. Food and Drug Administration has issued draft industry guidance for recommended maximum levels of fumonisin B1 in corn products intended for human and animal consumption. This status increases the importance of developing procedures for the isolation and surveillance of *F. verticillioides* and its toxins in maize.

In an effort to identify host resistance factors that could help prevent or control plant infections and fumonisin production, a variety of defense mechanisms exhibited by germinating kernels have been studied. For example, young corn seedlings synthesize and store high concentrations of chemical defense compounds (~3 mg/g fresh wt. at 6 days post-emergence). The compounds are the cyclic hydroxamic acids, DIMBOA (2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3-one) and DIBOA (2,4-dihydroxy-2H-1,4-benzoxazin-3-one), with the former occurring in greater concentrations (1, 3, 6). Wheat also produces both of these compounds, while rye produces just DIBOA. The plants initially produce these as stable, biologically inactive β-glucosides. Upon plant cell disruption, the glucosides are enzymatically converted to the biologically active aglycones by β-glucosidase. Free DIMBOA and DIBOA are highly reactive and spontaneously degrade to the corresponding benzoxazolinones, MBOA (6-methoxy-2-benzoxazolinone) and BOA (2-benzoxazolinone), respectively. Many insects, fungi, and bacteria are deterred or inhibited by these compounds, resulting in increased plant resistance (3). However, the main fungal inhabitant of maize, *F. verticillioides*, detoxifies MBOA and BOA within 24 h by actively metabolizing them into HMPMA [N-(2-hydroxy-4-methoxyphenyl) malonamic acid] and HPMA [N-(2-hydroxyphenyl) malonamic acid], respectively (4, 5).

The temporal and spatial congruence between production of cyclic hydroxamic acids by maize and infection by *F. verticillioides*, a fungus which is capable of detoxifying these compounds, suggests detoxification may be important for establishing *in planta* associations. These antimicrobials may protect the plant from colonization or pathogenic attack by non-detoxifying microbes. Alternatively, the ability to detoxify these or other phytoprotectants may allow for expansion of a fungus' host range. We have addressed several issues relating to fungal detoxification of the cyclic hydroxamic acids. Our primary goals were: 1) to further characterize the biology and chemistry of antimicrobial detoxification; 2) to identify genetic loci involved in antimicrobial detoxification; and 3) to determine the impact of antimicrobial detoxification on *in planta* associations.

The initial phase of this project involved screening many *Fusarium* species for their tolerance

or sensitivity to the fungistatic antimicrobial BOA. Until this survey, only a few species had been assessed. We screened 29 *Fusarium* species encompassing 127 strains and found 11 species with varying degrees of tolerance to 1.0 mg/ml BOA. We had previously determined that potato dextrose agar (PDA) amended with 1.0 mg/ml BOA could segregate tolerant strains from sensitive strains. The most tolerant species were *F. verticillioides*, *F. cerealis*, *F. subglutinans*, and *F. graminearum*. All four of these are mycotoxigenic fungi that can infect maize. Indeed, the majority of the 11 tolerant species identified are associated with maize and/or wheat, both of which are producers of the phytoprotectants. Within *F. verticillioides* alone, 56 strains were screened. All strains but one were very tolerant of BOA. The single sensitive strain, NRRL 25059, was isolated from banana in Honduras. Banana is not known to produce cyclic hydroxamic acids so the absence of selective pressure to maintain detoxification capacities may have resulted in the evolutionary loss of detoxification. Yet other banana isolates from Thailand were very tolerant of BOA.

To confirm that the tolerance to BOA exhibited by the 11 species was due to metabolic detoxification of the antimicrobials, a rapid thin-layer chromatography (TLC) procedure was developed. The procedure involved taking agar plugs from cultures grown on PDA amended with BOA and spotting the plugs to a TLC sheet containing UV_{254} indicator. After development and visualization under UV light, transformation of the antimicrobials to non-toxic metabolites was assessed. Tolerance appeared to be the result of metabolic detoxification. Likewise, the sensitivity of strains appeared to result from the inability to metabolically transform the antimicrobials. Utilizing the differential physiological capacity for detoxification of BOA, we developed a semi-selective medium that greatly enhances isolation of tolerant fungi such as *F. verticillioides* from field samples compared to the popular PCNB medium. Enhanced isolation partly results from greater suppression of common contaminating fungi that are sensitive to BOA.

The discovery of sensitive strain NRRL 25059 has allowed us to genetically characterize the fungal detoxification process. Sexual crosses and segregation analyses indicated that two genes, *Fdb1* and *Fdb2*, are involved in the detoxification pathway. Growth and physiological phenotypes support this. Only strains that have functional alleles at both loci are tolerant and able to detoxify the plant antimicrobials. If either *Fdb1* or *Fdb2* or both has a nonfunctional allele, then the strain is sensitive and unable to detoxify the antimicrobials. Physiological complementation assays suggest that an unidentified intermediate compound is produced in the detoxification pathway. Evidence suggests that *Fdb1* is involved in transformation of BOA into this intermediate, and the intermediate then serves as substrate for *Fdb2*, resulting in production of HPMA. Data are also supportive of a branch in the detoxification pathway at the intermediate, such that if *Fdb2* is mutated, then the intermediate is transformed into yet another metabolite.

Experiments were performed to assess the impact that detoxification of these phytoprotectants has on *in planta* associations such as infection, endophytic colonization, and virulence towards corn seedlings (*i.e.* seedling blight). In terms of virulence, detoxification of the cyclic hydroxamic acids is not necessary or sufficient to cause seedling blight, even though seedlings contain relatively high concentrations of these compounds. Similarly, fungal strains that were sensitive and unable to metabolize the phytoprotectants were still able to infect and endophytically colonize corn seedlings.

Thus, the significance of cyclic hydroxamic acid detoxification by *F. verticillioides* and other fungi is still uncertain. Given the dominance of the tolerant, detoxifying phenotype in

natural populations of *F. verticillioides*, the suggestion is that some sort of selective pressure is resulting in maintenance of detoxification. This pressure may be at the ecological level of primary colonization of corn stubble. If the compounds are present at significant levels in newly derived stubble, they may suppress the growth of sensitive fungi, thus precluding them as primary colonizers. *Fusarium verticillioides* and other tolerant species could easily metabolize the compounds and fully colonize the stubble. Future experiments will focus on this and other possible hypotheses.

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RAPID DETECTION OF FUMONISINS IN MAIZE USING FLUORESCENCE POLARIZATION

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A rapid, portable, immunoassay method was developed for fumonisins in maize using the principle of fluorescence polarization (FP). FP measurements are based upon the rate of rotation of a fluorescent tracer molecule (fumonisin labeled with fluorescein) in solution. Smaller molecules rotate faster and give lower polarization values than bigger molecules. Binding of the antibody to the tracer formed an immuno-complex that rotated more slowly and gave rise to higher polarization values. Depending upon the quantity of unlabeled fumonisin in the sample, a competition between unlabeled and fluorescently-labeled fumonisin occurred for the antibody and the polarization changed accordingly. The observed polarization was thereby related to the free fumonisin concentration in samples. An advantage of FP over other forms of immunoassays is that the polarization is independent of intensity and concentration and the assays can be conducted with colored or cloudy solutions. The FP assays were also simple, requiring only the mixing of a sample extract with antibody and tracer and measurement.

Samples of maize spiked with fumonisin B_1 (FB_1) were extracted with phosphate buffered saline (PBS). Extracts were then tested using two different FP protocols in order to gauge the impact of matrix effects upon the assay response. The protocols differed both in how the samples were handled and in what procedures were used to compile the data. With the first protocol each sample was used as its own blank, before addition of the tracer. With the second protocol all of the samples were compared to a single (buffer) blank. In the former case the "raw" polarization data for standards were used to generate a curve to which samples were compared. In the latter case the raw data were normalized to fit a scale range of 0 to 1 before generation of the standard curve. Using the latter method recoveries of FB_1 from spiked maize over the range of 0.5 to 20 ppm averaged 94%. An advantage of the second method was that, by using a buffer blank, insights could be obtained into the extent to which the matrix affected the range and shape of the response curve and less sample manipulation was required. However, the data needed an additional processing step (normalization) that was not needed with the first method.

Naturally contaminated maize samples were analyzed by both FP protocols and by a widely used HPLC method. For the HPLC assay a subsample of maize sample was extracted with acetonitrile/water and the fumonisins were isolated using strong anion exchange columns. A comparison with 48 naturally contaminated samples indicated a good correlation between the HPLC method and the first FP protocol (r^2=0.85), a good correlation between the HPLC method and the second FP protocol (r^2=0.88), and an excellent correlation between the two FP protocols (r^2=0.97). As with ELISAs the FP immunoassays are susceptible to both cross-reacting components and matrix effects. However, the matrix effects could be effectively controlled by either the type of blanking that was used or normalization of the data. The two FP protocols were performed by different personnel in different laboratories and the good agreement between them suggests the FP technology may be very robust. These results, combined with the speed and ease of use of the assays suggest that this technology has substantial potential as a screening tool for fumonisins in foods.

PROGRESS IN UNDERSTANDING THE BIOSYNTHESIS AND REGULATION OF FUMONISINS IN *FUSARIUM VERTICILLIOIDES*

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Fumonisins are a family of toxic fungal metabolites produced by *Fusarium verticillioides*, a fungus frequently found in corn worldwide (1). *F. verticillioides* has been associated with stalk, root and kernel rots in corn, but is also among the most common fungi found even in symptomless corn plants, and is considered by some to be an endophyte of corn (2). Fumonisins can be found at detectable levels in most samples of corn worldwide, but the level is generally low in high quality corn. Much higher levels can be found in visibly diseased and lower quality corn. The most effective control strategy to minimize fumonisin contamination in corn would be prevention of *Fusarium* infection and fumonisin production in the field and in storage. However, because of the close association of the fungus and corn, and because most isolates of *F. verticillioides* have the potential to produce high levels of fumonisins at the present time, this is not practical. In the long term, understanding the molecular biology of fumonisin production should help in the development of practical and specific controls.

Analysis of isolates of *F. verticillioides* isolated from various geographic locations has revealed that most strains have the potential to produce high levels of fumonisins when grown on corn in the laboratory. Most isolates produce primarily FB_1 with lesser amounts of FB_2, FB_3 and FB_4. Much less common are isolates that produce no FB_1 but accumulate high levels of FB_2 and FB_4 or FB_3 and FB_4. Only a very few isolates have been isolated that do not produce any measurable fumonisins when grown on corn in the laboratory. Classical genetic analysis of these isolates has identified three closely linked loci (*fum1*, *fum2*, *fum3*) that control fumonisin production (3). The ability to produce fumonisins is controlled by a single genetic locus, *fum1*. *Fum2*, and *fum3* control the ability to produce fumonisin homologs that lack hydroxyl groups at C10 and C5 respectively. Additional details of the fumonisin biosynthetic pathway have emerged through complementation and precursor feeding studies with strains with these altered fumonisin production phenotypes. A polyketide synthase (PKS) gene, FUM5 (4), required for fumonisin biosynthesis was identified and cloned using a PCR approach with degenerate PKS primers and cDNA from a fumonisin producing culture of *F. verticillioides*. Transformation of a fumonisin non producing (*fum1*-) strain with a cosmid containing FUM5 restored fumonisin production. Transformation of FUM5 reduced fumonisin production by over 99% in *F. verticillioides*. When the *fum1*- strain was transformed with FUM5 alone, fumonisin production was restored, indicating that the *fum1* mutation lies within the FUM5 gene. At least some of the fumonisin production genes are clustered in *F. verticillioides*. Several open reading frames (ORF's) up and down stream from FUM5 have been identified. Four of these ORF's are highly correlated with fumonisin expression. BLAST analysis suggests that these ORF's code for a cytochrome P450 monooxygenase/reductase, a dehydrogenase, an amino acid fatty acid transferase and a dioxygenase (5). The potential functions of these genes in fumonisin biosynthesis are being investigated.

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POTENTIAL FOR MINIMIZATION OF FUMONISIN LEVELS IN CORN USING FUMONISIN NON PRODUCING STRAINS

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Fumonisins are a family of toxic fungal metabolites produced by *Fusarium verticillioides*, a fungus frequently found in corn worldwide (1). *F. verticillioides* has been associated with diseases such as stalk, root and kernel rots in corn, but is also among the most common fungi found even in symptomless corn plants, and is considered by some to be an endophyte of corn (2). Fumonisins can be found at detectable levels in most samples of corn worldwide, but the level is generally low in high quality corn. Much higher levels can be found in visibly diseased and lower quality corn (1). The most effective control strategy to minimize fumonisin contamination in corn would be prevention of *Fusarium* infection and fumonisin production in the field and in storage. However, because of the close association of the fungus and corn, and because most isolates of F. verticillioides have the potential to produce high levels of fumonisins, at the present time this is not practical.

To identify the role, if any, fumonisins play in interactions between the corn plant and the fungus, we have conducted a series of field studies. In the first of these studies in 1993 and 1994 we observed that although *F. verticillioides* can be isolated from most of the kernels, even in highly contaminated corn, the overwhelming majority of the fumonisins (>90%) are found in damaged, visibly infected and abnormal kernels while the visibly healthy kernels contain only traces of fumonisins (3). In studies in 1996 and 1997 we tested the ability of *F. verticillioides* strains that produce unusual fumonisin homolog profiles to infect plants and cause disease (4). Plants were infected two ways, by placing toothpicks inoculated with a strain near the seed at planting or by inoculation of emerging silks with spore suspensions in Bilays medium.

Strains placed on toothpicks near the seed at planting could be recovered in the kernels of the ear at harvest. No significant differences in disease or total fumonisin levels were observed in these ears compared to control ears. However, infecting seeds at planting with strains of *F. verticillioides* that produce primarily either FB_2 or FB_3 impacted the profile of fumonisin homologs that were present in the harvested ears. Strains isolated from ears of plants infected at planting with fumonisin non producing strains retained that phenotype when grown in culture. Silk channel inoculation with wild type, FB_2 producing, FB_3 producing, or fumonisin non producing *F. verticillioides* strains all resulted in higher disease levels in the harvested ears compared to controls. Despite high disease levels, no fumonisins were detected in ears inoculated with a fumonisin non-producing strain. These data indicate that the ability to produce fumonisins is not required to infect plants or produce ear rot.

The lack of fumonisins in ears infected with the non producing strains suggests that when these strains effectively colonized the plant they competitively excluded fumonisin producing strains or prevent them from producing fumonisins. In kernels from plants treated at planting with the fumonisin non producing strain, low levels of fumonisins (not significantly different from those in control plants) were detected in the ears. These fumonisins may have resulted from co-infection of the plants by fumonisin producing strains. Because pools of kernels from 10 ears were analyzed for fumonisins, it could not be determined whether the fumonisins that were present in the samples came from all ears

in the sample or whether the fumonisins came only from some ears where the non producing strain was completely absent.

In 1999 we conducted a two site field test to rigorously test the importance in infection and ear rot disease of FUM5, a gene required for *F. verticillioides* to produce fumonisins. Two strains of *F. verticillioides* (a wild type fumonisin producing strain, and a FB_2 producing strain) and their corresponding FUM5- knockout strains were used. For each of the four strains and a water-treated control blocks of 10 plants were each treated by one of four ways: by toothpick treatment of seed at planting, by stalk inoculation, by spraying emerging silks, and by silk channel inoculation.

Fumonisin levels in ears inoculated via silk channel with the two FUM5- knockout strains were significantly lower than those in control plants which in turn were significantly lower than those from plants inoculated with fumonisin producing wild type and FB_2 producing strains. Fumonisin levels in ears from plants inoculated by silk spray with the FUM5 knockout strains were slightly lower, on average, than from control plants but the differences were not significant. Fumonisin levels were higher in ears sprayed with the fumonisin producing strains. The same trend was observed in the ears from the stalk inoculated plants, but the variability in fumonisin levels was much greater. No differences were observed for fumonisin levels in ears from plants inoculated via the seed at planting.

Analyses of data from the second field site in 1999 (conducted by Dr. Gary Munkvold, Iowa State University) and from a field test in 2000 are underway.

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REGULATION OF FUMONISIN BIOSYNTHESIS: THE CORN KERNEL HOLDS THE KEY

Charles P. Woloshuk and Won-Bo Shim. Department of Botany and Plant Pathology, Purdue University, West Lafayette, IN.

Fumonisins are a group of mycotoxins produced predominantly in the endosperm of corn kernels by the plant pathogenic fungus *Gibberella fujikuroi*. When grown in defined medium, the fungus produces fumonisin B_1 (FB_1) as early as 18 h in medium containing 1.25 mM or 2.5 mM ammonium phosphate. FB_1 production is repressed for 75 h and 125 h when mycelia is resuspended in media containing ammonium phosphate at 10 mM or 20 mM, respectively. Although we found that total FB1 production was greater in resuspension cultures grown in higher concentrations of ammonium phosphate, the accumulation was independent of the inoculum size and carbon/nitrogen ratio. The addition of ammonium phosphate to cracked corn cultures also repressed FB_1 production by 97 %, and persisted for at least three weeks. Thus, biosynthesis of FB_1 is regulated by a mechanism involving nitrogen metabolite repression, suggesting that control strategies that target the regulatory elements of nitrogen metabolism may be effective at reducing the risk of fumonisin contamination in food.

A mutant of the *G. fujikuroi*, FT563, affected in fumonisin B_1 (FB_1) biosynthesis was generated and was found to contain a disrupted gene named FIC1 (Fumonisin Inhibited on Corn). FIC1 contains an open reading frame of 1018bp, with one intron, and encodes a putative 319-amino-acid polypeptide. This protein has high similarity to UME3/SRB11/SSN8, a cyclin C of *S. cerevisiae*, and contains conserved motifs that are required for cyclin C function. Also similar to C-type cyclins, FIC1 was constitutively expressed during growth. Data suggest that when the fungus is grown on corn kernels, FIC1 is required for conidiation and expression of FUM5, the polyketide synthase gene involved in FB_1 biosynthesis. When FT536 mutant was grown in defined culture medium at pH3, conidiation, expression of FUM5 and FB_1 production were independent of FIC1. However, FIC1 was required for conidiation and FB_1 when grown in defined culture medium at pH 6. DNA sequence analysis of 800 genes expressed in WT and FT536 supports our conclusion that FIC1 plays a critical role as a regulator of genes involved in secondary metabolism and cell differentiation in *G. fujikuroi*.

Panel Discussion: Fumonisin Contamination of Corn

Chair: Ronald D. Plattner

Panel Members: Charles W. Bacon, Anthony E. Glenn, Ida E. Yates, Chris M. Maragos, and Charles P. Woloshuk

SUMMARY OF PRESENTATIONS: This year the topic of the Aflatoxin Elimination Workshop was expanded to include a session on Fumonisin Contamination of Corn. The Food Safety threat posed by the presence of fumonisin in corn has been an emerging issue since the initial report of fumonisin as a mycotoxin by a South African research group in 1988. In the twelve years since that initial report much information has emerged about the presence and toxicity of fumonisin in corn. It has become evident that fumonisins, a family of toxic fungal metabolites produced by *Fusarium verticillioides* (perfect state: *Gibberella fujikuori*) are present in corn world-wide and are unavoidable contaminants in food for human and animal consumption. This understanding, an international conference on the toxicology of fumonisin presented by ILSI (June 28-30,1999), a FDA workshop on Fumonisin Risk Assessment (January 10-11, 2000), and industry input lead the FDA to issue in the Federal Register a Draft Guidance for Industry: Fumonisin Levels in Human Foods and Animal Feeds to "recommend maximum fumonisin levels that the FDA considers adequate to protect human and animal health and that are achievable in human and animal feeds with the use of good agricultural and good manufacturing practices".

In the past decade ARS has conducted a strong research program designed to understand the processes leading to fumonisin contamination of corn and to develop strategies to eliminate or at least minimize the presence of fumonisins in the corn crop. In this session we had seven reports, three from the ARS laboratory in Athens GA, three from the ARS laboratory in Peoria IL, and one from Purdue University describing progress in this area.

Fumonisins are produced by *Fusarium verticillioides* (often previously called *Fusarium moniliforme*) and closely related species of section *Liseola*. These fungi are long known to be present in corn. They are associated with diseases such as stalk, rot and kernel rots, but are also found in symptomless corn plants. As reported by Charles Bacon, current thinking suggests that these fungi are biotrophic mutualists or endophytes of corn which actively colonize corn tissue establishing long-term associations. As endophytes, control of them in corn will be difficult. Dr. Bacon reported on his work on biological control of this fungus using an endophytic bacterium operating under the general mechanism of competitive exclusion. This biocontrol strain, identified as *Bacillus mojavensis*, has been patented for control of diseases of maize caused by fungi.

Ida Yates reported on a potential chemical based control approach. She reported her studies aimed at control of *F. verticillioides* (*moniliforme*) growth using an iodine based product, Plantpro 45™. She reported that her laboratory studies indicated that this approach merits further investigation into commercial application as a biologically compatible control agent and that it might find application as a pre-harvest control for seed rot in unfavorable growing seasons and for post-harvest control to prevent fungal growth and mycotoxin production in inadequately stored kernels.

Anthony Glenn reported on work at Athens on identification of host resistance factors in corn plants that have potential to prevent or control plant infections. It has been known

for some time that young corn seedlings synthesize high concentrations of chemical defense compounds such as the cyclic hydroxamic acids DIMBOA (2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3-one) and DIBOA (2,4-dihydroxy-2H-1,4-benzoxazin-3-one). These compounds are initially produced in plants as stable, but biologically inactive β-glucosides. These compounds are also found at very high levels in corn silks, a common route of fungal invasion of ears. When plant cells are damaged these inactive glucosides are enzymatically converted to the biologically active aglycones which can deter or inhibit many fungi and bacteria. However, generally, *F. verticillioides* strains can quickly metabolize the active compounds. Screens of various strains revealed differences in tolerance to BOA. Strain NRRL 25059 was used to genetically characterize that the fungal detoxification process involved two genes, *Fdb1* and *Fdb2*, both of which were necessary for BOA resistance. While the significance of the ability of *F. verticillioides* to detoxify cyclic hydroxamic acids is still uncertain, the dominance of the tolerant, detoxifying phenotype in natural populations of this fungus suggests that there may be selective pressure to maintain the ability to detoxify these compounds.

A rapid immunoassay method for detection of fumonisins in maize was reported by Chris Maragos. The novel method uses the principle of fluorescence polarization. The polarization measurement is based on the rate of rotation of a fluorescent trace molecule (fumonisin labeled with fluorescein) in solution. Smaller molecules rotate faster giving a lower polarization than larger molecules. When the tracer forms an immuno-complex by binding to the antibody its rotation is slowed giving a larger polarization value. The antibody, tracer and an aliquot of a simple corn extract are mixed in a tube. Competition of the tracer and the fumonisins in the extract for binding with the antibody thus provide an indirect measure of the level of fumonisins in the sample. The measurement is made in a simple portable device with only one moving part (a sliding compartment for the sample tube). Chris reported that using a simple protocol fumonisins could be detected in maize extracts over the range of 0.5 to 20 ppm. This device promises to be a rugged instrument that can be used by relatively untrained operators to generate fumonisin levels in corn samples, something that will be needed by the industry to comply with FDA guidance on acceptable fumonisin levels.

Ron Plattner gave two reports on the Peoria group's progress in understanding the biosynthesis and regulation of fumonisins in *F. verticillioides* and field studies in which corn was infected with novel *F. verticillioides* strains. The group has identified loci that are associated with the ability to synthesize fumonisins and is characterizing several genes involved in the complex fumonisin biosynthetic pathway. Fumonisins can be found at detectable levels in most corn samples, but the level is generally very low in high quality corn. The most effective control strategy to minimize fumonisin would be to minimize the amount of *Fusarium* infection in corn. However, because of the close, possibly endophytic nature of the fungus, at the present time this is not practical. Field work has revealed that the overwhelming majority of the fumonisins present in ears of corn are found in damaged, visibly infected and abnormal kernels, while visibly healthy kernels contain only traces of fumonisins. When plants are systemically infected with strains via infected toothpicks placed near the kernels at planting, the strains were recovered in the kernels of the ear at harvest. The ears had no significant differences in disease or total fumonisin levels from control ears. However, infecting seeds at planting with strains that produce primarily either FB_2 or FB_3 resulted in harvested ears with fumonisin homolog ratios like the strains with which they were infected. By contrast, infection by inoculation of emerging silks led

to higher disease levels in the ears compared to controls. Despite high disease levels, no fumonisins were detected in ears inoculated with fumonisin-nonproducing strains. This data indicates that the ability to produce fumonisins is not required to infect plants or produce ear rot. These tests suggest that biological control of fumonisin in corn by competitive exclusion with fumonisin non-producing strains of *F. verticillioides* might be possible if practical conditions that provide a suitable level of infection but do not induce disease symptoms that lower grain quality can be developed.

Charles Woloshuk reported on work done in his laboratory suggesting that FB1 biosynthesis is regulated by a mechanism involving nitrogen metabolite repression, suggesting this as a target regulatory element that may be effective at reducing the risk of fumonisin contamination in food. His laboraory identifed a gene in *G. fujikuroi* that inhibited fumonisin production. The gene FIC1 has high similarity to a cyclin C of *S. cerevisiae*. This gene was required for conidiation and production of FB1 in defined culture medium at pH 6 and supports their conclusion that FIC1 plays a critical role as a regulator of genes involved in secondary metabolism and cell differentiation in this fungus.

Summary of Panel Discussion: The panel took questions from the audience and each other and discussed the status of the effort to provide industry with workable protocols to protect the corn crop from fumonisin contamination. It is widely agreed that fumonisin contamination of corn is primarily a preharvest problem. Much promising research is being conducted to understand how fumonisins are synthesized and regulated by the fungus. A more complete understanding of this process may lead to the identification of effective targets for intervention in toxin synthesis in the field. These approaches will likely be based on biotechnology but viable chemical control strategies may also become apparent. The endophytic nature of *F. verticillioides* and its close association with corn will make elimination of fungus from the crop problematic. There is potential for effective biological control but more work will need to be done to demonstrate the safety and effectiveness of approaches. At the present time the best approach that can be taken to minimize fumonisin contamination is surveillance and care in selection of the highest quality corn possible for food uses. Inexpensive surveillance protocols will be helpful.

TRACKING *FUSARIUM VERTICILLIOIDES* INFECTION AND FUMONISIN ACCUMULATION IN NORTH CAROLINA CORN

B. J. Bush, M. L. Carson, M. A. Cubeta, W. M. Hagler, and G. A. Payne. Depts. of Plant Pathology and Poultry Science. North Carolina State University, Raleigh, NC.

Naturally infected corn kernels were harvested from two locations in North Carolina at weekly intervals during the growing season to determine the percent of kernels infected with *Fusarium verticillioides* and fumonisin content of the grain. One hybrid of intermediate resistance was used in 1999, while three hybrids ranging from more resistant to intermediate to more susceptible were planted in 2000. Kernels from the harvested ears were plated on a defined media and number of infected kernels was assessed. While the data is not completed for the 2000 season, several similarities exist in the data, including time of initial infection and timing of rapid increase in number of kernels infected. Fumonisin data from 1999 closely follows infection data and the same is expected for 2000. In summary, with the percent of infected kernels low early in the season, we hypothesize that early harvest of the grain would reduce fumonisin contamination.

EVALUATION OF INOCULATION METHODS FOR FUSARIUM EAR ROT OF CORN

Michael J. Clements[1], Donald G. White[1], Chris M. Maragos[2]. [1]Dept. of Crop Sciences, University of Illinois, Urbana, IL; [2]USDA-ARS Mycotoxin Research Unit, Peoria, IL.

Fusarium ear rot of corn caused by *F. verticillioides* (Sacc.) Nirenb. (Syn = *F. moniliforme* Sheldon), and *F. proliferatum* is of concern due to production of fumonisin B_1. Fumonisin B_1 is a mycotoxin that has been associated with clinical toxicoses of animals and humans. Four inoculation methods designed to screen for resistance to both ear rot and fumonisin production were evaluated on 14 commercial hybrids at Urbana, IL in 1999. The methods evaluated were (1) injection of a spore suspension into the side of the ear; (2) spray silks with a spore suspension at mid-silk and cover the ear with a shoot bag; (3) spray silks at mid-silk, cover the ear with a shoot bag, and spray silks a week thereafter; and (4) insertion of Fusarium colonized toothpicks into the silk channel. Visual ratings of ear rot severity ranged from 7 to 61% of the total ear rotted. Fumonisin levels ranged from none detected to 47ppm as determined by enzyme-linked immunosorbent assay (ELISA). Only method 1 was significantly different ($P<.01$) from an uninoculated control. This method appears to be useful for screening of resistant germplasm.

SESSION 2: CROP MANAGEMENT AND HANDLING, INSECT CONTROL AND FUNGAL RELATIONSHIPS

Moderator: **David Ramos**
Walnut Marketing Board

INSECT MANAGEMENT FOR REDUCTION OF MYCOTOXINS IN MIDWEST CORN - FY 2000 RESULTS

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Insect damage is frequently associated with high levels of mycotoxins in corn. Two objectives are currently being pursued oriented towards insect management for reduction of mycotoxins.

I. Plant Resistance

Field and lab studies in 2000 on insect resistance of corn inbred Tex6 (resistant to *Aspergillus flavus* and aflatoxin production) relative to B73 yielded results similar to those obtained in 1999. Only 8.8% of small (V3-V4) Tex6 plants were damaged (primarily by flea beetles) in field studies compared to 35.9% of B73 plants. Although incidence of milk stage kernel damage by inoculated corn earworm larvae was about the same for Tex6 and B73, only about 6 kernels per ear of Tex 6 were damaged compared to about 26 kernels per ear of B73, despite shorter silk channels of Tex 6 compared to B73, and similar lengths of ears with unfilled kernels. Silks removed from Tex6 ears killed 43.6% of corn earworms compared to 5.6% killed by feeding on B73 silks, but there was no difference in the effects of the two types of silks against fall armyworms this year. Protein extracts from Tex6 and B73 silks had similar activity against caterpillar species tested, but Tex6 silk extracts contained secondary chemicals that were highly active compared to B73 silk extracts. Chemical analysis of silks also indicated marked differences in secondary chemistry between B73 and Tex6 silks. The more active compound(s) appear to be uncommon in corn silk based on masses obtained using soft ion LC-MS at this point and searches of compound libraries.

Tobacco plants derived from two separate transformants designed to express an active maize ribosomal inactivating protein showed significantly enhanced resistance to feeding by corn earworms and cigarette beetle larvae, but not tobacco hornworm larvae (although a similar trend was observed). Leaves from younger plants reduced corn earworm feeding by about two fold and cigarette beetle larval feeding by about 1.5 fold compared to leaves from wild type plants. Feeding rates were significantly inversely correlated with RIP levels (which approached 100 ppm in some cases) in both constructs for corn earworms and for one construct for cigarette beetle larvae.

Transgenic tobacco overexpressing tobacco anionic peroxidase overall had fewer aphids per leaf or plant but increased levels of insect predators compared to wild type plants in field tests. At some sample intervals, 51.2% of wild type plants had more than 100 aphids per leaf compared to 12.5% of transgenic plants, while 56.2% of the transgenic plants had insect predators compared to 23.3% of wild type plants. Strategies were developed to match corn peroxidase clones with isozymes associated with disease resistance.

Genes potentially involved in insect resistance are being selected for transgenic testing. Studies with a Helios portable biolistic system indicated reasonable transient expression of a GUS reporter gene in leaves with minimized tissue damage when 1 mm particles were delivered at 2000 psi.

II. Field Management

It now appears monitoring of sap beetles in sweet corn will be the most likely way to gain grower interest in sap beetle monitoring related to mycotoxin management programs. Monitoring studies in sweet corn indicated traps and attractants developed for sap beetles were a useful alternative to scouting for the third year in a row.

Multiple organizations were involved in the first year of the comprehensive field mycotoxin management plan being evaluated in Central Illinois. ARS coordinated the program and provided corn and insect sampling and a predictive computer program, University Extension assisted growers in running the program and provided advisory bulletins as needed, a seed company analyzed leaf/husk samples for the presence of target fungi, a farm service organization and a vegetable company provided weather and insect sampling data, and a local grower organization assisted in locating cooperating growers for representative intensive field sampling. Bt corn is an important component of the program, but grower participation was limited due to lack of acceptability by end users that the growers normally sold their crop to. Insect damage was limited in milk stage, as was also predicted by insect trapping. The predictive computer program also indicated low probability of *Aspergillus* and moderate probability of *Fusarium* at silking (which was supported by tissue assays and milk stage ear sampling). The predictive computer program indicated no aflatoxin should occur, and fumonisin levels of 1-2 ppm may occur. Thus, despite high occurrence of the fungus in husks at milk stage (high rainfall occurred during silking), it was correctly decided that no additional control practices needed to be initiated and no early harvest needed to be done (with a target of < 2 ppm fumonisin and < 20 ppb of aflatoxin in mind). Harvest sampling indicated relatively low occurrence of symptomatic molded kernels (again more common in non or low Bt corn compared to high Bt corn planted in the same fields), which were associated with insect damage except for a few ears, and in most cases mean fumonisin levels ranged from around 1 to 1.5 ppm (exceptions were lower and most came from a hybrid not previously examined). Obtaining timely weather data was somewhat of a problem, but appears readily solvable.

Plans for 2001:

1. Continue investigating how insect resistance mechanisms work, useful combinations, and discover and evaluate new insect resistance mechanisms.

2. Continue to evaluate and refine the comprehensive field-based mycotoxin management plan in central Illinois.

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Dowd, P.F., Pingel, R.L., Rhul, D., Shasha, B.S., Behle, R.W., Penland, D.R., McGuire M.R. and Faron, E.J. II. 2000. Multiacreage evaluation of aerially applied adherent malathion granules for selective insect control and indirect reduction of mycotoxigenic fungi in speciality corn. J.Econ. Entomol. 93:1424-1428.

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Vega, F.E., Mercadier, G., Dowd, P.F. 1999. Fungi associated with the coffee berry borer *Hypothenemus hampei* (Ferrari)(Coleoptera: Scolytidae). Proceedings of the 18th International Scientific Colloquium on Coffee, Helsinki Finland, August 2-6. 1999. Association Scientifique International du Cafe. p. 229-237.

Whyte, A.C., Joshi, B.K., Gloer, J.B., Wicklow, D.T. and Dowd, P.F. 2000. New cyclic peptide and bisindolyl benzoid metabolites from the sclerotia of *Aspergillus sclerotiorum*. J. Nat. Prod. 1006-1009.

AFLATOXIN CONTROL IN PISTACHIOS: AGRONOMIC PRACTICES AND BIOCONTROL

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In order to determine the effect of delaying harvest on aflatoxin contamination, nut samples were collected on five dates between 14 September and 12 October 1999 in a commercial orchard. Over this four week period, the incidence of decay by *Aspergillus* species increased from 0.3 to 2.3% and the amount of general fungal decay of the kernel almost tripled. Navel orangeworm infestation (an important factor for aflatoxin contamination) increased from 1.8 to 14.2% during this period. However, the percentage of desirable nuts (hull intact and separated from a split shell) did not increase between 20 September and 12 October. So delaying harvest beyond 20 September would not increase the number of desirable nuts but would substantially increase the number of poor quality nuts with decayed or insect-damaged kernels.

The study on the effect of horticultural mineral oil (which is widely used to induce pistachio trees to break dormancy early) on the formation of early split nuts (the nuts most likely to be aflatoxin-contaminated) was completed. In 2000, a single application of Volck oil sprayed during January resulted in more early split nuts at harvest (4.5%) than spraying in March (2.6%). Although treatments differed significantly in their effect on early split formation in three out of the four years of this study, no specific treatment was consistently different than any other treatment.

The type of rootstock used affected the incidence of early split nuts in all four rootstock trials in 2000. In one trial in Fresno County, trees on the rootstocks *Pistacia atlantica* (4.7% of the nuts were early split nuts), PGI (4.0%), and PGII (4.0%) had a higher incidence of early split nuts at harvest than trees on UCB-1 (2.1%), whereas in another trial in Fresno County trees on PGII (1.8%) and *P. atlantica* (0.8%) had a higher incidence than trees on PGI (0.1%) and UCB-1 (0.1%). In the trial in Kern County, trees on PGII rootstock (0.9%) had a higher incidence of early split nuts than trees on *P. atlantica* (0.4%), PGI (0.3%) or UCB-1 (0.1%). And, in the trial in Madera County, trees on *P. atlantica* (16.6%) had a higher incidence of early split nuts than trees on PGII rootstock (10.3%), PGI (9.3%), and UCB-1 (7.2%). For all four trials, trees on *P. atlantica* had more early split nuts than trees on UCB-1.

Studies were initiated in 2000 on the effect of crop load on early split formation. In one study, the yield and incidence of early split nuts were determined for individual trees on four different rootstocks. In this study, there was no consistent relationship between tree yield and incidence of early split nuts. In another study, the total number of nuts, early split nuts, and leaves were counted on individual branches. The incidence of early split nuts was not related to total number of nuts, the total number of leaves, or the nut:leaf ratio for the branch. Crop load seems to have no or little effect on early split formation.

The effect of removing nuts left after harvest on navel orangeworm (NOW) infestation (a major factor in aflatoxin contamination of pistachio nuts) was investigated in two commercial orchards. In one orchard during winter pruning, clusters of nuts were removed

(a grower practice to reduce *Botryosphaeria* blight), while in the other orchard nuts were removed by poling (similar to the practice done in almond orchards). In the pruning experiment, the pruned trees had approximately the same incidence of NOW (26.8% of the early split nuts were infested) at harvest as the unpruned trees (27.6%). In the poling experiment, the poled trees (nuts removed during winter) had approximately the same incidence of NOW infestation (44.7%) at harvest as the trees that were not poled (46.9%). However, early split nuts on the poled trees had fewer pupae (1.3%) than those on trees that were not poled (2.6%), suggesting that the NOW infested the trees that were not poled before the poled trees. Nut samples have been collected from similar experiments in 2000 and are being evaluated.

In 2000 we initiated research on the biocontrol of aflatoxin-producing fungi using an atoxigenic strain of *A. flavus*. We have obtained 229 isolates of *A. flavus* strain L from California pistachio orchards. In a preliminary evaluation, 113 isolates did not produce aflatoxins. We are currently evaluating these isolates for production of cyclopiazonic acid.

Doster, M.A., Michailides, T.J., and Goldhamer, D.A. 2001. Insufficient irrigation during spring increases early hull splitting of pistachio nuts. California Agriculture 55: (In press).

Doster, M.A., Michailides, T.J., and Beede, R.H. 2000. Effect of rootstock on early hull splitting. Page 92 in California Pistachio Industry Annual Report, Crop Year 1999-2000. Fresno. [Full report: Pages 110-114 in California Pistachio Commission. 1999 Production Res. Rep. Fresno]

Beede, RH., Ferguson, L., Padilla, J., Gomes, N., Reyes, H.C., and Doster, M.A. 2000. Effect of rootstock and treatment date on the response of pistachio to dormant applied horticultural mineral oil. Pages 90-91 in California Pistachio Industry Annual Report, Crop Year 1999-2000. Fresno.

AFLATOXIN CONTROL IN FIGS: AGRONOMIC PRACTICES, ECOLOGICAL RELATIONSHIPS, AND BIOCONTROL

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The study on the effect of burying drip irrigation on fungal decay of Conadria figs was completed. The trees with buried drip irrigation did not have significantly fewer propagules of *Aspergillus* sect. *Flavi* in the soil or on the leaves than trees with surface drip irrigation. No decay of figs by fungi in section *Flavi* was found in the figs from the trees irrigated by buried drip, although this lack of decay was not statistically different than the incidence of decay by section *Flavi* in figs from surface-irrigated trees (0.003%). Furthermore, figs from trees irrigated by buried drip had significantly less decay by *Aspergillus* species (0.9%) and general fungal decay (8.3%) than the figs from the surface-irrigated trees (2.2 and 12.5%, respectively). The difference in fungal decay between the treatments was mainly due to the larger percentage of decay on the external fig surface for the surface-irrigated trees, because figs would land on areas of the soil wetted by the surface drip irrigation.

The relationships between various factors (such as densities of propagules in the soil or on leaves) on 27 August and the incidence of fungal decay of figs by aflatoxin-producing fungi at harvest (17 September) were determined by regression analysis on the means for ten commercial Calimyrna orchards. As the density of *Aspergillus* sect. *Flavi* in the soil increased, the incidence of decay by *Aspergillus* sect. *Flavi* also increased ($y=0.017x+0.008$; $r^2=0.419$). In addition, as the density of *Aspergillus* sect. *Flavi* on the leaves increased, the incidence of decay by *Aspergillus* sect. *Flavi* increased ($y=14.30x-0.04$; $r^2=0.640$). However, the amount of dust on the leaves was not as good for predicting the incidence of fruit decay by *Aspergillus* sect. *Flavi* ($y=-1.82x+0.83$; $r^2=0.271$). The level of aflatoxin contamination ranged from none to 5.9 mg/kg (depending on orchard) and was related to the incidence of fruit decay by *Aspergillus* sect. *Flavi*. The levels of *Aspergillus* sect. *Flavi* in the soil or on the leaves could be used as a rough guide to the severity of fruit decay by aflatoxin-producing fungi.

New fig selections have been developed by a breeding program that aimed at developing a Calimyrna-type fig that had the advantages of Calimyrna figs but not the disadvantages (high levels of decay, insect infestation, and aflatoxin contamination). The fruit harvested in 1999 from new fig selections in three commercial orchards had significantly smaller eyes (1.2-2.2 mm diameter, depending on orchard) than Calimyrna fruit from three orchards (2.9-3.9 mm) but about the same as Conadria fruit from one orchard (1.8 mm). These fruits of the new fig selections also had lower incidence of decay by *Aspergillus* sect. *Flavi* (0.00%) than Calimyrna fruit (0.00 to 0.95%, depending on orchard). In a research orchard, all 23 new selections had substantially smaller eye diameter of ripe fruit (0.0 to 3.6 mm, depending on selection) than Calimyrna (5.9 mm).

Fig shoots of ten new selections and Calimyrna were sprayed with a spore suspension of *A. flavus* in a research orchard. After inoculation, all except one of the new selections had lower incidences of decay caused by *A. flavus* (means ranged from 8.8 to 42.9%) than Calimyrna (70.4%). For noninoculated figs in the research orchard, as the eye diameter of the new fig selections decreased, the incidence of general fungal decay also decreased

(y=12.9x+19.4; r^2=0.291). These results support the idea that figs with small eyes will have less decay and less aflatoxin contamination than figs with large eyes, such as Calimyrna. These new fig selections with smaller eyes than Calimyrna show promise to substantially decrease aflatoxin contamination of figs.

In 2000 we initiated research on the biocontrol of aflatoxin-producing fungi using an atoxigenic strain of *A. flavus*. We have obtained 410 isolates of *A. flavus* strain L from California fig orchards. After preliminary evaluations for aflatoxin and cyclopiazonic acid production, 10 isolates have been selected for further evaluations. We are currently evaluating these 10 isolates for their vegetative compatibility group.

Doster, M., Michailides, T., Goldhamer, D., Doyle, J., and Morgan, D. 2000. Fungal Decay of Figs: Effects of Buried Drip Irrigation and New Cultivars on Fruit Decay and Prediction of Smut in Calimyrna Figs. Proceedings California Fig Institute Research, Crop Year 1999. Fresno. 15 pp.

ECONOMIC INPUT FOR AN 'EXPERT MANAGEMENT SYSTEM' TO MINIMIZE RISK OF AFLATOXIN CONTAMINATION OF MAIZE

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All aspects of a Corn Aflatoxin Management System (CAMS) have been completed except the economic inputs that will maximize profits to the producer while at the same time reduce or minimize the risk of aflatoxin contamination of his crop. The system has two separate but related economic components. The first is a Preplant Phase consisting of an itemized budget for the crop that projects production costs. The second is a Growing Season Phase that monitors actual costs as they occur so they can be reconciled with projections of assessed expected revenue.

The primary factors that influence grain contamination by aflatoxin in the Preplant Phase are:

1. Evaluation of irrigated vs. dryland production.
2. Rotation history of the production field.
3. Knowledge of soil characteristics.
4. Selection of hybrid to be grown.
5. Planting date options.
6. Long-term weather outlook.

The primary factors that influence grain contamination by aflatoxin during the Growing Season Phase are:

1. Frequency and amount of rainfall.
2. The build-up of insect populations.
3. Temporary or long-term development of drought conditions.
4. Disease and miscellaneous weather factors.
5. Timing of input application as influenced by weather or price changes of inputs and product.

The producer has limited control of fixed costs for equipment, depreciation, interest on long-term loans, taxes, insurance, overhead and management. Other costs are highly variable from year to year, and often also under limited control. These include: seed, fertilizer, herbicides, insecticides, repairs, fuel, crop insurance, irrigation, labor, drying and interest on short-term loans. All costs are monitored and totaled as they are determined or incurred, and assessed against anticipated revenue for assistance in variable input decisions. This process is accomplished by use of a spreadsheet listing all operational costs that can in turn be compared with anticipated revenues determined for any time frame during development of the crop. Initial testing of the software will be conducted during the 2001 growing season. The system should be ready for use by several growers beginning in early spring.

DETECTION OF WHOLE CORN KERNELS CONTAMINATED WITH AFLATOXIN BY NEAR INFRARED SPECTROSCOPY

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Pioneer 3394 corn kernels were harvested in 1998 from ears that were wound-inoculated with *Aspergillus flavus* NRRL A-27837 in the late milk to early dough stage of kernel maturity at the University of Illinois River Valley Sand Farm, Kilbourne, IL. Shortly after harvest on October 19, 1998, the corn kernels were examined under a black light and separated into the following categories based on their BGYF characteristics: (1) intact kernels with BGYF over most of the kernel, (2) intact kernels with BGYF limited to the germ region, (3) intact kernels with BGYF limited to the lower germ/tip cap, and (4) intact non-BGYF kernels. Friable kernels and fragments were not included in this study as they are usually removed by existing cleaning equipment at grain elevators. A total of 500 kernels were used for this study, 50 each from the above four categories, and 300 randomly selected kernels from the same lot without examination under a black light. Transmittance spectra (500 to 950 nm) and reflectance spectra (550-1700 nm) were obtained for each of these kernels. Afterwards, each kernel was analyzed for aflatoxin following the USDA-FGIS Aflatest affinity chromatography procedures.

Kernels were classified as aflatoxin negative if the measured aflatoxin was below 10 ppb, otherwise they were considered aflatoxin positive. Two methods were investigated for classifying kernels as aflatoxin positive or negative: discriminate analysis and partial least squares (PLS) regression. Classification features for the discriminant analysis procedure were one or two ratios of absorbance values. An exhaustive search from a database of all possible ratios of absorbance values was performed to determine the best subset of one or two ratios. Features used for the PLS regression procedure were obtained by mathematically transforming the whole spectra into independent score values.

Classification based on reflectance spectra was sensitive to the orientation of the kernel while classification using transmittance spectra was not. Discriminate analysis using one or two ratios of absorbance values from the transmittance spectra gave slightly better results than full spectra PLS analysis using either transmittance or reflectance spectra. More than 96% of kernels were correctly classified as containing either high (>100 ppb) or low (<10 ppb) levels of aflatoxin. About 50% of kernels with measured aflatoxin levels between 10 ppb and 100 ppb were classified as aflatoxin negative. These results indicate that this technology can potentially be used to automatically and rapidly detect high levels of aflatoxin in single whole corn kernels. Instrumentation that utilizes one or two ratios of absorbance values from transmittance spectra can be practically implemented in a high speed sorting device. This technology should provide the corn industry with a valuable tool for rapidly detecting and sorting aflatoxin contaminated corn.

SUBSAMPLING IN PISTACHIOS

Thomas F. Schatzki and Natsuko Toyofuku. USDA, ARS, WRRC, Albany, CA

Analysis of nut lots for aflatoxin involves the withdrawal of a random selection of a lab subsample from the lot sample (~10 kg) and for practical reasons the subsample should be ~10 g. One desires the subsample to have the same mean aflatoxin concentration as the lot sample and to have a coefficient of variation no larger than ~30 %. We show that this requirement is solely a function of the particle size in the lab sample. For pistachios one obtains maximum particle sizes of ~170 for clean lots ranging up to 370 for dirty lots.

Subsampling requires grinding to reduce particle size. Two methods are now in common use. In dry grinding the lot sample is pulse ground or ground with dry ice to produce a free flowing powder. This is the traditional method in use in the U.S. Europe has now adopted a slurry grinding technique in which the lot sample is mixed with liquid (typically water) to produce as slurry of finely ground particles. A number of operational parameters can be used: time of grinding, method of grinding, liquid type (slurry only), liquid ratio, among others. Neither method seems to have been thoroughly explored, particularly the slurrying method.

Analysis of the commutation methods can take two approaches. One can study the particle size distribution as a function of the experimental parameters and compare it with the theory presented above. Alternatively, one can take the pragmatic approach and study the statistical parameters (mean and variance) of the aflatoxin concentrations obtained. With support of the Pistachio Commission of California, we have commenced a study with both approaches.

We have a Hobart VCM (vertical cutter mixer) which is capable of grinding 10 kg of in-shell pistachios with the required dry ice. A free flowing powder is obtained. Virtually all the commutation occurs in the first 6 min of grinding. We have also purchased a Silverson DX slurry mixer (identical to what is in use in Europe). Experiments have been run with water slurries with water/nut ratios of 1/1-2/1 (the former allows little mixing, but seems to be in use in some European labs, the latter mixes much better and allows direct extraction with MeOH); with times ranging over 15-60 min (a fairly steady state is reached after 60 min), and with two different cutting heads. The analysis of particle size distributions have been done by microscopy (really a sampling, rather than distribution, method), by laser light scattering and by dry sieving (Hobart only). No aflatoxin results have been obtained so far, since virtually all of the initial work was done on lots containing little or no aflatoxin.

While our work is still tentative, for reasons discussed below, several encouraging conclusions can be drawn. 1. The slurry method produces smaller particle sizes than the grinding method. 2. The slurry method appears to be capable of producing subsamples for which most of the particles fall below 400, in good agreement with theory. 3. The required parameter settings appear not to be critical.

Our results are so far still preliminary for several reasons. 1. It has been difficult to obtain adequate quantities of high aflatoxin pistachios. This is a matter of time, mostly. 2. The interpretation of particle size distributions is problematic, particularly with respect to laser light scattering. This appears to be an instrument problem and will probably require an

instrument overhaul. In addition, an ad hoc interpretation of light scattering by flat particles (shell fragments) will be required. 3. The theoretical work, described above, needs to be generalized to account for particles of varying sizes and aflatoxin content. The road to this is quite clear and no difficulties are expected. 4. A clear comparison to the European and some U.S. methods needs to be established.

Panel Discussion: Crop Management and Handling, Insect Control and Fungal Relationships

Chair: Pat Dowd

Panel Members: Mark Doster, Themis Michailides, Tom Pearson, Tom Schatzki, and Neil Widstrom

Summary of Presentations: This session included somewhat diverse presentations involving specific insect management, composite management strategies for field use in tree nuts, figs and corn, and sampling and sorting methodology. Taken together, the presentations indicated a multitarget management strategy involving both pre- and postharvest tactics is likely to be most helpful in reducing mycotoxin problems in different commodities.

Pat Dowd (Dowd, Berhow, and White) reported past studies have indicated many common mechanisms for both insect and fungal resistance, and cross resistance can occur. Insect resistance in the *Aspergillus flavus*/aflatoxin resistant inbred Tex6 was compared to the susceptible inbred B73 in a two year lab and field study. Although there were no significant differences in kernel resistance for caterpillars and sap beetles tested in excised kernel studies, Tex6 showed both leaf and silk resistance compared to B73. Small Tex6 plants had significantly less flea beetle damage, and silks were significantly more resistant to three caterpillar species in lab and field studies.

Themis Michailides (Michailides and Doster) reported that delaying harvest substantially increased the number of decayed and insect-damaged nuts. The type of rootstock used and the application of horticultural mineral oil during winter affected the incidence of early split nuts at harvest; however, crop load had little effect. Removing nuts left after harvest had only a slight effect on navel orangeworm infestation. In 2000 research was initiated on the biocontrol of aflatoxin-producing fungi using an atoxigenic strain of *A. flavus*.

Mark Doster (Doster and Michailides) reported figs from trees irrigated by buried drip had significantly less decay than figs from surface-irrigated trees. In ten commercial Calimyrna orchards, as the density of *Aspergillus* sect. *Flavi* in the soil (or on leaves) increased, the incidence of decay also increased. Figs of new fig selections have smaller eyes and less decay by aflatoxin-producing fungi than Calimyrna figs. In 2000 research was initiated on the biocontrol of aflatoxin-producing fungi using an atoxigenic strain of *A. flavus*.

Neil Widstrom (Widstrom, Lamb and Williams) reported a Corn Aflatoxin Management System (CAMS) will maximize producer profits and reduce or minimize the risk of crop aflatoxin contamination. The two economic components being developed consist of a Preplant Phase with an itemized budget that projects production costs and a Growing Season Phase that monitors accumulating costs and reconciles them with projections of expected revenue. The system uses a spreadsheet for continuous comparison of operational cost and anticipated revenues. Software testing will begin during the 2001 season.

Tom Pearson (Pearson, Dowell, Wicklow, Xie and Magirang) reported that transmittance spectra (500-950 nm) and reflectance spectra (550-1700) were analyzed to determine if they could be used to distinguish aflatoxin contamination in single whole corn kernels. Spectra were obtained on whole corn kernels exhibiting various levels of bright greenish-yellow fluorescence. Afterwards, each kernel was analyzed for aflatoxin. More than 95% of kernels

were correctly classified as containing either high (>100 ppb) or low (<10 ppb) levels of aflatoxin.

Tom Schatzki (Schatzki and Toyofuku) reported investigating the subsampling error experienced when a sample is commutated (homogenized) before drawing a subsample for chemical analysis. A quantitative relationship was shown between the particle size after homogenization and the variance of subsample aflatoxin levels. While still rough, this relation indicates that a variance of 10% (coef. var.=30%) can be expected if the particle size < 400μ, assuming a 10g subsample and a contamination rate of 10^{-5}. Preliminary experimental results agree with these predictions.

Summary of Panel Discussion: Torbert Rocheford asked Pat Dowd if it was possible to screen around 100 lines of corn so that mapping of the Tex6 insect resistance could be done. Pat indicated it was rather labor intensive, but probably doable and he would discuss it further with Torbert and Don White.

Tom Griffith asked Themis Michailides and Mark Doster if the soil in fig or pistachio orchard was being fumigated. They indicated the soil in fig orchards is not fumigated, but the soil in pistachio orchards is treated with methyl bromide (which will eventually be removed from the market). Kanniah Rajasekaran asked Themis Michailides and Mark Doster if there were alternatives to the mineral oil that was used to bud break that maybe wouldn't cause early splitting of pistachios. They indicated that the Volk mineral oil was also used for scale control. Although some testing had been done with Dormax as a substitute for the other material for bud breaking, it also seemed to cause early split problems. Ken Damannn asked Themis Michailides and Mark Doster if the mechanism behind the mineral oil's ability to induce bud break in pistachios was known. They indicated the oil advances bud break by about a week to 10 days and that the mechanism was being studied, but no definite conclusions were available yet. Themis indicated that it may be the oil is causing the pistachio nut kernels to develop too rapidly, which would cause the splitting. Kanniah Rajasekaran then asked Themis Michailides if there was any knowledge about the graft transmissible factor that affected early split susceptibility in some varieties of pistachio. Themis indicated the reason for this was presently unknown but it is being investigated. Abhaya Dandekar asked Mark Doster if the reason Mission figs typically had low aflatoxin values was that the figs were resistant to the fungus. Mark indicated it was thought that the low aflatoxin levels were due to the very small eyes of the Mission figs, which did not allow spores to enter very readily. Abhaya Dandekar then asked Mark Doster and Themis Michailides whether there might be problems with gene recombination between toxic and atoxigenic strains of *A. flavus* being considered for biological control in fig or pistachio orchards. They indicated that the work of Peter Cotty with his atoxigenic strains indicated that the chances of recombination were very low.

Shane Cochran asked Tom Pearson about rate of false positives that occurred during the transmittance method of IR detection of corn kernels infected by *Aspergillus flavus* or *Fusarium* spp. fungi and aflatoxin or fumonisin levels when aflatoxin values were in the 10 to 100 ppb range. Tom indicated the error range was rather large, from 55% to 75%, although samples having more or less aflatoxin had error rates of only around 1%. Shane Cochran then asked if dust in the samples would have any effect on the accuracy of the rating due to interference with transmission or reflectance. Tom indicated that dust would not contribute to the error rate. Charlie Woloshuk asked Tom Pearson if kernels infected by fungi other

than *Aspergillus* or *Fusarium* would be detected in a similar manner (and thus act as false positives). Tom indicated that the transmission or reflectance curves were pretty similar for *Aspergillus* and *Fusarium*, so it would probably not be possible to distinguish between infection by mycotoxin producing fungi and other fungi. Pat Dowd asked for someone from industry to comment on the practicality of using Tom Pearson's method for sorting corn, which Tom had also requested during his presentation. Kyd Brenner indicated it was probably impractical for most of the continuous corn streams due to time constraints, but may have some use for some of the "static" batches, such as white or waxy corn. Don Wicklow commented that some high volume sorting was being done with a commercial machine (Satake) and that maybe it would be adaptable for this type of sorting. Tom indicated that the Satake machine, because it is reflectance based, would have orientation problems and not be usable, but that there is another high volume commercial machine that uses transmission detection that may be adaptable for corn. Shane Cochran asked Tom Pearson if maybe the false positives were related to chemical differences. Tom indicated it appeared to be due more to physical problems. Merle Jacobs asked Tom Pearson if the sorter may also work on nuts such as almonds or walnuts. Tom indicated that it has been tested with almonds and there is a good chance it will work with almonds and pistachios. Tom indicated that finding nuts with high levels of aflatoxin to run as test samples is a problem, and Tom Schatzki indicated this has also been a problem with peanuts. Charlie Woloshuk asked if it was practical to maybe work with 100 or so kernels/nuts at time as a batch analysis method. Tom indicated he thought that it would be more economical to inspect kernels one at a time with a small feeder. Victor Sobolev indicated that they have been doing transmission studies with peanuts, and that the oil content or quality has a major effect on transmission, and was wondering if Tom was looking at chemical factors as well. Tom indicated it was probably worth doing, although oil has absorbance peaks at 900 and 1200 nm, whereas the aflatoxin contaminated kernels absorb less in this region. The major change in the kernels appears to be a physical process, where they become more porous.

Sylvia Hua asked Neil Widstrom what the most expensive costs were for his monitoring program. Neil indicated that fertilizer was one of the most expensive inputs (which is important for high yields), but that the program would indicate at which point adding more fertilizer would no longer result in economically useful yield increases. He also indicated water was an expensive input. Sylvia Hua then asked how the management program was being evaluated (in regard to mycotoxin levels). Neil indicated that many states have laboratories that will evaluate mycotoxin levels of corn, and many grain elevators do it also. Joe Dorner asked Neil Widstrom what points the growers could do something based on what the program is telling them. Neil indicated that there are several points in the program that allow for changes at preplant, such as deciding on hybrids (one with a tight husk and nonfloury kernel would be desirable). Once the growing season is in progress, the grower can make choices on whether to use the corn as silage, for animal feed, or whether it would be good enough to be used as food grade corn. Neil indicated the program now allows growers to determine the optimum use once aflatoxin reaches a certain level of contamination, and that as levels increase, the number of options decreases.

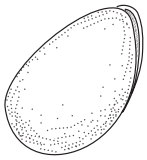
RESPONSE OF CHANNEL CATFISH TO DIETS CONTAINING AFLATOXIN CONTAMINATED MOLDY CORN

Bruce B. Manning, Meng H. Li, and Edwin H. Robinson. National Warmwater Aquaculture Center, Mississippi State University, Stoneville, Mississippi.

Aflatoxins are a group of mycotoxins produced by the mold organism *Aspergillus flavus*, which develops in feed grains and oil seeds such as corn and cottonseed that are commonly used in catfish feeds. After initial concerns about aflatoxins some 35 years ago, the United States FDA set an action level of 20 ppb total aflatoxins that can appear in foods and feeds for humans and animals. Since that initial action, modifications to that regulation have been implemented for corn and cottonseed meal designated for use in feeds for certain categories of livestock and poultry that have been shown through research to be less sensitive to aflatoxin. These regulations permit the use of corn and cottonseed meal with up to 300 ppb aflatoxin in feeds for these animals. About 10 years ago, research demonstrated that channel catfish are very tolerant to dietary aflatoxin B_1 (AFB_1). Catfish fed levels of pure AFB_1 up to 2,154 ppb had no significant reduction in weight gain. A significant reduction in growth was observed for catfish fed 10,000 ppb pure AFB_1. Incidence of hepatocellular carcinoma (HCC) was not observed in these catfish after 10 weeks of exposure to AFB_1 as has been previously reported in rainbow trout exposed to 20 ppb or less dietary AFB_1. Recently, to more thoroughly evaluate the effect of aflatoxin on channel catfish, moldy corn containing a high concentration (550 ppb) of aflatoxin was incorporated into practical diets and fed to fingerling catfish in two experiments. Experiment 1 consisted of feeding catfish (initial weight 7.1 g/fish) four diets containing 20 % or 40 % of two lots of corn; one with no apparent mold contamination, which was designated as clean corn (CC), or corn heavily contaminated with mold designated as moldy corn (MC). Each diet was fed twice daily to five 80-L aquaria of 20 fish each for 12 weeks. Body weights were determined every four weeks. At termination, hematocrits of 10 fish from each aquarium were determined. For experiment 2, three diets containing either 50 % of CC or MC, or a combination of 25 % CC and 25 % MC were prepared by extrusion-cooker processing and fed to fingerling catfish maintained in 1/10 acre ponds once daily for 130 days. At that time, a sample of 100 fish per pond was weighed; 90 fish were returned to the pond and 10 euthanized for hematocrit, liver weight, liver score determinations. Results of the experiments indicate that feeding diets containing aflatoxin from moldy corn does not affect catfish body weight gain, feed consumption, feed efficiency, survival, hematocrit, or liver weight. Liver scores indicated that there were no apparent liver abnormalities. Final levels of aflatoxin for diets used in experiment 1 were: 20 % CC, 9 ppb; 40 %CC, 18 ppb; 20 % MC, 110 ppb; and 40 % MC, 220 ppb. Levels for experiment 2 were: 50 % CC, 1 ppb; 25 % CC-25 % MC, 47 ppb; 50 % MC, 88 ppb. Diets used in experiment 1 were prepared by cold extrusion. In experiment 2, levels of aflatoxin in the mixed feeds were reduced approximately 2.5 times after exposure to the heat of extrusion-cooker processing. Extrusion-cooker processing is routinely used to manufacture commercial channel catfish feeds that float.

Session 3: Potential Use of Natural Products for Prevention of Fungal Invasion and/or Aflatoxin Biosynthesis in Crops

***Moderator*: Bob Klein**
California Pistachio Commission



EFFECT OF ALPHA-CAROTENE VOLATILE DEGRADATION PRODUCTS, D-TALOSE AND GLYCEROL ON AFLATOXIN B_1 PRODUCTION BY *A. FLAVUS*

Robert A. Norton, USDA-ARS, National Center for Agricultural Utilization Research, Mycotoxin Research Unit, Peoria, IL

Corn germ is a major site of aflatoxin B_1 formation in kernels infected by *Aspergillus flavus*. The main nutrients for the fungus in germ are the triglycerides (TGs) comprising corn oil. Oleic acid is the second most abundant fatty acid in corn oil. Factors affecting the growth and aflatoxin B_1 (AFB_1) production from TGs therefore can have a significant effect on the total toxin produced in the infected kernel. Results presented below concern two aspects of TG metabolism: the effect of glycerol, released when TGs are metabolized, on AFB_1 production from TGs or free fatty acids and the effect of sucrose, which occurs at levels of several percent in germ, on inhibition of TG metabolism by D-talose. This extends work reported last year that showed that talose was able to completely inhibit growth on TGs at submilligram concentrations. Additional results are also presented regarding work from last year that suggested the possibility of secondary inhibition.

The effect of medium containing 5% free oleic acid and either glycerol or glucose as the primary carbon source was compared. Glycerol increased net aflatoxin production from free oleic acid steadily as the concentration increased from 11 to 286 mM. AFB_1 content at this concentration was 207 mg/ml more than the level for oleic acid alone (37.6 mg/ml) and 49 mg/ml more than for 5% triolein alone. Glucose first depressed AFB_1 then increased it to a maximum over the control of 20 mg/ml and finally depressed toxin again at 286 mM. Growth was similar for both compounds over the range tested. Glycerol added at 56.7 mM to 2% glucose medium had the same effect as an equivalent amount of added glucose; when added to 5% triolein AFB_1 increased by ca. 9% (17.8 ug/ml). Therefore the effect of glycerol on oleic acid is not because it is a superior substrate by itself for aflatoxin production. However when 2% glucose was added to 5% oleic acid and 56.7 mM glycerol AFB_1 fell to 24 mg/ml, i.e. the sharp increase was abolished by sucrose. However sucrose does not inhibit AFB_1 formation from oleic acid, as it does toxin formation from triolein. When equivalent amounts of triolein (28.3 mM) and oleic acid (85 mM) were used as a carbon source triolein increased the production of AFB_1 from free oleic acid from 6 ug/ml for the free acid to 153.5 mg/ml, 85 ug/ml over the baseline level of 68.7 mg/ml (62.7 mg from 2.5% triolein plus 6 ug for the free acid). In contrast when the medium contained free glycerol at 56.7 mM and 170 mM free acid the increase was ca. 46 mg/ml (at twice the concentration of both glycerol and oleic acid used for the mixed TG/free acid experiment). Thus the bound glycerol released during TG metabolism appears to be at least twice as effective in increasing AFB_1 synthesis from free oleic acid as added glycerol.

D-talose in the range 22.4 - 1241 mg/ml was tested in medium containing 10% corn oil (CO medium), 5% sucrose (S medium) or 5% sucrose plus 10% corn oil (SO) as carbon sources. AFB_1 production on CO was 95% inhibited at 249 mg/ml talose and completely inhibited at 556 mg/ml. Growth declined sharply at the higher level as well. Inhibition of AFB_1 was similar on both S and SO media; toxin fell by 45% and 40%, respectively, at 1241 mg/ml. AFB_1 on SO was higher than on S due to the oil but toxin concentration fell in parallel on both media indicating that the TG contribution to aflatoxin was not affected by the high

level of talose. Growth was not affected on either S or SO by talose at any level. Sucrose as concentrations of 1.25%, 2.5% and 5% were tested with 10% corn oil and talose over the same range as above to determine if lower levels would allow inhibition. However no increase in inhibition of either growth or AFB_1 by talose was found at these concentrations. Conclusion: sucrose prevents the inhibition of TG metabolism exhibited when TG is the carbon source and a sucrose concentration in medium lower than 1.25% is required for this inhibition.

Secondary inhibition is defined here as inhibition in mycelium growing in medium without inhibitor that has been infected by, and has continuity with, mycelium growing from medium containing inhibitor. Last year I reported that α-carotene and delphinidin exhibited this characteristic. However that work assumed the compounds tested were not volatile. The method used two discs close enough together that the bottom disc with inhibitor could infect the top disc without inhibitor. This method was modified to determine if volatile compounds from the inhibitors could account for the inhibition found. Two discs were mounted on separate pins and separated by ca. 1.5 cm, one received medium and spores and the other medium and inhibitor. Aflatoxin production in discs exposed to α-carotene volatiles in the volatile inhibition (VI) test was about one third of discs infected by secondary infection (SI). Delphinidin produced no inhibition on SI discs in this experiment but VI discs were inhibited 39%. Therefore the effect of volatiles from the inhibitors can account for last years results. However there is another aspect of that work which is worth noting: the previous experiment removed the inhibitor-containing discs at daily intervals and the secondarily-infected discs were placed in fresh vials. Even though the source of inhibition was removed shortly after infection of the uninhibited disc significant inhibition was still observed. This suggests that if the infecting mycelium is initiated in an inhibiting environment, and the inhibitor subsequently removed, it can continue to be inhibited even if the mycelium undergoes considerable additional growth.

In summary, this year's work underscores the complex interactions which occur in the metabolism of seed nutrients by *A. flavus*, especially in the production of aflatoxin. Growth is frequently resistant to inhibition by corn metabolites but toxin production seems to be much more susceptible to reduction, including different engergy sources and their relative concentrations. This is illustrated by this year's data showing the key role the glycerol moiety of triglycerides may play in the production of aflatoxin from fatty acids and its interaction with sucrose as well as by the effect of glucose on talose inhibition of triglyceride metabolism.

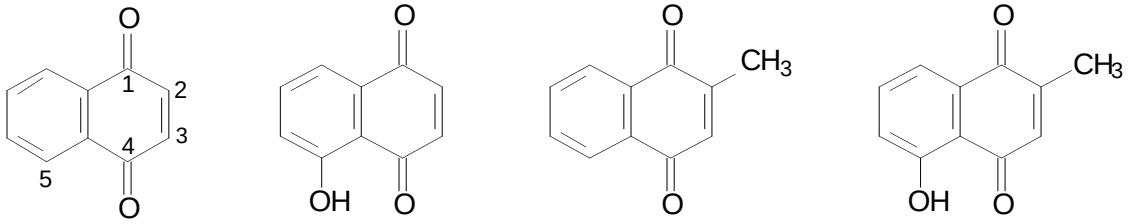
It is with deep sadness we mention that Bob passed away on November 20, 2000, shortly after presenting his talk at this year's aflatoxin workshop. Bob had been a contributor to these workshops for over a decade. He will be dearly missed.

ANTI-AFLATOXIGENIC ACTIVITY OF WALNUT CONSTITUENTS

Russell J. Molyneux[1], Noreen Mahoney[1], Bruce C. Campbell[1], Gale McGranahan[2] & Jim McKenna[2]. [1]Western Regional Research Center, ARS-USDA, Albany, California; [2]Department of Pomology, University of California, Davis, California.

The potential role of endogenous constituents of tree nuts as inhibitors of growth of *Aspergillus flavus* or aflatoxigenesis is being investigated. The objective is to ensure that aflatoxin levels are reduced below the current European Community requirement of 2ppb aflatoxin B_1, and thus to preserve the export markets for tree nuts, which currently exceeds $1 billion.

Bioactive constituents, situated in specific tissues of a tree nut, may serve a protective function dependent on both location and concentration. The primary barrier to attack by external organisms is the hull, which is known to contain a structurally related series of naphthoquinones, including 1,4-naphthoquinone **(1)**, juglone **(2)**, 2-methyl-1,4-naphthoquinone **(3)**, and plumbagin **(4)**. The effect of these compounds on *A. flavus* germination and aflatoxigenesis was investigated *in vitro*.[1] All of the quinones delayed germination of the fungus and were capable of completely inhibiting growth at higher concentrations. The most potent were 2-methyl-1,4-naphthoquinone and plumbagin, which were similar to each other in activity with germination delayed to 40 hours at a concentration of 20ppm and no growth occurring at 50ppm.



(1) **(2)** **(3)** **(4)**

The influence of these naphthoquinones on biosynthesis of aflatoxin B_1 by *A. flavus* grown on potato dextrose agar (PDA) was also investigated. After 7 days incubation, the aflatoxin produced over the same range of concentrations used in the germination inhibition study was measured. As expected, with all four of the quinones no measurable amount of the toxin was detected when fungal growth was completely inhibited. However, at levels 10ppm below that required for complete inhibition of germination, juglone, 2-methyl-1,4-naphthoquinone and plumbagin only reduced aflatoxin to 88%, 26% and 61% of controls, respectively. Moreover, at lower concentrations the latter two quinones had a stimulatory effect on aflatoxin production, which was most pronounced for 2-methyl-1,4-naphthoquinone, amounting to 347% of control at 20 ppm and 312% at 30 ppm. It may be hypothesized that the naphthoquinones have a regulatory effect on certain genes in the gene cluster responsible for aflatoxin biosynthesis and further investigation of their effect on specific genes is therefore warranted.

The kernels of tree nuts have previously been examined with respect to their ability to inhibit aflatoxin production and walnuts have been shown to be considerably more inhibitory than either almonds or pistachios. Cultivar differences were also significant, with no detectable aflatoxin being produced by 'Tulare', whereas at the other extreme 'Chico' produced 28 μg/plate of aflatoxin B_1. Additional experiments with the 'Tulare' cultivar have shown that neither growing location nor rootstock produce significant changes in aflatoxin production. Experiments with separated seed coat and kernel has demonstrated that the anti-aflatoxigenic activity is located in the seed coat. This is advantageous since the seed coat is derived from the maternal parent and can therefore be controlled in breeding experiments. Sequential extraction of the seed coat with solvents of increasing polarity has shown that the lipophilic fraction has no activity and that the highest activity is in the aqueous extract. Further research will focus on identification of the active constituents.

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Acknowledgements

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IN VITRO EFFECTS ON AFLATOXIN B1 BIOTRANSFORMATION BY NATURALLY OCCURRING COMPOUNDS

Sung-Eun Lee[1], Bruce C. Campbell[1], Russell Molyneux[1], Shin Hasegawa[1] and Hoi-Seon Lee[2]. [1]USDA, ARS, WRRC, Albany, CA; [2]Div. Biotechnology, ChonBuk National University, Chonju, Republic of Korea.

The most potent of the aflatoxins found in food, from a toxicological and carcinogenic standpoint, is aflatoxin B1 (AFB1). Toxicity of aflatoxin actually results from its conversion, or biotransformation, to toxic and carcinogenic compounds by enzymes, cytochromes P450 and reductases. The presence of cytochromes P450 and reductases vary greatly among animal species, with different groups of animals making different types of biotransformation products from AFB1. The biotransformation product that affects human health is mainly aflatoxin B1-8,9-epoxide (AFBO) and that of agricultural animals, such as cattle and poultry, includes AFBO and aflatoxicol (AFL). Our goal is to find inexpensive, safe and fairly uncomplicated approaches to inhibiting the biotransformation of AFB1 to toxic products.

We originally discovered a number of anthocyanidins in the seed coat of walnuts. These anthocyanidins are known antioxidants that inhibit biotransformation of AFB1 to AFBO. This lead us to suspect that other safe, natural compounds were available in common foods that could inhibit biotransformation of AFB1. We examined a large number of natural products from common types of food and spices. We developed bioassays using mouse liver as a model for humans in view that mouse liver contains the same family of cytochromes P450 as humans in production of AFBO. We used chicken liver as an obvious direct bioassay. Chicken liver mainly converts AFB1 into AFL, which is acutely toxic to poultry liver causing a more immediate death than AFBO-based toxicity.

Over 75 natural products were tested. A large number of compounds were discovered that inhibit the cytochrome P450 epoxidation of AFB1 to AFBO by mouse liver homogenates. Most of the active compounds fall in the class of structures known as coumarins and flavones. Some compounds were found that are active at a pharmacological level (<1 ppm). Of particular interest are the compounds angelicin, found in celery, and flavone and galangin, found in galanga root. Notably, many of the compounds, such as the flavones and coumarins, active in inhibiting the cytochrome P450 epoxidation in mouse liver did not inhibit chicken liver reduction of AFB1 to AFL. The most active compound in inhibiting biotransformation of AFB1 to AFL was curcumin, an herbal compound found in tumeric root. Curcumin had no effect in preventing epoxidation, however, by mouse liver homogenates. However, these compounds were only examined in *in vitro* tests. Further, dietary or nutritional studies are needed to truly determine if they would be active in the liver where the actual transformations occur.

Finally, while aflatoxin is not a significant problem in the United States, it remains one in a number of developing countries where a hot and humid environment combined with poor storage facilities can contribute to infection of food products by aflatoxigenic fungi. In addition, many of these countries also have a relatively high incidence of viral hepatitis, which exacerbates the damage to liver caused by aflatoxin. Some of the compounds we identified as inhibitors of biotransformation of aflatoxin are found in common food items. Increased levels of these compounds could be easily included in the diets of people in areas where aflatoxin is of a high risk. Also, a simple compound, such as curcumin, could be added to chicken, duck or turkey feed in such regions to reduce aflatoxicosis of poultry and fowl.

DEVELOPMENT OF SEMIOCHEMICAL-BASED CONTROL OF THE CODLING MOTH, A KEY PEST IN THE INFECTION OF WALNUTS BY *ASPERGILLUS* SPP.

Douglas M. Light[1], Katherine M. Reynolds[1], Ronald G. Buttery[1], Gloria Merrill[1], James Roitman[1], Bruce C. Campbell[1], Alan L. Knight[2] Clive A. Henrick[3], Dayananda Rajapaska[3], Scott Lingren[3], and Bill Lingren[3]. [1]USDA, ARS, Western Regional Research Center, Albany, CA; [2]USDA,ARS, Yakima Agricultural Research Laboratory, Wapato, WA; [3]Trécé, Inc., Salinas, CA

The chief avenue by which *Aspergillus* species invade tree nuts is through insect feeding wounds, primarily by moth larvae. Our effort to eliminate aflatoxin is to diminish and manage insect-caused nut damage using novel, species-specific control systems based on host-plant semiochemicals.

Here we report our progress toward developing semiochemical-based control systems for the walnut – codling moth – *Aspergillus* complex. Great progress has been made in developing new population monitoring and direct control techniques for the codling moth (CM), the key pest of walnuts. Over the last three years we have reported the discovery and resolution of a pear-derived volatile that has many special properties rivaling those of the CM sex pheromone. These pheromone-like properties include high potency and attractiveness, species-specificity, stability, and longevity. Fundamentally different from the CM pheromone, this kairomone strongly attracts both males and females. It is this attraction of females that provides unique and powerful opportunities to control CM populations and damaging infestations. The ability to delineate and temporally target control practices to coincide with female egg laying and subsequent larval hatch is critical to successful control tactics and diminishes needless insecticide spraying and food contamination.

ARS has joined in a CRADA with Trécé, Inc. to develop this kairomone as a monitoring lure for optimal and judicious timing of insecticide applications and as a lure for direct control techniques of mass-trapping and attracticide, or "attract and kill." Jointly ARS and Trécé have filed for a patent on use of this kairomone.

This year's research progress has established and refined the optimal active attractive ingredient, dose, and longevity of the lure. Numerous cooperators have collected data with the intent to correlate CM capture rates and flight pattern elicited by the kairomone with occurrence and degree of nut damage and mold invasion and development.

Further this year, studies were initiated to develop and use this kairomone in direct control strategies of mass-trapping and attract and kill, wherein the attractant is combined with a "soft" safer insecticide. Chemists at Trece, Inc. designed and developed a paste formulation that incorporated the attractant and soft insecticide in a synthetic matrix. This formulation matrix was demonstrated to: 1) protect the lure and toxicant from heat and UV decomposition and 2) provide longevity and effectiveness for more than six weeks. The attract and kill paste formulation when applied as globs (44 mg and 440 mg) effectively attracted both sexes of CM and readily provoked moths to touch and contact the formulation. This contact with the formulation caused an acceptable rate of adult CM mortality and significant reduction or control of oviposition or egg deposition.

Future endeavors will pursue the development and use of the kairomone in control strategies of mass-trapping, attract and kill glob formulations, and attract and kill droplet formulations using micro-encapsulation technologies.

ROLE OF LIPID METABOLISM IN *ASPERGILLUS* DEVELOPMENT

Richard A. Wilson and Nancy P. Keller. Texas A&M University, College Station, Texas.

Aspergillus spp. grow and produce aflatoxin (AF) on lipid rich seed. The primary fatty acids found in seed are linoleic, oleic and palmitic acid. Unsaturated fatty acids (*i.e.* linoleic acid) and their derivatives are known to affect sporulation, sclerotial production, cleistothecia production and mycotoxin production in *Aspergillus* species (Calvo *et al.* 1999). The primary effect is to induce asexual sporulation in *Aspergillus* spp., possibly by mimicking the effect of endogenous sporogenic factors called *psi* factors that are derived from linoleic acid. Depending on the configuration of the lipid moeity, it can either inhibit AF production or possibly extend AF production. For example, in plants, lipoxygenase (LOX) enzymes convert linoleic acid into either 9*S*-HPODE or 13*S*-HPODE hydroxylated derivatives. Both products increase sporulation of the fungus. However, 13*S*-HPODE delays aflatoxin (AF) production, while 9*S*-HPODE increases AF gene transcription. (Burows *et al.* 1997).

Due to these observations, our lab is interested in identifying fungal and plant genes involved in fatty acid metabolism. We have identified and characterized a gene in *A. nidulans*, *odeA*, whose product converts oleic acid to linoleic acid (Calvo *et al.*, in preparation). Mutants with this gene disrupted cannot produce linoleic acid and lack the psi factors derived from this fatty acid. They also have reduced growth, reduced sporulation and altered fatty acid content compared to the wild type. When grown on seed however, they produce the same number of spores as the wild type presumably by obtaining linoleic acid from the seed. This strongly suggests that linoleic acid contributes to the overall fitness of *Aspergillus*. Currently we are working on disrupting this gene in *A. flavus* and *A. parasiticus* to study the effect on AF production *in situ*.

Our lab has also identified *LOX* genes in peanut (Burows *et al.* 2000) and corn (Wilson *et al.*, in preparation) that are responsive to *Aspergillus* infection. We propose that 9*S*-HPODE producing LOX enzymes contribute to AF contamination and that 13*S*-HPODE producing LOX enzymes could decrease the amount of AF produced in seed. We have found a 9*S*-HPODE producing corn *LOX*, *cssap 92*, that is induced by *Aspergillus* to high levels in susceptible corn lines but not in resistant corn lines. This gene may be a putative biomarker for resistance to AF contamination.

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

Chair: Deepak Bhatnagar

Panel Members: Bruce C. Campbell, Late Robert A. Norton, Russell J. Molyneux, Douglas M. Light, and Richard A. Wilson

SUMMARY OF PRESENTATIONS: The presentations in this session focused on natural products (usually of plant origin) that effect the growth, development or biosynthesis of aflatoxins by *Aspergillus* spp. The natural products examined in this session were predominantly the result of the metabolism of certain primary plant products, such as starches, sugars and fatty acids which could play a significant role in the onset of aflatoxin synthesis and/or the development processes in the fungus.

Before opening up the panel discussion, the panel chair took a few minutes to try to put this session into perspective. He summarized efforts of all researchers in eliminating preharvest aflatoxin contamination as developing three lines of defense that will work in concert. (See Figure, p. 87). Because our experience over the years has told us that there is no "magic bullet" for solving the aflatoxin problem, a number of strategies will have to be utilized to ensure a healthy crop, free of aflatoxins:

The First line of defense is one where attempts are being made to prevent the toxigenic fungus from reaching the crop. And this is being achieved by biological control (using preferably native, atoxigenic strains), and effective and targeted cultural management practices.

The Second line of defense is designed to work if the fungus does get onto the crop. Attempts are being made to prevent the fungus from growing in the seed. Strategies to achieve this include enhancing host resistance by either breeding for resistance or genetically engineering host/non-host resistance traits into the crops affected by preharvest toxin contamination.

The Third line of defense is expected to be activated if the fungus does grow into the seed. Once the fungus has inhabited the seed, it is undesirable for the fungus to make toxins in the seed (*e.g.* embryo in the case of corn). Once again, preventing the fungus from making the toxin can be achieved by enhancing host resistance, targeted at factors that inhibit toxin synthesis.

Host resistance can be achieved by identifying as many biochemical makers as can be found in either the host plant or non-host plants. These factors are the ones that affect either fungal growth or toxin production. Plant breeders have genetically mapped lines that show resistance factors. These lines can be used initially to identify the resistance traits at the molecular level.

Biochemical markers can be identified in many ways. Proteins inhibitory to fungal growth or toxin production can be identified by comparing protein profiles of resistance versus susceptible germplasm (*e.g.* work at SRRC, Robert Brown and Colleagues[1]) or by following the inhibitory trait in protein fractionation (*e.g.* Gary Payne's lab[2], SRRC[3], Charles P. Woloshuk's lab[4]). Or, one can identify metabolites of various biochemical pathways that exhibit the desired effect of toxin production/growth or fungal development ([5,6]). Ultimately

the task would be to identify the proteins responsible for generating these desired metabolites, leading to characterization of genes responsible for the production of inhibitory metabolites. And that is where this session fits in.

For example, several years ago Hampden Zeringue at SRRC identified several cotton boll metabolites that inhibited fungal growth with or without affecting toxin synthesis ([5,6]). A number of those metabolites, not only had an effect on fungal growth but also on fungal development, including inhibiting sporulation ([7,8]); other metabolites enhanced sporulation.

A number of these metabolites came from the lipid metabolism[9], particularly products of the lipoxygenase pathway, and now Nancy Keller's lab (in the talk by Richard Wilson) elaborated on the involvement of lipoxygenase (LOX) in fungal development. The earlier work from that lab suggested that linoleic acid contributes to the overall fitness of *Aspergillus.* And the more recent work from the lab involved characterization of the lipoxygenase gene from peanut and corn; genes that were found to be responsive to *Aspergillus* infection. These gene could be used as biochemical markers. It should, however, be noted that the LOX involvement in resistance may not be that simple. Jay Mellon's research entitled "Does lipoxygenase activity explain low aflatoxin accumulation in soybeans?" has shown that soybean resistance to aflatoxin accumulation in his study was more a function of seed viability and seed coat integrity than the presence of LOX. On the other hand, this may be true in soybean, but not applicable to crops such as corn and peanuts.

Moreover, Bob Norton, using his suspended disc assay, has shown in the last few years that there may be a complex interaction between lipid metabolism and sugar breakdown in seeds. His work underscores the complex interactions which occur in the metabolism of seed nutrients by *A. flavus*, especially in the production of aflatoxin. Growth of the fungus is frequently resistant to inhibition by corn metabolites but toxin production seems to much more likely be reduced, and more sensitive to different energy sources and their relative concentrations. This is illustrated by data showing the key role the glycerol moiety of triglycerides may play in the production of aflatoxin from fatty acids and its interaction with sucrose as well as by the effect of glucose on talose inhibition of triglyceride metabolism.

With the studies on the effects of lipid metabolism (Keller and Norton) and identification of sugar utilization cluster next to the aflatoxin biosynthetic pathway cluster in *Aspergillus parasiticus*[10], it may be possible to understand these complex interactions between different energy sources and their effects on toxin synthesis.

Then there are other metabolites from host crop such as walnuts which could be used in the third line of defense. The potential role of endogenous constituents of tree nuts as inhibitors of growth of *Aspergillus flavus* or aflatoxigenesis is being investigated (presented by R. Molyneux USDA/WRRC). Bioactive constituents, situated in specific tissues of a tree nut, may serve a protective function dependent on both location and concentration. The primary barrier to attack by external organisms is the hull of the walnut, which is known to contain a structurally related series of naphthoquinones, including 1, 4-naphthoquinone, juglone, 2-methyl-1, 4-naphthoquinone, and plumbagin. All of the quinones were found to delay germination of the fungus and were capable of completely inhibiting growth at higher concentrations. The most potent compounds were 2-methyl-1, 4-naphthoquinone and plumbagin, which were similar to each other in activity with germination delayed to 40 hours at a concentration of 20 ppm and no growth occurring at 50 ppm.

Additionally, metabolites have been used for the first line of defense to prevent insect vectors from carrying the fungus to the crop. The goal in that study (presented by D. M. Light, USDA, WRRC) is to develop a new technology of using host-plant volatiles (HPVs), semiochemicals that monitor or disrupt the population levels, flight pattern, mating and oviposition of female moth pests of tree nuts. This investigation is on HPVs that attract female coding moth (CM), an insect pest of English walnuts. However, codling moths prefer to attack apples and pears over English walnuts. Thus, these scientists have discovered a novel and potent, pear volatile that attracts only CM, primarily females, in a walnut orchard context. This novel HPV attractant has been used in new, effective and precise semiochemical monitoring and control systems.

And finally, if all else fails, and we still have aflatoxin in the commodities, we can build the fourth line of defense. The goal of this research (presented by B. Campbell, USDA/WRRC) is to find inexpensive, safe and fairly uncomplicated approaches to inhibiting the biotransformation of AFB1 to toxic products. These researchers originally discovered a number of anthocyanidins in the seed coat of walnuts. These anthocyanidins are known antioxidants that inhibit biotransformation of AFB1 to the epoxide, AFBO. A large number of natural products were examined from common types of food and spices, and out of these over 75 natural products were tested. A large number of compounds were discovered that inhibit the cytochrome P450 epoxidation of AFB1 to AFBO in *in vitro* assays using mouse liver. Most of the active compounds fell in the class of structures known as coumarins and flavones. The most active compound in inhibiting biotransformation of AFB1 to AFL was curcumin, an herbal compound found in tumeric root. Further, dietary or nutritional studies are needed to truly determine if these compounds would be active in the liver where the actual transformations occur.

Panel Discussion: Included here are the most significant items discussed by the panel.

The discussion was initiated by Bruce Campbell, who wanted to know if Indian curry was a good dietary item for detoxification of various toxins such as aflatoxins. Deepak Bhatnagar replied that cumin and turmeric are integral parts of curry, and are included in the mixture to provide antiseptic properties and enhance the digestibility of the food. Both Bruce and Deepak highly recommended including "Indian curry" in the diets, particularly based on Bruce's and Sung-Eun Lee's preliminary results!!!

Pat Dowd indicated that a National Academy of Sciences report on natural products suggested that consumer groups were concerned about the toxic effects of flavonoids. R. Molyneux responded by saying that natural products could be labeled as "natural toxins" based on the concentration, because it is the dose that makes the compound toxic. Therefore, selection of any natural product for use in food or feed should be based on realizing the desired effect at extremely low concentrations.

It was pointed out that seed coats of almost all the nuts have antioxidants. But, as Themis Michailides pointed out that the nut kernel is the susceptible part for toxin production. So, if the seed coat barrier is broken down, the nut would be prone to toxin contamination, even if a cultivar shows resistance because of seed coat characteristics.

S. T. Hua advised the group that her poster entitled "Inhibitory effects of lactones on aflatoxin- producing *Aspergillus flavus*" contains results of her research where she has characterized the inhibitory effects of some natural products (aroma compounds). Also, Sadiq Tuzun drew attention to his work on the effects of specific natural products (plant extracts) enumerated on his poster entitled "Use of plant extracts for pre- and post-harvest control of aflatoxin".

The late Bob Norton was asked about the mechanism of glycerol-enhanced utilization of free oleic acid as a carbon source by the fungus to produced aflatoxins; and glucose, which is otherwise a good carbon source for the fungus to produced toxins, blocks the effect of glycerol- lipid interaction. Bob did not have any explanation except to say that glucose effects cAMP - dependent signalling in cells. And that may be what is responsible for these observed effects.

Charles Woloshuk observed that R. Wilson's results demonstrated that a mutation in *odeA* gene reduced sporulation, because the fungi were not able to convert oleic acid to linoleic acid, and the *psi* factors derived from this fatty acid were lacking. However, when the fungi were grown on seed, the fungi were apparently able to utilize linoleic acid from the seed because sporulation was restored to wild-type levels. But were the *psi* factors also elevated? This correlation needed to be verified, if *psi* factors are verified to be involved in sporulation.

To all the above arguments about linoleic acid enhancing fungal growth and toxin production, Corley Holbrook reminded the audience that his field studies with peanuts demonstrated that low linoleic peanut lines had no bearing on the level of colonization of peanut shells or seeds by *A. flavus* group fungi or on preharvest aflatoxin contamination in peanut[11]. He wondered if the mechanism in the field was different from *in vitro* studies observed by the Keller lab, or a different lipoxygenase pathway existed in peanuts. To these queries, Wilson responded that they do not understand the complete mechanism, and incrementally collected information is being put together to obtain an answer.

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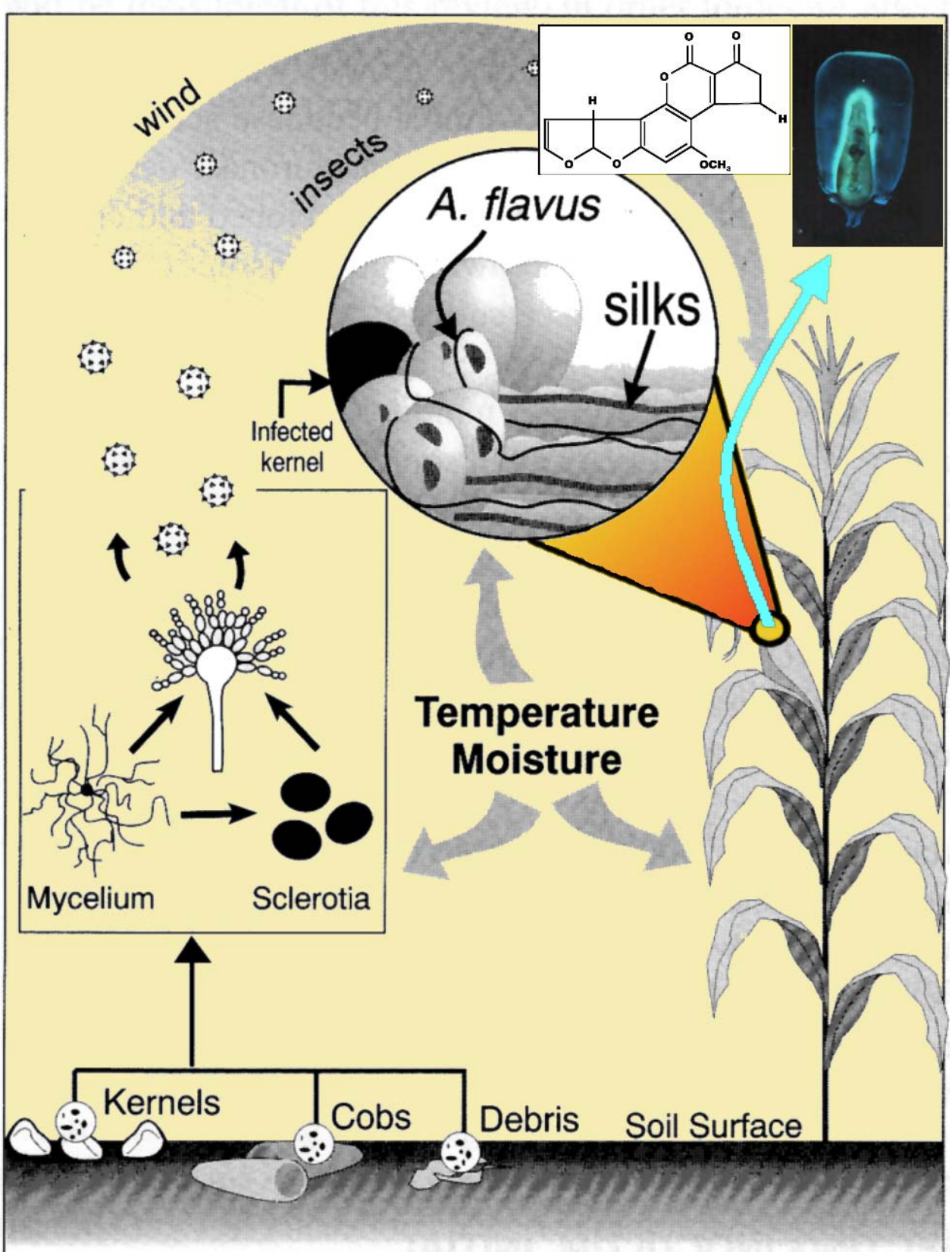

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PROTEOMICS ANALYSIS OF KERNEL EMBRYO AND ENDOSPERM PROTEINS OF CORN GENOTYPES RESISTANT OR SUSCEPTIBLE TO *ASPERGILLUS FLAVUS* INFECTION

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Aflatoxins are carcinogens produced by *Aspergillus flavus* and *A. parasiticus* during infection of susceptible crops such as corn, and cottonseed. Though resistant corn genotypes have been identified, the incorporation of resistance into commercial lines has been slow due to the lack of selectable markers. Here we report the identification of potential markers associated with resistance in corn using a proteomics approach. Embryo and endosperm proteins from two resistant and several susceptible genotypes have been compared using large format 2-D gel electrophoresis. Preliminary comparisons of reproducibly detected embryo protein spots have found both quantitative and qualitative differences between resistant and susceptible genotypes. Twelve protein spots with major qualitative or quantitative differences have been sequenced using ESI-MS/MS and Edman degradation after trypsin digestion. Based on peptide sequence homology analysis, these potential resistance markers are belonging to the following several categories: storage proteins (globulin 1 and globulin 2), late embryogenesis abundant proteins that are related to drought or desiccation (LEA3 and LEA14), water stress or osmo-stress related proteins (WSI18, aldose reductase, glyoxalase), and heat stress related protein (HSP16.9). The possible functions of these proteins in host resistance against fungal infection and drought tolerance are also discussed.

AFLATOXIN REDUCTION BY LIBERTY HERBICIDE TREATMENT OF LIBERTY LINK CORN

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Liberty (glufosinate-ammonium, AgrEvo USA, Wilmington, DE) was previously shown to inhibit *Aspergillus flavus* radial growth, aflatoxin production in liquid culture, and aflatoxin contamination in a kernel screening assay. Field studies were conducted at Baton Rouge and Winnsboro, LA, to determine the effects of herbicide rate and time of application on aflatoxin contamination. Hybrids were pinbar inoculated 20 days after mid-silk. Liberty (.096 0r .47 kg ai/ha) was applied 40 or 60 days after mid-silk to eleven Liberty Link hybrids. The mean aflatoxin concentrations across hybrids for each of the four treatments were significantly ($P < 0.05$) less than the control (51-74%) at Baton Rouge. Three of the four treatments were significantly less than the control (16-41%) at Winnsboro, a high stress environment. Results indicate Liberty at high concentration applied 40 or 60 days post mid-silk is effective in decreasing aflatoxin contamination in Liberty Link corn.

INHIBITORY EFFECTS OF LACTONES ON AFLATOXIN-PRODUCING *ASPERGILLUS FLAVUS*

Sui-Sheng T. Hua, Henry Shu and Nancy Han. USDA, ARS, Western Regional Research Center , Albany, CA

Lactones are molecules consisting of a carbon ring with an oxygen atom. They are formed by intramolecular esterification between the hydroxyl and carboxylic groups of a hydroxy fatty acids. Many species of yeast can produce γ-decalactone and δ-decalactone *via* biotransformation of fatty acids. γ-Decalactone possess a characteristic peach aroma and δ-decalactone provides a pleasant coconut scent. These two compounds are commercially available and are used as food additives to modify flavor and aroma. A study has been conducted to assess their potential for controlling *Aspergillus flavus* and aflatoxin contamination of food.

Suspensions of *A. flavus* spores were prepared to 10^5 or 10^6 or 10^7/ml. 5ml of spores were inoculated in the center of a PDA plate containing either γ-decalactone or δ-decalactone. The growth of the fungus was determined by measuring the diameter of the colony on the agar plate. The inhibitory effects of the two compounds on fungal germination and hyphae elongation were monitored by viewing with a microscope.

The *in vitro* inhibitory activity of test compounds on *A. flavus* was shown to be dependent on both inoculum concentration and time. This provides clear evidence that the effectiveness of inhibition decreases with increasing density of spore inoculum. Growth inhibition was further reduced on prolonged incubation. γ-Decalactone is more active than δ-decalactone. When the concentrations of γ-decalactone were above 400 ppm in PDA and spore inocula were applied at 10^5 spores/ml, germination and mycelial growth were completely constrained. This inhibition was sustained over a long time period. Microscopic observation indicates that both spore germination and germ tube elongation were blocked at and above 500 ppm of γ-decalactone.

γ-Decalactone has the potential to be used as a natural product for controlling *A. flavus*. The ability to produce γ-decalactone may provide one of the many criteria for the selection of yeasts as biocontrol agents.

GAS CHROMATOGRAPHY/MASS SPECTROSCOPY ANALYSIS OF THIN LAYER CHROMATOGRAPHY BANDS FROM CORN KERNEL PERICARP WAX ASSOCIATED WITH RESISTANCE TO *ASPERGILLUS FLAVUS* INFECTION/AFLATOXIN PRODUCTION

Shirley V. Gembeh, Robert L. Brown, Thomas E. Cleveland, and Casey Grimm. USDA-ARS-Southern Regional Research Center, New Orleans, LA.

Kernel pericarp wax of corn breeding population GT-MAS:gk has been associated with resistance to *Aspergillus flavus* infection and aflatoxin production. Previously, kernel wax of GT-MAS:gk was compared to kernel wax of 3 susceptible genotypes. Thin layer chromatography (TLC) of wax from these genotypes showed a band unique to GT-MAS:gk and a band that was absent in the GT-MAS:gk, but present in the three susceptible lines. GT-MAS:gk kernel wax was also shown to inhibit *A. flavus* growth, and to be at least twice as abundant as wax on susceptible kernels. The present investigation compared GT-MAS:gk wax resistance-associated traits to twelve susceptible corn genotypes. Results confirmed the unique TLC band in GT-MAS:gk kernel wax and a TLC band unique to the twelve susceptible genotypes. Wax abundance on GT-MAS:gk kernels was not associated with resistance in this study, however, GT-MAS:gk wax was the only one, among the genotypes tested, to inhibit *A. flavus* colony diameter. Gas chromatography-Mass spectroscopy (GC-MS) analysis determined a large quantity of ethyl-hexadecanoate and phenol-like compounds to be present in the GT-MAS:gk unique band, but a predominance of butyl-hexadecanoate in the "susceptible" unique band. Further investigation is needed to clarify the relationship of these identified compounds to *A. flavus* growth inhibition.

EPICUTICULAR WAX COMPOSITION AND AFLATOXIN LEVELS OF DEVELOPING CORN KERNELS OF SUSCEPTIBLE AND RESISTANT GENOTYPES

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Previous work indicated a role for pericarp wax in conferring resistance of the corn population GT-MAS:gk to aflatoxin contamination. Resistant (GT-MAS:gk, T115) and susceptible (Deltapine G4666, RX938, Pioneer 3154, and Pioneer 3136) genotypes were grown and selfed during 1999. Ears were harvested at 2, 3, 4, 5, 6, 7, 8, 9 wks post selfing. Epicuticular waxes were extracted and characterized using GC-MS. Kernels from both wax-extracted and non-wax-extracted ears of wks 3-9 were inoculated with *A. flavus* in a kernel screening assay and aflatoxin production was measured. In wks 3-5, wax extraction decreased aflatoxin production for all genotypes except T115 and Pioneer 3136. This suggests non-wax-extracted kernels had chemical component(s) that encouraged fungal growth and aflatoxin production. In contrast, the non-wax-extracted kernels of wks 7-9 of all genotypes (except wk 9 of T115) produced less aflatoxin compared to the extracted kernels. In GT-MAS:gk, RX938, Pioneer 3154, and Pioneer 3136, extracting wax from wk-8 kernels resulted in the highest concentration of aflatoxin amongst the extracted kernels of each genotype across all weeks observed. This may indicate that wk 8 is most vulnerable to *A. flavus* contamination without epicuticular wax protection. Germination tests indicated that kernels from wks 6-9 of GT-MAS:gk, T115, Deltapine G4666, and Pioneer 3154, and wks 7-9 of RX938 and Pioneer 3136 germinated. Our data supports the role of a live corn embryo in reducing aflatoxin production. The GC-MS results showed that long-chain alkanes, of 21- to 35-carbons, and sterols represent a major fraction of epicuticular waxes in all genotypes at all developmental stages. Long-chain alkanes decreased as kernels of each corn genotype mature. In contrast, sterols increased as kernels mature. No individual wax component correlated with aflatoxin production.

BIOTRANSFORMATION OF AFLATOXIN: REDUCING ITS HEPATOCARCINOGENICITY

Sung-Eun Lee, Kathleen L. Chan and Bruce Campbell, USDA, ARS, Western Regional Research Center, Albany, CA.

A large number of natural products from common types of food and spices were examined to determine their ability to inhibit the enzymatic biotransformation of aflatoxin B_1 (AFB_1) to active toxic and carcinogenic substances. Other compounds, mainly substances known as 'antioxidants', predicted to inhibit biotransformation of aflatoxin B_1, were also examined. We used the livers of mice and chickens as a source of the biotransforming cytochromes P450 and reductases. Mouse liver contains very similar types of cytochromes P450 as humans and produces the same epoxidation reaction converting aflatoxin B_1 into aflatoxin-8,9-epoxide (AFBO), a compound that can be directly toxic to the liver, but can also react with DNA leading to liver cancer. Chicken liver on the other hand, converts aflatoxin B_1 into aflatoxicol (AFL), a compound that is directly toxic to the liver causing a condition known as acute aflatoxicosis. Aflatoxicosis leads to a more immediate death, as occurred during the original 'Turkey X Disease'.

Seventy-five natural products were tested using *in vitro* assays and HPLC and LC/MS analysis of metabolites. A large number of compounds were discovered that inhibit the cytochrome P450 epoxidation of AFB_1 to AFBO in mouse liver). Most of the active compounds fall in the class of structures known as coumarins and flavones. Some compounds were found that are active at a pharmacological level (<1 ppm). Of particular interest are the compounds angelicin, found in celery, and flavone and galangin, found in citrus. Interestingly many of the compounds, such as the flavones and coumarins, active in inhibiting the cytochrome P450 epoxidation in mouse liver did not inhibit chicken liver reduction of AFB_1 to AFL. The most active compound in inhibiting biotransformation of AFB1 to AFL was curcumin, a common herbal compound found in tumeric root. Further, dietetic or nutritional studies are needed to truly determine if these compounds are active inhibitors of aflatoxin biotransformation *in vivo*.

KEYNOTE ADDRESS

Julie G. Adams
Almond Board of California

FOOD SAFETY AND INTERNATIONAL CHALLENGES

Julie G. Adams, Almond Board of California, Modesto, CA

When you think back 20 years ago, there was a low level of awareness about food safety issues and certainly no significant concerns about contaminants. With improvements in technology and better communication, we've seen increasing attention to the foods we eat and they way crops are produced. Mad cow disease, dioxin contamination, salmonella in eggs and allergy awareness have raised consumer concerns. European consumers have little or no confidence in the information being provided by government, and do not believe foods are being sufficiently regulated.

From the commercial point of view, we are dealing in bulk commodities with risks associated with differing import procedures depending on the destination and possible demurrage or re-shipment costs if goods are rejected. But regulatory authorities are having to respond to an increasing level of consumer awareness. With the expansion and speed of Internet information, consumers are being confronted with data that has not been properly reviewed before it is suddenly in front of them. Add to that continuing concerns over GMO's combined with the initiatives by consumer advocacy groups and environmental extremists, and you have perceptions and legislation shaped by sound bites.

To address consumer concerns, regulators are trying to control down the supply chain, using source-directed measures and a "Farm to Fork" approach. The difficulty is that improvements in analytical methods mean that we are trying to measure ever-decreasing amounts. The problem is that there is a belief that detection equals risk.

International food legislation is being addressed in two main areas – Codex Alimentarius and the European Union. Codex is jointly administered by the UN Food and Agricultural Organization and the World Health Organization. Its objective is to establish standards for foods moving in international trade which protect the health of consumers and facilitate international trade. Relying on advice from a joint expert committee, standards are to be based on sound scientific evidence.

At the same time these discussions are taking place in Codex, the EU has also been going through a harmonization exercise among its member governments. Through the EU Working Group of Experts, a number of contaminants are under review. The discussion and priorities are being driven largely by consumer food safety perceptions and concerns.

In looking at the international aflatoxin levels which have been established, the EU adopted the limits agreed in Codex of 15 ppb total (8 B_1). HOWEVER, these ONLY applied to peanuts destined for further processing. For other nuts, which were not part of the Codex consideration, the EU established a level of 10 ppb total (5 B_1). For consumer-ready products, all limits are at 4 ppb total (2 B_1). The difficulty is that the EU's sampling and analytical plan is extremely rigorous, and exposes the trade to considerable risk of rejections.

So where does that leave the industry?

Not all nuts are the same, but are being held to the same standard as peanuts. Also, port authorities differ in each country – in terms of the commodities they are concerned about

and how they apply the legislation. Even more difficult is the fact that customers are expecting their suppliers to meet the lower aflatoxin limits, to minimize their own exposure to on-shelf recalls. It is likely to become even more confusing, since aflatoxin has set the precedent for the way mycotoxins will be dealt with. Depending on the view of import authorities, rejections are a significant risk – maybe the goods can be reshipped to origin, but some commodities have been destroyed on the basis that they present a consumer health risk.

In the past, a rejection in one country was an isolated event. But now a Rapid Alert System has been initiated where results of import controls and rejections is circulated among all member governments. This was the procedure that resulted in the 1997 Iranian pistachio ban. Now, California did gain in that it was able to supply pistachios in Europe to replace Iranian product. HOWEVER, the ban has now been lifted and the overall market is still down 50%. Consumers do not necessarily think in terms of California or Iranian origin – they just know that pistachios were linked to cancer. Perceptions, definitely, but incredibly difficult to change. In the first 9 months of 2000, we are still seeing a high proportion of rapid alerts (and considerable focus still on nuts). Given the move toward Ochratoxin and Fumonisin concerns, it is very likely that controls on figs, raisins and cereals will increase.

So the real question is whether or not science makes the difference. In a word, no.

Media headlines focus on the fear – Food Giant to Phase out GM Ingredients; Salmonella Outbreak Linked to Fast Foods; Shell Shock; and on it goes. The article may in fact be relatively balanced, but most consumers do not get beyond the headlines.

What that really means is that science is without a doubt the foundation for addressing food safety concerns – but it is not the only basis for decisions. What is happening in the political and consumer environment will influence how much confidence there is in the food supply. As Europeans take more of a precautionary approach to food, standards are being viewed in terms of what might happen in the future. As scientists, you know there is no such thing as zero risk or absolute assurances that a health issue will not arise in the future. But those are not the messages consumers – or governments – want to hear. So the burden is on suppliers to ensure their messages are clear and concise, with data that can be supported. There also has to be a long-term commitment to getting to know the issues and the authorities involved. None of these issues are resolved quickly – aflatoxin was being addressed for more than 10 years in Codex.

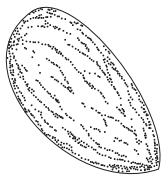
With all that said, the upcoming issues are going to continue to be in mycotoxin control. The EU has just notified the WTO of their intent to set standards for spices; discussions are also underway both in Codex and the EU to set limits for Ochratoxin. The main commodity under discussion has been cereals, but the UK has been intent on including dried vine fruit. Also under review are micro specifications for a number of foods, and the possibility of a global rapid alert system.

The fact is we need to pay close attention to what is happening in the international environment if we are going to be successful exporters. It is not enough to assume because consumers in the US accept science and research, that the same situation will exist abroad. We are dealing with perceptions for the most part, not scientific fact. But perceptions are more difficult to address – and to change.

Science has a crucial role in all of this, but has to be considered in line with other factors. The key is for continued cooperation among research and industry, to ensure that the appropriate data is being generated to demonstrate a commitment to quality and consumer safety. There are so many questions that need to be answered – how foods are produced, what are the effects of processing, whether or not contamination occurs and how it can be controlled. The challenge is to ensure consumer perceptions remain positive about the foods we produce and export. To do that, we need sufficient information and research to document procedures and demonstrate diligence at all stages of production. The ultimate objective is to build credibility for the industry, ensuring smooth commercial flows and continued confidence in safe, healthy foods.

SESSION 4: CROP RESISTANCE—GENETIC ENGINEERING

***Moderator*: Lynn Jones**
National Cottonseed Products Association



NOVEL INHIBITOR FOR CONTROLLING *ASPERGILLUS FLAVUS* GROWTH AND AFLATOXIN PRODUCTION ON CORN

C. P. Woloshuk and Ahmad Fakhoury. Botany and Plant Pathology, Purdue University, West Lafayette, IN

Our research has focused on the identification of metabolites in corn kernels that are involved in the induction of aflatoxin biosynthesis. These metabolites may very well be susceptibility factors that are inherently part of the interaction between *Aspergillus flavus* and corn. Altering the production or the availability of these metabolites through methodologies such as breeding, chemicals, bioengineering or biocontrol would ultimately lower aflatoxin contamination. An aflatoxin-inducing activity was identified in kernel extracts colonized by *A. flavus*. The inducing activity was determined to be glucose, maltose and maltotriose, produced by the extracellular α-amylase of *A. flavus.* Disruption of the α-amylase gene (*amy1*) in *A. flavus* by homologous recombination resulted in the failure to produce aflatoxin on starch and degermed kernels. These data suggest that α-amylase is involved in the colonization of the corn kernel and subsequent aflatoxin production in the endosperm tissue.

Can inhibition of α-amylase be part of a resistance mechanism in corn? Support for this mechanism is a 14-kDa protein found most abundantly in resistant corn genotypes. The protein was found to be a trypsin/α-amylase inhibitor that inhibits spore germination and fungal growth. These data support our hypothesis that inhibiting the activity of the α-amylase produced by *A. flavus* may be an effective strategy for controlling aflatoxin production.

We screened for inhibitors of *A. flavus* α-amylase in over 200 protein extracts from different plant sources. Inhibitory activity was detected in extracts from *Lablab purpureus* and *Sapindus drummondii*. A 36-kDa protein purified from the legume *L. purpureus* inhibited the activity of *A. flavus* α-amylase. The protein also inhibited spore germination and hyphal growth. α-Amylase was inhibited between pH 5 and pH 8, with optimum activity at pH 7.2. We also determined that enzyme inhibition is by competition with the starch substrate. The inhibitor was also active against the α-amylases from *A. oryzae* and *Magnaporthe grisea*, but did not inhibit the α-amylase from corn, human saliva and porcine pancreas. In contrast to the corn 14 kDa trypsin/amylase inhibitor, the *L. purpureus* protein did not inhibit trypsin. A comparison of specific activities suggests that the *L. purpureus* protein is 37 times more active than the 14 kDa inhibitor. The N-terminus amino acid sequence of the α-amylase inhibitor revealed similarity to a lectin from *Phaseolus acutifolius* and to an α-amylase inhibitor from *Phaseolus lunatus*. The inhibitor from *L. purpureus* agglutinated red blood cells, confirming its lectin nature. Introducing the gene encoding the α-amylase inhibitor into corn may enhance resistance to *A. flavus*.

TRANSGENIC COTTONS TO COMBAT PREHARVEST AFLATOXIN CONTAMINATION: AN UPDATE

Kanniah Rajasekaran[1], Jeffrey W. Cary[1], Tom J. Jacks[1], Caryl A. Chlan[2], and Thomas E. Cleveland[1]. [1]USDA-ARS, Southern Regional Research Center, New Orleans, LA and [2]University of Louisiana, Lafayette, LA.

We continue to evaluate and characterize the following three gene constructs (haloperoxidase, D4E1 and corn trypsin inhibitor) in transgenic tobacco and cotton plants for antifungal activity against the aflatoxin producing fungus, *Aspergillus flavus*. Results on the effectiveness of two of the gene constructs, *viz*. a nonheme chloroperoxidase gene from *Pseudomonas pyrrocinia* (CPO-P) and a gene encoding a synthetic peptide D4E1 have already been published (Rajasekaran *et al*. 2000, Cary *et al*. 2000). We have since demonstrated the inheritance of the antifungal/antimicrobial trait in R_1 and R_2 progenies of the original transformants. During the past year, we have made significant efforts to understand the mode of action of these two gene products *in planta*. Enzyme analyses have indicated that peroxidation by hydrogen peroxide (Jacks *et al*. 2000) of alkyl acids, including acetate, was not the basis for the antifungal activity in transgenic tobacco expressing the CPO-P. Other possibilities such as the enzyme's hydrolase or esterase activities are now being explored. The synthetic peptide, D4E1, has been shown to inhibit the growth of nearly 25 microbial pathogens, including phytopathogens belonging to four classes *viz*. Ascomycetes, Basidiomycetes, Deutromycetes, and Oomycetes. With regard to characterization of transgenic plants expressing D4E1, we are yet to surmount the technical difficulties in quantifying this small peptide of 17 amino acids. Our industry collaborators are continuing to devise a suitable method through Matrix-Assisted, Laser Desorption/Ionization Time-Of-Flight (MALDITOF) Mass Spectroscopy or developing antibodies specific to the peptide for measuring and evaluating the peptide in transgenic plants. Meanwhile, we have continued to generate transgenic cottons carrying the CPO-P or D4E1 or the corn trypsin inhibitor gene (Chen *et al*. 1999). We are also using a novel green fluorescent protein (GFP) – expressing *A. flavus* strain to evaluate the mode of invasion in transgenic and control cotton bolls and seeds (Rajasekaran *et al*. 1999; Cary *et al*. 2000). Availability of the GFP *A. flavus* strain makes it possible for us to study the ecology, etiology, toxicology and epidemiology of this saprophytic fungus, the control of which cannot be evaluated as a plant pathogen. Recently, we have also initiated several cotton transformation experiments with new constructs from our industry collaborators to overcome the freedom-to-operate (FTO) and potential proprietary issues so that commercially-viable products could be developed. An update from all these fronts will be summarized in this presentation.

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GENETIC ENGINEERING OF PEANUT FOR REDUCTION OF AFLATOXIN CONTAMINATION

Peggy Ozias-Akins[1], Hongyu Yang[1], Evelyn Roberson[1], Yoko Akasaka[1], Robert Lynch[2]. [1]Department of Horticulture and [2]USDA-ARS, University of Georgia Coastal Plain Experiment Station, Tifton, GA.

Through genetic engineering of peanut, we continue to focus on three levels of protection against aflatoxin contamination: the entry of spores through insect-damaged tissues (using a *Bt* toxin gene), the growth of the fungus after entry (using a peroxidase gene and an antifungal peptide gene), and the aflatoxin biosynthetic pathway (using a lipoxygenase gene). In addition, we have continued to explore the feasibility of using a mercury resistance gene as a selectable marker.

***Bt* toxin**. We are now in our fourth year of field testing with *Bt*-peanut to determine the stability of insect resistance in peanut lines containing a *cryIA(c)* gene and the ability of this *Bt* toxin to impact not only lesser cornstalk borer damage, but also aflatoxin contamination. One transgenic peanut line containing a *cryIA(c)* gene (74 plants) was planted along with three control lines - one which segregated from the transformed line, one which is a different hygromycin-resistant line that does not contain a *Bt* gene, and the untransformed background genotype, MARC I. The plot was inoculated with *Aspergillus* and infested with lesser cornstalk borer as well as subjected to drought stress. Harvest will take place in early November after which pods will be scored for insect damage and seed samples will be taken for aflatoxin analysis.

Eleven other *Bt* toxins in addition to *cryIA(c)* are being tested for their toxicity to lesser cornstalk borer. The assay system and concentrations for comparison with *cryIA(c)* have been determined, and the replicated tests with additional toxins will be initiated this fall.

Peroxidase and peptide D4E1. The second of our three-tiered transgene approach to reducing aflatoxin contamination is to determine if over-expression of an anionic peroxidase gene and a peptide gene can singly or in combination reduce the growth or penetration of invading fungus. Transgenic plants over-expressing tomato anionic peroxidase (*tap1*) have been recovered and analyzed at the RNA and protein levels. One plant with detectable transcript and an anionic peroxidase unique to the transgenic peanut plants has shown a tendency for reduced insect larval feeding on foliage. Leaf extract from this same plant, when incubated with germinated *Verticilium dahliae* spores, causes a reduction in spore survival and mycelial growth. A more recently regenerated *tap1* transgenic line shows peroxidase expression and is fertile. This line is segregating for the peroxidase gene and segregating progeny can be used to test the effect of peroxidase gene expression on both insect and fungal resistance.

A single line that shows stable and high levels of expression of the ubi3-D4E1 fusion gene has been propagated in the field. Efficacy tests with leaf extract and inoculation of immature seeds have not shown any indication of a reduction in fungal growth. It is possible that the D4E1 peptide is not properly cleaved from the ubiquitin protein or that it does not accumulate to sufficient levels to demonstrate fungal toxicity.

Lipoxygenase. Twenty-three out of 26 hygromycin-resistant embryogenic lines tested by PCR amplified the *lox1* gene. Expression was analyzed in somatic embryos because the

soybean gene was introduced into peanut under the control of a carrot embryo-specific promoter (DC3). Twenty-one of the 23 PCR-positive lines were tested for expression of *lox1* at the RNA level, but in only one was transcript detectable. When the transcript size from the one positive line was compared with a hybridizing transcript from soybean immature zygotic embryos, the size was considerably smaller. In addition, we have tested seeds from PCR-positive transgenic peanut plants for expression of the *lox1* gene and have found that none of those seeds containing the gene produced detectable transcript. It seems likely that this DC3 promoter is not active in peanut, and that the one line where we see a transcript that is altered in size may be due to rearrangement and insertion in the proximity of an endogenous promoter. Future work with lipoxygenase will be carried out with expression from the potato ubiquitin promoter which we have shown to confer high levels of expression in young leaves, pod walls, seed coat, immature and mature cotyledons.

Mercury resistance. A codon-modified mercuric ion reductase gene (*merA*) has been introduced into peanut under the control of an actin 2 promoter from *Arabidopsis.* Initial attempts to select for mercury resistance after bombardment with this construct were unsuccessful; however, this cassette in combination with a hygromycin resistance gene allowed selection of transgenic, hygromycin resistant lines that were resistant to mercury after regeneration of whole plants. Analysis of RNA expression of *merA* revealed that low expression occurs in embryogenic tissues and that expression is greatly elevated in leaf tissues. The *merA* gene was reengineered under the control of the ubiquitin promoter and mercury resistant, PCR-positive lines have been recovered after selection on mercuric chloride. Mercury resistance may provide a unique proprietary selectable marker system for peanut transformation.

GENETIC ENGINEERING AND BREEDING OF WALNUTS FOR CONTROL OF AFLATOXIN

Abhaya M. Dandekar[1], Gale McGranahan[1], Patrick Vail[2], Russell Molyneux[3], Noreen Mahoney[3], Charles Leslie[1], Sandie Uratsu[1] and Steven Tebbets[2]. [1]Department of Pomology, University of California, Davis, CA; [2]Horticultural Crops Research Laboratory, Fresno, CA; [3]USDA, ARS, Western Regional Research Center, Albany, CA.

The tree nut crops in California, including walnuts, represent a 1.5 billion dollar industry, a significant amount of which is exported and thus threatened by additional regulations regarding aflatoxin contamination. Our strategy for reducing aflatoxin contamination has three goals: 1)developing insect resistant plants by reducing infection opportunities through insect damaged tissues; 2) reducing the ability of tissues to support *A. flavus* growth and or toxin production and 3) identifying the component(s) in the seed coat of the walnut variety 'Tulare' that cause inhibition of aflatoxin production by *A. flavus*. The experimental strategy to achieve these three goals involves gene testing, breeding and genomics based gene discovery approaches.

For engineering resistance to insect pests we have focused on the *cry*IAc gene from *Bacillus thuringiensis*. We are continuing analysis of tissue obtained from field grown material and continuing work on the comparison of the expression of *cry*IAc using two promoters; CaMV35S and Ubi3 (from potato). In addition to leaf tissues, this year we were able to analyze some nuts early in the season. Currently we have 128 trees in the field in Davis and 111 in Fresno. The plants are doing well and are over 7 feet tall in most cases. Several of the lines flowered this year and nuts were harvested a few weeks ago. The feeding analysis revealed that both constructs, CaMV and Ubi, gave significant results compared to control untransformed walnut tissues. The *cry*IAc gene regulated by the CaMV35S promoter in transformed walnut leaves gave consistently higher mortality and delayed larval development compared to the same gene regulated by the Ubi3 promoter. Activity was higher in young leaves than in mature leaves for both promoters. In fruit (early season whole nuts), CaMV35S again produced better results than the Ubi3 promoter.

In addition to insect resistance, engineering resistance to *A. flavus* is an important objective. The strategy is not only to limit the ability of this fungus to grow in walnut tissues but to inhibit its ability to make aflatoxin. After trying antifungal genes from other sources (*i.e.*, chitin binding proteins, SAR8.2 , PGIP and RIP) we have turned our attention to some of the endogenous proteins in walnut kernels that may have antifungal activity. One of these proteins is vicillin. Vicillins are 7S oligomeric seed storage proteins utilized during germination and have been described in legumes, cotton, cacao. These proteins have antifungal activity and have been shown to interfere with germination of spores/conidia of *Fusarium solani*, *F. oxysporium*, *Phytophtora capsici*, *Neurospora crassa*, *Ustilago maydis* and *Botrytis cinerea*. The vicillins have also been shown to display anti-insect activity. For example they slow development and survival of the stored product pest *Callosobruchus maculatus*. A possible mode of action is strong binding with chitin containing structures found in fungal cell walls and midgut of insects. We have previously reported the cloning of the walnut vicillin and have made two vectors for expression *in planta*. The walnut vicillin is expressed as a precursor, so one construct expresses the full length cDNA and the other construct expresses the mature protein. Both expression cassettes are regulated by the CaMV35S promoter and were inserted into a binary vector, creating two transformation vectors

pDU99.3906 (full length) and pDU99.3841 (mature protein). Tobacco was transformed with these two vectors to evaluate inhibition of aflatoxin and antifungal activity. Transgenic tobacco lines were obtained expressing the mature construct and preliminary bioassays with *A. flavus* revealed two transgenic tobacco lines that significantly inhibited aflatoxin production as compared to control untransformed tobacco.

Our strategy for identifying the natural sources of resistance to aflatoxin synthesis in walnut kernels has two components. The first is to evaluate the genetics of the trait and the second is a gene discovery strategy to identify the genetic component. The major objective is to develop molecular markers for screening progeny and breeding selections at the seedling stage to identify those that will bear nuts with increased resistance to *A. flavus* growth and toxin production. Studies conducted at the USDA facility at Albany (Mahoney *et al*., unpublished) have shown the presence of at least two sources of natural resistance to *A. flavus* growth and toxin production in walnut kernels. The most active component appears to be present in the seed coat of the walnut variety 'Tulare' and results in the complete inhibition of toxin production. A second and more general component has an antifungal property that limits *A. flavus* growth and toxin production in walnut kernels. The following seedling populations have been generated: Chico x Chico (n=75), Tulare x Tulare (n = 76) Tulare x Chico (n = 38). In terms of aflatoxin production these correspond to: high x high, low x low and high x low. This year we were able to obtain some seed from trees after test crosses as they came into production. Analysis of the seed, should indicate whether the factor is inherited as a single gene or multiple genes. We are currently making cDNA libraries from the seed coat of kernels obtained from the walnut varieties 'Tulare' and 'Chandler'. Genomics based approaches will be used to identify genes responsible for the active component.

PROGRESS TOWARDS THE DEVELOPMENT OF COTTON WITH ENHANCED RESISTANCE TO *A. FLAVUS*

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We are interested in developing cotton that is resistant to the fungal pathogen *Aspergillus flavus* through genetic engineering. This approach relies on identification and construction of promoter/structural gene fusions to introduce potential anti-pathogen genes into cotton. Our current efforts have focused on the characterization of the genes and gene products for a natural cotton defense gene, characterization of cotton promoters that are expressed in response to pathogen attack, and continued analysis of transgenic plants that contain a haloperoxidase gene regulated by the CMV 35S promoter.

Last year we presented some preliminary information concerning the chitinase gene family in cotton. Based on genomic Southern blot experiments, we estimate that there are ~4 copies of the chitinase gene per haploid cotton genome. Northern and western blot studies have clearly shown that expression of at least some of these genes is induced by ethylene. Three different representatives of the cotton chitinase gene family have been isolated including one genomic clone and two cDNA clones. All three clones encode Class I chitinase sequences with predicted molecular weights of ~31,000.

The chitinases of cotton were purified using a chitin affinity column. 2-D gel separations of the purified cotton chitinase were tested for cross reactivity with anti-cotton chitinase serum, or stained with Coomassie blue or the periodic acid schiff reagent. Three isoelectric isomers with pIs of 5.8, 6.2 and 7.0 were identified on the stained gel, and all three cross reacted with the chitinase antibody. The protein with a pI of 7.0 stained with the periodic acid Schiff reagent. Because the genomic clone encodes a potentially glycosylated mature protein with a molecular weight of ~ 31,000 and a predicted pI of 7.0, we believe that the genomic clone is expressed. Thus, the promoter from the genomic clone is a viable candidate for generating promoter/structural gene constructs that respond to pathogens. The purified chitinase protein fraction was tested for its activity against *A. flavus in vitro*. Although the results are preliminary, there is evidence that the purified cotton chitinase fraction has an inhibitory effect.

We are characterizing the cotton chitinase promoter prior to its use to drive expression of structural genes to confer resistance to *A. flavus*. Towards this goal, we have generated 6 different deletions in the 300 bp region immediately 5' of the translational chitinase start. Three of these promoter segments have been subcloned into pBI221 upstream of the β-glucuronidase gene to test for transient expression in wounded tissue (particle bombardment assays). All three promoter deletions can be expressed (reporter gene activity is detected) but it appears that the Ethylene Response Element /H box promoter deletion has higher levels of expression. In the future, we plan to retest the expression of reporter genes driven by these promoter fragments in stable transformation studies when the binary vectors are complete.

Based on results presented at this meeting in the last several years, three types of structural genes have been identified as most active against *A. flavus*: haloperoxidase, the lytic peptides

and the trypsin inhibitor from maize. Last year, we continued the regeneration of transgenic cotton plants that contain a bacterial haloperoxidase gene whose expression is regulated by the constitutive CaMV 35 S promoter. Bioassays were performed with leaf extracts from 10 plants that contain this construct. Four plants, designated KH-2, 3, 9 and 10, inhibited germination of *A. flavus* spores. These results are preliminary and have not yet been shown to be statistically significant due to extreme experimental variation. As more material becomes available for testing, these experiments will be repeated.

In summary, we have characterized the cotton chitinases at the DNA and protein levels. The genomic clone that is the source of our promoter segments appears to be expressed in response to ethylene. The purified cotton chitinase protein may be effective against *A. flavus*, however additional studies are needed. The cotton chitinase promoter is currently being cloned into binary vectors to drive the expression of structural genes for future generation of transgenic cotton with enhanced resistance to *A. flavus.* Current studies of transgenic cotton that contain a constitutively expressed bacterial haloperoxidase gene show that leaf extracts from some of these plants inhibit *A. flavus* spores from germinating.

TRANSFORMATION WITH GENES ENCODING PEPTIDYL MIM®, AS A MEANS OF REDUCING AFLATOXIN CONTAMINATION IN PEANUT

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Transformation of plants with genes encoding various toxic peptides has been proposed as a means of combating fungal pathogens. Among the most interesting approaches involves the use of wholly synthetic Peptidyl Membrane Interactive Molecules (MIM®), small peptides, some of which closely mimic both the structure and anti-microbial activity of naturally occurring molecules such as cecropins. These peptides, which are the proprietary property of Demegen, Inc., have been designed to have several advantages over their naturally occurring analogs, however. They are highly active against an array of both fungal and bacterial pathogens. Importantly, they display relatively low toxicity in mammalian systems, making them appropriate for use in crop plants intended for human or animal consumption. Because they are synthetic and can be prepared artificially in limited quantities, it is easier to conduct *in vitro* tests with them than with naturally occurring proteins that are frequently difficult and expensive to purify.

We have prepared transgenic peanut plants using a gene encoding D5C, an alpha-helical peptide with a molecular weight of approximately 4000. A synthetic transgene encoding this peptide, driven by the ubiquitin 7 promoter from potato, was transferred into embryogenic cultures of peanut cv. 'NC7' by microprojectile bombardment. This plasmid also carried a transgene encoding hygromycin phosphotransferase, permitting selection of transgenics on hygromycin, using published protocols. A total of 128 putative transgenic plant lines were recovered.

Analysis of these plants by polymerase chain reaction (PCR) revealed the presence of the CaMV promoter and D5C coding sequences in fifteen lines, while another eight lines carried only the hygromycin phosphotransferase selectable marker. Neither Northern blot analysis for the presence of D5C mRNA, nor Western blot analysis for the peptide indicated expression of the transgene in any of the D5C transgenic lines. Southern hybridization analysis indicated that all fifteen lines carried numerous integrated copies of the transgene, a condition frequently associated with transcriptional silencing. Further, Southern blot results revealed that while fifteen independent plant lines had been recovered, they were derived from only two independent transformation events.

Primary transgenics were allowed to self-pollinate and set R1 pods. Only 12 of 15 transgenics carrying the D5C transgene set seed, and 7 of 8 HPT-only transgenics set pods. While all of the fertile plants appeared to set normal pods, plants carrying the D5C transgene set significantly fewer pods than did plants which carried only the HPT selectable marker gene. This result is consistent with a reduction in fertility associated with the presence of D5C. This suggests that the peptide may inhibit fertilization.

Analysis of R1 and R2 progeny indicated that the transgenes were inherited by progeny of the primary transgenics, and variation in Southern blot patterns showed that segregation

had occurred in at least some of the progeny, resulting in the presence of fewer copies of the transgene. It has been observed that such genetic simplification can lead to expression of previously silenced transgenes. However, neither Northern nor Western analysis gave evidence of expression of D5C in any progeny plants tested in either the R1 or R2 generations.

While no evidence of transgene expression was observed, it is possible that the R2 progeny are expressing D5C at very low levels. Therefore, representative R2 progeny from each of the original fifteen D5C lines are currently undergoing testing in the "minirhizotron" system to determine whether the transgene reduces infection by *Aspergillus flavus*. In this assay, a strain of *A. flavus* transformed with a gene encoding a green fluorescent protein (GFP) is introduced into barrels in which the transgenic peanut plants are growing. The barrels are equipped with transparent pipes which penetrate the barrels horizontally at intervals. A digital camera equipped with a UV light source can be introduced into these pipes to observe the underground parts of the plants. UV excitation causes the *A. flavus* to fluoresce, allowing direct observation of fungal colonization or infection of the plants. Results of these assays are pending at the time of this writing.

The apparent failure to produce transgenic peanut expressing high levels of the antifungal peptide D5C, and the apparent reduction in fertility of plants carrying the D5C transgene lead to concern that phytotoxicity of the peptide might impede the recovery of transgenic plants that express substantive levels of the peptide, and could complicate seed production. If this were true, it may be inherently impossible to produce commercially useful transgenic plants that express efficacious levels of the defensive peptide. It seemed of critical importance to determine whether this might be the case before additional attempts were made to produce transgenics with this or any other Peptidyl MIM®. Therefore, two types of experiments were devised to measure the potential phytotoxic effect of these peptides.

In the first set of experiments, cultured tobacco cells were transformed by microprojectile bombardment. These cells are likely to be especially sensitive to toxic effects because of their unusually high rate of division, and are therefore especially well suited to the detection of adverse effect of the Peptidyl MIM®. Cultures were treated using combinations of plasmids, one of which, delivered into all cultures, carried a gene encoding neomycin phosphotransferase (NPT II). A second plasmid was also transferred into the cells. In half of the treatments, this second plasmid carried a gene encoding D5C, while in the other half of treatments, the second plasmid carried a gene encoding bacterial *beta*-glucuronidase (GUS). Bombarded cells were then subjected to selection on media containing kanamycin. Surviving colonies of cells were picked and maintained independently. A random selection of 100 colonies was picked from each treatment, *i.e.*, 100 bombarded with NPT II and GUS, and 100 treated with NPT II and D5C. Colonies were then analyzed by PCR for the presence of either NPT II and GUS, or NPT II and D5C, as appropriate.

Of 100 colonies transformed with NPT II and GUS, 72 carried the GUS transgene. Of 100 colonies transformed with NPT II and D5C, only 14 carried the gene encoding D5C. These results are consistent with a depression in transformation frequency caused by expression of D5C in cells.

A second set of experiments was designed to determine if a therapeutic window could be obtained with four different Peptidyl MIM®. "Therapeutic window", as it is used here, refers to the difference between the amount of peptide required to inhibit the growth of *A.*

flavus, and the amount of peptide required to inhibit the growth of plant cells. If the amount needed to inhibit the growth of the fungus is less than the amount required to inhibit plant cell growth, then a therapeutic window can theoretically be achieved. The peptide is then an appropriate candidate for use in plant defense.

In order to determine if a therapeutic window can be achieved, pure Peptidyl MIM® was added in various concentrations to cultures of tobacco cells and to germinated spores of *A. flavus*, and the cultures were assessed for evidence of inhibition. Peptide was added to Nt-1 tobacco suspension cultures at the time of culture initiation. All cultures were initiated from a single log-phase culture, and all cultures were prepared using identical media composition and volume. Tobacco cultures with peptide were incubated at 22°C with agitation, and were harvested, dried and weighed three days after initiation (mid-log).

Peptide was applied to *A. flavus* cultures after spores germinated in standard fungal media. Peptide was added to desired level, and cultures were incubated at 22°C for three days. Cultures were assessed for inhibition of growth three days after addition of peptide.

Four Peptidyl MIM® were tested. Three are α-helical molecules whose structure is somewhat analogous to that of the cecropins. These included D5C (MW=4001.16), D5C1 (MW=5228.2), and D2A21 (MW=2775.8). The fourth compound, D4E1 (MW=2611.87), has a β-pleated sheet structure.

D5C1 retarded growth of *A. flavus* only slightly at a concentration of 10mM, but retarded growth of tobacco cells at a concentration of 5mM. D5C also affected fungal growth slightly at 10mM, but at this concentration the growth of tobacco cultures was retarded severely. D4E1 arrested growth of *A. flavus* at a concentration of 5mM, but had no measurable effect on the growth of plant cells at this concentration. This peptide slowed tobacco cell growth at concentrations above 10mM. Finally, D2A21 was not tested independently against *A. flavus* in this study. However, data provided by Demegen, Inc. suggest that this peptide retards fungal growth at concentrations below 20mM. Our test showed that plant cell growth was unaffected at concentrations below 20mM.

These data indicate that a suitable therapeutic window can probably be established with peptides D4E1 and D2A21. D5C and D5C1 may be too toxic to plant cells to be used effectively in plant defense.

The data reported here indicate that some Peptidyl MIM® can be used effectively as part of a comprehensive strategy to reduce *A. flavus* contamination of peanut. However, it is important to note that all of the compounds examined do retard plant cell growth at certain concentrations, and could therefore reduce the yield of plants expressing them. Expression of the peptides under control of wound-inducible or tissue-specific promoters may minimize this effect, and allow the use of the more toxic compounds in plant defense. All new compounds should be tested to determine whether a therapeutic window can be achieved before launching protracted and costly transformation efforts. Finally, it is important to examine as many compounds as possible to identify those that have the broadest possible therapeutic window.

Panel Discussion: Crop Resistance- Genetic Engineering

Chair: Jeffrey Cary

Panel Members: Charles Woloshuk, Kanniah Rajasekaran, Peggy Ozias-Akins, Abhaya Dandekar, Caryl Chlan and Arthur Weissinger.

Summary of Presentations: Five of the six presentations dealt with the development of transgenic crops expressing a variety of antifungal/antiinsecticidal proteins and peptides while one presentation discussed the potential of utilizing an α-amylase inhibitor to inhibit fungal growth and aflatoxin production.

Charlie Woloshuk discussed the role in aflatoxin production of *A. flavus* α-amylases that breakdown starch in the endosperm tissue of corn kernels. He theorized that one resistance mechanism in corn might be the production of amylase inhibitors that are specific for fungal amylases. Protein extracts from over 200 plant sources identified one extract, from *Lablab purpureus*, that produced an α-amylase inhibitor that was 37 times more active than the previously reported 14 kDa trypsin/amylase inhibitor isolated from corn. He discussed the potential of this gene as a candidate for introduction into corn to enhance resistance to *A. flavus*.

Peggy Ozias-Akins gave an update on her studies on transgenic peanut expressing antifungal/antiinsecticidal proteins and peptides. Data presented on the fourth year of field tests of *Bt*-peanut for lesser cornstalk borer resistance showed apparent stability of the transgene expression. Pods were to be scored for insect damage and seed assayed for aflatoxin contamination following harvest in November. One peanut plant transformed with an anionic peroxidase (*tap1*) gene construct was found to actively express *tap1* and contain what appeared to be the tomato anionic peroxidase. Leaf extracts of this plant demonstrated inhibition of spore survival and growth of *Verticillium dahliae*. A single line of peanut demonstrated high levels of expression of an ubiquitin-D4E1 synthetic peptide fusion gene. However efficacy tests failed to show reduction in fungal growth, possibly due to incomplete cleavage of the peptide from the ubiquitin protein. A number of somatic embryos transformed with a soybean lipoxygenase (*lox1*) gene failed to demonstrate expression of the gene. It was hypothesized that this was due to expression of *lox1* being under the control of a carrot embryo-specific promoter that does not function well in peanut. Future work will place *lox1* under the control of the potato ubiquitin promoter that is highly active in many peanut tissues. She also discussed the successful use of an ubiquitin promoter-mercury resistance (*merA*) gene construct as a selection system in peanut for which she will have proprietary rights.

Arthur Weissinger discussed his lab's work on transformation of peanut with antifungal peptides to reduce aflatoxin contamination. Data presented on the transformation of peanut with the antifungal peptide D5C showed that very few transformed lines were obtained and none demonstrated expression of the transgene. In addition, R1 progeny of plants known to harbor the D5C gene set fewer pods than control plants suggesting that D5C may inhibit fertilization. The R2 progeny are currently undergoing testing for ability to inhibit fungal growth using a "minirhizotron" system and a GFP-expressing *A. flavus* strain. Experiments performed with tobacco cells to measure potential phytotoxic effects on transgenic plants expressing D5C indicated that there was a reduction in transformation frequency possibly due to expression of D5C. However, comparison of concentrations of 4

different peptides with respect to levels needed to inhibit growth of *A. flavus* and that which retarded growth of cultured tobacco cells showed a "therapeutic window" for 2 (D4E1 and D2A21) of the 4 peptides could be obtained that did not significantly harm the host cells. Art stated that all new compounds should be tested for an efficacious "therapeutic window" prior to costly and laborious transformation efforts.

Kanniah Rajasekaran discussed the latest developments with regard to transformation of tobacco and cotton with antifungal gene constructs. Data obtained from *in vitro* antifungal assays of extracts of tobacco R1 and R2 progeny expressing either haloperoxidase (CPO-P gene) or synthetic peptide D4E1 showed stable inheritance of antifungal activity. Studies performed in an effort to elucidate the mode of action of the haloperoxidase *in planta* ruled out the production of peroxyacids as the basis for antifungal activity. Other possible avenues of inhibition being looked at include the enzyme's hydrolase or esterase activity. Efforts continue on the determination of levels of production of D4E1 peptide in transgenic plants. Both MALDI-TOF and numerous western blotting protocols are being tried in an effort to determine levels of D4E1 production in transgenics. Rajah also stated that a number of cotton plants putatively transformed with D4E1 and CPO-P are being regenerated and readied for analysis. Additionally, a GFP-expressing, toxigenic *A. flavus* is being used to evaluate the manner in which this fungus invades wound-inoculated closed bolls and open mature bolls. It was also noted that collaboration between the USDA aflatoxin lab in New Orleans and Dow AgroSciences has enabled the construction of vectors harboring D4E1 and CPO-P that are free of freedom-to-operate issues.

Caryl Chlan has characterized three Class I cotton chitinases at the DNA and protein levels. The genomic clone that is the source of the chitinase gene promoter segments appears to be expressed in response to ethylene. The purified cotton chitinase protein may be effective against *A. flavus*, however she stated that results were preliminary and additional studies are needed. The cotton chitinase promoter is currently being cloned into binary vectors to drive the expression of structural genes for future generation of transgenic cotton with enhanced resistance to *A. flavus*. Current studies of transgenic cotton that contain a constitutively expressed bacterial haloperoxidase (CPO-P) gene under the control of the CaMV 35S promoter show that leaf extracts from some of these plants inhibit *A. flavus* spores from germinating. As additional plant material becomes available, more assays will be performed to confirm these preliminary bioassay results.

Abhaya Dandekar reported on his research with Gale McGranahan and others for development of walnut that is resistant to *A. flavus* infection that involves both genetic engineering of antifungal/antiinsecticidal genes as well as identification of natural sources of resistance to aflatoxin production and fungal growth found in walnut kernels. Studies of walnut tissue expressing the *B. thuringensis cry*IAc gene under the control of either the CaMV 35S or potato ubiquitin3 promoter demonstrated increased mortality in feeding studies with codling moth larvae *vs*. control plant tissues. Better mortality and activity were seen with the *cry*IAc gene under the control of the CaMV promoter. A cloned walnut vicillin gene was transformed into tobacco and extracts from two lines expressing vicillin were shown to inhibit aflatoxin production by *A. flavus*. In addition, collaboration with Noreen Mahoney at USDA, Albany has identified at least two sources of natural resistance to *A. flavus* in walnut kernels. The most active component appears to be present in the seed coat and results in complete inhibition of toxin production. Crosses of walnut varieties demonstrating high (Chico) and low (Tulare) levels of aflatoxin production were performed to determine if the factor is inherited as a single gene or multiple genes.

Summary of Panel Discussion: Bob Sacher asked Caryl Chlan where the cotton chitinase she had cloned and characterized is normally accumulating in the plant. Caryl stated that the chitinase is probably targeted to the vacuole. Bob asked if she thought it would be effective against *A. flavus* if it accumulates in the vacuole. Caryl stated that it should be as invasion by the fungus should disrupt vacuoles but she may also want to delete the C-terminus of the chitinase, which would probably result in cytosolic accumulation. Sadik Tuzun stated that Caryl Chlan's chitinase work is a good approach to identifying potential antifungal genes for expression in cotton. He felt that host proteins should be looked at first for efficacy and then proteins from other plants and microbes.

Bob Sacher also asked the panel in general if plant lipoxygenase(s) that play a role in the upregulation of aflatoxin production in the fungus are really needed by plants and if not, why not just genetically knock them out. Peggy Ozias-Akins stated that no one has determined what the effects would be if these particular lipoxygenases were inactivated but she would like to try this sort of experiment in peanut.

Jay Mellon directed a question to the panel concerning the efficacy of pathogenesis related (PR) proteins from plants against *A. flavus*. Mike Hasegawa stated that PR proteins such as osmotin (PR-5) have not worked against *A. flavus* in previous experiments, however proteins such as wheat PR-4 have shown some inhibitory effects against the fungus in *in vitro* assays.

Bruce Campbell asked Abhaya Dandekar if he had any thoughts on how to get the public to accept genetically modified walnut. He replied that commercialization is still quite a ways off for walnut expressing transgenes such as *B. thuringensis cry*IAc and that the role of gaining public acceptance of these types of crops will require a concerted effort on the part of the food industry. All agreed that it will take some time and a strong effort at educating the public on the advantages to the consumer, and not just the farmer, of genetically modified crops. Pat Dowd commented that governmental regulatory agencies are keeping close tabs on corn *Bt* studies. He said that the overall outlook may be better with respect to regulatory agency approval but it is the consumers that still have the clout. Abhaya said that chemical agents are currently not working well (resistance being observed) so the transgenics should be an alternative, at the very least, being used as a "trap crop". Themis Michailides also asked Abhaya why freeze-dried leaf tissue was used in codling moth larvae feeding studies instead of fresh leaf tissue. Abhaya replied that it was a problem having sufficient quantities of fresh leaf tissue over the course of the experiment whereas freeze-dried material is essentially fresh at all times. This problem doesn't exist when using walnut kernel tissues because there is so much more material to work with.

USE OF A GREEN FLUORESCENT PROTEIN-EXPRESSING *ASPERGILLUS FLAVUS* STRAIN TO STUDY FUNGAL INVASION OF COTTON

Jeffrey W. Cary, Kanniah Rajasekaran, and Peter J. Cotty. USDA, ARS, Southern Regional Research Center, New Orleans, LA.

A strain of aflatoxigenic *A. flavus* genetically engineered to express the green fluorescent protein (GFP) from the jellyfish, *Aequora victoria*, was used to study invasion and aflatoxin production in mature cotton bolls. Aflatoxin contamination of cottonseed is thought to occur in two phases. The first phase of infection and aflatoxin contamination occurs prior to seed maturity and is usually associated with insect damage to the boll, particularly by pink bollworm. The second phase occurs after seed maturity, and involves direct infection of seed by *A. flavus*. This process is favored by moist, warm conditions during boll maturity, harvesting, and storage. Currently, little is known about the infection process with respect to the second phase of contamination. In particular, we are interested in determining if the fungus interacts with the cotton fiber and to what extent, point of penetration of the fungus into the seed, time course and extent of invasion and toxin formation in the seed. Preliminary observations using fluorescence microscopy of open bolls inoculated with a GFP-expressing *A. flavus* indicated the following with regard to fungal invasion and toxin production: 1) Fungal invasion appears to be rapid within the boll; infection of the distal seeds was not significantly different from the apical seeds within a locule. 2) Seed infection appears to proceed from the chalazal end to the micropylar end. 3) The seed coat is colonized rapidly (48-72 h), with penetration into the seed apparently occurring through the "chalazal plug" within 24-48 h of infection. 4) Once the fungus has penetrated, hyphal spread appears to progress along the seed coat-cotyledon interface and within about 48-72 h the cotyledon shows signs of fungal infection. 5) GFP in the cotyledon could be detected at about 48 h followed closely by aflatoxin production beginning at about 72 h and reaching high levels (200 ppm) by 168 h. Additional observations include detection of high levels of GFP in immature sclerotia that form on the surface of locule fibers at about 96 h post infection. GFP fluorescence was quenched by sclerotial melanization that was shown to proceed from the basal end of the sclerotium to the growing end. Also, fungal conidiation was observed on the fiber surfaces. Higher magnifications than available for this preliminary study will be required to better determine the extent of the fungus-fiber interaction and point of entry through the seed coat.

DOES LIPOXYGENASE ACTIVITY EXPLAIN LOW AFLATOXIN ACCUMULATION IN SOYBEANS?

Jay E. Mellon and Peter J. Cotty; USDA, ARS, Southern Regional Research Center, New Orleans, LA

Commercial losses from contamination by aflatoxin, a potent carcinogen produced by the fungus *Aspergillus flavus*, typically occur in corn, cottonseed, peanuts, and tree nuts. Reports of aflatoxin contamination in soybeans also exist, though levels tend to be lower than in more susceptible crops. Fatty acid oxidation products catalyzed by lipoxygenase (LOX) are known to inhibit aflatoxin biosynthesis. In order to further test the hypothesis that LOX-catalyzed metabolites are largely responsible for low aflatoxin levels in *A. flavus*-infected soybeans, a study was initiated using soybean lines with and without LOX activity. Seed treatments included whole seed, broken seed, and autoclaved seed. Seed samples were inoculated with *A. flavus* AF13 and incubated for 5 days at 31°C. Aflatoxin B_1 was determined by a thin layer chromatography / densitometer method. Lipoxygenase activity was determined with a qualitative, colorimetric dye-linked assay. Whole soybean seed samples produced significantly less aflatoxin B_1 than broken seed samples, regardless of LOX presence. LOX was detected in broken seed (seed fragments) of LOX-producing lines. Autoclaved seed lacked LOX activity and accumulated levels of aflatoxin similar to broken seed. Non-autoclaved whole seed generally produced less aflatoxin than autoclaved whole seed, regardless of LOX activity. There were no obvious differences in aflatoxin accumulation between whole soybean seeds with LOX versus whole seeds without LOX. The data in this study suggests that soybean resistance to aflatoxin accumulation was more a function of seed viability and seed coat integrity than of LOX presence.

CONFIRMATION OF THE QTL REGIONS FOR AFLATOXIN RESISTANCE BY EVALUATING TAILS OF THE VA35XMP313E MAPPING POPULATION IN MULTIPLE ENVIRONMENTS

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The Va35xMp313E population is derived from a resistant by susceptible cross for aflatoxin accumulation. This population of 216 F3 families was evaluated at Mississippi State, MS over three years (1997,1998,1999). Davis *et al*., (1999) reported QTL analysis for this population based on 1997 and 1998 data. Further investigation of this population in other locations was needed to confirm the stability of QTL over locations before embarking on a marker-assisted selection project. To facilitate the evaluation in multiple locations the most informative subset of 47 lines with the highest and 47 lines with the lowest best linear unbiased predictors for aflatoxin accumulation based on three years data were selected as the tails of the population. These lines along with Va35 and Mp313E were included in a trial with three replications grown in Weslaco, TX; Stoneville, MS; and Mississippi State, MS in 2000. Ears were needle inoculated with a suspension of *Aspergillus flavus*. Aflatoxin analysis was done using the Vicam Aflatest (Watertown, MA). Aflatoxin accumulation means were 602.2 ppb for Weslaco, TX; 106.5 ppb for Stoneville, MS; and 517.0 ppb for Mississippi State, MS. An experimentwise significance level of 23.58 likelihood ratio was determined using 1000 permutations of the data by QTL Cartographer. The QTL regions that were significant at this level were bins 1.07-1.09, 3.04, 4.03 and 9.07. Of the significant QTL, only bin 1.07-1.09 overlapped between locations. The aflatoxin lowering effect of region 1.07-1.09 was contributed by Va35. When QTL beneath the significance level were considered more overlaps appeared, most notably in regions previously identified by Davis *et al*., (1999) (bins 2.07, 4.06 and 6.00).

The regions that show the most promise for marker-assisted selection are those on chromosome 4. In these QTL regions the aflatoxin lowering effect is donated by MP313E and is genetically additive.

Davis, G. L. , G. L. Windham, and W. P. Williams. 1999. QTL mapping of genes that influence aflatoxin B_1 level. Maize Gen. Conf. Abstr. 40.

AFLATOXIN AND INSECT RESPONSE OF NEAR-ISOGENIC *BT* AND NON-*BT* COMMERCIAL CORN HYBRIDS IN SOUTH TEXAS

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Nine pairs of near-isogenic *Bt* (Mon 810, Yieldgard, Monsanto company)/ Non*Bt* commercial corn hybrids were obtained from six companies to assess *Bt* gene influence on (1) amount and type of insect injury (2) pre-harvest aflatoxin content at maturity, (3) agronomic performance, and to determine (4) potential relationships among these assessments. All hybrids were planted (18-20 K/ac target population) in an RCB design with nine single row replicates of each hybrid and near-isogenic hybrids in adjacent rows. Test locations were two Orelia soil sites at the Texas Agricutural Experiment Station (TAES) at Corpus Christi (CC), TX in 1999 and 2000 and a dryland location (60 miles N) at TAES, Beeville (BEE), TX in 2000. The CC sites were limited irrigation (Irr) with drip irrigation as needed until silking and dryland (Dry) with a high potential for drought stress. Late March planting dates were 3-4 weeks later than normal to maximize potential for drought stress. Inoculum (autoclaved corn kernels colonized by a high aflatoxin-producing *A. flavus*, NRRL3357) was distributed between treatment rows at the rate of 1 kg dry seed equivalent/ 200' when the first hybrids reached mid-silk at any site. Insect pest data included whorl, ear (Widstrom method) and stalk tunneling injury measurements. Ears were harvested after kernel moisture in all hybrids was below 15%. Threshed grain from the nine replicates of each hybrid were pooled into three composite replicates as follows: Reps (1,2,3), (4,5,6), and (7,8,9). All grain from each composite replicate, usually ninety ears, was ground in a Romer mill and a subsample was analyzed for aflatoxin content (ppb) using the Vi-Cam Aflatest P immunoassay system. Naturally-occurring insect pest populations, corn earworm (CEW) and fall armyworm (FAW), caused significantly lower average ear injury on *Bt* hybrids at all sites and years but injury ratings were still high (*Bt* vs Non*Bt*, 4.0 vs 4.5 CCDry and 3.5 vs 4.4 CC-Irr in 1999 and 7.0 vs 8.6 CC-Dry, 7.3 vs 8.9 CC-Irr and 6.3 vs 8.8 BEE in 2000). CEW was predominant in 1999 and both insects had high populations in 2000. The higher numbers of FAW (three times those of 1999) contributed to a near-doubling of ear injury in *Bt* hybrids compared to 1999. Almost no stalk tunneling insects were present but FAW did cause some ear shank and stalk tunneling injury at CC in 2000. Relationship of ear insect injury to aflatoxin content was difficult to determine but there was some association depending on location and year. Contrast analysis of all *Bt* versus Non-*Bt* hybrids showed significantly higher pre-harvest aflatoxin content in the Mon 810 *Bt* hybrids at both CC sites in 1999 (*Bt* vs Non*Bt*, 1136 vs 601 ppb CC-Dry and 423 vs 243 ppb CC-Irr). Some uniformity factor across Mon810 *Bt* hybrids may have influenced the significantly higher aflatoxin accumulation under the environments at CC in 1999. At those same CC sites in 2000, Non*Bt* hybrids generally had a slightly higher aflatoxin content but paired hybrid comparisons showed the differences were nonsignificant. Across all hybrids the average aflatoxin content was actually higher in *Bt* hybrids than Non*Bt* at both sites (*Bt* vs Non*Bt*, 1399 vs 1166 ppb CC-Dry and 1078 vs 979 ppb CC-Irr) but contrast analysis indicated that the differences were nonsignificant. At BEE in 2000, contrast analysis showed that Non*Bt* hybrids had significantly higher aflatoxin content than *Bt* hybrids (*Bt* vs Non*Bt*, 187 vs 347 ppb). Contrast analysis and paired hybrid comparisons showed that the Mon 810 *Bt* hybrids had significantly higher yields (average127-972 lb/ac more) than Non*Bt* across sites and years but there was no relationship between aflatoxin content and yield or any

other agronomic factor evaluated. Similar ear insect injury at CC and BEE in 2000 indicated that other factors, probably timing and severity of drought stress, may have had a greater contribution to aflatoxin levels observed. Drought stress at CC became moderate to severe at 2 wk past mid-silk stage of most hybrids but only near maturity at BEE. Differences in aflatoxin content between hybrid pairs indicated individual hybrid vulnerability was the primary factor influencing aflatoxin accumulation under drought stress environments but reduced insect injury by *Bt* or other means may further reduce aflatoxin content.

CLONING OF GENES ENCODING PEPTIDES WITH ANTIFUNGAL ACTIVITY AGAINST *ASPERGILLUS FLAVUS*

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Under favorable conditions, *Aspergillus flavus* invades peanuts, cottonseed, corn and certain nuts and produces aflatoxins that are extremely toxic chemicals for human and animals. Identification of antifungal peptides with activity against *A. flavus* could lead to the development of biotechnological or biological control strategies for controlling aflatoxin contamination, and, for example, increase plant resistance to fungal invasion through genetic engineering. In order to identify new genes encoding potent antifungal peptides, we have isolated a strain of *Bacillus subtilis* AU195 that inhibits *A. flavus* growth *in vitro.* We have purified two peptides analogs of bacillomycin D from the culture filtrate of *Bacillus subtilis* strain AU195. Bacillomycin D belongs to the iturin group of cyclic lipopeptides, which are synthesized nonribosomally by peptide synthetase. Comparison of several genes encoding peptide synthetases have revealed a modular structure for this class of enzyme, the order of the modules defining the sequence of residues in the peptide product. Iturins shared a common amino acid sequence Asx-Tyr-Asx and recently the genes encoding the mycosubtilin iturin synthetases have been cloned (Duitman *et al*., 1999). Using primers, designed according to the mycosubtilin synthetase genes, we have amplified by PCR and cloned the asparagine activation module of bacillomycin D. We used this PCR amplified fragment to screen a *lambda* phage genomic library and identified several clones which are currently under sequencing. Cloning of these bacterial antifungal genes will not only allow us to express the genes in other bacteria and plants but also to modify the activity of the peptide and create new antibiotics.

Duitman, E. H., Hamoen, L. W., Rembold, M., Venema, G., Seitz, H., Saenger, W., Bernhard, F., Reinhardt, R., Schmidt, M., Ullrich, C., Stein, T., Leenders, F., and Vater, J. 1999. The mycosubtilin synthetase of *Bacillus subtilis* ATCC6633: a multifunctional hybrid between a peptide synthetase, an amino transferase, and a fatty acid synthase. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 13294-9.

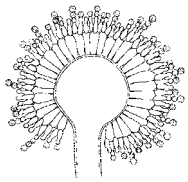
A CORN *LOX* GENE, *CSSAP 92*, IS DIFFERENTIALLY EXPRESSED IN DIFFERENT CORN LINES DURING *A. FLAVUS* COLONIZATION

Richard A. Wilson[1], Harold W. Gardner[2] and Nancy P. Keller[1]. [1]Texas A&M University, College Station, Texas. [2]USDA, ARS, National Center for Agriculture Utilization Research, Peoria, Illinois.

Corn kernels are highly susceptible to *Aspergillus* infections and aflatoxin contamination. The plant lipoxygenase (lox) enzymes are thought to play a role in the *Aspergillus* / plant interaction, and our lab is involved identifying and characterising *lox* genes that are expressed in the corn embryo. Screening nine non-homologous cDNA clones for embryo-specific expression, we identified one *lox* clone, *cssap 92*, that is clearly expressed in response to *A. flavus* infection of corn lines susceptible to aflatoxin contamination, but is repressed during the infection of corn lines resistant to aflatoxin. Moreover, this *lox* gene is expressed differently in different corn lines in response to infection by *Fusarium verticillioides*. Over expression of the *cssap* 92 gene and analysis of the reaction products of the resulting enzyme shows it preferentially adds oxygen to the 9th carbon of its linoleic acid substrate to give 9*S*-HPODE. This derivative of linoleic acid is known to increase aflatoxin gene expression in *Aspergillus* species.

SESSION 5: MICROBIAL ECOLOGY

***Moderator*: Phil Wakelyn**
National Cotton Council



VARIATION IN *ASPERGILLUS OCHRACEUS*

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Ochratoxins are of increasing concern in foods, especially in wheat, coffee, beer and wine, cacao, legumes, and dried fruits. European countries are placing stringent limits on ochratoxins in commodities. Little is known about *A. ochraceus* and ochratoxins compared to *A. flavus* and aflatoxins. We are using this understanding of *A. flavus* to develop hypotheses about the *A. ochraceus* group. These hypotheses include: 1) the fungus is ubiquitous in crops and fields; 2) the same species is responsible for contaminating many crops; 3) there is great variation in toxigenicity among isolates; 4) toxigenic isolates tend to make more sclerotia than atoxigenic isolates; and 5) sclerotia contain high concentrations of toxin.

Results to date: 1) Our data, and previously published data by Doster and Michailides, suggest that *A. ochraceus* is as common as *A. flavus* is in tree nuts and in tree nut orchards. *Aspergillus melleus* is also common. 2) Many of the *A. ochraceus* group fungi we have isolated from tree nuts and coffee fit poorly with described species. Recent studies have shown that *A. ochraceus* and *A. melleus* are polyphyletic and the *A. ochraceus* group is polyphyletic. Apart from the *A. ochraceus* group, ochratoxin production has been reported in three sections of *Aspergillus* as well as in *Penicillium verrucosum*. Furthermore, different fungi have been reported to cause ochratoxin contamination in different commodities. The situation is confusing (and confused). 3) We have detected ochratoxin production in 0 of 23 isolates of *A. ochraceus*, 4 of 7 isolates of *A. elegans*, and 1 of 6 isolates of *A. melleus*. 4) We have found great variability within species in sclerotium size and number of sclerotia per plate. Since none of our *A. ochraceus* isolates have produced ochratoxin, no correlation with toxin has been observed. 5) Preliminary data show that ochratoxin can be highly concentrated in sclerotia even when mycelia and culture media contain very low concentrations. We are now sequencing several genes to help define species concepts in this group, we are testing ochratoxin production in other isolates, and we are trying to establish which fungi contaminate different crops with ochratoxins.

EFFECT OF RAINFALL ON THE MOVEMENT OF BIOCONTROL CONIDIA IN PEANUT FIELDS

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The application of nontoxigenic strains of *Aspergillus flavus* and *A. parasiticus* to peanut fields effectively reduces aflatoxin in peanuts. Conidium-producing inoculum is applied to the soil surface under the peanut leaf canopy where soil moisture and high relative humidity result in extensive sporulation of the fungus. Because of the subterranean fruiting of peanuts, biological control may depend upon the movement of conidia to the pod zone (upper 6 cm) before the pods become susceptible to invasion by *Aspergillus* species.

The movement of conidia in soil was examined following natural rainfall in plots within two peanut fields with different soil types. During the growing season, 24 and 29 cm of rainfall was recorded in the two fields, with the highest single rainfall in both fields being approximately 9 cm. Conidia of nontoxigenic *A. flavus* and *A. parasiticus* remained near the soil surface and showed minimal downward movement. Lateral movement of conidia occurred with surface water runoff, which washed conidia along the peanut furrows for up to 100 meters downstream from the plot boundary. In contrast, conidial densities in soil decreased sharply to <50 CFU/g at 1 to 10 meters from the plot boundary either upstream along the furrows or in directions perpendicular to the peanut rows.

In another experiment with a third soil type, the downward movement of conidia was further examined under controlled conditions of a line-source sprinkler irrigation system. Irrigation resulted in decreasing amounts of applied water with increasing distance from the sprinkler line source. The treated plot was protected from natural rainfall and was submitted to two separate irrigation events, each of which gave a gradient of approximately 0 to 6 cm of water. Conidia remained near the soil surface and downward movement was relatively insensitive to the amount of water applied. There was no significant increase in movement following the second irrigation event.

This study demonstrates that nontoxigenic *A. flavus* and *A. parasiticus* remain within the pod zone of peanuts where they would be most effective in reducing aflatoxin through competition with native toxigenic populations. Conidia of *Aspergillus* species are highly hydrophobic and this property is likely responsible for their retention in the upper soil layers despite repeated rainfall and sprinkler irrigation. Characteristics of soil, such as the pore size diameter, may also influence downward movement. The presence of biocontrol conidia in the upper soil layers is also important for aerial crops such as corn and cottonseed in which conidia dispersed from soil by wind and insects comprise the primary inoculum. The loss of inoculum through water runoff may be a factor in the effectiveness of biocontrol strains under some field conditions.

A SIMPLE TECHNIQUE FOR PRODUCING AFLATOXIN BIOCONTROL FORMULATIONS

Joe W. Dorner and Richard J. Cole. USDA, ARS, National Peanut Research Laboratory, Dawson, GA

Application of nontoxigenic strains of *Aspergillus flavus* and *A. parasiticus* to soil around peanut plants effectively reduces aflatoxin contamination. Previous studies have utilized biocontrol inoculum prepared by solid-state fermentation. This process involves sterilizing the substrate to be used as the inoculum base (such as rice or other small grain), inoculating with the desired strain of *Aspergillus*, incubating for a 1-2 day period, and drying the inoculum at a relatively low temperature so as not to kill the fungus. This process has worked well for producing an effective biocontrol formulation, but it is not amenable to large-scale production because of the time necessary to produce each batch of inoculum and the costs associated with sterilization and drying.

We have investigated various alternatives to traditional solid-state fermentation for production of biocontrol formulations and have developed a method that can be scaled up simply. The technique eliminates the need for sterilization and drying, and equipment is available that would provide for the production of inoculum at a rate of several tons per hour. Such capacity will be needed if this biocontrol technology is adopted on a large scale in the future.

The process involves coating small grains, such as hulled barley, with conidia of the *Aspergillus* strain to be used for biocontrol. The conidia can be purchased at minimal cost from sources in Japan that specialize in production of *Aspergillus* conidia for food fermentations. The conidia are added to soybean oil at a ratio of 1 g of conidia per 100 g of oil to produce a suspension of about 1×10^8 CFU/g. The suspension is evenly sprayed onto hulled barley at a rate of 1.5% by weight of oil to barley using readily available seed coating equipment. Finally, the barley is coated with diatomaceous earth at 2.5% by weight to produce a free flowing formulation containing about 1×10^6 CFU/g. Upon exposure to moisture in the peanut fields, the conidia germinate, grow on the barley, and produce secondary conidia at a level of about 1×10^9 CFU/g. These conidia are disseminated into the soil where they compete with toxigenic strains. The cost of all raw materials used in this process is about $0.12 per pound of finished formulation.

Field plot experiments were conducted to compare coated formulations using hulled barley and rice with a formulation of rice produced by solid-state fermentation. Sixteen plots (18 x 50 ft) were inoculated with 50 lbs/acre of each formulation consisting of a mixture of nontoxigenic strains of *A. flavus* and *A. parasiticus*. Sixteen uninoculated plots served as controls. Peanuts were exposed to natural, late-season drought conditions conducive to aflatoxin contamination. Significant reductions ($P < 0.05$) in aflatoxin were achieved with all formulations, and the treatments were not significantly different from each other. Reductions were 77.0%, 80.5% and 86.6% for coated rice, coated barley, and solid-state fermented rice, respectively.

UTILIZING ATOXIGENIC STRAINS OF *A. FLAVUS* TO MANAGE AFLATOXINS IN COMMERCIAL COTTON: PART I. COMMERCIAL SCALE MANUFACTURE OF INOCULUM

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Aspergillus flavus, the causal agent of aflatoxin contamination of cottonseed, is a complex species composed of many distinct vegetative compatibility groups (VCGs). Isolates belonging to different VCGs may produce widely different quantities of aflatoxins. Certain isolates produce no aflatoxins. Some of these atoxigenic strains can competitively exclude aflatoxin-producing strains during crop infection and thereby reduce aflatoxin contamination. In both greenhouse and field-plot tests atoxigenic strain efficacy has repeatedly been demonstrated. Communities of *A. flavus* vary among fields in aflatoxin-producing capacity. Field-plot tests suggested applications of atoxigenic strains may provide long-term reductions in the aflatoxin-producing potential of fungi resident in treated fields. This has been confirmed by commercial field tests undertaken since 1996 under Experimental Use Permits (EUP) issued by the EPA.

A solid formulation was developed in which whole wheat seeds are sterilized and colonized by an atoxigenic strain. Since its first use in 1989, the process for manufacturing this formulation has been gradually scaled-up to a laboratory process that allowed production of 11,000 lb. of atoxigenic strain material manufactured from 1996 through 1998. When produced optimally, the formulation performs well under severe on-farm conditions, has multiple year stability, and tolerates exposure to 70° C.

The Arizona Cotton Research and Protection Council (ACRPC) initiated development of a facility to produce commercially useful quantities of atoxigenic strain inoculum in 1998. The first phase of development sought to sterilize, inoculate, incubate, and dry the material in one piece of equipment. To this end, a used Littleford polyphase processor was acquired and modified to permit forced air-drying. Internal plows of the polyphase processor severely damaged the wheat and sterilization was difficult to achieve. This resulted in poor quality product that didn't compare well to laboratory produced material in consistency, microbiology, and survival in non-sterile flooded soil. Furthermore, product drying (target moisture 6%) was not achieved in the polyphase processor and a static bed dryer was developed to dry the product. The first phase ended with abandonment of the polyphase processor.

For the second phase of development separate pieces of equipment were designed for sterilization-inoculation and for incubation-drying. A jacketed 5 ft^3 twin-shell vacuum dryer was acquired for sterilization and inoculation. The twin-shell produced good quality inoculated wheat that was incubated and dried in modified 50-gallon drums. This process was adapted to a 30 ft^3 double-cone vacuum dryer. The double-cone was used to successfully sterilize and inoculate 1,500 lb. batches of wheat. Product from the double-cone was transferred through a sterile transition to a static incubator/bed-dryer that was designed and fabricated for the process. The bed-dryers were steam sterilized prior to receiving the inoculated wheat that was subsequently incubated within the vessel and dried by forcing

hot air through screens beneath the wheat. The dry wheat was packed directly into bulk bags. Product manufactured by this process had good microbiological and structural characteristics. However, the product did not survive as well as laboratory manufactured product in flooded non-sterile soil. In order to increase product performance and survival in field soil, improvement of wheat seed colonization will be one goal of the next phase in process development.

Several crop substrates (*i.e.* sorghum, millet, rice, rye) work well in atoxigenic strain formulations. Each has advantages and disadvantages for use in modifying *A. flavus* communities. The equipment and process described above should be readily adaptable to many substrates.

The goal of this work is development of a process that grower cooperatives can apply to production of atoxigenic strain inoculum. Successful completion will result in a non-proprietary public sector technology that can be altered and advanced through future public and private sector efforts. The technology may be readily adapted to other fungal biocontrol agents and may provide a new avenue for the utilization of diverse biocontrol agents by communities of growers.

UTILIZING ATOXIGENIC STRAINS OF *A. FLAVUS* TO MANAGE AFLATOXINS IN COMMERCIAL COTTON: PART II. FIELD ASPECTS

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Commercial field tests conducted in Arizona have shown *Aspergillus flavus* AF36, an atoxigenic strain of *A. flavus*, to be a potentially useful tool in the long-term management of aflatoxin contamination of cottonseed. A partnership involving the Arizona cotton industry through The Arizona Cotton Research and Protection Council and the USDA Agricultural Research Service has produced an organizational structure designed to address the concept of area-wide aflatoxin management utilizing atoxigenic strain technology. The year 2000 marks the second season of commercial scale application of AF36 upon test areas ranging from 1,500 to 5,600 acres of cotton. This report outlines the current status of the program in Arizona as well as numerous elements necessary for future expansion and refinement of atoxigenic strain technology.

1999 Program Activities: During the 1999 crop season three organized treatment zones were established in Yuma, Maricopa and Pinal Counties. Within these areas a total of 10,388 acres received applications of AF36. Treated fields were separated from untreated control fields by one mile buffer zones. Pretreatment soil samples were taken from treated and control fields to determine baseline levels of *A. flavus* strains. Following crop harvest and ginning, seed samples from AF36 treated fields were collected at participating cotton gins for analysis of fungal displacement on the crop. Additionally, where possible, toxin levels resulting from commercial seed tests were obtained for comparisons. Results of the 1999 program were variable both within and between areas. Overall, AF36 applications had the greatest influences in Yuma and Pinal Counties. In Pinal County AF36 averaged 1.8% of the *A. flavus* soil community prior to treatment as compared to 39.6% one year post treatment with a high value of 88.3%. AF36 composed an average of 65.9% of the *A. flavus* on the crop indicating significant displacement of aflatoxin producers. Seventy five percent of Stanfield evaluation fields produced seed with commercial aflatoxin test results of 20 ppb or less. Yuma results displayed similar positive effects of AF36 treatments, but were complicated by multi-year influences in fields treated during prior years. Two fields that received applications for the first time in 1999 had 8.3% of their resident *A. flavus* composed of AF36 prior to treatment and 71% one year later. After ginning, AF36 averaged 72% of the *A. flavus* on the crop from treated fields.

Applications of AF36 had the smallest influence on *A. flavus* communities in the Paloma treatment area of Maricopa County. In evaluation fields, AF36 composed 4.2% of the *A. flavus* soil community pretreatment and 24.6% one year after treatment (high value of 40.2%). AF36 incidence on the crop averaged 36.3% of the total *A. flavus*. The poor showing in the Paloma area was attributed to two factors: 1) batches of AF36 product with poor quality; 2) poor canopy development. Poor canopy development resulted in reduced cotton yields and inadequate conditions for AF36 growth and spread. Forty percent of Paloma fields tested 20 ppb or less in commercial aflatoxin analyses.

One site in Maricopa County produced dramatic and consistent results in all phases of analysis. Prior to treatment soil samples showed 12.2% of resident *A. flavus* composed of AF36. After ginning AF36 composed 89% of the *A. flavus* on the crop. Commercial aflatoxin

analyses detected no aflatoxin in the crop (first pick) and 8 ppb on ground gleaned cotton collected after spindle picking. In addition, one-year post treatment soil analyses indicated 89% of the resident *A. flavus* composed of AF36.

2000 Program Activities: Improvements in processing, incubation, and drying equipment allowed expansion of AF36 program activities. Addition of a treatment area near Laveen in Maricopa County increased program coverage by 5,600 with a total of 16,094 acres being treated in 2000. Crop (seed) analyses will be run on evaluation fields from all treatment areas after ginning and will be compared to commercial aflatoxin test results. Air collection data from six Burkard cyclone air samplers continued to be generated from both treated and control fields in an effort to characterize influences of AF36 applications on levels of *A. flavus* in the air. Progress was made in addressing the multitude of variables influencing area-wide program effectiveness. Continued efforts must focus on improved field delivery systems as well as agronomic practices designed to enhance AF36 activity.

EPA Registration: Discussions with EPA confirmed that full registration of AF36 will require completion of avian, mammalian and bee toxicity tests. The bee study was completed in August 2000 and the results are being analyzed for submission to EPA. Plans are to complete avian and mammalian tests by spring 2001. An Expansion of the existing EUP for AF36 to 95,000 acres has been requested for 2001.

BIOLOGICAL CONTROL OF AFLATOXIN CONTAMINATION IN ALMOND AND PISTACHIO

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Aflatoxin contamination of peanut, corn, cotton and tree nuts (almond, pistachio, walnut) results from a number of factors. The major aflatoxin-producing fungus, *Aspergillus flavus,* is opportunistic and has a broad ecological niche. Under field conditions it readily infects organic debris and proceeds to reproduce copiously. The annual combined market value for tree nut crops approaches $2 billion per year in California. Domestic and certain export markets presently allow maximum levels of aflatoxin B_1 in edible nuts to be 20 and 4 ppb, respectively. Even very low levels of infection of nuts by *A. flavus* can result in aflatoxin levels above these mandatory standards. Managing aflatoxin contamination of tree-nuts *via* biological control of *A. flavus* may be the most promising approach currently available.

For effective biological control insights derived from microbe- microbe interactions and microbial ecology are mandatory. We are developing methods of using saprophytic yeasts to control *A. flavus* in tree nut orchards and techniques for effectively establishing biocontrol agents in the field. A visual bioassay for screening saprophytic yeasts inhibiting both fungal growth and aflatoxin production has been invented by this laboratory (Hua *et al*. 1999). A few strains of yeasts out of several hundred isolates were shown to reduce aflatoxin production of *A. flavus* by about 100 fold. Some of these yeasts form pseudo hyphae which enable them move to the nutrients. The utilization of a wide range of carbohydrates by the yeasts may improve their ability to compete with the fungi. We have conducted experiments to test the biocontrol activity of one effective yeast against *A. flavus* on almond leaves. The results indicate that this particular yeast strain competes well against *A. flavus* reducing the total fungal spore number by 40-80%.

Saprophytic yeasts have been demonstrated by many researchers to effectively control several fungal pathogens such as *Penicillium expansum* and *Botrytic cinerea*. Commercial biocontrol products consisting of yeast formulation are now available to prevent fruit rot. Yeasts are not known to produce toxic compounds and are generally considered safe for use in food. We are now looking forward to a cooperative association with industry partners and growers to field-test these promising saprophytic yeasts as biocontrol agents against *A. flavus* in tree nut orchards.

Hua, S.-S. T., Baker, J. L. and Flores-Espiritu, M. 1999. Interactions of saprophytic yeasts with a *nor* mutant of *Aspergillus flavus*. Appl. Environ. Microbiol. 65: 2738-2740.

TWO NEW AFLATOXIGENIC SPECIES FROM *ASPERGILLUS* SECTION *FLAVI*

Stephen W. Peterson, USDA, Agricultural Research Service, Microbial Properties Research, National Center for Agricultural Utilization Research, Peoria, IL.

Two new species of *Aspergillus* are described and each species produces aflatoxins. *Aspergillus pseudotamarii* Ito, Peterson, Wicklow & Goto is known only from two collections, one is from South America (probably Argentina) in 1923, and the other in Japan, 1995. This species resembles *Aspergillus tamarii*, but unlike *A. tamarii*, *A. pseudotamarii* produces aflatoxin B. Colony color differs between *A. pseudotamarii, A. tamarii* and *A. caelatus*. Conidia of *A. tamarii* and *A. pseudotamarii* both have the characteristic double wall in which the outer wall can be separated from the conidium by gentle crushing under a coverslip. However, *A. tamarii* isolates typically grow at 42° C, while isolates of *A. pseudotamarii* do not. Phylogenetically, *A. pseudotamarii* is a distinct species on the basis of the concordance of gene trees generated from beta tubulin, calmodulin, ITS and 28S r-DNA and norsolorinic acid reductase DNA sequences. The rarity of isolation of this new species suggests that it is not a major component of the soil fungus population. It may have microhabitat requirements, but this will only be determined when we have a greater sampling of isolates from nature.

Aspergillus bombycis Peterson, Ito, Horn & Goto is described from 8 isolates collected from insect rearing houses in Japan and Indonesia. This species produces alfatoxins B and G and cyclopiazonic acid. At this time, we believe that *A. bombycis* is an associate of silkworm larvae, having been isolated from larvae and the frass and dust present in the silkworm rearing houses. In the same locales, we have also isolated *A. nomius* and *A. flavus*. Soil samples taken from the same regions and screened for the presence of *A. flavus* group fungi have not revealed the presence of *A. bombycis* anywhere but in close association with silkworm larvae. *Aspergillus bombycis* isolates grow at 37° C but not at 42° C, while *A. nomius* isolates grow at 42° C and *A. flavus* isolates grow at 45° C. The vesicular stipe of *A. bombycis* is smooth, compared to the rough stipes of *A. nomius* and *A. flavus*. On the basis of DNA sequence analysis of beta tubulin, calmodulin, norsolorinic acid reductase, and ITS and 28S r-DNA, *A. bombycis* is a genetically distinct species and our data show no evidence of cryptic meiotic recombination. *Aspergillus nomius* is the sibling species of *A. bombycis*, and comparisons of the same unlinked genetic loci suggests that cryptic meiotic recombination occurs in this species.

Numerous surveys of soil samples for the presence of *A. flavus* group have been conducted around the globe. *Aspergillus nomius* is rarely encountered and it is rarely encountered as a contaminant of commodities. It is found with some regularity on the cadavers of insect larvae. *Aspergillus bombycis* likewise has been found associated with insect larvae but not in the soil or crops of adjacent areas. While anecdotal in nature, this evidence suggests the possibility that *A. nomius* and *A. bombycis* are primarily insect pathogens and not a serious concern in regard to the spoilage and aflatoxin contamination of our food supply. More thorough studies of the habitat requirements of these two species could establish the danger that each species poses for agriculture and establish the potential that these species may have for bio-control of insect pests.

Panel Discussion: Microbial Ecology

Chair: Paul Bayman

Panel Members: Bruce Horn, Joe W. Dorner, Sui-Sheng Sylvia Hua, Stephen W. Peterson, Phil Wakelyn.

SUMMARY OF PRESENTATIONS: The presentations covered a wide range of topics, from applied to theoretical. Four of the talks dealt with practical aspects of using atoxigenic strains of *A. flavus* for biocontrol of aflatoxin contamination of crops. These talks covered methods of production of inoculum, ways it can be most effectively applied, and how rainfall disperses spores of the biocontrol strains. Another talk covered the potential of native yeast strains for biocontrol of *A. flavus* and aflatoxins in tree nut orchards. One talk dealt with a newly described species in the *A. flavus* group, and its relationship to the common species. Another talk covered the *Aspergillus ochraceus* group and ochratoxins in tree nuts.

SUMMARY OF PANEL DISCUSSION: Peter Cotty's talk was presented by Larry Antilla. Larry had to leave early to catch a flight, so Phil Wakelyn agreed to answer questions about Peter and Larry's work in the panel discussion.

The discussion alternated between three main themes: practical aspects of biocontrol with atoxigenic *A. flavus* strains, systematics of the *A. flavus* group, and the potential of yeasts for biocontrol of *A. flavus* and aflatoxins. Steve Peterson and Bruce Horn discussed their newly described species in the *A. flavus* group. When asked if he had considered describing his new species at the subspecies level rather than at the species level, Steve Peterson replied that it fit the critera of the phylogenetic species concept.

Joe Dorner, Phil Wakelyn and Bruce Horn offered some estimates and perspectives on application costs using atoxigenic biocontrol strains.

Sylvia Hua elaborated on plans for applications of biocontrol yeasts to tree nut orchards. Male pistachio inflorescences were mentioned as a good point of inoculation.

The question that generated the most interest was posed by Gary Gray: how feasible is the use of atoxigenic strains of *A. flavus* to control aflatoxin in tree nuts? Various panel and audience members addressed this question. Several factors were mentioned, including low frequency of *A. flavus* in tree nuts, the perennial and arboreal nature of the crop, and the benefits of using native strains from California tree nut orchards as opposed to strains that had been proven effective in other areas. Concern over potential of aspergilliosis in aerial application of the atoxigenic spores in the tree canopy was mentioned. Aerial application of atoxigenic strains is probably unfeasible from this health-safety standpoint.

MOLECULAR CHARACTERIZATION OF AFLATOXIN BIOSYNTHESIS GENES IN AFLATOXIGENIC *ASPERGILLUS* SPP. OTHER THAN *A. FLAVUS/PARASITICUS*

Jeffrey W. Cary, Maren A. Klich, Shannon M. Brennan, and Cecily A. Bennett. USDA, ARS, Southern Regional Research Center, New Orleans, LA.

Until recently, it was thought that only *Aspergillus parasiticus* (*A. p.*), *A. flavus* (*A.f.*) and *A. nomius* produced aflatoxin (AF). The genes responsible for AF production are known to be present as a gene cluster in these fungi. In *A. nidulans* (*A. n.*) the genes responsible for synthesis of sterigmatocystin (ST) have also been shown to be clustered though they are not arranged in the same order as their AF gene cluster counterparts. Recently, a strain of *A. ochraceoroseus* (*A.o.*, section *Circumdati*) was isolated and shown to produce AF. In addition, two isolates of *A. tamarii* (*A.t.*, now classified as *A. pseudotamarii*) were also found to produce AF. The objective of this research was to identify genes involved in AF production from *A.o.* (SRRC 1432) and *A.t.* (SRRC 2420), and if possible, determine their origin based on homology to either *A. p.* or *A. n.* AF/ST genes. Southern hybridization analysis indicated that *A.t.* AF genes were highly homologous to *A.p.* AF gene probes while *A.o.* DNA showed little or no homology with either *A.p.* or *A.n.* probes. Optimum AF production for *A.o.* was obtained during static culture at 25°C using nitrate as the main nitrogen source. TLC analysis of metabolites showed that *A.o.* and *A.t.* produced only AFB1. Oligonucleotide primers designed to *A.p.* AF genes successfully PCR amplified the homologs from *A.t.*, but failed to yield products from *A.o.* DNA. Degenerate primers designed from highly homologous regions of the *A. p.* and *A. n.* pathway regulatory gene, *afl*R, and the structural genes, *ver*B/*stc*L and *avnA* were used to PCR amplify both *A. o.* cDNA and genomic DNA. A product showing a high degree of homology to *verB/stcL* was obtained, while no product was obtained that showed significant deduced amino acid identity to *aflR* or *avnA* .

MONITORING *ASPERGILLUS FLAVUS* AF36 AND S STRAIN INCIDENCE IN THE DESERT SOUTHWEST

D. M. Bigelow[1], T. V. Orum[1], P. J. Cotty[2], and M. R. Nelson[1]. [1]Department of Plant Pathology, University of Arizona, Tucson, AZ; [2]Southern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, New Orleans, LA.

In the early 1990's, researchers demonstrated that an atoxigenic isolate of *Aspergillus flavus* (designated AF36) that occurs naturally in Arizona could effectively displace aflatoxin producing strains of *A. flavus* in the field. After a succession of field trials, up to hundreds of acres, the Arizona Cotton Research and Protection Council (ACRPC) treated 12,000 acres with AF36 in Arizona during 1999. ACRPC is seeking a biopesticide registration for AF36 that would permit area-wide treatment of all cotton fields within Arizona. To provide baseline data for monitoring of potential long-term impacts of the AF36 treatment program on the community structure of *A. flavus*, soil samples were collected from fields at ten locations distant from treatment areas prior to the 1999 cotton-field treatments. Locations were designated "sentinel sites". The *A. flavus* resident in samples was quantified and the incidence of AF36 was determined. The incidence of the highly toxigenic *A. flavus* S strain was also determined. *Aspergillus flavus* AF36 was found naturally occurring at all "sentinel sites" except Aguila, AZ and San Simon, AZ. The range of incidence was 0-7.2%, with the highest area being Magma, AZ. Maps of S strain incidence estimated by spatial averaging (kriging) from data derived from soil samples collected from 1998 and 1999 were prepared. The incidence of the S strain in Arizona is inversely correlated with elevation. At lower elevations, S strain incidence is frequently greater than 40%, while at elevations above 2,500 feet, S strain incidence is uniformly below 20%. The sentinel sites will be monitored in the future to detect changes in *A. flavus* communities resulting from area-wide treatment of cotton fields with AF36.

ECOLOGY OF *ASPERGILLUS* IN MISSISSIPPI DELTA SOILS UNDER CORN PRODUCTION: AFLATOXIN INOCULUM PRODUCTION

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Corn is becoming a major crop for the Mississippi Delta, however potential economic losses due to aflatoxin is a concern. Soils from 16 fields with diverse histories of corn production, soil types, and tillage management were sampled. From these soils, propagules of total fungi, *Fusarium* spp. and *Aspergillus* spp. were enumerated and a collection of fungi was established. Production of aflatoxin by aspergilli was screened by reaction of colonies to ammonia vapor. Toxin levels were quantified by ELISA, and confirmed by TLC and LC/APCI/MS. Propagules of *Aspergillus* spp. and *Fusarium* spp. ranged from log (10) < 2.5 to 4.3 and 3.6 to 5.4 cfu g^{-1} soil respectively. *F. moniliforme* represented about 30 % of the fusaria with all *F. moniliforme* isolates producing fumonisins. Although *A. flavus* was isolated from all soils, higher propagules were typically recovered from soils with greater corn production history. Higher populations of *A. flavus* were associated with no-till compared to respective conventional-tilled soils. The ammonia color change assay of 242 *A. flavus* isolates indicated about 53% of the isolates produced aflatoxins. ELISA assays confirm aflatoxin production by 100% of the positive isolates (100 to 24,300 ng g^{-1} fungal biomass) and 25% of negative isolates (160 to 1,880 ng g^{-1}). An inoculum potential of aflatoxin-producing fungi is present in most Mississippi Delta soils studied.

DESERT LEGUMES: AFLATOXIN CONTAMINATION OF NATURAL *ASPERGILLUS FLAVUS* RESERVOIRS IN THE SONORAN DESERT

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Legumes are abundant and important plants in desert habitats. One function of legumes in desert ecosystems is the contribution of organic matter (plant debris) to the soil structure. This plant debris is frequently a good microhabitat for *Aspergillus flavus*, an aflatoxin-producing fungal species. Debris and pods of four common legumes of the Sonoran desert were tested to assess the potential for aflatoxin production and accumulation in plants other than traditional crops. A total of 245 samples of plant debris and pods were analyzed for colony forming units and 2,868 isolates were characterized. Eighty-seven percent of samples were positive for *A. flavus* with both the L and S strain present in some samples. Inoculation tests designed to establish the suitability of desert legume pods and pod parts as substrates for aflatoxin production revealed a potential for aflatoxin accumulation. Pods collected from desert habitats were also analyzed for aflatoxin. Fifteen pod samples out of 68 tested were positive, with levels ranging from 1 to over 2000 ppb. Results indicate that *A. flavus* is a frequent colonizer of common desert legumes and that significant amounts of naturally occurring aflatoxin can accumulate in legume pods. Because legumes are important wildlife food sources in deserts, aflatoxins could detrimentally impact desert fauna.

MOLECULAR EVIDENCE FOR A RETROTRANSPOSON IN *ASPERGILLUS FLAVUS*

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Retrotransposons are mobile genetic elements that move to new chromosomal locations via RNA intermediates. Retrotransposons can generate mutations by inserting within or near genes. These transposable elements could contribute to genetic variation in fungi under natural conditions. Long-terminal repeat (LTR) retrotransposons have been found in a wide range of filamentous fungi. The LTR transposons encode a number of proteins, specified by three major genes called *gag*, *pol* and *int*. The *gag* gene encode a peptide of 457 amino acids exhibiting a potential zinc finger domain. The *pol* gene encodes a polypeptide with protease, reverse transcriptase, RNaseH, and endonuclease domains. Preliminary DNA sequence analysis shows an alignment of the putative *gag* sequence with those corresponding to *gag* genes from several other fungal transposons. Work in progress indicates a *pol* sequence is also present on this clone.

The genomic clone pAF28 was previously isolated from *Aspergillus flavus.* It has been used as a molecular hybridization probe on Southern blots (RFLP) to distinguish the genotypes in numerous strains of *A. flavus* found in pistachio and almond . The resulting phenogram indicates a very diversified *A. flavus* population. Through DNA sequence data, we hypothesize that pAF28 may consist of a transposable element.

Pathogenic fungi isolated from the field often show changes in their phenotype upon culturing, including morphological changes such as colony color or shape, loss of pathogenicity, and reduction of spore production. This may result from active transposition in field isolates. Mutations caused by retrotransposon insertions within or in close proximity to genes result in gene activation or alteration of the expression pattern of genes. Many gene promoters contain fragments of retrotransposons that now contribute to that gene's regulation. Evidence is accumulating slowly that many classes of transposable elements have been transferred horizontally between species.

A better understanding of the role of retrotranspons in *A. flavus* could generate new insights leading to the development of new strategies for controlling aflatoxin contamination in corn, cotton seed, peanut and tree-nut.

INTERACTIONS AMONG *FUSARIUM VERTICILLIOIDES*, INSECT PESTS, AND *ASPERGILLUS FLAVUS* IN TRANSGENIC AND CONVENTIONAL MAIZE HYBRIDS

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Insect pests of maize play a major role in the development of Fusarium and Aspergillus ear rots and subsequent accumulation of mycotoxins in maize grain. Several insect species, including the European corn borer (*Ostrinia nubilalis*) and the corn earworm (*Helicoverpa zea*) have been shown to influence infection of maize by *Fusarium* spp. and *Aspergillus flavus*. In previous studies we have observed consistently that control of the European corn borer with the use of transgenic *Bt* maize hybrids results in reductions in Fusarium ear rot severity and fumonisin concentrations in the grain. *Bt* hybrids currently available in the U.S. are of five different types: 176, BT11, CBH351, DBT418, and MON810. *Bt* proteins in these hybrids are effective against the European corn borer and partially effective against the corn earworm. The most effective control of insect damage to the maize kernels occurs with BT11, CBH351, and MON810. Because of their effectiveness against insects, these types of *Bt* maize also demonstrate the most effective control of *Fusarium* infection and fumonisin accumulation. In 1998 and 1999, we studied the interactions among *Fusarium* spp., *A. flavus*, the European corn borer, and the corn earworm on *Bt* maize hybrids and near-isogenic conventional hybrids in the field. We recorded the severity of insect feeding and visible ear rot; internal infection of the kernels by *Fusarium* spp. and *A. flavus*; and samples of grain were analyzed for aflatoxins, fumonisins, and deoxynivalenol (L.G. Rice, USDA-APHIS National Veterinary Services Laboratory, Ames, IA, or Romer Labs, Union, MO). In 1998, results were consistent with previous years in terms of fumonisins, but there were no Aspergillus ear rot symptoms or aflatoxins. In 1999, the natural population of European corn borers was lower than usual but there was an unusually high natural infestation with corn earworms, and this resulted in more damage to the *Bt* maize ears than we have observed in previous years. Fusarium ear rot severity was generally low, and Aspergillus ear rot symptoms occurred only in the inoculated treatment. In general, the differences between *Bt* and non-*Bt* hybrids were less pronounced than in previous years. Insect infestation did not significantly affect the severity of Aspergillus ear rot. There were no significant differences in Aspergillus ear rot between *Bt* hybrids and non-*Bt* hybrids except that the MON810 hybrid had significantly lower severity than did its conventional counterpart. The BT11, CBH351, and MON810 hybrids had lower severity of Fusarium ear rot than their conventional counterparts in the insect-infested treatments. Infection by *Fusarium* species consisted primarily of *F. verticillioides*, with some other species. Fumonisin and aflatoxin concentrations generally were not significantly different between *Bt* hybrids and their conventional counterparts. The most unusual result was that *A. flavus* inoculation caused a significant increase in Fusarium ear rot severity and fumonisin concentrations. Deoxynivalenol concentrations were low and there were generally no significant differences within the pairs of hybrids.

SESSION 6: CROP RESISTANCE— CONVENTIONAL BREEDING

***Moderator*: Bob Sacher**
Hunt Wesson, Inc.

CHARACTERIZATION OF THE STRUCTURAL, BIOCHEMICAL AND MOLECULAR BASIS FOR ENDOCARP, SEED AND AND SEED-COAT BASED RESISTANCE TO PREHARVEST AFLATOXIN CONTAMINATION IN ALMOND

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Aflatoxin contamination represents a double threat to U.S. almond production. Aflatoxins are reported to be highly toxic and carcinogenic. In addition, approximately 75% of the $1 billion almond crop is exported, primarily to European countries which have imposed very low to zero import tolerance levels for aflatoxins. Our research has shown that vulnerability to preharvest aflatoxin contamination of almond occurs during a well defined period of fruit development and is associated with insect damage to the kernel. High levels of aflatoxin control have been achieved in genotypes selected for an integrated insect/fungal resistance within almond mesocarp, endocarp and seed tissue. Large scale field testing in northern, central and southern California growing regions demonstrated effective control in 1998-2000. Individual resistance mechanisms including endocarp histogenesis and seed/seedcoat suppression of toxin formation, have been analyzed using NMR and light microscopy, and genomic/protenomic techniques to help identify genetic selections and orchard management practices effective in reducing vulnerability in current varieties, as well as optimizing breeding (selection) efficiency for resistant varieties presently being developed. The genetic manipulation of seed coat structure has proven particularly effective as it controls both the insect pest and disease infection and has shown a high response to selection. Early results from field studies suggest that genetic control of an '*argentia*' type shell with the desirable thin, yet highly sealed endocarp is controlled by as few as two to three genes. Developmental studies of the early stages of endocarp development have further shown that later breakdown of endocarp integrity is frequently associated with abnormal development of the tissue surrounding vascular bundles feeding the funiculus. Structural weaknesses associated with his abnormal development lead ultimately to endocarp fracture at the time of hull split with further breakdown associated with mechanical harvest process. Structural weaknesses at the sites are also associated with higher levels of insect damage to the kernel. Improved knowledge of the mechanism of endocarp breakdown should lead to more efficient genetic screening methods as well as opportunities for the cultural management of endocarp splitting in current varieties. Related studies have shown initial high levels of insect resistance in bitter almond genotypes, while the evaluations of effect of seed coat thickness on fungal infection had given inconclusive results.

cDNA libraries have been constructed from both seedcoat and embryo tissue of the susceptible variety Nonpareil and the more resistant variety Mission. These libraries will be used to assess the genetic control for both aflatoxin production suppression and seed and seed coat based disease and insect resistance.

Gradziel, T., N. Mahoney and A. Abdallah. 2000. Aflatoxin production among almond genotypes is unrelated to either kernel oil composition or *Aspergillus flavus* growth rate. HortScience 35:937-939.

CORN VARIETAL RESPONSE FOR NUMBERS OF AFLATOXIN CONTAMINATED BGYF KERNELS

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The seed coat or testa, underlying the pericarp, is a natural barrier and structural impediment to *A. flavus* infection of the maize kernel germ. Microscopic tears in the seed coat represent a critical susceptibility factor allowing *A. flavus* infection and aflatoxin contamination of otherwise undamaged grain (Smart *et al.*, Phytopathology 80:1287-1294). Previous cooperation with a corn seed company revealed that the harvested grain from *A. flavus* wound-inoculated ears of elite inbreds showed variation for the frequency of undamaged bright greenish yellow fluorescent (BGYF) kernels when examined under ultra violet light (365 nm). The fluorescent kernels are evidence that *A. flavus* has penetrated seed coat defenses and infected the germ. Numbers of BGYF kernels have been related to levels of aflatoxin contamination of grain sampled following harvest (Dickens & Whittaker, J. Am. Oil Chem. Soc. 58:973A-975A). Selecting for reduction in the frequency of BGYF kernels in conventional maize breeding programs may result in lines with reduced susceptibility to seed coat tearing and thus provide protection against *A. flavus* and possibly other kernel molds.

In 1999 the same set of 43 commercial corn hybrids and 34 inbreds evaluated in 1998 were also grown near Monmouth, IL, and hand-pollinated ears were wound-inoculated with a potent aflatoxin-producing strain of *A. flavus* NRRL A-27837 isolated from corn grown in Illinois (Wicklow, Plant Dis. 83:1146-1148). Kernels in grids surrounding (but not including) the woundsites were collected and these kernel samples separated into BGYF and non-BGYF portions of each sample. The number of BGYF kernels/grid for the different hybrids in 1999 ranged from 0.3 to 4.1 (ave. = 1.7), confirming that there is genetic variability for susceptibility to invasion of the germ by *A. flavus*. 1999 results also confirmed that these differences in the number of BGYF kernels/grid among hybrids can be related to the use of certain inbreds. Correlation between the number of BGYF kernels observed vs. the aflatoxin level was poor for both hybrids and for the estimated effects of inbreds in both years. Aflatoxin levels for the portion of each sample represented by the BGYF pick-outs recorded a low of 10 ppb for BGYF kernel pick-out samples in 1999 (all hybrids, ave.= 837 ppb). In this example, NRRL A-27837 produced sufficient kojic acid during kernel infection to elicit BGY fluorescence but very little aflatoxin. In 1999 the grain sampled from all 43 hybrids had aflatoxin less than 20 ng/g in the portion of the sample without BGYF kernels.

The 'aflatoxin susceptible control' B73 x Mo17 averaged 0.6 and 1.1 BGYF kernels/grid in 1998 and 1999, respectively. Equivalent numbers of BGYF kernels were recorded for B73 x Mo17 in previous variety trials with a corn seed company at Bloomington, IL (1990,1991), Union City, TN (1990, 1992), and Weslaco, TX (1992). At the same time, aflatoxin values for these grain samples ranged from lows of 8 ppb and 2 ppb following late plantings (June 7, 1998 and May 29, 1999) at Monmouth, IL and a high of 1497 ppb for Weslaco, TX (1992). These results suggest B73 x Mo17 produces a consistent 'maximum number' of kernels susceptible to seed coat tearing across environments. The extent to which these B73 x Mo17 susceptible kernels become contaminated with aflatoxin is largely a function

of prevailing day/night temperatures during the period of kernel maturation.

We plan to study the differences in BGYF kernel frequency in the progeny of breeding populations made by crossing lines that are relatively high (poor) for BGYF kernels with lines that are low (good), to see whether good lines can be recovered, and whether these "good" lines tend to result in reduced aflatoxin levels from hybrids made with these lines.

IDENTIFICATION AND CHARACTERIZATION OF NEW CORN KERNEL TRAITS ASSOCIATED WITH RESISTANCE TO *ASPERGILLUS FLAVUS* INFECTION/AFLATOXIN PRODUCTION

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Advances have been made in the identification and characterization of corn kernel traits, both pericarp and subpericarp, that are associated with resistance to *Aspergillus flavus* infection/aflatoxin production. Resistance-associated properties of kernel pericarp wax of corn population, GT-MAS:gk, previously demonstrated in a study involving three susceptible genotypes, were further confirmed in a comparative investigation between GT-MAS:gk and twelve susceptible corn lines. These properties include: 1) resistance- or susceptibility-associated bands in corn kernel pericarp wax separated by thin layer chromatography (TLC) and, 2) GT-MAS:gk wax *in vitro* inhibition of *A. flavus* growth. Not confirmed was an association between wax quantity and resistance, previously observed in the earlier study. Gas chromatography/mass spectroscopy analysis of pericarp wax of GT-MAS:gk and the susceptible lines revealed high levels of ethyl hexadecanoate and phenol-like compounds, and low levels of butyl hexadecanoate in the GT-MAS:gk unique TLC band. This was in contrast to the high levels of butyl hexadecanoate, and low levels of ethyl hexadecanoate and phenol-like compounds observed in the "susceptible" unique band. Further investigation of resistance-associated chemical compounds may yield markers useful to breeders developing aflatoxin-resistant corn lines.

In another wax investigation, developing kernels of corn genotypes (including GT-MAS:gk) three to nine weeks post-pollination, were stripped of wax or not stripped, and then inoculated with *A. flavus* and subjected to the kernel screening assay (KSA). Results showed higher levels of aflatoxins in younger unextracted kernels than in extracted ones. Also, wax in younger kernels (3 to 6 weeks old) had a higher ratio of long chain hydrocarbons compounds to sterols, whereas, the reverse was true for older kernels (7 to 9 weeks old). An investigation of this type may assist in identifying pericarp wax components inhibitory or stimulatory to aflatoxin production.

Proteome analysis of kernels of two resistant genotypes, Mp420 and MP313E, and of several susceptible corn lines was performed and several potential resistance-associated constitutive proteins were identified. These proteins belong to several categories: storage proteins (globulins 1 and 2), late embryogenesis abundant proteins related to drought stress (LEA3 and LEA14), osmo-stress related proteins (WSI18, aldose reductase, glyoxalase), and heat stress related proteins (HSP16.9). The identification of stress-related proteins potentially associated with resistance expands the previous model of kernel resistance, that primarily centered around antifungal traits. These discoveries and future characterization of these proteins may yield new resistance markers that make possible, the deployment of a gene pyramiding strategy for developing commercially useful, aflatoxin-resistant corn germplasm.

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MAIZE RESISTANCE TO AFLATOXIN IN TEXAS

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Preharvest aflatoxin (AF) contamination is one of the main limitations for corn production in Texas, causing enormous economic losses. The combined crop loss due to AF epidemics in 1988, 1989, 1990, 1995, 1996 and 1998 surpassed one billion dollars. At present, there are no elite inbred lines resistant to AF that can be used directly in commercial hybrids. There is limited information about the performance and the efficiency of known sources of resistance under field conditions in Texas. Furthermore, the majority of these sources lack agronomic performance and adaptation that preclude their direct use in commercial hybrids. In 1999, we tested aflatoxin-resistant candidate inbreds and hybrids using artificial inoculation in Weslaco (WE), Corpus Christi (CC) and College Station (CS). The material under evaluation had different backgrounds: sources of aflatoxin resistance, subtropical and tropical germplasm with good husk cover and grain quality, and elite temperate germplasm with good yields and loose husks. Based on the 1999 data, a diallel among the most resistant yellow (Mp420, Mp715, Tx601, FR2128, Tx772, CML326, CML285) and white inbreds (Tex6, CML322, CML176, CML269, CML384, Tx124, Tx114) was conducted in the same three locations during 2000. The silk channel technique was used at CS and WE, while in CC, *A. flavus*-colonized corn kernels were placed on the soil surface between treatment rows around mid-silk stage. Drought and heat stress was induced by late planting and limited irrigation. GCA (General Combining Ability) effects for AF contamination were estimated using Griffing method IV.

Significant differences among the hybrids and among inbred GCAs were detected in all three locations. At WE the average AF was 382.9 ppb (range: 44.3 ppb to 1235 ppb) and 408.3 ppb (range: 17.8 ppb to 2147.5 ppb) for the white and yellow hybrids, respectively. The hybrids with less AF were CML269/Tx124 and CML269/CML176 (both white), and Tx772/Mp715 and Tx772/CML326 (both yellow). The best GCA for inbreds was Tx772, CML285 and Mp715 (all yellow) and CML269, CML322 and Tex6 (all white). At CC the average AF was 195.3 ppb (range: 23.3 ppb to 1233.3 ppb) and 571.5 ppb (range: 106.7 ppb to 2400.0 ppb) for white and yellow hybrids, respectively. The hybrids with less AF were CML269/CML176 and CML176/Tex6 (both white), and DKXL269 and FR2128/Mp715 (both yellow). The best GCA for inbreds was CML326 and Mp715 (both yellow) and CML176, CML322 and Tex6 (all white). At CS the average AF was 89.4 ppb (range: 15.5 ppb to 383.0 ppb) and 110.3 ppb (range: 22.0 ppb to 789.9 ppb) for white and yellow hybrids, respectively. The hybrids with less AF were CML343/Tx114 and CML384/Tx114 (both white), and TX772/Mp715 and Tx772/CML326 (both yellow). The best GCA for inbreds was Tx772 and CML326 (both yellow) and CML176 and CML322 (both white). The most promising inbreds for the Texas growing conditions are CML322, CML269, CML176 among the whites and CML326, Tx772, CML285 and Mp715 among the yellows. Some of these inbreds have shown desirable agronomic characteristics in Texas. Our future plans are: to evaluate further the most promising inbreds, to characterize their resistance factors, to combine resistant factors from different resistant sources, to transfer the resistance to elite inbreds parents, to study the influence of pollen source in AF development, and to screen additional elite and exotic germplasm.

IDENTIFICATION AND PYRAMIDING OF GENES/MARKERS ASSOCIATED WITH CORN EAR RESISTANCE TO INSECTS AND *ASPERGILLUS FLAVUS* FOR CONTROL OF PREHARVEST AFLATOXIN CONTAMINATION IN THE SOUTH: A RESEARCH PROGRESS REPORT

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The field aflatoxin contamination process is known to be influenced by numerous factors, several of which cannot be controlled by the producer. A recurring theme in southern grown corn, however, is the influence of insects on ultimate contamination levels. The effect of husk tightness and coverage, subsequently has an important influence on aflatoxin contamination, especially as it relates to prevention of entry by insects into the ears of southern grown hybrids. Tight husks that cover the ear completely are necessary to insure silk feeding while the corn earworm enters the ear and prevention of larvae normal growth by silk antibiotic compounds (maysin). The research in our lab is focusing on investigation of the biotic/abiotic factors affecting host resistance to aflatoxin production and breeding corn lines which adapt to the South with reduced aflatoxin contamination. In this year, we conducted research on evaluation of drought tolerance of corn germplasm in the cages and the fields, comparison of the relationship of drought tolerance and aflatoxin production in different commercial corn hybrids in irrigated and non-irrigated fields, and the second year field evaluation of two F2:3 mapping populations for silk antibiotic compound, husk coverage and aflatoxin production. A new synthetic population has been generated in this summer to combine/pyramid the different traits.

We evaluated 20 inbreds and 2 synthetic materials from Spain, and 2 inbreds and 6 populations from CIMMYT for drought tolerance in two cages with 4 replications and in the field. Two inbreds from Spain and 2 populations from CIMMYT were excellent in drought tolerance. We conducted multi-location field evaluation on drought tolerance and aflatoxin production in 13 commercial hybrids and one cross of GT-MAS:gk and Mp313E. There were three locations and each location had 10 replications for each hybrid with two treatments, irrigation and non-irrigation in the summer of 2000. Aflatoxin analysis has been completed for one location at Midville, Georgia. GT-MAS:gk x Mp313E was floating on the top for both irrigated and non-irrigated plots with lowest aflatoxin levels, 2.7 and 42.9, respectively. The followings were Dekalb 668, Pioneer 33K81 and Dekalb 687, all 3 hybrids having excellent drought tolerance rated as 8, 8, and 7 by each company, respectively (1=poor and 9 excellent).

A two-year field experiment to evaluate selected genotypes inoculated with *A. flavus* and infested with corn earworm revealed that significant variation exists among the genotypes for aflatoxin contamination and corn earworm damage. The protection of maize ears against aflatoxin contamination was primarily dependent on resistance to fungal infection, ear-feeding insects, and excellent husk coverage and tightness. A major QTL (*p1*) identified on chromosome 1S had an effect of 54.0%, 42.1%, and 28.3% on the phenotypic variability for concentrations of silk maysin, 3'-methoxymaysin+apimaysin, and chlorogenic acid, respectively. A new marker, *csu1066* on chromosome 2C, had an effect of 20.4% on the

variability for silk chlorogenic acid concentrations but had less effect on silk maysin and 3'-methoxymaysin+apimaysin. Markers/QTLs for husk phenotypic traits and total aflatoxin concentrations have been determined, but more detailed mapping for these chromosomic regions will be necessary to locate more precise markers/QTLs for husk traits and aflatoxin production. Realizing the complexity of the aflatoxin-corn system and the factors affecting aflatoxin contamination, we are directing our program toward marker-assisted breeding to enhance or improve general genetic resistance to ear-feeding insects and invasion by *Aspergillus* spp. in the south adapted corn lines with the characters of tight ear shucks, ears that turn down and hard textured kernels.

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IDENTIFICATION AND SELECTION OF MOLECULAR MARKERS ASSOCIATED WITH RESISTANCE TO AFLATOXIN PRODUCTION IN MAIZE, AND VALUE OF INBRED CI2 AS RESISTANCE SOURCE

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Inheritance of resistance to Aspergillus ear rot and aflatoxin with the resistant corn inbred CI2 in crosses with the susceptible inbred B73 was studied in 1998-99. Generations evaluated included the resistant and susceptible parent, F_1, F_2, backcross to the resistant parent, backcross to the susceptible parent, F_3, and backcross to the susceptible parent selfed. Primary ears were inoculated and visually estimated for ear rot severity. Aflatoxin production was quantified using an ELISA technique. The warm and dry summer of 1998 favored Aspergillus ear rot and aflatoxin production whereas, the environmental conditions of 1999 did not. In both years there was no significant differences between the F_1 values and the calculated midparent. Generation by environment interaction was high mostly due to the differences in environment between the two years. In 1998 epistasis was the most important gene action. In 1999 additive gene action was most important. Broad and narrow sense heritabilities for both ear rot and aflatoxin production were higher in the F_3 generation than the backcross susceptible self generation. Pearson's and Spearman's correlation coefficients calculated between Aspergillus ear rot ratings and aflatoxin values for F_3 and backcrossed to susceptible self families were not significant in 1998 and were highly significant but low in 1999. We have determined that CI2 has very limited possibilities as a usable source of resistance because of low heritabilities, lack of dominant gene action, and relatively low levels of resistance compared to other resistant inbreds. Consequently, we decided not to perform molecular marker analysis on the CI2 x B73 BC1S1 and F2:3 populations that had been evaluated, sib mated to increase seed, and DNA isolated.

Diallel analysis of resistance sources evaluated for four years from 1995 to 1998 indicated Oh516 is a very promising new source of resistance. Oh516 was very comparable to Tex6 for inhibition of aflatoxin among the set of F1s evaluated. Oh516 appears complementary to Tex6 as the Oh516 x Tex6 F1 had one of the lowest levels of aflatoxin over the four year study. A (Oh516 x B73) B73 Backcross-1 S1 population with over 300 families has been developed for QTL mapping this new source.

We have detected QTL for resistance to aflatoxin production from Tex6 x B73 F2:3 and backcross to B73 S1 mapping populations. The QTL on the long arm of chromosome 5 explains 25% of the variation in the BC1S1 mapping population in one year. This is the largest QTL we have detected thus far. We are using the very conservative PLAB QTL software which performs composite interval analysis. We also detected a QTL for resistance to aflatoxin production in this region in the F2:3 population the same year. This QTL explains 8% of the variation. The finding of a QTL in the same region in the two populations supports the validity of the QTL. We have also detected QTL for resistance to aflatoxin production using single factor analysis of variance on chromosomes 2, 4, 5, 6, 7, 9 and 10. However we place less weight on these associations than PLAB QTL analysis. We detected a number of single factor analysis associations on chromosome 5 which supports the QTL detected on chromosome 5 with PLAB QTL analysis. The major QTL on 5L was detected in 1997 data but not in 1996. In 1996 levels of aflatoxin production were low and there was limited

variation among families, making it difficult to detect QTL in general. However this points out the value and need for QTL since in years when there are not good levels of expression for aflatoxin production, the QTL associations established in years favorable for detection can be used for marker assisted selection.

We are performing Near-Isogenic Analysis of the Tex6 QTL for resistance to aflatoxin production on chromosome 3 and 5 to confirm the QTL and initiate Marker Assisted Backcrossing to B73. Tex6 x B73 F3 families heterozygous for markers in the region of these QTL were selfed last winter in Hawaii and sublines developed. Replicated evaluations were performed this past summer. These lines have been inoculated and scored for Aspergillus ear rot, molecular marker genotyping and aflatoxin determinations are being performed presently. The homozygous sublines with chromosome regions confirmed with QTL for resistance to aflatoxin production will be backcrossed this winter to B73 and the commercially elite inbred Fr1064. We are also performing marker assisted backcrossing with (Tex6 x B73)B73 BC1S1 families that have low levels of aflatoxin production and have chromosome regions associated with QTL. These lines are being sent to Hawaii for backcrossing.

We are performing molecular marker assisted backcrossing of the two major chromosome 4 QTL for resistance to aflatoxin production from Mp313E . We genotyped (Mp313Ex FR1064) Fr1064 BC1 plants throughout the long arm of chromosome 4 and backcrossed plants containing the two QTL regions to Fr1064. The BC2 plants will be backcrossed again in Hawaii to produce BC3 generation. We are also using this molecular marker genotyping to develop a (Mp313E x FR1064) Fr1064 BC2 S1 mapping population that contains the Mp313E chromosome 4 QTL in an approximately 87.5% FR1064 background. This will enable confirmation QTL detected in Mp313E x Va26 F2:3 in an adapted, commercially elite Stiff Stalk genetic background.

We have molecular marker genotyped a series of 37 advanced experimental lines that were developed with conventional crossing and selection procedures designed to introgress resistance from Tex6 and LB31 resistance sources into more elite backgrounds. These lines have been selected for yield performance and resistance to aflatoxin production. We performed marker assays in regions where QTL had been detected previously with multiple regression analysis and recently with PLAB QTL. We have identified chromosome regions that appear to have been selected and introgressed from the resistance sources into the more agronomically elite genetic backgrounds. The presence of the introgressed segments provide some independent support of the validity of QTL detected in segregating populations. These genotyped advanced experimental lines will serve as useful donor lines for marker assisted selection into elite germplasm since the segments have already been introgressed into more elite backgrounds and withstood the selection for grain yield and aflatoxin resistance.

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PREHARVEST AFLATOXIN CONTAMINATION IN DROUGHT TOLERANT AND DROUGHT INTOLERANT PEANUT GENOTYPES

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Peanuts become contaminated with aflatoxins when subjected to prolonged periods of heat and drought stress. The effect of drought tolerance on aflatoxin contamination is not known. The objectives of this research were to evaluate preharvest aflatoxin contamination in peanut genotypes known to have drought tolerance and to determine the correlation of drought tolerance characteristics with aflatoxin contamination. Twenty genotypes with different levels of drought tolerance were grown in Yuma, AZ (a desert environment) and under rain protected shelters in Tifton, GA. Two drought tolerant genotypes, PI 145681 and Tifton 8, and an intolerant genotype, PI 196754 were selected for further examination in a second experiment with two planting dates at Tifton. Drought and heat stress conditions were imposed for the 40 d preceding harvest. The drought intolerant genotype had greater preharvest aflatoxin contamination than the check cultivar, Florunner. Both drought tolerant genotypes had less preharvest aflatoxin contamination than Florunner in these tests. Significant positive correlations were observed between aflatoxin contamination and leaf temperature and between aflatoxin contamination and visual stress ratings. Leaf temperature and visual stress ratings are less variable and less expensive to measure than aflatoxin contamination. Leaf temperature and visual stress ratings may be useful in indirectly selecting for reduced aflatoxin contamination in breeding populations. In separate studies, we have examined the accessions in the peanut core collection for other physiological traits that may be related to drought tolerance. None of these traits appeared to be correlated with aflatoxin contamination. We have also been screening several breeding populations for resistance to preharvest aflatoxin contamination under heat and drought stress conditions. Several breeding lines have been observed that have low aflatoxin contamination and high relative yield.

ASPERGILLUS INFECTION OF PEANUT IN RELATION TO POD DEVELOPMENT STAGE AND WATER DEFICIT, AND SCREENING FOR MECHANISMS OF RESISTANCE TO *ASPERGILLUS*

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Genetic differences in aflatoxin resistance have been found among peanut cultivars and methods have been developed to screen peanut germplasm for aflatoxin resistance. It is difficult to screen germplasm directly for aflatoxin resistance because *Aspergillus* spp. infection occurs mostly in plants that have been weakened by stress, particularly drought, and because even susceptible genotypes have relatively few pods that are infected and contaminated under stress. Thus, rather than attempting to screen germplasm directly for aflatoxin resistance, it may be possible and far more efficient to develop aflatoxin resistant germplasm indirectly, that is through screening for drought resistance or some other trait that confers aflatoxin resistance. Some newly developed tools that may enable us to indirectly screen for resistance to *Aspergillus* and drought include combined uses of minirhizotrons and *Aspergillus flavus* mutants that produce a green fluorescent protein (GFP).

Our long-term research objectives are:

1. To quantify the relationships among susceptibility to *Aspergillus* spp. infection, pod developmental stage, and intensity of water deficit in peanut.

2. To identify varietal differences in root and pod growth traits that contribute to drought resistance among peanut genotypes, and the relationship of these traits with resistance to *Aspergillus* spp. infection.

3. To develop a method for screening transgenic peanut lines for resistance to *A. flavus* infection using a GFP *A. flavus* strain and *in situ* observation with a minirhizotron system.

4. To observe *A. flavus* infection of peanut roots and pods *in situ* using fluorescence under a microscope.

5. To ascertain the relationship between root exudates, development of *A. flavus* populations, water deficit, and aflatoxin resistance.

6. To develop more efficient methods to screen indirectly for aflatoxin resistance in peanut.

Genotypic differences in root distribution and plasticity were related to both drought and aflatoxin resistance. Though highly beneficial, a large root system alone is not enough for thorough water extraction during periods of low water availability. Plastic root system responses to water deficit ability, such as increased root growth in deeper soil layers, are also important. Water deficit increased susceptibility to preharvest *A. flavus* infection and aflatoxin contamination. Results showed greater *A. flavus* population growth under water deficit conditions. However, minimal seed infection was observed after harvest. Other experiments are still in progress.

Panel Discussion: Crop Resistance—Conventional Breeding

Chair: Thomas M. Gradziel

Panel Members: Don Wicklow, Zhi Yuan Chen, Javier Betrán, Baozhu Guo, Torbert R. Rocheford, Donald G. White, C. Corley. Holbrook and Keith T. Ingram.

The discussion dealt with comments and questions from the audience with a particular emphasis on research methods used to assess resistance. Crops discussed included maize, peanut and almonds. The consensus of the discussants was that the required levels of field resistance to aflatoxin contamination required an integrated approach to disease control. At the conclusion of the discussion, however, it was pointed out that such integrated approaches inherently are more difficult to pursue in breeding programs and may have unanticipated consequences on important agronomic traits including yield.

MULTI-LOCATION EVALUATION OF SINGLE CROSS MAIZE HYBRIDS FOR AFLATOXIN CONTAMINATION

W. Paul Williams[1], Gary L. Windham[1], Martha C. Willcox[1], Hamed K. Abbas[2], Javier Betrán[3], Donald G. White[4], Steven H. Moore[5], Rick Mascagni[6], Neil W. Widstrom[7], Kenneth E. Damann[8]. [1]USDA-ARS, Mississippi State, MS; [2]USDA-ARS, Stoneville, MS; [3]Texas A&M University, College Station, TX; [4]University of Illinois, Urbana, IL; [5]Louisiana State University, Alexandria, LA; [6]Louisiana State University, St. Joseph, LA; [7]USDA-ARS, Tifton, GA; [8]Louisiana State University, Baton Rouge, LA.

Infection of maize with *Aspergillus flavus* and the subsequent accumulation of aflatoxin in grain are chronic problems in the South and sporadic problems in the Corn Belt. Aflatoxin is a potent carcinogen that adversely affects both humans and livestock. Grain with aflatoxin levels exceeding 20 ng / g is banned from interstate commerce. Some countries have imposed even more stringent standards on imported grain. A potentially effective and highly desirable method of reducing aflatoxin contamination is the use of genetically resistant hybrids. Public maize breeding programs in Georgia, Illinois, Mississippi, and Texas are currently evaluating germplasm to identify and develop sources of resistance that can be used in producing aflatoxin resistant maize hybrids. Insect damage, drought stress, high temperatures, and other environmental factors are frequently associated with aflatoxin contamination of maize. Differences in levels of aflatoxin contamination among environments make identification of resistant germplasm difficult. This investigation was undertaken to evaluate hybrids from breeding programs in Illinois, Mississippi, and Texas for resistance to aflatoxin accumulation. Hybrids were evaluated for aflatoxin in replicated experiments conducted at Weslaco, TX; St. Joseph, LA; Alexandria, LA; Stoneville, MS; Starkville, MS; Tifton, GA; and Urbana, IL. Mp313E x Mp420 and Mp313E x Mp494 had relatively low levels of aflatoxin contamination at all locations. CML269 x CML332 also performed well at most locations. Generally, the hybrids developed in Illinois were not well adapted to the South and sustained levels of aflatoxin contamination comparable to susceptible check hybrids.

REACTION OF CORN INBREDS TO *ASPERGILLUS FLAVUS*/AFLATOXIN CONTAMINATION IN THE FIELD

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Aflatoxin is a naturally occurring toxin produced by the fungus *Aspergillus flavus*. This toxin is the most potent carcinogen found in nature. In the United States, aflatoxin contamination of corn grain occurs sporadically in the Midwest, but is a chronic problem in the southeastern U.S. In 1998, a major aflatoxin epidemic in corn occurred in the Southeast. The most desirable method of aflatoxin control is through host plant resistance to *A. flavus* infection and subsequent aflatoxin accumulation. Unfortunately, no commercial corn hybrids have been identified that are resistant to aflatoxin contamination. Progress has been made in identifying sources of resistance. Corn inbreds Mp420, Mp313E, and Mp715 have resistance to *A. flavus* and have been released by the USDA Agricultural Research Service at Mississippi State, MS. However, other sources of resistance are needed to help eliminate aflatoxin contamination in corn. The objective of this study was to evaluate inbred lines for aflatoxin contamination and identify additional sources of resistance.

Corn inbreds were grown in 1998 (one test), 1999 (two tests), and 2000 (one test) at Mississippi State University's Plant Science Research Farm. The top ear of each plant was inoculated with the *A. flavus* isolate NRRL 3357 seven days after midsilk (50% of the plants in a plot had silks emerged) using the side needle technique. Ears were harvested by hand ca. 63 days after midsilk and dried at 38°C for 5 days. Ears were then machine shelled, and grain samples were ground using a Romer mill. Aflatoxin contamination in 50-gram subsamples from each plot was determined using the Vicam Aflatest. Aflatoxin data were transformed to stabilize the variance of the data. Data were subjected to analysis of variance with the SAS general linear models procedure. Means were compared by the least significant difference test at P=0.05.

Aflatoxin contamination in the inbreds was extremely high in 1998. Levels ranged from 139 to 21,090 ng/g. Extremely high temperatures and drought conditions contributed to the high levels of aflatoxin. In 1999, aflatoxin contamination ranged from 14 to 1,278 ng/g in one test and from 17 to 1,070 ng/g in the other test. Resistant checks Mp313E and Mp715 had low levels of aflatoxin in 1999. In 2000, aflatoxin contamination ranged from 227 to 7,503 ng/g. Inbreds that showed promise as new sources of resistance included Mp494, Mp92:673, Mp80:04, and Mp81:112. These inbreds and others with low levels of aflatoxin contamination in the 2000 growing season will be included in future tests to confirm their resistance to aflatoxin contamination.

MARKERS ASSOCIATED WITH SILK ANTIBIOTIC COMPOUNDS, HUSK COVERAGE, AND AFLATOXIN CONCENTRATIONS IN TWO MAIZE MAPPING POPULATIONS

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Maysin and related compounds, such as apimaysin, 3'-methoxymaysin, and chlorogenic acid, have been determined to be important maize (*Zea mays* L.) produced antibiotic compounds against corn earworm (*Helicoverpa zea* Boddie), but, to be effective under field conditions, silk antibiotics should be accompanied by a good husk coverage. The objective of this work was to identify molecular markers associated with synthesis of maysin and related compounds in a maize population and determine if they could affect husk tightness. A total of 102 probes were used to screen for RFLP polymorphisms and were used to genotype 250 F_2 individuals. Silks and husks of 250 $F_{2:3}$ families, each with 3 replications, were evaluated and aflatoxin levels were also measured. Two major QTL were identified for the synthesis of maysin and related compounds, the already known *p1*, on the short arm of chromosome 1, and a novel one on the interval *csu1066-umc176* on genomic region 2C-2L. A QTL for husk tightness was located near to *p1*. The functional allele for *p1* and the favorable allele for husk tightness were in repulsion linkage. Therefore, in a marker-assisted selection program for increasing silk antibiosis against corn earworm, markers for silk antibiotic synthesis, *p1, csu1066*, and *umc176* should be accompanied by marker *csu3* to avoid the negative effect on husk tightness of some genotypes carrying functional *p1* alleles. Efforts should be made to convert the RFLP-markers into PCR-based markers for user friendly application in marker-assisted breeding. Based on one year aflatoxin data of 1999, a two locus model accounted for 24.7% of the phenotypic variance for aflatoxin contamination, two-thirds of which was attributable to the epistatic interaction of regions on chromosomes 1S and 2L. The recombination of progenies with chromosome region 1S from GTA1 and 2L from GT119 gave the lowest aflatoxin concentrations.

The data we present on QTL analyses for husk coverage and aflatoxin production are setting the stage for progress toward our long-term goal. However, our field studies demonstrated that prevention of ear-feeding insects and husk coverage are essential for reduction and/or elimination of aflatoxin contamination in southern U.S. produced corn; requirements are different from these for corn production in the U.S. corn belt. The protection of maize ears against aflatoxin contamination is primarily dependent on resistance to fungal infection, resistance to ear-feeding insects, and excellent husk coverage and tightness.

EVALUATION OF CORN GERMPLASM TOLERANCE TO DROUGHT STRESS AND EFFECTS ON AFLATOXIN PRODUCTION

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Corn is an important crop in the Coastal Plain Region as feed for livestock and poultry and a rotation crop with other high-value crops such as peanut and cotton. Of all agricultural commodities produced on the Coastal Plain, the production of corn falls far short of the actual demand in the region. This is partially due to rapid expansion of the poultry industry on the Coastal Plain. The primary reduction in corn acreage on the Coastal Plain has occurred for dryland corn production. Three factors have been primarily responsible for this decline: drought which reduces both yield and quality of corn produced, aflatoxin contamination of the grain which dramatically reduces the grain quality and is exacerbated by drought stress, and insect damage which both reduces yield and enhances aflatoxin contamination. Thus, unpredictable yields as a result of drought, insect damage, and aflatoxin contamination of the grain are important factors limiting the expansion of corn production in the Coastal Plain Region.

Our research objective are screening and identification of corn germplasm resistant to drought stress and resistant to ear-feeding insects and aflatoxin formation. We conducted studies in the cages and fields to evaluate the drought tolerance of corn germplarm from CIMMYT and Spain. Two inbreds from Spain and 2 populations from CIMMYT survived from extreme drought with excellent drought tolerance. Multi-location field evaluation on drought tolerance and aflatoxin production have demonstrated that drought tolerant commercial lines, in general, had lower aflatoxin contamination in drought condition (such as Dekalb 668, Pioneer 33K81 and Dekalb 687). The positive association of drought tolerance and lower aflatoxin production is encouraging. This data is based on one location study and two other location samples have been in processing.

MARKER ASSISTED BACKCROSSING AND IDENTIFICATION OF CHROMOSOMAL SEGMENTS ASSOCIATED WITH RESISTANCE TO AFLATOXIN PRODUCTION IN MAIZE

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See platform summary from Rocheford and White pages 153-154 in this section.

SOURCES OF RESISTANCE TO AFLATOXIN PRODUCTION IN MAIZE

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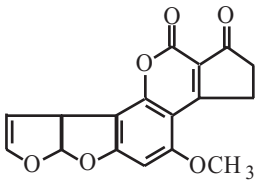
Drought-tolerant maize genotypes (Huffman, Z08-004, Tuxpan, PH 9, NRC 5348, Chunco, Saint Croix, and Arizona) were compared in the field and laboratory to toxin-resistant GT-MAS:gk and Yellow Creole. SDS-PAGE, scanning electron microscopy of kernel wax, amount of kernel wax, *Aspergillus flavus* kernel colonization, Aspergillus ear rot, insect damage, aflatoxin production, and their relationships were examined. SDS-PAGE showed the presence of a 14 kDa trypsin inhibitor in kernels of all genotypes except Chunco, which contains a protein of a larger MW. The 14 kDa trypsin inhibitor protein content in these genotypes was as high as in GT-MAS:gk and Yellow Creole. Scanning electron microscopy revealed that Arizona, Huffman, and Chunco genotypes had abundant wax deposits on kernel surfaces and the amount of pericarp wax was equal or above that from GT-MAS:gk and Yellow Creole. Differences in Aspergillus ear rot ratings, fungal colonization, and insect damage by the corn earworm were observed in all drought-tolerant maize genotypes as well as in controls. KSA assays showed that aflatoxin levels in inoculated drought-tolerant genotypes differed significantly from those in GT-MAS:gk and in Yellow Creole (LSD = 576). In the field where corn was grown under drought stress conditions, aflatoxin levels in the inoculated genotypes also differed significantly from those of GT-MAS:gk or Yellow Creole (LSD = 1389). Pearson correlation coefficients were significant between ear rot ratings and insect damage ($r = 0.75$; $P = 0.01$), and between Aspergillus ear rot and aflatoxin levels ($r = 0.54$; $P = 0.05$). Based on the parameters studied, there are indications that these genotypes are potential good sources of resistance to aflatoxin contamination.

POSTERS: REGULATION OF AFLATOXIN BIOSYNTHESIS



VARIABILITY IN NITROGEN REGULATION OF AFLATOXIN PRODUCTION BY *ASPERGILLUS FLAVUS* STRAINS

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Several studies have demonstrated that nitrate is inhibitory to aflatoxin production by *A flavus*. AflR and AflJ, proteins encoded by divergently transcribed genes in the aflatoxin biosynthetic gene cluster are involved in the positive control of aflatoxin pathway genes. The *aflR-aflJ* intergenic region contains GATA-motif binding sites recognized by AreA, the positively acting, wide-domain regulatory protein that mediates nitrate utilization and other processes. S strain isolates of *A. flavus* collected in Benin, (S_{BG} phenotype, aflatoxin B and G-accumulating) have five GATA sites, while S strain isolates collected in North America, (S_B phenotype, aflatoxin B-accumulating) have seven sites. We hypothesized that nitrogen sources affecting the activity of AreA might alter expression of both *aflR* and *aflJ*, and thereby the accumulation of aflatoxin. Compared to ammonium salts medium, aflatoxin production was inhibited 4 to 10-fold for the S_{BG} isolates and 0 to 4-fold for the S_B isolates when cultures were grown on buffered nitrate medium. When either S_B or S_{BG} isolates were grown in urea, addition of nitrate was not inhibitory to either. The level of *aflJ* mRNA was higher in nitrate than in ammonium salts medium for the S_B isolates, but lower for the S_{BG} isolates. However, the level of *aflR* mRNA was slightly higher in nitrate than in ammonium salts under the experimental conditions for isolates with either phenotype. These results suggest that increased transcription of both *aflR* and *aflJ* in nitrate medium may partially offset other potential inhibitory effects of nitrate on aflatoxin biosynthesis.

INHIBITION OF *ASPERGILLUS* GROWH BY A MAIZE RIBOSOME INHIBITING PROTEIN

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The maize endosperm ribosome-inactivating protein, RIP, is a potent translational inhibitor with a putative role in plant defense. RIP was tested for antifungal activity against *Aspergillus flavus* and *Aspergillus nidulans* with a microculture assay in which fungal conidia treated with RIP or control proteins were monitored over time. Growth of the maize pathogen *A. flavus* was inhibited by RIP. However, the organism could overcome this inhibition by producing a new hyphal tip that led to a branched phenotype. When conidia from a closely related non-pathogenic species, *Aspergillus nidulans*, were treated with RIP, we observed a striking decrease in hyphal proliferation, followed by lysis. The lysis and branching phenotypes both occurred at the transition from pre-divisional to post-divisional growth. To determine whether the presence of RIP is important for fungal infection in the field, ears segregating for the opaque-2 mutation were inoculated with an *A. flavus* strain expressing GFP and individual kernels were examined for fluorescence. Mutant kernels had higher levels of fluorescence than normal kernels. These results suggest that the antifungal activity of maize RIP plays a role in defense of the kernel against fungal invasion.

CLONING AND FUNCTIONAL ANALYSIS OF *AVFA* AND *OMTB* GENES IN AFLATOXIN BIOSYNTHESIS IN *ASPERGILLUS PARASITICUS*, *A. FLAVUS* AND *A. SOJAE*

Jiujiang Yu[1], Charles P. Woloshuk[2], Deepak Bhatnagar[1] and Thomas E. Cleveland[1]. [1]Southern Regional Research Center, USDA, Agricultural Research Service, New Orleans, LA and [2]Department of Botany and Plant Pathology, Purdue University, West Lafayette, IN.

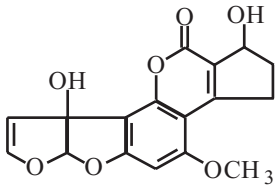
Aflatoxins are a family of polyketide derived secondary metabolites of several *Aspergilli*, but commonly produced by the fungi *Aspergillus flavus* and *A. parasiticus*. Amongst these, aflatoxin B_1 is one of the most potent natural carcinogens known. Aflatoxin contamination of the agricultural commodities is, therefore, not only a serious health hazard but an economic issue as well. The biosynthesis of aflatoxins (B_1, G_1, B_2, and G_2) is a multi-enzyme process controlled genetically by over 20 genes. These aflatoxin pathway genes are clustered in a 70-kb DNA region in *A. flavus* and *A. parasiticus* under the control of the positive regulatory gene, *aflR*, and another gene *aflJ*. In this study, we report the identification and characterization of the *avfA* gene, which was found to be involved in the conversion of averufin (AVF) to versiconal hemiacetal acetate (VHA), in *Aspergillus parasiticus* and *A. flavus*; a copy of *avfA* gene was also cloned from a non-aflatoxin producing strain *A. sojae*. Complementation of an averufin-accumulating, nonaflatoxigenic mutant strain of *A. parasiticus*, SRRC 165, with the *avfA* gene cloned from *A. flavus*, restored the ability of the mutant to convert AVF to VHA and to produce aflatoxins B_1, G_1, B_2, and G_2. Sequence analysis revealed that a single amino acid replacement from aspartic acid to asparagine disabled the function of the enzyme in the mutant strain SRRC 165. The *A. parasiticus avfA* was identified to be a homolog of previously sequenced, but functionally un-assigned transcript, *stcO*, in *A. nidulans* based on sequence homology at both nucleotide (57%) and amino acid (55%) levels. In addition to *avfA*, another aflatoxin pathway gene, *omtB*, encoding for an *O*-methyltransferase involved in the conversion of demethylsterigmatocystin (DMST) to sterigmatocystin (ST) and dihydrodemethylsterigmatocystin (DHDMST) to dihydrosterigmatocystin (DHST), was cloned from *A. parasiticus*, *A. flavus*, and *A. sojae*. The *omtB* gene was found to be highly homologous to *stcP* from *A. nidulans*, which has been reported earlier to be involved in a similar enzymatic step for the sterigmatocystin formation in that species. RTPCR data demonstrated that both the *avfA* and *avfA1* as well as *omtB* genes in *A. parasiticus* were expressed only in the aflatoxin-conducive medium. An analysis of the degrees of homology for the two reported genes between the *Aspergillus* species *A. parasiticus*, *A. flavus*, *A. nidulans* and *A. sojae* was conducted.

ADHA IN *ASPERGILLUS PARASITICUS* IS INVOLVED IN THE CONVERSION OF 5'-HYDROXYAVERANTIN TO AVERUFIN

Perng-Kuang Chang, Jiujiang Yu, Kenneth C. Ehrlich, Stephen M. Boue, Beverly G. Montalbano, Deepak Bhatnagar, and Thomas E. Cleveland. Southern Regional Research Center, ARS, USDA, New Orleans, LA.

Two routes for the conversion of 5'-hydroxyaverantin (HAVN) to averufin (AVF) in the synthesis of aflatoxin have been proposed. One involves the dehydration of HAVN first to give the lactone, averufanin (AVNN), which is then oxidized to give AVF. Another requires dehydrogenation of HAVN to 5'-ketoaverantin, the open chain form of AVF, which then cyclizes spontaneously to AVF. In this study, we isolated a gene, named *adhA*, from the established aflatoxin gene cluster of *Aspergillus parasiticus* SU-1. The deduced ADHA amino acid sequence shared homology with short-chain alcohol dehydrogenases from bacteria, yeast, fungi, plants and vertebrates. ADHA also contained two conserved motifs of the dehydrogenases, a glycine-rich loop, GXXXGXG, that is necessary for interaction with $NAD^+/NADP^+$ and the motif, YXXXK, that is found at the active site. *A. parasiticus* SU-1, a strain that produces aflatoxins, contained a second copy of *adhA* (*adhA1*), whereas *A. parasiticus* SRRC 2043, a strain that accumulates *O*-methylsterigmatocystin (OMST), had only one copy. Disruption of the *adhA* gene in SRRC 2043 resulted in strains that predominantly accumulated HAVN. This result suggests that ADHA is involved in the dehydrogenation of HAVN to AVF. These *adhA* disruptants still made small amounts of OMST and, after prolonged time in culture, accumulated other metabolites including AVNN. One possible explanation for the accumulation of OMST is that overlapping dehydrogenase activities in the *adhA* disruptants can carry out the conversion of HAVN to AVF, but with lower efficiency. AVF then would be converted to OMST by the remaining aflatoxin pathway enzymes. The formation of AVNN might result from dehydration of HAVN followed by lactonization as the growth medium becomes more acidic. Thus, AVNN would merely be a shunt metabolite of the aflatoxin biosynthetic pathway.

Posters: Detection, Analysis, and Extraction of Aflatoxins



SIMPLE CLEANUP METHOD FOR DETERMINATION OF AFLATOXINS IN PEANUTS

Victor S. Sobolev and Joe W. Dorner, USDA, ARS, National Peanut Research Laboratory, Dawson, GA

The cost of existing methods for aflatoxin analysis used by the peanut industry and the aflatoxin research community remains high. The majority of the methods currently in use employ proprietary, antibody-based cleanup columns or immunoassays. The advantages of these methods are that they are selective and sensitive, but they cost substantially more than traditional minicolumn methods, which lacked the desired selectivity, sensitivity, and reliability. The purpose of this work was to develop a simple, inexpensive chemical cleanup procedure for quantitative determination of aflatoxins in peanuts. Aflatoxin is extracted from a ground peanut sample with a methanol-water mixture, and after a cleanup step on two minicolumns packed with aluminum oxide and florisil, aflatoxin is quantified with any appropriate analytical technique including fluorometry, TLC, or HPLC. Fluorometric quantitation using simple, inexpensive instruments would suit the needs of most interested institutions. The analysis time of the method using fluorometric quantitation of aflatoxins did not exceed the analysis time of current immunoaffinity column methods. It does not require any vacuum or pumping devices. The detection limit, accuracy, and precision are comparable with the antibody-based methods. The combined cost of suggested minicolumns is about $0.50, which is a substantial savings compared with commercial test kits. The method is also applicable for aflatoxin analysis in corn and cottonseed. Recovery of aflatoxin B_1 (quantified by HPLC) from peanuts spiked at 5-25 ppb was 68.8-82.4%; at 25-250 and 1000-3000 ppb it was 87.8-96.7% and 91.9-98.7%, respectively. The detection limit was extrapolated to be 1 ppb.

DETECTION OF OCHRATOXIN BY LC/MS/MS: AN ALTERNATIVE DETECTOR FOR USE WITH THE AOAC OFFICIAL METHOD SAMPLE PREPARATION PROTOCOL?

Ronald D. Plattner, Mycotoxin Research, National Center for Agricultural Utilization Research ARS/USDA, Peoria IL

Ochratoxins which were discovered in 1965 as secondary metabolites of *Aspergillus ochraceus* have emerged in recent years as a significant mycotoxin concern. Ochratoxin A is frequently measured in commodities and food products because it is made by several species of fungi, is nephrotoxic, carcinogenic and implicated in the etiology of Balkan endemic nephropathy, and occurs naturally in many commodities including cereal grains, green coffee beans, peanuts, mixed feeds, and raisins. The AOAC official method for Ochratoxin A in corn and barley uses extraction, solid phase extraction cleanup, and liquid-liquid partition followed by reverse phase HPLC with detection by fluorescence with excitation at 333nm and emission at 460nm. An alternative approach to detection is LC/MS. We recently used this detection method to confirm the occurrence of Ochratoxin A in dust collected from a problem household. Because better signal to noise is available using LC/MS/MS, lower detection limits can be achieved for low level positive samples than were possible by LC/MS alone. Positive samples are easily confirmed in the full scan mode from injections of less than 1 ng of ochratoxin. These detection levels are compatible with the official method and affords an alternative detector response that is a highly selective and based on a different principle than the normal fluorescence method and may be useful either as an alternative to fluorescence detection or for confirmation of positive samples.

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V

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X

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Z

L’amandier en fleurs, Pierre Bonnard, 1945